

**Towards high-throughput analysis of
biomolecules using LED-induced
fluorescence and zone-electrophoresis based
methods**

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In loving memory of Richard K. Radcliffe. Thanks for always encouraging me to aim high, work hard, and achieve greatness.

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Abstract

Peptide hormone secretions play a key role in many biological processes and elucidating their secretion profiles is an important step for understanding mechanisms involved in disease pathology. To investigate these processes, analytical tools are required that can probe biomolecules with high spatial and temporal resolution. This often requires analysis methods to interface with sampling techniques with low flow rates and small sample volumes. This work focuses on the development of novel low-cost, automated analytical tools for highly sensitive and selective assays for biomolecules.

Capillary electrophoresis (CE) applications are well-suited for this aim due to low sample volume requirements, fast analysis, and automation capabilities. In this dissertation, methods for enhancing CE detection are explored specifically with the goal of minimizing detection limits of a CE-immunoassay. CE-immunoassays combine the advantages of CE with the sensitivity and selectivity of immunoassays where antibodies are used for targeted analysis of biomolecules. Initially, a low-cost, miniature 3D printed LED-IF detector was designed and optimized for high performance CE separations in a commercial CE instrument for CE of immunoassays and CE-TDA analysis for monitoring a common biomolecule conjugation reaction.

Since CE-immunoassays can suffer from poor analyte peak resolution in the time domain, Fourier-transform detection methods were investigated. Specifically, the 3D printed LED-IF detector was integrated into a six-detector array to increase the number of detection points in the electropherogram. This array convolutes the electropherogram with a Shah function for Shah convolution Fourier-transform (SCOFT)-CE. Once the convoluted electropherogram undergoes a Fourier-transform, the frequency domain will contain characteristic frequencies that correspond to each analyte. Multiphysics simulations illustrate the advantages of SCOFT when combined

with continuous multiple injection methods. This work presents a continuous injection method termed Sine-injection Fourier-transform (SIFT). SIFT, on its own, has been shown in simulation to enhance peak resolution for analytes in the frequency domain, but an even larger enhancement in resolution and S/N was observed when combined with a multi-detector array.

This dissertation explored the applications of paper substrates for zonal electrophoresis. Microfluidic paper-based analytical devices (μ [micro]-PADs) were constructed and characterized using various low-adsorption paper types to determine electrokinetic properties and performance for fluorescence detection.

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Chapter 1

Introduction

1.1 Biological motivation

Autism spectrum disorder (ASD), a developmental disorder characterized by social interaction and communication deficits and the presence of repetitive behaviors and interest [1], has been estimated to have increased in prevalence among eight year old children from 1 in 150 children in 2002 to 1 in 59 children in 2014 [2]. While the increase in prevalence may be a result of inconsistent methods for monitoring and diagnosing ASD, studies have consistently revealed a ratio of 4:1 for males diagnosed with ASD in relation to females and the average age for early diagnosis was 53 months [2]. Symptom heterogeneity and the lack of a bio-marker creates a challenge for early diagnosis.

Endogenous opioid signaling from the pituitary gland has been under investigation for understanding ASD because of its role in social attachment [3, 4]. In fact, endogenous opioid peptide β -endorphin has been identified as a potential biomarker of ASD, but studies to measure β -endorphin levels in blood and CSF have not been conclusive in determining if an up-regulation or down-regulation of the peptide results in symptoms of ASD [5]. Therefore, we hypothesize that the dysregulation of β -endorphin secretion leading to ASD is a temporal dysregulation rather than a simple up- or down-regulation of the peptide. In order to properly examine how the secretion profile of β -endorphin changes over time, new tools must be developed to assay the neuropeptide hormone with high temporal resolution.

Interestingly, endogenous opioids have been found to interact with the magnocellular neurosecretory system in the hypothalamus which is responsible for the synthesis of oxytocin [6]. Oxytocin, another peptide neuromodulator, has also been found to have implications for symptoms of ASD due to the role of oxytocin in social bonding and lower plasma levels of oxytocin

found in autistic subjects compared to controls [5]. Therefore, investigation of the temporal neurosecretory relationship between β -endorphin and oxytocin may also unveil critical information for understanding the role of these neuromodulators in the pathophysiology of ASD.

1.2 Analytical challenges

In order to obtain relevant information for the secretory relationship between β -endorphin and oxytocin, it is paramount that sampling occurs from the pituitary gland, the origin of secretion for both peptides [6, 7]. This can be achieved by placing a microdialysis or perfusion probe in the pituitary tissue in an animal model or from on-line pituitary tissue cultures. While this work does not progress to working with biological tissue cultures or animal models, sampling methods are an important consideration for developing an adequate analytical measurement tool to provide the desired temporal resolution.

The fundamental challenge for assay development of small peptide hormones in a biological sample containing many other peptides is the ability to selectively measure only the peptides of interest. Immunoassays provide this selectivity by capitalizing on the affinity of an antibody for a particular antigen. Enzyme-linked immunosorbent assays (ELISA) and radio immunoassays (RIA) are most common, and while these methods are well-established, sensitive, and selective, they require time consuming multi-step procedures with long incubation times in addition to the use of large sample volumes. Since flow rates for microdialysis or on-line culture perfusion are slow, as low as $100 \mu\text{L min}^{-1}$, the volume requirement can limit the temporal resolution of the assay. Therefore, it is advantageous to use a tool that is capable of analysis using small volumes of samples. To achieve this aim, this work utilizes capillary electrophoresis, and other zone electrophoresis methods, to develop new tools for profiling neuropeptide hormones with high temporal resolution.

1.3 Capillary electrophoresis instrumentation and applications

1.3.1 Introduction to capillary electrophoresis (CE)

Capillary electrophoresis (CE) is an analytical separation method where analytes are exposed to a high electric field (100-500 V cm⁻¹) in narrow-bore capillaries (25-100 μm inner diameter). The analytes are then separated by differential migration times based on a charge-to-size ratio [8]. Modern CE was first introduced in 1981 by James Jorgenson as a high-efficiency separation method for biological sample components [9]. In summary, a narrow-bore capillary is filled with a background electrolyte and each end is placed in a buffer well with an electrode (Fig. 1.1). An applied electric field will result in electrophoresis. The advantages of CE include fast analysis times, high separation efficiency, ease of automation, and low sample volumes. The ability for CE to complete analysis with small sample volumes is ideal for development of peptide assays with high temporal resolution. CE injections can be <100 nL, depending on the injection mode and time of injection.

The governing forces of capillary electrophoresis are electroosmosis and electrophoresis. When an electric field is applied across a capillary filled with an electrolyte solution, contents are driven from anode to cathode in bulk, plug-like flow via electroosmosis. This plug-like flow is called electroosmotic flow (EOF) and is dependent on the electroosmotic mobility of the background electrolyte which fills the capillary. EOF is the result of the formation of an electronic double layer (EDL) that forms along the capillary wall when the pH > 2. When the silica capillary wall becomes deprotonated at low pH, the negative charge on the capillary wall attracts cations from the background electrolyte which results in formation of the EDL (Figure 1.1).

Electroosmotic mobility (μ_{eof}) is described by:

$$\mu_{eof} = \frac{\epsilon \zeta}{\eta} \quad (1.1)$$

where ϵ is the dielectric constant, ζ is the zeta potential, and η is the dynamic viscosity of the background electrolyte. The negative potential is greatest at the capillary wall, which is what drives formation of the EDL. After EDL formation, the potential felt by species in the center of the

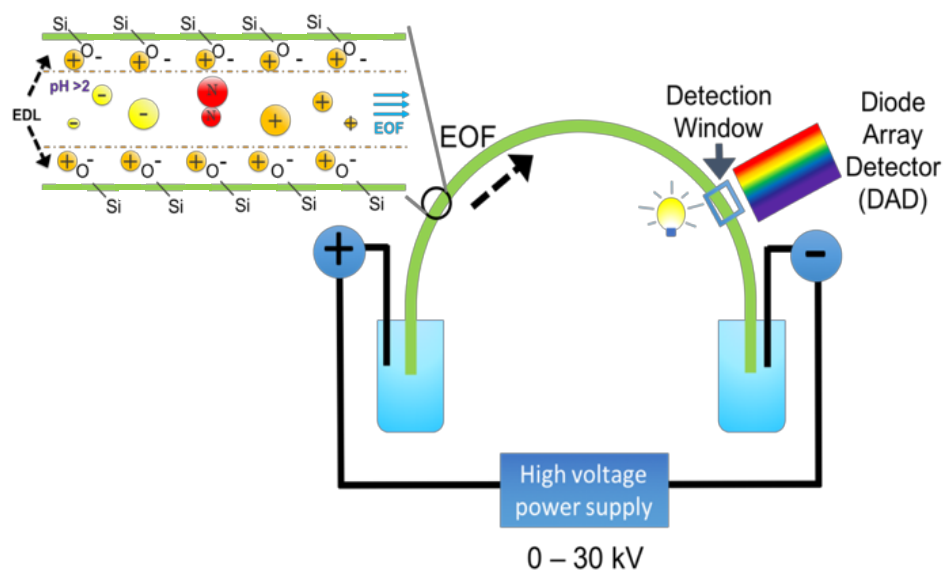


Figure 1.1: Schematic for CE instrumentation operating under positive polarity with ultra-violet-absorbance detection

capillary is lower than the species closer to the wall. As depicted in Figure 1.2, the EDL consist of a Stern layer and the diffuse layer. When the capillary surface is negatively charged, the Stern layer consist of tightly packed cations that aligned against the capillary wall. Since the Stern layer masks some of the surface potential, the second layer of ions that form is less tightly packed. This second layer is called the diffuse layer. The potential is still high enough to maintain a higher density of cations than in the bulk solution, but not high enough to pack as tightly and remain as stationary as the Stern layer. The magnitude of ζ is the potential at the edge of the slipping plane of the EDL, which is the interface between the EDL and bulk flow. The velocity of electroosmotic flow, ν_{eof} , is a function of the product of μ_{eof} and the applied electric field, E , as seen in Equation 1.2.

$$\nu_{eof} = \mu_{eof} E \quad (1.2)$$

Electrophoresis is the force which results in separation of sample components by a charge-to-size ratio upon addition of an electric field. Each species will have an electrophoretic mobility, μ_{ep} , defined in Equation 1.3,

$$\mu_{ep} = \left(\frac{q}{6\pi r} \right) \quad (1.3)$$

where q is the charge of a given species, and r is the hydrodynamic radius (or Stokes radius). Upon application of an electric field, cations will migrate towards the cathode, while anions will migrate towards the anode. Since μ_{ep} is a function of the charge-to-size ratio, small cations will migrate faster than larger cations and neutral molecules will only migrate with the EOF with conventional capillary zone electrophoresis. The total mobility of a species, known as the apparent mobility, μ_{app} , is the vector sum of both the EOF and electrophoresis forces,

$$\mu_{app} = \mu_{eof} + \mu_{ep} \quad (1.4)$$

so, as long as μ_{eof} is larger than μ_{ep} , all species, including anions, will migrate from anode to cathode. Thus, migration order will go from small cations, large cations, all sizes of neutral molecules, large anions, to small anions, as illustrated in Figure 1.1.

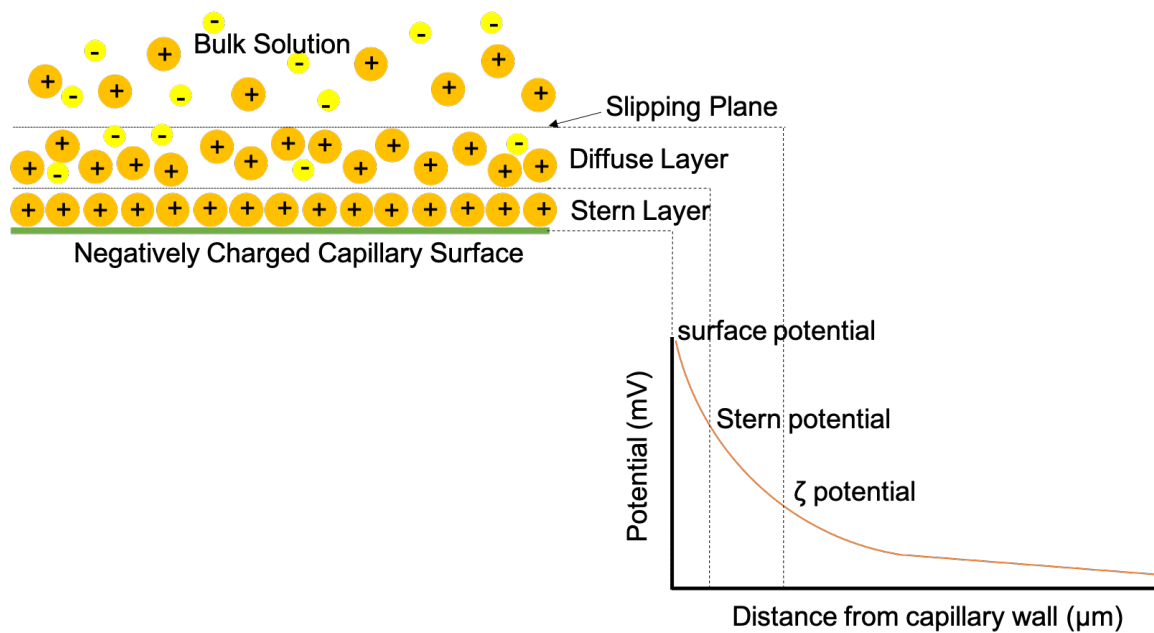


Figure 1.2: When the surface of the capillary becomes charged, an electronic double layer (EDL) forms. The potential at the slipping plane is known as the ζ -potential. The magnitude of the ζ -potential directly effects the electroosmotic mobility of the background electrolyte when an electric field is applied during capillary electrophoresis.

Similar to EOF, the electrophoresis can also be expressed as a velocity vector,

$$\nu_{ep} = \mu_{ep}E \quad (1.5)$$

Similar to apparent mobility in Equation 1.4, the apparent velocity can be described both by combining Equations 1.2 and 1.5 to give:

$$\nu_{app} = \nu_{eof} + \nu_{ep} \quad (1.6)$$

and in terms of the applied electric field:

$$\nu_{app} = \mu_{app}E \quad (1.7)$$

Equation 1.7 illustrates that the total velocity of each species in a sample mixture is dependant on the μ_{eof} , μ_{ep} and the magnitude of the applied electric field, where higher fields leads to faster separation times.

The high efficiency of CE can be explained by the van Deemter equation:

$$H = A + \frac{B}{u} + Cu \quad (1.8)$$

where H is plate height, u is flow rate, A corresponds to eddy diffusion, B corresponds to longitudinal diffusion, and C corresponds to mass transfer. For chromatography, the minimization of plate height gives maximized separation efficiency. Capillary zone electrophoresis is performed in an open tubular capillary, so the A term of Eq.(1.8) can be eliminated, and since there is no stationary phase, the C term can also be eliminated; thus, reducing Eq.(1.8) to:

$$H = \frac{B}{u} \quad (1.9)$$

Therefore, the separation efficiency for CE is maximized with increasing flow rate, which from Equation 1.7, can be achieved by increasing the applied electric field.

While, in theory there would be no upper limit to flow rate by simply increasing the electric field. In practice, however, increased electric fields will eventually lead to Joule heating which will disrupt the current (due to boiling of the background electrolyte) and uniformity of the electric field, E , along the length of the capillary, L . The applied electric field is defined as the applied voltage per unit length of the capillary:

$$E = \frac{V}{L} \quad (1.10)$$

where V is the applied voltage. This defines an upper limit to the applied field for a CE separation as an electric field applied that gives an applied voltage which results in a current outside of the Ohmic region (i.e. where a plot of Ohm's law (Eq.(1.11)) is no longer linear). Ohm's law is defined as:

$$V = IR \quad (1.11)$$

where I is the current and R is the resistance. The deviation from the Ohmic region of Eq.(1.11) occurs when the current is high and Joule heating results in increased resistance that is not linearly dependent on applied voltage. Current density, J , is given by:

$$J = \sigma E \quad (1.12)$$

where σ is the conductivity of the background electrolyte. The current density, Eq.(1.12), can be minimized by reducing conductivity of the background electrolyte or by decreasing cross-sectional area of the capillary since current density is the electric current per unit cross-sectional area of the capillary. Thus, to achieve the highest separation efficiency without Joule heating, smaller bore capillaries and low conductivity background electrolytes are advantageous.

1.3.2 CE instrumentation and modes of detection

CE instrumentation requires two buffer wells, two electrodes, a high-voltage power supply, and a detection region. For the work discussed in this dissertation, unless otherwise noted, CE was performed in an Agilent HPCE 1600 CE instrument which is equipped with a diode array detector for absorbance measurements in the ultra-violet and visible regions. While it is most common for commercial CE instrumentation to include detection capabilities for UV-absorbance detection, CE

detection modes can include fluorescence, electrochemical, or mass spectrometry.

1.3.2.1 UV-Visible absorbance detection

Commercial CE instruments routinely are equipped with capabilities of UV-Visible absorbance detection. A halogen lamp is directed through a detection window on the capillary and the absorbance is calculated from the percentage of light that was transmitted through the capillary. The experimental measurement is transmittance, T :

$$T = \frac{I}{I_0} \quad (1.13)$$

which is the measured by the ratio of intensity of light collected (I) to the intensity of incident light (I_0). Absorbance is then calculated by Eq. 1.14:

$$A = -\log(T) \quad (1.14)$$

A relationship between the absorbance and concentration of a species is linear and described by the Beer-Lambert law:

$$A = \varepsilon b c \quad (1.15)$$

where ε is the molar absorptivity coefficient of a species at a particular wavelength, b is the pathlength, and c is the concentration of the absorbing species. An advantage of absorbance detection is that it can be a universal, label-free detection method. There are a wide-variety of species that absorb at a given wavelength. While this is advantageous, it simultaneously serves as a caveat as it is a less selective detection method and often results in a high background.

Another caveat to absorbance detection methods is the small capillary diameters. Since the fused-silica capillaries used for capillary electrophoresis are typically on the order of 25-100 μm in diameter, the pathlength is very small and thus absorbance is small, so the difference between incident light and light transmitted is very small. This leads to a lack of sensitivity for absorbance detection methods when working with very low concentrations of analyte.

1.3.2.2 Fluorescence detection

Fluorescence detection has been a popular option for overcoming the challenges of absorbance detection for capillary electrophoresis. Fluorescence is a less common mechanism for relaxation, thus lowering the background for a fluorescence measurement. While molecules may absorb at a wide variety of wavelengths, excitation for a fluorescence process will be specific according to the fluorophore. In general, this is described by Eq. 1.16:

$$S_0 = h\nu_{ex} = S_1 \quad (1.16)$$

where S_0 and S_1 are the ground and excited states of the fluorophore, respectively, and $h\nu_{ex}$ is the photon energy where h is Plank's constant, and ν_{ex} is the frequency of the excitation light. While absorbance detection is the ratio of transmitted and incident light (Eq. 1.13), fluorescence efficiency is given by the ratio of emitted and absorbed photons (Eq. 1.17).

$$\Phi = \frac{\text{emitted photons}}{\text{absorbed photons}} \quad (1.17)$$

This ratio is known as the quantum yield, Φ and is inherent to the electronic properties of each fluorophore. Fluorescence detection of dilute solutions is also linearly proportional to fluorophore concentration according to Eq. 1.18:

$$I_f \approx I_0 \Phi A \quad (1.18)$$

where I_f and I_0 represent the intensity of fluorescence emission and intensity of incident light, respectively. Since absorbance, A , is required for fluorescence to occur, and by substituting Eq. 1.15 into Eq. 1.18:

$$I_f \approx I_0 \Phi (\epsilon b c) \quad (1.19)$$

it becomes apparent that fluorescence intensity is linearly dependent on concentration, but also dependent on the pathlength. However, unlike for absorbance measurements, small pathlengths for fluorescence detection can still be used for high sensitivity measurements of dilute solutions if the quantum yield for the fluorophore of interest is very high. Fluorescence emission can also be controlled, to an extent, by the intensity of incident light. Because of this, lasers are a common

choice for fluorescence excitation.

Auto-fluorescence of molecules is extremely rare, providing enhanced sample selectivity, but fluorescent tags are often required. However, fluorescent tags can provide selectivity by placing fluorescent tags only on molecules of interest.

1.3.3 Capillary electrophoresis immunoassay (CE-IA)

Capillary electrophoresis immunoassays (CE-IAs) combine the selectivity of an immunoassay with the many advantages of CE including speed, automation, and ability to assay small sample volumes. The feasibility of a CE-IA was demonstrated by Nielsen and coworkers in 1991 by illustrating the ability to separate human growth hormone (HGH) from an antibody complex using UV detection [10] and the utility was expanded to fluorescence detection by the Kennedy group in 1993 using fluorescently tagged antibodies [11]. A CE-IA for a specific analyte (antigen) involves using an antibody that has a specific affinity for the antigen. The binding of antibody to the antigen should yield a change in electrophoretic mobility for the immunocomplex from the free antibody. The binding reaction between antibodies and the antigen is generalized as follows:



Ag is the antigen, Ab^* is the fluorescently labeled antibody concentration, and $Ag - Ab^*$ is the immunocomplex. The equilibrium expression is then:

$$K = \frac{[Ag - Ab^*]}{[Ag][Ab^*]} \quad (1.21)$$

where K is the equilibrium constant for the antigen-antibody binding reaction. Quantification of the analyte is measured directly from the amount of complex formed because antibody is added to the sample in excess to ensure complete complex formation with all the sample antigen. Therefore, the detection limits of this format are related to the sensitivity of fluorophore detection and how well the immunocomplex peak can be resolved from the free-labeled antibody.

A typical immunoglobulin G (IgG) antibody, the most common antibody found in blood, is approximately 150 kDa while the neuropeptides we are interested in measuring are around 1-4 kDa, thus the immunocomplex is 151-154 kDa. Since CE separates based on a ratio between size and charge, the separation between the immunocomplex and free antibody is not trivial.

To overcome this challenge, it is common to use a competitive immunoassay format, where antigen is fluorescently tagged. Now the binding reaction becomes more complex and there are two equilibria at play. First, the equilibrium between the labeled antigen and antibody,



and then the equilibrium between the sample antigen and antibody,



where Ag^* is the labeled antigen, Ag is the sample antigen and Ab is unlabeled antibody and $Ag^* - Ab$ and $Ag - Ab$ represent the labeled and unlabeled immunocomplex, respectively. The sample antigen and fluorescently tagged antigen will compete for the binding sites on the antibody, so a known amount of limiting antibody concentration is added to the mixture. The amount of antigen in the sample can be quantified using the ratio of bound-labeled antigen and free-labeled antigen. As the amount of unlabeled antigen in the sample increases, the amount of labeled antigen-antibody complex formed will decrease.

For the competitive format, there are two binding reactions for which the equilibrium conditions are important to consider. First, the expression for the labeled antigen:

$$K_{labeled} = \frac{[Ag^* - Ab]}{[Ag^*][Ab]} \quad (1.24)$$

and the expression for the antigen in the sample:

$$K_{sample} = \frac{[Ag - Ab]}{[Ag][Ab]} \quad (1.25)$$

Since our detection system is dependent on the labeled reaction, where we will use the ratio of $[Ag^* - Ab]$ (bound) to $[Ag^*]$ (free) for quantification, we are limited by the dissociation constant of the $Ag^* - Ab$ interaction. To understand this, we will start by looking at a binding curve for an antibody-antigen interaction see in Figure 1.3. When the percentage of bound antigen is plotted against the total antigen concentration, the result is a sigmoid, where the dynamic range of the plot is on the order of 50% binding. In other words, the immunoassay is most sensitive when half of the labeled antigen has formed a complex with the antibody. At this point of half saturation, since antibody is added in limiting amounts, $[Ab] = [Ag^* - Ab]$. The equilibrium expression from Figure 1.3 then becomes

$$K_{eq} = \frac{[Ab]}{[Ag^*][Ab]} \quad (1.26)$$

which simplifies to

$$K_{eq} = \frac{1}{[Ag]} \quad (1.27)$$

The dissociation constant, K_D , of the antibody for the labeled antigen is the inverse of equilibrium value from Equation 1.24, so

$$K_D = \frac{[Ag^*][Ab]}{[Ag^* - Ab]} \quad (1.28)$$

and at half saturation,

$$K_D = [Ag^*] \quad (1.29)$$

so this reaction is most sensitive when we are looking to detect concentrations very similar to the K_D of the antibodies. This sets a practical limit for this immunoassay format to detection limits on the order pM concentrations as the minimum dissociation constant for antibodies is about 10^{-12} [12].

In order to use the labeled antibody to quantify unlabeled antibody, the concentrations of Ag^* and Ag must be similar. If Ag is too high, then $Ag - Ab$ complex will be promoted and $Ag^* - Ab$ complex will be inhibited. This will prevent quantification of Ag since both the bound and free peaks should be dynamic for sensitive quantification. The reverse case is also an issue, where Ag^* is too high. When Ag^* is too high, $Ag^* - Ab$ complex formation is promoted and unlabeled $Ag - Ab$ complex formation is inhibited. Ag cannot be quantified if it does not form a

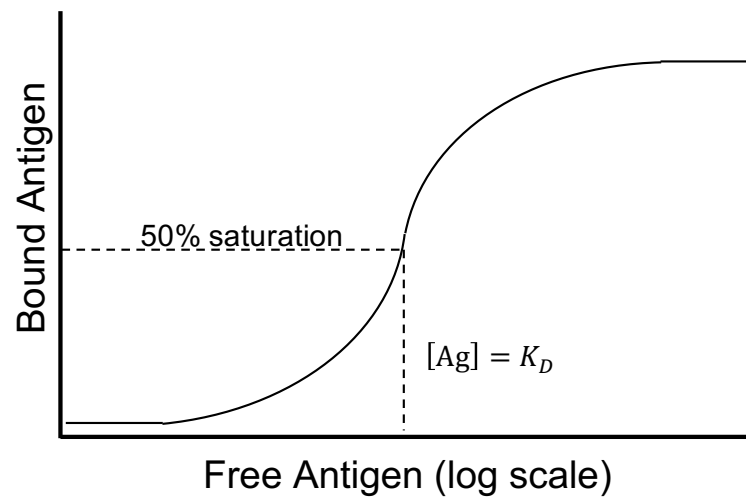


Figure 1.3: A saturation curve is obtained when bound antigen concentration is plotted against a log scale of free antigen concentration. At 50% saturation, where the antigen has formed an immunocomplex with 50% of the antibody, the free (unbound) antigen concentration is equal to the dissociation constant of the antibody-antigen binding reaction.

complex with the added antibody. Thus, for this competitive immunoassay format, the sensitivity relies on K_D of the antibody-antigen reaction $\approx [\text{Ag}] \approx [\text{Ag}^*]$. Therefore, it is probable that a higher sensitivity measurement and lower limit of detection can be achieved using the direct assay format. A focus of this dissertation is the development of new tools specifically designed to tackle the antibody-immunocomplex resolution challenge while simultaneously maximizing detection sensitivity using LED-induced fluorescence detection.

1.3.4 Taylor dispersion analysis (TDA)

Taylor dispersion analysis (TDA), first described in 1953 [13], allows the absolute determination of diffusion coefficients by evaluating the change in temporal variance of an analyte peak as it is transported via pressure-driven laminar flow. The fact that TDA allows absolute determination enables calibration-free determinations of diffusion coefficients which can be used for peak identification. TDA requires flow conditions in which the rate of advection is significantly greater than the rate of analyte diffusion and that analytes reside in this condition long enough for measurable diffusion to occur. The first condition is met by satisfying the requirement that the Péclet number (P_e) is greater than 69 [14]. For a cylindrical capillary, P_e is defined as:

$$P_e = \frac{uR_c}{D} \quad (1.30)$$

where u is the linear flow rate, R_c is the channel radius, and D is the diffusion coefficient for the molecule under interrogation. The second condition is met when $\tau > 1.4$, where τ is defined as:

$$\tau = \frac{Dt_R}{R_c^2} \quad (1.31)$$

where t_R is the time in which the analyte spends in the appropriate Taylor flow condition determine from satisfying $P_e > 69$. Equation 1.31 illustrates that analysis times in TDA can be significantly reduced by reducing R_c , which has motivated the use of fused silica capillaries as flow systems for TDA.

TDA can be performed using only a single detection point, but this dissertation will specifically use dual-point detection TDA. For dual-point detection TDA, the differences in migration time

and variance at the two detection points are used to calculate the diffusion coefficient using Equation 1.32 [14]:

$$D = \frac{R_c^2(t_2 - t_1)}{24(\sigma_2^2 - \sigma_1^2)} \quad (1.32)$$

where t_1 and t_2 correspond to migration time of peak in the first and second detector respectively, and σ_1 and σ_2 corresponds to the variances of peaks as species pass each detector. Then, the hydrodynamic radius can then be determined using Equation 1.33, the Stokes-Einstein equation:

$$R_H = \frac{k_B T}{6\pi\eta D} \quad (1.33)$$

where k_B is the Boltzmann constant, T is the temperature, η is the dynamic viscosity of the solution, and R_H is the hydrodynamic radius of the detected analyte.

1.3.4.1 CE-TDA

The advantages of using CE instrumentation for TDA analysis are many, and are presented in Chapter 2. One key advantage, however, is the ability to perform CE and TDA in tandem. Traditional TDA allows for absolute determination of species in flow; however for mixtures, the diffusion coefficient measured by TDA will be a weighted average between all the components of the mixture that give rise to a signal on the detector. By adding a CE separation step, mixture components can be separated before TDA, and each component of a mixture that is resolved in the CE step can then undergo size analysis via TDA. Size analysis after the separation can be used as a peak identification metric and could be particularly useful, as described in this dissertation, for chemical reaction monitoring.

1.4 Frequency encoded capillary electrophoresis detection

Improving signal-to-noise and analyte resolution are perpetual analytical challenges for capillary electrophoresis (CE) detection methods and are a key focus for this dissertation. Fourier transform-based techniques have been developed to address these challenges, including Shah Convolution Fourier Transform (SCOFT) [15, 16, 17, 18, 19], two-beam fluorescence cross-correlation [20], Hadamard Transform CE [21, 22], and Fourier Transform CE (FT-CE) [23]. Each of these

techniques thrives on the idea of using multi-point excitation for detection along the distance of the separation channel and that resolution in the time domain is not required for resolution in the frequency domain. Each method used a different approach to creating a mask that produces multiple detection windows. SCOFT employs a physical mask over the separation channel, Hadamard Transform CE uses an injection pattern to produce a non-physical mask based on a Hadamard matrix, two-beam fluorescence cross correlation uses multiple beams and multiple detectors, and FT-CE simultaneously uses five separation channels that are physically offset in a manner such that the detection window across the five channels simulates a mask detection a different lengths of the channel. These approaches allow enhancement of both S/N and analyte resolution from detection, rather than changing separation conditions or modifying excitation source or fluorophore.

1.4.1 Shah and sine and convolution Fourier-transform for CE

SCOFT was originally demonstrated by imaging the detection region of a cylindrical capillary with a micro-machined mask placed on top to convert a single detection region into 55 detection points [15]. Because of the detection mask, the time-domain electropherogram is convoluted with a Shah function which can be converted into frequency-domain through the Fourier-transform. The Shah function, also known as a Dirac comb, $\text{III}(x)$ is a sequence of evenly spaced delta functions $\delta(x)$:

$$\text{III}(x) = \sum_{n=-\infty}^{\infty} \delta(x - n) \quad (1.34)$$

As the analyte moves through the detection region, a blinking frequency is now observed. The blinking frequency is observed by a signal produced by the fluorescent analyte as it passes between the nodes of the delta functions and is directly related to the velocity of each analyte. The signal for each detection point is summed and collected into a single signal transducer, so the electropherogram for a single species consist of a series of Gaussian peaks for each detection point (Fig. 1.4-A). Adding multiple detectors increases the number of times an analyte is viewed by the detector (Fig. 1.4-B) to enable multiplexing and enhanced S/N. Thus, there will be a characteristic frequency observed for each analyte velocity, so analytes with different electrophoretic mobilities

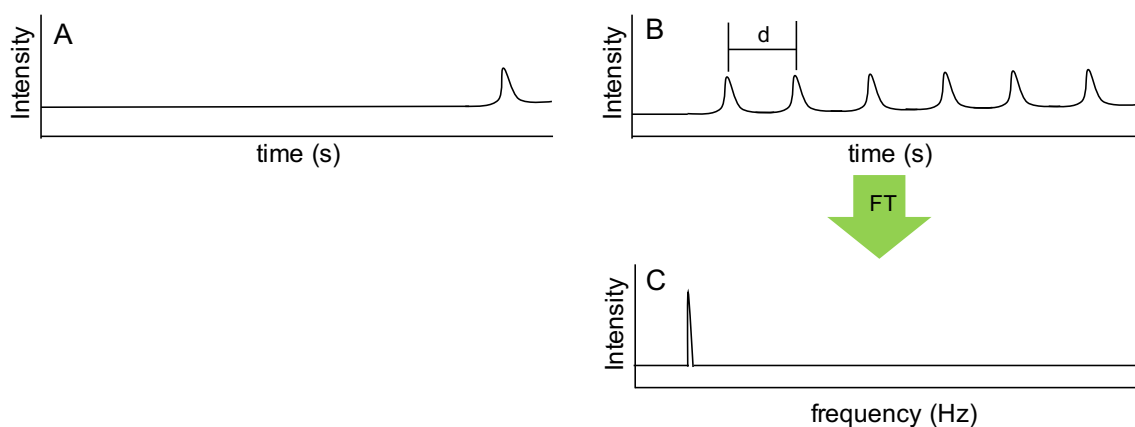


Figure 1.4: Panel A illustrates an electropherogram of a single analyte with a single detector. When the detection region is convoluted by a Shah function, multiple detection points are observed and a peak is observed for each detection spot as illustrated in panel B. When the convoluted electropherogram undergoes a Fourier-transform, the frequency will correspond to the velocity of the analyte moving through the detection region. Thus, a characteristic frequency dependant on the apparent mobility of a species during electrophoresis can be observed for each unique apparent mobility.

will then resolve in the frequency domain when the the electropherogram undergoes a Fourier-transform (Fig. 1.4-C). The characteristic frequency will be dependent on both analyte velocity and the spacing between detection slits. Since the frequency domain resolution is increased with longer interference patterns, a increased number of detection slits is advantageous and decreased spacing between detection slits will lead to higher temporal resolution.

Another parameter found to be advantageous for enhancing S/N with Fourier-transform based detection was increasing the number of injections [17, 23]. This dissertation work extends the idea of multiple injections to increase the interference pattern and enhance resolution by presenting a continuous injection technique. By continuously injecting sample in a pulsatile fashion, high temporal resolution can be achieved and enhanced S/N will result from an increased number injection plugs.

1.5 Microfluidic devices for zonal electrophoresis applications

1.5.1 Advantages of using microfluidic devices

Microfluidic devices provide many of the same advantages of capillary electrophoresis including automation, reduced sample consumption and fast analysis making them advantageous for biological analysis. In addition, lab-on-a-chip style microfluidic devices further enable portability, multiplexed injection techniques, integration of on-line reaction chemistry required for biological analysis such as fluorescent conjugation. Electrophoretic separations in a microchip are subject to the same physics described above for CE. In fact, microchip electrophoresis is commonly still referred to as CE even though the electrophoresis is occurring in a microchannel as opposed to a capillary.

1.5.2 Electrokinetic gating for electrophoresis injections

Electrokinetic gating provides a mechanism for manipulating flow in a microdevice to deliver small, reproducible sample plugs into the separation channel [24]. Electrokinetic gating in a cross-t chip is illustrated in Fig 1.5. Flow is driven from both a sample inlet and buffer inlet,

simultaneously. When an electric field applied across the gating channel is higher in magnitude than the electric field across the electrophoresis channel, all the sample is driven to a waste port before entering the separation channel. When the potential across the gating channel floats (the connection is disrupted), sample is allowed to move into the separation channel. The time in which the gating potential is floating will correspond to the injection time.

1.5.3 Simulation-aided device design

Sample manipulation and separations in a microfluidic device are highly dependent on device geometry. Specifically, in this work, device geometry must be optimized for sample manipulations driven by electrokinetics. In this dissertation, microdevice design was investigated using COMSOL Multiphysics to simulate geometry and field effects before lengthy fabrication procedures. COMSOL Multiphysics uses finite element analysis to solve systems partial differential equations that can be coupled to combine the effects of multiple physics. To investigate flow manipulation by electrokinetics in a microdevice, the coupled partial differential equations used for the simulation described the electric field, electroosmotic flow, and transport of dilute species in a particular device geometry. Optimization of parameters such as required applied potential and channel length were achieved for obtaining a desired electric fields for successful electrokinetic gating. These simulations have demonstrated comparable performance to actual experimental results (Fig. 1.5).

1.5.4 Device fabrication techniques

1.5.4.1 Microfabrication for glass microfluidic devices

Due to optical properties and high potential for electroosmotic flow, glass is well-suited for fabrication of microfluidic devices that will be used for electrophoresis applications. Similar to the surface chemistry in a fused-silica capillary, at neutral to high pH, the microchannel walls will become negatively charged thus facilitating EOF when an electric field is applied.

Glass microdevices are fabricated using UV-photolithography- a process depicted in Fig. 1.6. A photomask is designed using CAD software. The photomask is then printed as a high resolution

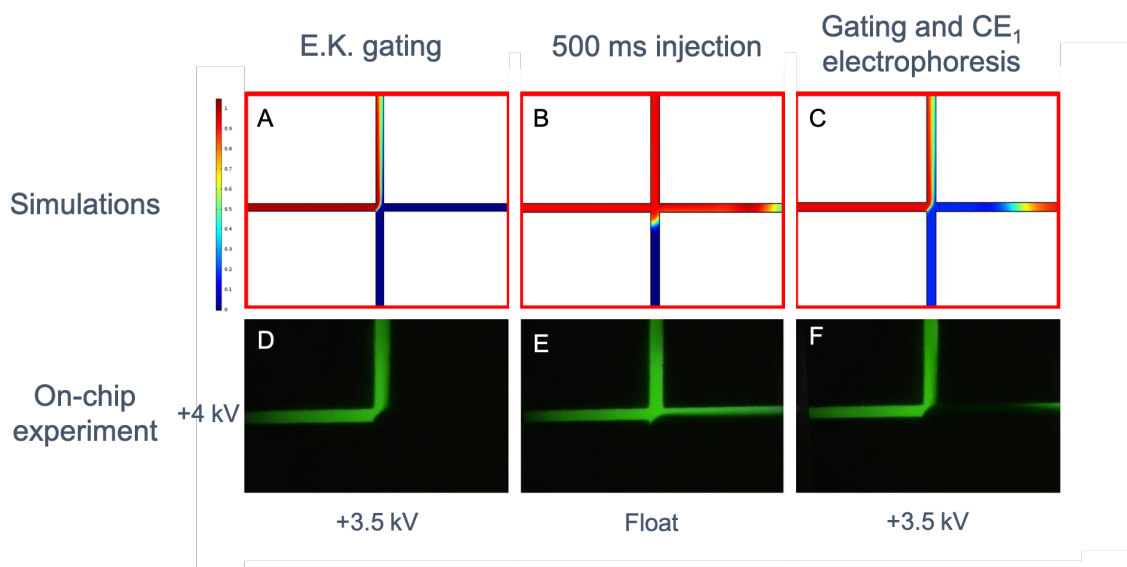


Figure 1.5: Electrokinetic gating in a cross-t device is driven by modulating the electric fields across the intersection. Appropriate conditions required can be simulated (A-C) and then performed in practice (D-F). In both simulation and experiment, the horizontal channel corresponds to sample inlet and separation channel (left-to-right) and the vertical channel is the buffer inlet (bottom-to-top). During a gating condition (A/D), sample (red/green) is continuously introduced at the sample inlet, but is driven out to waste before entering the separation channel but a higher flow rate of buffer (blue/dark) across the intersection. This higher flow rate is driven by a higher magnitude of applied field across the intersection than from the sample inlet to waste across the separation channel. When the voltage at the buffer inlet floats, the field is no longer present and thus sample is permitted to enter the separation channel. Once the field is returned, the sample is again driven out to waste. The volume of sample plug injected into the separation channel directly corresponds to the length of time in which the buffer inlet remains in the float condition.

film and placed above a glass substrate coated with a layer of chrome and a layer of positive photoresist. The positive photoresist is a photactive polymer that becomes soluble in a developer solution upon UV irradiation. The mask protects the photoresist everywhere except in the region containing fluidic channel geometry. After exposure, the chrome layer is exposed where the microchannels will be etched. The device is then bathed in a chrome-etch solution to dissolve the chrome layer where the channels will be placed. At this point, the microdevice is placed in a hydrofluoric acid solution where micron sized channels are uniformly etched in all directions where the chrome was previously removed. The width and depth of the resulting microchannels is a function of the time in which the device is left in the acid solution. After the channel etching is complete, the remaining photoresist and chrome are removed, channel dimensions are measured using a profilometer, and access port holes are drilled. The glass containing the fluidic network and a cover glass slide are then cleaned with an acid piranha bath followed by base piranha bath before being thermally bonded for 10 hours. The protocol used in this dissertation work was based on a protocol developed by the Roper Lab in 2010 [25].

1.5.4.2 Other fabrication methods

While glass provides many advantages including optimal silanol chemistry for EOF and optical transparency for fluorescence detection platforms, the entire fabrication process can be lengthy. Depending on resources available, it can take several days to obtain the photomask after designing the device geometry in CAD. In addition, the hazardous fabrication process is labor intensive and time consuming. For these reasons, alternative fabrication strategies have been developed. A common replacement for glass in rapid microdevice fabrication protocols is poly(dimethylsiloxane) (PDMS) [26]. PDMS can be poured over a master- a mold containing the device geometry. Once a single mold is created, either by UV-photolithography using negative photoresist on a silicon wafer [26] or by 3D printing [27], it can be re-used numerous times for quick, reproducible production of PDMS micro-structures. The PDMS containing microchannel geometry can be adhered to a glass cover slide to still reap advantages of glass chemistry desired for electrophoresis and electroosmosis.

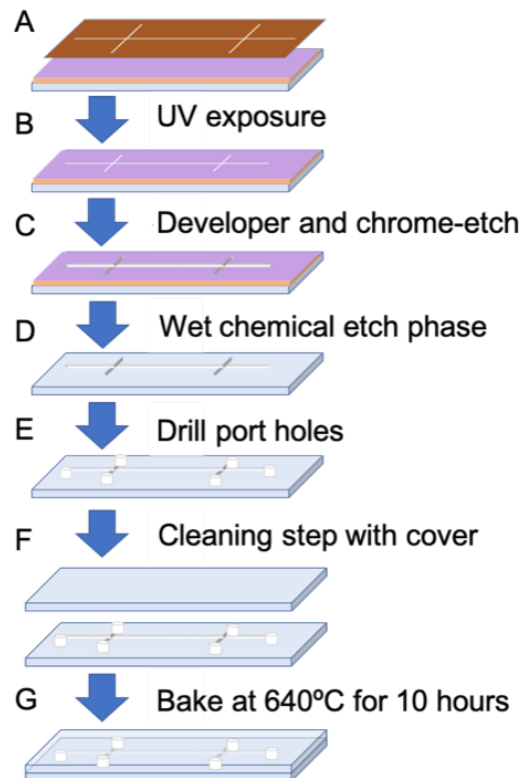


Figure 1.6: Microfluidic channels can be fabricated on a glass substrate using photolithography. A) The blank glass substrate is coated with chrome (orange) and photoresist (purple) and a mask containing fluidic geometry is placed on top. B) After UV exposure, the photoresist becomes soluble in the regions exposed, so chrome is exposed according to the fluidic geometry. C) The chrome is then etched away, exposing the glass substrate. D) An HF etching bath will etch the microfluidic channels into the glass and then remaining photoresist and chrome is removed. E) Port holes are drilled before F) cleaning the microchip and a blank of similar size in a piranha bath. G) Finally, the microchip and glass cover are thermally bonded.

1.5.5 Zonal electrophoresis in paper-based microfluidic platforms

Paper is one of the oldest substrates for chromatography [28, 29, 30] and initially gained popularity for electrophoresis due to low-cost [31]. However, with the development of gel electrophoresis and modern day CE methods capable of higher resolution and sensitivity, the popularity of paper as an electrophoretic substrate declined. With the growing interest in designing miniaturized lab-on-a-chip devices for point-of-care analysis or field studies, paper has re-emerged as a popular choice for electrophoresis [32, 31, 33]. In order to fully realize the capabilities of paper substrates for high resolution electrophoresis, sample manipulation abilities must be investigated for various paper substrates. There are a wide variety of paper types to choose from- including nitrocellulose membranes, commercial filter paper, and chromatography paper [31]. This dissertation presents a study to characterize low-adsorption paper substrates and their potential for electrokinetic sample manipulation and sensitivity for fluorescence detection of common fluorescent dyes.

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Chapter 2

A miniature 3D printed LED-induced fluorescence detector for capillary electrophoresis and dual detector Taylor dispersion analysis

A version of this chapter was originally submitted by Laura D. Casto, Kevin B. Do and Christopher A. Baker. Only minor modifications were made.

Laura D. Casto, Kevin B. Do and Christopher A. Baker, “A miniature 3D printed LED-induced fluorescence detector system for capillary electrophoresis and two-point detection Taylor dispersion analysis” *Submitted*, 2018

Additional materials for Chapter 2 are provided in Appendix [A](#).

2.1 Abstract

Taylor dispersion analysis (TDA) provides absolute determination of diffusion coefficients for analytes ranging from small molecules to particulate matter. TDA has seen a resurgence in recent years, as modern commercial capillary electrophoresis (CE) instrumentation is well equipped to meet the precision flow requirements of TDA. Discontinuous flow velocities, which occur during sample injection, can lead to substantial inaccuracies in single-point detection TDA. Dual-point detection allows TDA to be carried out under continuous flow in the volume between the detection points, but dual-point fluorescence detection has not previously been feasible within the confines of commercial CE instrumentation. Here, we describe a compact LED-induced fluorescence detector designed for on-line, dual-point capillary detection within a commercial CE system. The 3D printed detector houses an inexpensive LED excitation source, a bandpass excitation filter, an integral 3D printed pin hole collimator, and a ball lens which collects fluorescence emission. Multivariate optimization of operating conditions yielded a detection limit of 613 ± 13 pM for CE of fluorescein disodium salt solution in borate buffer. The miniature size of the device allowed integration of two detectors within a commercial CE system without modification to the instrument, thereby enabling dual detector assays including TDA and CE-TDA. Monitoring of the bioconjugation reaction between fluorescein isothiocyanate (FITC) and a model protein illustrate the utility of direct, calibration-free size determination, which enabled the resolution of fluorescence originating from free FITC from that of protein-bound FITC. TDA detection coupled to CE enabled the determination of peak identities without the need for standard solutions.

2.2 Introduction

Taylor dispersion analysis (TDA) allows the absolute determination of diffusion coefficients by evaluating the change in temporal variance of an analyte peak as it is transported via pressure-driven laminar flow. Taylor first described the principles of TDA in 1953,[1] yet the technique has seen a resurgence in recent years as the utility of commercial capillary electrophoresis (CE) instrumentation for performing TDA has become evident [2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18]. TDA requires flow conditions in which the rate of advection is significantly greater than the rate of analyte diffusion, which is satisfied by a Peclet number (Pe) greater than 69 [14]. Pe is defined for cylindrical flow channels as:

$$Pe = \frac{uR_c}{D} \quad (2.1)$$

where u is the linear flow rate, R_c is the channel radius, and D is the diffusion coefficient for the molecule under interrogation. TDA further requires that analytes reside in this flow condition for a time (t_R) sufficient to satisfy $\tau > 1.4$, where τ is defined:

$$\tau = \frac{Dt_R}{R_c^2} \quad (2.2)$$

Equation 2.2 illustrates that analysis times in TDA can be significantly reduced by reducing R_c , which has motivated the use of fused silica capillaries as flow systems for TDA. Equation 2.1 suggests that there is no upper limit to u , since the criterion for TDA is $Pe > 69$. Practically, however, the length of capillary tubing required to achieve an appropriate t_R at high flow rates effectively limits the upper bound of appropriate u values. Thus, precise flow rate control is needed in TDA, which is achieved via precision control of applied pressures (ca. 1 - 100 mbar). As such, commercial CE instrumentation is ideally equipped to achieve the appropriate flow conditions for TDA in fused silica capillaries with inner diameters of ca. 10 - 100 μm .

The use of commercial CE instrumentation also enables online integration of CE separations with TDA, which offers increased information content over CE analysis alone [19]. CE-TDA has been presented as an alternative for size-exclusion chromatography and dynamic light scattering

methods [20, 21, 22, 23, 24]. While TDA allows calibration-free determinations of diffusion coefficients for species ranging from small molecules to particulate matter, inaccuracies of as much as 40% have been observed when TDA is performed with a single detection point [25]. These errors arise from the injection volume, pressure ramping, and discontinuous flow velocities that result from sample injection and transfer of the capillary inlet to a buffer vial before commencing laminar flow. Inaccuracies due to flow conditions can be overcome using dual point detection TDA [26, 27, 28, 29, 30], in which diffusion coefficients can be determined under continuous laminar flow via differences in peak variance observed at two locations along the capillary length. Dual-point detection in commercial CE instrumentation has been achieved by looping the capillary such that it passes the detection region twice [31], but this has only been demonstrated with UV absorbance detection. Integrating dual-point fluorescence detection into commercial CE instrumentation will enable rapid sizing of molecules with lower detection limits and enhanced selectivity as compared with UV absorbance detection.

Increasing availability and low cost of 3D printing is impacting the development of next-generation microanalytical technologies by enabling rapid, inexpensive production of laboratory equipment and instrumentation, and facilitating the digital distribution of these technologies. Much work in this area has focused on 3D printing of fluidic architectures [32, 33, 34], but somewhat less attention has been given to 3D printing as a tool for integrating optical detection systems. Prikryl and Foret described a 3D printed fluorescence detection system for capillary separations that utilized a bifurcated optical fiber for excitation and emission collection [35]. Fiber-coupling enabled the implementation of this detector within a commercial capillary cassette for CE analysis. Cecil et. al. presented a 3D printed UV absorbance detection system that incorporated an LED source and diode detector directly into the compact detection head [36].

Here, we describe a 3D printed fluorescence detector design based on orthogonal optical axes for excitation and collection, with integrated excitation source and optical filters. The resulting low cost, 3D printed LED-induced fluorescence detection system has a critical volume of ca. 6 cm³, making it sufficiently compact for dual detector integration within the air-cooled capillary cartridge of an Agilent G1600 CE instrument. Design considerations of focused versus collimated excitation

are described, along with the optimization of operating conditions to yield a fluorescence LOD of $613 \pm 13\text{pM}$ for fluorescein dye. Dual detector integration is demonstrated, and the utility of this system for TDA and CE-TDA applications is illustrated via monitoring of the bioconjugation reaction between bovine serum albumin and fluorescein isothiocyanate.

2.3 Materials and methods

2.3.1 Reagents and materials

Glycine and bovine serum albumin (BSA) were Fisher bioreagents brand from Fisher Scientific (Suwannee, GA). Sodium tetraborate, fluorescein disodium salt, fluorescein isothiocyanate isomer I (FITC), and remaining amino acids were ACROS Organics from ThermoFisher Scientific (Waltham, MA). All aqueous solutions were prepared using Milli-Q water purified with a Milli-Q purification system (Millipore, Bedford, MA).

2.3.2 3D printing

A QIDI Technology Dual Extruder Desktop 3D printer was used to print the detection head with 1.75 mm diameter acrylonitrile butadiene styrene (ABS) filament. There are two main components of the device design: (1) the body of the detection head, which houses the optical components and the capillary, and (2) the LED holder to secure the excitation source inside the detection head. Since the body of the detection head has smaller design features, a scaling factor of 1.125 was applied in the CAD design to compensate for thermal expansion effects during cooling for ABS filament. No scaling factor was required for the LED holder component. After scaling, the two components were merged and exported as a .stl file for print. Replicator G was used to generate a G code and drive the 3D printer. The extruder temperature was set to 260° C, and the bed temperature was 110° C. The device was printed with 35% object infill, 0.1 mm layer height, and 30 mm/s feed rate. To improve print quality and facilitate lift-off of the finished print, a 4 mm thick glass sheet was placed on the build platform and sprayed with hairspray (Aqua Net, Lornamead, Inc., Tonawanda, NY) before printing.

2.3.3 LED modulation

A high-power single-color blue LED (XPEBBL-L1-0000-00301-SB01, Opulent Americas, Raleigh, NC) mounted on a starboard was used in the final optimization of the detector experiments. The LED was powered using a DC adjustable power supply (TP3003E, TekPower, Montclair, CA). A multi-meter was placed in series with the LED circuit to measure applied current. The LED was modulated at a frequency of 32.69 Hz using the TTL out-put of a lock-in amplifier (SR830, Stanford Research Systems, Sunnyvale, CA) and a high voltage relay switch (5501-12-1, Coto Technology, Mansfield, TX). Since the LED was modulated with a 50% duty cycle on a quick timescale, the current readout on the multi-meter was 50% of the actual magnitude of applied current.

2.3.4 Detection head assembly and optical components

One-piece fittings (C360-100, LabSmith, Livermore, CA) were used to secure the capillary to the plastic device, ensuring the detection window was situated appropriately with respect to excitation and collection optical axes. LED excitation light passed through a 6.3 mm diameter 490 ± 5 nm bandpass filter (W4489, Omega Optical, Inc., Brattleboro, VT) and was directed to the detection window of the capillary, initially via 5 mm diameter fused silica ball lens, and later via a 3D printed passive collimator discussed further in the following section. Fluorescence emission was collected via a second 5 mm ball lens which coupled emission light to a fiber optic bundle (02-540, Edmund Optics Inc., Barrington, NJ) for transmission to an off-device photomultiplier tube (PMTSS2, Thorlabs Inc., Newton, NJ) fitted with a 532 ± 5 nm emission filter (FL532-10, Thorlabs Inc., Newton, NJ) unless otherwise specified. A lock-in amplifier (SR830, Stanford Research Systems, Sunnyvale, CA) was used to modulate the LED excitation and demodulate the PMT signal before data acquisition via a USB data acquisition module (USB-6001, National Instruments, Austin, Texas). An assembly diagram can be found in Appendix A (Fig. A.2 and A.1).

2.3.5 Capillary electrophoresis

Capillary electrophoresis experiments were performed with an Agilent G1600 3D Capillary Electrophoresis System, controlled with Agilent ChemStation software. LED-IF data acquisition software was written in-house using LabView™2016 Version 16.0 (National Instruments, Austin TX).

Separations were performed in a 100 μm i.d./ 360 μm o.d. fused-silica capillary (35 cm total length, 19 cm effective length). Each morning, the capillary was flushed with 1 M sodium hydroxide, water, and 0.1 M sodium hydroxide for 10 min each followed by 20 min of the background electrolyte. Before each injection, the capillary was rinsed with 0.1 M sodium hydroxide for 30 s and the background electrolyte for 1 min. Samples were injected electrokinetically with an applied voltage of 30 kV for 1s unless otherwise specified. For capillary zone electrophoresis experiments, the background electrolyte solution was 10 mM borate buffer, pH 8.5. Electrophoresis was carried out with an applied voltage of 15 kV (429 V cm^{-1}). All separations were achieved in less than 5 minutes.

2.3.6 Experimental Design and Response Surface Methodology

Response surface methodology[37] using JMP Pro 13 statistical analysis software (SAS Institute Inc., Cary, NC) was used to conduct a multivariate optimization of LED current and PMT gain. The optimization goal was to maximize the signal-to-noise ratio for a sample of 250 nM fluorescein disodium salt in 10 mM borate buffer, pH 8, using electrokinetic injection and electrophoresis conditions described above. Preliminary experiments were used to identify an initial design space using trial-and-error. First-order modeling of the original design space and the path of steepest ascent method was used to locate an optimal design space in which a second-order surface model was fit to identify the combination of factors which yield a maximum signal-to-noise response.

2.3.7 FITC conjugation

Amino acids were labeled with FITC based on a reaction described previously [38, 39]. Amino acids were combined with FITC in a 10:1 molar ratio for final concentrations of 1 mM amino acids and 100 μM FITC. The mixture was diluted 20-fold before electrophoresis experiments.

For fluorescent conjugation of BSA, FITC and BSA were dissolved in 20 mM sodium tetraborate buffer, pH 10.3 and combined in a 1:10 molar ratio FITC:BSA with a final concentration of 500 nM FITC.

2.3.8 Taylor dispersion analysis

Taylor dispersion experiments were carried out in a 35 cm 50 μm i.d. capillary with fluorescence detectors placed at 14 and 20 cm from the inlet. Before each run, the capillary was rinsed with 1 M sodium hydroxide and water for 30 s each and then filled with 20 mM sodium tetraborate buffer, pH 10.3, for 1 minute. The sample was injected with 50 mbar for 60 s, and then TDA was carried with 16 mbar pressure. Operating conditions and detector placement were determined based on Equations 2.1 and 2.2. The first derivative of the sample plug rear was used to determine migration times and peak variances. The differences in migration time and variance at the two detectors were then used to calculate the diffusion coefficient using Equation 2.3 [14]:

$$D = \frac{R_c^2(t_2 - t_1)}{24(\sigma_2^2 - \sigma_1^2)} \quad (2.3)$$

where t_1 and t_2 correspond to migration time of peak in the first and second detector respectively, and σ_1 and σ_2 corresponds to the variances of peaks as species pass each detector. Then, the hydrodynamic radius can then be determined using Equation 2.4, the Stokes-Einstein equation:

$$R_H = \frac{k_B T}{6\pi\eta D} \quad (2.4)$$

where k_B is the Boltzman constant, T is the temperature, η is the dynamic viscosity of the solution, and R_H is the hydrodynamic radius of the detected analyte.

CE-TDA experiments were carried out with the same capillary and rinse conditions described previously for TDA. The sample was injected with 50 mbar for 5 s, and electrophoresis was performed with an applied voltage of 15 kV for 60 s. The sample buffer was diluted with water such that for CE-TDA, sample buffer concentration was 10-fold less than the background electrolyte to

promote sample stacking. After electrophoresis, TDA was carried out under the same conditions as described above.

2.4 Results

2.4.1 3D printed detector design

The capillary, LED excitation source, and optical fiber bundle for collecting fluorescence emission were situated on orthogonal axes within a miniature (*ca.* 6 cm³) 3D printed detector body, which was compact enough to be situated within an Agilent CE capillary cassette. To maximize fluorescence signal collected and minimize interference from excitation light, the device mimics a traditional fluorimeter optical path by collection fluorescence emission 90° from the excitation path as illustrated in Figure 2.1A. The detector includes housing for the LED excitation source, the excitation bandpass filter, a ball lens for fluorescence emission collection, and an optical fiber to carry fluorescence signal to the PMT module (Fig. 2.1C).

2.4.2 Design of the excitation optical path

A significant advantage to designing analytical instrumentation for fabrication by 3D printing is the ability to share the hardware by digital distribution. Therefore, designing a fixed optical system architecture that required no end-user alignment was an important consideration for effectively transferring optimized performance along with the distributed hardware. To pursue this, we investigated two configurations of the excitation optical path (Fig. 2.2): (1) focused excitation light and (2) collimated excitation light. For the focused light path, a 5 mm ball lens was used to focus excitation light on the capillary detection window. Figure 2.2B shows that the fluorescence signal produced via the focused excitation configuration was dependent on the accurate placement of the excitation focusing lens. A 500 μm shift in lens position toward the capillary resulted in a 13-fold reduction in signal intensity for fluorescein solutions. The axial resolution of typical consumer grade FDM 3D printers is on the order of 100-300 μm ; thus, printer-to-printer error may result in sufficient misalignment to significantly influence detector performance. Therefore, the ball lens was replaced with two co-axial 3D printed pinholes to create a collimator. The Soller collimator is a spatial rejection filter in which uncollimated excitation light is rejected, creating a collimated

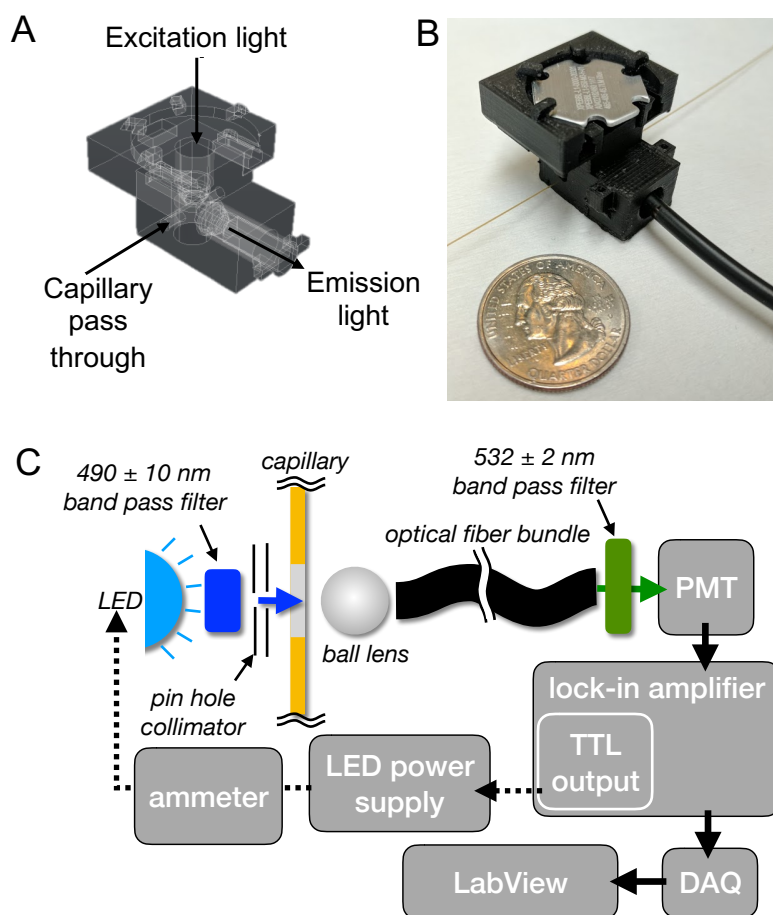


Figure 2.1: Fluorescence detector design. A) The excitation light, capillary pass through, and emission collection axes are mutually orthogonal. B) A photograph of the assembled detector illustrates the detectors critical volume of approximately 6 cm³ C) Schematic of the optical and signal pathways.

beam incident on the capillary detection window. Since the optimal applied LED current for the focused excitation path design was very low relative to the maximum current rating, significantly higher output intensities were possible from these LED sources, and intensity of excitation light passing the rejection filter was not a major concern. While this format offers advantages of reduced cost, reduced complexity, and reduced sensitivity to alignment and fabrication processes, it also increases the illuminated volume relative to that of the focused excitation design. The increase in illuminated volume had potential to increase the detection volume, which would contribute to band broadening. To compare uniformity of illumination and detection volume between focused and collimated excitation paths, peak metrics from CE separations of three amino acids were compared (Fig. 2.3). Table A.1 displays the comparison of theoretical plate count and peak asymmetry, which were used as metrics for comparing detection volume and uniformity of excitation within the detection volume, respectively. No significant difference between the focused and collimated excitation paths were observed, but the collimated pathway offers the advantage of eliminating dependence on optical alignment.

Table 2.1: Peak metrics for CE of amino acids

		Focused Excitation	Collimated Excitation
<i>N</i>	Arg	3183 ± 89.6 (3%)	3418 ± 150 (4%)
	Gly	5836 ± 231 (4%)	6086 ± 173 (3%)
	Gln	5830 ± 174 (3%)	5507 ± 195 (4%)
<i>B/A</i>	Arg	1.32 ± 0.02 (1.3%)	1.32 ± 0.05 (3.6%)
	Gly	1.35 ± 0.15 (11%)	1.25 ± 0.13 (10%)
	Gln	1.27 ± 0.10 (8.3%)	1.14 ± 0.10 (8.5%)

2.4.3 Collection path and optimization of operating parameters

To efficiently collect fluorescence emission, a ball lens was used to couple the emission to an optical fiber. The ball lens was placed on the emission axis such that the effective focal point of the lens was approximately in the center of the capillary. This arrangement maximized the collection of both fluorescence and scattered excitation light, which motivated the use of a narrow band emission bandpass filter (532 ± 2 nm). Response surface methodology [CITE] was used to

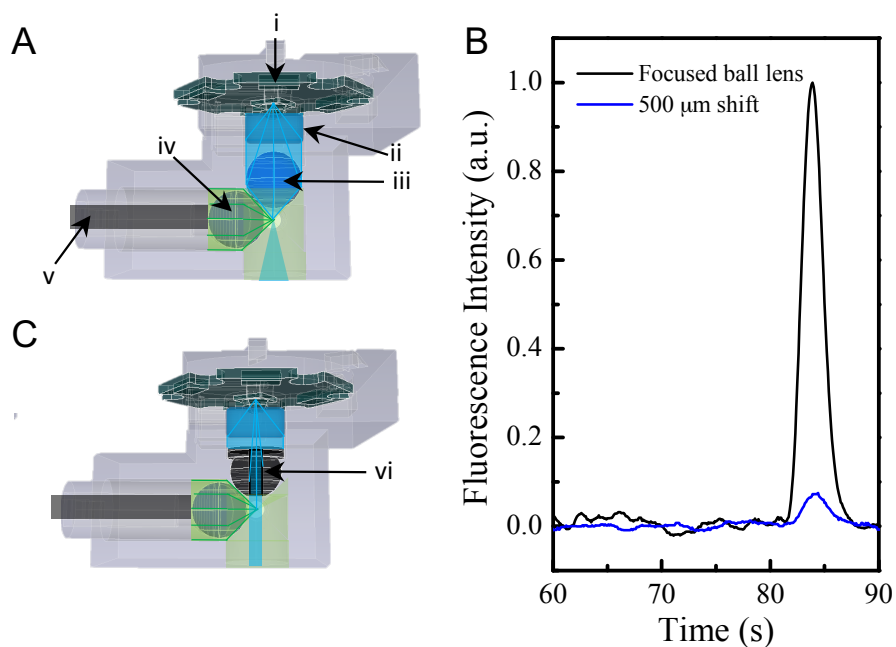


Figure 2.2: Focused and collimated excitation paths A) Cross-section of the detector with focused excitation light. (i. LED, ii. Excitation band-pass filter, iii. Ball lens for focusing excitation, iv. Ball lens for collecting fluorescence emission, v. optical fiber bundle) B) CE of 250 nM fluorescein excitation ball lens in the ideal positions (solid trace) and with the lens shifted axi-ally 500 m out of position (dashed trace). C) Cross-section of the detector with collimated excitation light. (vi. Coaxial 3D printed pinholes for collimated excitation)

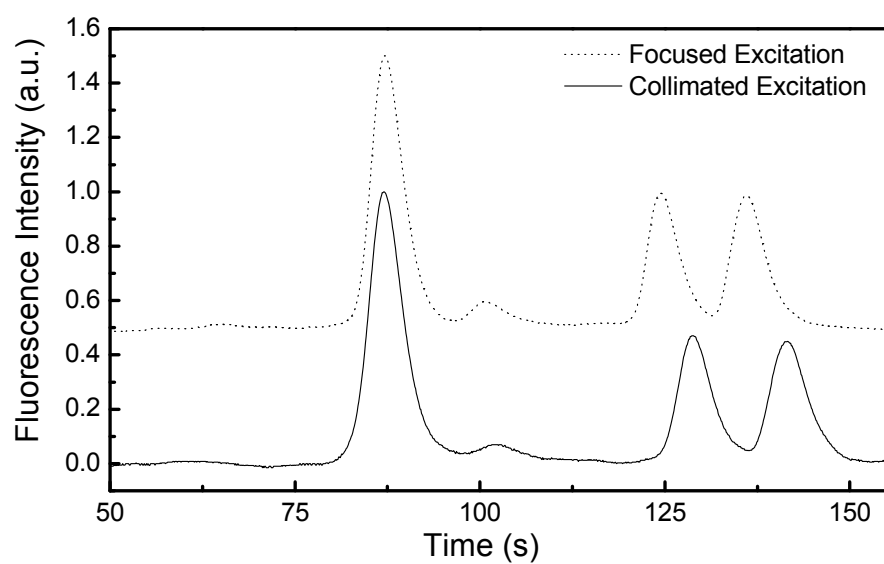


Figure 2.3: CE separations with focused and collimated excitation Separations of three FITC-conjugated amino acids were used to compare the performance of the detector between focused (dashed trace) and collimated (solid trace) excitation paths.

optimize the operating parameters of the detection system. S/N was modeled as a function of LED current (mA), and PMT gain (V) for a fluorescein solution. Response surface methodology allowed for determination of the relationship between multiple factors (PMT gain and LED current) and a response (S/N) with fewer experiments than independent single variable optimizations. Standard least squares regression analysis of the experimental data produced a response surface function, where the optimum combination of factors is found at the maximum of the response surface. In this case, the optimization goal was to maximize S/N . The operating conditions (factor values) were determined to be 123.6 mA applied current to the LED and 0.6143 V for the PMT control voltage to give maximum S/N (Fig. 2.4A). A calibration curve was constructed with fluorescein solutions ranging from 2.5 to 100 nM fluorescein disodium salt in 1 mM borate buffer, pH 8, using 10 mM borate pH 8 as the background electrolyte (Fig. 2.4B). A limit of detection was calculated to be 613 ± 13 pM based on linear regression of a calibration curve where L.O.D. is 3 times the standard deviation of the blank divided by the sensitivity.

2.4.4 Dual detector integration for TDA and CE-TDA analyses

The miniature detector design described above enabled two detectors to be situated inside the capillary cassette for dual-detection TDA and CE-TDA analyses (Fig. 2.5A). TDA was used to monitor the progress of the bioconjugation reaction between FITC and BSA (Fig. 2.5B). Initially, a diffusion coefficient of $484 \pm 294 \mu\text{m}^2 \text{s}^{-1}$ was observed, which corresponds to a hydrodynamic radius of 0.62 ± 0.28 nm. This is in agreement with previous characterizations of the hydrodynamic radius of FITC [40]. As the reaction proceeds, the apparent diffusion coefficient reduces to $186 \pm 70 \mu\text{m}^2 \text{s}^{-1}$ (1.43 ± 0.45 nm) after 5 minutes of reaction. Since TDA reports the average diffusion coefficient of an analyte mixture [13], the observed decrease in diffusion coefficient illustrates the progress of the reaction from a state of predominantly free FITC toward the complete reaction in which all fluorescent signal can be attributed to FITC-BSA. Finally, beyond 90 min, TDA analysis reveals a steady-state diffusion coefficient of $60.5 \pm 7.8 \mu\text{m}^2 \text{s}^{-1}$, corresponding to a hydrodynamic radius of 4.10 ± 0.54 nm, agreeing well with previous reports of the hydrodynamic radius of BSA [27].

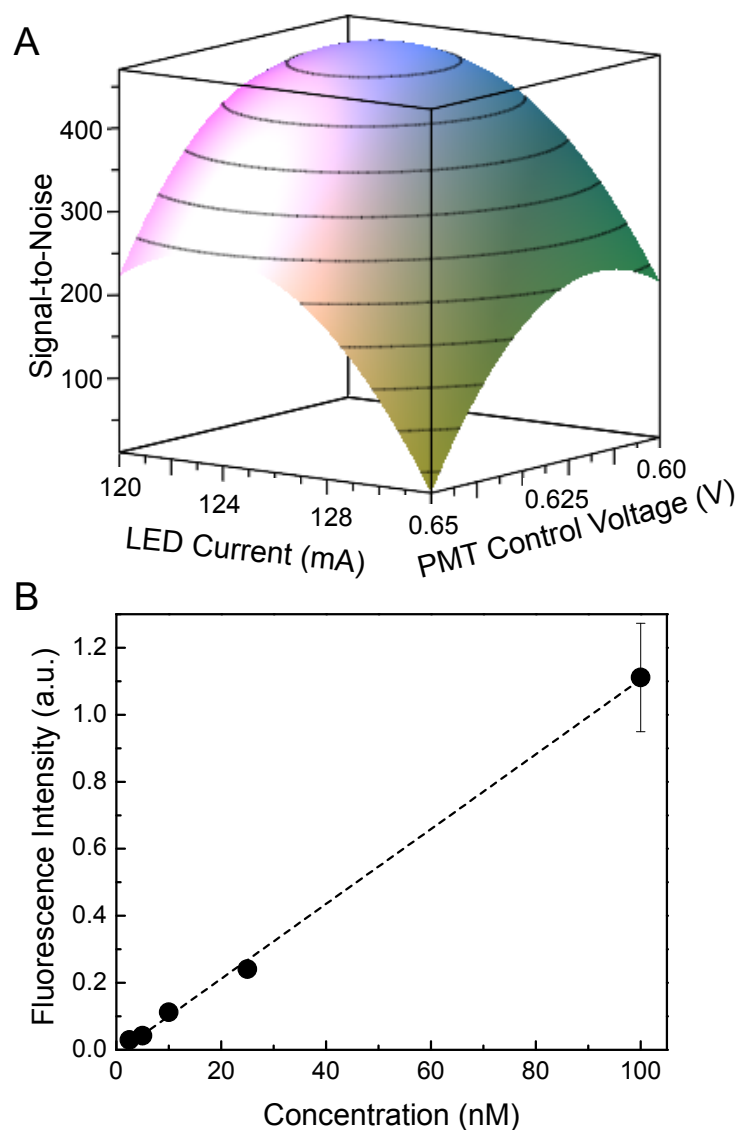


Figure 2.4: Optimization of detector operation. (A) A response surface of S/N of 250 nM fluorescein as a function of PMT Gain Control Voltage and LED Current was used to determine optimal operating conditions. (B) Calibration using optimized operating conditions gave a calculated LOD of 613 ± 13 pM fluorescein disodium salt solution in borate buffer.

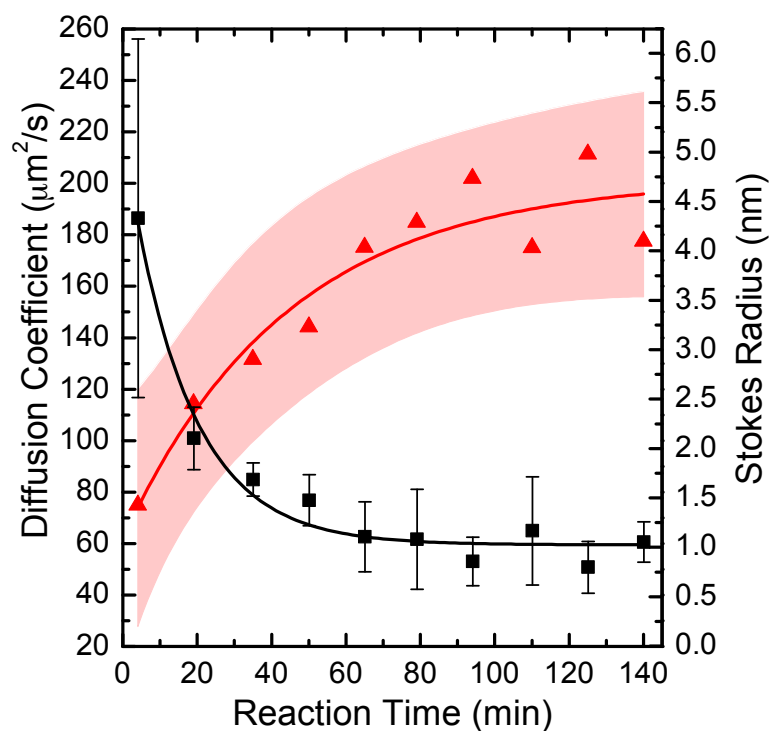


Figure 2.5: FITC-BSA reaction monitoring by dual-point detection TDA. The apparent diffusion coefficient (black squares) of a FITC-BSA mixture decreases as the reaction proceeds, with a corresponding increase in apparent Stokes radius (red triangles). Solid lines are arbitrary exponential fits. Error bars represent 1 standard deviation from the mean of $n = 3$ experiments. Shaded region represents the 95% prediction interval for the fit of Stokes radius data.

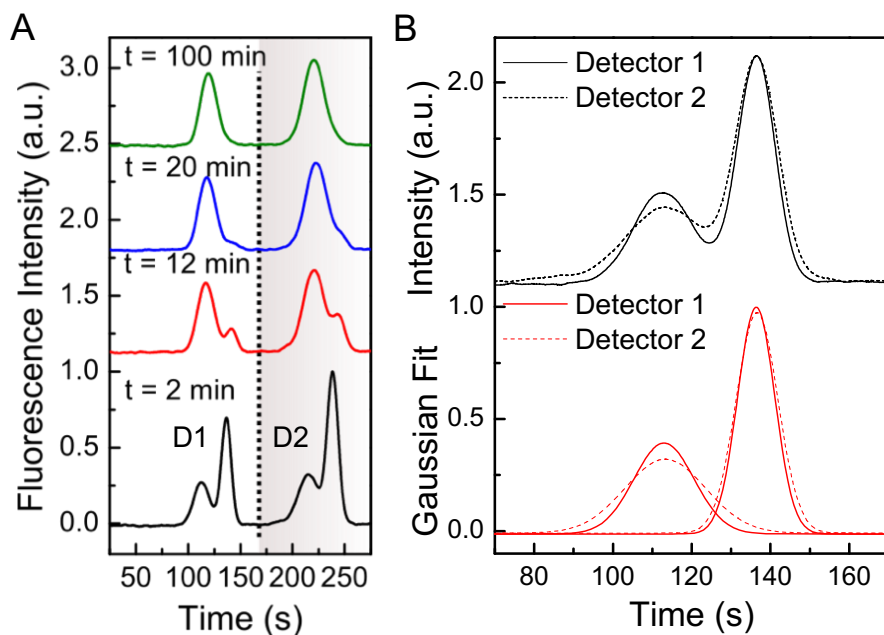


Figure 2.6: FITC-BSA reaction monitoring by CE-TDA A) Time course CE-Taylorgrams for bioconjugation reaction monitoring. The dotted line separates signal originating from detector 1 (D1) and detector 2 (D2). B) TDA analysis of $t = 2$ min trace yields Stokes radii of 4.4 and 0.54 nm for the early and late eluting peaks, respectively. Black traces are experimental data, red traces are Gaussian fits. Solid traces originate from D1, dashed traces originate from D2.

Integration of the miniature fluorescence detectors within commercial CE instrumentation also facilitated analysis via CE-TDA. By online coupling of CE with TDA, the FITC-BSA reaction mixture was separated prior to TDA, enabling size determination of each resulting electrophoretic zone. Figure 6A displays four CE-Taylorgrams that correspond to various reaction times. At 2 minutes of reaction time, electrophoresis yielded two zones, hence the Taylorgram had four total peaks in two groups which corresponds to the two detection points. By 100 minutes of reaction time, the separation no longer produces two zones, suggesting that the reaction has run to completion. Although these results indicate that reaction monitoring by CE alone is possible in this case, via electrophoretic resolution of FITC and FITC-BSA, it also illustrates a powerful capability for standard-free peak determination in CE-TDA. Figure 2.6 shows the overlay of signals (black) at detectors 1 and 2 (solid and dashed, respectively), and the corresponding Gaussian peak fits (red). The band broadening of separated zones was analyzed by TDA, and Stokes radii of 4.4 and 0.54 nm were determined for peak 1 and peak 2, respectively. Thus, the determination that peak 1 corresponded to FITC-BSA while peak 2 corresponded to free FITC is possible by CE-TDA without the need for standards. The results from TDA are summarized in Table A.1.

2.5 Conclusion

We have developed a low-cost, 3D printed capillary fluorescence detection system sufficiently miniaturized to enable dual detector integration with commercial capillary electrophoresis instrumentation. The optimized detector was used to demonstrate the application of 3D printed detection system to CE separations, and dual detector TDA and CE-TDA for bio-conjugation reaction monitoring.

The 3D printed LED-induced fluorescence detector does not achieve the sensitivity of laser-induced fluorescence systems. Further work will focus on improving detection limits by rejecting LED scatter via additional spatial and spectral filtering incorporated directly into the 3D printed architecture. Additionally, the use of a fiber-coupled PMT for signal transduction reduces the cost and size efficiencies of the detection system. In future developments, we aim to incorporate the latest generation of miniaturized silicon photomultipliers for on-board signal transduction. Still,

the LOD of the present system ($613 \pm 13\text{pM}$ for fluorescein) is sufficient to enable numerous high sensitivity fluorescence assays by CE.

The ability to integrate the miniature detection system into commercial CE instruments harnesses the precision flow control of such instruments to realize dual fluorescence detector TDA as a detection modality for CE separations. CE-TDA with fluorescence detection performed using commercial CE instrumentation will facilitate access to rapid, simple, and automated interrogation of binding interactions, direct and absolute size determinations in electrophoretically resolved zones, and the design of assays that improve specificity via electrophoretic and size-based resolutions.

2.6 Acknowledgements

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Chapter 3

A 3D-printed LED-IF detector array for Shah convolution Fourier-transform capillary electrophoresis

A version of this chapter was originally prepared by Laura D. Casto, Claire M. Smith, and Christopher A. Baker. Only minor modifications were made.

Laura D. Casto, Claire M. Smith, and Christopher A. Baker, “A 3D-printed LED-IF detector array for Shah convolution Fourier-transform capillary electrophoresis” *In preparation* (2019).

3.1 Abstract

Improving signal-to-noise (S/N) and analyte resolution is a common challenge for capillary electrophoresis (CE) separations. Increasing S/N increases sensitivity and reduces detection limits, while increased resolution improves selectivity. Both improvements are critical for investigating chemical mechanisms of biological processes. A crucial step in elucidating the underlying mechanisms of disease pathology is developing analytical technologies for characterizing temporal dynamics in biological processes. Specifically, hormone secretion dynamics play major roles in the pathology of a variety of diseases. The main goal of this work is to develop a low-cost CE detection system that will enable fast analysis of biological samples to probe temporal dynamics of hormone secretion with high sensitivity, high selectivity, and high temporal resolution.

3.2 Introduction

CE is an analytical separation technique that enables fast (*ca.* sec. - min.) and selective analysis of small sample volumes (*ca.* 1 - 100 nL). CE is readily coupled to on-line cell culture or perfusion systems for real-time hormone profiling from *in vitro* or *in vivo* biological systems. To selectively measure a specific protein or peptide, CE-immunoassays (CE-IAs) are often used. For CE-IAs, an antibody is used to target the analyte of interest. Performing CE-IAs with fluorescence detection was described by Schultz and Kennedy in 1993 [1]. When using fluorescence detection formats, the antibody can be labeled with a fluorophore and the electropherogram will yield two zones—one corresponding to the immunocomplex and one corresponding to the free-labeled antibody. Because of the nature of CE, and the size similarities between free antibody (*ca.* 150 kDa) and immunocomplex (*ca.* 151-155 kDa) when measuring small peptide hormones, this format is sometimes a difficult separation to achieve. This is often by-passed by using a competitive

immunoassay format, where the analyte is fluorescently labeled instead of the antibody. In this case, the electropherogram still yields two zones. However, now one corresponds to the immunocomplex (ca. 151-155 kDa) and the other to the unbound analyte (ca. 1-5 kDa), so the separation is easier to achieve. However, this competitive format highly dependent to an equilibrium and is most sensitive when the concentration of target analyte is on the same order of magnitude of the dissociation constant, K_D , of the antibody where a good K_D value is around 1 nM. Therefore, we are limited in how low of a detection limit that can be reached when using a competitive format and the direct approach, where the antibody is fluorescently labeled, is preferred. For the direct approach, the limit of detection is only limited by the sensitivity of fluorophore detection and resolution between the bound and free-labeled antibody zones during electrophoresis. These challenges can be addressed by maximizing the S/N and resolution. Resolution is often addressed by modification of separation conditions, but the approach offered in this chapter is capable of enhancing both S/N and resolution through the detection system.

Frequency encoded detection methods such as Shah convolution Fourier-transform (SCOFT) [2, 3, 4, 5, 6] and Fourier transform-CE (FT-CE) [7] have been shown to improve both S/N and analyte resolution in CE. Both techniques have a similar working principle stating that S/N scales as the square root of the number of detectors. Employing multiple injections has been demonstrated to further improve the S/N. In SCOFT, a comb-like mask is placed over the detection region of a CE microchip creating a periodic on/off pattern in the detected fluorescence emission, producing a frequency encoded electropherogram [2]. Analytes of greater electrophoretic mobility move through the masked region at a higher velocity, thus producing a periodic fluorescence signal of higher frequency than that of lower mobility analytes. Fourier-transform of the time domain, frequency encoded electropherogram produces a frequency domain spectrum where peaks correspond to the electrophoretic mobility of their respective analytes. The drawback to SCOFT is that the entire electrophoresis channel must be imaged simultaneously, resulting in significant signal loss due to efficiency of optical coupling between the channel and detector. In addition, the micro-machined mask requires lengthy photolithography protocols that can be hazardous and costly.

The Baker group has designed a low-cost, miniature LED-IF detection system for CE that can easily be extended into a detection array. Each detection window has dedicated optics for excitation and collection of fluorescence emission to enhance collection efficiency of each signal and improve S/N for a single injection of sample. This work presents a 3D printed LED-IF six-detector array for facile integration on a fused silica capillary for high sensitivity CE detection.

3.3 Materials and methods

3.3.1 Materials and reagents

Glycine was Fisher bioreagents brand from Fisher Scientific (Suwannee, GA). Sodium tetraborate, fluorescein disodium salt, fluorescein isothiocyanate isomer I (FITC), and remaining amino acids were ACROS Organics from ThermoFisher Scientific (Waltham, MA). All aqueous solutions were prepared using Milli-Q water purified with a Milli-Q purification system (Millipore, Bedford, MA).

3.3.2 3D printing

A QIDI Technology Dual Extruder Desktop 3D printer was used to print the detection head with 1.75 mm diameter acrylonitrile butadiene styrene (ABS) filament. The device design and print protocol were similar to the protocol discussed in Ch. 2. Briefly, Replicator G was used to generate a G code and drive the 3D printer; and the extruder temperature was set to 260° C, while the bed temperature was 110° C. The device was printed with 20% object infill, 0.1 mm layer height, and 30 mm/s feed rate. To improve print quality and facilitate lift-off of the finished print, a 4 mm thick glass sheet was placed on the build platform and sprayed with hairspray (Aqua Net, Lornamead, Inc., Tonawanda, NY) before printing.

3.3.3 LED modulation

Six high-power single-color blue LEDs (XPEBBL-L1-0000-00301-SB01, Opulent Americas, Raleigh, NC) mounted on a starboard were used in these experiments. The LEDs were connected in series and powered using a DC adjustable power supply (TP3003E, TekPower, Montclair, CA) and a multi-meter, placed in series with the LED circuit, was used to measure applied current. The

modulation circuit used is described in Ch. 2.

3.3.4 Detection head assembly and optical components

A six-detector array was 3D printed to deliver and collect both excitation light and fluorescence emission to six evenly spaced capillary windows (Fig.??). The spacing between detection windows was 1.4 cm apart. Similar to the assembly described in Ch. 2, LED excitation light passed through a 6.3 mm diameter 490 ± 5 nm bandpass filter (W4489, Omega Optical, Inc., Brattleboro, VT) and was directed to the detection window of the capillary for all six detection windows via a 3D printed coaxial pinhole for collimation. Fluorescence emission was collected via a second 5 mm ball lens which coupled emission light to a fiber optic bundle (02-540, Edmund Optics Inc., Barrington, NJ) for transmission to an off-device photomultiplier tube (PMTSS2, Thorlabs Inc., Newton, NJ). All six fiber bundles were coupled into the same PMT. The PMT was fitted with a 520 ± 5 nm (FB520-10, Thorlabs Inc. Newton, NJ) emission filter to reduce the magnitude of scattered excitation light delivered to the transducer. A lock-in amplifier (SR830, Stanford Research Systems, Sunnyvale, CA) was used to modulate the LED excitation and demodulate the PMT signal before data acquisition via a USB data acquisition module (USB-6001, National Instruments, Austin, Texas). An assembly diagram for an individual detection head can be found in Appendix A (Fig. A.2).

3.3.5 Capillary electrophoresis

Capillary electrophoresis experiments were performed with an E series DC to HV converter (E50, EMCO High Voltage Corporation, Sunnyvale, CA) to supply high voltage and platinum wire electrodes. LED-IF data acquisition software was written in-house using LabView™ 2016 Version 16.0f2 (National Instruments, Austin TX). Fig. 3.1

Separations were performed in a 100 μm i.d./ 360 μm o.d. fused-silica capillary (35 cm total length, 19 cm effective length). Samples were injected hydrostatically by raising the inlet vial for 30 s. The background electrolyte solution was 10 mM borate buffer, pH 8.5, and samples were in 1

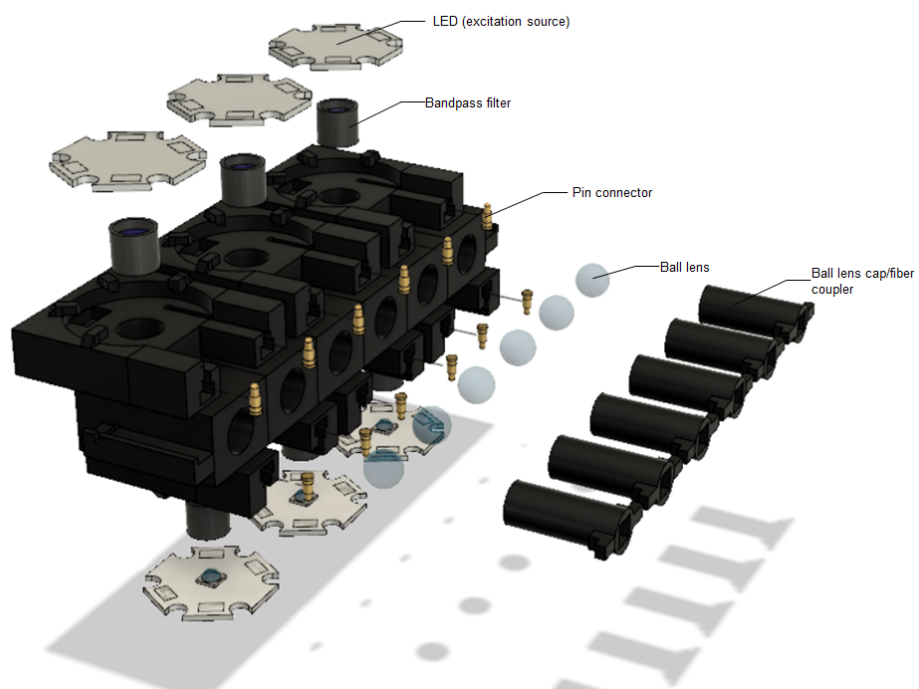


Figure 3.1: The multi-detector array includes six separate detection points using six individual starboard LEDs, excitation filters, emission collection lenses, and six fiber optic outputs.

mM borate buffer, pH 8.5. Electrophoresis was carried out with an applied voltage of 15 kV (430 V cm^{-1}).

3.3.6 Multiphysics simulations

COMSOL Multiphysics Microfluidics Module (Version 5.3a, COMSOL, Inc., Burlington, MA) was used to solve coupled partial differential equations for electric field and transport physics in the capillary using finite element analysis.

3.4 Results

A six-detector array was used to compare the electropherogram for fluorescein and a fluorescein and fluorescein isothiocyanate mixture from a single detection point (Fig. 3.2-A) to those with six detection points when the multi-detection electropherogram (Fig. 3.2-B) undergoes a Fourier-transform (Fig. 3.2-C).

It is evident that six detection points did not affect the peak resolution between two analytes not well-resolved in the time domain. Resolution was calculated to be 0.7 for both the time and frequency domains. This can be attributed to a significant reduction in the number of detection points from SCOFT using a micro-machined mask with 55 slits to this six-detector array. Due to the size of each detector, the number of detectors that can be included will reach a practical limit due to the length of the capillary. As the capillary length increases, a higher applied voltage will be required to attain the appropriate electric field for electrophoresis. To overcome this limitation without adding more detectors or increasing capillary length, a continuous injection method can be employed to increase the number of times the sample passes through the detection region 3.3 A. It was presented that multiple injection plugs would enhance S/N in traditional SCOFT literature [4, 7]. Therefore, by employing a continuous sample injection method, the S/N and resolution could be further improved.

Multiphysics simulations demonstrate the viability of a continuous injection method for enhancing resolution and S/N. Fig. 3.3 illustrates how the combination of multiple detectors and continuous injection method can significantly enhance resolution and S/N.

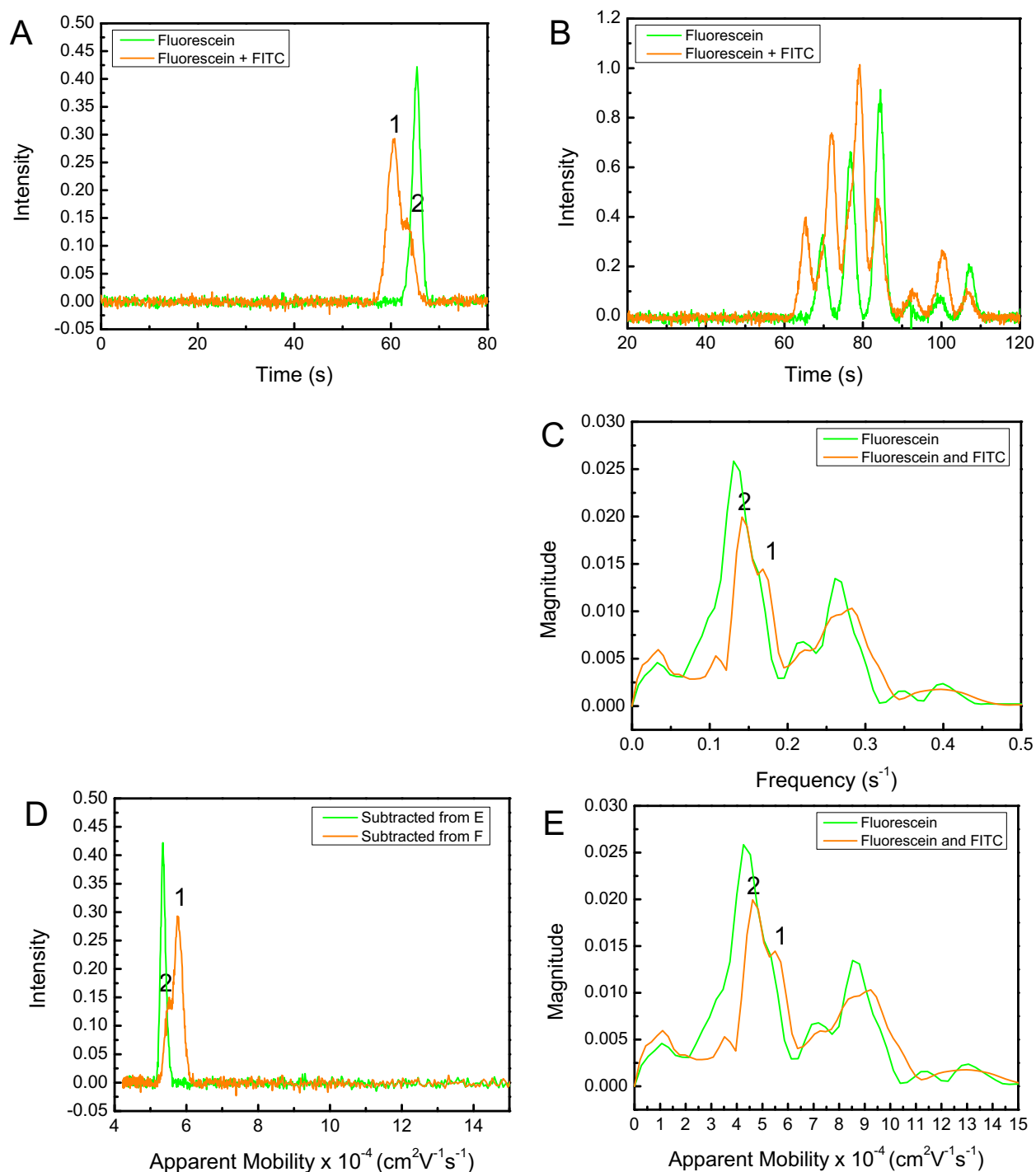


Figure 3.2: Panels A and B are the time domain electropherograms for single and multiple detectors, respectively. Panel C is the Fourier-transform of B, to convert the electropherogram into the frequency domain. Panels D and E correspond to the electropherograms in A and B, respectively, with the x-axis being apparent mobility of the analytes. For all panels, the peaks labeled 1 correspond to FITC and peak 2 corresponds to fluorescein.

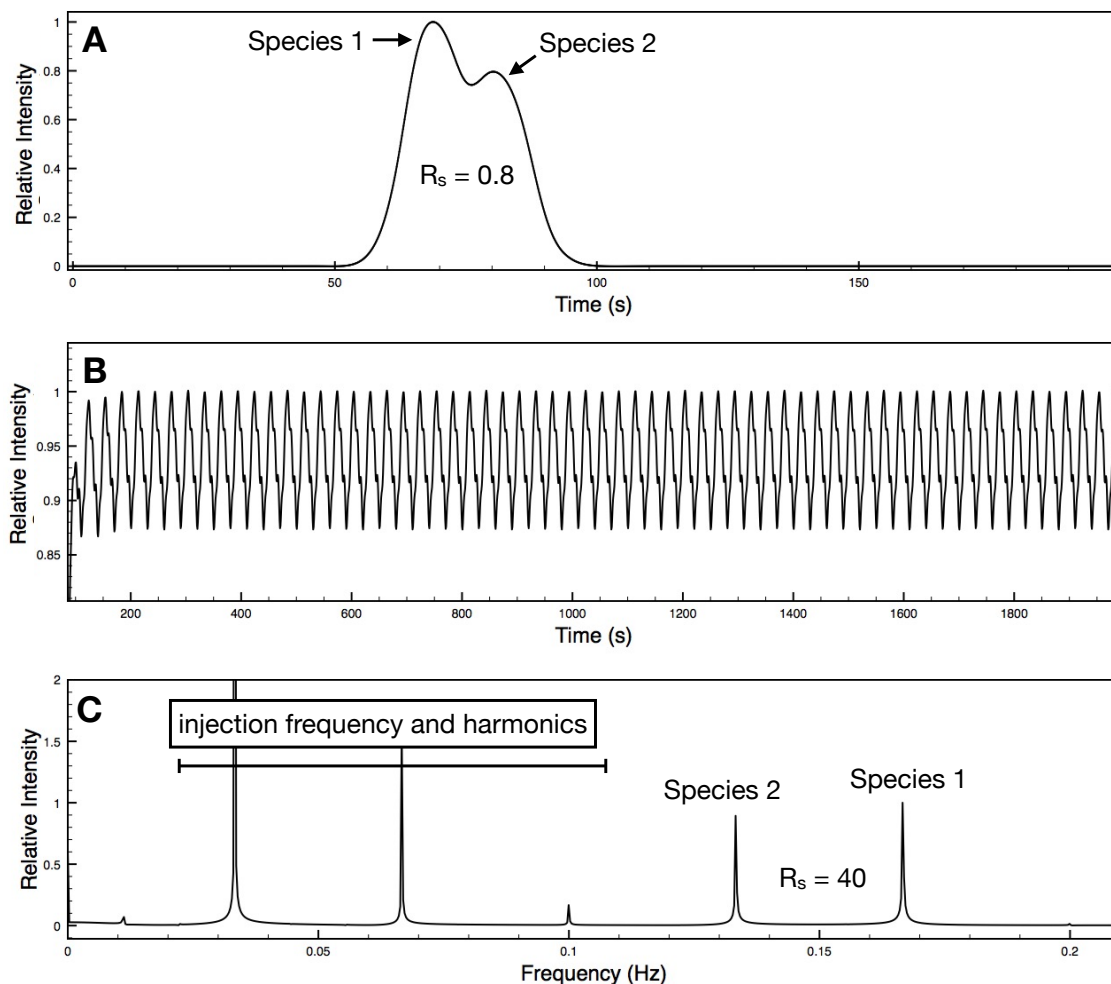


Figure 3.3: A) Conventional electropherogram from single-point detection with single injection
 B) Time domain signal using a continuous square wave injection at 0.03 Hz and multiple detectors.
 C) Frequency domain from Fourier-transform of the time domain from panel B.

3.5 Conclusion

This chapter presented a low-cost 3D printed multi-detector array for SCOFT-CE. It was found that while the detector array alone will not enhance S/N, the combination of multiple detection points with a continuous injection method could significantly enhance peak resolution and S/N.

The continuous injection method presented in Fig. 3.3 was based on pulsed injection where analytes were injected every 30 s. This leads to a large fundamental peak at the injection frequency and a significant amount of harmonics in the frequency domain. In practice, these harmonics could overshadow the analyte peaks and hinder the ability to resolve or adequately quantify analytes. It also be noted that the sampling time to achieve the 50-fold increase in resolution was one hour. It is desirable to enhance resolution and S/N with shorter analysis times to achieve high temporal resolution measurements, so a new continuous injection method is presented in Ch. 4.

3.6 Acknowledgements

This work was supported by laboratory start-up funds from The University of Tennessee. The authors would also like to acknowledge Kevin Do for contributing his expertise in designing illustrations of the detector array.

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Chapter 4

Sine-injection Fourier-transform enhanced capillary electrophoresis

4.1 Abstract

Improving signal-to-noise (S/N) and analyte resolution are perpetual challenges in capillary electrophoresis (CE) separations. Fourier-transform based detection methods have proven to be useful for both enhancement of S/N and resolution to provide better sensitivity and higher measurement selectivity. This work presents a multiplexed injection method termed sine-injection Fourier-transform (SIFT) where a continuous sine-wave injection pattern is used for convolution of the electropherogram with a sine wave. Proof-of-concept for SIFT-CE has been modeled using multiphysics simulations and initial experiments for expansion into practical use have been performed.

4.2 Introduction

Fourier-transform detection methods such as Shah convolution Fourier-transform (SCOFT) [1, 2, 3, 4] and Fourier transform-CE (FT-CE) [5] discussed in Ch. 3 provide enhanced sensitivity and analyte resolution for CE separations by multiplexing using multiple detection points. It has been shown that employing multiple injections has been demonstrated to further improve the S/N [6].

This work aims to employ continuous injection to enhance throughput and maximize the advantages of SCOFT by continuously injecting sample at a particular frequency. Upon Fourier-transform, in addition to peaks corresponding characteristic analyte frequencies, there will be a peaks corresponding to the injection frequency. To minimize appearance of harmonics for the injection frequency, the injection profile will take the shape of a sine wave- thus proposing a new technique for enhanced analyte resolution in CE detection: Sine-injection Fourier transform (SIFT). Sine convolution has been presented as a superior alternative to SCOFT [7] and by convolution of the injection profile rather than the detection region, we reduce the need for high efficiency optical coupling, micro-machined masks, or detector arrays which can extend the ability for facile integration of SIFT with a variety of common CE detection methods including mass spectrometry. Although, as presented in this work, combination of SIFT and multi-detector arrays will further provide S/N enhancement.

4.3 Materials and methods

4.3.1 Materials and reagents

Fluorescein disodium salt was ACROS Organics from ThermoFisher Scientific (Waltham, MA). All aqueous solutions were prepared using Milli-Q water purified with a Milli-Q purification system (Millipore, Bedford, MA). Schott borofloat microfluidic blank substrates and substrates coated with chrome and 5300A AZ1500 positive photoresist were purchased from Telic Company (Valencia, CA). Microchem Corp MF-319 developer (NC9713727) and Transene Inc. Chromium Etchant (NC9820871) were purchased from Fisher Scientific (Waltham, MA).

4.3.2 Multiphysics simulations

Multiphysics simulations were performed using COMSOL Multiphysics 5.3a (COMSOL, Inc., Burlington, MA) using the Chemical Reaction Engineering Module. The capillary is modeled in one dimension as a line segment with length, L , equal to 35 cm, using built-in parameters for water as the material. Two types of physics are used for investigating the feasibility of SIFT-CE for improvement of signal-to-noise and resolution— Electrostatics and Transport of diluted species. The model uses one-way coupling between studies such that the Electrostatics physics is solved in a stationary study and then the solution is used as the initial conditions for a time depended study of transport physics.

4.3.3 Microchip electrophoresis

Microchip electrophoresis was performed in a cross-t borofloat microchip with ca. 50 micron i.d. channels (8.4 micron depth). The separation channel is 35 cm in length while the cross-t channel for gating is 1.5 cm. Platinum electrodes were placed in each of the four wells and potential was applied with a Spellman high voltage power supply (SL50P150, Spellman High Voltage Electric Corporation, Plainview, NY). The applied field in the separation channel was ca. 100 V/cm.

4.3.4 Sine wave modulation

Pulse-width modulated sine-wave injection profiles were created using a function/ arbitrary waveform generator (SDG1025, Siglent Technologies, Solon, Ohio). The underlying square wave was produced at 30 Hz while the sine wave was delivered at a frequency of 0.5-1 Hz.

4.4 Results

4.4.1 Working principle of SIFT-CE

SIFT-CE is a Fourier-transform based method that uses continuous injection of a sample to enhance analyte resolution. Because of the shape of the injection profile, the electropherogram is convoluted with sine wave. The working principle for SIFT-CE of two species using a single detection point is illustrated in Fig. 4.1. This illustration was produced using COMSOL Multiphysics to simulate two-species of similar electrophoretic mobilities that are not well resolved in the time-domain of a conventional electropherogram (Fig. 4.1-B inset). The SIFT electropherogram for two species will consist of wavelets containing the injection frequency and a characteristic frequency for each of the two species. A beat frequency where the two species migrate passed each other as they migrate through the capillary is observed (Fig. 4.1-A). A Fourier-transform of the electropherogram results in peak resolution between the two analytes (Fig. 4.1-B). Increasing the number of detection points by employing SCOFT detection method rather than single-point detection will result in an enhancement of S/N (Fig. 4.1-C).

4.4.2 SIFT-CE simulations using COMSOL Multiphysics

Multiphysics modeling enabled simulation-aided optimization of SIFT-CE operation parameters. The importance of detector placement and the effect on magnitude injection frequency in the frequency domain was investigated Fig. 4.2). As seen in Fig. 4.2-A, when the detector is placed to close to the inlet, the electropherogram is overwhelmed by the 1 Hz injection frequency from the sine wave and the characteristic frequencies of the analytes will not be observed in the frequency domain (Fig.4.2-F). As the detection point is moved further away from the injection inlet, the analyte frequencies become more apparent and thus S/N is enhanced in the frequency domain. Detector placement would need to be optimized for each separation system depending on the

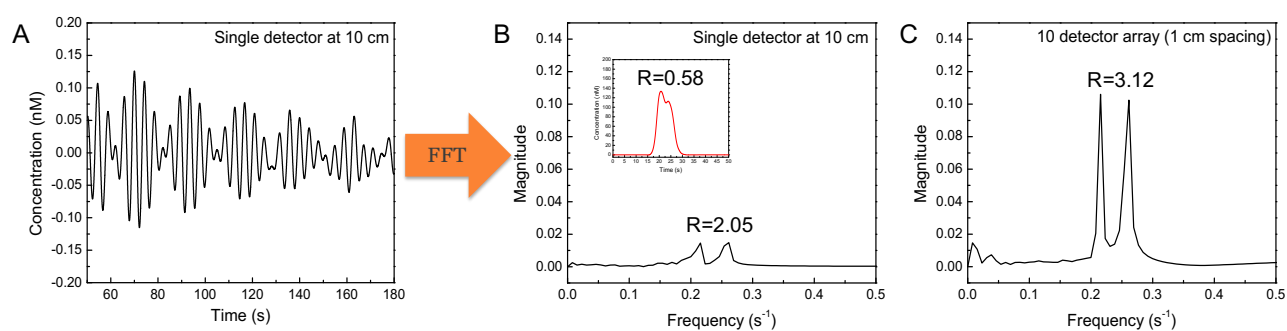


Figure 4.1: (A) The SIFT electropherogram (time-domain) for two species using a single detection point undergoes a Fourier-transform (B) to show enhanced resolution over a single injection technique (inset). (C) By substituting single-point detection with an array of detectors, the resolution is further improved.

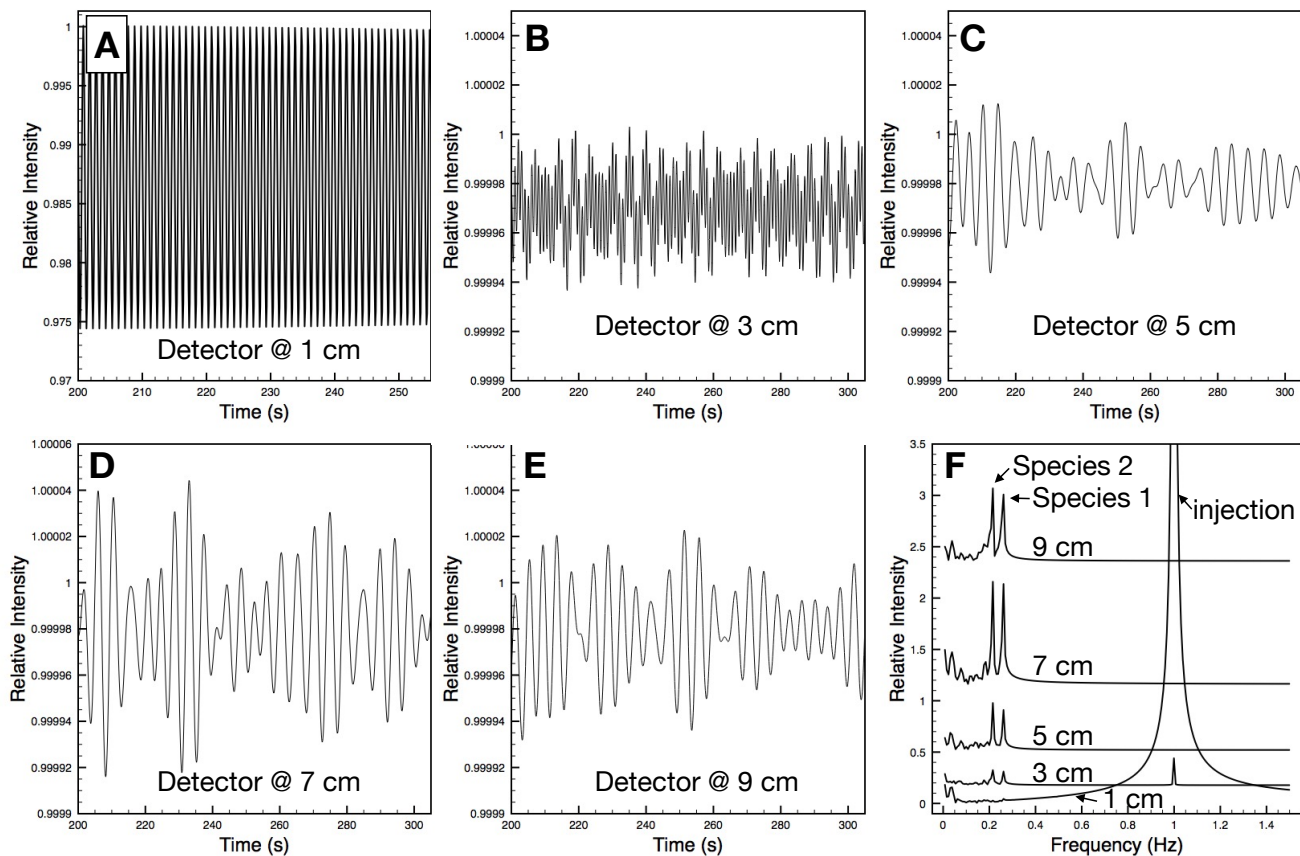


Figure 4.2: Multiphysics simulations show the time domain signal of a 1 Hz sine wave injection pattern with a single detection point at 1, 3, 5, 7, and 9 cm from the capillary inlet for A-E, respectively. F) The resulting frequency domain spectra show that the analyte-specific oscillation frequencies are independent of detector placement and can be easily separated from the 1 Hz injection frequency.

apparent mobilities of the analytes of interest. Also, it is important to note that these simulations were performed without accounting for the electroosmotic flow (EOF) vector. However, since EOF is a constant plug-like flow that will be uniform for all species, when EOF is present, analytes will move at a higher frequency than without EOF so it will be important, in practice, to ensure the detection point is sufficiently far away from the inlet of the separation channel to eliminate the contribution of the injection frequency to the electropherogram.

4.4.3 Sine-wave injections

We investigated the viability of performing SIFT-CE on a microchip using an inverted epifluorescence microscope equipped with fluorescein excitation and emission optics to perform single-point detection. A sine wave injection pattern was achieved using pulse-width modulation. This works by creating a digital to analog conversion of injection pulses to a sine wave for delivery via electrokinetic gated injections, discussed in Ch. 1). To confirm this pulse-width modulation was producing the desired sine wave, an electrokinetic sine wave injection with 30 Hz pulse width modulation and 0.5 Hz sine wave introduced fluorescein into the microfluidic separation channel (Fig. 4.4-A). When the electropherogram undergoes a Fourier-transform, a sharp peak is observed at 0.5 Hz (Fig. 4.4-B) which confirms the desired sine wave is being delivered successfully. However, a second peak to correspond to the fluorescein was not observed. It is possible that fluorescein is migrating too slowly with an applied field of only 100 V/cm. Also, the presence of EOF could be hindering our ability to resolve the fluorescein specific frequency from the injection profile. Decreasing the injection frequency could provide sufficient time for the analyte specific frequency to resolve from the injection profile. For further experiments, however, EOF should be suppressed in the separation channel, and the applied field should increase.

4.5 Conclusion

This work presents SIFT as a continuous injection method for high throughput analysis with enhanced analyte resolution and S/N as a complimentary method to SCOFT for capillary electrophoresis detection. Simulations demonstrate the proof-of-concept for SIFT as it applies to capillary electrophoresis and initial experiments illustrate the viability for performing a continuous sine-wave injection profile. While this is not a complete demonstration for SIFT proof-of-concept

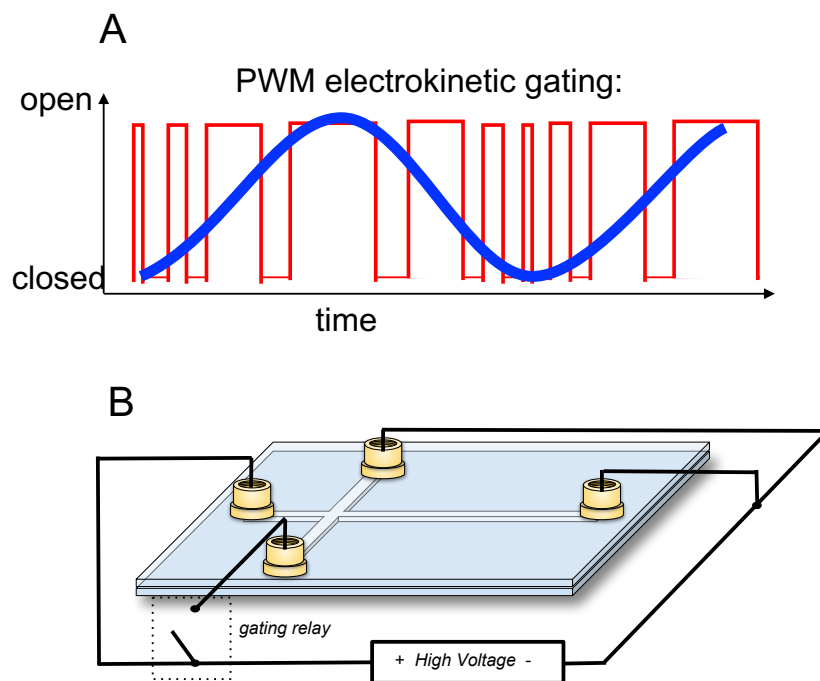


Figure 4.3: A) A sine wave injection pattern can be achieved by using pulse width modulation where fast injection pulses are much faster than the fluidic circuitry for how electrokinetic gating occurs. This produces a digital (injection pulses) to analog (injection waveform) conversion. B) Microfluidic platform used for testing sine injection waveforms with single-point detection.

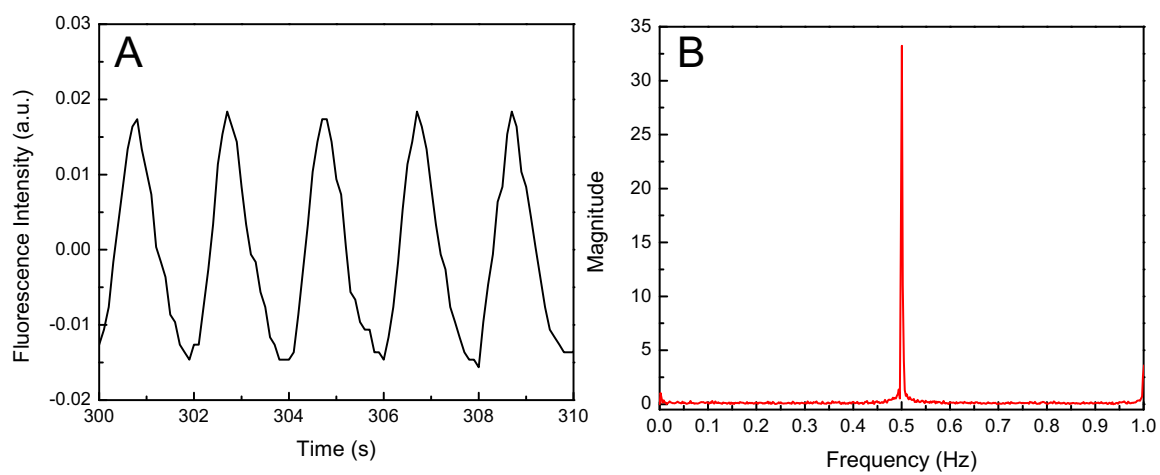


Figure 4.4: A) A sine wave injection pattern can be achieved by using pulse width modulation and electrokinetic gating. B) A Fourier-transform of the pulse-width modulated sine wave confirms that the sine wave is being produced at the desired frequency of 0.5 Hz.

in practice, this work provides a foundation for optimizing SIFT conditions and expanding SIFT-CE into practical applications.

4.6 Acknowledgements

The authors would like to acknowledge support from COMSOL, Inc. This work was supported by laboratory start-up funds from The University of Tennessee.

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Chapter 5

Characterization of low adsorption filter membranes for electrophoresis and electrokinetic sample manipulations in microfluidic paper-based analytical devices

A version of this chapter was originally published by Laura D. Casto, Jennifer A. Schuster, Claire D. Neice and Christopher A. Baker. Only minor modifications were made.

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Additional materials for Chapter 5 are provided in Appendix [B](#).

5.1 Abstract

The emergence of microfluidic paper-based analytical devices (μ PADs) has renewed interest in paper as a substrate for chemical separations and analysis. The availability of engineered filter membrane materials effectively broadens the definition of paper as a substrate material, and presents the opportunity to utilize their engineered properties in chemical analyses. Here we evaluate a selection of low adsorption filter membrane materials for their efficacy in achieving zonal electrophoretic separations of amino acids within μ PADs. Cellulose acetate (Whatman OE66), cellulose ester (MF-Millipore), and polyvinylidene fluoride (Durapore PVDF) substrates were evaluated for their performance in electrokinetic μ PADs, including establishing microfabrication parameters, characterizing Joule heating, and establishing fluorescence detection limits. Heating-limited electric fields in the range of 230-350 V cm⁻¹ were achieved, and fluorescence limits of detection of ca. 3 nM were observed in both green (fluorescein) and red (Nile blue) fluorescence channels for OE66 substrates. Electrophoretic separations of a three amino acid mixture were demonstrated in PVDF and OE66 μ PADs, while relatively high rates of electroosmotic flow in MF-Millipore substrates enabled electrokinetic flow gating in this material. These studies demonstrate the efficacy of zonal electrophoresis in μ PADs made from low adsorption substrates, and highlight design considerations for the development of similar μ PAD systems.

5.2 Introduction

Paper has a long history as a substrate for analytical separations, from the origins of paper chromatography in the 19th century [1], to early examples of paper-supported electrophoresis in the 1930s [2]. The low cost and reduced analysis volumes achieved in paper substrates are as valuable in addressing modern analytical challenges as they were in the first half of the twentieth century, however paper was largely replaced in electrophoresis applications with the emergence of hydrogel materials like polyacrylamide [3], and high resolution techniques such as capillary electrophoresis [4]. Subsequently, capillary electrophoresis played a role in the evolution of microfluidic lab-on-a-chip systems [5, 6], many of which utilize electroosmotic flow and other electrokinetic sample manipulations to automate sample handling and analysis.

The emergence of microfluidic paper-based analytical devices (μ PADs) [7, 8] has renewed interest in paper as a substrate material for chemical analysis. Most commonly, μ PADs take advantage of passive transport by capillary action in the paper substrate, which reduces cost and complexity of instrumentation. For example, a recent report describes high sensitivity detection of the cancer marker alpha-fetoprotein from human serum in a wax printed μ PAD device [9]. This device enabled an automated enzyme-labeled immunoassay, utilizing only capillary flow within the paper substrate for analyte and reagent mass transport, and achieved a detection limit of 1 ng mL⁻¹ (*ca.* 15 pM). In addition to lateral flow immunoassays, distance-based readout schemes are becoming popular in μ PAD assay designs [10, 11, 12]. Chen et al. combined immunoassay and distance readout techniques in a μ PAD to achieve detection of carcinoembryonic antigen with a 2 ng mL⁻¹ (*ca.* 10 pM) detection limit [13]. This device utilized a sandwich immunoassay in which capture antibodies were immobilized at the μ PAD channel inlet, and excess HRP-labeled detection antibodies were washed away along a paper channel by capillary action. The length of the eluted band of detection antibodies was inversely correlated with the concentration of carcinoembryonic antigen captured in the sandwich assay. These examples illustrate that creative assay design and inventive approaches to quantitative detection are required to overcome the limited versatility of sample handling achievable using only capillary action. Increasing the versatility of fluidic

transport in μ PADs, therefore, may enable new approaches to assay design.

μ PADs utilizing active fluidic transport by electroosmosis and electrophoresis have emerged in recent years [14, 15]. For example, ion concentration polarization (ICP) has been utilized as a sample enrichment technique in several μ PAD devices [16, 17, 18]. ICP occurs when an ion depletion region develops near the interface of free solution and nanoporous ion selective membranes, enabling electrophoretic focusing in localized regions of high electric field. This mechanism is particularly attractive in paper substrates since ion selective membranes are readily formed within the porous substrate by solution deposition of polymer materials like Nafion [19]. Sample enrichment is one example of how μ PAD assays may become more versatile with the addition of active, electrokinetic sample transport.

A recent review by Breadmore and coworkers described the past and present landscape of electrophoresis in paper substrates, and identified a need for systematic study of substrate performance for electrokinetic sample manipulations in μ PADs [20]. One promising opportunity in this area is the availability of modern engineered filter membrane materials that broaden the definition of paper as a substrate. The next generation of μ PADs may exploit the properties of these engineered materials for new approaches to assay design and detection readout. Reduced sample adsorption on low binding filter membrane materials, for example, may offer advantages for performing zonal electrophoresis in μ PADs.

Here, we evaluate a selection of low adsorption filter membranes for their performance in electrokinetic μ PADs. Microfabrication procedures are established, and Joule heating characteristics are evaluated. We compare these materials for their performance in fluorescence detection and separations by zonal electrophoresis. Finally, we demonstrate electrokinetic gating in a paper substrate with suitable electroosmotic flow characteristics.

5.3 Materials and methods

5.3.1 Materials and reagents

Whatman™ cellulose acetate membrane filters (OE 66 ST, 10404170, 0.2 μm pore size) were from GE Healthcare Life Sciences (Pittsburgh, PA). Durapore™ polyvinylidene fluoride (PVDF) membrane filters (SVLP04700, 5 μm pore size), and MF- Millipore™ mixed cellulose ester membranes (GSWP04700, 0.22 μm pore size; and SMWP04700, 5 μm pore size) were from Merck Millipore Ltd. (Tullagreen, Carrigtwohill Co. Cork, IRL). Sodium tetraborate, fluorescein disodium salt, fluorescein isothiocyanate isomer I, Nile blue A, arginine, and glutamine were ACROS Organics from ThermoFisher Scientific (Waltham, MA). Glycine was Fisher bioreagents brand from Fisher Scientific (Suwanee, GA). Tris(hydroxymethyl)amino-methane was from Sigma Chemical Co. (St. Louis, MO). 25 mm $\times$ 75 mm $\times$ 1.1 mm borosilicate microscope slides were purchased from Fisher Scientific (Suwanee, GA). All solutions were prepared in Milli-Q (Millipore, Bedford, MA) 18 M Ω cm deionized water.

5.3.2 μ PAD fabrication and assembly

Paper microchannels were cut from filter membranes using a 30 W CO₂ direct laser writing instrument (VLS 2.3, Universal Laser Systems, Scottsdale, AZ). Filter membranes were secured at the edges to the cutting platform using clear tape to prevent substrates from moving under nitrogen flow from the laser cutting system. Microfluidic networks were designed in Auto-CAD and cut on the laser cutter in vector mode. Linear energy density (LED) is given in units of J mm⁻¹ and is the ratio of the laser power (J s⁻¹) and linear speed (mm s⁻¹) [21]. Three unique power and speed combinations were used to achieve each value of linear energy density reported. Microfabricated paper devices were imaged via stereo microscope, and dimensions were measured by pixel counting using ImageJ software [22].

500-700 μm thick PDMS sheets (HT6240, Stockwell Elastomers, Inc., Philadelphia, PA) and 1 mm thick borosilicate glass microscope slides were used to laminate the paper microchannels into a glass-PDMS-paper-PDMS-glass laminate structure (Fig. 5.1). Binder clips provided

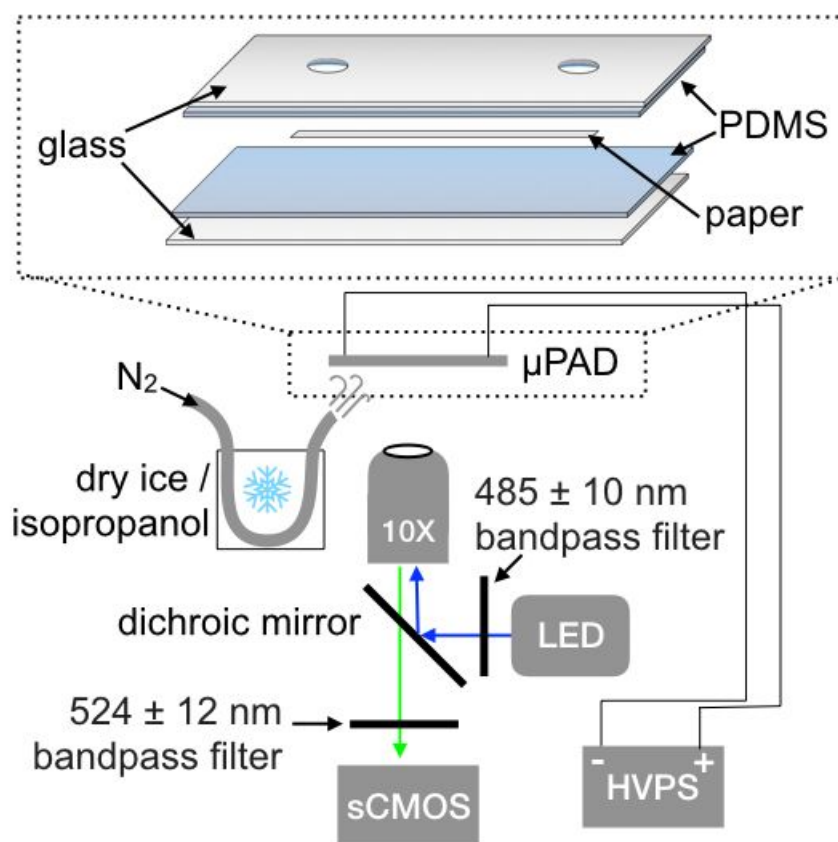


Figure 5.1: Schematic diagram of experimental apparatus. The laminated μ PAD device (inset, top) was imaged on the stage of an inverted fluorescence microscope via 10 $\times$ objective. A broadband light-emitting diode source provided fluorescence excitation, with emission collected by sCMOS camera. A high voltage power supply (HVPS) was used to apply electrical potential. Active cooling was accomplished using nitrogen flow cooled via dry ice and isopropanol bath. The green fluorescent filter set shown was utilized in all experiments, with an alternate red fluorescent filter set (not shown) used to determine fluorescent limits of detection for Nile blue.

sufficient clamping force to compress the PDMS around the paper substrate such that little or no dead volume was evident at the edge of the paper microchannel upon visual inspection via 10 $\times$ microscope objective.

5.3.3 Joule heating characterizations

Linear channels with lengths ranging from 1-4 cm were cut from the respective substrates, assembled as described above, and filled with 15 mM borate buffer (pH 8). Electrical potential was applied across the length of the channels to give electric field strengths in the range of 0-700 V cm⁻¹. Each potential application was held until measured electrical current stabilized to within $\pm 5\%$ (typically 1-3 minutes), and the stable current was recorded. Plots of current vs. electric field strength were prepared, and the heat-limited condition was defined as the field strength at which the coefficient of correlation for the least squares linear regression of current vs. applied potential dropped below 0.98, and continued to deteriorate with additional data points at higher field strengths.

5.3.4 Fluorescence characterizations

μ PAD channels were wetted with either buffer or fluorescent dye solution, and fluorescence detection was carried out by an inverted fluorescence microscope (Nikon Eclipse Ti-U, Nikon Instruments Inc., Melville, NY) using a high intensity LED for fluorescence excitation (SOLIS3C, Thorlabs, Newton, NJ), and sCMOS for image capture (Zyla 4.2, Andor USA, South Windsor, CT). Cube-mounted fluorescence filter sets were used to excite and collect green (96 226, Nikon Instruments Inc., Melville, NY, λ_{ex} = 480 nm, λ_{em} = 535 nm), and red (M358745 Semrock, Rochester, NY, λ_{ex} = 635 nm, λ_{em} = 680 nm) fluorescence. Green fluorescent dye solution was 5-1000 nM fluorescein disodium salt in 10 mM TRIS buffer (pH 8). Red fluorescent dye solution was 5-1000 nM Nile blue in 10 mM TRIS buffer (pH 8). Calibration plots were constructed from grayscale counts vs. dye concentration (see Fig. B.1 in Appendix B), and the limit of detection was defined as the standard deviation of the blank multiplied by three, divided by the slope of the

calibration line.

5.3.5 Electrophoretic separations

Amino acids were labeled following a procedure described previously [23]. Briefly, arginine, glycine and glutamine, were combined with a limiting concentration of FITC to final concentrations of 1 mM for arginine and glutamine, and either 1mM or 2mM glycine and 100mM FITC in 20mM sodium tetraborate (pH 9.5). The variation in glycine concentration was used to help identify migration order, and limiting FITC concentration ensured that no removal of excess FITC was necessary. The reaction was protected from light, and placed on a rotator at room temperature overnight. The concentrated solution of FITC-labeled amino acids was diluted 200-fold in 10 mM TRIS buffer (pH 8) to a final concentration of 500 nM total FITC content.

μ PADs consisting of individual linear channels of 4 cm in length and 1 mm in width were assembled as described above. One buffer reservoir was filled with background electrolyte (BGE) of either 10 mM TRIS, pH 8.0 or 10 mM sodium borate, pH 8.5 and the channels were left to fill by capillary action. While the channel filled, 500 nL of sample was manually pipetted onto the unwetted end of the paper channel to form the sample plug for electrophoretic separation. Fluorescence detection was carried out using the inverted microscope system illustrated in Fig. 5.1. Peak profiles were extracted from recorded videos of migrating fluorescent bands by defining a region of interest within the paper channel using ImageJ software, and plotting grayscale intensity vs. time. Baseline drift was subtracted from electropherograms using Origin Pro (OriginLab Corporation, Northampton, MA) and signal intensity values were normalized to give each electropherogram a maximum intensity of 1 relative unit.

5.3.6 Electrokinetic gating

μ PADs with cross-t geometries were prepared as described above. All channels of the cross-t geometry were 1 cm in length, which simplified control of the applied field strengths in the complex geometry. Applied voltage was 400 V, to achieve a field strength of 200 V cm⁻¹ in all

channels of the device. BGE was 10 mM sodium borate, and sample was 250 nM fluorescein in BGE.

5.4 Results

The reemergence of paper as a substrate for chemical analysis, in the form of μ PADs, presents the opportunity to utilize modern filter membrane materials for their specific engineered properties. Here, we investigated a selection of three low adsorption filter membrane materials. MF-Millipore (MF) is a hydrophilic filter membrane of mixed cellulose ester composition. Polyvinylidene fluoride (PVDF) is a polymer membrane material commonly employed in biological and analytical chemistry filtration applications. Whatman OE 66 (OE66) is a filter membrane material of cellulose acetate composition. Low adsorption filter membranes were chosen for their potential to enable electrokinetic transport minimally influenced by sample-substrate adsorption. We hypothesized that this property would enable effective zonal electrophoresis within these materials.

5.4.1 μ PAD fabrication

Wax printing is a common fabrication procedure for capillary-driven μ PADs. We elected instead to laser cut microchannels from the filter membrane substrates using a CO₂ direct write laser for two reasons: (1) cutting away unused areas of the membrane substrate reduced the total thermal mass of the device, aiding in heat dissipation; and (2) differences in wettability of the various membrane materials may lead to variability in channel dimensions and geometries patterned by wax printing.

Since laser cutting is an ablation process, the thermal breakdown properties of the substrate materials play a role in the resulting dimensions of the microchannels. Thus, we characterized the ablated dimension, termed the cut width, as a function of LED for the low adsorption filter membrane materials (Fig. 5.2). For all materials a similar trend was observed, increasing cut width with increasing LED up to a maximum above which no increase is observed with increasing LED. This trend is similar to that observed with direct laser ablation of glass microfluidic channels [21]. Large variance in cut width was observed, especially in the LED regime above maximum cut

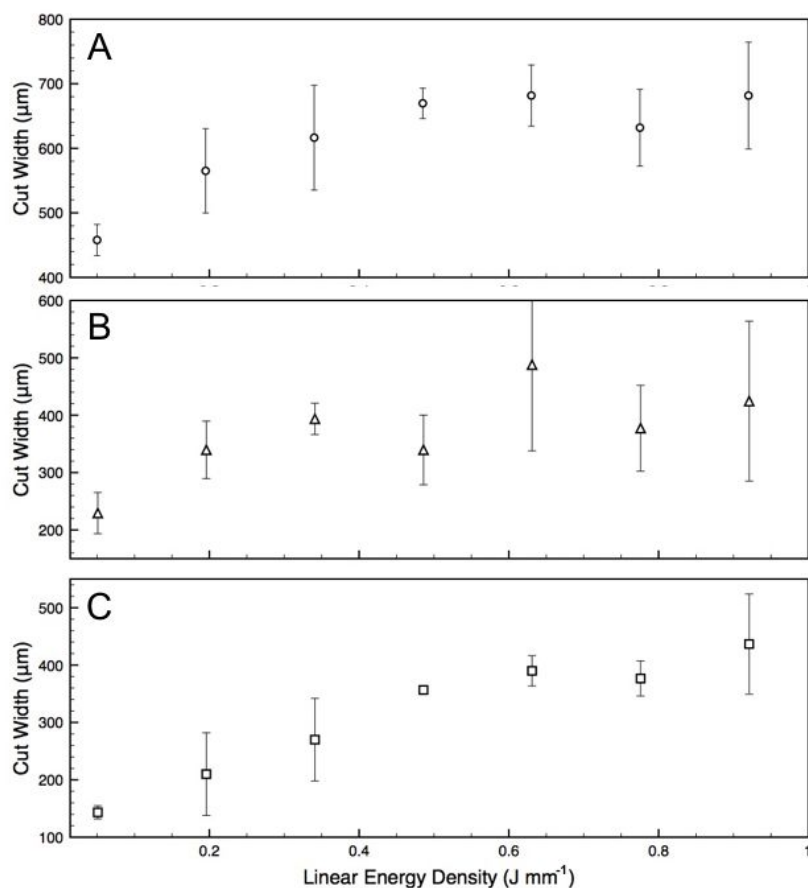


Figure 5.2: Characterization of laser ablation. Cut width as a function of linear energy density was evaluated for (A) MF; (B) PVDF; and (C) OE66. Cut width was determined via digital imaging and pixel counting. Maximum cut width was observed between 0.3 and 0.6 J mm⁻¹ for all materials, above which ablated dimension do not increase. Error bars represent ± 1 standard deviation from $n \geq 3$ replicate experiments. Linear energy density was modulated by varying laser speed, laser power, or both, with 3 unique power/ speed combinations at each value of linear energy density.

width. We hypothesize that large variances originates from the substrates being thermally labile, possibly resulting in uncontrolled combustion processes at the micron scale. This effect is most pronounced in PVDF substrates. For all materials tested here, maximum cut width was achieved at LED values between 0.3 and 0.6 J mm⁻¹. Interestingly, in all cases the maximum observed cut width was greater than the focused spot size of the CO₂ laser, which was *ca.* 125 μm. This finding suggests that radiant heat occurring secondary to that within the area of laser incidence participates in the ablation of the membrane materials.

Since cut width describes the amount of paper ablated in the fabrication process, and channel dimensions are defined by the paper left behind after ablation, cut width will play a role in the lower limit of achievable channel dimensions. A theoretical lower limit can be conceived as a function of the variance of cut width and the precision of laser positioning, however the practical lower limit was encountered before the influence of these factors became evident. In practice, channels with dimensions smaller than *ca.* 250 μm tended to curl up and protrude into the path of the advancing laser, resulting in fabrication failure. To avoid this, all further characterizations were carried out on μPAD devices with channel widths of 1 mm.

5.4.2 Joule heating characterization

Joule heating was recognized as a limiting factor in early examples of paper electrophoresis [24]. μPADs offer similar advantages for heat management to those found in other microfabricated electrophoresis platforms; specifically, reduced current due to small channel dimensions, reduced thermal mass, and increased heat dissipation due to increased surface area to volume ratios. The potential for poor heat conduction via the fibrous organic substrates, however, presents a unique challenge in μPADs. Plots of current vs. applied potential were used to determine the electric field strength at which Joule heating changes the conductivity of the system. Illustrated in Fig. 5.3, Joule heating was evaluated with the μPAD channels operated in three conditions: exposed to ambient air, within the laminated μPAD, and the laminated μPAD with the addition of active cooling by a flow of cooled nitrogen.

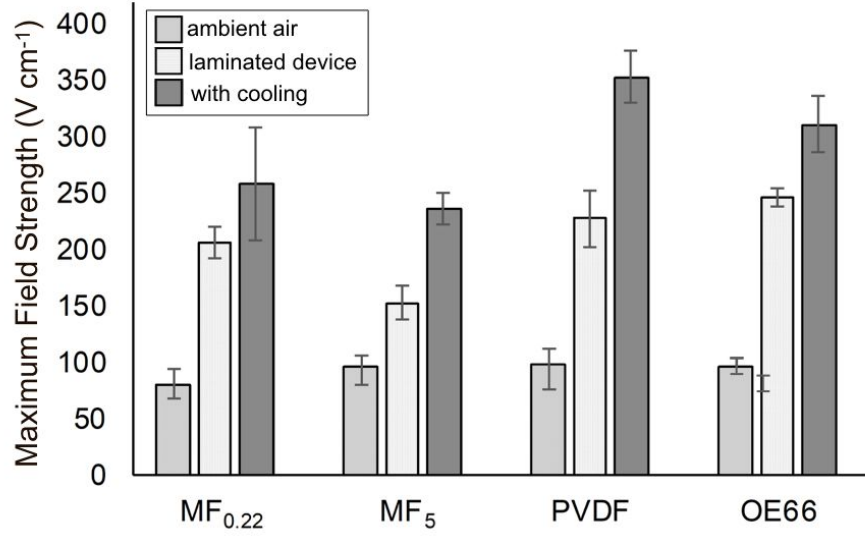


Figure 5.3: Joule heating-limited field strength vs. μ PAD substrate material. Heating-limited electric field strengths were determined via plots of current vs. applied potential. “Ambient air refers to paper channels operated in open air with no means of cooling except convective heat transfer to the ambient lab air. “Laminated device refers to device operated in the PDMS and glass lamination illustrated in Fig. 5.1, but without nitrogen cooling. “With cooling refers to devices operated exactly as shown in Fig. 5.1. MF substrates were evaluated with 0.22 and 5 μ m pore sizes (MF_{0.22} and MF₅, respectively). Within each substrate, all cooling conditions are statistically different as validated by *t*-test ($p \leq 0.05$), except laminated and cooled conditions for MF_{0.22} ($p=0.099$). Error bars represent ± 1 standard deviation from $n \geq 3$ replicate experiments.

Statistical evaluation by two way ANOVA revealed that the heating trends observed for OE66 and PVDF are statistically similar, with 95% confidence. Trends observed for MF₅ and MF_{0.22} were also statistically similar with 95% confidence; however, the OE66/ PVDF trends differed from MF₅/ MF_{0.22} trends with statistical significance at 99% confidence ($p < 1 \times 10^{-7}$). For all substrates, the limiting electric field when operated in ambient air was *ca.* 100 V cm⁻¹. In ambient air and at fields greater than 100 V cm⁻¹ all microchannels followed a similar failure mode of thermal breakdown or spontaneous ignition. Lamination of the μ PAD channels improved heat dissipation, as the glass microscope slides acted as heat sinks. To observe the relative contribution of pore size to heating and heat dissipation, MF substrates were evaluated at 0.22 μ m and 5 μ m pore diameters, both being 75% porous (MF_{0.22} and MF₅, respectively). In the laminated device without cooling, a decrease in the heat-limited electric field was observed for MF₅ as compared to MF_{0.22}. This difference may suggest that increased surface area within the porous substrate contributes to conductive heat dissipation, however further study is needed to isolate this heat transfer mechanism. With the addition of active cooling, differences in heat dissipation due to pore size were no longer evident, suggesting that the rate of heat transfer at the airglass interface had a greater effect under these conditions than the rate of buffer-to-substrate heat transfer. With laminating and active cooling, heat-limited electric field strengths in the range of 230-350 V cm⁻¹ were achievable, with PVDF and OE66 performing similarly and achieving higher limiting fields than those of MF.

In general, studies of Joule heating in low adsorption filter membrane μ PADs demonstrated that with lamination and active cooling these devices can be operated at electric field strengths between those typical of slab gel electrophoresis (<100 V cm⁻¹) [25] and those typical of capillary and microchannel electrophoresis (>500 V cm⁻¹) [26].

5.4.3 Fluorescence characterization

Electrophoretic separations often employ fluorescence detection, but in this regard μ PADs present the challenge of scatter and autoluminescence from the opaque substrate. To assess the potential for fluorescence detection, we characterized limits of detection (LOD) for fluorescence measurements

conducted within these membrane materials, illustrated in Fig. 5.3. Fluorescence in green and red emission channels were assessed using fluorescein and Nile blue, respectively. Fluorescein detection limits were 98 ± 9 nM and 41 ± 3 nM in MF and PVDF substrates, respectively. These measurements were limited by relatively high intensity of luminescence or scatter from the MF and PVDF substrate as compared with OE66, which showed a limit of detection for fluorescein of 2.7 ± 0.1 nM. We observed no indication of significant photobleaching during imaging, which involved fluorescence excitation of the sample for approximately 30 seconds per measurement. Background intensities were substantially reduced with red illumination for all substrate materials, leading to reduced Nile blue detection limits as compared to fluorescein for MF and PVDF substrates (11 ± 2 nM and 4.0 ± 0.5 nM, respectively). Although background intensity was substantially reduced with red illumination of OE66, the fluorescence sensitivity, as defined by the calibration slope, was also substantially reduced for Nile blue as compared to fluorescein. As a result, OE66 detection limits were slightly higher for Nile blue (3.5 ± 0.3 nM) as compared to fluorescein. Importantly, reduced fluorescence sensitivity appears to be a property of the dye molecule, as reduced sensitivity was observed in all substrates for Nile blue as compared to fluorescein.

As anticipated, the intensity of background luminescence presents a challenge for high sensitivity fluorescence detection in these substrate materials. Reduced background luminescence in OE66 gives an advantage for fluorescence detection in the green emission channel. Importantly, these LOD characterizations illustrate relative differences in detection limits between substrate materials, but not absolute achievable limits. Camera gain and exposure time were not optimized for maximum signal-to-noise ratios in these studies, and therefore lower absolute detection limits may be possible. Still, the trends observed here suggest OE66 is favorable for electrokinetic μ PADs employing fluorescence detection.

5.4.4 Electrophoresis and electrokinetic gating

The incorporation of zonal electrophoresis into μ PAD designs is expected to enable complex analyses that utilize fast, efficient, and high resolution separations. Depending on the degree of

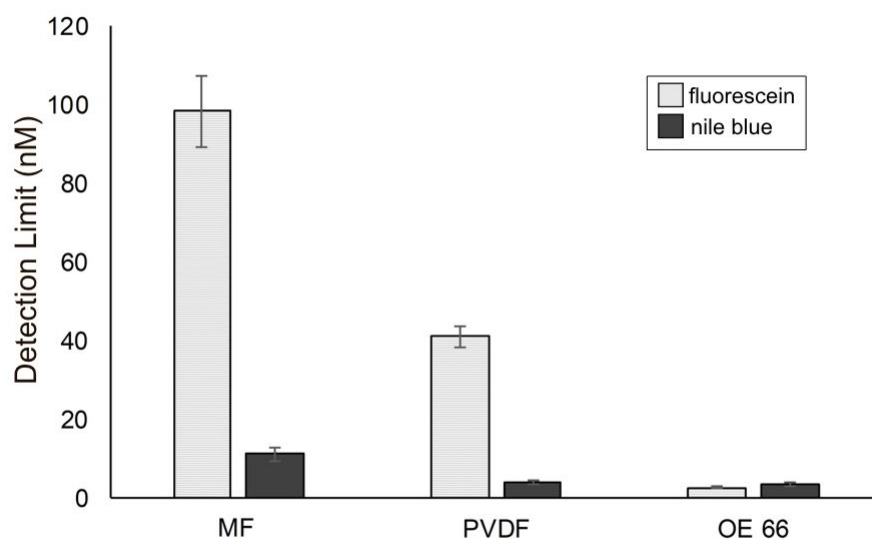


Figure 5.4: Fluorescein and Nile blue detection limits. Calibration curves were prepared using triplicate measurements in the concentration range of 0-500 nM fluorescent dye, and limit of detection was defined as $3 \times$ the standard deviation of the blank measurement divided by the slope of the calibration curve. Error bars represent ± 1 standard error of the estimate for the linear regression of the best fit calibration line.

interaction, analyte-substrate adsorption within paper channels may result in peak asymmetry, band broadening, and ultimately the loss of zonal separation. To evaluate the efficacy of zonal electrophoresis in low adsorption filter membranes, a fluorescently labeled amino acid mixture of FITC-arginine, FITC-glutamine, and FITC-glycine was separated in the μ PAD devices. Effective separation was not possible in MF substrates, which demonstrated high electroosmotic flow velocities in the direction from anode to cathode. As a result, a complex and irreproducible pattern of bands was observed migrating past the detection point in under 4 minutes (see Fig. B.2 in Appendix B). While these rates of electroosmotic flow velocity do not preclude effective zonal electrophoresis, optimization of device geometry and applied field strength would be needed to assess the feasibility of zonal separations in MF.

Fig. 5.5A illustrates representative amino acid separations performed in PVDF substrates using BGE of 10 mM TRIS (solid lines) or 10 mM sodium borate (dashed lines). Low rates of electroosmotic flow velocity required these separations to be conducted in the direction from cathode to anode. While zonal separation is clearly observed in the PVDF substrate using TRIS BGE, resolution is lost with sodium borate BGE. The mechanism by which resolution is lost in borate BGE is unclear, however we hypothesize that a low velocity of electroosmotic flow is present in opposition to the direction of electrophoresis. In this case, the higher ionic strength of sodium borate as compared with TRIS may reduce double layer thickness and zeta potential, as observed in other model systems [27], thereby reducing EOF velocity. The expected result would be overall faster electromigration, as observed, and potentially reduced resolution. More work is needed to investigate this mechanism further, however this study demonstrates the importance of buffer selection for separations and other electrokinetic sample manipulations carried out within the high surface area environment of paper substrates.

Representative amino acid separations in OE66 μ PADs are shown in Fig 5.5B. Similar to PVDF, low rates of EOF required separations to be performed in the direction from cathode to anode. Unlike PVDF, resolution is observed in both TRIS and sodium borate BGE, albeit with reduced resolution in borate. OE66 substrates are demonstrated to be less sensitive to the mechanisms that lead to degraded resolution in PVDF μ PADs utilizing borate BGE.

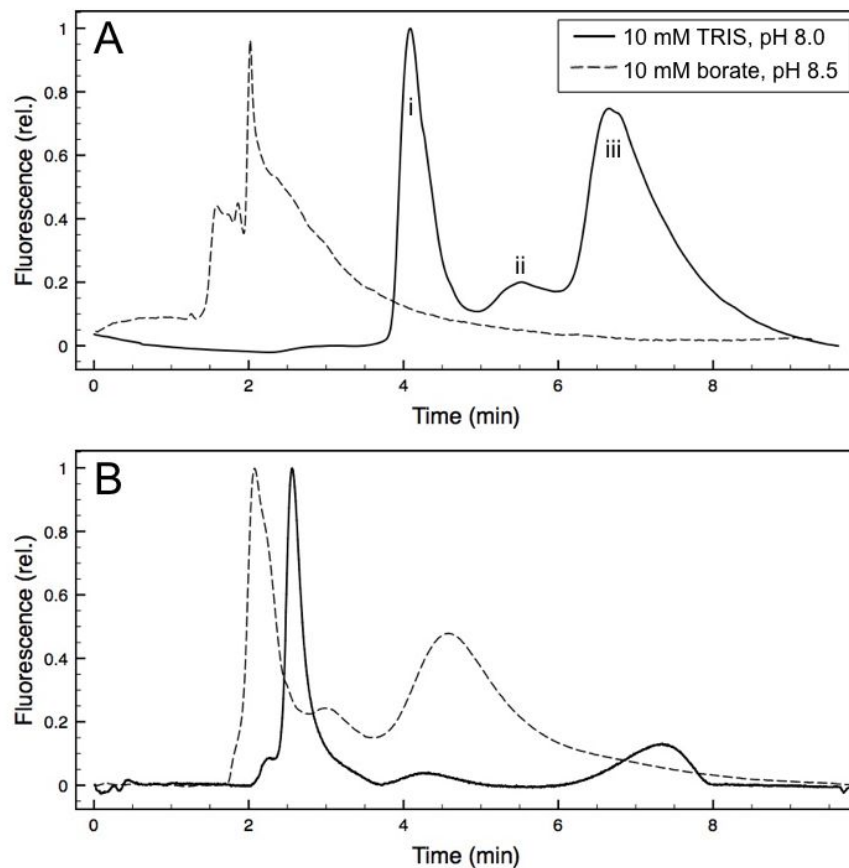


Figure 5.5: Representative electropherograms in PVDF and OE66 μ PADs. (A) Electropherograms of arginine (i), glutamine (ii), and glycine (iii) in PVDF using TRIS (solid) or borate (dashed) BGE. (B) The same mixture components as part A, separated in an OE66 μ PAD using TRIS (solid) or borate (dashed) BGE. Fluorescence was normalized to relative units for each electropherogram.

Migration time reproducibility was substantially improved in PVDF as compared to OE66 substrates. Migration time RSDs were 12% and 19% for arginine and glutamine (leading and trailing peaks, respectively) in OE66 with TRIS BGE. In PVDF with TRIS BGE, RSDs for arginine and glutamine migration times were 3.8% and 3.4%, respectively.

Peak asymmetry calculations offer insight to the degree of analyte-substrate interactions that negatively impact zonal electrophoresis separations in these substrates. Peak asymmetry values for arginine and glycine in PVDF substrates with TRIS BGE were 2.0 ± 0.3 and 1.9 ± 0.5 , respectively. In OE66 substrates with TRIS BGE, these values were 1.7 ± 0.3 for arginine, and 0.6 ± 0.5 for glycine which showed substantial peak fronting. Glycine fronting was reduced in borate BGE giving peak asymmetry values of 1.4 ± 0.1 . While calculated asymmetry values for arginine in OE66 substrates increased with borate BGE to 2.3 ± 0.4 , it is unclear whether this is the result of analyte-substrate adsorption or if it emerges from reduced resolution between the principle arginine peak and a trailing shoulder observed in both BGE conditions. Overall, peak asymmetry in these experiments was somewhat higher than one would expect for ideal zonal electrophoretic separations in an open tubular format. This is to be expected, as the porous paper substrate materials contain increased surface area as compared with open tubular microfluidic devices. Still, peak asymmetry does not prevent electrophoretic resolution of the principle amino acid peaks utilized in this study.

Although high EOF velocities in MF substrates were detrimental to electrophoretic separation, EOF is useful for achieving complex fluid transport schemes in microfluidic devices [28]. Electrokinetic gated injection is often utilized to restrict or initiate flow of a reagent in microfluidic devices. Fig. 5.6 illustrates that EOF in MF substrates is sufficient to achieve electrokinetic gating under applied electric fields of 200 V cm^{-1} . Importantly, these gating manipulations required several seconds to transition between the gated (Fig. 5.6A) and floated (Fig. 5.6B) configurations, which is significantly longer than typical sub-second injection times achievable in glass microfluidic devices by this gating mechanism. Upon reapplication of the appropriate potential scheme, gating was re-established and a sample injection plug was observed migrating along the right hand channel (not shown), completing the conventional electrokinetic gated

injection procedure. Although electrokinetic sample manipulation occurred on slower time scales to those typical for glass microfluidic devices, EOF in MF is nonetheless sufficient to facilitate electrokinetic flow gating in μ PADs even under modest electric field strengths limited by Joule heating.

Direct measurements of EOF velocity are clearly needed to understand the mechanisms observed in these studies; however, such measurements are not trivial within the high surface area environment of porous substrates. While measuring migration time of a neutral dye is a straightforward approach for determining EOF velocity in capillaries [29, 30], in paper substrates the potential influence of dye-substrate partitioning is not readily isolated from EOF velocity, hindering such measurements. Attempts were made to determine EOF velocity by the buffer dilution method [31], but these studies were not conclusive due to irregular and irreproducible current drift observed during these experiments. As a result, direct observation of EOF velocities was not achieved in this work, warranting further study.

5.5 Conclusion

To increase the versatility of sample handling and assay design in μ PADs, we evaluated the electrokinetic and electrophoretic performance of μ PADs fabricated from low adsorption filter membrane substrates. The Joule heating-limited electric fields achieved in these μ PADs were lower than those typical for open-tubular glass microfluidic devices, however zonal electrophoresis and electrokinetic flow gating were possible even at modest electric field strengths. Low adsorption membrane materials were considered for their potential to enable zonal electrophoretic separations minimally perturbed by the effects of analyte-substrate adsorption. Peak asymmetry observed in these studies suggest that detrimental analyte-substrate interactions do occur in these substrates, but they are not sufficient to prevent resolution of the model amino acid mixture employed in these studies. In comparing the selection of low adsorption filter membrane materials studied here, OE66 offers clear advantages for fluorescence detection and electrophoretic separation. MF demonstrates a substantial degree of electroosmotic flow, which may be useful in designing electrokinetic sample transport schemes in μ PADs, but will likely require design optimization to

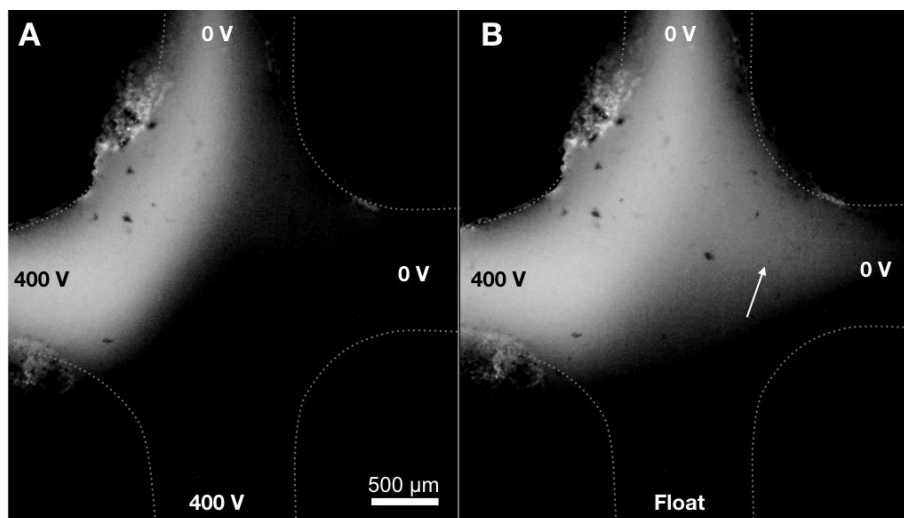


Figure 5.6: Electrokinetic gating in an MF μ PAD. Electrokinetic gating is demonstrated in a μ PAD with MF substrate. (A) In the gated configuration, under applied fields of *ca.* 200 V cm^{-1} , electroosmotic flow of buffer (from bottom) is sufficient to shunt the flow of fluorescent sample (from left) towards the top channel. (B) In the floated configuration, potential is withheld (i.e. float potential) at the buffer inlet to allow the buildup of sample flow into the channel at the right, indicated by the arrow. Float potential was held for 4 seconds to achieve the sample flow shown here.

achieve zonal electrophoretic separations.

While the present work is not exhaustive in surveying all available low adsorption filter membrane materials, these studies lend design insights toward the development of new μ PAD technologies that utilize electrokinetic sample manipulations and electrophoretic separations to achieve increased complexity of analysis from low cost microfluidic instrumentation.

5.6 References

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Chapter 6

Conclusions and Future Work

New tools are necessary for interrogating biological systems with high temporal resolution. A focus of this work has been development of micro-systems that use zonal electrophoresis and fluorescence detection for sensitively and selectively probing proteins, peptides or oligonucleotides. The analytical challenges addressed in this dissertation include detection sensitivity, electrophoretic peak resolution, and ability to work with small sample volumes. Capillary electrophoresis (CE) offers advantages of reduced sample volumes, rapid analysis times, and increased automation capabilities as compared to many other separations technologies. These advantages are particularly desirable for advancing towards real-time analysis of neuropeptide hormone secretion profiles from on-line cell cultures.

6.1 3D printed LED-induced fluorescence detection

Chapter 2 presents a 3D-printed LED-induced fluorescence detector that can be integrated into commercial capillary instrumentation. The detector was found to have a detection limit of 613 ± 13 pM for fluorescein disodium salt. The small size of the printed detector (ca. 6 cm³) makes it small enough to fit inside the capillary cassette of an Agilent CE instrument for integrating fluorescence detection with CE. Work discussed in Chapter 2 heavily emphasized the optimization of the excitation axis, but future work will include optimization of fluorescence collection—integrating mirrors, lenses or more expensive optics for improving sensitivity and detection limits.

6.2 CE-TDA-immunoassays

The fluorescence detector presented in Chapter 2 was also used for dual-detection TDA and CE-TDA. The small size enabled facile integration of two devices inside of the capillary cassette. It was

demonstrated that CE-TDA could enable calibration free peak identification between two proteins of different sizes. Future work will include the development of a CE-TDA immunoassay technique for multiplexed profiling of neuropeptides from complex biological samples. All peptides in a sample will be fluorescently labeled and separated by CE. The separated bands will then analyzed via online post-column TDA immunoassay, which specifically identifies components via a shift in their hydrodynamic radius upon antibody binding. This technique offers the advantages of CE, with the detection sensitivity of fluorescence detection, and the specificity of immunoassays.

6.3 Sine-injection Fourier-transform enhanced CE

Ch. 4 of this dissertation presented the foundation for a new multiplexing injection method for CE term Sine-injection Fourier-transform (SIFT). Simulations demonstrated its utility for enhancing analyte resolution and demonstrated ability to enhance S/N when combine with a multi-detector array. Future work includes an investigation into the relationship between apparent mobility of analytes and the optimal detector placement for a single-point detection protocol. This will be important for ensuring the injection frequency does not overwhelm the characteristic analyte frequencies of the electropherogram. However, as shown in Fig. 4.2-F, moving the detection spot too far away from the inlet will result in a decline in S/N, so optimal placement is crucial. In addition to further simulations, once these conditions have been determined, the proof-of-concept for SIFT must be demonstrated in experiment.

Once SIFT-CE is performed in practice, it will be desirable to integrate the multi-detector array discussed in Ch. 3. We propose to achieve this by integrating a fused silica capillary with the microchip

There is a possibility that EOF, as discussed in Ch.4, could negatively impact the ability to resolve the analyte frequencies from the injection frequency in the Fourier-transform of the electropherogram. In this case, there will be a need to eliminate EOF in the separation channel. However, since EOF is critical to performing the electrokinetic gated injections necessary for delivering continuous sine-wave injection patterns, we will want to eliminate EOF in the separation channel while still using an unmodified cross-t microchip for injections.

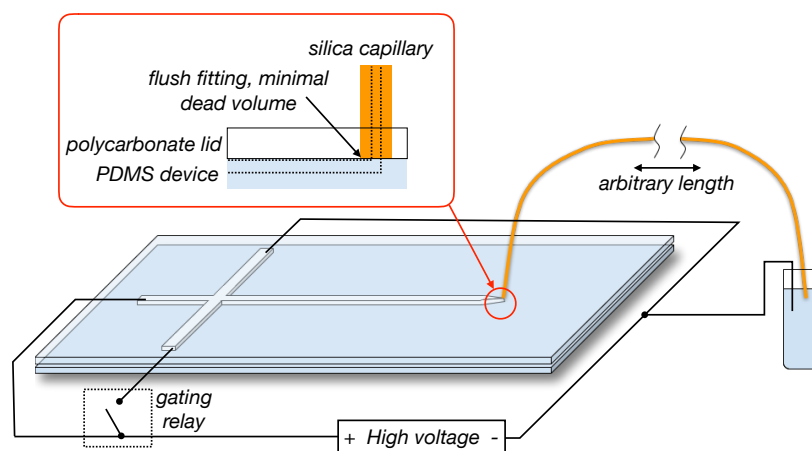


Figure 6.1: The microchip will interface with a fused-silica capillary which could have a modified surface for reduced EOF. The microchip will be used to modulate a sine wave injection profile and the capillary will contain a multi-detector array for SCOFT detection.

Appendices

A Additional Materials for Chapter 2

The following figures and table are meant to accompany the article in chapter 2, originally published in the Electronic Supplementary Information by Laura D. Casto, Kevin B. Do, and Christopher A. Baker. Minor modifications were made.

“A miniature 3D printed LED-induced fluorescence detector system for capillary electrophoresis and two-point detection Taylor dispersion analysis” *Submitted*, 2018

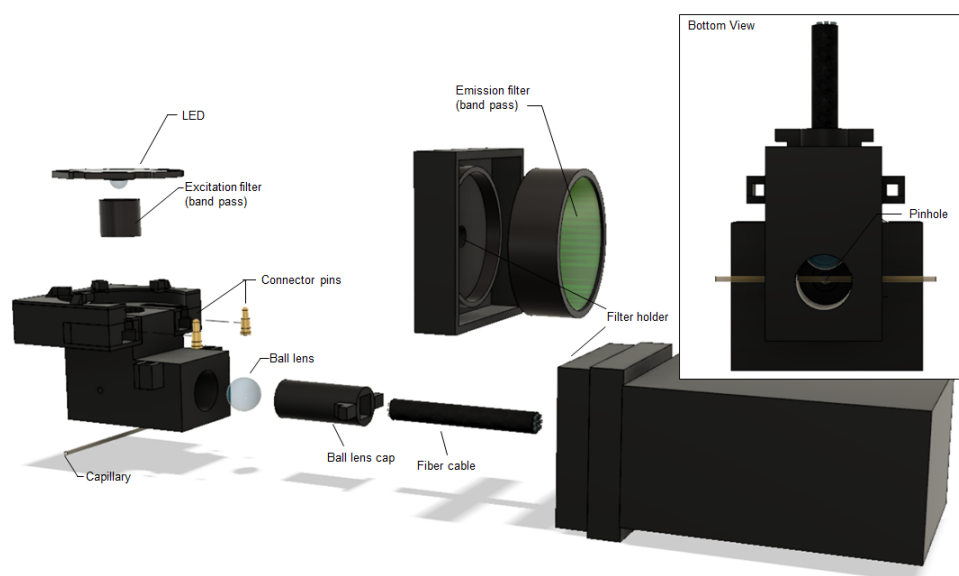


Figure A.1: Table of Contents Figure

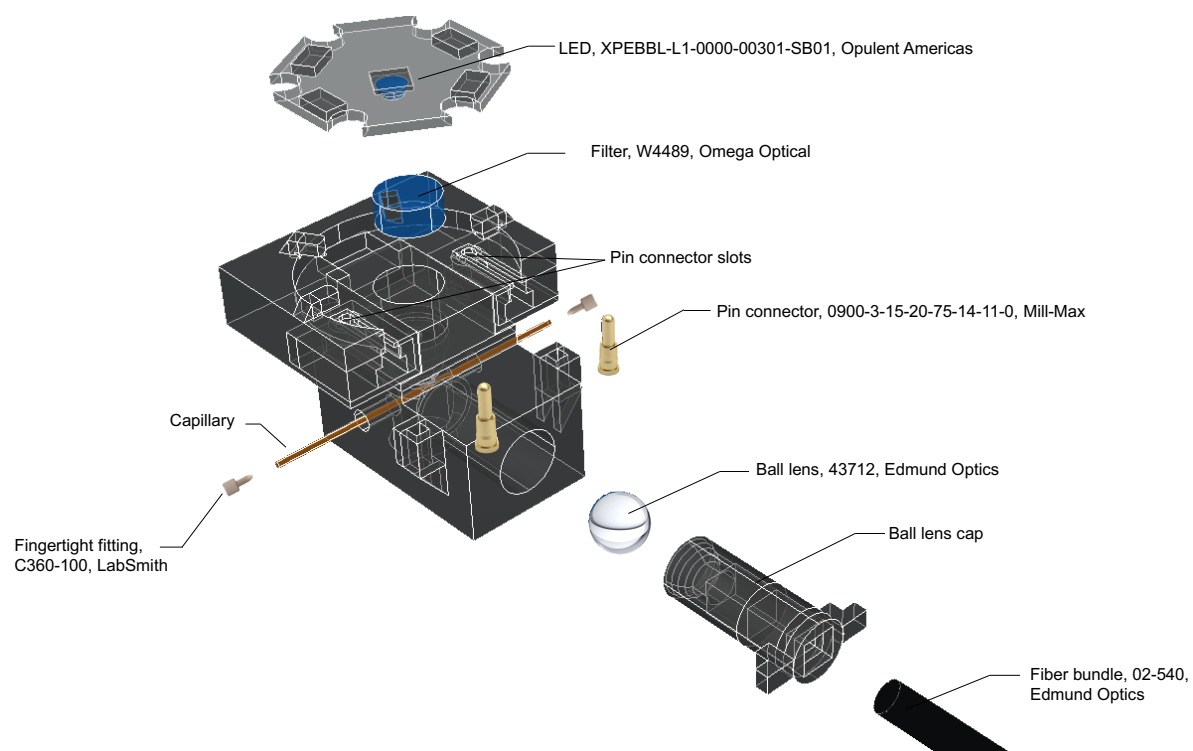


Figure A.2: Assembly diagram for LED-IF detector

Table A.1: TDA results from CE-TDA FITC-BSA reaction

Peak Number	Reaction Time (min)	Diffusion coefficient ($\mu\text{m}^2/\text{s}$)	Stokes Radius (nm) Radius (nm)	Peak ID
1	2	55.57	4.42	BSA
2	2	453.4	0.54	FITC
1	12	61.69	3.98	BSA
2	12	419.8	0.58	FITC
1	20	58.07	4.23	FITC-BSA
2	20	—	—	—
1	100	60.22	4.08	FITC-BSA
2	100	—	—	—

B Additional Materials for Chapter 5

The following figures are meant to accompany the article in chapter 4, originally published in the Electronic Supplementary Information by Laura D. Casto, Jennifer A. Schuster, Claire D. Neice, and Christopher A. Baker.

“Characterization of low adsorption filter membranes for electrophoresis and electrokinetic sample manipulations in microfluidic paper-based analytical devices,” *Anal. Methods*, 2018, **10**, 3616-3623.

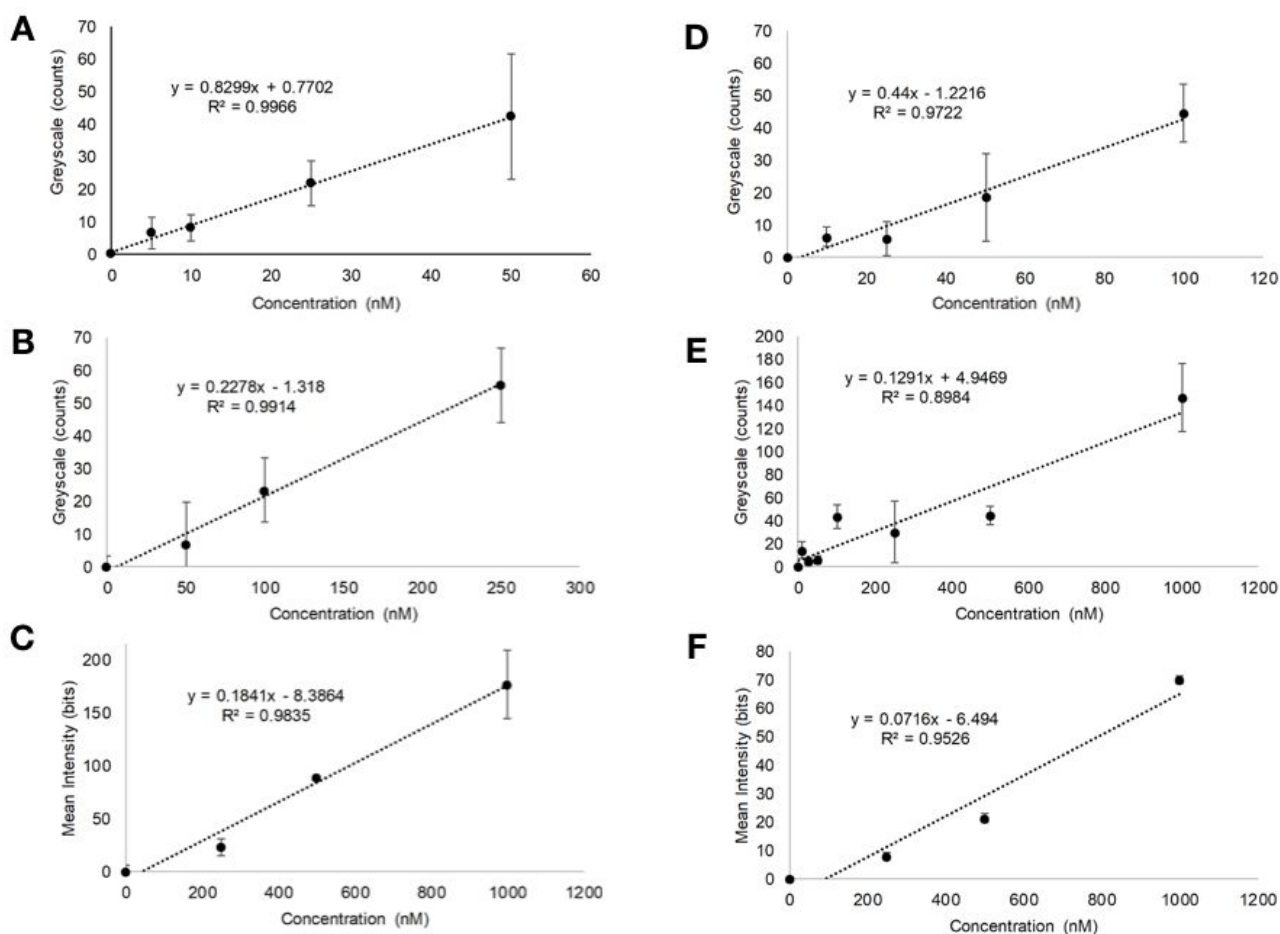


Figure B.1: Fluorescence calibration plots. Blank-subtracted fluorescence calibration plots utilized for detection limit calculations. A. Fluorescein on OE66 B. Fluorescein on PVDF C. Fluorescein on MF D. Nile blue on OE66 E. Nile blue on PVDF F. Nile blue on MF

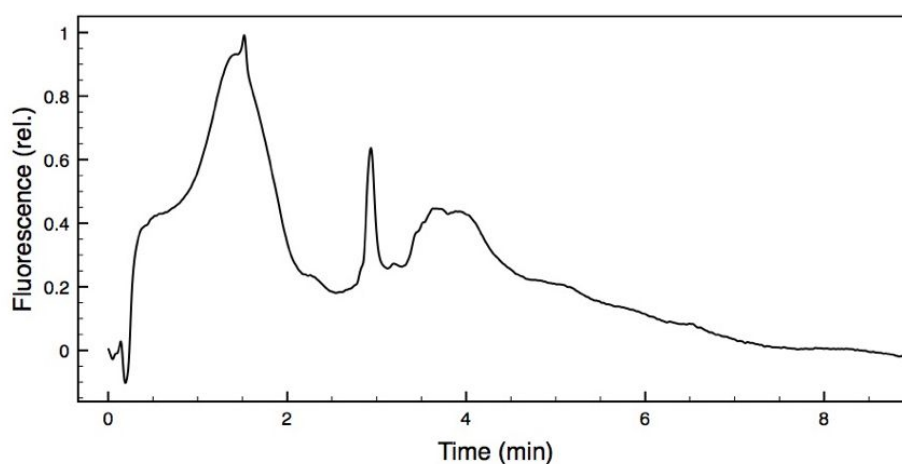


Figure B.2: Representative electropherograms in MF μ PADs. Electropherograms in both TRIS and borate BGEs were irreproducible and complex, but the representative trace here demonstrates their common appearance: a complex peak profile migrating entirely before 4 minutes. We hypothesize this results from relatively high rates of electroosmotic flow which prevents electrophoretic resolution on these time scales. Further device optimization may improve separation performance and reproducibility.

Vita

Laura Dawn Casto grew up in Putnam County West Virginia and graduated from Hurricane High School in 2009. She earned her Bachelor of Science in Chemistry and Mathematics from West Virginia Wesleyan College in Buckhannon, West Virginia. During her undergraduate career, she completed two consecutive Summer Undergraduate Research Experiences at West Virginia University with Dr. Lisa Holland working on aptamer-based assays and characterizing a thermally responsive smart gel as a separation medium for microchip electrophoresis which inspired her to pursue a career in research and attend a graduate program for training in Analytical Chemistry. In August 2013 she joined the Department of Chemistry at the University of Tennessee and joined the research group of Dr. Christopher A. Baker in August of 2015. In the Baker group, her research was motivated by the development of neuropeptide assays with high temporal and spatial resolution using capillary and microchip electrophoresis. This work has resulted in peer-reviewed publications, numerous presentations at national, regional, and local conferences, several poster awards including the *Margaret “Tina” Riedinger Societal Impact Award* at the 2nd Annual Women in STEM Research Symposium at UTK and *Best Poster* at the 2018 Department of Chemistry Board of Visitors Meeting. She was also the recipient of the Eastman Fellowship in Analytical Chemistry for the Summer of 2018. Throughout her time in the UTK Chemistry Department, she was consistently involved with the Association of Chemistry Graduate Students, serving as the President, Vice President, and Career Development Chair, as well as serving on the department recruitment and seminar committees as the graduate student representative. Laura will receive her Ph.D. in Analytical Chemistry in May 2019.