Polymer Microchips for Capillary Electrophoresis and Electric Field Gradient Focusing of Biomolecules

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POLYMER MICROCHIPS FOR CAPILLARY ELECTROPHORESIS AND ELECTRIC FIELD GRADIENT FOCUSING OF BIOMOLECULES

by

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ABSTRACT

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Doctor of Philosophy

Polymeric materials have seen increasing use as microfluidic device substrates due to their low cost and the simplicity of templated fabrication procedures. I showed that poly(methyl methacrylate) (PMMA) microdevices could be enclosed in a boiling water bath, which allowed the seal to form more quickly than in conventional approaches, and enabled microchannels to remain hydrated throughout the bonding process. Microchip capillary electrophoresis (μ-CE) devices were fabricated using water-based enclosure, and a mixture of fluorescently labeled amino acids was separated in 30 s in these microchips.
To create more robust capillary electrophoresis (CE) microdevices with improved separation performance, phase-changing sacrificial materials were developed for solvent bonding of polymer microchips. Devices were fabricated by filling channels in embossed PMMA with a heated liquid that formed a solid sacrificial layer at room temperature. The sacrificial material prevented the bonding solvent and softened PMMA from filling the channels. Once the sealing step was finished, the sacrificial layer was melted and removed, leaving enclosed microchannels. These solvent-welded devices withstood internal pressures >2,200 psi, and 300 CE runs were performed on a single microchip without any loss of separation performance. Furthermore, CE separations of peptides and amino acids were completed in ~10 s, with peak efficiencies of 43,000 theoretical plates.

Electric field gradient focusing (EFGF), which uses a combination of pressure-driven flow and an electric field gradient to separate charged species according to their electrophoretic mobilities, was explored for protein analysis. Capillary-based EFGF devices were characterized; mixtures of four proteins were resolved, band focusing dynamics were studied, and analytes were enriched 10,000-fold.

EFGF was miniaturized further to a microfluidic platform. Phase-changing sacrificial layers were employed to interface an electric field gradient
enabling semi-permeable copolymer with microchannels. Because of decreased channel dimensions, EFGF microchips produced narrower bands and yielded threefold higher resolution compared with capillary-based devices.

Beyond providing improved performance for polymer-based \( \mu \)-CE and EFGF, the advances in microchip fabrication technology presented here should be applicable broadly in interfacing microfluidics with hydrogel structures, for example in sample pretreatment.
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1 INTRODUCTION

1.1 CHEMICAL SEPARATIONS*

The analysis of complex chemical mixtures plays a significant, although frequently behind-the-scenes, role in virtually all branches of science, including fields as diverse as molecular biology [1, 2], pharmaceutical development [3, 4], forensics [5], space exploration [6, 7], and environmental monitoring [8]. The components of chemical mixtures frequently need to be separated to enable the quantification and identification of individual species. Linear techniques like chromatography and electrophoresis, in which fractionation is achieved by the differential migration of analytes along a column, dominate the field. Indeed, separations employing liquid chromatography alone are run approximately 1 million times per day [9].

An exciting advance in analytical separations during recent years has been the development of micromachined devices [10-12]. These microfluidic systems are created using technology and equipment developed by the semiconductor industry for the fabrication of integrated circuits (ICs). Using this methodology, which will be described in detail in Section 1.3, channels with cross-sectional features of just a few micrometers [13] can be made with complex geometries, and minute sample volumes can be separated very rapidly and efficiently.

* Sections 1.1 and 1.2 are modified from an article in J. Sep. Sci. 2005, 28, in press. Reprinted with permission.
Sample introduction in linear separations is critical, as the length of the injected plug directly impacts the resolution of the final separated bands. Because of the need for narrow sample plugs, and since the concentration of each analyte decreases during the course of a separation as bands broaden, a preconcentration step [14-21] is frequently necessary to allow the detection of more dilute analytes.

1.2 ELECTRIC FIELD GRADIENT FOCUSING

1.2.1 Introduction to Electric Field Gradient Focusing

The group of separation techniques that enables analytes to be concentrated while they are separated has been termed equilibrium-gradient methods [22]. These employ a gradient in an external field or another property (e.g., pH) to cause analytes to focus at unique, stable equilibrium positions along a separation column, regardless of original analyte location. Density gradient sedimentation [23] and isoelectric focusing [24-27] are well-known equilibrium-gradient methods that employ gradients in the density of the separation medium and pH, respectively, and have made important contributions to biological analysis. Capillary isoelectric focusing (CIEF), for example, has been used to perform very high-resolution separations of proteins according to isoelectric point (pI), with peak capacities exceeding 4,000 [28].
A relatively new equilibrium-gradient method, known as electric field gradient focusing (EFGF), was first reported by Koegler and Ivory in 1996 [29]. As the name implies, EFGF utilizes a gradient in electric field along the length of a separation column. The electrophoretic force, which drives analytes of like charge from an appropriately designed region of higher to lower electric field, is opposed by a constant, pressure-driven bulk fluid flow. Because electrophoretic velocity equals the product of electrophoretic mobility and electric field, each analyte slows as it moves to the location in the column where the analyte electrophoretic velocity and the velocity of the bulk fluid flow sum to zero. Thus, a mixture of analytes that is introduced into an EFGF system will focus into discrete bands according to their electrophoretic mobilities. Figure 1.1 is a schematic representation of an EFGF separation of two components.

Although newer and less well developed than CIEF, EFGF is potentially a more general technique, as analytes must only be charged rather than amphoteric to be focused. EFGF has additional distinct advantages over CIEF and other protein analysis methods that make it very attractive and deserving of additional attention. For example, proteins can be focused in EFGF to concentrations far higher than those attainable using CIEF (>50 mg/mL) [30], and proteins with extreme pI values that are inaccessible by CIEF can also be
Figure 1.1. Schematic representation of separation by EFGF. Arrows are vectors representing the linear velocity of the constant buffer flow (black) and the electrophoretic velocities of analytes with high (dark gray) and low (light gray) electrophoretic mobilities ($\mu$). Analytes form stationary focused bands at the locations where their electrophoretic velocities equal the velocity of the buffer in the opposite direction. Analytes are analyzed. Furthermore, in EFGF the maximum sample volume is not limited to the volume inside the focusing column; sample can be introduced continuously, enabling a higher degree of enrichment. Theoretical and experimental advances that have taken place since EFGF was first reported [29] are discussed below, with the cited articles grouped according to the approach used to establish the electric field gradient.

1.2.2 EFGF Theory

Giddings and Dahlgren [22] provided a general theoretical treatment of equilibrium-gradient methods, which is applicable to EFGF [31]. These equations hold true when molecular diffusion is the sole source of band broadening in the system. The force, $F(x)$, on a charged species in an EFGF device can be described
as a Taylor expansion about the equilibrium position, \( x = x_0 \), where second order and higher terms can be neglected for narrow bands:

\[
F(x) \equiv F(x_0) + \left. \frac{dF(x)}{dx} \right|_{x=x_0} (x - x_0) \tag{1.1}
\]

Because \( F(x_0) = 0 \) at the equilibrium position, Equation (1.1) describes a Hooke’s law force in which the effective Hooke’s law constant is:

\[
k = \left. \frac{dF(x)}{dx} \right|_{x=x_0} \tag{1.2}
\]

The potential energy of this Hookian well is:

\[
E = \frac{1}{2} k (x - x_0)^2 \tag{1.3}
\]

which, when used in the Boltzmann distribution equation gives the concentration, \( c(x) \), relative to the concentration at the equilibrium point, \( c_0 \).

\[
c(x) = c_0 \exp \left( - \frac{k(x - x_0)^2}{2RT} \right) \tag{1.4}
\]

Equation (1.4) represents a Gaussian distribution around \( x_0 \), with the standard deviation, \( s \), given in (1.5).

\[
s = \sqrt{\frac{RT}{k}} \tag{1.5}
\]

Resolution, \( R_s \), is thus expressed as

\[
R_s = \frac{\Delta x}{4} \sqrt{\frac{k}{RT}} \tag{1.6}
\]
where $\Delta x$ is the difference in location between 2 bands. Peak capacity, $n$, is

$$n = \frac{L}{4\sqrt[4]{kRT}}$$  \hfill (1.7)

In equations (1.6) and (1.7), $\bar{k}$ is the average Hooke’s law constant for the bands being considered, and $L$ is the channel length. Because $\Delta x$ is inversely proportional to the force gradient (which in turn is proportional to $\bar{k}$), resolution is inversely proportional to the square root of the force gradient, while peak capacity is directly proportional to the square root of the force gradient. This theoretical treatment has important implications for EFGF device design, because it shows that resolution and peak capacity cannot be improved simultaneously under static conditions.

In an effort to enable separations with both high peak capacity and high resolution, Tolley et al. [32] proposed EFGF systems with electric field gradients that change from being larger at the low-field region to smaller at the high-field end of devices. The sample components are first focused in the region of low electric field and concentrated into narrow bands that are ordered according to electrophoretic mobility. One of the operating parameters, either applied voltage or fluid counterflow velocity, is then adjusted gradually such that the equilibrium positions of the analytes shift toward the high-field end of the device. Because the electric field gradient is smaller at the high-field end, the
resolution of the bands increases as they enter this region. Importantly, the focused bands remain near equilibrium even as their positions shift, thus limiting Taylor dispersion (which results from the parabolic velocity profile in laminar flow) that would occur upon changing the electric field abruptly and eluting the bands from the channel. Hence, this theoretical treatment indicates one way to improve separation performance with EFGF.

1.2.3 EFGF Based on Changing Cross-Sectional Area

The first reported setup for EFGF, explored by Koegler and Ivory [29, 33], used a channel of changing cross-sectional area (CSA) to establish an electric field gradient. For constant electrolyte concentration, the electric field along the length of the channel, \( E(x) \), is related to the current, \( I \), buffer conductivity, \( \sigma \), and CSA normal to the field, \( A(x) \), by:

\[
E(x) = \frac{I}{\sigma A(x)} \tag{1.8}
\]

Simply varying the CSA of an entire focusing column would indeed produce a gradient in electric field, but the linear velocity of the opposing counterflow through the channel would change at exactly the same rate as the electric field, thus preventing focusing from taking place. In contrast, if the changing CSA channel is divided into two regions with a semipermeable barrier that enables the passage of current-carrying buffer ions but restricts the protein analytes and
bulk fluid flow to a region of fixed CSA, a constant counterflow linear velocity and a gradient in electric field can be established simultaneously.

EFGF was first applied to preparative-scale focusing [29, 33]. An open cylinder of poly(methyl methacrylate) (PMMA) was machined such that the interior had a changing CSA along its length (Figure 1.2). A packed, 6.4-mm-diameter dialysis tube provided the fixed-width protein focusing column, which was mounted in the center of the PMMA cylinder. Counterflow was generated by pumping run buffer through the packed focusing column using a syringe pump, and Pt electrodes were applied to the ends of the device for electrical contact. Because of the ~cm dimensions of the changing CSA region, buffer outside the focusing column was circulated through a glass heat exchanger to control Joule heating. Using this design, Koegler and Ivory were able to focus hemoglobin, while in another experiment, they focused and separated two myoglobin (Mb) variants that differed in oxidation state and charge. While this pioneering work effectively demonstrated the principle of EFGF, fabrication challenges and design limitations were evident. Peak asymmetry was attributed to temperature variations in the channel, edge effects at the dialysis tubing-packing interface, and imperfections in the tubing shape. In addition, focusing times were 6–10 hours, enrichment factors of just 2–3 were obtained, and on-
column visual observation was used to detect these colored proteins. In another setup, focused bands were eluted from the column and through a UV detector, but pumping the proteins from their equilibrium positions in the absence of electric field reduced resolution.

1.2.4 EFGF in a Conductivity Gradient

Greenlee and Ivory were the first to explore EFGF using gradients in buffer conductivity to establish an electric field gradient [34]. When a flowing buffer of higher conductivity is dialyzed against a pumped lower conductivity buffer, a concentration gradient is established; then, if a voltage is applied along
the length of a column having such a concentration gradient, the electric field changes according to:

$$E(x) = \frac{I}{\sigma(x)A}$$  \hspace{1cm} (1.9)

The device fabricated to test this approach consisted of two PMMA substrates, each machined with 10-cm-long channels that were 800 μm wide and 500 μm deep. A cellulose sheet membrane having a molecular weight cutoff (MWCO) of 6,000 Da was sandwiched between the two PMMA pieces, which were bolted together such that the channels were aligned and separated by the membrane. The higher conductivity buffer in the experiments was pumped through one of the channels, with recirculation and cooling used to control Joule heating caused by the large channel dimensions. The electrodes were in contact with the higher conductivity (gradient enabling) buffer, which allowed electrolysis products to be pumped from the device without entering the focusing channel. The lower conductivity buffer was introduced into the focusing channel with a syringe pump, and proteins were injected through a sample loop coupled to the syringe pump.

Experiments using hemoglobin and dyed bovine serum albumin (BSA) as model analytes showed that the focused proteins generally formed contiguous, non-overlapping bands similar to those observed in isotachophoresis. Greenlee
and Ivory hypothesized that either secondary effects resulting from the conductivity gradient were causing isotachophoresis to occur, or the focused proteins were perturbing the buffer conductivity and decreasing the electric field locally, which allowed the bands to stack next to each other without significant overlap. To overcome this stacking phenomenon, the difference in conductivity between the run buffer and the gradient-enabling (high conductivity) buffer was decreased. This produced a shallower electric field gradient, which caused proteins to focus separately without stacking together, although band width was broader.

To further improve the performance of conductivity gradient EFGF, 45-μm-diameter beads were packed in the focusing column. This greatly enhanced the resolution between bands by both decreasing peak widths and increasing the spacing between the focused analytes. With a packed column, a hemoglobin sample was resolved into four distinct isoforms, which had formed a single band in the free-solution focusing experiments.

Another setup for performing EFGF using a conductivity gradient was presented by Wang et al. [35]. Fused silica capillaries were connected to each end of a 6-cm-long, 200-μm-inner diameter (I.D.) hollow dialysis fiber (MWCO: 10,000 Da). The assembly was inserted coaxially inside a larger capillary (535 μm I.D.), enabling higher conductivity buffer (100 mM Tris, pH 8.7) to be pumped
through the smaller capillaries and the hollow fiber, while lower conductivity buffer (1 mM Tris, pH 8.7) was introduced through the larger capillary, surrounding the exterior of the dialysis fiber. A gradient in conductivity inside the hollow fiber along its length was established as the 100 mM Tris diffused to the outer capillary. A UV detector was connected to the smaller capillary at the high-field end of the device, allowing proteins to be monitored as they eluted from the hollow fiber.

As a result of the relatively high MWCO (10,000 Da) and the thin (8 μm) walls of the hollow fiber membrane, dialysis was extremely efficient, such that the electric field gradient was very steep in the first ~0.5 cm of the dialysis fiber, and essentially flat for the remaining ~5.5 cm. With such a steep electric field gradient, proteins should focus into very narrow bands, but peak resolution will suffer. However, if the applied potential was decreased during the run, proteins with lower electrophoretic mobilities were dislodged from the step-gradient region and eluted from the column with the pressure-driven flow, while higher mobility proteins remained focused in the fiber. Further decreases in the applied potential caused the remaining sample components to elute from the channel individually in the order of increasing electrophoretic mobility.

Using this setup, a BSA peak that had been focused at 5 kV and eluted at 3 kV was compared with an identical sample plug that was simply flushed
through the column with no applied voltage. Importantly, the focused peak was narrower and showed enrichment relative to the flushed plug, although the concentration factor was not quantified. Also, when a 5-fold larger sample volume was introduced, the signal of the focused protein band increased roughly proportionately, while the peak width remained nearly unchanged. Finally, a binary protein mixture consisting of Mb and BSA was focused at 8 kV (Figure 1.3). When the voltage was decreased to 5 kV, the Mb eluted while the BSA remained focused. Upon further decreasing the voltage to 3 kV, the BSA eluted as well, demonstrating a dynamic EFGF separation of the two proteins.

Figure 1.3. Separation of Mb and BSA using voltage-controlled elution in a conductivity gradient EFGF device based on a hollow dialysis fiber. Reprinted with permission from [35]; copyright 2003 Elsevier B. V.
While the voltage-dependent elution method enabled controlled fractionation of different proteins, the peak widths were not diffusion limited. Because the electric field profile over most of the hollow fiber was essentially flat, hydrodynamic dispersion caused band broadening as the proteins moved from the focusing region to the detector. A shorter dialysis fiber, better matched to the ~0.5 cm length involved in gradient formation, would reduce the out-of-gradient distance the proteins traveled to the detector, and should thus decrease dispersion.

1.2.5 Digital Field Gradient Focusing

Another method for creating gradients for EFGF, termed digital field gradient focusing (DFGF), used a computer-controlled array of individually addressable electrodes [30]. A focusing column (8 cm long × 500 μm × 1 mm) was machined into a PMMA block and was packed with 4.5 μm particles. A dialysis membrane separated the channel from the electrode array, which was immersed in a trough of recirculating buffer that was purged of electrolysis products and cooled to control Joule heating. Electrical contact was thus supplied to the focusing channel while direct interaction between analytes and electrodes was avoided. The 50 Pt electrodes were evenly spaced along a 2.5-in. region of the channel, and each was connected to a computer interface for applying and monitoring the voltages at each electrode.
With a colored protein sample, up to 4 species were separated at once in this system. Moreover, the resolution between selected proteins could be improved during a run by decreasing the slope of the gradient. As preparative separations were the main focus of the work, protein enrichment to beyond 50 mg/mL was demonstrated. The precise gradient control afforded by DFGF was critical, as the conductivity in the channel can change significantly when the concentration of a protein band approaches that of the run buffer.

A key strength of this system was the operational flexibility afforded by the computer-controlled electrodes. The gradient could be altered during a run to improve the resolution between bands, and while only linear electric field gradients were tested, more sophisticated profiles could be developed easily. Such capability would allow individual bands to be eluted while others remained focused, or differentiation of proteins having similar electrophoretic mobilities.

More recently, Myers and Bartle [36] developed a DFGF system in PMMA with a smaller, 2.5-cm-long, 100-μm-wide, and 1-mm-deep focusing channel. A 1-mm-thick porous glass membrane separated the electrodes from the analytes, and a monolith was polymerized in the focusing channel to reduce dispersion. A syringe pump provided the opposing flow, and the electric field gradient was established by five individually controllable gold electrodes. The electrodes were
in a trough with 1 mM Tris, pH 8.7 buffer, while the run buffer was 50 mM Tris, pH 8.7. Prestained model proteins were analyzed, with on-column visualization for detection. With a nonlinear voltage profile, a 7-protein mixture was separated into 6 tentatively identified bands. The effects of having only 5 electrodes (compared to 50 in earlier work [30]) and a probable conductivity gradient formed by the unequal buffer concentrations, were not characterized. Importantly, the smaller channel dimensions combined with the polymer monolith produced narrower (~1 mm) bands than previous DFGF work.

1.2.6 Temperature Gradient Focusing

A markedly different means of establishing electric field gradients for analyte focusing has been reported by Ross and coworkers [37-39], who have explored buffers with electrical conductivities that change as a function of temperature. Thus, a temperature gradient also creates an electric field gradient when a voltage is applied along a column. This approach to EFGF, called temperature gradient focusing (TGF), creates electric field gradients without the use of membranes, which simplifies fabrication and makes possible the focusing of small analytes that can traverse typical semipermeable barriers.

Ross and Locascio [37] first explored TGF and found that, of the various buffers tested, equimolar mixtures of Tris and boric acid were most effective, as a strong temperature dependence of conductivity was observed. Interestingly, that
dependence changed markedly as a function of buffer concentration. The conductivity of 9 mM Tris/9 mM boric acid increased by a factor of ~2 from 10–80 °C, while the conductivity of 900 mM Tris/900 mM boric acid decreased by ~30% over the same temperature range. The higher concentration buffer, which had a conductivity similar to 20 mM carbonate, was used for TGF studies. Polycarbonate microchips having straight, 30-μm-deep channels were utilized for most of the TGF experiments, and the temperature along the column was regulated externally using affixed, heated or cooled copper blocks. Temperature gradients were established in a 2-mm-long channel section, and flow was provided by a combination of electroosmosis and pressure resulting from uneven buffer reservoir levels. By applying ~1 kV potentials along the 2.3-cm-long column and regulating the copper blocks to differ in temperature by 30–70 °C, a wide variety of analytes could be separated, including fluorescent dyes, amino acids, oligonucleotides, and polystyrene particles. GFP isoforms were also resolved using a similarly designed capillary-based setup. Moreover, 8 nM Oregon Green 488 carboxylic acid was concentrated more than 10,000-fold over a period of 100 min in TGF, which was the highest degree of enrichment that had been achieved by a single preconcentration method. Finally, TGF has been accomplished without external temperature control, simply by taking advantage of Joule heating in a PMMA microchip with a channel whose CSA changed
abruptly. In the absence of Joule heating, the electric field and bulk fluid velocity should change proportionately as the channel cross section varies. However, because the Joule heating produces higher temperatures in the narrower portion of the channel when high voltage is applied, the proportionality is disrupted and TGF can take place. As a demonstration, 8 μM Oregon Green 488 carboxylic acid was trapped at the region where the channel cross-section changed and was enriched more than 300-fold in 190 s. These different analyses demonstrate the broad applicability of TGF.

TGF has also been applied in DNA hybridization assays in two different modes [39]. In the first, a single-stranded target DNA was focused in a temperature gradient. Then, a fluorescently labeled peptide nucleic acid (PNA) probe was added to the buffer and flowed through the focused target DNA band. If hybridization occurred, a new band corresponding to the PNA/DNA duplex formed in the temperature gradient, while if the PNA probe and target DNA were noncomplementary, the probe simply passed through the focusing region to the waste reservoir. Experiments using complementary and noncomplementary DNA/PNA pairs demonstrated that TGF could both identify the matching sequence and increase the target DNA concentration ~240-fold. The second nucleic acid assay used the temperature gradient to focus DNA into a concentrated band and then perform melting experiments to identify single-base
mismatches. A PNA probe and target DNA were hybridized off-chip and then focused at the cooler side of a temperature gradient. Next, the bulk fluid velocity was increased gradually to shift the equilibrium position of the focused duplex toward the warmer region. When the focused band reached its melting temperature, the neutral, fluorescently labeled PNA probe was freed from the target DNA and carried from the channel by bulk fluid flow. Fully complementary DNA/PNA duplexes melted at higher temperatures than ones with single-base mismatches and migrated further into the warm region of the gradient before the PNA fluorescence disappeared. This temperature scanning approach allowed testing for a single nucleotide polymorphism in the transmembrane conductance regulator gene that is responsible for cystic fibrosis. The TGF-determined melting temperatures of the wild type and mutant duplexes differed by ~8 °C, in close agreement with conventional results.

TGF can also separate optical isomers [38] in an approach analogous to chiral capillary electrophoresis (CE). The D- and L-enantiomers of dansyl glutamic acid were first focused into a single band, and then a chiral selector, γ-cyclodextrin, was flowed through, causing the enantiomers to focus at different places in the channel, with resolution comparable to chiral CE. A urine sample spiked with fluorescently labeled enantiomers of the drug baclofen hydrochloride was analyzed, and the enantiomers were well separated (Figure
1.4A), although the resolution was worse than in a comparable focusing experiment performed in buffer solution (Figure 1.4B). Finally, this technique was used to determine the presence of trace enantiomeric impurities by detecting dansyl-D-glutamic acid in the presence of a 2,000-fold molar excess of dansyl-L-glutamic acid.

1.2.7 EFGF Conclusion

To date, a number of approaches have been explored for analyte focusing in EFGF, including using channels of changing CSA, conductivity gradients caused by the diffusion of buffer ions across a membrane, individually addressable electrode arrays, and buffers whose conductivities change as a function of temperature. EFGF has shown great promise in two areas: sample enrichment by as much as 10,000-fold [37, 40], and separating compounds with very close electrophoretic mobilities [29, 30, 34, 37, 38]. Reported peak capacities

![Figure 1.4. Chiral TGF of the fluorescently labeled (R)(+) and (S)(−) enantiomers of the drug baclofen in (A) urine and (B) buffer. Adapted with permission from [38]; copyright 2004 American Chemical Society.](image)
have not yet exceeded ~10 total analytes resolved, although theory indicates that the attainable peak capacities should be similar to those of linear separation techniques [22, 32]. This discrepancy between theory and experiment is likely due to sources of band broadening other than diffusion. To realize the full potential of EFGF for both analyte concentration and high peak capacity separation, miniaturization will be crucial, as shrinking the focusing channel dimensions both enhances Joule heat dissipation and reduces the Taylor dispersion caused by the counterflow (see Section 1.3.1). Approaches for miniaturizing EFGF to a capillary-based and microchip format are presented in Chapters 4 and 5 of this dissertation, respectively.

1.3 ELECTROPHORESIS

1.3.1 Introduction to Electrophoresis

Another separation technique that benefits greatly from miniaturization is electrophoresis. First described by Tiselius in 1930 [41], electrophoresis is the migration of charged species through a current-carrying medium in the presence of an electric field, with cations moving toward the cathode and anions migrating toward the anode. Gel electrophoresis, in which analyte molecules traverse a supporting medium such as polyacrylamide or agarose [42], has enjoyed continuous use for nearly 50 years as a powerful separation technique.
for proteins, peptides, and nucleic acids [43]. Because the gels are typically >1 mm in the smallest dimension, only low potentials (e.g., ~100 V) can be used, resulting in analysis times of several hours. An additional drawback to gel electrophoresis using conventional equipment is the limited ability for automation, as separated bands must be stained inside the gel for visualization or physically excised from the gel for further analysis.

To overcome the limitations of slab gel electrophoresis and develop a faster, automated separation technique, Mikkers et al. [44] introduced, and then Jorgenson and Lukacs [45] further developed capillary zone electrophoresis (CZE), in which the separation medium contained only buffer, with no supporting gel. Narrow-bore capillaries were used, which enhanced the dissipation of Joule heat and made the application of higher voltages possible relative to slab gel techniques. Because of the high resolution, fast analysis times, and the decreased hazardous waste streams relative to those generated with liquid chromatography (LC), CZE has become a widely used separation technique that is in many ways complementary to LC [46].

1.3.2 Theory of CZE

The speed, $v$, at which a charged species travels in electrophoresis is determined by the applied electric field, $E$, the electrophoretic mobility of the species, $\mu_{ep}$, and the electroosmotic mobility, $\mu_{eo}$ [45]:
\[ v = (\mu_{ep} + \mu_{eo})E = (\mu_{ep} + \mu_{eo})V / L \]  

(1.10)

In equation 1.10, \( V \) is the applied potential and \( L \) is the separation distance. The migration time, \( t \), is given by:

\[ t = L / v = L^2 / (\mu_{ep} + \mu_{eo})V \]  

(1.11)

In the ideal case, where molecular diffusion is the sole source of band broadening during separation, the variance, \( s^2 \), of the Gaussian band will be:

\[ s^2 = 2Dt = 2DL^2 / (\mu_{ep} + \mu_{eo})V \]  

(1.12)

where \( D \) is the analyte diffusion coefficient. The separation efficiency, in terms of the number of theoretical plates, \( N \), is then given by:

\[ N = L^2 / s^2 = (\mu_{ep} + \mu_{eo})V / 2D \]  

(1.13)

The remarkable result of this simple derivation is that separation efficiency is independent of length and time, and depends largely on the applied potential.

In equations (1.10)–(1.13), \( \mu_{eo} \) is a proportionality constant relating electroosmotic flow (EOF) velocity to electric field strength. EOF results from charges immobilized on the surface of a capillary attracting ions of the opposite charge from the bulk solution, creating a potential known as the \( \xi \)-potential [47]. A layer of tightly bound counterions, called the Stern layer, cannot completely mask the surface charge, so an adjacent layer of less tightly bound counterions also forms. When an electric field is present, the counterions in this diffuse layer
migrate toward the appropriate electrode and drag the bulk solution as well, inducing a plug-like flow in the channel with a velocity proportional to the electric field strength.

The Debye-Hückel-Henry theory approximates electrophoretic mobility as:

$$\mu_{ep} = q / 6r \pi \eta$$  \hspace{1cm} (1.14)

where $q$ is the net charge of the species, $r$ is the Stokes’ radius, and $\eta$ is the buffer viscosity [46]. Analyte mass, $m$, and $r$ are related by:

$$m = (4/3)\pi V r^3$$  \hspace{1cm} (1.15)

In equation (1.15), $V$ is the partial specific volume of the analyte. Thus, while $E$ and $\mu_{eo}$ are the same for all species present in a mixture, $\mu_{ep}$ depends on the properties of individual analytes and provides a basis for separation.

### 1.4 MINIATURIZATION OF CHEMICAL SEPARATIONS

#### 1.4.1 Motivation for Miniaturizing Chemical Separations

Although separation efficiency is independent of column length for CE as shown in equation (1.13), separation time is not [equation (1.11)]. Therefore, for fast, efficient separations, high voltages must be applied over short distances, necessitating the use of large electric fields. As electrical current passes through a buffer-filled capillary, Joule heat is generated. Manz and Eijkel [48] developed
the relationship between channel dimensions and the maximum electric field, $E_{\text{max}}$, that can be applied in a channel without overheating:

$$E_{\text{max}} = \frac{1}{h} \sqrt{\frac{2}{\pi \sigma}}$$  \hspace{1cm} (1.16)

In equation (1.16), $h$ is the smallest cross-sectional dimension of a rectangular channel. It follows from equation (1.16) that to increase the electric field without overheating, either $\sigma$ or $h$ must be reduced. Decreasing $\sigma$ will have less of an effect, and when $\sigma$ is too low or the analyte concentrations are too high, the analytes themselves can alter the local conductivity of the solution and cause peak asymmetry [45]. Therefore, decreasing channel dimensions is the single most effective means of achieving fast, efficient electrophoretic separations.

In the case of EFGF, where pressure-driven laminar flow is used to provide a counterforce and enable focusing to occur, miniaturization has the additional benefit of decreasing Taylor dispersion [40]:

$$D_t = D + \frac{\theta^2 d^2}{192 D}$$  \hspace{1cm} (1.17)

In equation (1.17) $D_t$ is the modified dispersion coefficient that takes into account both Taylor dispersion and molecular diffusion, $u$ is the average flow velocity, and $d$ is the diameter of a cylindrical column. Thus, by decreasing channel diameter ($d$), $D_t$ approaches the fundamental diffusion limit ($D$).
1.4.2 History of Microfluidic Analysis

Terry et al. reported the first chemical separations application of the microfabrication techniques that were originally developed by the IC industry [49]. They created a miniaturized gas chromatograph on a silicon wafer, complete with an integrated injection valve and thermal conductivity detector. Unfortunately, little notice was paid by the separations community, and it was not until the early 1990s, when Harrison and Manz began microfabricating CE devices [50-53], that the field began to grow. A typical microchip CE (μ-CE) device such as those described by Harrison and Manz consisted of a glass substrate with photolithographically patterned and chemically etched channels (see Sections 1.3.4 and 1.3.5.1) that was thermally annealed to a glass cover plate. The cover plate had drilled holes that served as buffer reservoirs, enabling fluidic and electrical contact with the microchannels.

Initial μ-CE experiments showed the separation of fluorescein and calcein in ~5 min [50, 51] and later fluorescently labeled amino acids in just 15 s [52, 54]. Ramsey et al. then refined μ-CE by developing a “pinched” sample introduction scheme [55] that enabled extremely small (<100 pL) sample plugs to be injected reproducibly into a separation column. A schematic depiction of the “pinched” injection scheme is shown in Figure 1.5.
Figure 1.5. Schematic depiction of “pinched” sample injection in μ-CE, shown for negatively charged analytes. (A) Sample (gray) is driven electrokinetically from reservoir (a) to reservoir (c) where a positive voltage (+) is applied. Reservoirs (b) and (d) are grounded (0) to prevent analyte diffusion into the separation channel that connects reservoirs (b) and (d). (B) A positive bias is applied to reservoir (a) and a higher voltage (++) is applied to reservoir (d), causing only the sample present in the channel intersection to be injected into the separation channel where CE takes place.

1.4.3 Advances in Microfluidic Analysis

Since those early papers, numerous reports on microfluidic analysis have appeared in the literature, many of which have been reviewed by Manz and coworkers [10-12]. While not all of the progress in the field can be discussed here, some significant advances indicative of the current state of sophistication and performance are presented below.
Integration of sample handling steps, increasing throughput, and generating portable devices for DNA analysis [56] are some areas in which microfluidic systems are developed most fully. Capillary array electrophoresis (CAE) [57-59], in which parallel separation lanes are used to analyze multiple samples simultaneously, was originally adapted to a microchip format by Woolley et al. [60], who used a 12-channel CAE chip to determine HFE gene variants from multiple individuals in a single analysis that took only 160 s. For detection, the device was placed on a translation stage and scanned repeatedly through a confocal fluorescence system. More recently, CAE microchips in a radial layout have been used [61]. The sample is injected near the perimeter of a circular device, and the separation in each channel proceeds toward the center. A radial CAE device has been used to simultaneously sequence 95 M13mp18 samples in 24 min with an average read length of 430 bases and 99% accuracy [62], which was a 5-fold increase in separation throughput compared with commercial CAE instruments available at the time. A rotary confocal fluorescence scanner detected the separated DNA fragments in the microchannels near the center of the device. A similar radial device has been used to simultaneously test 384 samples for HFE gene mutations in just 7 min with 98.7% accuracy [63].
Microfluidic systems have also been employed to create integrated genetic analyzers. A recent publication demonstrated the arrangement of all of the supporting hardware (high voltage power supplies, pumps, confocal fluorescence detector, etc.) into an 8 in. × 10 in. × 12 in. portable box that interfaced with a removable glass microdevice [64]. The microchip itself was capable of performing polymerase chain reaction (PCR) to amplify specific DNA sequences by rapidly cycling the temperature of a 200 nL chamber containing the appropriate template DNA, individual nucleotide triphosphates, polymerase enzymes, and oligonucleotide primers. Integrated heaters were used for temperature cycling, and elastomeric valves connected the PCR chamber to the injection arm of the CE device where separation took place. A number of analyses using whole bacterial cells was performed using the portable PCR/CE setup, including the identification of toxic E. coli strains with a detection limit of just 2–3 cells. Detection of the pathogen S. aureus and determination of its methicillin resistance status were also performed using the portable genetic analyzer. All experiments were completed in less than 30 min, including the time required for PCR amplification.

While the above examples clearly demonstrate the power of microfluidic technologies for DNA testing, similarly noteworthy advances have occurred in other areas as well. Jacobson et al. [65] used microchip CE to separate a binary
mixture of fluorescent dyes in just 0.8 ms, and Culbertson and coworkers [66] employed a microchip with a 25-cm-long, spiral separation channel to resolve 19 fluorescently labeled amino acids in 165 s using micellar electrokinetic chromatography. The maximum peak efficiency could be obtained when the device was operated in CZE mode; 1.1 million theoretical plates were achieved in 46 s for dichlorofluorescein.

In biomedical research, Roper et al. have used microfluidic devices to continuously monitor *in vitro* insulin secretion from pancreatic cell clusters called islets of Langerhans [67]. Perfusate from the islets was mixed on-chip with anti-insulin antibody and fluorescently labeled insulin and then separated electrophoretically. The ratio of bound-to-free labeled insulin was used in the competitive immunoassay to quantify insulin secretion from the sample. Assays were performed automatically every 15 s over a 30 min period and gave 3 nM detection limits. This setup provided a promising means for testing the viability of islets, which can be transplanted to treat type 1 diabetes. Although the above examples describe just a few microchip applications, they clearly demonstrate that the technology has expanded the realm of possibilities for chemical analysis.

### 1.4.4 Photolithography

Photolithography refers to the process by which a photon-sensitive material (e.g., photoresist) on a surface is exposed selectively to a light source
through a patterned photomask [68, 69]. For a positive photoresist, portions that are exposed to light become soluble and are removed in a developer solution, transferring the pattern from the photomask to the substrate. For negative photoresists, only the exposed regions remain on the surface after immersion in developer. Photolithography enables the selective doping of different regions of semiconducting materials such as Si, making possible the fabrication of transistors, capacitors and resistors required for ICs. Additionally, patterning and etching of thin films such as silicon dioxide, silicon nitride and metals, also crucial for IC fabrication, has been enabled by photolithography. Persistent but extremely costly improvements to photolithographic processes have improved patterning resolution to the point where minimum widths of IC components have shrunk to below 100 nm for today’s consumer-grade computer processors [70]. These shrinking dimensions have been largely responsible for the dramatic improvements in speed and performance in microprocessors. Because micromachining technology for fluidic microchip fabrication also relies heavily on photolithography, the basic processes involved are briefly discussed below.

1.4.4.1 Positive photoresists

Positive photoresists are generally spin-coated onto a wafer surface at several thousand revolutions per minute to form a ~1-μm-thick film. The temperature of the wafer is then elevated to drive off solvent required for spin-
coating the photoresist. The most commonly used positive photoresist for micromachining applications is the two-component DQN resist [71, 72]. DQN consists of photosensitive diazoquinone ester (DQ) which complexes to novolak resin (N), a copolymer of phenol and formaldehyde. DQN photoresists are sensitive to the 365, 405, and 435 nm lines in a Hg arc lamp, a typical light source. Upon exposure, the DQ sensitizer that renders the photoresist insoluble in basic solution is photolysed and becomes reactive with water to form a carboxylic acid. Once this chemical reaction takes place, the exposed portion of the photoresist dissolves in a basic developer solution at a rate of 100–200 nm/s, while the unexposed regions dissolve at just 1–2 nm/s [72]. Pattern transfer is thus enabled by dissolving selective portions of the photoresist.

1.4.4.2 Negative Photoresist—SU-8

The most commonly used negative photoresist for micromachining is SU-8, which has the ability to form very thick (~1 mm), high-aspect-ratio features in a single layer [71]. SU-8 is a multifunctional epoxy resin consisting of low molecular weight polymers, and is sensitive to UV light, X-rays and electrons. When SU-8 is exposed to the appropriate source, the polymer chains cross-link to form a chemical- and temperature-resistant material with high mechanical strength, while unexposed regions are soluble in organic developer solutions. Because it can form such tall structures, SU-8 can be used directly to make
microchannels for microfluidics applications [73]. More commonly, SU-8 serves as a template for creating patterns in other materials, which can then serve as microdevice substrates [74-78].

1.4.4.3 Photomasks

While state-of-the-art IC fabrication requires photomasks made using high-resolution electron-beam lithography [71], a variety of photomasks can be used for typical micromachining applications. Quartz or glass plates with a thin film of chromium covered with a photoresist layer can be processed using a pattern generator (PG). A mask layout created using computer-aided design (CAD) software is saved in a format compatible with the PG. The PG, consisting of a light source and a moving camera system with a small aperture and a shutter, exposes the photoresist on the photomask in a series of “flashes”. A typical 5 in. × 5 in. photomask may require 10,000 to >100,000 “flashes” to create. The exposed features on the photomask are then developed and removed (if a positive photoresist was used) and the chromium under the dissolved photoresist is etched away selectively. Once the remaining photoresist is stripped and the substrate is cleaned, the finished photomask consists of glass with an optically opaque pattern of chromium. When placed in contact with or in proximity to a photoresist-covered wafer, the photomask transmits light and exposes the wafer only where the chromium has been removed, enabling pattern
transfer. Alternatively, a photomask can be imaged onto a wafer surface using
demagnifying optics, which results in the transferred pattern being $5\times-10\times$
smaller than the features on the mask itself [71]. If a PG is not available and
feature sizes are larger than $\sim10$ μm, an inexpensive photomask can be created by
saving a CAD design as a Post-Script file and printing the design onto
transparency film using a high-resolution (e.g., 3,600 dpi) printer [74].

1.4.5 Materials

As interest in microfluidics has grown, a wide variety of materials have
been explored as substrates. Three major classes of devices: glass and quartz, the
elastomer poly(dimethylsiloxane) (PDMS), and thermoplastics such as PMMA
and polycarbonate are discussed below, including typical fabrication schemes,
and advantages and disadvantages associated with each.

1.4.5.1 Glass and Quartz

Figure 1.6 provides a schematic overview of typical methods for glass
microdevice fabrication [79]. First, a thin layer of amorphous silicon (blue) is
deposited on a clean glass wafer (light blue, side view) as shown in Figure 1.6A.
Next, positive photoresist (red) is spin-coated on top of the amorphous silicon
layer (Figure 1.6B). The channel design is exposed to UV light through a
photomask, and the exposed photoresist is solubilized and removed in a
developer solution (Figure 1.6C).

The amorphous silicon that is exposed by the removal of photoresist is etched away in a CF$_4$ plasma (Figure 1.6D), while the rest of the wafer is protected from the plasma by the remaining photoresist. The underlying glass in the pattern of the original photomask is etched isotropically in HF (Figure 1.6E). The photoresist and amorphous silicon are then removed from the etched glass wafer, which is thermally bonded to another piece of glass at ~600 °C for several hours, forming enclosed microchannels (Figure 1.6F). Glass microfluidic devices have also been created using just photoresist as an etch mask for the HF solution [55, 80-83], but channels can only be etched to a depth of ~10 μm before the photoresist degrades and ceases to protect the underlying glass. For applications where the optical properties of glass are not suitable (i.e.

Figure 1.6 Fabrication schematic for a glass microdevice. See text for description.
optical detection below ~350 nm) fused silica substrates have been used [84]. However, fused silica etches more slowly than soda lime or borosilicate glass, making more robust etch masks necessary to achieve a given channel depth, and temperatures of ~1100 °C are necessary for thermal bonding.

Glass is an attractive microchip material for several reasons. First, the surface chemistry that has been developed for fused silica capillaries can be transferred to glass microchannels with little or no modification. Thus, glass microdevices can be functionalized readily with chromatographic stationary phases [85] or passivated to modify EOF or reduce adsorption of analyte molecules [80]. Also, unlike many polymers, glass is compatible with organic solvents [78]. Thermally bonded glass devices can be very robust, withstanding up to 2,000 psi internally without delamination [86], although pressure limits of several hundred psi are more typical [87]. Finally, glass has a higher thermal conductivity than most polymeric materials [88], which enhances its ability to dissipate Joule heat.

Unfortunately, microchip-quality glass is more expensive than alternate materials [89], making it less desirable for single-use applications. Also, as shown in Figure 1.6, each glass device must be patterned and etched individually, which requires heavy reliance on clean room facilities where photolithography takes place. The relatively long times required for etch mask deposition, chemical
etching, and bonding make device fabrication a laborious, sometimes multi-day process. It is true that several devices may be incorporated into a single wafer, and multiple substrates can be processed simultaneously without a significant additional time requirement, but much of the present work with microfluidic systems is exploratory in nature, requiring prototyping of just a few devices, and thus limiting the efficacy of parallel processing. Finally, bonding of glass devices is still a difficult, low-yield process, requiring extremely clean, flat substrates [90] and expertise on the part of the researcher.

1.4.5.2 PDMS

The use of PDMS as a fluidic microchip substrate was pioneered by Effenhauser et al. [91] and Whitesides and coworkers [74-77] as a material that is simple and inexpensive to use and that does not require clean room facilities. Device construction uses a process called rapid prototyping [74] that is outlined in Figure 1.7. First, a layer of SU-8 photoresist (yellow, Figure 1.7A) is spin-coated on a wafer (blue). The substrate is exposed to a light source (black arrows) through an inexpensive transparency photomask, forming a cross-linked structure in the shape of the channel design (Figure 1.7B). The photoresist is developed, removing unexposed regions (Figure 1.7C), and the SU-8-patterned wafer then serves as a mold for casting PDMS (green, Figure 1.7D). The PDMS is
cured thermally, removed from the SU-8 mold, and sealed to another substrate to form an enclosed microfluidic network (Figure 1.7E).

Because of the simplicity and low cost of this process (4 kg of PDMS costs ~$100), and because clean room conditions are not necessary for rapid prototyping, microfluidics research has become more widely accessible. Also, rapid prototyping can take a design from a CAD drawing to an enclosed PDMS device in less than a day [74]. The patterned PDMS can be sealed reversibly to another piece of PDMS, glass, or other materials via van der Waals interactions; however, the seal cannot withstand internal pressures greater than 5 psi [76]. Following treatment of the surface in an oxygen plasma, PDMS can bond irreversibly to glass or another oxidized PDMS substrate [75]. Plasma oxidation is believed to add silanol groups.
to the surface, which can then bond covalently to other silanol groups by a condensation reaction.

Unfortunately, because of the elastomeric nature of PDMS, low pressures must still be used even when the substrates are sealed irreversibly, as elevated pressures distort the channel shapes. Also, PDMS is very hydrophobic, which makes filling the devices with aqueous solutions and eliminating air bubbles from the channels a difficult procedure. PDMS swells in many organic solvents [78], limiting its use largely to aqueous applications. The hydrophobicity of PDMS also cause band broadening, adsorption, and irreproducible results when hydrophobic samples or analytes with hydrophobic moieties such as proteins [93-97] are analyzed. A variety of PDMS surface treatments have been reported [93, 95, 98, 99], but many detract from the otherwise simple fabrication procedures, and the reported results are generally inferior to those demonstrated with glass devices. Despite these drawbacks, the use of PDMS continues to grow as analytical chemists, either by necessity or by choice, strive to implement simple, inexpensive methods.

1.4.5.3 Thermoplastics

Another group of materials that enables low-cost, templated fabrication is thermoplastics. A number of plastics have been explored as microdevice substrates, such as Zeonor 1020 [100], polystyrene [101, 102], thermoset polyester
[96, 97], PC [103, 104], and PMMA [105-111], although PC and PMMA are most commonly used. PC is more solvent resistant and thermally conductive than PMMA, but has no optical transmission below 400 nm and suffers from high autofluorescence in the visible wavelength range [112]. PC must also be processed at higher temperatures and is more hydrophobic than PMMA, making its use inappropriate for some analyses. This section will focus on PMMA, because it was used for the work in this dissertation, although much of the discussion applies to thermoplastics in general.

Thermoplastics are produced for a wide variety of commercial uses. Thus, care must be taken when purchasing material for microfluidics research to ensure that any additives present will not interfere with the application [89]. For instance, pure PMMA transmits visible light and UV radiation down to 280 nm, but PMMA developed for use as Plexiglas windows generally has UV absorbers that prevent the transmission of light below 400 nm. Other polymer additives can increase background fluorescence, which is otherwise very low in PMMA [112].

While PMMA has been patterned for microfluidics applications using injection molding [106], laser ablation [113], and X-ray lithography [107, 108], hot embossing from a template master [105] is implemented most commonly, as the process is simple, reproducible, and not equipment intensive. Si wafers can be
patterned photolithographically and etched to serve as templates for hot embossing as shown in Figure 1.8. First, a silicon wafer (dark blue, Figure 1.8A) is oxidized in a tube furnace in an atmosphere of oxygen and water to grow a layer of silicon dioxide (light blue). Next, a film of photoresist (red, Figure 1.8B) is spin-coated on top of the silicon dioxide layer. Exposure and development of the photoresist provide a surface pattern on the wafer as shown in Figure 1.8C.

The SiO$_2$ layer is etched away from all regions not protected by photoresist (Figure 1.8D). The remaining SiO$_2$ masks the pattern from an aqueous KOH solution, used in anisotropic etching of the silicon from the surface of the rest of the wafer (Figure 1.8E). What remains is a silicon template with protruding

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**Figure 1.8.** Silicon template microfabrication. Additional description in text.
features with trapezoidal cross-sections that can be used to pattern microchannels in PMMA.

Once a suitable template has been created, patterning the PMMA substrate [105] is straightforward and is described in Figure 1.9. The template (blue, Figure 1.9A) with protruding features is clamped to a blank piece of PMMA (white) and placed in an oven at a temperature above the softening point of PMMA (~105 °C). Once the assembly has reached the appropriate temperature, additional pressure is applied, which forces the softened PMMA to conform to the features of the template (Figure 1.9B). The assembly is removed from the oven, allowed to cool to room temperature, and the PMMA is separated from the template (Figure 1.9C). This imprinting procedure has been used to pattern at least ~70 PMMA pieces from a single Si template [114]. Finally, the patterned PMMA is bonded to another substrate to enclose the channels (Figure 1.9D), as described below.

As with glass devices, a patterned PMMA substrate can be thermally bonded to a blank piece of PMMA to create microcapillaries [105]. Thermal sealing is accomplished by pressing the blank and patterned substrates together above the glass transition temperature of the polymer [105], where the polymer chains from each of the PMMA pieces are mobilized and intertwine, effecting the
thermal bond. The challenge in forming PMMA devices by thermal annealing is that it is difficult to apply sufficient pressure at the appropriate temperature to cause a strong bond to form without having the channels collapse or deform severely, which limits the success rate of this critical step. When conditions are controlled precisely to avoid channel distortion during substrate annealing, the resulting bond is generally weak, such that high internal pressures (>200 psi) must be avoided, and even moderate mechanical shock such as dropping a device a short distance can cause the pieces to separate. To circumvent the difficulties associated with thermal bonding, a variety of other sealing approaches have been explored, including thermal lamination of Mylar films [106], applying adhesive tape [115], and reversibly sealing PDMS slabs.

Figure 1.9. Si-templated imprinting and subsequent bonding of PMMA substrates. Additional description in text.
Unfortunately, all of these approaches result in composite microchannels. The different $\xi$-potentials for the two surfaces can cause a significant loss in the number of theoretical plates for electrically driven separations, thus limiting the analytical capabilities of the devices [116, 117]. Clearly, a robust bonding approach for polymer microchips that enables all channel surfaces to be composed of the same material would be ideal, and would allow plastic microdevices to achieve their potential more fully as inexpensive, yet effective, analytical platforms.

1.5 DISSEMINATION OVERVIEW

In my research, I have worked to develop improved bonding methods for PMMA microchips that enable all surfaces of the microchannels to be composed of the same material. Two distinct approaches, thermal bonding in a boiling water bath, and solvent bonding with the use of phase-changing sacrificial layers are presented as Chapters 2 and 3 of this dissertation, respectively. $\mu$-CE of peptides and amino acids was used to characterize microchannels created using the new bonding methods. My work with EFGF is then discussed in Chapters 4 and 5. Chapter 4 describes a capillary-based EFGF setup that enabled the separation of fluorescently labeled and natively fluorescent protein standards. Sample enrichment factors of 10,000 were achieved for a model protein using
capillary-based EFGF devices. Chapter 5 presents results with EFGF microchips, which, like solvent bonded microchips, were also created using phase-changing sacrificial layers, and provided narrower bands and improved resolution relative to the capillary-based devices. Finally, conclusions and future directions, including a promising new approach for analyte focusing, are discussed in Chapter 6.
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2 THERMAL BONDING OF POLYMERIC CAPILLARY ELECTROPHORESIS MICRODEVICES IN WATER*

2.1 INTRODUCTION

The need for low-cost, high-throughput, automated analyses has driven the emerging trend toward miniaturization of chemical and biochemical characterization methods. Rapid growth in the field of photolithographically patterned microfluidic devices built on planar substrates provides a striking example of the great potential of miniaturized analysis [1-3]. Not only can microfluidic devices offer smaller dimensions compared to traditional analytical methods, but also more sophisticated device layouts, relative to conventional approaches, are enabled by computer aided design methods.

Glass was initially the material of choice for capillary electrophoresis (CE) microdevices [4-6], but polymeric substrates are now being used with increasing frequency [7]. The emerging use of plastics can be attributed largely to advantages in biocompatibility, and increased flexibility with respect to fabrication methods and substrate dimensions [7]. Polymeric substrates have been patterned using laser ablation [8], X-ray lithography [9], injection molding [10], and imprinting from master templates [11, 12]. A number of polymeric materials have been used to create microfluidic devices, including polycarbonate

[13, 14], poly(methyl methacrylate) (PMMA) [11], SU-8 photoresist [15], Zeonor 1020 [16], and the elastomer poly(dimethylsiloxane) (PDMS) [17-19].

A key step in the fabrication of microfluidic devices is the generation of capillaries by affixing a cover plate to a substrate containing microfabricated channels. Several methods for bonding PMMA substrates have been demonstrated, including thermal bonding in a convection oven [11, 20] or with heated weights [9, 21], thermal lamination [10], adhesive tape [22], and PDMS films [12]. Of these methods, thermal bonding approaches are especially desirable, as they allow formation of microcapillaries with a uniform surface composed entirely of the same polymeric material. On the other hand, when multiple materials define a channel, the resulting inhomogeneities in ξ-potential can cause band broadening due to differing electroosmotic flow velocities along the varied materials comprising the channel’s cross-section [23, 24].

I demonstrate here a low-cost technique for thermally bonding PMMA substrates using a boiling water bath. This method shares all the advantages of other thermal bonding techniques, while also enabling the microfluidic system to remain hydrated throughout the bonding process.
2.2 EXPERIMENTAL SECTION

2.2.1 Microfabrication

The CE microdevices were made by imprinting a PMMA piece with a photolithographically patterned and chemically etched silicon template, and then bonding a planar top plate to the imprinted PMMA substrate. Conventional procedures for photolithographic patterning and wet chemical etching were used to create the silicon template [25]. Briefly, a 0.8 μm layer of silicon dioxide was grown on 4” diameter silicon <100> wafers (TTI Silicon, Sunnyvale, CA) (Figure 1.8A) at 1110 °C using a tube furnace in an atmosphere of oxygen that had been bubbled through water. Next, Microposit S1813 (Shipley, Marlborough, MA) positive photoresist was spin-coated on the wafers at 3,000 rpm for 60 s (Figure 1.8B), resulting in a film thickness of 1.3 μm. The wafers were baked on a hot plate at 110 °C for 60 s to improve adhesion of the photoresist to the surface and drive off residual solvent. The photoresist was then exposed to UV radiation through a patterning mask using a PLA-501F (Canon, Tokyo, Japan) contact mask aligner with a Hg arc lamp source at 3 mW/cm² for 30 s. The photomask was generated using the mask layout software CleWin (WieWeb Software, The Netherlands), and printed onto transparency film using a 3600 dpi printer at the BYU Print and Mail Production Center. Following exposure, the wafer was immersed in 20% aqueous Microposit 351 developer (Shipley) for 80 s, which
removed the exposed regions of photoresist, as illustrated in Figure 1.8C. The substrate was rinsed and placed in an oven for 30 min at 150 °C to harden the remaining photoresist. A subsequent 20 min etch in 10% buffered HF was used to remove the silicon dioxide layer from the areas no longer protected by photoresist (Figure 1.8D). Finally, the pattern was transferred to the silicon substrate by wet etching in 40% aqueous KOH solution for 35 min at 70 °C (Figure 1.8E). Photographs of the completed Si template are shown in Figures 2.1A and 2.1C.

The elevated features in the etched Si template were embossed into 1/8” thick PMMA (Plaskolite, Columbus, OH) substrates (Figure 1.9A–C) in a convection oven at 140 °C to form channels in a procedure adapted from earlier work by Locascio et al. [11]. First, the Si template and PMMA substrate were sandwiched between glass microscope slides followed by aluminum blocks, and the assembly was held together tightly enough using two 2” C-clamps to prevent sliding of any of the pieces. This package was placed in the oven for 10 min to soften the PMMA. The setup was carefully removed, the clamps were tightened 1/8 turn, and the apparatus was returned to the oven for another 10 min. The tightening procedure was repeated once more and, after a final 10 min in the oven, the substrate was removed, the template and imprinted substrate were allowed to cool to room temperature, and the clamps were loosened.
Access holes were drilled using a 5/64” drill bit to form each of the four buffer reservoirs in the imprinted substrate (Figure 2.1B). The patterned PMMA sheet was then bonded to an unimprinted 1/16” thick PMMA piece (Figure 1.9D) by clamping the substrates together with approximately 0.2 ft-lbs of torque on two standard 2” C-clamps. The same glass slides and aluminum blocks used to sandwich the PMMA substrate during imprinting were used to transfer the applied pressure from the clamps to the substrates. The entire assembly was then immersed in a boiling water bath for 1 h, which bonded the imprinted and blank substrates.

**Figure 2.1.** Photographs of Si and PMMA microstructures. (A) Photograph of an entire silicon template used to imprint microchannels into PMMA. (B) Picture of a completed PMMA microdevice. Reservoirs are (1) analyte reservoir, (2) buffer reservoir, (3) analyte waste reservoir, and (4) waste reservoir. Channel lengths from reservoirs 1 through 3 to the injection intersection region are 0.5 cm, and the distance from reservoir 4 to the injection region is 2.2 cm. (C) Zoomed image of the injection region of the Si template. (D) Zoomed photograph of the injection region in an imprinted and bonded PMMA microdevice. Scale bars represent 1 cm in (A) and (B) and 400 μm in (C) and (D).
together. Residual air in the assembly could be eliminated by wetting the substrates prior to clamping.

2.2.2 Microdevice Characterization

To determine the strength of the bond between the two PMMA substrates that comprise the microdevices, 2” × 3” sheets of ¼” thick PMMA were solvent-welded using Weld-On 3 (IPS, Compton, CA) to both the top and bottom faces of several devices. Solvent bonding was used because it was expected and observed to provide a stronger seal than the bond enclosing the device channels. A container holding weights was hung from the bottom of the assembly, and the top solvent-bonded sheet served as a handle to lift the device and weighted container. For each trial, the device with attached weights was lifted off the ground for 10 s, after which it was lowered and more weight was added. The highest weight for which the assembly remained intact was divided by the bonded area of the device to calculate the maximum applied pressure before failure.

An Alpha-step 200 stylus profilometer (Tencor, Mountain View, CA) was used to measure the cross-sectional dimensions of the raised features in the silicon template, and of the imprinted channels both before and after water-based bonding. Surface profiles were obtained with 2 μm lateral resolution. I also used a Leica DMLM (Wetzlar, Germany) semiconductor inspection microscope.
and a Nikon 995 (Tokyo, Japan) digital camera to measure cross-sectional dimensions by imaging the structures side-on.

2.2.3 Separation and Detection of Amino Acids

Glycine, asparagine, and phenylalanine were purchased from Sigma (St. Louis, MO) and diluted in pH 9.0, 30 mM boric acid buffer, which also served as the run buffer. The buffer solution was passed through a 0.2 μm filter (Pall, East Hills, NY) prior to use. The amino acids in each solution were fluorescently labeled by conjugating fluorescein 5-isothiocyanate (FITC, Sigma) to the free amine group [26]. Briefly, 100 μL of 6 mM FITC in acetone was combined with 300 μL of a 3 mM solution of each amino acid and allowed to react overnight in the dark at room temperature. After labeling was complete, each amino acid conjugate was diluted to 3 μM in boric acid buffer.

Channels were filled by micropipetting 10 μL of run buffer into reservoirs 1, 2, and 3 (Figure 2.1B) and applying vacuum to reservoir 4, after which reservoir 4 was also filled with 10 μL of run buffer. Vacuum was then applied to reservoir 1 to remove its contents, and the reservoir was filled with 10 μL of a mixture containing 1 μM of each of the FITC-labeled amino acids in run buffer. Platinum wires inserted into the buffer reservoirs provided electrical contact. I used a “pinched” injection scheme [5] in a double-T injector [27] having a volume of ~150 pL, with a sample injection time of 30 s. The “pinched” injection
approach minimizes broadening of the sample plug. During injection, reservoirs 1, 2, and 4 were grounded and reservoir 3 was maintained at +1.2 kV. For separation, reservoirs 1 and 3 were at +1.2 kV, reservoir 2 was grounded, and reservoir 4 was at +2.0 kV.

The 488 nm line from an air-cooled Ar ion laser (Laser Physics, West Jordan, UT) was passed through a 10× beam expander (Newport, Irvine, CA) and directed into an inverted optical microscope (TE300, Nikon) through a 488 nm excitation filter (D488/10, Chroma, Brattleboro, VT) to a 20×, 0.45 NA objective. The beam spot was focused within the separation channel approximately 1 mm before reservoir 4 (Figure 2.1B). Fluorescence was collected with the same objective, passed through a 505LD dichroic filter (Chroma) and a D535/40 bandpass filter (Chroma), and then out of focus fluorescence was removed by confocal spatial filtering with a 1000 μm diameter pinhole. Photons passing through the spatial filter impinged on the window of a Hamamatsu HC120-05 (Bridgewater, NJ) photomultiplier tube. Detector signal was amplified and filtered with an SR-650 preamplifier (Stanford Research Systems, Sunnyvale, CA), and then digitized with a PCI-6035E (National Instruments, Austin, TX) analog to digital converter controlled by LabVIEW (National Instruments) software running on a Dell (Round Rock, TX) personal computer. The sampling rate for data collection was set in the software to be 10 Hz.
2.2.4 Safety Information

The 40% aqueous KOH and 10% buffered HF used as etchants in this work are both corrosive. To avoid skin or eye contact with these solutions, safety goggles, a face shield, and elbow-length nitrile gloves should be used. The voltages used for electrophoretic injection and separation can cause electric shock, so appropriate precautions, such as current limiting settings on power supplies and isolation of electrical leads, should be taken.

2.3 RESULTS AND DISCUSSION

Profilometry data and optical microscopy measurements indicate that the raised features in the Si template were 22 μm tall, and the top and base widths were 20 and 48 μm, respectively. The trapezoidal shape is due to the anisotropy of the KOH etchant, which etches silicon at an angle of 54.7° [28]. I determined the etch rate of Si <100> in 40% KOH at 70 °C to be approximately 36 μm/h, which agrees with published results [29]. The imprinted PMMA devices had features whose depths agreed to within 1 μm, and whose widths agreed to within 2 μm of the Si template. Comparison of four imprinted PMMA channels, both before bonding and in bonded and subsequently separated surfaces, revealed minimal distortion to channel dimensions during the bonding process. Channel depth decreased an average of 0.2 μm with a standard deviation of 0.1
μm, and channel width decreased an average of 1 μm, with a standard deviation of 2 μm. Stylus tip geometry was a limiting factor in our ability to accurately profile the embossed channel interior in PMMA.

The imprinting procedure was done in three steps to avoid undue stress on the Si template, and to allow use of the master in embossing many devices. Very light pressure was used when the template and blank PMMA substrate were first brought into contact, and not until the PMMA was heated above its softening temperature was increased pressure applied to imprint PMMA channels. After imprinting, when the clamped assembly was removed from the oven and cooled to room temperature, the patterned PMMA substrate easily separated from the Si master. I have used this approach to imprint at least 65 PMMA substrates without damaging the Si template.

Figures 2.1A and 2.1C are photographs of a Si template used to create CE microdevices in PMMA. Figure 2.1B shows a photograph of an entire water-bonded CE microdevice, and Figure 2.1D displays a zoom image of the intersection of the sample inlet and waste channels with the separation channel in this same device. The strength of the bond formed between the imprinted and blank PMMA substrates in enclosing the channels was evaluated as described in Section 2.2.1. For the four devices tested, the average maximum applied pressure before bond failure was 130 kPa with a 95% confidence interval of 10 kPa. Similar
experiments on four air-bonded devices yielded a mean bond failure pressure of 60 kPa with a 95% confidence interval of 50 kPa. Substrates with higher bond strength could be achieved by either method, but channel integrity was often compromised. Devices formed by our thermal bonding approach are thus sufficiently strong to enable repeated microfluidic fillings, and use of the same chip for multiple CE experiments.

As further evidence of the utility of devices bonded using this approach, I have performed CE analysis on a mixture of fluorescently labeled amino acids. Figure 2.2 depicts an electropherogram of the separation of FITC-labeled glycine, asparagine, and phenylalanine. Separation and detection were performed as described in Section 2.2.2. Peaks were identified by repeating the separation using samples spiked with a 5-fold excess of one of the amino acids. The three amino acids were separated in just over 30 s, with resolutions of 1.1 between the glycine and asparagine peaks and 0.74 between the asparagine and phenylalanine peaks. The numbers of theoretical plates for the glycine, asparagine, and phenylalanine peaks were 6700, 4700, and 5200, respectively; these values correspond to 310,000, 210,000, and 240,000 plates/m, respectively. Our results clearly demonstrate that CE microdevices made from imprinted PMMA substrates bonded in water can be used to achieve high resolution electrophoretic separations.
Figure 2.2. Electropherogram of FITC-labeled amino acids separated using a water-bonded PMMA microdevice. Peaks are (a) glycine, (b) asparagine, and (c) phenylalanine. Potentials for injection and separation are described in the Section 2.2.2.

Bonding of PMMA microfluidic substrates in boiling water rather than a convection oven offers several advantages. First, the temperature stability and uniformity of boiling water are excellent, which enables bonding reproducibility. Moreover, the boiling temperature of water is close to the glass transition temperature of PMMA (105 °C) [30], which enables bonding under conditions where the polymer is sufficiently rigid to avoid collapse of channels during enclosure. Water-based bonding of PMMA substrates should also facilitate the permanent incorporation of molecular-size-selective membranes into microfluidic devices. Although a microfluidic microdialysis device has been
demonstrated for cleanup and fractionation in sample introduction to a mass spectrometer [31], this setup requires the use of nuts and screws to hold membranes between channel-containing substrates. Ideally, a membrane should be physically incorporated within a microfluidic system, to avoid the need for large external hardware to hold the device together. However, one of the difficulties with inclusion of size-selective membranes in thermally bonded microdevices is that dialysis membranes swell upon hydration [32, 33]. Our preliminary results indicate that the swelling of dry cellulose dialysis membranes incorporated into microfluidic devices will either push apart the two PMMA layers upon hydration, or lead to blockage of the microfluidic pathways, either of which will destroy device functionality. On the other hand, hydrated dialysis membranes incorporated into microfluidic devices through water-based bonding should avoid membrane swelling-induced device damage.

2.4 CONCLUSIONS

I have shown that PMMA substrates can be bonded together to form microfluidic devices by clamping a blank piece to a substrate containing imprinted microchannels and heating the assembly in a boiling water bath. Rapid, high-resolution CE separations of FITC-labeled amino acids were successfully carried out on devices fabricated using this method. Bonding in
boiling water enables the entire device to remain hydrated throughout the assembly process, which should make possible the facile, permanent incorporation of membranes into microfluidic systems. Membrane-based functionality in microchip analysis could then open the door to implantable, *in vivo* microdialysis sampling and analysis [34].
2.5 REFERENCES


3 PHASE-CHANGING SACRIFICIAL MATERIALS FOR SOLVENT BONDING OF HIGH-PERFORMANCE POLYMERIC CAPILLARY ELECTROPHORESIS MICROCHIPS∗

3.1 INTRODUCTION

As the lab-on-a-chip field has developed, polymers such as poly(dimethylsiloxane) (PDMS), poly(methyl methacrylate) (PMMA) and polycarbonate (PC) have been employed increasingly as device substrates [1-3] in favor of glass, which was originally used almost exclusively [4]. This shift toward polymeric substrates has likely occurred because of two factors. First, the templated procedures used to create microchips in polymers allow a single photolithographically defined master to be used to pattern numerous devices [5, 6], thus decreasing the need for cleanrooms and other costly instrumentation. Second, the polymeric materials themselves are typically less expensive than microchip-quality glass, and lower costs per device should facilitate the development of disposable microfluidic systems [3].

Despite these attractive features of polymeric materials, glass remains the substrate of choice for very fast [7] or high-performance [8, 9] microchip CE. This performance gap is due in part to the convenience of adapting the well-characterized chemistry of fused silica capillaries for surface modification in a

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wide array of glass microchip applications. Also, the thermal conductivity of
glass is higher than that of commonly used polymers (e.g., PMMA, PC and
PDMS) [10], which provides better dissipation of Joule heat and enables higher
electric fields in microchannels in glass substrates.

Unfortunately, glass microchips must be patterned and etched
individually in a cleanroom, and the thermal annealing of glass substrates to
enclose microcapillaries generally takes place in a furnace at >400 °C for several
hours [1]. Moreover, special care must be taken to ensure that the bonded
surfaces are extremely clean and lacking even small particulates, or thermal
bonding will not be successful [11]. Low- and room-temperature glass bonding
approaches that avoid high-temperature processing have been reported [12-15],
but the resulting adhesion is weaker than in thermally sealed devices, and even
greater care must be taken to ensure that the surfaces are extremely clean and
flat.

To avoid a sealing step for microcapillary enclosure and to create sub-μm
features, sacrificial techniques have been explored. In these methods a channel
design is patterned on top of a bulk substrate, and a thin film of a different
material is deposited over the entire surface, covering the patterned design. Next,
the sacrificial material under the deposited layer is etched away [16, 17] or
thermally decomposed [18-21], leaving microcapillaries defined by the cover
layer and the base substrate. While these sacrificial methods have successfully created devices without a thermal bonding step, the fabrication protocols are involved, and templated procedures are not possible because each device is patterned individually.

Phase-changing materials (typically waxes) have been incorporated into fluidic microchips to create micropumps [22], membrane actuators [23], and valves [24, 25], but these materials have not been used as sacrificial layers in microdevice fabrication. In addition, Liu et al. [24] recently used a solvent-assisted thermal bonding method to seal PC substrates at ~200 °C, but the large feature dimensions (>300 μm deep) made it unnecessary to protect the channels from the bonding solvent.

Here I report a general approach for solvent bonding of polymeric substrates using a phase-changing sacrificial material. Microchannels are imprinted in PMMA with a silicon template and filled with a liquid, which forms the sacrificial layer upon solidification. After solvent bonding, the device is heated above the melting temperature of the sacrificial material to enable its facile removal as a liquid. These solvent-bonded substrates can withstand internal pressures >2,250 psi, much higher than thermally bonded PMMA. I demonstrate the functionality of solvent-bonded microfluidic systems by performing rapid and high-resolution CE separations of fluorescently labeled
amino acids and peptides. These separations compare favorably with glass microchip CE of peptide mixtures [26-28], and surpass those previously done on polymer microchips [29-32] in terms of both speed and efficiency. I have performed separations in electric fields as high as 1,500 V/cm, the highest reported to date for polymer microchips. Finally, a single device was used for more than 300 runs over a three-month period without a decrease in separation performance, demonstrating long device lifetimes.

3.2 EXPERIMENTAL SECTION

3.2.1 Microfabrication

Microchips were made by imprinting raised features from photolithographically patterned and anisotropically etched silicon templates into PMMA substrates using previously described methods [33]. Imprinted PMMA substrates (Acrylite OP-3, Cyro, Rockaway, NJ) were 1.75" × 1" × 1/8" and had 3-mm-diameter reservoir holes aligned with the channel ends. A CO₂ laser cutter (C-200, Universal Laser Systems, Scottsdale, AZ) was used to excise the PMMA substrates from larger sheets and create the reservoir holes. A clean, flat, 1/16"-thick piece of PDMS (Sylgard 184, Dow Corning, Midland, MI), which had been cured according to the manufacturer’s specifications, was sealed to the patterned side of a PMMA substrate (Figure 3.1A), temporarily forming enclosed channels.
Figure 3.1. Solvent bonding to create microfluidic systems in polymers. (A) A PDMS slab (dark grey with white crosshatching) is sealed to an imprinted PMMA substrate (grey), temporarily forming enclosed microchannels. (B) The assembly is heated, and liquid paraffin wax (grey with vertical lines) fills the microchannels. (C) The device is cooled to solidify the wax (grey with horizontal lines), and the PDMS slab is removed and placed on the opposite side of the PMMA to protect the device exterior. The patterned side of the PMMA is then coated with acetonitrile (black). (D) A second, blank PMMA piece, which also has PDMS protecting its exterior, is pressed against the acetonitrile-coated PMMA for 2 min to effect bonding. (E) The device is heated to melt the sacrificial layer, which is removed by a combination of applied vacuum and dissolution in cyclohexane.

The PMMA/PDMS assembly was mounted on a glass microscope slide and placed on a heating block at 85 °C (above the melting temperature of the paraffin wax) for 30 s. A pipet was used to quickly transfer melted paraffin wax (melting point: 65 °C; Service Assets, Newport Beach, CA) from a heated vial to three of the reservoirs before the melted wax could cool and solidify. After filling three reservoirs with melted wax, vacuum was applied for 1–2 s at the fourth reservoir to ensure that all channels were filled (Figure 3.1B) and no air pockets were present, after which the PMMA/PDMS assembly was removed from the hot plate.
and cooled to room temperature. Next, the channels were inspected under a microscope. If a small amount of paraffin wax had solidified beyond the channels, the imprinted PMMA substrate was left in contact with the PDMS until the paraffin wax outside the channels had dissolved in the PDMS, which was then removed. PDMS was sealed to the non-imprinted side of the PMMA and to a blank piece of 1/16”-thick PMMA to prevent the solvent from contacting the device exterior. Acetonitrile (200–400 µL) was pipetted directly onto the channel-containing substrate (Figure 3.1C) to cover the entire surface, and the unpatterned PMMA was placed in contact with the solvent-coated substrate and held together with an applied pressure of 2 psi for 2 min to effect bonding (Figure 3.1D). Effective bonding was feasible with as little as 3 µL of acetonitrile per cm² (~35 µL/device); with volumes over 400 µL/device, excess solvent sometimes flowed from the bonding interface and impaired the optical clarity of the surface. The PMMA pieces were brought together at an angle as shown in Figure 3.1D to allow any air bubbles to escape out the side. After the designated time, the applied pressure was released, and the pieces of PDMS were peeled from the device. To remove the sacrificial layer from the microchannels (Figure 3.1E), 10 µL of cyclohexane were pipetted into each of the reservoirs, and the device was placed on the heating block until the paraffin wax melted. Vacuum was applied at one of the reservoirs to begin removing paraffin wax from the
channels, after which that reservoir was refilled with cyclohexane. The same procedure was repeated at each of the reservoirs, and the device was cooled to room temperature. To ensure that all residual sacrificial material was removed, the channels were soaked in cyclohexane for >5 min before vacuuming all liquid from the device. Complete removal of the paraffin wax was verified by the absence of air bubbles upon filling the channels with water, as air bubbles typically became trapped at any points in the channel where the hydrophobic wax was still present. A schematic of the microchip layout and channel dimensions is shown in Figure 3.2.

3.2.2 Bond Strength Determination

To measure the internal pressure that could be applied to solvent-bonded substrates, I threaded a hole to accept a 5/16"–24 brass fitting in a 1" × 1" × 1/2" piece of PMMA. The threaded piece was solvent bonded to a 1" × 1" × 1/4" PMMA substrate using the same solvent, applied pressure and time as for microchips. For comparison, I also thermally bonded substrates of the same dimensions by clamping the pieces together and placing them in an oven at 107 °C. After 30 min the thermally bonded PMMA was cooled and evaluated to ensure bonding completeness. If voids were found, the substrates were reclamped, and the bonding procedure was repeated. The brass fitting, which connected the PMMA to a N₂ gas cylinder via 1/16" copper tubing, was threaded
into the bonded assemblies. The copper tubing was branched to allow a pressure transducer (MSP-300, Measurement Specialties, Fairfield, NJ) with a linear response between 0 and 2,500 psi to be connected. The regulator on the gas cylinder was opened gradually, increasing the internal pressure in the bonded substrate until either the pieces separated or the maximum pressure of 2,250 psi was reached.

3.2.3 Separation and Detection of Amino Acids and Peptides

The amino acids were from ICN Biomedicals (Aurora, OH), and the peptides were

Figure 3.2. Schematic of microchip layout, showing (A) channel lengths and reservoir numbers, and (B) approximate cross-sectional dimensions.
from Sigma-Aldrich (St. Louis, MO). Each analyte was individually diluted in pH 9.2, 10 mM carbonate buffer, which was passed through a 0.2 μm filter (Pall, East Hills, NY) prior to use. The amino acids and peptides in each solution were labeled fluorescently using fluorescein-5-isothiocyanate (FITC; Molecular Probes, Eugene, OR) [34]. For amino acids, 200 μL of 6 mM FITC in dimethylsulfoxide (DMSO) were combined with 600 μL of a 3 mM solution of each amino acid. For peptides, 200 μL of a 2 mM solution of each peptide were combined with 50 μL of 6 mM FITC in DMSO. All solutions were allowed to react at room temperature in the dark for at least 24 h; longer times (up to 5 days) enabled the reaction to go to completion such that the unreacted FITC peak was eliminated.

Prior to use, microchip channels were filled with 10 mM carbonate buffer, pH 9.2, having 0.5% (w/v) hydroxypropyl cellulose (HPC; average MW: 100,000; Sigma-Aldrich). The HPC served to minimize electroosmotic flow (EOF) and analyte adsorption to the channel walls [35-37]. Channels were filled by micropipetting 16 μL of the buffer into reservoirs 1, 2, and 3 (Figure 3.2) and applying vacuum to reservoir 4, after which reservoir 4 was also filled with 16 μL of buffer. To load samples in the injection well (reservoir 1) when HPC-containing buffer was used as run buffer, vacuum was applied to reservoir 1 to remove its contents, and the well was filled with 16 μL of amino acid or peptide sample in 10 mM carbonate, pH 9.2. To run a separation without HPC in the
buffer, the HPC-containing buffer was vacuumed from the device; the channels and reservoirs 2–4 were filled with pH 9.2, 10 mM carbonate buffer; and reservoir 1 was filled with 16 μL of sample. For injection, reservoirs 1, 2, and 4 (Figure 3.2) were grounded, and reservoir 3 was maintained at an injection voltage ranging from +300 V to +1250 V (depending on the separation voltage used) for at least 20 s. During separation, reservoirs 1 and 3 were held at the injection voltage, reservoir 2 was grounded, and a potential between +1.0 and +4.5 kV was applied at reservoir 4. The injection and separation voltages for each run are indicated in the corresponding figure legends. All peaks were identified by spiking.

The laser-induced fluorescence system has been described previously [33]. Briefly, excitation of the fluorescently labeled amino acids and peptides was achieved with the 488 nm line from an air-cooled Ar ion laser, which was focused ~500 μm from the end of the separation channel using a 20×, 0.45 NA objective. Fluorescence was collected with the same objective, and stray light was removed by confocal spatial filtering with a 200-μm-diameter pinhole. A photomultiplier tube detected photons passing through the pinhole, and the detector output was recorded on a computer at 100 Hz.
3.2.4 Safety Information

The 40% aqueous KOH and 10% buffered HF used as etchants to micromachine the Si templates are both corrosive. To avoid skin or eye contact with these solutions, safety goggles, a face shield, and elbow-length nitrile gloves should be worn. The voltages used for electrophoretic injection and separation can cause electric shock, so appropriate precautions such as current-limiting settings on power supplies and isolation of electrical leads should be taken. Solvents for bonding PMMA and dissolving paraffin should be used in a fume hood. The high pressures used to test bond strengths pose a projectile hazard. A face shield should be worn, and the bonded substrates should be secured inside a metal container during testing.

3.3 RESULTS AND DISCUSSION

After filling the microchannels with the sacrificial layer (see Section 3.2.1 and Figure 3.1), I sometimes observed that a small amount of paraffin wax had solidified outside of the imprinted channels (Figure 3.3A). This was likely due to the hydrophobic interaction between the paraffin wax and the PDMS, and bonded devices made directly from these substrates typically had poorly defined channels (Figure 3.3B) that led to reduced separation efficiency. This problem was largely eliminated by having the liquid sacrificial material in contact with PDMS for as little time as possible (removing substrates from the heating block 1-
2 s after first introducing the liquid paraffin wax). Furthermore, residual paraffin wax outside the channel regions dissolved into the PDMS [38] within 1-2 h, leaving well-defined channels filled with the sacrificial layer. Figure 3.3C shows the same channel region presented in Figure 3.3A, after 80 min in contact with the PDMS; essentially all the undesired sacrificial material had dissolved in the PDMS. Alternatively, the accumulation of sacrificial material outside of the channels could likely be eliminated by employing a less hydrophobic sealing material than native PDMS, such as O₂ plasma-oxidized PDMS [6]. Figure 3.3D shows the channel intersection region of a solvent-bonded CE microchip made from the imprinted substrate from Figure 3.3A and 3.3C. The small amount of topography visible in the channels, caused by the granularity of the solidified wax, did not affect performance, as CE separations in such devices had symmetric peaks with reproducible theoretical plate counts. I am currently exploring alternate sacrificial materials that have less granularity upon solidification.

Figure 3.4 shows cross-sections of patterned features at various stages of microchip fabrication. A small indentation in the top of the sacrificial layer, which is caused by paraffin shrinkage upon solidification, is visible in Figure 3.4B. Profilometry indicates that the magnitude of shrinkage is less than ~10% of the channel cross-sectional area; these smaller dimensions are retained in the
Figure 3.3. Photographs of the double-T injector region of a device. (A) PMMA/PDMS assembly just after filling the channels with sacrificial material; some paraffin wax had solidified outside the channels. (B) A poorly defined microchannel resulting from solvent bonding without removing excess wax. (C) The same PMMA/PDMS assembly as (A), but 80 min later when excess sacrificial material had dissolved in the PDMS, leaving well-defined channels. (D) A completed microchip made from the PMMA substrate in (A,C) after solvent bonding and sacrificial layer removal.

final bonded devices (Figure 3.4C). Additionally, if the PDMS is left in contact with the wax-filled substrate at room temperature, wax from the channel dissolves into the PDMS at a rate of ~1 μm/h. Importantly, I have not observed the total collapse of channel features during solvent bonding; such channel deformation occurs more commonly when thermally bonding polymers. I have also found the phase-changing sacrificial layer and solvent bonding approach to be successful with much shallower, 7-μm-deep channels.

My comparison of the bond strength in thermally and solvent-bonded substrates yielded the following results. For solvent-bonded PMMA, all three test
devices withstood the maximum tank pressure (2,250 psi) without separating. In contrast, three thermally bonded PMMA devices failed at 145, 232, and 222 psi, giving an average failure pressure of ~200 psi. These tests show that solvent-bonded PMMA can withstand at least an order of magnitude higher internal pressure than thermally bonded PMMA. The ability to withstand pressures >200 psi is valuable for the replacement of viscous sieving media [39] commonly used in capillary gel electrophoresis of DNA and proteins. I tested the solvent-bonded microchips by separating a mixture of amino acids at different applied voltages.

Figure 3.4. Channel cross-sectional photomicrographs at various fabrication stages. (A) Imprinted channel. (B) Imprinted channel filled with wax and having the PDMS removed. (A-B) were obtained by scoring and then fracturing the substrates, which led to some roughness in the surrounding bulk PMMA. (C) Bonded device after wax removal; the slightly roughened appearance around the channel perimeter was the result of using a diamond-tipped circular saw to obtain the cross-section. The scale bar in (C) applies to all images.
to find the range that provided the highest theoretical plate numbers. Figure 3.5 shows the separation of FITC-labeled glycine, asparagine, phenylalanine and arginine at potentials ranging from 1.0 kV–4.5 kV. At 4.5 kV, the four peaks are baseline resolved, and the separation is completed in just 8 s. The highest theoretical plate numbers were obtained between 2.5–3.5 kV, as shown in Figure 3.6.

Figure 3.5. Electropherograms of a mixture of FITC-amino acids at different separation voltages (shown on figure). The injection voltages from bottom to top were: +300 V, +450 V, +600 V, +750 V, +900 V, +1050 V, +1150 V, and +1250 V. Amino acid concentrations were 75 nM, and the run buffer was 10 mM carbonate, pH 9.2, with 0.5% (w/v) HPC. Peaks are: (a) Gly, (b) Asn, (c) Phe, and (d) Arg.
The plateauing of theoretical plate numbers and their eventual decrease at higher potentials may be largely due to the 280-μm offset (center-to-center) in the double-T injector rather than Joule heating or other fundamental limits. For example, the width at half height of the glycine peak in the 4.5 kV separation in Figure 3.5 is only 320 μm, making the injected sample plug length the most significant contributor to peak breadth.

To minimize electroosmotic flow and prevent analyte adsorption [35-37], the channels were filled with buffer containing 0.5% (w/v) HPC prior to each run. In some cases, the run buffer also contained 0.5% HPC (e.g., Figure 3.5). I evaluated the migration time reproducibility for CE in HPC-free buffer by

![Figure 3.6.](image)

**Figure 3.6.** Theoretical plates vs. applied voltage for the amino acid separations shown in Figure 3.5. Legend: Gly (♦), Asn (■), Phe (▲), Arg (●).
running ten replicate injections of the amino acid mixture at 1 min intervals. The migration time for FITC-Arg had a relative standard deviation (RSD) of 0.9% for 10 consecutive runs, indicating that the adsorbed polymer coating was stable over that time. Furthermore, over two days with the channels flushed and refilled multiple times, the RSD was 1.5% for 25 runs. A representative separation performed in a channel that was treated with HPC, but filled with HPC-free buffer, is shown in Figure 3.7B. For comparison, a separation of the same mixture run in HPC-containing buffer is also shown. Theoretical plate numbers were not significantly different for the separations in the two solutions, but the selectivity changed slightly for some analytes, most likely due to increased buffer viscosity when HPC was present. For this reason, FITC-Asn and FITC-Phe were fully resolved when HPC was added to the run buffer (Figure 3.7A), but not when HPC was absent (Figure 3.7B). Passivating channel walls with an additive and then running the separation without that additive present in the run buffer should be useful where such buffer components would interfere with detection (e.g., mass spectrometry).
Figure 3.7. Separation of FITC-labeled amino acids in 10 mM carbonate buffer, pH 9.2 with (A) and without (B) 0.5% (w/v) HPC in the run buffer. Peaks are: (a) Gly, (b) Asn, (c) Phe, (d) FITC, and (e) Arg. The injection voltage was +800 V, and the separation voltage was +3.0 kV.

To further demonstrate the suitability of solvent-bonded CE microchips for high-performance biological analyses, I separated FITC-labeled peptides in HPC-containing buffer (Figure 3.8). The separation performance was similar for the peptides and amino acids; peak (a) in Figure 3.8 has a theoretical plate
number of 43,000, corresponding to \(1.7 \times 10^6\) plates/m. I also separated the peptides in buffer lacking HPC (not shown), and the plate numbers and resolution were similar to those in Figure 3.8.

The excellent performance of these PMMA microchips in CE clearly demonstrates the usefulness of the solvent bonding technique. I applied electric fields nearly twice as high as those previously reported in PMMA microchips [40], which enabled separations with \(>40,000\) theoretical plates in \(~10\) s. I believe that higher fields are possible in our devices because the robust bonding is more resistant to dielectric breakdown at elevated voltages. Furthermore, a single

![Figure 3.8](image)

**Figure 3.8.** Separation of FITC-labeled peptides. Peaks are: (a) FLEEI; (b) FA; (c) FGGF; (d) Leu enkephalin; (e) angiotensin II, fragment 3-8; (f) angiotensin II; and (g) GGYR. The buffer composition and voltages were the same as in Figure 3.7A, and the concentration of all peptides was 110 nM.
device was used for >300 separations over the course of 3 months with no degradation of separation performance. These results demonstrate a significant advance in fabrication technology that should make polymer substrates more attractive for a broad range of microchip analyses.

A key advantage of the phase-changing sacrificial layer solvent bonding approach is that it should be generalizable to other combinations of polymeric substrates, sacrificial materials, and bonding solvents. The requirements for application to new systems are (1) a sacrificial material that has a melting point below the glass transition temperature of the chosen polymer, and (2) a bonding solvent that can dissolve the polymer substrate but not the sacrificial material. I am currently exploring appropriate combinations of solvents and sacrificial materials to create microfluidics in other polymeric materials, which should broaden the application of this technique.

3.4 CONCLUSIONS

I have shown that polymeric microchips can be created by using a sacrificial material to protect channel integrity during solvent bonding. This phase-changing sacrificial layer fabrication method is simple to implement, and tests show that solvent-bonded devices can withstand >10-fold higher internal pressures than thermally bonded substrates. CE separations of FITC-labeled
amino acids and peptides were successfully carried out in solvent-bonded devices in as little as 8 and 15 s, respectively, with theoretical plate numbers exceeding 40,000 for both analyses. Finally, devices can be operated at electric fields $>1,500$ V/cm and can be used for hundreds of electrophoretic separations without any change in performance. Solvent bonding with phase-changing sacrificial layers should help to overcome some of the previous limitations of polymer microfluidic devices and make them more attractive for chemical analyses.
3.5 REFERENCES


4 CAPILLARY ELECTRIC FIELD GRADIENT FOCUSING OF PROTEINS BASED ON SHAPED IONICALLY CONDUCTIVE ACRYLIC POLYMER*

4.1 INTRODUCTION

To date, electric field gradients have been established for electric field gradient focusing (EFGF) in four different ways (see Section 1.2): changing cross-sectional area (CSA) [1, 2], conductivity gradients based on the dialysis of buffer ions across a membrane [3, 4], buffers having strongly temperature-dependent conductivities [5-7], and individually addressable electrode arrays [8, 9]. EFGF based on changing CSA is particularly attractive because of the promise of optimizing the electric field gradient profile by simply varying the geometry of the changing CSA region. In contrast, it is much more difficult to control the shape of electric field gradients when dialysis of buffer ions is used [3, 4]. Similarly, when temperature gradients have been employed, the conductivity of the reported buffer systems only changes by ~30% over the usable temperature span [5], thus limiting the range of analyte electrophoretic mobilities that can be focused simultaneously. Electrode arrays offer considerable control over the electric field gradient, but at the cost of complex electronic circuitry [8]. The original setup for EFGF based on changing CSA, reported by Koegler and Ivory

* Sections 4.2 and 4.3.6, and Figures 4.1 and 4.3 are reproduced with permission from Anal. Chem. 2004, 76, 5641-5648. Copyright 2004 American Chemical Society
[1, 2], was used for preparative-scale separations of colored proteins. The devices had 6.4-mm-diameter focusing columns, which required the buffer in the changing CSA regions to be recirculated and cooled to avoid excessive Joule heating. The analytical performance was limited, as the separation of just two protein species required 6–10 h to complete, and only modest protein enrichment factors of ~3 were achieved. Clearly, EFGF based on changing CSA merited further optimization.

This chapter reports on my work to characterize a capillary-based EFGF system that was originally developed by Dr. Paul Humble [10]. With miniaturized channel dimensions, it was expected that separation performance could be improved, as Taylor dispersion decreases with channel diameter, and Joule heat can be dissipated more easily (see Section 1.3.1). As with previous EFGF work, the development of novel fabrication techniques was required to interface the focusing channel with the gradient-enabling region, as was the creation of a new semi-permeable copolymer (SPC). Below I describe initial EFGF experiments that resulted in proteins forming extremely narrow but unresolved bands, modifications of device design that enabled the improved resolution of proteins, and the use of a capillary-based EFGF system to concentrate proteins by a factor of ~10,000.
4.2 EXPERIMENTAL SECTION

4.2.1 Instrumentation

Laser-induced fluorescence detection of focused proteins was accomplished in three different ways for the various experiments. In all cases, the 488 nm line from an air-cooled Ar ion laser was passed into an inverted optical microscope (TE300, Nikon, Tokyo, Japan) through an excitation filter (D488/10, Chroma, Brattleboro, VT) to an objective [11]. Fluorescence was collected with the same objective, passed through a 505 LD dichroic filter (Chroma) and an E515LPm long-pass filter (Chroma). Color micrographs were obtained by passing the excitation beam unexpanded into a 4×, 0.12 N.A. objective and imaging the collected fluorescence onto a Nikon Coolpix 995 digital camera. For preconcentration experiments, the laser beam was expanded to ~1.6 cm using a 10× beam expander (Newport, Irvine, CA) and passed through the same 4× objective, resulting in a more focused beam spot with a diameter of ~400 μm. Fluorescence data were collected as TIFF images using a cooled CCD camera (Coolsnap HQ, Roper Scientific, Tucson, AZ). To detect focused bands along the length of the entire column, the separation channel was scanned through a fixed detection volume by connecting the microscope stage to syringe pump (PHD 2000, Harvard Apparatus, Holliston, MA) that served as a one-dimensional translation system. The laser beam was expanded to 1.6 cm and passed through a
20×, 0.45 N.A. objective. After traversing the long-pass and dichroic filters, the light was filtered spatially to remove out-of-focus fluorescence with a 200-μm-diameter pinhole. Photons passing through the pinhole were detected at a Hamamatsu HC 120-05 (Bridgewater, NJ) photomultiplier tube. The detector signal was amplified and filtered with a SR-560 preamplifier (Stanford Research Systems, Sunnyvale, CA) and then digitized with a PCI-6035E (National Instruments, Austin, TX) analog-to-digital converter controlled by LabVIEW (National Instruments) software running on a personal computer. The sampling rate for data collection was set in the software at 10 Hz.

### 4.2.2 Materials and sample preparation

Recombinant, enhanced green fluorescent protein (GFP; Clontech, Palo Alto, CA) and R-phycoerythrin (R-PE; Polysciences, Warrington, PA) were diluted in 20 mM, pH 8.7 Tris buffer (Sigma-Aldrich, Saint Louis, MO). All buffer solutions were prepared using purified water from a Barnstead EasyPure UV/UF system (Dubuque, IA) and passed through a 0.2-μm filter (Pall, East Hills, NY) prior to use. Lysozyme (Sigma) was dissolved in 20 mM, pH 7.4 Tris buffer to a concentration of 100 μM. A 10-fold molar excess of Oregon Green 488-maleimide (Molecular Probes, Eugene, OR) in dimethylsulfoxide was added to the lysozyme solution and allowed to react in the dark at room temperature for 2 h. To remove unconjugated label, 3 mL of Tris buffer, pH 8.7 were added to 100 μL of the
fluorescently labeled lysozyme solution, and the mixture was placed in the upper chamber of a Microsep (Pall) centrifugal device with a molecular weight cutoff of 1,000 Da. The Microsep device was centrifuged at 4,000 rpm at 4 °C for 2 h, which forced buffer and free label into the lower chamber, but retained lysozyme in the upper chamber. After centrifugation, the lysozyme-containing solution in the upper chamber (approximately 100 μL) was collected. Hemoglobin (Sigma) was fluorescently labeled using a FluoroTag FITC Conjugation Kit (Sigma) according to the instructions of the manufacturer and isolated from excess label as described above for lysozyme preparation.

All the monomers used to construct the ionically conductive polymer were obtained from Aldrich and used as received. The ionically conductive membrane was a UV-polymerized random copolymer hydrogel containing the following components: 27 wt% hydroxyethylmethacrylate (HEMA), 23 wt% methylmethacrylate (MMA), 19 wt% 100 mM Tris buffer (pH 8.7), 18 wt% poly(ethylene glycol) acrylate (PEGA), 12 wt% poly(ethylene glycol) diacrylate (PEGDA), and 1 wt% 2,2-dimethoxy-2-phenyl-acetophenone, which served as the photoinitiator. These components were mixed together to create a transparent prepolymer solution.
Fused silica capillary tubing (150 μm I.D.) was obtained from Polymicro Technologies (Phoenix, AZ). To suppress electroosmotic flow, capillaries were coated with poly(vinyl alcohol) using an established protocol [12].

4.2.3 Device Fabrication

A 120-μm-diameter nichrome wire was used to form the separation channel through the interior of the ionically conductive polymer. A cavity for casting the polymer slab was cut through a 1.5-mm-thick piece of acrylic sheet (Acrylith OP-3, Cyro, Rockaway, NJ) using a CO₂ laser cutter (C-200, Universal Laser Systems, Scottsdale, AZ). Channels for attaching sections of fused silica capillary were also laser cut into this top piece of acrylic sheet extending from the high- and low-field sides of the cavity. The wire was threaded through two ~5-cm-long, 150-μm-I.D. capillaries, which were placed in the laser-machined channels with the nichrome wire suspended through the center of the cavity used to form the polymer slab (Figure 4.1A). The top plate was then thermally bonded to a solid acrylic bottom plate by clamping the two pieces together and placing them in an oven at 107 °C for 15 min. Next, the cavity in the top plate was filled with prepolymer solution, and the device was placed under a UV lamp (Model 5000, Dymax, Torrington, CT) for 5 min to effect polymerization. The wire was then withdrawn from the device, leaving a hollow separation channel between the two short capillaries and through the interior of the ionically
Figure 4.1. Schematics showing (A) an exploded view of the components used to construct the ionically conductive polymer-based EFGF devices and (B) an assembled device. (C) Schematic of a completed device with buffer reservoirs that hold electrodes and tubing connected to the low-field capillary for syringe pump attachment. (D) Photograph of a finished EFGF device. Scale bar is 1 cm.

The separation channels produced in this manner were 4 cm long and ~110 μm in diameter.

The short fused silica capillaries on both ends of the separation channel facilitated the attachment of the device to pumps and buffer reservoirs. The low-field capillary was connected to a syringe pump (PHD 2000, Harvard Apparatus) (Figure 4.1C). The electric field gradient was established using a Stanford Research Systems high voltage power supply (Model PS 350) attached to platinum wire electrodes. The electrodes that served as the anode were placed in buffer reservoirs at the low-field end of the ionically conductive polymer (Figure
4.1C) or placed directly in contact with the polymer at the low-field end. The cathode was placed at the high-field end of the ionically conductive polymer or in a buffer reservoir attached to the capillary extending from the high-field end of the separation channel (Figure 4.1C). The applied potentials ranged from 500 to 2000 V, and the resulting currents were generally between 10 and 60 μA. A photograph of a capillary EFGF device is shown in Figure 4.1D.

4.2.4 Sample Introduction

The proteins used in EFGF experiments were either fluorescently labeled or natively fluorescent when excited at 488 nm. Analytes were introduced into the EFGF separation channel using electrokinetic injection or by including the analytes in the buffer being pumped through the separation channel. For electrokinetic injection, the cathode was placed in a buffer reservoir attached to the high-field capillary (Figure 4.1C). During injection, the buffer reservoir was replaced with a reservoir containing the analytes, and +1 kV was applied for 5–30 s. After injection, the analyte reservoir was replaced with the buffer reservoir. Once proteins were observed entering the separation channel, the syringe pump was activated to create the hydrodynamic counterflow. For continuous sample loading, analytes were included in the buffer being pumped through the column. In this case, analytes entered the EFGF channel at the low-field end of the device and moved toward the high-field end until reaching their focusing position.
Continuous loading was used for protein concentration experiments, although a disadvantage of this approach was that protein was continually being introduced into the EFGF channel, making it difficult to distinguish individual bands when multiple proteins were present. It is also possible to introduce analytes into the low-field capillary using a chromatographic injection valve, but this technique was not explored here.

4.3 RESULTS AND DISCUSSION

4.3.1 Analyte Stacking

Initially, rather than immersing the end of the high-field capillary in a buffer-containing vial that served as the cathode (Figure 4.1C), electrical contact was provided by placing electrodes in drops of buffer on top of the narrowest and widest parts of the SPC. The peak widths observed using this setup were narrower than expected; a photomicrograph of one such GFP band is shown in Figure 4.2A. Given GFP’s electrophoretic mobility at pH 8.7 ($1.7 \times 10^{-4}$ cm$^2$/Vs) [10] and diffusion coefficient ($8.7 \times 10^{-7}$) [13], the average linear flow velocity (0.018 cm/s) and the applied potential (800 V), a linear decrease in electric field, providing a gradient of 25 V/cm$^2$, should give a peak with a standard deviation of 1.6 mm and width of nearly 1 cm [2]. However, the observed peak widths of ~100 μm are consistent with an electric field gradient of 2,000 V/cm$^2$, indicating
Figure 4.2. Fluorescence micrographs of focused proteins. (A) GFP focused to a ~150 µm band. (B) R-PE (yellow) and GFP (green) stacked into a contiguous band. Scale bars are 250 µm. For both runs, buffer was 20 mM Tris, pH 8.7, the applied potential was 800 V, and the counterflow rate was 50 nL/min. 

poor correlation between the designed and observed electric field gradient. Furthermore, changing the voltage or bulk fluid velocity enabled the focused protein to translate along the channel, but the width of the peak did not change significantly, indicating that the electric field gradient was larger than expected along the entire length of the channel. One possible reason for the difference between the designed and observed gradient is that the small buffer reservoirs and the relatively long run times quickly depleted most of the buffer ions from the focusing column. Once this occurred, the focused protein itself made a large contribution to the local conductivity in the column [14], causing a well in the electric field profile to form. The observation that when multiple proteins were introduced, the bands stacked next to each other and could not be resolved (Figure 4.2B) also corroborates the ion depletion explanation. Simply increasing the size of the buffer reservoirs should eliminate ion depletion, enable the
resolution of multiple species, and result in peak widths that are consistent with those predicted theoretically.

4.3.2 Protein Separation

In subsequent work, the small buffer droplets on top of the membrane were replaced by large (~400 μL) buffer reservoirs at the low-field end and a buffer-filled vial interfaced with the high-field capillary. Although broader peaks were observed, the resolution of different proteins into separate bands became possible. Because the proteins could no longer be monitored within the viewing range of the microscope objective, the scanning detection setup described in Section 4.2.1 was employed. Figure 4.3 shows a mixture of natively fluorescent and fluorescently labeled proteins focused along the length of an EFGF device.

![Figure 4.3. EFGF of a four-protein mixture. Applied potential was +1,000 V, counterflow rate was 30 nL/min, and the run buffer was 20 mM Tris, pH 8.7.](image)
This separation was repeatable, as similar results were obtained with ~10 different devices. While this work made it clear that the capillary-based EFGF setup could focus multiple proteins, further characterization of the system was necessary. Research to verify the reproducibility of the scanning detection system, study the dynamics of protein focusing, improve separation performance, and explore EFGF for sample enrichment is reported below.

### 4.3.3 Characterization of Scanning Detection System

Because the scanning detection method was crucial to studying focusing dynamics and observing the changes in peak position and shape that take place under different operating conditions, it was critical to verify that focused proteins in the column could be probed reproducibly. This was especially important since the translation setup was assembled from equipment not originally designed for that purpose. Figure 4.4 shows 3 sequential scans of a focused R-PE band. The stage was translated for each scan at 5.1 cm/min, and detection sampling was at 10 Hz. The 3 peaks are nearly identical, which shows that the scanning system can indeed be effective for recording and comparing peak intensities, widths, and absolute positions along the column length. To align scans accurately after collecting the data, the highly fluorescent polyimide coating on the fused silica capillaries served as an indicator to locate the
Figure 4.4. Sequential scans of a focused R-PE band to verify the reproducibility of the scanning confocal fluorescence detection setup. These repeated scans also show the excellent stability of the R-PE band over the ~2 min time period.

4.3.4 Focusing Dynamics

The whole-column scanning detection setup enabled a more detailed study of band focusing dynamics in EFGF. Figure 4.5 shows sequential scans of an R-PE band that had been introduced electrokinetically as a broad plug from the high-field end of a device and focused into a temporally stable peak by ~7 min. This focusing time was ~10-fold faster than had been reported in preparative-scale EFGF [1-3, 8], and was likely the result of the higher electric fields that were applied with the smaller column.
Figure 4.5 Sequential scans of an EFGF channel showing R-PE focus into a narrow (~3 mm) band. The applied potential was +500 V, the counterflow rate was 50 nL/min and the buffer was 20 mM Tris, pH 8.7. A stable band formed in ~7 min.

4.3.5 Improving Protein Resolution

Another device design optimization to enable higher resolution separations entailed dropping the entire voltage over just the focusing channel, instead of across the connecting capillaries as well. Thus, rather than providing electrical contact at a buffer vial attached to the high-field capillary, I designed devices with large (0.5 mL) buffer reservoirs attached directly to the narrowest region of the SPC. Figure 4.6 shows a separation of the same protein mixture used in Figure 4.3. The bandwidths decreased by a factor of ~2, while the spacing between peaks also increased, providing a 3–4-fold improvement in resolution.
The increased resolution and spacing between peaks indicate a shallower electric field gradient. The range of electrophoretic mobilities that could be focused simultaneously in the device also decreased, as GFP and R-PE were no longer focused in the channel under these conditions. However, if Taylor dispersion and diffusion were the sole sources of broadening, then as the electric field gradient decreased, the peaks should have become wider. Instead, the observed peaks became narrower with this design, indicating that another source of band

**Figure 4.6.** Comparison of separation performance (A) in the original device design and (B) in a device where the electric field in the high-field capillary was eliminated. Dashed lines indicate the same proteins in the different devices.
broadening was reduced. It is likely that electroosmotic flow due to the voltage drop across the high-field capillary had caused additional dispersion in the separation in Figure 4.3. With this dispersion source gone, a shallower electric field gradient could increase resolution without broadening peak widths.

4.3.6 Sample Concentration

An important attribute of EFGF and other focusing techniques is the ability to concentrate analytes. To determine the degree of concentration that could be achieved in capillary-based EFGF devices, a calibration curve was created using GFP samples of known concentration. Each sample was pumped through the EFGF device at a flow rate of 100 nL/min, and fluorescence images were obtained at three positions along the channel using a cooled CCD camera with an integration time of 50 ms. The intensity values of the 20 brightest pixels in the images were averaged to provide each data point on the calibration curve. The signal intensity increased linearly between 150 nM and 1.5 μM, which was the concentration range used in constructing the calibration curve (Figure 4.7).

Preconcentration experiments were performed by operating the device as described in Section 4.2.4, with a +2 kV applied potential and a counterflow of 30 nL/min. The syringe pump that provided the counterflow was filled with 18 pM GFP, which was pumped continuously into the channel. The highest observed signal gains were obtained after the GFP was focused into a narrow band for 40
min, resulting in a GFP concentration of 180 nM and an enrichment factor of 10,000 ± 2,500 with a 95% confidence interval.

The enrichment factor reported here is equivalent to that published by Ross and Locascio [5] using temperature gradient focusing, which reportedly surpassed any other sample concentration methods. Taken together, these two demonstrations show the tremendous power of analytical equilibrium gradient methods for sample enrichment. Moreover, concentration factors beyond those reported here should be attainable with this design, simply by allowing dilute protein to focus at its equilibrium position for longer periods of time. Because the rate of sample concentration is determined by the counterflow, which introduces the dilute protein into the focusing channel, similar levels of concentration could be achieved more quickly by increasing the counterflow rate, and increasing the
applied voltage correspondingly. When multiple proteins are simultaneously separated and concentrated, one must be careful to ensure that the local conductivity in the channel is not altered by the focused protein bands, as analyte stacking could occur (see Section 4.3.1).

4.4 CONCLUSION

This chapter describes my work using capillary EFGF devices based on changing CSA. Initial experimentation with the devices produced narrower-than-expected focused protein bands, but when multiple species were present, the bands did not resolve. By increasing the size of the buffer reservoirs and changing the electrode configuration to eliminate the electric field in the connecting capillaries, high-resolution separations of model proteins were achieved. A novel scanning detection system was developed to enable the visualization of proteins focused along the column length, which also made possible the study of focusing dynamics. In addition, by continuously introducing protein into the focusing channel via the counterflow-providing run buffer over a 40 min period, concentration factors of $\sim10^4$ were obtained. These results demonstrate that EFGF could be a potentially powerful tool for proteomic analysis.
4.5 REFERENCES


5 FABRICATION OF ELECTRIC FIELD GRADIENT FOCUSING MICRODEVICES WITH IN SITU-POLYMERIZED SEMI-PERMEABLE MEMBRANES

5.1 INTRODUCTION

While microfluidic devices have made possible extremely fast [1] and high-performance [2-4] chemical separations, perhaps the most significant promise of lab-on-a-chip technology is the ability to combine multiple sample handling and analysis steps onto a single platform [5-8]. Such integration can significantly decrease the total analysis time, rather than that for separation alone, especially with complex samples that require extensive pretreatment. For example, microfluidic mixers and reactors have been combined on a microchip capillary electrophoresis (μ-CE) device having 6 parallel separation lanes to perform multiple immunoassays in ~1 min [9]. In another case, μ-CE systems with polymerase chain reaction chambers, complete with on-chip heaters, temperature sensors and valves, have enabled genotyping from whole bacterial cells in <10 min [10].

Size-selective membranes have been incorporated in microfluidic devices for various sample preparation and manipulation steps. For example, Smith and coworkers [11] created a microdialysis system that sandwiched commercially available sheet membranes between microchannel-containing substrates,
allowing samples to be purified from interfering high- and low-molecular weight species prior to being introduced into a mass spectrometer. In a similar setup, affinity microdialysis was performed on-chip, where antigen-antibody complexes were retained by a sheet membrane while smaller, unbound components were removed [12]. The purified complexes were then exposed to counterflowing air through a second membrane, which concentrated the sample in solution through evaporation. Nanoporous, track-etched polycarbonate membranes have been used to interface intersecting microchannels on different substrates [13-15]. Analyte transport between the channels was controlled by applying electric fields across the membranes, enabling selected fractions from one channel network to be driven electokinetically through the nanopores and introduced into the opposing channel structure. Samples have been injected and fractions collected across a membrane using this approach, showing considerable control in analyte manipulation. Khandurina et al. [16, 17] demonstrated size-selective barriers for concentration of DNA prior to electrophoretic separation. Microchannels in a μ-CE injection region were connected electrically through small pores in a thin sodium silicate layer. DNA molecules that were driven electrokinetically to the sodium silicate membrane were too large to pass through, and over time the concentration of the trapped DNA increased ~100-fold. The enriched sample plugs were then separated electrophoretically. Zhang
and Timperman [18] employed a similar membrane-based preconcentration system, but with a sandwiched nanocapillary array. Rather than pore size, charge played the dominant role in analyte trapping, as the 10–50 nm pores were much larger than the molecules that were enriched. While these examples demonstrate the broad applicability of membrane-based microsystems to various modes of sample pretreatment and manipulation, most utilized commercial sheet membranes sandwiched between microfluidic device substrates. Such configurations have limited device geometries and are constrained by the properties of available materials.

The ability to polymerize semi-permeable barriers in situ in microfluidic networks adds design flexibility and enables membranes with a variety of properties to be explored. Recently, a dialysis system that incorporated an in situ-polymerized membrane was reported by Kirby and coworkers [19]. 280-μm-wide channels were filled with a prepolymer solution having an appropriate photoinitiator, and a shaped laser beam was focused into a plane to effect spatially controlled polymerization. This produced a membrane that divided the channels in two along their lengths, allowing dialysis to take place between countercurrent flows. Membrane properties could be altered by tailoring the prepolymer composition, but a complicated optical setup was required, and
repeated laser exposures in the channels with fresh monomer solution were necessary to complete polymerization.

Electric field gradient focusing (EFGF) is an analytical technique that is facilitated by having a semi-permeable membrane interfaced with a separation column [20-24]. A detailed description of EFGF has been published [25]. Briefly, a gradient in electric field, combined with a constant-velocity, pressure-driven flow in the opposite direction, causes charged analytes to focus into stationary bands along the column according to electrophoretic mobility. Chapter 4 reports a capillary-based EFGF design that interfaces an *in situ*-polymerized semi-permeable copolymer (SPC) of changing cross-sectional area (CSA) with a ~100-μm-diameter focusing column [24]. The SPC enables the electric field to change along the channel length, as current-carrying buffer ions can pass through, but the bulk fluid and protein analytes cannot. The focusing column was formed by polymerizing the SPC around a wire (sacrificial material) in a well of changing CSA. Following polymerization, the wire was pulled out from the SPC, leaving an open, cylindrical column connected to capillaries at either end of the SPC.

Although this approach allowed for smaller-dimension devices than previous membrane-incorporating EFGF designs [20-22, 26], several limitations were also apparent. For example, further column miniaturization is impractical, as smaller wires are more fragile and difficult to use. In addition, a diameter mismatch
between the focusing channel and the capillaries would reduce resolution if analytes are to be eluted from the column. Improved EFGF fabrication methods that avoid these challenges while enabling smaller channel dimensions would be valuable.

In Chapter 3, I described a fabrication procedure for solvent bonding of polymer microdevice substrates [27]. Imprinted microchannels in PMMA were filled with a phase-changing sacrificial layer (PCSL), after which solvent was applied to the surface. A cover plate was placed in contact with the patterned substrate to allow a robust seal to form, and then the PCSL, which prevented solvent from filling the microchannels during bonding, was melted and removed. More generally, PCSL placeholders could be used in other microfluidics applications, such as interfacing membranes with microchannels.

Here I demonstrate a simple technique for in situ polymerization of membranes in microdevices, based on the PCSL approach developed in Chapter 3 for making enclosed microfluidic networks [27]. The procedure involves casting a prepolymer solution over PCSL-filled microchannels, followed by sacrificial material removal after membrane polymerization. Microfluidic EFGF (μ-EFGF) devices based on changing CSA were fabricated using PCSLs for semi-permeable membrane incorporation. The smaller dimensions of μ-EFGF devices
relative to other changing CSA-based EFGF platforms result in decreased
dispersion caused by pressure-driven flow and narrower analyte bands.

5.2 EXPERIMENTAL SECTION

5.2.1 Sample preparation and materials

All buffer solutions were prepared using purified water from a Barnstead
EasyPure UV/UF system (Dubuque, IA) and passed through a 0.2-μm filter (Pall,
East Hills, NY) prior to use. Peptide standards (Sigma-Aldrich, St. Louis, MO)
were labeled fluorescently [28] by combining 200 μL of a 2 mM solution of each
peptide in 10 mM, pH 9.2 carbonate buffer with 50 μL of 6 mM fluorescein
isothiocyanate (FITC; Molecular Probes, Eugene, OR) in dimethylsulfoxide. The
mixture was allowed to react at room temperature in the dark for at least 3 days
prior to use. R-phycoerythrin (R-PE; Polysciences, Warrington, PA) and
recombinant, enhanced green fluorescent protein (GFP; Clontech, Palo Alto, CA)
were used after dilution in run buffer.

The in situ-polymerized SPC for μ-EFGF devices was similar in
composition to the SPC employed for capillary-based EFGF [24]. The prepolymer
solution consisted of 34 wt% hydroxyethylmethacrylate, 24 wt%
methylmethacrylate, 17 wt% 100 mM Tris buffer (pH 8.1), 21 wt% poly(ethylene
glycol) acrylate, 3 wt% ethylene glycol dimethacrylate, and 1 wt% 2,2-
dimethoxy-2-phenyl-acetophenone (photoinitiator). All reagents for the SPC were obtained from Sigma-Aldrich and used as received. The PMMA for device substrates was Acrylite OP-3 (Cryo, Rockaway, NJ), and the paraffin wax PCSL (melting point: 65 °C) was from Service Assets (Newport Beach, CA).

### 5.2.2 EFGF Microdevice Fabrication

Silicon wafers were patterned photolithographically and wet etched; these substrates served as templates for hot embossing PMMA, as described in Chapter 2 [29]. Figure 5.1 illustrates the different steps of μ-EFGF device fabrication, and more detailed views of these systems are presented in Figure 5.2. The patterned PMMA (Figure 5.1A, white) had straight microchannels that were

![Figure 5.1](image)

**Figure 5.1.** Fabrication procedure for *in situ* polymerization of membranes in μ-EFGF devices. Scale bar in (G) is 250 μm. Additional description is in the text.
3 cm long (Figure 5.2), with trapezoidal cross-sections that were 30 μm deep and a width that increased from 40 to 80 μm from bottom to top.

A flat, 2-mm-thick piece of the elastomer poly(dimethylsiloxane) (PDMS; Sylgard 184, Dow Corning, Midland, MI) had two 500-μm-diameter through holes set 3 cm apart. The PDMS (Figure 5.1A, gray) was sealed reversibly to the imprinted PMMA piece such that the drilled holes aligned with the channel ends. The temperature of the PDMS/PMMA assembly was raised to 85 °C on a heating block, and 10 μL of melted paraffin wax PCSL (Figure 5.1B, white with gray stripes) were transferred quickly from a heated vial to one of the holes in the PDMS piece. Vacuum was applied to the other opening to fill the channels.

**Figure 5.2.** Schematic depiction (left, exploded view) and photograph (right) of an EFGF microchip. In the photograph, buffer reservoirs and the microchannel were dyed for enhanced visualization. Scale bar on the photograph is 1 cm. Additional description is in the text.
with melted PCSL. Next, the assembly was transferred to another heating block at 35 °C for 3 min to solidify the PCSL (Figure 5.1C, gray with white stripes), followed by cooling to room temperature. Cooling the devices in two steps prevented the deposition of solid PCSL in regions beyond the microchannels [27]. The PDMS piece was peeled from the surface, and a PMMA cover plate with a region of changing CSA cut from its center with a CO₂ laser cutter (C-200, Universal Laser Systems, Scottsdale, AZ) was aligned with the imprinted, protected PMMA as shown in Figure 5.1C and Figure 5.2, left. In addition to the changing CSA pattern, rectangular buffer reservoirs and a 0.9-mm-diameter circular hole for connecting tubing to provide pressure-driven flow were cut from the PMMA cover plate. The substrates were clamped together and epoxy (No. 14250, Devcon, Danvers, MA) was applied around the perimeter of the assembly. Four holes, one at each of the device corners, were drilled through the cover plate to prevent air pockets from forming when the prepolymer solution was added. Melted paraffin wax was pipetted and then solidified in the pump access hole and the high-field reservoir, and rectangular PDMS plugs were inserted into the low-field reservoirs (Figure 5.2). Approximately 400 μL of prepolymer solution (Figure 5.1D, black) were pipetted into the changing CSA region, also filling the interstitial space between the two PMMA substrates. The prepolymer-containing device was mounted on a copper block cooled to 4 °C.
and placed under a 320 W Hg arc lamp (Model 5000, Dymax, Torrington, CT) for 5 min to polymerize the SPC (Figure 5.1E, gray with white cross-hatching). The cooled block prevented the paraffin from melting during polymerization. Next, the device was heated to 85 °C to melt the PCSL, which was removed from the channel and reservoirs by applying vacuum (Figure 5.1F). After the EFGF microchip was cooled to room temperature, the channels were flushed with hexanes (EM Science, Darmstadt, Germany) to dissolve residual paraffin, and the PDMS plugs that defined the low-field buffer reservoirs were removed. Finally, an 8-in.-long piece of flexible tubing (0.9 mm O.D.) was inserted into the pump access hole and sealed in place with epoxy (Figure 5.2, right). A photomicrograph of a completed EFGF microchannel is shown in Figure 5.1G, and an image of an entire μ-EFGF device is depicted in Figure 5.2, right.

5.2.3 EFGF Microdevice Operation

To operate the EFGF microchips, a 100 μL gas-tight syringe (Hamilton, Reno, NV) was filled with run buffer (20 or 100 mM Tris, pH 8.1, containing 0.5% w/v hydroxypropyl cellulose), connected to the flexible tubing, and placed in a syringe pump (PHD 2000, Harvard Apparatus, Holliston, MA), enabling the counterflow-providing run buffer to be introduced into the channel at rates as low as 0.4 nL/min. Two Pt electrodes were connected to a high-voltage power supply and inserted into the low-field reservoirs (Figure 5.2), which were filled
with run buffer. A grounded Pt electrode was placed in the high-field buffer reservoir (Figure 5.2). For analyte introduction, the counterflow was interrupted, and the high-field reservoir was filled with sample dissolved in buffer. The mixture was injected electrokinetically for ~30 s at an applied potential of 500 V, after which the power supply was turned off. The injection time and voltage could be varied to accommodate different sample concentrations or analyte electrophoretic mobilities. Following injection, the sample was pipetted from the high-field reservoir, and the well was rinsed and refilled with run buffer. The applied potential and counterflow were then adjusted to focus the proteins or peptides into discrete bands.

5.2.4 Instrumentation

Detection of focused analytes in the column was accomplished as described in Section 4.2.1 [24]. Briefly, micrographs were obtained by passing the 488 nm line of an Ar ion laser unexpanded into a 4×, 0.12 N.A. objective of an inverted microscope (TE300, Nikon, Tokyo, Japan), and imaging the resulting fluorescence with a digital camera (Coolpix 995, Nikon). I converted the photomicrographs to electropherograms by averaging the fluorescence intensity along the focusing column using the image processing program ImageJ 1.34s (National Institutes of Health, USA). Noise resulting from laser speckle and
reflections from the SPC was filtered from the electropherograms by boxcar averaging.

When focused bands could not be imaged in a single exposure, the devices were scanned through a confocal detection point using a translation stage. For scanning detection, the laser beam was passed through a 10× beam expander prior to being focused with a 20×, 0.45 N.A. objective. The collected fluorescence was filtered spatially with a 200-μm-diameter pinhole and detected at a photomultiplier tube (PMT; HC 120-05, Hamamatsu, Bridgewater, NJ).

5.2.5 Microchip Capillary Electrophoresis

μ-CE experiments were performed in solvent-bonded PMMA microchips. Device fabrication, channel dimensions, and operating procedures are described in Chapter 3 [27]. The separation distance was 2.5 cm, and the channel cross-sectional dimensions were the same as for EFGF microchips (see Section 5.2.2). The run buffer was 100 mM Tris (pH 8.1) with 0.5% w/v hydroxypropyl cellulose, the injection potential was +300 V, and the separation potential was +1.0 kV.

5.3 RESULTS AND DISCUSSION

For the PCSL approach to be effective for in situ membrane incorporation, the solid sacrificial material must not be soluble in either the prepolymer solution
or the polymerized membrane. The monomer mixture used herein could be placed in contact with solid paraffin wax in a microchannel for >20 min without any observable dissolution taking place at the microscopic level. In contrast, other potential PCSLs such as poly(ethylene glycol) dissolved readily in the prepolymer and could not be used. While the prepolymer solution and paraffin wax made an appropriate combination for these μ-EFGF experiments, other PCSLs could be explored for interfacing alternative SPCs with microchannels.

Initial experiments that applied solvent bonding [27] to affix the PMMA substrates together prior to adding the prepolymer frequently resulted in air pockets forming at the PMMA-membrane junction at the low-field end of the μ-EFGF devices. While these bubbles did not form in every solvent-bonded microchip, the fabrication yield was sufficiently low that alternatives were pursued. I found that when the SPC served both as an ionically conductive membrane to provide an electric field gradient and as an adhesive to bond the PMMA cover plate to the patterned PMMA substrate, air pockets were not observed at the PMMA-membrane junction. For this design, the thin layer of SPC extending beyond the changing CSA region made current leakage a possible concern. However, the SPC was much thinner in the adhesive region compared to the electric field gradient formation area (<10 μm vs. >1 mm), and the SPC was
~100 times less conductive than the run buffer solution [24], so current leakage effects were minimal and EFGF was feasible.

Figure 5.3A shows the separation of two natively fluorescent proteins, R-PE and GFP, in an EFGF microchip. These same species had been analyzed previously in capillary-based EFGF devices (Figure 5.3B) [24], which allowed performance to be compared. Average peak widths in the microchip separation were over fourfold narrower than those in the capillary-based devices, and resolution was increased threefold. Although the comparison between the two

![Figure 5.3. Separation of R-PE and GFP in (A) a µ-EFGF device and (B) a capillary-based EFGF system. In (A), 20 mM Tris, pH 8.7 was used, and the counterflow rate and applied potential were 20 nL/min and +1,000 V, respectively. For (B), the run buffer was 5 mM Tris, pH 8.7, the counterflow rate was 30 nL/min, and the applied potential was +2,000 V. Plots were obtained from photomicrographs as described in Section 5.2.4. Maximum fluorescence intensities were normalized to be the same in (A–B).](image)
platforms is not perfect because buffer composition and run conditions were somewhat different, the decreased peak widths in the μ-EFGF experiment in the presence of an approximately equivalent electric field gradient (based on similar peak spacing) indicate that dispersion is reduced in μ-EFGF systems. This observation is consistent with the expectation that as cross-sectional channel dimensions shrink, Taylor dispersion decreases [25].

With a lower applied voltage that created a shallower electric field gradient, it was possible to concentrate and separate a mixture of fluorescently labeled peptides that had electrophoretic mobilities spaced more closely than the natively fluorescent proteins in Figure 5.3. For comparison, the peptides were analyzed by μ-EFGF (Figure 5.4A) and μ-CE (Figure 5.4B) at the same initial concentrations and using the same run buffer. Peak resolution calculations for the two analyses, provided in Table 5.1, indicate comparable overall separation performance for peak pairs (a–b) and (b–c). The resolution between peaks (c) and (d) was considerably higher in the μ-EFGF study, presumably due to a shallower electric field gradient toward the high-field end of the device. The μ-CE precluded the use of electric fields above ~300 V/cm. In Chapter 3, I showed a

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<td>μ-EFGF</td>
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<td>μ-CE</td>
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Table 5.1. Resolution between adjacent peaks for the separations shown in Figure 5.4.
Figure 5.4. Separation of fluorescently labeled peptides by (A) μ-EFGF and (B) μ-CE. Peaks are (a) FLEEI, (b) FGGF, (c) angiotensin II, fragment 3–8, and (d) GGYR. Initial peptide concentrations were 50 nM, and the run buffer was 100 mM Tris, pH 8.1 with 0.5% (w/v) hydroxypropyl cellulose added to suppress electroosmotic flow. The separation potential was 200 V in (A) and 1,000 V in (B). The counterflow rate in (A) was 5 nL/min.

higher resolution separation of fluorescently labeled peptides in 10 mM carbonate buffer with an electric field of 1,000 V/cm [27]. Importantly, the μ-EFGF separation demonstrates an improvement in resolution over previous
EFGF results as a result of decreased dispersion in the smaller channels, and the performance is comparable to μ-CE.

The ability of μ-EFGF to concentrate analytes is also evident in Figure 5.4. The maximum output signal for FITC-FLEEI was 1.00 V for μ-EFGF with the PMT bias set to -650 V, and 0.11 V for μ-CE with a PMT bias of -950 V. When PMT signals are normalized for the different bias settings, the μ-EFGF results indicate a concentration enhancement of ~150-fold relative to μ-CE, which is especially notable given the <10 min total analysis time. In these experiments, the gain in the signal to noise ratio was <150-fold, due to higher noise levels associated with on-column scanning detection in μ-EFGF compared to stationary point detection in μ-CE. To reduce the noise in the scanning setup, improved spatial filtering would be used to avoid detection of SPC background fluorescence. Alternatively, focused peaks could be eluted past a point detector [23].

5.4 CONCLUSIONS

Here, a simple method for the in situ polymerization of semi-permeable membranes in microfluidic devices is reported. Channels are first filled with a phase-changing material, which forms a protecting sacrificial layer upon solidification. A prepolymer solution is then poured over the filled
microchannels and UV polymerized. Once the SPC is formed, the PCSL is melted and removed from the microchannels. \( \mu \)-EFGF devices were constructed using this fabrication approach, and because the channel dimensions were smaller than those of previous EFGF setups based on changing CSA, Taylor dispersion was reduced, and narrower focused bands were formed. EFGF of natively fluorescent proteins was demonstrated with improved resolution compared to earlier work (Chapter 4). Fluorescently labeled peptides were also focused with 150-fold enrichment and comparable separation to \( \mu \)-CE. This general fabrication approach is adaptable to other applications beyond EFGF that require semi-permeable membranes to be interfaced with microchannels, and as such should provide a useful tool for the development of integrated microfluidic systems.
5.5 REFERENCES


6 CONCLUSIONS AND FUTURE WORK

6.1 CONCLUSIONS

6.1.1 Improved Bonding Methods for Channel Enclosure in Polymer Microdevices

For this dissertation, two new methods, water-based enclosure and solvent bonding, were developed for sealing poly(methyl methacrylate) (PMMA) substrates to enclose microchannels. In Chapter 2, I showed that clamped PMMA pieces could be bonded thermally in a boiling water bath [1], rather than in a convection oven [2]. Water bonding has advantages over conventional thermal bonding in terms of improved temperature stability and the higher thermal conductivity of water compared with air, which allows sealing to take place in a shorter period of time. Additionally, bonding in water enables applications that require a microfluidic network to stay hydrated throughout the bonding process.

While polymer microchannel enclosure using a boiling water bath instead of an oven has certain advantages, the solvent bonding technique [3] developed in Chapter 3 should have broader appeal. It is well established that an appropriate solvent can form a strong weld between polymer pieces [4]. The challenge is that open microchannels become filled with softened polymer when exposed to solvent during the sealing process, and blockage occurs. The phase-changing sacrificial layer (PCSL) approach described in Chapter 3 enables a
microfluidic network to be protected from solvent while enclosure takes place, after which the sacrificial material is easily melted and removed. Solvent-bonded PMMA microchips made using this procedure could withstand internal pressures >2,200 psi, more than an order of magnitude greater than thermally sealed devices. Furthermore, higher electric fields could be applied in solvent-bonded microchips, compared with thermally sealed devices, as alternate current paths in the more robust microchips formed less readily. Indeed, faster, higher-resolution electrophoretic separations of peptides and amino acids were performed than had been demonstrated previously in polymer microdevices, and the separation quality was comparable to that obtained in glass microchips. Thus, solvent-based sealing enables the advantages of polymer microdevices, such as low cost for single-use applications, to be realized without sacrificing separation performance.

6.1.2 Miniaturized Electric Field Gradient Focusing (EFGF) Devices Based on Changing Cross-Sectional Area (CSA)

EFGF was explored as an analysis platform that allows proteins to be concentrated and separated simultaneously. EFGF, which uses a gradient in electric field and a constant, pressure-driven flow to cause analytes to focus along a column according to electrophoretic mobility, benefits from decreased column dimensions, as Taylor dispersion is smaller and Joule heat dissipation is
enhanced [5]. A capillary-based changing CSA EFGF device having a ~100-μm-diameter focusing column was developed in Chapter 4 [6]. The channel was surrounded by a shaped, semi-permeable hydrogel, which allowed an electric field gradient to be established and protein analytes to be confined within the focusing column. I used these capillary-based devices to separate proteins in mixtures and concentrate a model analyte ~10,000-fold in 40 min. A scanning laser-induced fluorescence detection system, which allowed focused proteins to be detected on-column, was developed and used to study EFGF dynamics. R-phycoerythrin was found to focus into a stable band in ~7 min.

EFGF based on changing CSA was further miniaturized to a microchip format in Chapter 5. I used PCSLs developed for solvent bonding of polymer microdevice substrates (Chapter 3) [3] to interface an ionically conductive hydrogel with an imprinted microchannel, enabling electric field gradient formation. Fourfold narrower bands, compared with those observed in capillary EFGF, were formed in these devices as a result of the decreased channel dimensions. Natively fluorescent proteins and a mixture of fluorescently labeled peptides were focused. It should be possible to generalize this in situ polymerization approach for use in other microfluidic devices that require membranes for integrated sample pretreatment.
While the research to develop new techniques for polymer microchannel enclosure may seem somewhat unrelated to EFGF, the microdevice bonding work was in fact inspired by EFGF fabrication needs. Thermal bonding in water was explored initially as a means of incorporating hydrated sheet membranes in EFGF microchips, because membranes in convection-oven-bonded PMMA swelled when they became hydrated, causing the substrates to separate. Eventually *in situ*-polymerized size-selective hydrogels replaced bonded membranes for EFGF gradient formation.

Similarly, the PCSL approach that enabled solvent bonding of PMMA microchips was inspired by a desire to further miniaturize capillary EFGF to a microchip format. For capillary-based EFGF devices, the gradient-enabling semi-permeable copolymer (SPC) was cast around a wire, which was later removed to leave an open focusing column running through the SPC. The wire thus served as a crude sacrificial material in device fabrication, but the approach was difficult to miniaturize further. Using PCSLs in microdevices provided a way to interface smaller channels with SPCs, and the PCSL fabrication method was also found to be useful for solvent bonding polymer microdevices.
6.2 FUTURE DIRECTIONS

6.2.1 Improvements to Solvent-Based Bonding

Paraffin wax proved to be an effective sacrificial material for protecting PMMA microchannels when acetonitrile was used as the bonding solvent. However, paraffin is slightly granular when it solidifies (see Section 3.3), and that granularity translates into reduced smoothness in bonded devices. Such surface topography can make it more difficult to remove air bubbles from channels and can affect separation performance in extreme cases. Also, paraffin shrinks upon solidification (see Section 3.3), which can lead to enclosed microchannels that have a different cross-sectional shape from their original imprinted design. An ideal sacrificial material should be able to withstand a variety of solvents and form a non-granular solid with little change in volume. Screening of compounds that undergo phase transitions from solid to liquid at temperatures between 25–70 °C should reveal new PCSLs with better properties than paraffin wax. In addition, solvent bonding should be applied to other device materials. Recent work with paraffin wax and acetonitrile as the PCSL and bonding solvent, respectively, has shown promise for sealing polycarbonate microchips [7], but further characterization is necessary.
6.2.2 Alternative Equilibrium-Gradient Methods

EFGF is predicted by theory to be an extremely powerful analytical tool, providing high peak capacity and resolution, straightforward elution, large-volume injection, and the ability to enrich proteins to concentrations far higher than those attainable by isoelectric focusing. Unfortunately, experimental progress toward developing practical EFGF instrumentation has been hindered by challenges associated with establishing electric field gradients along a column that supports a constant, pressure-driven counterflow. Indeed, considerable difficulty has stemmed from the use of membranes to separate electric field gradient generating features from fixed-width focusing channels. Importantly, the incorporation of membranes into microfluidic devices has led to the development of new fabrication procedures. However, factors affecting transport through the membranes are not well characterized or understood fully at present [8]. Thus, EFGF devices based on changing CSA sometimes build up membrane transport-induced concentration gradients, causing discrepancies between predicted and observed electric field gradients. A newer EFGF technique, temperature gradient focusing (Section 1.2.6), does not require the use of membranes and can be miniaturized easily, but this approach has prominent drawbacks. For instance, binary buffer systems at ~1 M concentrations are required to produce only a 30% change in conductivity over a 60 °C temperature
span [9], which limits the range of analyte mobilities that can be focused simultaneously. Thus, an equilibrium-gradient method that avoids the use of membranes and does not require temperature gradients would be of great benefit.

In a paper that inspired the first EFGF work, O’Farrell presented a focusing technique called counteracting chromatographic electrophoresis (CACE) [10], which utilized an electrophoretic force opposed by hydrodynamic flow. In contrast to EFGF, the electric field along the CACE column was approximately constant, but the solute effective hydrodynamic velocity changed abruptly at the interface between two regions of packed gel-permeation chromatography media having different size-exclusion properties. With an appropriate electrophoretic counterforce, the net force on a protein reversed direction when the analyte hydrodynamic velocity changed at the packing interface, causing the protein to become trapped.

If the effective hydrodynamic flow velocity of a group of proteins were to change by, for example, an order of magnitude at the boundary between different media, then multiple proteins could be focused at the interface, provided their electrophoretic mobilites differed by less than a factor of 10. Under static electric field conditions such as O’Farrell described, the focused bands would increase in concentration but not be resolved during continual
sample infusion. However, as Wang et al. have shown [11], adjusting the applied potential can cause proteins to elute individually from their equilibrium position in order of electrophoretic mobility. Thus, a protein mixture can be focused in a CACE column at a given potential, and if the voltage is lowered gradually, the electrophoretic force for each protein will eventually become insufficient to counter the fluid flow, causing each analyte to be eluted as a function of potential, as shown schematically in Figure 6.1. Figure 6.1A shows three analytes (1–3) with different electrophoretic mobilities focused at the interface between two different packing materials. In Figure 6.1B, the applied potential has been lowered such that (1) becomes untrapped and begins to elute to the right in the direction of the hydrodynamic flow, while (2) and (3) remain focused. In Figure 6.1C, an additional decrease in electric field increases the velocity of (1) and starts the elution of (2), while (3) will remain trapped until the voltage decreases further.

Voltage-scanning CACE merits both theoretical and experimental exploration, as this approach should enable analyte enrichment and provide high-resolution separations if the potential is ramped appropriately. The separation mechanism is also independent of column length, so detection could take place adjacent to the focusing point in very short and easily microfabricated
Figure 6.1. Schematic depiction of voltage-scanning CACE. Numbered analytes increase in electrophoretic mobility from 1 to 3. On the right, vectors depict electrophoretic velocities ($V_{E1}$, etc); flow vectors, $F$, representing the protein velocity in the absence of an electric field, are shown in blue. Colored arrows in the focusing columns represent the net velocities of the different proteins in each region. The relative electric field, $E$, is shown above the column and does not change in the different media. Additional description is in the text.

Importantly, CACE would eliminate the need for interfacing channels with ion-permeable membranes.

In general, equilibrium-gradient methods continue to offer promise for high-resolution separations of trace biological compounds. Hence, efforts to develop new techniques like voltage-scanning CACE, and to improve performance in established methods (e.g., EFGF) could both have a broad impact on separation science. Furthermore, as shown in this dissertation, fabrication
improvements aimed at enhancing equilibrium-gradient methods, such as the development of PCSLs for interfacing membranes with microchannels, can have unforeseen but valuable uses that extend well beyond their original intended applications.
6.3 REFERENCES


