Microelectrophoresis system utilizing conductivity detection analyzing biological molecules

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MICROELECTROPHORESIS SYSTEM UTILIZING CONDUCTIVITY DETECTION ANALYZING BIOLOGICAL MOLECULES

A Dissertation

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by

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Ω Dedication Ω

I dedicate my dissertation in loving memory of my beloved father, Earl Daniel Galloway, Sr. and to my living mother Carrie Bell Taylor Galloway. I thank the heavens for granting me a father and mother who pronounced and demonstrated the "true" values of LIFE and the wisdom that has been instilled in my spirit. You both have said, "With The Lord, You Can Never Go Wrong", "The Trinity (Father, Son, and Holy Spirit) Will Always Be With You", and "Whatever You Shall Ask Mother Earth, In Due Season She Will Pour Out The Blessings From Heavens Window". Daddy, although your flesh is not with me, I know that your spirit lives within me. On the day you passed, I will NEVER forget that beautiful blue jay that came to my rescue to let me know that you are in the hands of God. Still today, that blue jay returns as a reminder. I will ALWAYS treasure the moments that God has granted us here on this earth. When God's Will for my life is finished here on earth, I know that our spirits will meet again, this time in everlasting love. Thank you for being such a God-giving father and a great husband to my strong and vibrant mother. You are still greatly loved and missed by the family and the presence of life.
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Symbols and Abbreviations

α………………….. selectivity factor
amols…………… attomoles
bp…………………. base pair
°C………………… degree celsius
CE………………… capillary electrophoresis
CEC……………… capillary electrochromatography
C_D……………… double layer cell capacitance
CGE………………… capillary gel electrophoresis
cm…………………… centimeter
CMC……………… critical micelle concentration
COMOSS………… collocated monolith support structures
C_p……………… parallel cell capacitance
CZE………………… capillary zone electrophoresis
dNTP…………… deoxynucleoside triphosphates
E…………………… electric field
ECD……………… electrochemical detection
EOF……………… electroosmotic flow
et.al……………… and others
FSE……………… free-solution zone electrophoresis
G………………… conductivity response
h………………… hour
HARM……………… high-aspect-ratio microstructures
i.d……………… inner diameter
i.e………………… for example
ITP………………… isotachophoresis
J………………… joule
k’……………….. retention factor
kV……………… kilovolts
L…………………… length
LC………………… liquid chromatography
LIF……………… laser-induced fluorescence
LOD……………… limit of detection
m………………… meter
M………………… molar
MΩ……………… megaohms
MEKC…………… micellar electrokinetic chromatography
MEMS…………… microelectromechanical system
min……………… minute
mL……………… milliliter
mm……………… millimeter
mM……………… millimolar
MS……………… mass spectrometry
N………………… plate numbers
η………………… viscosity
ng…………………… nanogram
nL…………………… nanoliter
nm…………………… nanometer
nM…………………… nanomolar
nmols……………… nanomoles
ns………………… nanosecond
o.d…………………… outer diameter
OT…………………… open-tubular
P…………………… power
Pa………………. pascal
PC…………………… poly(carbonate)
PCR……………… polymerase chain reaction
pL…………………… picoliter
PMMA……………. poly(methyl methacrylate)
q…………………… charge
RP-IPOCCEC………… reverse-phase ion-pair open channel capillary electrochromatography
Rs…………………… resolution
s………………. seconds
SDS……………… sodium dodecyl sulfate
SEM……………… scanning electron microscopy
SFM……………… scanning force microscopy
$\sigma^2_{ads}$………… adsorption variance
$\sigma^2_{det}$………… detector variance
$\sigma^2_{diff}$……… diffusion variance
$\sigma^2_{inj}$………… injection variance
$\sigma^2_{temp}$………… temperature variance
$\sigma^2_{tot}$………… total variance
S/N……………… signal-to-noise ratio
TEAA……………. triethylammonium acetate
Tg…………………. glass transition
tm………………. migration time
tmc………………. micelle migration time
tR………………. retention time
$\mu$A……………… microamps
$\mu$app…………… apparent mobility
$\mu$eof…………… electroosmotic mobility
$\mu$ep…………….. electrophoretic mobility
$\mu$L……………. microliters
$\mu$m……………. micrometer
$\mu$s…………… microsecond
$\mu$TAS………… micro total analytical system
UV………………. ultraviolet
V…………………… volts
$\nu$app…………… apparent velocity
$\nu$eof…………… electroosmotic velocity
\( \nu_{cp} \) ................. analyte velocity
\( \zeta \) ...................... zeta potential
Abstract

Microfabrication technology has proven to be a valuable tool for creating polymer-based devices utilized in chemical and biochemical assays. Although, reducing the size of the device allows for short analysis times and reduces the reagent demand to ultrasmall volumes (< 1 nanoliter), a resulting consequence is the constraint placed on the limits of detection associated with the detector hardware required for readout. To overcome such constraints, laser-induced fluorescence (LIF) is often employed as a detection method as it provides low detection limits, which approach the single molecule level. Unfortunately, most LIF systems do not offer the benefits of miniaturization, with the detector components (i.e. laser, optics, filters) often times requiring a much larger footprint compared to the device. Another readout strategy that has shown promise for these devices is conductivity detection. Detection can be accomplished using either conventional-size or microfabricated electrodes, which can be integrated on the device. Although conductivity has been commonly used to detect inorganic or small organic species, the potential for detection of biological species has received little attention. In this work, an integrated conductivity detector was developed for the analysis of amino acids, peptides, proteins, and oligonucleotides (double-stranded DNA). Using the detector, mass detection sensitivities in the range of $10^{-18} - 10^{-21}$ moles were achieved. To increase the throughput of the system a state-of-the-art, multichannel device with a conductivity array detector was devised. This device, which consists of a 16-channel fluidic network and a printed circuit board, is geared toward automating three-processing steps onto a single fluidic platform including purification, preconcentration and detection for downstream parallel processing.
Chapter 1 Capillary Electrophoresis (CE)

1.1 Origin of Capillary Electrophoresis (CE)

This chapter introduces the origin of capillary electrophoresis (CE), the driving force behind CE, optimization parameters, separation modes and principles, as well as the fabrication of miniaturized CE devices, and the transduction modes commonly employed for CE.

Capillary electrophoresis (CE) has a long history of development dating back to the late 1880's. During that time, many experiments were performed using glass 'U'-shaped tubes in the upright configuration with electrodes (platinum foil) connected to each of the tubes' arms. In the 1980's, Jorgenson and Lukacs convincingly demonstrated the power of CE after a high efficiency separation resulted after applying an electric field across a narrow-bore fused silica (glass) capillary tube. With rapid advances being made in different modes of CE, including capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC) and capillary electrochromatography (CEC), commercial CE apparatus became available toward the end of the 1980's.

CE is an analytical technique complementary to liquid chromatography (LC), in that both involve the separation of analytes in a sample mixture. Contrary to LC, the benefits of CE include; (1) miniaturization of the operating platform; (2) faster analysis times; (3) reduced sample volumes (typically a few nanoliters (nL)); and (4) provides separation efficiencies up to two orders of magnitude greater than LC.

As stated above, the operating platform of CE is less complex than LC due to the absence of an injector, pumps, pressure gauges, mixers, and flow splitting valves. CE consists of six primary units including a high voltage power supply, injection system (autosampler), capillary, anode and cathode reservoirs, and an online detector (see Figure 1.1). To allow for online
detection, a window is formed by burning, scraping with a razor, or chemical leaching with an acid a section of the polyimide coating on the capillary.

**Figure 1.1.** Representation of CE analysis.\textsuperscript{14} Electrically charged and neutral analytes suspended in the carrier electrolyte migrates along with the stronger moving EOF toward the cathode (detection end) when an electric field is applied across the capillary. Cationic analytes are first detected, then neutral analytes (though not separated), and lastly the anionic analytes.

### 1.2 CE Driving Force

The driving force behind CE that causes the unidirectional transport of electrically charged and neutral analytes within the boundaries of the capillary is the EOF and the analytes' electrophoretic mobility. EOF is a bulk flow of liquid that results from the double layer produced at the interface of the capillary wall and liquid of opposing charges when a voltage using two electrodes is applied across the capillary.\textsuperscript{12-13, 15} An important feature of the EOF is the distribution of an uniform, flat flow profile (see Figure 1.2A). Under the influence of a flat flow profile, all analytes' are swept along at the same velocity irrespective of their charge and cross-sectional position within the capillary. This is in contrast to a parabolic flow profile.
commonly seen in pressure-driven systems, such as LC (see Figure 1.2B). Because of pressure differences across the column, analytes' in the center of the column move at a faster velocity than those located near the column wall. The differences in these velocities create a non-uniform flow that leads to band broadening defined as the spreading of analyte zones as they are transported through the capillary or column (see section 1.3 for details).

![Figure 1.2. Cross-sectional view of (A) flat and (B) parabolic flow profiles.](image)

The linear velocity of the EOF ($v_{eof} \text{ m s}^{-1}$) can be described as:

$$v_{eof} = \frac{\varepsilon \xi}{4\pi \eta} E = \frac{\sigma}{\kappa \eta} E \quad (1.1)$$

where $\varepsilon$ is the dielectric constant of the solution (C$^2$ J$^{-1}$ m$^{-1}$), $\eta$ is the solution viscosity adjacent to the wall (N s m$^{-2}$), $E$ is the electric field (V m$^{-1}$), $\sigma$ is the charge density on the capillary wall (C m$^{-2}$) and $\kappa$ is the double layer thickness (m), and $\xi$ is the zeta potential (V) defined as the
potential difference between the capillary-solution interface (potential of the diffuse double layer). Variables that can affect the zeta potential include solution pH, ionic strength, dielectric constant, and viscosity. The electroosmotic mobility \( \mu_{\text{eof}} \) is given by:

\[
\mu_{\text{eof}} = \frac{v_{\text{eof}}}{E} \quad (1.2)
\]

There are two straightforward ways to measuring the electroosmotic mobility. One approach is the current monitoring method wherein the motion of a fluid is monitored by a change in the current flow as the electrolyte in the capillary is displaced by an electrolyte of the same pH, but of different ionic strength. Another approach is recording the elution time of a neutral marker carried under the action of only the EOF and has negligible interaction with the capillary wall.

1.3 Band Broadening

As mentioned earlier, band broadening is the spreading of analyte zones as they migrate through a capillary or column. The amount an analyte zone spreads is given by the total variance \( \sigma_{\text{tot}}^2 \), which is proportional to peak width at one-half of its height \( w_{1/2} = 2.354 \sigma_{\text{tot}} \). The total variance is equal to sum of all variances given by:

\[
\sigma_{\text{tot}}^2 = \sigma_{\text{inj}}^2 + \sigma_{\text{diff}}^2 + \sigma_{\text{temp}}^2 + \sigma_{\text{ads}}^2 + \sigma_{\text{det}}^2 + \sigma_{\text{o}}^2 + \ldots \quad (1.3)
\]

where the subscripts refer to band broadening caused by injection \( (\text{m}^2) \), diffusion \( (\text{m}^2) \), temperature gradients (Joule heating) \( (\text{m}^2) \), adsorption \( (\text{m}^2) \), detection \( (\text{m}^2) \), and other sources such as electromigration dispersion (also known as "sample overloading") \( (\text{m}^2) \), and nonuniform flow profile, respectively. From the above equation, these sources of band broadening can lead to concurrent loss of efficiency \( N \) (plate numbers) and resolution \( (R_s) \) described as;
where \( L \) is the capillary or column length (m), \( \Delta \nu \) is the difference in zone velocities (m s\(^{-1}\)) and \( \nu \) is the average zone velocity (m s\(^{-1}\)). During electrophoresis, band broadening is mainly dominated by diffusion related to longitudinal and radial molecular diffusion and convective (thermal) diffusion. Longitudinal diffusion is diffusion parallel to the direction of the analyte migration while radial diffusion is perpendicular to the direction of migration. Longitudinal and radial molecular diffusion are mainly influenced by the analyte diffusion coefficient, which is the rate an analyte diffuse in a liquid. In general, smaller molecules (i.e. amino acids) have higher diffusion coefficients than larger molecules (i.e. proteins, DNA). Therefore, it is possible to obtain higher plate numbers for larger molecules described as:

\[
\mathcal{N} = \frac{(\mu_{ep} + \mu_{coef}) V}{2D} \tag{1.5}
\]

where \( \mu_{ep} \) is the analyte electrophoretic mobility (m\(^2\) V\(^{-1}\) s\(^{-1}\)), \( V \) is the applied voltage (V), and \( D \) is the diffusion coefficient (m\(^2\) s\(^{-1}\)).

In addition to molecular diffusion, the other major band broadening factor is convective diffusion. Convective diffusion results from Joule heating, generated by the passage of current, which warms the solution due to resistive heating. Consequently, band broadening results from analytes in the warmer, center of the capillary migrating faster than those located near the cooler wall. One main approach to minimizing convective diffusion is reducing the capillary diameter.

The sources of band broadening can never be totally eliminated, but their impact on the chromatographic efficiency and resolution can be controlled by appropriate design of the instrumental equipment and by careful selection of the operational parameters.
1.4 Optimization Parameters in CE

1.4.1 Capillary

Capillaries are fused silica tubes that can range in size, typically having dimensions of 25 - 100 centimeters (cm) in length and 10 - 300 micrometers (µm) in inner diameter (i.d.). Polyimide coatings are used to increase the mechanical strength of the capillary since bare silica is extremely fragile. Smaller i.d. capillaries have higher surface area-to-volume ratios wherefore heat, generated by the passage of current through the capillary, can be effectively dissipated through the walls of the capillary to prevent severe, thermally-induced band broadening. Thus, higher voltages necessary for both high efficiencies and rapid separations can be adopted. Assuming that heat generated in this manner, or Joule heating, is efficiently dissipated, then the electrical power dissipated per unit length of the capillary is given by:

$$\frac{P}{L} = \frac{\kappa Cr^2 V^2}{L^2}$$

(1.6)

where $\kappa$ is the electrolyte molar conductance (m² mol⁻¹ Ω⁻¹), $C$ the electrolyte concentration (mol m⁻³), and $r$ is the radius of the capillary (m). Clearly, the use of smaller i.d. capillaries, long capillaries, or a combination of the two can dissipate heat quickly. However, the use of a longer capillary can result in longer analysis times. An alternative is active cooling of a shorter length capillary.

1.4.2 Surface Modification

The inner wall of a capillary contain silanol (SiOH) groups, which can influence the EOF in several ways. At high pH, these groups are deprotonated (SiO⁻) whereby hydrated cations from the electrolyte are attracted to the fixed negative charges on the capillary surface from which a double layer is produced at the capillary-solution interface. The cations in this layer are not
dense enough to preserve electroneutrality; thus a mobile layer of cations will form. Upon application of the electric field, the mobile layer will migrate toward the cathode and, owing to viscous drag, transports the bulk liquid (EOF) inside the capillary. At pH values < 3, silanol ionization is repressed to the extent that the EOF is negligible. It is also possible to vary the EOF by chemically modifying the capillary wall with dynamic,\textsuperscript{24-25} charged hydrophilic,\textsuperscript{26-27} or hydrophobic\textsuperscript{28-29} coatings. These coatings can either reduce or eliminate the EOF and analyte-silanol interactions, and in some cases reverse the direction of the EOF (toward the anode). An ideal wall coating is stable under conditions required for separation, preferably over a broad range of electrolyte pH.

### 1.4.3 Injection Modes

To preserve the high efficiency capabilities of CE, the injection mode should be capable of delivering a volume of sample into the capillary efficiently and reproducibly to avoid significant broadening of the analyte zones in the sample mixture.\textsuperscript{30-31} Because of this, hydrodynamic and electrokinetic injection modes are commonly used (see Figure 1.3). In hydrodynamic modes, a positive pressure is applied at the injection end of the capillary causing a volume of sample to be forced into the capillary, whereby the sample injected is a true representation of the sample mixture. The quantity injected (\(V_{\text{inj}}\)) is a function of several variables as shown below;

\[
V_{\text{inj}} = \frac{\Delta P \pi C \cdot t \cdot d^4}{8 \cdot \eta \cdot L}
\]  

(1.7)

where \(\Delta P\) represents the pressure differences across the capillary (N m\(^{-2}\)), \(C\) is the sample concentration (mol m\(^{-3}\)), \(t\) is the injection time (s), and \(d\) is the capillary i.d. (m). Equation 1.7 shows that the sample volume can be manipulated by varying the injection time and/or the
pressure difference, but should be carefully optimized to avoid zone broadening as a result of the parabolic profile that is characteristic of a pressure-driven flow (see Figure 1.2B).\textsuperscript{13}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.3.png}
\caption{Illustration of hydrodynamic and electrokinetic injection modes. After injection, the electrolyte vial replaces the sample vial and the separation process can begin.}
\end{figure}

For electrokinetic injection, a voltage is applied at the injection end of the capillary while the other end is maintained at ground. The quantity injected ($V_{inj}$) is described as:

\begin{equation}
V_{inj} = \frac{V \pi C t \tau^2 (\mu_{ep}^+ + \mu_{EOF})}{L} \tag{1.8}
\end{equation}

where $V$ is the injection voltage (V) and $t$ is the injection time (s) (i.e. time over which the injection voltage is applied). As seen from the above equation, the analytes in the sample enters the capillary by a combination of their mobilities and the EOF. This means that during electrokinetic injection, two types of sample biasing is likely to occur.\textsuperscript{32} One type occurs in the event that faster moving analytes are injected in larger quantities than the slower moving
analytes due to differences in their mobilities. Another is related to the conductivity difference between the sample and carrier electrolyte solutions. Since the analytes' mobilities and electroosmotic mobility are different in different solutions, changes in the absolute amount injected can result. Nevertheless, sample biasing can be resolved using the sample stacking technique, which reduces the width of the injected sample zone before separation. In this technique, the sample is suspended in a low ionic strength electrolyte and is injected into the capillary filled with the same electrolyte, but of higher ionic strength. Under these circumstances, analytes rapidly migrate to the interface between the lower and higher conductivity zones. Upon reaching the interface, the analytes then slowly stack into a narrow band.

1.4.4 Electrolyte pH and Ionic Strength

The pH of the electrolyte is a variable that can affect the charge on the capillary surface, and thus the zeta potential is affected resulting in differences in the EOF. At low pH, the zeta potential is lower due to less silanol ionization. As a result, the EOF will decrease. At high pH, the zeta potential is greater, causing the EOF to increase. Modifying the pH can also result in altering the mobilities of analytes' that are sensitive to pH.

The electrolyte ionic strength or concentration can modify the EOF by increasing or lowering the zeta potential. Higher ionic strength electrolytes lowers the zeta potential because of a compression in the double layer thickness. As a result, the EOF will decrease. The use of high ionic strength electrolytes can give rise to Joule heating in the capillary owing to the larger conductivity of the electrolyte solution. If the heat is not effectively dissipated, it can initiate changes in the electrolyte pH or result in sample decomposition (i.e. denaturation of proteins).
1.4.5 Organic Modifiers

Organic modifiers are organic solvents such as acetonitrile, methanol, ethanol, or 2-propanol, that possess different dielectric constants and viscosities. These solvents can be used to increase the solubility of analytes that are insoluble in an aqueous media or as an electrolyte additive to improve the selectivity of a separation system, for example capillary electrochromatography (CEC).\textsuperscript{9-11} As a consequence, both the EOF and the mobility of the analytes' are affected. Keeping the electrolyte concentration constant, the EOF generally increases with increasing organic solvent in the electrolyte.\textsuperscript{36-38} Crego and others reported this trend relates to modifications in the dielectric constant to viscosity ratio of the electrolyte, which can affect the zeta potential.\textsuperscript{39-40}

1.5 CE Separation Modes and Principles

1.5.1 Capillary Zone Electrophoresis (CZE)

Capillary zone electrophoresis (CZE) is the simplest form of CE and is often termed free-solution electrophoresis (FSE). CZE provides an electrophoretic technique for separating charged analytes.\textsuperscript{2-6, 41} The separation relies principally on differences in the analytes' mobilities, which is dependent upon the analyte size and charge at a given pH. The mobility (\(\mu_{ep}, \text{m}^2 \text{V}^{-1} \text{s}^{-1}\)) of a charged analyte (for spherical particles) is described as;

\[
\mu_{ep} = \frac{q}{6\pi \eta r} \quad (1.9)
\]

where \(q\) is the charge on the analyte (C) and \(r\) is the radius of the analyte (m). Equation (1.10) shows that the velocity (\(v_{ep}, \text{m s}^{-1}\)) of a charged analyte is directly related to the electric field given by;

\[
v_{ep} = \mu_{ep} E \quad (1.10)
\]
The time \((t_m, s)\) taken for the charged analyte to migrate through the capillary is represented by the following equation;

\[
    t_m = \frac{L}{(v_{ep} + v_{eof})} \tag{1.11}
\]

where \(L\) is the effective capillary length, which is the length of the capillary from injection to the detector. The sum \((v_{ep} + v_{eof})\) is equal to the apparent velocity \((v_{app}, \text{ m s}^{-1})\). The apparent mobility \((\mu_{app}, \text{ m}^2\text{ V}^{-1}\text{ s}^{-1})\) is given by;

\[
    \mu_{app} = \mu_{ep} + \mu_{eof} \tag{1.12}
\]

### 1.5.2 Micellar Electrokinetic Chromatography (MEKC)

Micellar electrokinetic chromatography (MEKC) can be used to separate electrically charged analytes, but is mainly devoted to the separation of neutral analytes, which are not separable by CZE. To carryout MEKC, a surfactant, such as sodium dodecyl sulfate (SDS), is employed as an electrolyte additive.\(^7\)\(^8\) A surfactant is a detergent that possesses amphiphilic properties, in which the head group is polar and the tail group is nonpolar, usually an alkyl chain. At concentrations above the critical micelle concentration (CMC), which in the case of SDS is 8.27 mM, the tail \((12\text{- carbon chain})\) will align toward the center and the head \((\text{sulfate})\) group along the surface forming spherical micelles. These micelles create a slower-moving pseudostationary phase, whereby neutral analytes can distribute between the SDS micelle hydrophobic interior and the faster-moving surrounding aqueous phase (see Figure 1.4). This partitioning behavior contributes to the separation in MEKC.
Figure 1.4. Schematic representation of a MEKC system. Shown is the EOF migration, anionic SDS micelle migration, and partitioning behavior of the electrically neutral analyte (S) between the SDS micelle and surrounding aqueous phase under normal capillary wall conditions.

The EOF is the electrically-driven pump that drives the aqueous phase toward the cathode, whereas the anionic SDS micelles are attracted to the anode. At a pH where there is an appreciable EOF, SDS micelles are eventually swept in the same direction as the cathodic EOF.

To determine the analyte retention time, the retention factor \( k' \) is preferred since it accounts for the two moving phases and is given by:

\[
k' = \frac{t_R - t_0}{t_0 (1 - t_R / t_{mc})}
\]

where \( t_0 \) is the time (s) of an analyte that has little or no interaction with the SDS micelles, \( t_R \) is the retention time (s) of an analyte migrating between \( t_0 \) and \( t_{mc} \), and \( t_{mc} \) is the retention time (s) of an analyte that is totally solubilized by the micelles. Retention behavior of neutral analytes is governed by hydrophobicity, given the more hydrophobic analyte eluting later in the micellar phase \( t_{mc} \). The resolution (\( R_S \)) between the analytes can be expressed as;
\[ R_s = \left( \frac{N^{1/2}}{4} \right) \left( \frac{\alpha - 1}{\alpha} \right) \left( \frac{k'_2}{k' + 1} \right) \left( \frac{1 - t_0 / t_{mc}}{1 + (t_0 / t_{mc})k'_1} \right) \] (1.14)

where \( N \) is the plate numbers, \( \alpha \) is the selectivity factor, \( k'_2 / k'_1 \), and the subscripts 1 and 2 refer to retention factors of earlier and later eluting peaks.

1.5.3 Capillary Electrochromatography (CEC)

Capillary electrochromatography (CEC) is considered a hybrid of CE and LC, whereby the retention mechanisms and selectivity afforded by LC are retained. However, the pressure-driven flow of LC is removed and replaced by the EOF. The EOF serves as a pump to propel mixed organic/aqueous solvents (mobile phase) and electrically charged and neutral analytes through a packed or open-tubular (OT) capillary.\(^9-11, 42-44\) Unlike OT capillaries, packed capillaries normally contain microparticles coated with various types of LC stationary phases,\(^{40}\) particularly an alkyl-bonded (hydrocarbon) stationary phase.\(^{42, 44-45}\) Although, packed capillaries impart a significantly larger sample capacity (higher surface area) relative to open-tubular capillaries; the integrity of the microparticles dictates the capillary performance, which is ultimately controlled by the packing technique.\(^{40, 46-47}\) Capillaries that have been poorly packed can lead to the development of eddy dispersion, which can contribute to asymmetric peak shapes, poor resolution, and low efficiencies. Consequently, several techniques have been developed for the fabrication of packed capillaries via pressure, pseudoelectrokinetic, carbon dioxide, centripetal forces, and gravity.\(^{40}\) One of the major problems in packing capillaries is the retaining frits, which are used to restrict the packing material to the inside of the capillary. Unfortunately, the presence of frits can introduce side effects such as nonspecific (secondary) interactions, gas-bubble formation during CEC analyses, increased backpressure during LC analyses, increased
fragility of capillaries, column-to-column reproducibility problems, and poor column performance due to disruption of the structure of the separation medium.

In CEC using a packed capillary, the cathodic EOF is mainly contributed from the localized double layer around the silica microparticles, since most of the area is provided by the microparticles, with negligible contribution from the capillary wall (see Figure 1.5).

![Figure 1.5. Schematic representation of a CEC system. Shown is the EOF migration and partitioning behavior of electrically charged or neutral analytes (S) between the alkyl-bonded stationary phase and mobile phase (MP, organic modifier) under normal capillary wall conditions. Electrically charged analytes are separated by a combination of mobility differences and partitioning between the stationary phase and mobile phase, similar behavior observed in LC. To increase the hydrophobic interaction between the charged analyte and the stationary phase, an ion-pairing agent can be employed as an electrolyte additive. An ion-pairing agent is often a cationic or anionic salt with an alkyl chain of various lengths. For example, quaternary ammonium alkyl halides are cationic salts used as pairing agents for anions (i.e. DNA) and sodium alkyl sulfates are anionic salts used as pairing agents for cations. These agents act as a counter-ion to form a neutral ion-pair complex with the electrically charged analyte to transfer the analyte from a polar aqueous phase to a nonpolar alkyl-bonded stationary phase. As for...](image-url)
neutral analytes in CEC and LC, the separation is based on the partitioning between the two phases. The separation principles for both electrically charged and neutral analytes also apply in CEC using an OT capillary with the exception of having the stationary phase affixed to the capillary wall.\textsuperscript{11, 43-44, 51} El Rassi and coworkers synthesized a silica-based stationary phase comprised of two layers. The sublayer contained sulfonic acid groups, which were tethered to the capillary wall.\textsuperscript{52} The sublayer was then used as a scaffold to covalently attach a toplayer containing octadecyl (C18) groups. The sulfonic acid groups were not end-capped, whereby unreacted silanol groups contributed to the EOF. Presence of these residual negative charges on the capillary wall lead to mixed retention mechanisms including cation exchange and hydrophilic/hydrophobic phase partitioning, all which contributed to reduced chromatographic efficiency.

In CEC as in LC, there are several dispersion processes that can contribute to band broadening. The influences of these processes on capillary and/or column plate height (quantitative measure of chromatographic efficiency) are additive. Extracolumn effects caused by instrumental factors, such as injection and detection can also cause observed band broadening. However, these extracolumn effects were reported to be negligible.\textsuperscript{40} Equation (1.15) is the van Deemter equation that describes these dispersion processes. These processes are eddy diffusion, (first term), longitudinal molecular diffusion (second term), and mass transfer between two phases (third term) given by;

\[ H = 2\lambda d_p + 2 \gamma \frac{D_m}{\nu} + \frac{k^2 d_p^2}{16 (1 + k^2) D_m} \nu \]  

\text{(1.15)}

where \( \lambda \) is the constant which is almost close to 1, \( d_p \) is the particle diameter (m), \( \gamma \) is the factor which is related to the diffusion restriction by column packing, \( D_m \) is the analyte diffusion
coefficient in the mobile phase \( (m^2 \text{ s}^{-1}) \), and \( v \) is the flow velocity \( (\text{m s}^{-1}) \).

Horvath and coworkers reported band broadening associated with eddy diffusion was smaller in CEC than in LC by a factor of 2-4\(^{40} \). This effect was attributed to the EOF flat flow profile reducing multipath band dispersion. In LC, band broadening associated with mass transfer was higher than in CEC if the average pore size > 300 Å. Band broadening due to maldistribution and mass transfer is smaller when the mobile phase flow is driven by voltage as in CEC, rather than by pressure as in LC.

### 1.6 Microfabricated CE Device and Transduction Modes

Microfabricated CE devices, commonly termed microelectromechanical system (MEMS), enables downscaling and integrating various experimental steps of the assay onto a single fluidic platform to analyze ultrasmall volumes of material (< 1 nL). One of the main driving force behind the development of these devices is the dramatic speed increase for analysis than a conventional platform with comparable separation performance.\(^{53-57} \) Devices fabricated in glass or polymers have confirmed their high application potential in the areas of biochemical and chemical analysis. These areas have included separation techniques such as electrophoresis, gel electrophoresis, isotachophoresis, electrokinetic chromatography, and electrochromatography.\(^{53-67} \) In addition, the versatile nature of these devices to perform multiplexed polymerase chain reactions (PCR) is particularly interesting because of its potential widespread application in molecular biology and clinical laboratories for in-depth understanding of genetics and disease processes.\(^{68-69} \) PCR, relative to cloning, is an ubiquitous protocol involving rapid enzymatic amplification of specific DNA sequences via repeated cycles of denaturing, oligonucleotide primer annealing, and DNA polymerase extension (see Figure 1.6).\(^{70-74} \)
Figure 1.6. PCR amplification protocol.

Prior to performing PCR amplification, a molar excess of oligonucleotide primers is mixed with the DNA template, deoxynucleoside triphosphates (dNTPs), and DNA polymerase in the appropriate electrolyte. The DNA template is initially heated to ensure that the template strands are effectively denatured into single strands. The temperature is cooled to a low enough temperature for the annealing of oligonucleotide primers that are bound at the 5′ terminus. While the 3′ terminus is determined by the position at which the DNA polymerase finishes its synthesis at optimum temperature forming new strands (PCR product) of the original template. This process of denaturing, annealing, and polymerase extension can be repeated many times (cycles) resulting in an exponential accumulation of strands that increase the probability of the amplified target regions of DNA having the correct identity.

1.6.1 Microfabrication Technology

Over the years, significant progress has been made in the design of CE devices from simple, single channel structures to the more complex, array channel structures for high-throughput analysis. These devices are typically designed with reservoirs (cathode, anode, sample, waste) and micrometer-sized channel sections (channel tail, cross 'T' or offset 'T', separation channel) of various widths and depths. Specifically, the small cross section of the separation
channel and large thermal mass of the device allows Joule heat to be dissipated efficiently when operating at high electric fields. To this effect, rapid separations and high efficiencies are obtainable in this format.

Earlier CE devices were fabricated in glass by means of standard photolithography and subsequent chemical wet-etching. Motivations of using glass stems from the fact it exhibits excellent optical properties pertinent for ultrasensitive fluorescence detection and its surface structure is similar to conventional capillary tubes producing comparable EOFs and immobilization chemistries. Disadvantages associated with glass-based devices include the expensive cost of the material, the use of hydrofluoric acid (HF) for etching, and high temperature annealing (∼600°C) for assembling the device, all which can limit the feasibility of rapid prototyping. In addition, the isotropic nature of the etching process results in shallow, semicircular channels. Because of this, it is rather difficult for these channels to accommodate fiber optics or capillaries for interconnecting devices. Finally, careful manual handling is imperative considering the fragile nature of the device.

To circumvent the prerequisites associated with glass-based devices, considerable attention has been reallocated toward developing these devices in polymers, which are less-expensive to fabricate than glass-based devices. A major advantage of polymers is that multiple devices can be produced more rapidly from a single master with only minimal use for clean room facilities. In addition, polymers offer a wide range of mechanical and thermal properties that can potentially make them an ideal material for electrophoretic-based assays. In particular, poly(methyl methacrylate) (PMMA) and poly(carbonate) (PC) both possess a thermal conductivity in the range of 0.1 - 0.2 W m⁻¹ K⁻¹, similar to glass. This is a variable which indicates effective dissipation of heat while preserving system performance. The dielectric
strength of PMMA has been reported to be $10^4$ V cm$^{-1}$ comparable to glass, while PC can sustain at least $10^3$ V cm$^{-1}$ for electrophoretic separations without material breakdown effects. The native surface of both PMMA and PC is negatively charged, often attributed to carboxylate (COO$^-$) functionalities, which are the charged groups most likely present in polymers containing ester (COO$^-$) or carbonate (OCOO$^-$) groups (see Figure 1.7). The presence of these groups hold potential for modification to vary the EOF in addition to changing the surface properties to minimize swelling/dissolution induced by organic solvents and analyte-wall interactions, which contributes to peak distortion resulting in reduced chromatographic efficiency.

![Chemical structures of (A) PMMA and (B) PC.](image)

**Figure 1.7.** Chemical structures of (A) PMMA and (B) PC.

Polymer-based devices can be fabricated via LIGA techniques, a German acronym for lithography, electroplating, and molding whereby PMMA is exposed to X-ray radiation (see Figure 1.8). LIGA is advantageous when developing channels with extremely high-aspect-ratio (depth:width) microstructures (HARMs), which can assist in system integration and reducing the size of the device. First, the desired device topography is designed using a computer-aided program (CAD), and is subsequently transferred to an optical mask (chromium-coated quartz plate with a positive photoresist layer). Chromium serves to block ultraviolet (UV) rays during the development of the X-ray mask. The X-ray mask is prepared on a resist-coated Kapton® film with the desired device topography transferred to the film during UV radiation.
The X-ray mask is aligned over a layer of PMMA bonded onto a stainless steel plating base and this assembly is exposed to X-rays to form microstructures. After removal of exposed PMMA, the voids are filled with Ni using an electroplating bath. The Ni electroform molding die (master or embossing tool) is then refined with the unexposed PMMA intact to serve as a support for the raised Ni microstructures while mechanical polishing. Hence, the most expensive step is the fabrication of the Ni master, which can be used to make many replicates in different polymeric materials. The Ni master is mounted together with the planar polymeric wafer in an embossing system vacuum chamber. Vacuum is essential to prevent formation of air bubbles due to entrapment of air in small cavities. It also drives out water vapors that could be present in the polymeric wafer during the molding process. Furthermore, it sustains the lifetime of the Ni master by preventing corrosion of the metal surface at elevated temperatures. The Ni master and the polymeric wafer are heated above the glass transition ($T_g$) of the polymeric material. Once the appropriate temperature has been reached, the Ni master is brought into contact with the polymeric wafer at a controlled force over a period of time. While applying the embossing force, the raised Ni microstructures are replicated in the polymeric wafer. This step can be repeated numerous times for different polymeric materials with minimal replication errors. The vacuum chamber is then slowly cooled below the $T_g$. Afterwards, the Ni master and embossed polymeric wafer are mechanically driven apart. To assemble the device, a cover plate of the corresponding polymeric material is placed over the embossed polymeric wafer and the two pieces are thermally annealed together at a temperature dependent upon the $T_g$ of the polymeric material.
Laser-induced fluorescence (LIF) is so far the most popular detection scheme for CE devices because of its exquisite sensitivity with detection limits approaching the single-molecule level.\textsuperscript{57, 81-83} Most LIF systems do not offer the benefits of miniaturization, with the detector components (i.e. laser, optics, filters) often times requiring a much larger footprint compared to the device. In addition, alignment procedures for the detector hardware can be time-consuming. LIF is selective for analytes that contain fluorophores, which have the ability to fluoresce under excitation by the laser-operating wavelength. For nonfluorescent analytes, the use of derivatization or labeling reactions to convert the analytes to fluorescent entities is necessary.
An alternative for nonfluorescent analytes is indirect LIF, wherein a fluorophore present in the electrolyte is displaced by the nonfluorescent analyte. Indirect LIF is also applicable for analytes that are not so easily derivatized. Nevertheless, indirect LIF offers poor detection limits, which is partly due to the fact that existing laser sources can be unstable, yielding a noisy background signal.\(^8^4\)

Mass spectrometry (MS) has been coupled to CE devices through an electrospray ionization tip.\(^8^5\)\(^-^8^7\) MS has the advantage to acquire structural information for a broad range of non-labeled analytes in complex sample mixtures. Unfortunately, commercially available MS systems offer limited compatibility with device miniaturization. Moreover, the detection limit and sensitivity is comparable to that obtained for UV absorbance detection.

Electrochemical detection (ECD) including amperometric, potentiometric, and conductivity, have become an attractive choice in devising miniaturized electrophoretic systems such that sample injection, separation, and detection processes are all integrated into one device. In these systems, detection can be accomplished using conventional-size\(^5^6, 5^8-5^9, 8^8-9^0\) or microfabricated electrodes,\(^9^1-1^0^0\) that are composed of gold (Au), platinum (Pt), palladium (Pd), copper (Cu), or carbon (carbon ink, carbon paste, carbon fiber). The configuration and placement of the electrodes relative to the separation channel generally results in rapid response times, favorable sensitivity and limits of detection. However, alterations in the detector response and unwanted zone broadening can result from interferences between the separation voltage and the electrode potential. In this case, electrical decoupling of the separation voltage and detection electronics is required. Electrical decoupling can be accomplished by placing the electrode into the sidewalls adjacent to the separation channel or at the exit end of the channel.\(^5^5, 5^8, 1^0^1\) Another consideration is the adsorption of analytes to the electrode surface, which can lead to impaired
performance and high-background currents. The presence of high-background currents results in poor signal-to-noise ratio (S/N), which describes the quality of an analytical technique and performance of its instrumentation. To preserve optimum performance, periodic cleaning of the electrode surface by polarization followed by reverse polarization is one option. Another option is insulating the electrode with a thin dielectric layer of titanium dioxide, tantalum pentoxide, silicon nitride, or silicon carbide.\textsuperscript{102} Insulating the electrode can, however, decrease the sensitivity and also, thermal breakdown of the insulating material is likely to occur.

Amperometric and potentiometric detection systems are comparable to LIF; they are selective such that the target analyte must be intrinsically electroactive or an electroactive species must be appended to the analyte to perform electrochemical reactions (Faradaic processes) at the electrode surface. The primary advantage of these detection systems is the good selectivity, in that only electrochemically active species produce a response. However, this selectivity can be turned into a disadvantage, since it strongly limits the applicability of the detection system.

Conductivity is often considered an indirect detection method that has the ability to transduce any charged analyte irrespective of whether or not it contains a fluorophore or electroactive species. The only requirement is that the conductance of the migrating analyte zone be different from the carrier (background) electrolyte. The performance of conductivity detection can be limited by fluctuations in high conductivity electrolytes, which aids in the formation of gas-bubbles. In this case, low conductivity electrolytes are preferable. Measurement of the conductance can be performed via a computer-controlled bipolar pulsing waveform technique first developed by Enke and coworkers, in which one electrode is maintained at virtual ground while a bipolar pulse waveform is applied to the other electrode.\textsuperscript{103} Bipolar pulse waveform
consists of consecutive constant voltage pulses of equal magnitude but opposite in polarity ($\pm V$) at an ideal frequency, which is appropriately chosen to avoid Faradaic processes. The current flowing between the electrodes is measured at the end of the second pulse, which is considered an accurate representation of the solution Ohmic resistance over a dynamic range of $100 \Omega - 1M \Omega$.

1.7 Miniaturized CE System

The operating platform for a miniaturized CE system is consisted of a high voltage direct current power supply, high voltage relay (switch box), microfluidic device, conductivity detector, data acquisition board, and a computer to display the data output (see Figure 1.9). The board controls the switching signal of the relay with outputs distributed to each solution reservoir configured on the device. In addition, the board controls the electronic circuitry, which is responsible for producing a bipolar pulse waveform for conductivity measurements.

Figure 1.9. Block diagram of microelectrophoresis system apparatus.

Incorporated into the instrument design are hardware devices and software techniques such as ground and shielding, sample-and-hold amplifier, differential amplifier, analog and digital filtering, modulation, and signal averaging. These devices and techniques can remove and/or attenuate the noise without significantly affecting the detector response, thus, improving the S/N.
For operation of the microfluidic device (see Figure 1.10), points (ii), (iii), and (iv) on the device are filled with the carrier electrolyte solution and the sample is manually loaded into point (i). The sample is introduced electrokinetically into the cross offset 'T' by applying a high voltage to point (ii) while points (i), (iii), and (iv) are maintained at ground for the appropriate amount of time to completely fill the cross offset 'T'. The high voltage is then switched to point (iv) while point (iii) is at ground and ~ 10 - 20% of the high voltage is applied to points (i) and (ii) to minimize sample leakage into the separation channel. Analytes will begin to form zones as they migrate toward the finish line format conductivity detection.

**Figure 1.10.** Representation of miniaturized CE system performed in reverse mode (detection end at the anode). Also shown is a simple electrical bipolar waveform model of the capacitively coupled 2-electrode conductivity detector. \( C_P \) is the parallel cell capacitance, \( C_D \) is the series cell capacitance (double layer capacitance), \( R \) is the cell resistance, \( R_F \) is the feedback resistor, and DA is the differential amplifier.
Upon entering the T-cell, via a capacitively coupled 2-electrode detector, a brief pulse of positive polarity is applied to one electrode and $C_P$ will charge quickly to the positive potential. During some time, the charge on $C_P$ will exponentially decay and the current flowing through the resistor will drop slightly due to charging of $C_D$. To lessen the likelihood of double layer formation at the electrode surface, the polarity of the second pulse is reversed to discharge $C_D$. $C_P$ will charge quickly to the negative potential. As the charge on $C_P$ begins to exponentially decay over time, $C_D$ will take the same charge but with the negative sign. This causes a decrease in current until at the end of the second pulse prior to the reverse polarity, the cell current is measured. At the time of the measurement, the current through $C_P$ is 0 and the entire voltage drop across the cell is the same like on $R$. This potential difference, representative of the cell solution resistance, follows through a feedback resistor, processed by the differential amplifier, converted to a voltage and subsequently reported at an analog output pin, which is displayed as a negative or positive chromatographic peak on the computer. A negative peak will result if the analyte possesses a conductance below that of the carrier electrolyte, otherwise a positive peak will result.

1.8 References


14. www.ucg.ie./ncbes/capillary_electrophores.html


Chapter 2
Chemical Modification of Polymeric Surfaces Used in the Construction of Microanalytical Devices

2.1 Introduction

Polymers have gained increasing popularity for microelectromechanical systems (MEMS) in analytical chemistry primarily due to their ease of fabrication, inexpensive costs, and increasing versatility.1-4 These systems generally take the shape of microanalytical devices that have been fabricated from a variety of different polymers including poly (dimethylsiloxane) (PDMS), poly (methyl methacrylate) (PMMA), and poly (carbonate) (PC), poly (amide) (PA), and poly (ethylene) (PE). Fabrication techniques commonly used for such devices from polymers include X-ray lithography,5 "soft" lithography,6 ultraviolet lithography,7 hot embossing,8 imprinting,9 laser ablation,10 and injection molding.11 In addition, many of these fabrication techniques can be used to produce high-aspect-ratio microstructures (HARMs) with minimal replication errors, which is typically difficult to obtain using wet isotropic etching in glass.12-13

Although considerable progress has been achieved in the design and development of polymer-based devices in the past few years, modification procedures for polymeric surfaces is still in its infancy. Efforts have been made in attaching biomolecules on UV-modified14 and amine-modified15 polymer surfaces. In addition, self-assembled monolayers (SAMs) of colloidal gold particles on amine-modified polymeric surface has been demonstrated.16 As for glass, being chemically similar to conventional capillary tubes maintain many of the surface modification procedures already established using organosilanes,17 covalent,18-19 noncovalent,20 and dynamic coatings.21

In this chapter, we described solution-based modification procedures applicable for polymer-based substrates, such as PMMA. The choice of PMMA stems from the fact that this material
has been used extensively as a resist in lithographic applications for MEMS and the presence of a functional group, methyl ester (COO−), for potential modification. To corroborate the success for differences in the surface properties after chemical modification, wettability and microscopy studies were performed. In addition, characterization of the bulk properties were conducted using a current monitoring procedure22 to determine the electroosmotic mobility and its directional flow dependence on electrolyte pH. The electroosmotic mobility is known to be a function of several parameters with one of the most important parameters being the surface charge on the channel wall.23-24

2.2 Experimental Details

2.2.1 Microchannel Fabrication

Commercially available PMMA sheets (Goodfellow, Berwyn, PA) were mechanically etched on their edges (4 cm x 100 µm x 100 µm). For electrolyte reservoirs, holes (1 mm in diameter) were drilled ~ 5 cm apart through the cover plate. After chemical modification, the fluidic channel (open channel format) was covered with the corresponding cover plate and the two pieces were clamped between two glass plates and thermally bonded together at 107°C for 12 min in a GC oven. After thermal bonding, cylindrical PMMA reservoirs (1 mm in diameter) were epoxyed onto the two predrilled holes.

2.2.2 Preparation of N-lithiodiaminoethane and N-lithiodiaminopropene

Synthesis of N-lithiodiaminoethane and N-lithiodiaminopropane has been previously described.25-26 A colorless homogenous liquid of dry 1,2-diaminoethane (6 mmol, 99%, Aldrich, Milwaukee, WI) was added in a 100 mL round bottom flask that was air-tight sealed and purged under nitrogen for 20 min. After purging, n-butyl lithium (1 mmol, 2M in cyclohexane, Aldrich) was added dropwise (via syringe) at ambient temperature to the colorless liquid to afford a dark
purple product, N-lithiodiaminoethane. After 3 h, the product remained in the purged, sealed flask for no longer than 1 week. Similarly, a yellowish brown product, N-lithiodiaminopropane, was evident after the addition of n-butyl lithium to purged 1,3-diaminopropane (99%, Aldrich) at ambient temperature for 3 h. After 3 h, the product remained in the purged, sealed flask for no longer than 1 week.

2.2.3 Amine (NH\textsubscript{2})-terminated PMMA

Before chemical modification, mechanically etched PMMA microchannels and cover plates were precleaned using HPLC grade 2-propanol (Aldrich), 18 MΩ•cm water (Barnstead, Dubuque, IA) and purged under nitrogen for 20 min in an air-tight vessel. After 20 min, the PMMA surfaces were introduced to N-lithiodiaminoethane or N-lithiodiaminopropane (via syringe) for a given period of time followed by subsequent washes with 2-propanol, 18 MΩ•cm water, and later dried under a stream of nitrogen (see Figure 2.1).

![Synthetic chemical scheme for the amine-terminated PMMA.](image)

**Figure 2.1.** Synthetic chemical scheme for the amine-terminated PMMA.

2.2.4 C18-terminated PMMA

Freshly prepared amine-terminated PMMA substrates were purged under nitrogen for 20 min in an air-tight vessel. After 20 min, amine-terminated PMMA surfaces were introduced to neat N-octadecane 1-isocyanate (98%, Aldrich) (via syringe) for 10 min followed by subsequent
washes with copious amounts of hexanes, toluene, acetone (Aldrich) and finally dried under a stream of nitrogen (see Figure 2.2).

**Figure 2.2.** Synthetic chemical scheme for the C18-terminated PMMA.

### 2.2.5 Sulfonate (SO₃⁻)-terminated PMMA

Sulfonate-terminated PMMA (see Figure 2.3) was prepared according to peptide synthesis procedures.²⁷

**Figure 2.3.** Synthetic chemical scheme for the sulfonate-terminated PMMA.
Freshly prepared amine-terminated PMMA substrates were immersed in a solution of 4-sulfobenzoic acid (1 mM, 95%, Aldrich), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (100 mM, EDC, 99%, Sigma, St. Louis, MO), and HEPES electrolyte (1M, pH 9.0, Fisher, Fair Lawn, NJ) for 3 h. After 3 h, the sulfonate-terminated PMMA surfaces were rinsed with copious amounts of 18 MΩ·cm water and subsequently dried under a stream of nitrogen.

2.2.6 Carboxyl (CO₂H)-terminated PMMA

Carboxyl-terminated PMMA was prepared similar to standard peptide coupling procedures.²⁷ Freshly prepared amine-terminated PMMA substrates were immersed in a solution of 1,9-nonanedioic acid (5 mM, 98%, Aldrich) and 1,3-dicyclohexylcarbodiimide (95 mM, DCC, 99%, Aldrich) in purged ethanol (Aldrich) for 3 h. Following this reaction, the carboxyl-terminated PMMA surfaces were rinsed with copious amounts of purged ethanol then dried under a stream of nitrogen (see Figure 2.4).

![Chemical Scheme Figure 2.4](image)

Figure 2.4. Synthetic chemical scheme for the carboxyl-terminated PMMA.
2.2.7 Surface Characterization

To follow differences in the PMMA surface properties after chemical modification, sessile drop water contact angles of flat PMMA sheets (15 mm x 15 mm x 3 mm) were performed with a VCA 2000 Goniometer (VCA, Billerica, MA). Using a syringe, 4 - 6 µL of 18 MΩ•cm water was placed on the air-side of PMMA and chemically modified PMMA surfaces at ambient temperature. The sessile drop of water was allowed to remain on the surface in the same position for a given period of time until the magnifying camera captured the image of the water droplet. Using software provided by the manufacturer, left and right contact angles of five separate water droplets were taken and averaged for each given substrate. If the measured contact angle is low then the surface is considered hydrophilic. The surface is considered hydrophobic if the measured contact angle is high. It was found in all cases that there were no differences in the left and right contact angles. Contact angles on polymeric surfaces can be influenced by the surface roughness, chemical heterogeneity, or swelling of the polymeric material. In light of this, a variation of the ninhydrin protocol in addition to the protocol described by Ichijima and coworkers were adopted in this study to quantitatively determine the coverage of amines populated on the PMMA surface.

At ambient temperature, scanning force microscopy (SFM) images of native PMMA, amine-terminated PMMA, and C18-terminated PMMA films (3 mm thick) were performed with a Digital Instruments Nanoscope III multimode atomic force microscope (Veeco Inc., Santa Barbara, CA) utilizing the D scanner (13 µm x 13 µm). The microscope was operated in contact-mode and images were flattened using Nanoscope software. Before imaging, the films were rinsed with HPLC grade 2-propanol (Aldrich, Milwaukee, WI), 18 MΩ•cm water (Barnstead, Dubuque, IA), and later dried under a stream of nitrogen.
The electroosmotic mobility and its directional flow in PMMA and chemically modified PMMA microchannels was determined using a current monitoring procedure that employed a discontinuous electrolyte system.\textsuperscript{22} For accuracy in the electroosmotic mobility, this procedure required less than 5\% difference in the electrolyte ionic strength\textsuperscript{31} to avoid its effects on the double layer thickness and electric field strength. The electric field was supplied by a Spellman high voltage power supply (CZ1000R, Plainview, NY) operated at a field strength of 150 V cm\textsuperscript{-1}. Studies were conducted at various pH values using either acetate (pH = 3.0, 5.0) or borate (pH = 7.0, 9.0, 11.0) electrolyte solutions. The microchannel, anode reservoir, and cathode reservoir were filled with the low ionic strength electrolyte (1 mM). Pt electrodes were placed in each reservoir and the current was monitored following application of an electric field, which served as the baseline current. One reservoir was emptied and replaced with the same electrolyte, but of higher ionic strength (2 mM). The electrical field was then applied to the reservoirs containing the low and high ionic strength electrolytes and the current was monitored until no change in the current was observed over the length of the microchannel. After the current had plateaued, the time to reach this plateau was secured from the plot from which the linear velocity (cm s\textsuperscript{-1}) could be calculated. Dividing the linear velocity by the electrical field strength (V cm\textsuperscript{-1}) produced the electroosmotic mobility (cm\textsuperscript{2} V\textsuperscript{-1} s\textsuperscript{-1}). The electroosmotic mobility was calculated and averaged after five consecutive runs at different pH values.

2.3 Results and Discussion

2.3.1 Wettability

The averaged contact angle of native PMMA was found to be 66 ± 2°, similar to the literature value of 67° for a highly ordered methyl ester-terminated monolayer.\textsuperscript{28} For the amine-terminated PMMA, the contact angle decreased to 33 ± 4°, indication of a hydrophilic surface
due to the presence of amine-terminating groups. This value is consistent with that obtained for self-assembled monolayers terminated with hydrophilic functional groups. Similar results were obtained with sulfonate-terminated PMMA and carboxyl-terminated PMMA, which were found to be $39 \pm 3^\circ$ and $33 \pm 2^\circ$, respectively.

Next, we attempted to react N-octadecane 1-isocyanate with the amine-terminated PMMA with the presumption that the resulting C18-terminated PMMA can be used in the development of a microanalytical electrochromatography device, similar to what has been demonstrated for glass-based substrates. When we compared the contact angle of the amine-terminated PMMA versus C18-terminated PMMA, it increased to $103 \pm 10^\circ$, consistent with its high degree of hydrophobicity and close in value to a close-packed methyl surface (ordered, long-chain n-alkanethiols on Au), which has been reported to be $113^\circ$.

2.3.2 Scanning Force Microscopy (SFM) Images

To characterize the surface roughness and chemical homogeneity of PMMA after chemical modification, 2 $\mu$m x 2 $\mu$m contact-mode topography images were performed in reference to the PMMA surface (see Figure 2.5).

![SFM images](image.png)

**Figure 2.5.** SFM images of (A) PMMA; (B) amine-terminated PMMA and (C) C18-terminated PMMA surfaces. The scan size is 2 $\mu$m x 2 $\mu$m and the Z-range is 10 nm.
As can be seen, the PMMA surface was relatively smooth without defects, which was incomparable to an overgrowth of defects observed for the amine-terminated and C18-terminated PMMA surfaces. Particularly, the C18-terminated PMMA surface appears as if it possesses fluid-like characteristics resembling to alkylsilane-coated silicon surfaces. As a result, a well-resolved SFM image of this surface was difficult to obtain.

The root-mean-square (RMS) roughness for PMMA was found to be 0.39 nm and the roughness factor (R) was calculated to be 1.002. The R factor was simply calculated from dividing the surface cross-sectional distance by the horizontal distance. As for the PMMA surface that was reacted with N-lithiodiaminopropane for 2 – 5 min, the R factor was determined to be 1.013 and the RMS roughness increased ~ 3.5 times higher (1.45 nm) than that of PMMA. It was found that the RMS roughness for PMMA exposed to N-lithiodiaminoethane was determined to be 1.80 nm, while the R factor was calculated to be 1.015. A possible explanation for the differences observed between the amine-terminated surfaces may be attributed to swelling/dissolution of the PMMA by the lithiated diamine and not the cyclohexane solvent used in the aminolysis reaction. It was found that this solvent does not affect the surface of the PMMA in any way. The R factor following C18-modification was 1.034 and the RMS roughness was found to be 2.80 nm, which was estimated to be ~ 1.6 – 2 times greater than that of amine-terminated PMMA surfaces.

Based on the SFM studies and the preliminary investigations of edge roughness, the amine surface coverage was estimated to be slightly less than 5 nmol cm\(^{-2}\), which was found to be ~ 6 times greater than that expected for a close-packed alkane monolayer. This significant difference in value is possibly the result of aminolysis reactions that occurred below the PMMA surface (~ 1.6 nm in depth).
2.3.3 Electroosmotic Mobility

Experimental data in Figure 2.6 shows the electroosmotic mobility and its directional dependence on electrolyte pH.

Figure 2.6. EOF profiles of PMMA and chemically modified PMMA evaluated over a pH range of 3.0 to 11.0 using acetate and borate electrolyte solutions. The field strength used (150 V cm\(^{-1}\)) was selected to minimize Joule heating in the microchannels, which measured 4 cm x 100 \(\mu\)m x 100 \(\mu\)m. Vertical error bars shown represent the standard deviation in the measurements.
It was found that the EOF in PMMA is nearly independent of electrolyte pH, opposite of what is seen for glass.\textsuperscript{5} For reference, electroosmotic mobility in PMMA was calculated to be $2.17 \pm 0.03$ to $2.58 \pm 0.01 \times 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ (positive value indicates EOF migrating from anode to cathode) over a pH range of 3.0-11.0, consistent with our previous data.\textsuperscript{5} The EOF in the amine-terminated PMMA was reversed (cathode to anode), consistent with a surface with an excess positive charge on its wall. In addition, the electroosmotic mobility for this surface decreased as the solution pH became more alkaline. This decrease is the result of accessible primary amines, which can be protonated at acidic pH or deprotonated at increasing pH. At pH values < 7.0, the carboxyl-terminated PMMA displayed an electroosmotic mobility that was slightly smaller than the amine-terminated PMMA, yet their directional flow were similar. However, at pH values > 5.0, the EOF reversed in direction migrating from anode to cathode comparable to that of PMMA. These observations of the carboxyl-terminated PMMA is attributed to both protonation of carboxyl- and amine-terminating groups at low pH resulting in excessive positive charges on the surface since the amines were used as a scaffold to convert the surface with carboxyl functionalities. At increasing pH values, an excess negative charge on the surface results from deprotonation of the carboxyl- and amine-terminating groups. As do of glass, ionizable silanol groups are protonated and deprotonated from lower to increasing pH. In the case of sulfonate-terminated PMMA, the EOF migrated from cathode to anode over all pH values. This is due to deprotonation of sulfonate-terminating groups as the solution pH increases resulting in a negatively charged surface.

We attempted to surpress or possibly eliminate the EOF by covalently attaching C18 alkyl chains to the amine-terminated PMMA. According to the results shown in Figure 2.6, it was found that the C18-terminated PMMA electroosmotic mobility was significantly smaller than the
amine-terminated PMMA at the high pH values. This is likely due to less charge on the C18-terminated PMMA surface.

2.3.4 Alkaline Hydrolysis

To investigate the hydrolytic stability of the amine-, carboxyl-, sulfonate-, and C18-terminated PMMA versus PMMA, these surfaces were exposed to 0.1 M NaOH for 1 h. After 1 h, EOF measurements were repeated using the strategy outlined previously at a pH 9.0. It was found that the direction of the EOF for the PMMA and terminated-PMMA surfaces remained unchanged. In addition, the electroosmotic mobility did not change significantly after exposure to 0.1 M NaOH for 1 h. For PMMA, the final electroosmotic mobility was found to be $2.47 \pm 0.08 \times 10^{-4}$ cm$^2$ V$^{-1}$ s$^{-1}$. This value is ~ 2 orders of magnitude lower than that reported for glass, following exposure to alkaline conditions.\textsuperscript{38} For the amine-terminated PMMA, the initial electroosmotic mobility was calculated to be $-1.13 \pm 0.02 \times 10^{-4}$ cm$^2$ V$^{-1}$ s$^{-1}$ at pH 9.0 versus the final electroosmotic mobility $-1.15 \pm 0.03 \times 10^{-4}$ cm$^2$ V$^{-1}$ s$^{-1}$ at pH 9.0. For the carboxyl-, sulfonate-, and C18-terminated PMMA, the electroosmotic mobility was calculated to be $2.19 \pm 0.11$, $1.25 \pm 0.06$, and $-0.75 \pm 0.05 \times 10^{-4}$ cm$^2$ V$^{-1}$ s$^{-1}$, respectively, versus the initial results observed in Figure 2.6 at pH 9.0. For comparison purposes, Morris and coworkers reported a nearly 10-fold increase in the electroosmotic mobility of a Vivak copolymer after exposure to 0.1M NaOH for 5 min.\textsuperscript{39}

2.4 Conclusions

We have defined and demonstrated the ability to chemically modify commercially available PMMA sheets to impart different surface properties to the material. As a result, the flow properties of the bulk solution were altered. Specifically, accessible amine sites on the PMMA surface served as a scaffold for further functionalization. This leads to the ability to perform
heterogeneous enzyme cleavage of DNA for microarray applications, metal deposition for microcircuitry applications, and creating hydrophobic surfaces appropriate for future electrochromatography applications. Overall, the electroosmotic mobility results were reasonably reproducible, which greatly increases the utility of PMMA in the construction of microanalytical devices.

2.5 References


Chapter 3

Contact Conductivity Detection in Poly(methyl methacrylate)-Based Microfluidic Devices for Analysis of Mono- and Polyanionic Molecules

3.1 Introduction

Microfabrication technology has proven to be a valuable tool for creating miniaturized devices for applications in many chemical and biochemical assays. The attractive features associated with these devices includes, potential for system integration in which various processing steps of the assay are included onto the fluidic platform, rapid analysis speeds, construction of highly multiplexed systems, the ability to reduce reagent consumption and the mass production of devices at minimal costs. As this technology continues to evolve, several areas will require further development to expand on existing capabilities such as, increasing system integration without sacrificing the benefits of a small footprint, reducing the cost, labor and time associated with fabricating devices and further reducing the size of the device. Indeed, significant progress has been made and continues to be made by many research groups in these areas. For example, efforts have focused on developing microfluidic devices in polymers.\(^1\)\(^-\)\(^15\) These polymer-based devices offer a variety of inexpensive fabrication methods that can be used to rapidly create devices inexpensively and in addition, these fabrication methods can be used to produce high-aspect-ratio microstructures (HARMs), which can assist in system integration and reducing the size of the device.

One of the consequences of reductions in size of the microfluidic device is the resulting constraint placed on the detector required for readout. For example, the sampling volume in many microfluidic devices is \(< 1\) nL and therefore, significant requirements on the limits of detection (LOD) associated with the detector hardware must be realized to analyze even modest concentrations of material in these platforms.
The readout strategy most commonly used for these devices has relied on laser-induced fluorescence (LIF) due to the fact that it provides exquisite sensitivity with detection limits approaching the single molecule level. Unfortunately, most LIF systems do not lend themselves to developing miniaturized systems, with the detector components often times requiring a much larger footprint compared to the microfluidic device. While several attempts have been made to fabricate miniaturized LIF detectors with integrated capabilities, LIF requires analytes that either show intrinsic fluorescence or can be readily associated with (either covalently or non-covalently) labeling chromophores.

Another readout strategy that has shown promise in microfluidic applications is electrochemical detection (ECD), such as amperometric or potentiometric detection systems. Attractive features associated with ECD includes the simple instrumentation required to carry out detection and the favorable sensitivity and limits of detection it offers. However, for amperometric and potentiometric detection, the target material must be intrinsically electroactive or if it is not, an electroactive species must be appended to the target molecules.

Conductivity detection can be considered an electrochemical technique as well, but has the ability to detect any analyte irrespective of whether it contains an electroactive species or not. The only requirement is that the migrating analyte zones possess a conductivity that is different from that of the carrier electrolyte. Another benefit of conductivity is that performance improves with smaller detection volumes, making it an attractive detector for microelectrophoretic separations, such as capillary zone electrophoresis (CZE). To demonstrate the low limits of detection that are obtainable using conductivity detection with CZE, Zare and coworkers described an integrated conductivity detector that possessed a detection volume of 30 pL and could detect $\sim 10^6$ lithium ions ($10^{-7}$ M).
Recently, reports have appeared describing the integration of contact conductivity into microfluidic devices.\textsuperscript{35-37} In these examples, the detector was fabricated to analyze analyte zones isolated via isotachophoresis (ITP) with the analytes consisting of small inorganic ions or low molecular weight organic ions. Guijt and coworkers described the fabrication of an integrated conductivity detector for zone electrophoresis separation of several inorganic ions and organic acids.\textsuperscript{38} In this device, Pt electrodes required for contact conductivity detection were vapor-deposited onto a glass cover slip and consisted of \(\sim 200\) nm thick electrodes spaced by \(25\ \mu m\) and configured orthogonal to the electrophoretic flow direction. Using a sinusoidal voltage waveform applied between the Pt electrodes, the concentration detection limits for several organic anions were determined to be in the range of \(5\ \mu M\). These same authors constructed an integrated contactless conductivity detector fabricated in a four-electrode geometry.\textsuperscript{39} To reduce analyte adsorption to the electrode surface causing electrode fouling, a silicon carbide insulating layer was deposited over the electrodes. A concentration detection limit of \(20\ \mu M\) was reported for \(K^+\) ions. Also in this report, the authors presented a one-dimensional zone electrophoretic separation of a two-component peptide mixture.

While conductivity detection can be used in miniaturized platforms for the analysis of a variety of species, improvements in its detection limit performance and its utility to detect analytes other than inorganic ions or small organic ions must be realized. For conductivity detection, the analytical response (\(G\), conductivity = \(S\) = siemens = \(\Omega^{-1}\)) is described through the following expression:

\[
G = \frac{(\lambda_+ + \lambda_-)C}{1000\ K} \quad (3.1)
\]
where \( \lambda_+ \) and \( \lambda_- \) (S cm\(^2\) mol\(^{-1}\)) are the limiting ionic conductance’s of cations and anions in solution, \( C \) is the concentration (mol cm\(^{-3}\)) and \( K \) is the cell constant (\( K = D/A \)) where \( D \) (cm) is the distance between the electrode pair and \( A \) (cm\(^2\)) is the area of the electrodes. Clearly, increasing the area of the electrodes and reducing the spacing between the electrodes can improve the detection limits of the conductivity measurement. In addition, one must reduce the contribution of Faradaic currents to the measured current. One approach to accomplish this is to use a bipolar pulse waveform.\(^{40-43} \) In this format, successive voltage pulses of equal amplitude and duration but opposite polarity are applied to the conductivity electrodes with the current passing between the electrodes measured at the end of the second pulse. If the pulse frequency is appropriately chosen with respect to the cell time constant (defined as the time to charge the double layer), the electrical double layer does not have sufficient time to form, which can minimize Faradaic reactions from occurring at the electrodes. Furthermore, since the bipolar pulses are of equal amplitude and time duration but opposite polarity, the measured current is effectively free from charging currents. Therefore, the measured current primarily results from solution Ohmic resistance.\(^{40} \) The attractive feature of this format is that the electrodes can be configured directly into the fluidic channel while maintaining the efficiency of the separation.

In this chapter, we wish to describe a simple, sensitive, bipolar-pulse, contact conductivity detector integrated directly into a PMMA-based microfluidic device for the detection of various mono- or polyanionic molecules (amino acids, peptides, proteins or oligonucleotides). The PMMA device was configured to separate the ionic materials using several different electrophoresis formats such as, free-solution zone electrophoresis (FSE) (amino acids and peptides), micellar electrokinetic chromatography (MEKC) (proteins) and reverse-phase ion-pair open channel capillary electrochromatography (RP-IPOCCEC) (oligonucleotides) in a 3 cm
effective length fluidic channel. To perform RP-IPOCCEC separations in the PMMA microfluidic device, the PMMA wall was chemically modified using chemistry described in Chapter 2 by attaching a C18 reverse-phase.\(^7\) By adding an ion-pairing agent to the carrier electrolyte, reverse-phase ion-paired separations of the oligonucleotides could be carried out using conductivity detection.\(^{44}\) Analysis of these materials reported herein will be described using contact conductivity detection. Sensitivity, limits of detection and stability of the conductivity detector will be discussed as well.

3.2 Experimental Details

3.2.1 Microfabrication of PMMA-Based Devices

Planar PMMA wafers (Goodfellow, Berwyn, PA), 133 mm in diameter, were embossed from Ni electroforms fabricated via LIGA techniques, which can be used to make many replicates from this single master.\(^{45}\) The sections below and in Chapter 1 describe the steps involved in fabricating the electroform (molding die) and the production of the final polymer microparts using hot embossing.

The device layout (see Figure 3.1A) was designed using AutoCAD (Autodesk Inc., San Rafael, CA), which was read by a GCA Mann 3600 Pattern Generator (Seattle, WA) to optically write the desired pattern to an optical mask. Microfeatures were printed with this generator on a 5" × 5" plate, which consisted of a chromium-coated quartz plate with a positive photoresist layer. The device possessed a 4 cm long x 15 \(\mu\)m wide separation channel (effective length = 3 cm). Guide channels for the Pt wires used to construct the integrated conductivity detector were 130 \(\mu\)m in width. Following development of the exposed resist, the plate was subjected to a Cr etching solution to produce the optical mask.
Figure 3.1. (A) Topographical layout of the assembled microfluidic device with an integrated conductivity detector. Injection channel length = 1.0 cm; separation channel 4.0 cm x 15 \( \mu \)m x \( \sim \) 85 \( \mu \)m (effective length = 3.0 cm). The solution reservoirs are: (1) sample reservoir; (2) electrolyte reservoir; (3) waste reservoir; and (4) receiving reservoir. (B) Optical micrograph of assembled device cut near the conductivity cell. (C) Optical micrograph of integrated conductivity detector (T-cell, electrode gap \( \sim \) 20 \( \mu \)m). In this micrograph, the cover plate was not assembled to the fluidic substrate. Working and reference electrodes possessed a 127 \( \mu \)m diameter and were placed 0.5 cm up stream from reservoir (4). (D) SEM of Ni electroform embossing die taken near (4).

The X-ray mask was prepared on a Kapton film with the desired device topography transferred to the film using optical proximity printing. A 25 \( \mu \)m thick Kapton film was stretched and glued to a Ti ring to which 50 \( \AA \) of Cr and 300 \( \AA \) of Au were deposited. SU-8 (negative photoresist, Microchem, Newton, MA) was spin-coated onto the Kapton film at 900 rpm for 20 s (Headway Research Inc., PWM101 Spinner). After a pre-bake for 1.5 h at 96°C, the optical mask was placed over the Kapton film and this assembly was exposed to UV light for 20 s (Oriel UV Exposure System, Stratford, CT). The unexposed SU-8 photoresist was removed after a 20 min post-bake at 96°C. The thickness of the photoresist (30-40 \( \mu \)m) was measured using a surface profiler (Alpha-Step 500, Tencor, San Jose, CA). Following development,
plasma cleaning of the Kapton film was performed to remove any remaining photoresist. Au was then electroplated onto the Kapton film at 2 mA cm\(^{-2}\) for 2 h.

The molding tool (see Figure 3.1D) was fabricated by electroplating Ni microstructures onto a stainless steel support, which served as the plating base. A 5" diameter, 3 mm thick polished stainless steel plate was activated using a C-12 activator solution (Puma Chemical, Warne, NC) followed by pre-electroplating in a NiCl bath to form a thin layer (< 5 \(\mu\)m) of Ni. Then, a 1 \(\mu\)m layer of PMMA (9% PMMA in methyl methacrylate, MMA) was spin-coated onto the plating base and baked at 180°C for 1 h. The spin-coated PMMA film was coated with MMA, which was used to bond a 3 mm thick PMMA sheet to this base. The PMMA sheet served as a positive resist during X-ray lithographic patterning. The PMMA was fly cut to slightly higher than the desired structure height to account for final polishing and device assembly. The PMMA/stainless steel plating base assembly was then exposed to an X-ray beam at the Center for Advanced Microstructures and Devices (CAMD, Baton Rouge, LA) with the X-ray mask positioned in front of the assembly. After exposure, the PMMA was developed in GG developer (60% 2-(2-butoxyethoxy)ethanol, 15% morpholine2-(2-butoxyethoxy) ethanol, 5% ethanolamine, 20% deionized water). The sample was then rinsed in a GG rinse solution (80% 2-(2-butoxyethoxy)ethanol, 20% deionized water). C-12 activator was again used to remove any oxides that may have formed on the exposed metal surfaces prior to final Ni-electroplating.

The desired Ni structures were plated out of a Ni-sulfamate bath onto the exposed areas of the stainless steel base. The plating was done at 55°C under continuous stirring at pH = 4.0. Following electroplating, the Ni parts were surface ground to remove excess Ni and to planarize the top of the features. The sample was then lapped to a mirror finish. The raised Ni structures were measured to be 95 \(\mu\)m tall by step profilometry following complete processing. Finally, the
unexposed PMMA was removed by dissolving in CHCl₃. A scanning electron micrograph (SEM) of the finished metal electroform used for embossing parts is shown in Figure 3.1D.

Embossing was performed using a PHI Precision Press model number TS-21-H-C(4A)-5 (City of Industry, CA). A vacuum chamber was installed into this press to remove air (pressure < 0.1 bar) so complete filling of the molding die could take place. The PMMA wafers were inserted into the press and the maximum area that could be patterned was 100 mm. Before molding, all residual water present in the polymer had to be removed. At ambient temperature, water adsorption to PMMA is ~ 0.4% and this amount must be reduced to 0.1% for proper embossing. Therefore, the PMMA wafers were baked in an oven at 80°C for 8 h. The die was coated with a release agent, MoldWiz (Axel, Woodside, NY), to improve demolding. During embossing, the die was heated to 150°C and pressed into the PMMA wafer with a force of 1000 lbs for 4 min. During this process, the die was heated to 160°C. After 4 min, the press opened after the polymer part was cooled. The PMMA wafer was maintained at 85°C throughout the demolding process.

### 3.2.2 Device Assembly with Integrated Conductivity Detector

The integrated conductivity detector (see Figures 3.1B and 3.1C for optical micrographs) was constructed from a pair of polished Pt wires (Scientific Instrument Services, Ringoes, NJ) with diameter of 127 µm that served as the working and reference electrodes. The wires were each cut to 2.5 cm in length and the end of each wire polished starting with a 600 µm grit sandpaper then moving down to 25 µm, 15 µm, 3 µm, and finally 1 µm diamond paste (Buehler, Lake Bluff, IL).

Between individual polishing, the wires were rinsed and sonicated for 2 min with 18 MΩ•cm water (Barnstead, Dubuque, IA). After polishing, the ends of the wires were inspected under a
20X stereomicroscope to ensure a blunt end had been formed and inserted into 130 µm wide guide channels embossed into the PMMA fluidic substrate. Through inspection of the wires under the stereomicroscope with a calibrated eyepiece, they were situated orthogonal to the electrophoretic channel and terminated with an end-to-end spacing of ~ 20 µm. Once situated properly, the wires were tacked down with a small amount of epoxy and the wires with the entire fluidic channels were covered with the corresponding cover plate that was clamped between two glass plates and thermal annealed at 107°C for 12 min in a GC oven.

3.2.3 Apparatus

Operating platform for the electrophoretic separations consisted of a high voltage power supply (CZE1000R, Spellman, Plainview, NY). The leads were connected to an in-house built high voltage relay (switching driven by a 5 V signal) with four outputs distributed to each of the solution reservoirs configured on the microfluidic device. The switching signal for the relay was generated using a universal data acquisition board (PCI-1200, National Instruments, Austin, TX). In addition, the applied voltage for the electrophoresis was controlled by one of the DAC outputs of this board.

The bipolar pulse waveform for the conductivity detector was generated by an in-house fabricated circuit (see Figure 3.2), which was controlled by a National Instruments controller board (Austin, TX). The pulse frequency (typically 5.0 kHz) was controlled by the timer on the board. The potential of one electrode was maintained at virtual ground while the potential at the other electrode was controlled by the bipolar waveform. To generate the bipolar waveform, a brief positive pulse period (typical pulse width, 100 µs) during which switch 1 (A) is turned on is immediately followed by a negative pulse period of the same length in which switch 1 (B) is turned on. The pulse width was selected to be short compared to the cell time constant, ~ 250 µs,
which was defined by RC where R is the solution resistance and C is the double layer capacitance. The current between the electrode pair was measured 30 ns prior to the rising edge of every bipolar pulse (± 0.5 V) at the input port and averaged over the electrophoresis sampling time (1 s) to improve signal-to-noise ratio (S/N). The resulting signal was processed by the adjustable gain amplifier (five resistors ranging from 1kΩ - 10MΩ) and passed to a sample-and-hold amplifier whereby the output was digitized by the board. Data acquisition and controlling software was written in Labview (National Instruments).

**Figure 3.2.** Schematic representation of the fabricated circuit for the capacitively coupled 2-electrode conductivity detector. See text for detailed description.

### 3.2.4 Microelectrophoresis

All electrophoretic separations were carried out at ambient temperature and performed in reverse mode (detection end at the anode). Injection was initiated by applying a high voltage to point (3) and grounding point (1) for the appropriate amount of time to completely fill the cross offset 'T' (see Figure 3.1A). Points (2) and (4) were also grounded during injection. The cross offset 'T' was designed to have a load volume of 425 pL (length = 250 µm). Calculation of the
injection plug variance ($\sigma_{\text{inj}}^2$) from $l_{\text{inj}}^2/12$, assuming a rectangular injection plug, yielded a value of $5.2 \times 10^{-5}$ cm$^2$ for $\sigma_{\text{inj}}^2$. For typical electrophoretic separations with this device design (see Figures 3.4 and 3.7), $\sigma_{\text{tot}}^2 \sim 3.0 \times 10^{-3}$ cm$^2$ and therefore, $\sigma_{\text{inj}}^2$ represents less than 2% of $\sigma_{\text{tot}}^2$. Following injection, a high voltage was switched to point (4) and (2) was grounded. Points (1) and (3) were set to 10% of the high voltage applied to point (4) and acted as pullback voltages to prevent sample leakage from these channels during the separation. (CAUTION: Electrophoresis uses high voltages and special care should be taken when handling the electrophoresis electrodes.)

### 3.2.4.1 Free-Solution Zone Electrophoresis (FSE)

FSE was carried out on the amino acids (Sigma, St. Louis, MO) using triethylammonium acetate (TEAA) carrier electrolyte (10 mM, pH 7.0, Fluka, Milwaukee, WI) or a solution of peptides (Sigma) using a phosphate carrier electrolyte (100 $\mu$M, pH 5.0, Sigma). The appropriate concentration of amino acids or peptides were made from stock solutions diluted in the carrier electrolyte and electrokinetically injected into the cross offset 'T' using the voltage pattern described above. Electrophoresis for the amino acids and peptides was performed at a field strength of 150 V cm$^{-1}$. The conductivity detector was operated at a frequency of 5.0 kHz and bipolar pulse amplitude of $\pm 0.5$ V.

### 3.2.4.2 Micellar Electrokinetic Chromatography (MEKC)

MEKC of the protein mixture (BioRad, Laboratories, Hercules, CA) used a TRIS HCl electrolyte (100 $\mu$M, pH 9.2) with 1% sodium dodecyl sulfate, SDS, above its CMC. The appropriate concentration of proteins were diluted in the carrier electrolyte and electrokinetically injected into the cross offset 'T' as described above. Electrophoresis for the proteins was
performed at 250 V cm\(^{-1}\). The conductivity detector was operated at a frequency of 5.0 kHz and bipolar pulse amplitude of ± 0.5 V.

### 3.2.4.3 Reverse-Phase Ion-Pair Open Channel Capillary Electrochromatography (RP-IPOCCEC)

RP-IPOCCEC separations of oligonucleotides were performed in open channels of the C18-terminated PMMA microfluidic devices using 25% acetonitrile in 75% aqueous phase containing 50 mM TEAA serving as the ion-pairing agent. The oligonucleotides analyzed were comprised of a low DNA mass sizing ladder (Gibco-BRL, Gaithersburg, MD), that consisted of 100, 200, 400, 800, 1200 and 2000 bp fragments. Injection was accomplished as described above with the field strength used for the separation equal to 100 V cm\(^{-1}\). The conductivity detector was operated at a frequency of 5.0 kHz and bipolar pulse amplitude of ± 0.5 V.

In order to conduct reverse-phase separations, the PMMA surface was chemically modified using chemistry developed in our laboratories\(^7\) and described in Chapter 2, prior to thermal bonding. Modification chemistry consisted of converting the methyl ester groups of the polymer backbone into an amine (NH\(_2\))-terminated surface by flooding the fluidic channel with N-lithiodiaminoethane (via syringe). After the reaction, the fluidic channel was rinsed with HPLC grade 2-propanol (Aldrich, Milwaukee, WI) and 18 M\(\Omega\cdot\text{cm}\) water. The amine-terminated PMMA substrate was then placed in an air-tight vessel, purged under nitrogen and later introduced to neat N-octadecane 1-isocyanate (98%, Aldrich) (via syringe). Following this reaction, the C18-terminated PMMA fluidic channel was washed with copious amounts of hexanes, toluene acetone (Aldrich) and subsequently dried under a stream of nitrogen.

### 3.2.5 Sessile Drop Water Contact Angles

At ambient temperature, contact angles of flat PMMA sheets (15 mm x 15 mm x 3 mm) were performed with a VCA 2000 Goniometer (VCA, Billerica, MA). Using a syringe, 4 - 6 \(\mu\text{L}\) of
18 MΩ•cm water was placed on the air-side of PMMA and chemically modified PMMA surfaces. Using software provided by the manufacturer, the contact angle values were calculated over an average of five measurements utilized for each water droplet.

3.2.6 Electroosmotic Mobility Measurements

Measurements of the electroosmotic mobility was determined using the current monitoring procedure described by Zare and coworkers. The procedure involved filling the entire microfluidic device with a low ionic strength electrolyte solution (9 mM for TEAA via native PMMA; 49 mM for TEAA via C18-terminated PMMA) at the appropriate pH. After filling the device, one reservoir was emptied and replaced with the same electrolyte, but of higher ionic strength (10 mM for TEAA via native PMMA; 50 mM for TEAA via C18-terminated PMMA). The electrical field was then applied to the reservoirs containing the low and high ionic strength electrolyte solutions and the current was monitored continuously. After the current had plateaued, the time to reach this plateau was secured from the plot from which the linear velocity (cm s⁻¹) could be calculated. Dividing the linear velocity by the electrical field strength (V cm⁻¹) produced the electroosmotic mobility (cm² V⁻¹ s⁻¹). Electroosmotic mobility values were calculated over an average of five consecutive runs in each device.

3.3 Results and Discussion

3.3.1 Integrated Conductivity Detector Fabrication

The detector was fabricated by inserting (using a 20X stereomicroscope) polished Pt wires into guide channels embossed in the fluidic substrate to accommodate the electrodes, which allowed reproducible lateral placement of the electrodes within the fluidic device. These guide channels were 130 μm wide and 95 μm deep. The Pt wires were 127 μm in diameter. Therefore, the wires were taller than the height of the microstructures. Attempts to make the
microstructures of the appropriate height to accommodate the circular wires completely, which would have allowed insertion of the Pt wires following assembly of the device, was found to be difficult due to the various grinding and lapping processes and the slight compression of the embossed microstructures during device assembly (structure height ~ 85 \( \mu \text{m} \) following assembly). For this reason, we decided to place the wires in an unassembled device and then thermally anneal the cover plate to the substrate once the wires had been properly positioned within the guide channels. For this to work properly, sealing of the cover plate around the electrodes was critical. Similar to the wire imprinting methods reported by Locascio and coworkers,\textsuperscript{2} it was found that the Pt wires during assembly formed impressions into the cover plate, depicted in Figure 3.1B (optical micrograph of an assembled device that had been microtomed (cut) across the device at the conductivity cell intersection). In addition, the cover plate formed an impression around the Pt wire, which allowed tight sealing not only around the electrode, but between the two polymer pieces as well. The versatility of polymers is very attractive in this particular case, because they can thermally deform around structures pre-inserted into the device at much lower and for laboratory use, more convenient temperatures.

In Figure 3.1C (optical micrograph), the width of the guide channels was somewhat wider than that of the wire potentially leaving unswept volumes near the detector, which could give rise to memory effects. However, as can be seen from Figure 3.1B, the walls of the guide channel were slightly compressed around the wires due to assembly and from inspection of our data, no memory effects were observed in the detector response.

\subsection*{3.3.2 Figures of Merit for Conductivity Detector}

We reported on the fabrication and characterization of a miniaturized contact conductivity detector,\textsuperscript{44} similar in design to that used herein, was operated in a bipolar pulse format. A mass
detection limit equal to 3.46 ng for KCl was found using direct conductivity detection. The conductivity detector analytical figures of merit were evaluated here by constructing a calibration plot covering the concentration range of 15 to 80 nM for alanine using a 10 mM TEAA carrier electrolyte (pH 7.0) as shown in Figure 3.3. The correlation coefficient ($R^2$) for this plot was determined to be 0.994. The concentration limit of detection (LOD) was determined by measuring the S/N for a series of electropherograms with the amino acid concentration adjusted near the anticipated LOD as determined from the regression plot. Inspection of this data indicated a concentration LOD of 8.5 nM at a S/N ~ 3. Based upon the known injection volume (425 pL), the mass detection limit for this concentration was calculated to be 3.4 amols.

![Figure 3.3](image)

**Figure 3.3.** Calibration plot for alanine constructed by integrating the area under each peak and averaging over five runs (vertical error bars) for a concentration range of 15 nM to 80 nM using 10 mM TEAA as the background carrier electrolyte (pH 7.0).

For comparison purposes, the mass detection limit for several amino acids analyzed by glass-based devices using indirect fluorescence detection has been reported to be 1.6 amols,
comparable to that reported herein. The LODs reported herein resulted primarily from a small spacing of the conductivity electrodes, implementation of the bipolar pulse waveform and signal averaging (~ 5000 data points). It should be noted that because we are using indirect conductivity detection and the carrier electrolyte conductivity is higher than that of the migrating analyte zone, the S/N depends intimately on the carrier electrolyte concentration, with lower concentrations of the carrier electrolyte improving LODs, since the fractional change of the signal will be large for a given amount of carrier electrolyte additive being displaced. However, the dynamic range of the response is reduced at lower concentrations of the carrier electrolyte.

3.3.3 FSE Analysis of Amino Acids

The FSE separation of four non-labeled amino acids was carried out in reverse mode, in which the injection end was cathodic and the detection end was anodic (see Figure 3.4).

![Figure 3.4](image.png)

**Figure 3.4.** FSE separation of 100 µM amino acid mixture consisting of (1) alanine, (2) valine, (3) glutamine, and (4) tryptophan in the PMMA device using indirect, contact conductivity detection. Electrophoretic conditions: carrier electrolyte was 10 mM TEAA (pH 7.0); 3 s electrokinetic injection time; field strength 150 V cm⁻¹ for the electrophoresis and the detector was operated at 5.0 kHz with bipolar pulse amplitude of ± 0.5 V.
While PMMA does show an EOF that runs from anode to cathode,$^4$ it is smaller than that observed for fused silica at this same pH. The electroosmotic mobility of this PMMA device measured using this carrier electrolyte was found to be $2.17 \pm 0.03 \times 10^{-4}$ cm$^2$ V$^{-1}$ s$^{-1}$ and changed minimally with pH over the range of 3-11, consistent with our previous data.$^4$ At the pH used for these separations, the amino acids exist in their anionic form and therefore, migrate toward the anode only if their electrophoretic mobility is greater than the electroosmotic mobility, which is the case here because all amino acids were observed by finish line format conductivity detection at the anode (see Figure 3.1A). The extended time required for migration of the amino acids to the detector indicates that their apparent mobility is small. The apparent mobility for alanine was measured to be $3.34 \times 10^{-5}$ cm$^2$ V$^{-1}$ s$^{-1}$, giving a value of $2.50 \times 10^{-4}$ cm$^2$ V$^{-1}$ s$^{-1}$ for its electrophoretic mobility. For comparison, the electrophoretic mobility of alanine was calculated to be $3.05 \times 10^{-4}$ cm$^2$ V$^{-1}$ s$^{-1}$ (electroosmotic mobility $= 8.7 \times 10^{-4}$ cm$^2$ V$^{-1}$ s$^{-1}$, taken from reference 49), from published data using a capillary and indirect fluorescence detection.$^{50}$

3.3.4 Separation of Peptides using FSE

To demonstrate the ability to detect the presence of other polyanionic species, several peptides were investigated via FSE as shown in Figure 3.5. All 9 peptide fragments were baseline separated within 250 s at a field strength of 150 V cm$^{-1}$. The conductivity response was again negative, indicating that the analyte zones possessed a conductance below that of the carrier electrolyte. We also investigated the stability of the detector response, since it has been shown that electrode fouling due to non-specific adsorption can degrade its performance.$^{51}$ As can be seen in Figure 3.5, the detector response degraded by ~ 50% after performing 35 separations on this device. We also note that the amino acid and protein assays (see Figures 3.4
and 3.6) produced similar results in terms of degradation of the detector response. However, for oligonucleotides assays (mid- and lower panels of Figure 3.7), no degradation in the response of the detector was observed over the lifetime of the device. Fortunately, the ease of fabrication via hot embossing and assembly of the device with its detector electrodes makes it simple to replace failed devices.

**Figure 3.5.** FSE separation of a peptide mixture (~ 0.23 \( \mu \)M total peptide concentration) consisting of (1) bradykinin, (2) bradykinin fragment 1-5, (3) substance P, (4) \([\text{Arg}^8]\)-vasopressin, (5) luteinizing hormone, (6) bombesin, (7) leucine enkephalin, (8) methionine enkephalin, and (9) oxytocin in a PMMA device using indirect, contact conductivity detection. Electrophoretic conditions: carrier electrolyte was 100 \( \mu \)M phosphate (pH 5.0); 3 s electrokinetic injection time; field strength 150 V cm\(^{-1}\) for the electrophoresis. Detector was operated at 5.0 kHz with bipolar pulse amplitude of ± 0.5 V.
3.3.5 MEKC Analysis of Proteins

Protein separations and detection represent a unique analytical challenge in light of recent efforts devoted to proteome research and the diverse nature of proteins. When proteins are separated by electrophoresis techniques using capillary or microfluidic devices, they can be detected via direct UV absorbance (only if the protein contains a chromophoric amino acid residue) or LIF following either covalent or non-covalent labeling with fluorescence being the preferred mode for most microfluidic devices.\(^{52-57}\) In this series of experiments, we were interested in determining if contact conductivity detection could be applied to our PMMA-based device for the analysis of proteins separated by MEKC (see Figure 3.6). From the electrophoregram, most of the protein components were sufficiently resolved, except for ovalbumin and serum albumin (RS = 0.41) as well as phosphorylase B and β-galactosidase (RS = 0.57). The calculated plate number (N) for myosin was found to be 2.54 x 10\(^4\) (calculated using, \(N = 5.54 \left(\frac{t_R}{w_{1/2}}\right)^2\)), which yielded a value of 1.17 x 10\(^{-4}\) cm for the height equivalent to a theoretical plate (H). Calculation of H assuming that the major contribution to band broadening is longitudinal diffusion produced a value of 1.33 x 10\(^{-4}\) cm, close to the observed plate height for this separation. Therefore, diffusion is the major zone broadening mechanism for our protein separation and not extra-column effects (finite injection plug length or detector length) or analyte-wall interactions.

The total protein concentration in Figure 3.6 was 1.7 \(\mu\)M, which resulted in signals above the background. The attractive feature of conductivity detection in this case is that the proteins did not require labeling with a fluorophore, which makes them readily, accessible for downstream processing, such as proteolytic digestion followed by mass spectral fingerprinting for
identification purposes. In addition, detection is non-destructive maintaining all material in an analyzable form.

![Figure 3.6](image)

**Figure 3.6.** MEKC separation of a protein mixture (1.7 μM total protein concentration with all proteins at similar concentrations within the mixture) consisting of (2) lysozyme, (3) trypsin inhibitor, (4) carbonic anhydrase, (5) ovalbumin, (6) serum albumin, (7) phosphorylase B, (8) β-galactosidase, and (9) myosin in the PMMA device using indirect, contact conductivity detection. Benzoic acid (1) was added to the mixture as an internal standard. Electrophoretic conditions: carrier electrolyte 100 μM TRIS HCl with 1% SDS (pH 9.2); 3 s electrokinetic injection time; field strength 250 V cm⁻¹ for the electrophoresis and the detector was operated at 5.0 kHz with bipolar pulse amplitude of ± 0.5 V.

### 3.3.6 RP-IPOCCEC Analysis of Oligonucleotides

We have developed a microreverse-phase liquid chromatography (μRP-LC) technique using a 15 cm long x 300 μm i.d. column packed with C18-coated particles to separate oligonucleotides comprised of a low DNA mass sizing ladder. To facilitate the separation, acetonitrile was used as the organic modifier in the carrier electrolyte containing the ion-pairing agent, TEAA. We
were interested in adopting this separation mode onto a single platform via a PMMA-based open channel device. Recently, a CEC separation of neutral organic dye molecules in a glass-based open channel device has been described.\textsuperscript{58} In this work, a stationary phase comprised of octadecylsilanes groups was covalently bonded to the glass surface via siloxane-based chemistry. Shallow channels were found to give smaller plate heights as predicted from theory for open channel chromatography.\textsuperscript{59-60} While our channels were not shallow, they were designed to be narrow (15 \( \mu \text{m} \)) to yield acceptable plate heights for the oligonucleotides assays (see Figure 3.7).

In the top upper panel of Figure 3.7, only one band appeared for DNA fragments of a low mass sizing ladder and in addition, the background signal from the conductivity detector was very unstable. The lack of separation in this case is due to; (1) DNA fragments electrophoretic mobilities are similar; and (2) relatively poor hydrophobic nature of the PMMA surface, allowing minimal partitioning (i.e., low retention factor) by the ion-paired DNA complexes to the PMMA “stationary phase”, therefore will predominately migrate with the EOF. The instability of the conductivity response was due to dissolution of the PMMA surface by acetonitrile attack. This observation was supported through inspection of blank PMMA sheets subjected to acetonitrile, which significantly altered the appearance of the polymer. To circumvent challenges associated with (1) and (2) listed above, the PMMA surface was chemically modified by covalently attaching a C18 monolayer. To validate the success for differences in the PMMA surface after chemical modification, we measured the sessile water contact angle of native PMMA versus C18-terminated PMMA as described in Chapter 2. It was found that the contact angle increased from 65 \( \pm 4^\circ \) to 103 \( \pm 11^\circ \), following C18-modification, indication of increased hydrophobicity due to the presence of C18 alkyl chains.\textsuperscript{7}
Figure 3.7. Analyses of a low DNA mass sizing ladder (400 ng mL\(^{-1}\)) on PMMA and C18-terminated PMMA devices using indirect, contact conductivity detection. The ladder consisted of (1) 100, (2) 200, (3) 400, (4) 800, (5) 1200 and (6) 2000 bp fragments. Electrophoretic conditions: 25% acetonitrile in 75% aqueous phase containing 50 mM TEAA (ion-pairing agent, pH = 7.4); 3 s electrokinetic injection time; field strength 100 V cm\(^{-1}\) for the electrophoresis and the detector was operated at 5.0 kHz with bipolar pulse amplitude of ± 0.5 V.
As can be seen in the mid- and lower panels of Figure 3.7, the individual DNA fragments were well resolved and the stability of the conductivity signal evident, indicating no dissolution of the PMMA surface. Apparently, the C18 layer protects the underlying PMMA from acetonitrile attack. Also, the sizing ladder peaks observed were negative due to their lower conductivity relative to the background carrier electrolyte, consistent with our previous data for this type of separation with conductivity detection.\textsuperscript{44}

For most CEC separations, the driving force is electrokinetic, which is the result of EOF from the fused silica capillary and the silica microparticles. In our case, the EOF for native PMMA migrates from anode to cathode that possesses a value similar to the free-solution electrophoretic mobility of DNA.\textsuperscript{4} Because the electrophoresis reported herein was performed in reverse mode, the electrophoretic mobility of the DNA would migrate counter to PMMA's EOF giving a relatively long analysis time. However, the electroosmotic mobility in the C18-terminated PMMA microfluidic device was measured to be $-1.92 \pm 0.07 \times 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$. The negative sign represents EOF migrating from cathode to anode due to the diamine foundation layer used to attach the alkyl-bonded C18 phase to the PMMA substrate.\textsuperscript{7} Unreacted surface amine-terminating groups result in an excess surface charge that is slightly positive, producing a reversed EOF compared to native PMMA. This results in DNA fragments moving in the same direction as the EOF (toward the anode). It should be noted as well that the presence of these positive charges on the PMMA wall could result in anion exchange at the surface in addition to hydrophilic/ hydrophobic phase partitioning, giving rise to mixed retention mechanisms. Under these circumstances, one typically observes lower N values compared to the absence of these mixed retention mechanisms. Calculation of N for the 800 bp fragment ($t_{1/2} = 6 \text{ s}, t_R = 89 \text{ s}$) was found to be $4.1 \times 10^4 \text{ m}^{-1}$ (calculated using, $N = 5.54 \left(\frac{t_R}{w_{1/2}}\right)^2$). As mentioned earlier, using a
C18 packed column (no mixed retention mechanism) for reverse-phase ion-pair separation, we were able to achieve a N value of $\sim 5.1 \times 10^4 \text{ m}^{-1.44}$. However, eddy effects produced by the packed column should significantly reduce the value of N contrary to the open channel format employed herein. Therefore, the mixed retention mechanism could be giving rise to reduced chromatographic efficiency. Elimination of these mixed retention mechanisms could be accomplished by end capping the residual amine-terminating groups, which would result in improved separation efficiency.

### 3.4 Conclusions

An integrated conductivity detector consisting of a pair of Pt electrodes was developed for a PMMA-based microfluidic device and used for the detection of various anionic materials separated by FSE, MEKC or RP-IPOCCEC. Attomole detection limits for alanine was demonstrated, comparable to those obtained with indirect fluorescence detection. Favorable detection limits resulted from bipolar pulse operation, signal averaging and small spacing of the electrode pair. In addition, direct analysis of several proteins was presented with no requirement for labeling. Using appropriately prepared PMMA surfaces; we also demonstrated RP-IPOCCEC analysis of oligonucleotides using conductivity detection. In our examples, contact conductivity detection was used and as such, slight electrode fouling was observed due to adsorption of analytes or other electrolyte components to the electrode surface. For obtaining quantitative information on a sample, it may thus be necessary to include an internal standard (such as benzoic acid added to the peptide mixture, see Figure 3.5) into the sample or consider contactless conductivity detection. Nevertheless, if only qualitative information is required, then slight degradation in the detector response is not an issue. In addition, due to the ease of fabrication of the polymer fluidic device and integration of the detector electrodes, replacement
of the device is relatively simple, quick and not prohibitively expensive when significant
degradation of detector response results in device failure. We also were able to demonstrate that
PMMA surfaces could be prepared with modified hydrophobic character, thus allowing the
surface to behave as a viable stationary phase for reverse-phase separations. Furthermore, the
C18-terminated PMMA surface was found to be stable upon exposure to acetonitrile. Utilizing
conductivity detection, a reverse-phase ion-pair separation of a low DNA mass sizing ladder was
shown. The ability to use reverse-phase chromatography with conductivity detection will serve
as a viable platform for isolating certain DNA fragments required for subsequent analysis with
high efficiency. From results presented, it is clear that conductivity detection can be used to
detect various polyanionic species separated by a variety of electrophoretic formats. While only
anionic species were analyzed, the conductivity detector should work for mono- and/or
polycationic species as well. Finally, due to versatility and sensitivity of conductivity detection
as well as its ease of implementation, it could potentially be used as a flow sensor in microfluidic
devices possessing a complex network of fluidic channels.

3.5 References


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Chapter 4

Contact Conductivity Detection of PCR Amplicons Analyzed in C18-Terminated Poly (methyl methacrylate)-Based Devices

4.1 Introduction

The Polymerase Chain Reaction (PCR) protocol is a valuable tool frequently applied for either DNA sequencing or molecular diagnostic applications. The protocol entails amplifying target regions of DNA enzymatically that can produce \( >10^5 \)-fold increases in the amount of starting target sequences.\(^1\) Following amplification, results of amplified target DNAs must be detected and validated, which is usually carried out via an electrophoretic analysis, such as capillary gel electrophoresis (CGE) with either UV absorbance or laser-induced fluorescence (LIF) detection.\(^3-10\) CGE not only serves to validate the correct production of the PCR amplicon, but also can be used to purify the amplicon prior to subsequent analysis, such as sequencing of the PCR amplicon or determining its allelic composition for mutational analysis. Purification of the PCR amplicon is required to remove unwanted salts, primers, and enzymes and is important if downstream processing of the amplicon is required, since the reagents present in the PCR cocktail can potentially interfere with subsequent steps.

Numerous strategies have been used to perform purification of the PCR amplicon including gel filtration,\(^11\) gel electrophoresis,\(^7\) ultrafiltration\(^12\) or ion-exchange.\(^13-14\) Unfortunately, these techniques require large amounts of solvents, are plagued by low sample recoveries, labor-intensive, can be time-consuming, and often cannot be easily automated. Generally, gel-based techniques require the amplicon to be excised from the gel matrix following separation.

An attractive alternative to the aforementioned purification techniques for the PCR amplicon is reverse-phase chromatography, which can readily purify the amplicon with high recoveries, typically exceeding 97%.\(^15-18\) Reverse-phase chromatography is conducive to automation,
making it amenable to implementation in high-throughput DNA sequencing applications. For most reverse-phase separations of DNA amplified via PCR, an ion-pairing agent is used in conjunction with a packed column containing for example, a C18 stationary phase. Common ion-pairing agents that have been employed are quaternary ammonium salts, such as tetrabutylammonium phosphate (TBAP) or triethylammonium acetate (TEAA).19-20

Conventional reverse-phase separations operate using liquid chromatographic (LC) equipment, which consists of dual reciprocating pumps to allow gradient elution, a liquid chromatographic column (10 cm length x 4.1 mm i.d.) and a UV detector to allow direct detection of the DNA. There are three potential shortcomings associated with LC equipment for PCR amplicon separations or purifications; (1) sample dilution due to usage of macro-bore columns; (2) large consumption of solvents; and (3) difficult to multiplex the assay (i.e., run many columns in parallel). In order to circumvent the limitations associated with (1) and (2) listed above, we have developed a microreverse-phase liquid chromatography (µRP-LC) technique interfaced with a miniaturized conductivity detector to isolate and detect the PCR amplicon.21 The packed column was 15 cm in length x 300 µm i.d. and the detector was constructed from two Pt wires that served as the working and reference electrodes. Detection was accomplished by applying a bipolar pulse waveform to the electrode pair from which the conductivity of the solution could be measured. Efficient separation of the PCR amplicon from the other reagents present in the PCR cocktail was achieved in less than 4 min with a retention factor of 2.5 and separation efficiency of $9.1 \times 10^3$ plates. Unfortunately, this chromatographic format is not feasible for high-throughput assays, which would require many separation columns running in parallel.
Recently, we reported a miniaturized electrophoretic system utilizing a microfluidic device fabricated in PMMA to perform reverse-phase ion-pair separations with an integrated conductivity detector to transduce the eluting polyanionic materials from the chromatographic channel. PMMA substrates were hot embossed from a Ni master to allow rapid production of microfluidic devices. In order to create a hydrophobic stationary phase, pretreatment of the PMMA surface by chemical modification was performed to produce monolayer coverage of C18 alkyl chains. The PMMA surface was unable to separate 100 to 2000 bp DNA fragments of a low mass sizing ladder. Yet the C18-terminated PMMA surface resulted in baseline resolution of the fragments with the additional benefit of protecting the underlying PMMA surface from acetonitrile attack included in the reverse-phase separation.

Conductivity detection is considered a universal electrochemical transduction mode that is attractive for a miniaturized electrophoretic system relative to UV absorbance or LIF detection systems. Indeed, the concentration detection limit for DNA by UV absorbance (260 nm) has been reported to be on the order of $10^{-4}$-10$^{-5}$ M. A prominent disadvantage of UV absorbance is the detection limit depends on pathlength, which can translate into poor detection limits for micro-based separations. While LIF offers exquisite sensitivity, labeling of the target does not simplify upstream and downstream processing steps of the analyte. Conversely, conductivity is attractive since no chromophoric species or labeling with a fluorophore is necessary. It has the ability to transduce the migrating analyte if it possesses a conductivity different than the carrier electrolyte and is amenable to small volume detection considering that small distances between the electrode pair can optimize the sensitivity.

In this chapter, we wish to report on the isolation/separation of DNA amplified via PCR by reverse-phase ion-pair open channel capillary electrochromatography (RP-IPOCCEC) with
contact conductivity detection. The electrophoretic platform was performed in a PMMA-based microfluidic device, whose walls were chemically modified with a C18 phase to permit effective partitioning of the ion-pair DNA complexes into the stationary phase. As part of the method development, the acetonitrile content in the carrier electrolyte was altered to optimize and examine the effects in terms of development time, separation efficiency, and separation resolution. Also, we investigated the effect of the acetonitrile content on the performance of the contact conductivity detector.

4.2 Experimental Details

4.2.1 Microfabrication

A Ni mold master was fabricated from LIGA techniques as described in Chapters 1 and 3, which was used for hot embossing the desired PMMA (Goodfellow, Berwyn, PA) polymer parts from the master.\textsuperscript{22,24} Hot embossing was performed using a PHI Precision Press model number TS-21-H-C(4A)-5 (City of Industry, CA). Prior to embossing, all residual water present in the polymer must be removed. At ambient temperature, water adsorption to PMMA is \(\sim 0.4\%\) and this amount must be reduced to 0.1\% for proper embossing. Planar PMMA wafers, 133 mm in diameter, were baked in an oven controlled at 80°C for 8 h. Afterwards, the PMMA wafer was mounted in a vacuum chamber (pressure < 0.1 bar) with the mold master coated with a releasing agent, MoldWiz (Axel, Woodside, NY) to improve demolding. The mold master was heated to 150°C and pressed into the PMMA wafer with a force of 1000 lbs for 4 min. During this process, the mold master was heated to 160°C. After 4 min, the embossed PMMA wafer was cooled and demolded from the mold master. Throughout the demolding process, the PMMA wafer was maintained at 85°C. The embossed PMMA wafer consisted of a 15 \(\mu\)m wide separation channel of 4 cm total length (effective length = 3 cm). The conductivity detector was
fabricated by inserting two 127 µm polished Pt wires (Scientific Instrument Services, Ringoes, NJ) into 130 µm wide guide channels embossed in the polymeric material. Pt wires were end-polished with 600 µm grit sandpaper followed by 25 µm, 15 µm, 3 µm, and 1 µm diamond paste (Buehler, Lake Bluff, IL). After each polishing, wires were rinsed and sonicated for 2 min with 18 MΩ•cm water (Barnstead, Dubuque, IA) and later inspected under a 20X stereomicroscope with a calibrated eyepiece to ensure proper end-polishing. The wires were placed 0.5 cm before the anode orthogonal to the electrophoretic flow and terminated with an end-to-end spacing of ~20 µm via manual manipulation with viewing under the microscope. The device was assembled by covering the wires and fluidic channels with the corresponding cover plate, which was placed between two glass plates and thermally annealed in a GC oven at 107°C for 12 min.

4.2.2 PMMA Surface Treatment

Prior to device assembly, the PMMA embossed substrate and cover plate were chemically modified via a synthetic scheme developed in our laboratories25 and described in Chapter 2. The PMMA fluidic channel was introduced to N-lithiodiaminoethane (via syringe) for a given period of time followed by subsequent washes with HPLC grade 2-propanol (Aldrich, Milwaukee, WI), 18 MΩ•cm water, and dried under a stream of nitrogen. Afterwards, the amine-terminated PMMA fluidic channel was exposed to neat N-octadecane 1-isocyanate (98%, Aldrich) (via syringe) for 10 min. Following this reaction, the C18-terminated PMMA fluidic channel was subsequently rinsed with copious amounts of hexanes, toluene, acetone (Aldrich) and finally dried under a stream of nitrogen.

4.2.3 Discontinuous Electrolyte System

EOF measurements were conducted by a current monitoring procedure,26 in which the change in current flow produced by a discontinuous electrolyte system served as an indicator of
the linear velocity under the application of an applied field. The procedure involved filling the entire microfluidic device with an electrolyte solution comprised of 24 mM triethylammonium acetate (TEAA) (Fluka, Milwaukee, WI) and replacing one reservoir with 25 mM TEAA. The electrical field was applied and the current was monitored until no change in the current was observed. The linear velocity (cm s⁻¹) could be calculated from the time in which this occurred. Dividing the linear velocity by the electric field (V cm⁻¹) produced the electroosmotic mobility (cm² V⁻¹ s⁻¹). While the electroosmotic mobility can be measured using the migration time of a neutral marker moving through the PMMA device (native or modified), it was not adopted in the present application because it may experience partitioning into the stationary phase producing errors in the electroosmotic mobility determination. While errors may be produced in the electroosmotic mobility deduced from employing a discontinuous electrolyte system due to ionic strength effects on the double layer thickness, these effects can be minimized by using less than 5% difference in the electrolyte ionic strength.²⁷

4.2.4 PCR Amplification Protocol

The 500 bp (PCR-1) and 1000 bp (PCR-2) amplicons of the λ-DNA template (Applied Biosystems, Foster City, CA) were amplified by PCR in a Perkin-Elmer GeneAmp PCR System 2400 thermocycler (Applied Biosystems). Amplification involved the following step-temperature program: 94°C for 1 min to completely denature the template and the cycling temperature steps were 94°C for 15 s, 55°C for 15 s, and 72°C for 1 min. These steps were repeated for the appropriate number of thermal cycles and then, the temperature was held at 72°C for 7 min to complete chain extension. To conclude the program, samples were cooled to 4°C. The PCR reaction cocktail contained 10 mM TRIS-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 200 µM each of deoxynucleoside triphosphates (dNTPs), 25 units mL⁻¹
AmpliTaq DNA polymerase, 1.0 µM each primer, and 10 ng mL⁻¹ of the template. Primer set PCR01 forward primer (5′-GATGAGTTCGTGTCCGTACAACTGG-3′) and a PCR02 reverse primer (5′-GGTTATCGAAATCAGCCACAGCGCC-3′) (Applied Biosystems) were used to amplify PCR-1. The primer set used to amplify PCR-2 was PCR03 forward primer (5′-GGATATGGGCCGCAGTGAGGAGAA-3′) (Life Technologies, Gaithersburg, MD) and the PCR02 reverse primer (5′-GGTTATCGAAATCAGCCACAGCGCC-3′).

4.2.5 DNA Analysis via RP-IPOCCEC

Instrumentation to perform RP-IPOCCEC is illustrated in Figure 4.1. PCR amplicons were loaded directly from the thermal cycler into the sample reservoir of the microfluidic device, while the other three reservoirs were filled with carrier electrolyte solution. Samples were electrokinetically loaded into the cross offset 'T' by applying a high voltage to the waste reservoir with the sample and other two reservoirs maintained at ground for 3 s. For electrophoresis, the high voltage was applied at the anode reservoir (cathode reservoir at ground) while 10% of the high voltage was applied to the sample and waste reservoirs to prevent sample leakage from these channels during the electrophoretic run.

All RP-IPOCCEC separations were performed in reverse mode (detection end at the anode) and conducted at different acetonitrile content in the carrier electrolyte containing 25 mM TEAA (pH 7.4). Conductivity measurements were performed using a bipolar pulse waveform generated by an in-house fabricated circuit (see Chapter 3), which was controlled by a National Instruments board (PCI-1200, Austin, TX)²¹⁻²². Pulse frequency was typically 5.0 kHz, and a pulse width of 100 µs. The pulse width was selected to be short relative to the cell time constant, approximately 250 µs, defined by the time to charge the double layer to minimize Faradaic currents that could be produced following double layer formation. The bipolar pulse waveform was applied to one
electrode while the other electrode was held at virtual ground. Current between the electrode pair was measured 30 ns prior to the rising edge of every bipolar pulse (± 0.5 V) and averaged over the electrophoretic sampling time (1 s) to improve signal-to-noise ratio (S/N). The data acquisition and controlling software was written in Labview (National Instruments).

Figure 4.1. Schematic diagram of the miniaturized electrophoretic system with a conductivity detector integrated into the C18-terminated PMMA device.

4.3 Results and Discussion

4.3.1 EOF in C18-terminated PMMA Device

Conventional CEC utilizes the EOF as the primary driving force for the mobile phase and ionic species through packed or open fused silica capillaries coated with C18 or other stationary phase chemically tethered to the support via siloxane chemistry. In a simple system using an uncoated capillary and no surfactants included in the electrolyte, the EOF velocity is
significantly greater than the velocity of most ionic species and as such, acts to promote the mass transport of all species toward the cathode. The EOF velocity \( \nu_{\text{eof}} \) can be calculated from the Smoluchowski equation as:

\[
\nu_{\text{eof}} = \left( \frac{\sigma \epsilon_0 \epsilon_r RT}{\eta 2F^2 I} \right)^{1/2} \frac{E}{I} \tag{4.1}
\]

where \( \sigma \) is the charge density \( (\text{C m}^{-3}) \) of excess ions in the Gouy-Chapman layer, \( \eta \) is the viscosity \( (\text{N s m}^{-2}) \) of the mobile phase, \( \epsilon_0 \) is electric permittivity of vacuum \( (\text{C}^2 \text{ J}^{-1} \text{ m}^{-1}) \), \( \epsilon_r \) is the dielectric constant of the mobile phase \( (\text{C}^2 \text{ J}^{-1} \text{ m}^{-1}) \), \( R \) is the gas constant \( (\text{kg m}^2 \text{ s}^{-2} \text{ mol}^{-1} \text{ K}^{-1}) \), \( T \) is the temperature \( (\text{K}) \), \( F \) is Faraday’s constant \( (\text{C mol}^{-1}) \), \( I \) is the electrolyte ionic strength \( (\text{mol L}^{-1}) \), and \( E \) is the applied voltage \( (\text{V}) \). From the above equation, excess charge on the surface wall plays a significant role in determining the electroosmotic mobility as well as its directional flow.

In the present case we are using a PMMA surface, which is known to have a substantially smaller electroosmotic mobility compared to fused silica (electroosmotic mobility = \( 2.03 \times 10^{-4} \) \( \text{cm}^2 \text{ V}^{-1} \text{ s}^{-1} \) at \( \text{pH} \sim 7.0 \) for PMMA; \( 17.80 \times 10^{-4} \) \( \text{cm}^2 \text{ V}^{-1} \text{ s}^{-1} \) at \( \text{pH} \sim 7.0 \) for fused silica).\(^{28}\) When chemically modified with an amine-scaffold, which was used to covalently attach a neutral (C18) phase, this system produced an electroosmotic mobility different to native PMMA. The measured electroosmotic mobility for this type of surface was found to be \( -1.27 \times 10^{-4} \) \( \text{cm}^2 \text{ V}^{-1} \text{ s}^{-1} \), where the negative sign indicates a cathodic EOF (pure aqueous borate electrolyte, \( \text{pH} \sim 7.0 \)).\(^{29}\) Figure 4.2 illustrates the electroosmotic mobility determined in the C18-terminated PMMA device as a function of acetonitrile content in the carrier electrolyte. The electroosmotic mobility was higher than our previous reported data (electroosmotic mobility = \( -1.92 \times 10^{-4} \) \( \text{cm}^2 \text{ V}^{-1} \text{ s}^{-1} \)) at \( \text{pH} \sim 7.4 \) (see Chapter 3),\(^{29}\) most likely due to a lower ionic strength carrier electrolyte.
Figure 4.2. EOF profile of C18-terminated PMMA device taken at different acetonitrile contents in the carrier electrolyte. The EOF profile was determined by monitoring a change in current versus time using 24 mM TEAA and 25 mM TEAA electrolyte solutions. Vertical error bars shown represent standard deviation in the measurements.

4.3.2 RP-IPOCCEC

Figure 4.3 depicts the RP-IPOCCEC analysis of a low DNA mass sizing ladder operated at a field strength of 333 V cm\(^{-1}\) using 30% acetonitrile in a 70% aqueous phase comprised of 25
mM TEAA. Only one peak was evident for the sizing ladder with no apparent separation. The lack of separation may have resulted from minimal interactions between the ion-paired DNA complexes and the stationary phase during the course of the elution process since lateral diffusion must carry the analytes to the wall of the device for effective partitioning into the stationary phase to affect the separation.

**Figure 4.3.** RP-IPOCCEC analysis of a low DNA mass sizing ladder (100 ng mL$^{-1}$) on a C18-terminated PMMA device utilizing indirect, contact conductivity detection. The sizing ladder consisted of 100, 200, 400, 800, 1200 and 2000 bp fragments. Electrophoresis was accomplished utilizing 30% acetonitrile in 70% aqueous phase consisting of 25 mM TEAA (ion-pairing agent, pH 7.4); 3 s electrokinetic injection time; field strength 333 V cm$^{-1}$ for the electrophoresis. The conductivity cell was operated at 5.0 kHz with bipolar pulse amplitude of ±0.5 V.

The short elution time and small diffusion coefficients associated with these polyanionic molecules minimizes the interaction time with the stationary phase. A lack of separation also indicates that the free draining behavior (molecular conformational state of the DNA molecule that results in the frictional coefficient dependence of the electrophoretic mobility to scale
directly with the number of bases comprising the DNA molecule; coupled to the fact that the charge of the DNA scales directly with the number of bases, the mobility in free solution is independent of base number) of the DNA remains intact even for these ion-paired complexes and therefore, they have the same effective electrophoretic mobility in free solution. From the apparent mobility in the absence of chromatographic retention, we can estimate a linear velocity of 0.25 cm s\(^{-1}\) at this field strength, giving an apparent mobility of \(-7.51 \times 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}\). We can calculate the free solution electrophoretic mobility of the ion-paired complexes using the electroosmotic mobility obtained from Figure 4.2 at 30% acetonitrile and the apparent mobility of these ion-paired complexes calculated above, which produced a value of \(-1.54 \times 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}\). The free solution electrophoretic mobility of the DNA with base pair numbers > 400 has been measured to be \(-4.35 \times 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}\). Our smaller value for the DNA electrophoretic mobility is most likely due to the presence of associated cationic species, triethylammonium, which neutralizes some of the negative charge found on the DNA fragment as well as adding mass to the fragment increasing its frictional coefficient (i.e., lower electrophoretic mobility). Figure 4.4 shows the results observed in Figure 4.3 were ameliorated by using a lower field strength. The sizing ladder separation was effectively developed within 75 s with near baseline resolution for all fragments comprising this ladder. The ability to do this separation within an effective length of 3 cm at 67 V cm\(^{-1}\) is rather surprising given the fact that in this open channel format, the analytes must laterally diffuse to the wall in order to interact with the stationary phase. As previously seen in Figure 4.3, the 2000 bp DNA fragment was not visible, whereas in Figure 4.4 the retention time for the 2000 bp DNA fragment was 69 s. Using this value and a diffusion coefficient of \(2.2 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}\) (determines the potential number of interactions of the fragment between the stationary phase and mobile phase), the average diffusional distance
was calculated to be 17.4 µm. At the present time, the differences observed in Figures 4.3 and 4.4 are uncertain. A possible explanation may be the altered diffusion coefficient observed by these ion-paired complexes at higher applied fields and the defined orientation they adopt when placed in a high field. Large DNA molecules at high fields are found to have enhanced diffusion coefficients due to orientational effects in this field, which may result in reduced lateral diffusion (rectified diffusion). At lower fields, the DNA is more randomly oriented.

Figure 4.4. RP-IPOCCEC analysis of a low DNA mass sizing ladder (100 ng mL\(^{-1}\)) (black line) and PCR-1 (500 bp) and PCR-2 (1000 bp) amplicon profiles at 0 and 10 PCR cycles (red lines) on a C18-terminated PMMA device utilizing indirect, contact conductivity detection. The sizing ladder consisted of (1) 100, (2) 200, (3) 400, (4) 800, (5) 1200 and (6) 2000 bp fragments. Electrophoresis was accomplished utilizing 30% acetonitrile in 70% aqueous phase consisting of 25 mM TEAA (ion-pairing agent, pH 7.4); 3 s electrokinetic injection time; field strength 67 V cm\(^{-1}\) for the electrophoresis. The conductivity cell was operated at 5.0 kHz with bipolar pulse amplitude of ± 0.5 V.

Also shown in Figure 4.4 is the electrophoregram for two PCR amplicons, which appeared at 45 and 54 s, for PCR-1 and PCR-2, respectively and possessed retention times that confirmed their size when overlaid on the sizing ladder electrophoregram. Under electrophoretic conditions employed here, the PCR amplicons were readily resolved and separated from other components found within the PCR cocktail such as dNTPs, salts, primers and polymerase.
We next attempted to carry out RP-IPOCCEC by only changing the acetonitrile content in the carrier electrolyte to optimize and examine the effect on DNA fragments retention times as shown in Figure 4.5.

**Figure 4.5.** RP-IPOCCEC analysis of low DNA mass sizing ladder (100 ng mL\(^{-1}\)) (black line) and PCR-1 (500 bp) and PCR-2 (1000 bp) amplicon profiles at 10 PCR cycles (red line) on a C18-terminated PMMA device utilizing indirect, contact conductivity detection. The sizing ladder consisted of (1) 100, (2) 200, (3) 400, (4) 800, (5) 1200 and (6) 2000 bp fragments. Electrophoretic conditions and conductivity cell operations are similar to those employed in Figure 4.4.
As expected, decreasing the acetonitrile content extended the retention time and PCR amplicons migrated between the sizing ladder fragments consistent with their expected sizes. At 20% acetonitrile, PCR-1 and PCR-2 amplicons were evident at 130 and 176 s and increased to 240 and 297 s with 5% acetonitrile. Surely, modifying the acetonitrile content in the carrier electrolyte presents an example of how to increase the speed and enhance the separation power for RP-IPOCCEC.

PCR amplicons in each case were produced from only 10 thermal cycles and discernible signals were generated from the contact conductivity detector. We can estimate the mass of DNA analyzed from knowledge of the fixed volume injector dimensions (425 pL) and the concentration of the PCR amplicons. The DNA concentration can be determined from the PCR amplification by;

\[
C_{DNA} = \frac{N \cdot (E_n)^2n}{N_a V}
\]

(4.2)

where \(N\) is the starting copy number of DNA molecules, \(E_n\) is the efficiency of a particular amplification step (depends on supply of dNTPs and integrity of polymerase enzyme), \(n\) is the number of PCR thermal cycles, \(N_a\) is Avogadro’s number, and \(V\) is the volume of the PCR reaction. Since we are using only 10 cycles for this calculation, \(E_n\) was assumed to be 1 for each cycle. Beginning with a starting copy number of \(1.9 \times 10^6\) (10 ng mL\(^{-1}\) of \(\lambda\)-DNA, 1.0 \(\mu\)L used in PCR reaction), the DNA concentration analyzed in the peaks pictured in Figures 4.4 and 4.5 was 15.9 pM. Taking into account an injection volume of 425 pL, this represents \(6.8 \times 10^{-21}\) mols. The S/N from the data presented was estimated to be 17, giving a mass detection limit equal to \(1.2 \times 10^{-21}\) mols at a S/N \(\sim 3\). To the best of our knowledge, this is the lowest detection limit reported to date using conductivity detection.
4.3.3 Effects of Acetonitrile

Figure 4.6 represents the effects of 5% to 30% acetonitrile content on the retention factor ($k'$) for several DNA fragments of the sizing ladder and PCR amplicons.

![Figure 4.6. Effects of acetonitrile content on retention factor of several DNA fragments of the low mass sizing ladder and PCR amplicons. Electrophoretic conditions and conductivity cell operations are similar to those employed in Figure 4.4.](image)

The separation process in CEC is determined by the partitioning between two immiscible phases as well as differences in the analytes' electrophoretic mobilities. The migration velocity ($\nu$, cm s$^{-1}$) of any charged analyte in CEC can be calculated from:

$$\nu = \frac{1}{1 + k'} (\nu_{ep} + \nu_{eof})$$ (4.3)

where $k'$ is the retention factor and the sum $\nu_{ep} + \nu_{eof}$ is equal to the apparent velocity ($\nu_{app}$, cm s$^{-1}$). In the present case, all of the ion-paired complexes possess similar electrophoretic mobilities (see Figure 4.3) and as such, the separation is determined primarily by selective
partitioning between C18 stationary phase and mobile phase. The retention factors were calculated by rearrangement of equation (3) to give;

\[ k' \left( \frac{\nu_{ep} + \nu_{eof}}{\nu} \right) - 1 = (4.4) \]

The velocity of all ion-paired complexes was calculated from the results depicted in Figure 4.3, where no stationary phase interactions were assumed and the linear velocity was taken from the results depicted in Figure 4.2. As can be seen from Figure 4.6, the retention factor decreased as the acetonitrile content was increased, similar to what has been observed in other studies using CEC with C18-based stationary phases and fused silica capillaries. While increasing the acetonitrile content does reduce the analysis time due to increases in the electroosmotic mobility, a faster moving EOF minimizes the partitioning of the ion-paired complexes into the stationary phase. Therefore, the ability to obtain baseline resolution can become more problematic (i.e., smaller differences in retention factor values for the analytes). In fact, at 30% acetonitrile, the retention factor value for the 400 bp fragment was found to be \( \sim 0.44 \), indicating minimal partitioning into the stationary phase. Thus, for short DNAs (< 400 bp) utilizing these electrophoretic conditions, acetonitrile compositions exceeding 30% can be less effective in performing the desired separation.

We also looked at effects of acetonitrile content in the carrier electrolyte on other chromatographic parameters such as the selectivity factor \( (\alpha) \), plate numbers \( (N) \) and resolution \( (R_s) \) and those results are presented in Figures 4.7 and 4.8. The selectivity factors were calculated for peak pairs 400/800 bp and 800/1200 bp fragments from the sizing ladder and for PCR-1/PCR-2 as well. The selectivity factor was simply calculated from the ratio of the two retention times of the peaks within a pair with the later eluting peak’s retention time placed in the
The plate numbers were calculated from the retention time and width of the peak at the base assuming purely Gaussian peak shapes. Resolution values were calculated from the plate number, selectivity factor, and retention factor values using the equation:

\[
R_S = \left( \frac{N^{1/2}}{2} \right) \left( \frac{\alpha + 1}{\alpha - 1} \right) \left( \frac{k_{ave}^{'}}{k_{ave}^{'^2} + 1} \right)
\]  

(4.5)

where \(N\) is the plate numbers, \(\alpha\) is the selectivity factor, and \(k_{ave}^{'}\) is the average retention factor for the particular peak pair. In Figure 4.7, the selectivity factor of all three peak pairs show a maximum at \(\sim 20\%\) acetonitrile, while the plate number decreased as the acetonitrile content increased with maximum plate number values occurring at 5\% acetonitrile.

**Figure 4.7.** The effects of acetonitrile content on the selectivity factor (dash lines) and plate numbers (solid lines) for the 400 bp/800 bp (■), PCR-1/PCR-2 (●), and 800 bp/1200 bp (▲) peak pairs. Electrophoretic conditions and conductivity cell operations are similar to those employed in Figure 4.4.

In all cases, the longer DNA fragments (800/1200 bp) produced larger plate number values due to their higher affinity for the stationary phase (see Figure 4.7). The fact that the plate numbers were higher for the longer DNA fragments also suggests that mass transfer artifacts into and out
of the stationary phase contributed minimally to the dispersion of the bands, most likely a direct consequence of the fact that the stationary phase is a monolayer. Inspection of Figure 4.8 shows the resolution decreased continuously as the amount of acetonitrile was increased due mostly to the loss of plate number as well as reductions in the retention factor.

![Figure 4.8](image)

**Figure 4.8.** The effects of acetonitrile content on the resolution for the 400 bp/800 bp (■), PCR-1/PCR-2 (●), and 800 bp/1200 bp (▲) peak pairs. Electrophoretic conditions and conductivity cell operations are similar to those employed in Figure 4.4.

We also monitored the S/N for several peaks in the electrophoregrams as a function of acetonitrile content in the carrier electrolyte (see Figure 4.9). We found no substantial changes in the S/N as the amount of acetonitrile increased when keeping the ionic strength of the carrier electrolyte constant. However, we noticed that S/N did degrade slightly when the ion-pairing agent concentration was increased in the carrier electrolyte, but this increase did improve the dynamic range of the response. Therefore, it seems that the composition of the carrier electrolyte in terms of the amount of acetonitrile can be altered to optimize the electrophoretic performance without significantly affecting the response elicited by the conductivity detector.
Figure 4.9. Effects of acetonitrile content on the signal-to-noise ratio. Electrophoretic conditions and conductivity cell operations are similar to those employed in Figure 4.4. Vertical error bars shown represent standard deviation in the measurements.

4.4 Conclusions

RP-IPOCCEC can be used as an effective tool for the isolation and/or purification of PCR amplicons. Due to the free draining behavior of ion-paired DNA complexes, separation is affected predominately by partitioning into the stationary phase, which in this case was a C18 wall-coated surface. Treatment of PMMA surfaces with a C18 stationary phase effectively separated 100-2000 bp DNA fragments in an open channel format when a low field strength was applied in addition to providing a protective layer for the underlying PMMA against acetonitrile attack. By varying the acetonitrile content in the carrier electrolyte, the electrophoretic performance could be altered, with maximum performance, as indicated by the plate numbers and resolution values, afforded by low amounts of acetonitrile. However, the electrophoretic development times did decrease with increasing acetonitrile due not only to the higher elution power of acetonitrile but also due to an increase in the electroosmotic mobility.
4.5 References


Chapter 5
Summary and New Developments for Future Growth

5.1 Summary

The fabrication and application of disposable, micromachined fluidic device has been demonstrated. Attractive features associated with the device described in this document include; the potential for system integration in which various processing procedures were implemented onto a single fluidic platform, rapid analysis speeds, reduced sample reagent consumption, and the ability to mass produce the device at minimal costs.

The detection protocol used for the device discussed herein was contact conductivity detection, in which the electrodes were in direct contact with the solution. The detector was constructed by placing two conventional-sized electrodes into the sidewalls adjacent to the separation channel to minimize interferences between the separation voltage and the electrode potential. The waveform applied to the electrode pair was a bipolar pulse waveform, which was used to reduce the charging current from the measurement so that the current recorded prior to the rising edge of every bipolar pulse is more representative of the solution conductance. From the results reported, the applicability of an integrated conductivity detector for direct analysis of amino acids, peptides, proteins, and oligonucleotides (double-stranded DNA) separated by a variety of electrophoretic formats was demonstrated. In addition, the use of conductivity detection allowed mass detection sensitivities in the range of $10^{-18} - 10^{-21}$ moles.

5.2 New Developments for Future Growth

We present to the best of our knowledge, a state-of-the-art, multichannel device with a conductivity array detector. This device, which consists of a 16-channel fluidic network and a printed circuit board, is geared toward automating three-processing steps onto a single fluidic
platform including purification, preconcentration, and detection for downstream parallel processing. The 16-channel fluidic network was produced from a Ni electroform molding die using hot embossing. The printed circuit board with metal electrodes serves as the cover plate for the supply of high voltage and for detection. Fabrication of the printed circuit board involved a photoresist procedure consisting of photoactivation and metal deposition, followed by a photoresist-free procedure involving UV light and electroless deposition processes. This approach eliminates the need for clean-room facilities and expensive apparatus required for vacuum deposition or sputtering.

5.2.1 Multichannel Fluidic Network

The first phase of microfabrication involved hot embossing the desired PC polymeric parts from a single Ni electroform molding die developed via LIGA techniques as described in Chapters 1 and 3. Employing LIGA techniques, raised Ni microstructures measured at 100 µm tall by step profilometry following the completion process were fabricated (see Figure 5.1).

![Figure 5.1](image)

**Figure 5.1.** SEM images of the multichannel Ni molding die taken of (A) array of separation channels; (B) cross offset 'T' for sample injection; (C) inlet flow channel near point of injection; (D) microfabricated diamond-shaped cavities; and (E) outlet flow channel near point of detection.
The embossing system consisted of a PHI Precision Press model number TS-21-H-C(4A)-5 (City of Industry, CA) equipped with an in-house built vacuum chamber to remove air (pressure < 0.1 bar) so that complete filling of the molding die could take place. Prior to embossing, planar PC wafers (Goodfellow, Berwyn, PA) (133 mm in diameter) were baked in an oven at 85°C for 12 h to remove all residual water present in the polymer. The molding die was coated with a releasing agent, MoldWiz (Axel, Woodside, NY), to improve demolding. During embossing, the molding die was heated to 185°C and pressed into the PC wafer with a force of 1150 lbs for 7 min. During this process, the molding die was heated to 195°C. After 7 min, the press was opened and the embossed PC wafer was allowed to cool. Following embossing, the PC wafer was cut to a 4" x 2" square and holes (1 mm in diameter) were drilled at their designated areas to serve as the fluidic reservoirs (see Figure 5.2).

**Figure 5.2.** (A) Topographical layout of the multichannel fluidic network. Injection channel length = 1.0 cm; separation channel = 4 cm x 230 µm x ~ 100 µm; inlet and outlet flow channel = 1 cm in length x 20 µm wide (outside-to-outside edge). The solution reservoirs are; (1) sample reservoir; (2) electrolyte reservoir; (3) waste reservoir; (4) and (4') 150 µm o.d. capillary insertions for interconnecting devices. (B) SEM image of the network taken near point of injection. (C) Configuration of the flow distributor before and after the separation channel. (D) SEM image taken of one separation channel that embodied 30 µm x 30 µm diamond-shaped collocated monolith support structures (COMOSS) (equally spaced ~ 20 µm). (E) SEM image of the network taken near point of detection.
The fluidic network consisted of 16 independent channels, whereby each channel corresponded to a unique separation lane. Dimensions of these channels were equal to provide a system in which the linear velocity at all points (i.e. inlet, separation, and outlet channels) are equal in magnitude. Situated in each separation channel were diamond-shaped support structures. These structures alleviate the need for conventional packing materials and in addition, these structures can provide a much higher surface area relative to the open-channel format adopted in our earlier work.

In an effort to increase the hydrophobic character of PC for future electrochromatography applications, the surface was UV-chemically modified using chemistry recently developed in our laboratories. Prior to surface modification, the PC substrates (Goodfellow, Berwyn, PA) were precleaned using HPLC grade 2-propanol (Aldrich, Milwaukee, WI), 18 MΩ·cm water (Barnstead, Dubuque, IA), and subsequently dried under a stream of nitrogen. PC surfaces were exposed to UV light (254 nm, 15 mW cm⁻²) for 50 min using an AB-M Series 60 Exposure System (ABM Inc., San Jose, CA). Sonication in 10% (v/v) solution of HPLC grade 2-propanol in 18 MΩ·cm was then performed for 15 min to remove any non-covalently adsorbed PC molecules. Following sonication, the UV-modified PC surface was rinsed with copious amounts of 18 MΩ·cm water and then dried under a stream of nitrogen. Next, the UV-modified PC substrates were immersed in a solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (50 mM, 99%, Sigma, St. Louis, MO), 1,2-ethylenediamine (15 mmol, Fisher, Fair Lawn, NJ), and phosphate electrolyte (100 mM, pH 7.0) for 4 h. The resulting amine-terminated PC surface was rinsed with copious amounts of 18 MΩ·cm, dried under a stream of nitrogen, and later introduced to neat N-octadecane 1-isocyanate (98%, Aldrich) (via syringe). Following this
reaction, the C18-terminated PC surface was rinsed with copious amounts of hexanes, toluene, acetone (Aldrich) and subsequently dried under a stream of nitrogen.

To determine differences in the PC surface properties after UV chemical modification, sessile drop water contact angles of flat PC sheets (15 mm x 15 mm x 3 mm) were performed with a VCA 2000 Goniometer (VCA, Billerica, MA). Using a syringe, 4 - 6 µL of 18 MΩ•cm water was placed on the air-side of PC and UV-chemically modified PC surfaces. Using software provided by the manufacturer, the contact angles of five separate water droplets were taken and averaged for each given substrate. The average contact angle of native PC was found to be 73 ± 1°. After the surface was exposed to UV light, the contact angle decreased to 35 ± 3°, consistent with our previous data. Also, this value is consistent with that obtained for self-assembled monolayers terminated with hydrophilic functional groups. Similar results were obtained with the amine-terminated PC, which was found to be 34 ± 2°. The contact angle for the C18-terminated PC was found to be 100 ± 9°, indicative of increased hydrophobicity.

5.2.2 Microfabricated Electrodes: Procedure I

The second phase of this project involved fabrication of a printed circuit board via photoactivation and metal deposition. Described below are the steps involved in fabricating metal electrodes on a PC cover plate for the potential use in supplying high voltage and conductivity detection.

The electrode topography (see Figure 5.3) was written using AutoCAD (Autodesk Inc., San Rafael, CA), after which the pattern was transferred to a 6" x 6" x .120" chromium-coated quartz plate (HTA Photomask, San Jose, CA). Prior to photoactivation and metal deposition, the PC substrate (AIN Plastics, Mount Vernon, NY) (6" x 6" x .059") was rinsed with copious amounts of HPLC grade 2-propanol and then with 18 MΩ•cm water, dried under a stream of nitrogen,
and placed in an oven at 85°C for 30 min. At this point, the substrate was placed into a thermal evaporator (Temescal, Fairfield, CA) in which 50 Å of Cr (seed layer) and 1000Å of Au were deposited. A S1813 (positive photoresist, Shipley, Somerville, NJ) was spin-coated onto the Au-coated PC substrate at 2000 rpm for 30 s (Headway Research Inc., PWM101 Spinner). After a pre-bake for 30 min at 90°C, the optical mask was placed over the resist-coated PC substrate and this assembly was exposed to UV light for 9 s (Oriel UV Exposure System, Stratford, CT). During UV exposure, the desired features on the optical mask were photo-directed onto the coated PC surface. The substrate was then developed (40 s) and rinsed with 18 MΩ•cm water, followed by air drying. After resist development, the exposed Au and Cr layers were etched away, revealing the desired electrode pattern.

5.3. Topographical layout of the printed circuit board designed with respect to the reservoirs on the fluidic network. The inlet end consisted of 72 electrodes (2.54 mm long x 1.78 mm wide), in which 36 electrodes are for high voltage and the other 36 electrodes for detection. The outlet end consisted of 16 electrodes (7.62 mm long x 2.0 mm wide) for the supply of high voltage.
5.2.3 Microfabricated Electrodes: Procedure II

As stated above, a printed circuit board was developed in our laboratories utilizing a photoresist-free procedure that involved UV light and electroless deposition processes. Prior to UV radiation and electroless deposition, the PC substrate (AIN Plastics, Mount Vernon, NY) was precleaned using HPLC grade 2-propanol, 18 MΩ•cm water, and then dried under a stream of nitrogen. UV radiation was carried out using an AB-M Series 60 Exposure System (ABM Inc., San Jose, CA). The sections below described the steps involved in fabricating metal electrodes on a PC cover plate for the potential use in supplying high voltage and detection.

A 6” x 6” x .059” PC substrate was situated under the optical mask and this assembly was exposed to UV light (254 nm, 15 mW cm⁻²) for 2 h. During the photoactivation process, the desired features on the optical mask were photo-directed onto the PC substrate. Sonication in 10% (v/v) solution of HPLC grade 2-propanol in 18 MΩ•cm water was then performed for 15 min to remove any non-covalently adsorbed PC molecules. The UV-modified PC surface was rinsed with 18 MΩ•cm water and then dried under a stream of nitrogen. Next, the UV-modified PC substrate was immersed for 6 h in a solution of 1,2-ethylenediamine (24 mmol, Fisher, Fair Lawn, NJ), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (50 mM), and phosphate electrolyte (100 mM, pH 7.0). The resulting amine-terminated PC surface was dried under a stream of nitrogen and immersed for 1 h in a filtered aqueous solution of hydrogen tetrachloroaurate (III) trihydrate (HAuCl₄) (1.0 mM, 99%, Aldrich) in glassware covered by Al foil. Sonication in 18 MΩ•cm water was then performed for 10 min to remove any unbound species, and the surface was later dried under a stream of nitrogen before being placed in an aqueous solution of sodium borohydride (NaBH₄) (0.1 M, 99%, Aldrich). A uniform raspberry-colored layer of the reduced Au (seed layer) appeared on the photopattern surface after ∼ 2 - 4
min and the surface was later rinsed with copious amounts of 18 MΩ•cm water then dried under a stream of nitrogen. Electroless deposition was then performed. First, the raspberry-colored PC substrate was immersed in a basic solution (pH 11.0) comprised of copper sulfate (CuSO$_4$) (0.02 M, Mallinckrodt Laboratory Chemicals, Phillipsburg, NJ), sodium potassium tartrate (NaKC$_4$H$_4$O$_6$) (0.12 M, Fisher) and formaldehyde (0.9 M, 37%, Aldrich) at ~ 60°C. Deposition took place for 5 - 20 min to form the Cu electrodes (see Figure 5.4). Au electrodes (not shown) were prepared by immersing the Cu electrodes in a gold sulfite solution ((Na$_3$Au(SO$_3$)$_2$)) (10 mM, pH ~ 6.0 - 7.0) with the addition of formaldehyde (0.7 M, Aldrich) for 3 – 15 min.

5.4. (A) Schematic demonstrating alignment of the pattern electrodes after complete device assembly. SC, separation channel; HV, high voltage electrode; PE, ground electrode; DE, conductivity detector (working and reference electrodes). (B) Top view of printed circuit and fluidic network.

As can be seen in Figure 5.4, the microfabricated metal electrodes on the PC cover plate were adapted to the shape of the fluidic reservoirs on the fluidic network to ensure best contact with the carrier electrolyte and sample solutions in the reservoirs. In addition, the electrode pattern was in agreement with the optical mask original design (see Figure 5.3). The configuration of the conductivity array detector was adopted to maintain a footprint relative to the size of the
fluidic network and this geometry is more favorable for device production, because of easier alignment with the separation channel (see Figure 5.5). The resistance of the Cu electrode (with a multimeter from one end of the prepared electrode for electrical connection to another) was measured to be in the range of 36 - 95 Ω that decreased to 11 - 20 Ω for Au. For comparison, Yan and coworkers reported a resistance in the range 30 - 50 Ω for Cu electrodes and 8 - 12 Ω for Au electrodes.7

**Figure 5.5.** Optical micrographs of Cu microfabricated electrodes on PC surface. Shown is the detection region of the working and reference electrodes for (A) first conductivity detector; (B) eighth conductivity detector; and (C) sixteenth conductivity detector. The electrodes were 50 µm in diameter with an end-to-end spacing of 25 µm.

The next attempt was to electroplate Au onto the exposed Cu metal surfaces at 1 mA cm⁻² using a 6" x 4" platinized titanium mesh (Technic, Irving, TX), which served as the anode. Prior to final Au-electroplating, the exposed metal surfaces were sonicated in 10% (v/v) solution of sulfuric acid (96 %, Fisher) in 18 MΩ•cm water for 2 min. The desired Au structures (∼ 1 µm thick) were plated out of an Au-sulfite bath onto the exposed areas of the metal surface. Plating was done at ambient temperature under continuous stirring at pH ∼7.0. The amount of Au in
solution was maintained at no less than 80% of its initial value. Unexpectedly, the results from this experiment showed the detection electrodes in the array overlapped as demonstrated in Figure 5.6 below.

![Image](image.png)

(A) (B) (C)

**Figure 5.6.** Optical micrographs of electroplated Au electrodes on PC surface. Shown is the detection region of the working and reference electrodes for (A) first conductivity detector; (B) eighth conductivity detector; and (C) sixteenth conductivity detector.

### 5.2.4 Electronics

Once the individual components are constructed, the multichannel device will be assembled using alignment markes on both the fluidic network and the printed circuit board. For detection, the bipolar pulse waveform for the conductivity array detector will be generated by an in-house fabricated circuit (see Figure 5.7), which is controlled by a National Instruments controller board (PCI-6071E, Austin, TX) programmed using Visual basic. The instrument outputs a bipolar pulse waveform that can be adjusted in amplitude. A 12 bit analog-to-digital converter (A/D) covers a range of 0 V - 2 V. The potential of one electrode is maintained at virtual ground while the potential at the other electrode is controlled by the bipolar waveform. To generate the bipolar waveform, a brief positive pulse period during which switch 1 (A) is turned on is immediately followed by a negative pulse period of the same length in which switch 1 (B) is turned on. The detector oscillates (3 - 6 kHz) and gathers instantaneous current readings between the electrode pair ~ 100 ns prior to the rising edge of every bipolar pulse (up to ± 0.5 V) at the input port. The readings can be averaged over the electrophoresis sampling time to improve the signal-to-noise ratio (S/N). The resulting signal from each detector in the array can
be processed by an adjustable gain amplifier (four resistors ranging from 1 kΩ - 1 MΩ) and passed to a sample-and-hold amplifier whereby the output is filtered by a low-pass filter with a cutoff frequency of .16 Hz. To further preserve signal integrity, the filtered output is transmitted to the board via a differential amplifier driving a shielded twisted pair. The received signal can be amplified by the board and converted to a 12-bit word.

5.7. Schematic representation of the fabricated circuit for the capacitively coupled 2-electrode conductivity array detector. See text for detailed description.

5.3 References


Vita

Michelle Galloway was born on October 8, 1974, in Cheraw, South Carolina, to Earl Daniel Galloway, Sr. of Clio, South Carolina, and Carrie Bell Taylor Galloway of Bennettsville, South Carolina. She has two older brothers, Earl and Darryl Galloway, an older sister, Regina Lowe-Easterling, a younger sister, Angela Galloway, and is a fraternal twin to Mitchell Galloway. She is the aunt of two nieces Shanetta Jacobs and Stephanie Easterling, and two nephews, Keldrick Williams and Javad Easterling.

In 1992, Ms. Galloway received her high school diploma from Marlboro County High School in Bennettsville, South Carolina. She later made her way to Johnson C. Smith University in Charlotte, North Carolina. While there she worked with former research advisor Dr. Derrick C. Tabor, Mrs. Jennifer Dickson, and colleague Ms. Kia Ghee for three consecutive years in the Minorities of Biomedical Research Program (MBRS) funded by the National Institutes of Health (NIH). Her research involved synthesizing and characterizing substituted dicarbocyanine dyes. During the summer of 1995, she worked in the field of polymer science with Dr. Joseph DeSimone at the University of North Carolina at Chapel Hill, in Chapel Hill, North Carolina. There she synthesized polymers and copolymers in supercritical carbon dioxide. A year later, she received her Bachelor of Science degree in chemistry with a minor in mathematics from Johnson C. Smith University. A few months following, she was accepted into the graduate program at Delaware State University in Dover, Delaware. While there she worked with former research advisor Dr. Donald Wilkinson in developing separation protocols for polynuclear aromatic hydrocarbons. In the summer of 1997, she worked at the United States Environmental Protection Agency, Air Pollution Prevention and Control Division (EPA), in Research Triangle Park, North Carolina. While there she worked with Dr. Gene Tucker and Ms. Bethany in
collecting, organizing, and statistically analyzing published and non-published data on emissions of volatile organic compounds from flooring materials, coatings, furnishings, machines, and personal activities. Later in May of 1998, she received her Master of Science degree in applied chemistry-environmental science from Delaware State University in Dover, Delaware.