DEVELOPMENT AND IMPROVEMENT OF CAPILLARY ELECTROPHORESIS METHODS AND INSTRUMENTATION

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In this work, I present the development and improvement of capillary electrophoresis (CE) methods and instrumentation. Included are advancements made for two-dimensional CE separations, the development and characterization of an online SERS detector for CE, an automated fraction collector for CE separations, a current sinking power supply for electrospray ionization mass spectrometry that is used with a CE instrument, and the development of an instrument and method for high through-put aptamer generation termed 2D-CE-SELEX.
This is for my family, especially my wife Rebecca. Their support and encouragement was constant and unwavering.
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CHAPTER 1:
INTRODUCTION

1.1 Capillary electrophoresis

Electrophoresis was first developed as a separation technique by the Swedish chemist Arne Tiselius who received the Nobel Prize in Chemistry for this development. Electrophoresis is a separation method that employs a direct current electric field to separate sample components based on the differential migration of charged species present in the sample. Electrophoretic separations are most often performed in two different formats. The first format is called gel, or slab, electrophoresis. Gel electrophoretic separations are performed on a thin, porous gel that contains an aqueous buffer. Samples are injected into wells that are formed in one end of the gel when it is cast. Once sample is introduced into a well, the electric field is applied and the sample components are separated by differential migration through the gel matrix. Once the separation is complete, the electric field is turned off and the separated sample components are visualized by staining.

The second format for performing electrophoretic separations is called capillary electrophoresis. As the name implies, this second format uses capillaries rather than gels to perform an electrophoretic separation. These capillaries are made of fused silica,
have narrow inner diameters (20 – 200 µm), and have outer diameters that range in size but tend to have dimensions similar to the diameter of a human hair. The capillaries have a high surface-area-to-volume ratio that allows rapid heat dissipation and enables the use of high electric fields. Samples are injected into one end of the capillary, an electric field is applied to the capillary, and samples are detected either on-column or postcolumn as they migrate through or out of the capillary.

1.1.1 Theory of capillary electrophoresis

1.1.1.1 Electrophoresis and resolution

The basis for electrophoretic separations is that an ion in an electric field will have a migration velocity, \( \nu \) (cm/s) that is equal to the product of the field strength, \( E \) (V/cm), and the electrophoretic mobility \( \mu_e \) (cm\(^2\) V\(^{-1}\) s\(^{-1}\)).

\[
\nu = \mu_e E \quad \text{Eq. 1-1}
\]

The ion’s size-to-charge ratio will affect its electrophoretic mobility and its migration time. The electrophoretic mobility is directly proportional to the charge that is present on an analyte and is inversely proportional to frictional drag forces that result from the ion moving through the separation medium (i.e. background electrolyte). As shown in Eq. 1-1, the migration rate of an ion is directly proportional to the field strength to which the ion is subjected. The field strength in turn is directly proportional to the applied voltage, \( V \), and inversely proportional to the length of the capillary, \( L \). Therefore
\[ v = \mu_e \times \frac{V}{L} \]  

Eq. 1-2 indicates that the application of high voltages to the separation capillary would be beneficial in the pursuit of rapid ionic migration, which would result in a fast separation.

While fast separations are useful, a separation that does not resolve two analytes of interest will not be as useful as a slower separation that achieves the necessary resolution. Resolution in separation techniques can be calculated for two peaks and is discussed below. Separation efficiencies are compared based on the number of theoretical plates a separation provides. The term “theoretical plates” comes from distillation separations where the number of plates present in the distillation apparatus dictated the efficiency of the distillation separation; the greater the number of plates, the greater the separation efficiency. Each plate in a distillation apparatus offers the opportunity for distillate to condense onto the plate and be distilled anew from the plate. As this process progresses, the distillate decreases in complexity, which results in the separation between sample components. Subsequent separation techniques have adopted the term theoretical plates to quantify the resolving power achievable in a separation. Separation efficiency in capillary electrophoresis is given by Eq. 1-3, where \( D \) is the diffusion coefficient of the solute (cm\(^2\)/s).\(^7\)

\[ N = \frac{\mu_e V}{2D} \]  

Eq. 1-3

As the number of theoretical plates increases, so does the resolution of the separation. Therefore, according to Eq. 1-3, applying high voltages for separations in capillary electrophoresis would be beneficial in achieving faster and more efficient separations.
electrophoresis will improve the resolution of the separation. It is important to note that in electrophoresis the number of theoretical plates, $N$, does not increase with the length of the capillary.\(^7\) This differs from chromatography, where increasing the length of the column will increase the number of theoretical plates for the separation.

1.1.1.2 Electroosmotic flow

Capillary electrophoresis has a force, other than electrophoresis, working when an electric field is applied to the capillary. That force is called electroosmotic flow. On a bare, or untreated, fused-silica, electroosmotic flow will “pull” the background electrolyte toward the cathode. Electroosmotic flow arises from the electric double layer that develops at the silica-solution interface. At pH values greater than 3, the wall of a silica capillary is negatively charged as a result of the ionization of the silanol groups (Si–OH).\(^1\) Cations in solution are attracted to the negatively charged capillary wall and form an electrical double layer. The cations in the double layer are attracted to the cathode, or negative electrode, and due to their solvation within the buffer, they pull or drag the bulk solution toward the cathode (Figure 1-1).\(^1\)

The flow profile that results from electroosmotic flow is essentially flat; whereas the flow profile of a liquid under pressure, as in liquid chromatography, has a flow profile that is laminar or parabolic (Figure 1-2). Since the flow profile in capillary electrophoresis is essentially flat, electroosmotic flow does not contribute to band broadening the way that laminar flow does in liquid chromatography.\(^1,\,7\)
In general, the rate of electroosmotic flow is greater than the rate of electrophoresis. For this reason, analytes will tend to be carried with the electroosmotic flow while also being separated by electrophoresis (for charged analytes). Neutral analytes in solution will also be carried by electroosmotic flow but they will not be separated by electrophoresis. Separation of neutral analytes in solution can be achieved with buffer additives such as cyclodextrins or surfactants.

The velocity of electroosmotic flow (EOF) is given by an equation similar to Eq. 1-1.

\[ v = \mu_{\text{EOF}} E \]  

Eq. 1-4
The velocity of an ion when taking into account the forces of electrophoresis and electroosmotic flow is given by Eq. 1-5.

\[ v = (\mu_{EOF} + \mu_e)E \]  

Eq. 1-5

The migration order of analytes in solution is straightforward once the forces acting on the ions are understood. Nonetheless, it is worthwhile to discuss the migration order as it pertains to the most common set-up in capillary electrophoresis. For example, when capillary electrophoresis is performed in positive mode the sample or analytes are introduced at the anode, or positive electrode. Sample introduction can be done by applying a pressure or applying a voltage to inject sample into the capillary.
Once sample has been introduced into the capillary a separation voltage is applied. Assuming the background electrolyte has a pH greater than 3, the capillary wall will have deprotonated silanol groups and the electroosmotic flow will carry the bulk solution towards the cathode, or negative electrode, where the detector is located. Detection can be done either on-column or postcolumn and will be addressed in Section 1.2. Figure 1-3 depicts the migration order that would result from separation conditions as discussed in this example. Any changes to the conditions in this example could impact the migration order. When predicting migration order it is important to take into account the size (hydrodynamic radius) and charge of the analyte under the separation conditions. The separation mechanism in free solution electrophoresis (capillary zone electrophoresis) is based on the size-to-charge ratio of the analyte. Figure 1-3(a) shows the migration order of sample components if the size of each analyte was equal and the charge on the analyte was the only difference. Similarly, Figure 1-3(b) shows the migration order of sample components if charge on each analyte was equal and the size of the analytes differed.

Generally, under electrophoresis, analytes with the smallest size-to-charge ratio will migrate the fastest toward the electrode with the opposite charge to what the analyte carries. Similarly, analytes with the largest size-to-charge ratio will migrate the slowest toward the electrode with the opposite charge to what the analyte carries under electrophoresis. Then, when taking into account electroosmotic flow, the analytes will be moving with or against the flow of the bulk solution, which will further contribute to the separation of the analytes. Neutral analytes will migrate together with only
Figure 1-3: Representation of the velocities of solutes under electrophoretic and electroosmotic influence. (a) is an example of migration order if solute size (hydrodynamic radius) remains constant and charge is varied. (b) is an example of migration order if solute size is varied and charge is constant. The magnitude of velocity of a solute corresponds to the length of the arrow next to the solute. The direction of motion is indicated by the direction of the arrow. The total migration velocity is shown in the lower panel of (a) and (b) for the respective examples. The positive electrode is to the left and the negative electrode is to the right. Adapted from Skoog et al.1
electroosmotic flow contributing to their migration. One can envision a situation where an analyte is very small, carries a large charge, and would oppose electroosmotic flow. In such a situation it is possible that the electrophoretic driving force for that analyte would overcome the bulk solution flow driven by electroosmotic flow and thus the analyte would never reach the detector.

As an example of a change to the separation conditions, if an analyte’s electrophoretic mobility overcomes the electroosmotic flow such that the analyte does not reach the detector, then switching from a positive polarity to a negative polarity configuration could result in detection of the analyte. This change would reverse the direction of electroosmotic flow and would result in the electrophoretic driving force of the analyte opposing the electroosmotic flow. Alternatively, it is possible to reverse the direction of electroosmotic flow by adding cationic surfactants to the background electrolyte that adsorb to the capillary wall and make the wall positively charged. Cationic surfactants are often used to speed the separation of anions.

The time it takes for a solute or analyte to migrate from the point of introduction to the detector is called the migration time, $t_m$.

$$t_m = \frac{l}{(\mu_e + \mu_{EOF})E} = \frac{lL}{(\mu_e + \mu_{EOF})V} \quad \text{Eq. 1-6}$$

In Eq. 1-6, $l$ is the length to the detector and $L$ is the total length of the capillary used in the separation. Most capillary electrophoresis set-ups use on-column detection, which
makes \( l \) less than \( L \). However, some set-ups detect analytes after they have exited the capillary and in those situations \( l \) is greater than \( L \).

As expressed in Eq. 1-6, the migration time depends on both electrophoresis and electroosmotic flow. In Eq. 1-3, an expression for calculating the number of theoretical plates was introduced. Eq. 1-7 expresses the number of theoretical plates produced by a separation in terms of the migration time, \( t_m \), and the peak width, \( W \), measured at the base of the peak.

\[
N = 16 \left( \frac{t_m}{W} \right)^2 \quad \text{Eq. 1-7}
\]

Eq. 1-7 can also be expressed in terms of the peak width at half height, \( W_{1/2} \), and is shown in Eq. 1-8 below. For a derivation of these equations see Skoog’s *Principles of Instrumental Analysis, 6th Edition*.\(^1\)

\[
N = 5.54 \left( \frac{t_m}{W_{1/2}} \right)^2 \quad \text{Eq. 1-8}
\]

Electroosmotic flow can be a powerful force in generating rapid separations with a large number of theoretical plates. However, electroosmosis is not always desirable in separations. Capillary wall modifications can minimize, eliminate, or reverse electroosmotic flow. These modifications will not be discussed in any depth here and the reader is directed to the literature for discussions on capillary wall modifications.\(^8 \text{–} 14\)

Another metric of the quality of a separation is the resolving power, or resolution, that is achieved during the separation. Resolution is a quantitative measure of the performance of the separation. Eq. 1-9 and Figure 1-4 show how to calculate resolution between two adjacent peaks in a separation. The resolution is defined as the
distance between the centers of two zones, \((X_2 - X_1)\), divided by the average width of each zone, \(\frac{1}{2}(W_1 + W_2)\). When the resolution is equal to 1.5 the peaks are said to be base line resolved.\(^{13}\)

![Diagram](image)

Figure 1-4: Schematic showing the parameters used to calculate the resolution of a separation using Eq. 1-9.\(^{15}\)

\[
Resolution = \frac{X_2 - X_1}{\frac{1}{2}(W_1 + W_2)}
\]

Eq. 1-9

1.1.2 Capillary electrophoresis separation modes

When considering a particular separation technique (e.g. chromatography, electrophoresis, etc.) it is important to consider the differences in physical properties
between the analytes that you wish to separate. Regardless of the separation technique utilized there will be parameters that can be adjusted to optimize the separation. In this section, the different modes of capillary electrophoresis will be discussed. In capillary electrophoresis some of the parameters that can be varied include capillary length, capillary inner diameter, applied electric field, background electrolyte composition, and chemical modifications to the capillary wall.

1.1.2.1 Capillary zone electrophoresis (CZE)

Capillary zone electrophoresis (CZE), also known as free-solution electrophoresis, is the simplest mode of capillary electrophoresis. In CZE, a homogenous background electrolyte fills the separation capillary. Analytes are separated in an applied electric field based on their size-to-charge ratio. The examples discussed in Figure 1-3 are illustrative of the separation mechanisms at work in CZE. Neutral analytes will not be separated from one another as they travel with the EOF and are not affected by electrophoresis. Therefore, CZE is used for the separation of charged analytes. By maximizing the differences in velocities of analytes and minimizing zone dispersion, or band broadening, the resolution of the separation can be maximized.  

1.1.2.2 Micellar electrokinetic capillary chromatography (MECC)

In micellar electrokinetic capillary chromatography (MECC), a surfactant is used that has a hydrocarbon tail and a polar head group. When dissolved in solution above a concentration called the critical micelle concentration (CMC), the surfactant will
assemble into micelles such that the hydrocarbon tails face each other and the polar head groups face the solvent (water). CMCs vary based on the chemical make-up of the surfactant. The micelles resemble spherical aggregates of ions. Micelles are capable of incorporating or solubilizing nonpolar molecules by partitioning into the hydrocarbon core of the micelle. This partitioning constitutes the presence of a second phase in the separation. The first phase is the polar background electrolyte, which can be called a mobile phase. The second phase is the nonpolar pseudostationary phase of the micelle’s inner hydrocarbon core. Most surfactants used in MECC have a large negative charge and will oppose the electroosmotic flow of a positive mode separation (sample introduced at the positive electrode). In this system, the polar background electrolyte flows towards the detector while the nonpolar micelles migrate away from the detector. This opposition of forces acting on the nonpolar neutral analytes will produce a separation of the previously unresolved sample components.

1.1.2.3 Capillary isoelectric focusing (cIEF)

Capillary isoelectric focusing, cIEF, is employed when the sample contains amphiprotic molecules that are capable of both donating and accepting a proton. For example, amino acids and proteins contain a weak carboxylic acid group and a weakly basic amine group. A separation that employs cIEF will separate molecules in a sample based on their pI, which is the pH at which the molecule carries no charge and is neutral. cIEF is performed by filling the separation capillary with carrier ampholytes. Ampholytes are
amphoteric compounds that often contain carboxylic and amino groups. A mixture of ampholytes is typically purchased from a commercial source for use in cIEF. After introduction into the capillary, the ampholytes generate a pH gradient across the length of the capillary. When the amphiprotic sample molecules are introduced to the ampholyte filled capillary and a separation voltage is applied, analytes migrate to the point along the length of the capillary that corresponds to their pl. Once a molecule reaches its pl, migration ceases and the molecule is “focused”. Once all of the molecules have been focused to their respective pls, the next step is to mobilize the focused bands. Since migration will have ceased for all of the molecules present in the capillary, they must be forced to pass the detector by a mobilization step. Mobilization of the focused samples can be done by either applying a pressure to the capillary such that the samples are pushed past the detector or by changing the solution in one of the electrode compartments. During the focusing step, the pH gradient has become stable and by changing the solution in one of the electrode compartments that pH gradient is disrupted and migration will begin again.

Capillary coatings are often used in cIEF for two reasons. First, the capillary coating will serve to decrease or eliminate EOF, which allows for longer focusing times and ultimately higher resolution of neighboring pl bands. Second, the molecules separated using cIEF are often proteins that can stick to the untreated, bare silica wall of the separation capillary. The capillary coating will minimize the likelihood of sample molecules adsorbing to the capillary wall and causing band broadening.
In general, cIEF has three stages of the separation. First, the pH gradient is established within the capillary using carrier ampholytes. Second, the charged analytes are focused to their pI within the capillary at which point their migration ceases due to the loss of their charge. Finally, after the analytes have been focused, the separated analytes are mobilized for detection at one end of the capillary.

1.1.2.4 Capillary sieving electrophoresis (CSE)

Capillary sieving electrophoresis, CSE, is also known as capillary gel electrophoresis, CGE. This separation method is analogous to conventional slab-gel electrophoresis. In CSE a sieving matrix, or gel, is introduced to the capillary in the presence of background electrolyte. The sieving matrix forms small pores that serve as a physical barrier to the progress of sample through the capillary. The size of the pores in the sieving matrix depends on the sieving matrix chosen. Small molecules will negotiate the sieving matrix at a faster rate than larger molecules. The sieving action employed by CSE is helpful in separating macromolecules such as proteins, DNA fragments, and oligonucleotides. Migration time often increases linearly with the log of the molecular weight of the macromolecule.

1.2 Sample detection

In the majority of capillary electrophoresis set-ups, all of the separated sample components pass a common point. This attribute of CE systems is similar to chromatographic systems and many chromatography detectors have been adapted for
detection of sample in CE. There are at least four performance metrics to take into account when choosing a detector that is appropriate for a given experiment. Those performance metrics include sensitivity, selectivity, linear range, and noise.

1.2.1 On-column versus postcolumn considerations

Many factors must be taken into account when choosing a detector for CE separations. One of the factors that must be considered is whether on-column or postcolumn detection will be the most appropriate. There are, of course, advantages and disadvantages with each type of detection. This section is by no means an exhaustive comparison between on-column and postcolumn detection. Rather, this section sets forth a few advantages and disadvantages of each.

On-column detection is perhaps the most straight-forward and many commercial instruments utilize on-column detection. This method is straight forward because on-column detection often requires less modification. As an example I will consider optical detection schemes for comparison between on-column and postcolumn detection.

On-column optical detection, such as absorption or fluorescence, is accomplished by removing the polymer coating from the outside of a section of the capillary and defines an optically transparent section of the capillary. This optically transparent section of the separation capillary is placed in the path of the optical detector. Light is then focused onto the capillary and sample is detected as it passes the optical window and a detector response is registered. The on-column set-up is easy to use and can be used with postcolumn manipulations of the separated samples, such as
fraction collection. However, on-column detection tends to have a poorer limit of detection than what can be achieved with postcolumn optical detection. This higher limit of detection arises from the behavior of light at the air-capillary and capillary-liquid interfaces. When the light is focused onto the capillary scatter arises from the curvature of the outer wall of the capillary tubing, which can decrease sensitivity. Additionally, there is a refractive index change when changing mediums from air to the fused-silica capillary that will contribute to light scatter. Capillary tubing that has a flat outer surface is available commercially that can decrease the scatter attributed to the air-capillary interface. However, the capillary-liquid interface is unavoidable when utilizing on-column detection. There is yet another refractive index change when the light passes from the fused-silica capillary to the liquid solution that fills the capillary. Each refractive index change contributes to a decrease in sensitivity.

Postcolumn optical detection is a topic that our group is uniquely qualified to discuss. The Dovichi group has used a postcolumn optical detection scheme for nearly three decades. The postcolumn scheme utilized has produced exceptional limits of detection and a large dynamic range. A general schematic of the postcolumn optical detection scheme used in the Dovichi group is shown in Figure 1-5.

As discussed above, there are refractive index changes present within a postcolumn set-up as well. However, with the postcolumn set-up shown in Figure 1-5 the refractive index changes are all remote to the area where sample detection is occurring. The first refractive index change at the air-cuvette interface is not proximate to the sample stream that is exiting the capillary. Similarly, the cuvette-sheath liquid
interface is not in direct contact with the sample stream exiting the capillary. Finally, the sheath liquid composition is identical to the separation medium exiting the separation capillary. Therefore, no refractive index change is observed at the sheath-liquid-sample interface.

Figure 1-5: Postcolumn sheath flow cuvette for optical detection. Adapted from Cohen et al.27

1.2.2 Laser-induced fluorescence (LIF)

Coupling capillary electrophoresis with laser-induced fluorescence (LIF) offers the highest sensitivity and the greatest dynamic range available. Our group has demonstrated single molecule detection28 and a dynamic range of nine orders of magnitude25 using the postcolumn detection scheme described in Section 1.2.1 and Figure 1-5.

Analytes detected by LIF must be labeled with fluorogenic reagents if they do not fluoresce naturally. Some common fluorogenic reagents include O-phthalaldialdehyde
(OPA), naphthalene-2,3-dicarboxaldehyde (BQCA), 3-(2-furoyl)quinolone-2-carbaldehyde (FQ), and Chromeo™ dyes.\textsuperscript{16} Depending on the application, the need for a fluorescent label can be detrimental to the questions being investigated by a given experiment. Additionally, LIF does not directly provide structural information from the detector response. In some applications inferences can be made about the structure of an analyte based on the detector response. However, these inferences are often based upon comparisons between sample runs and standard runs. Standards are not available for all applications and component identification can be difficult using LIF detection.

1.2.3 Ultraviolet–Visible spectroscopy (UV-Vis)

Ultraviolet-visible (UV-Vis) absorbance detectors are the most widely used detectors in CE. The main reason UV-Vis is such a popular detection scheme is due to its easy and early adaptation to CE applications from high performance liquid chromatography (HPLC) instrumentation. Additionally, it is believed that at least 65\% of all compounds analyzed by chromatography can absorb light at 254 nm, and more than 90\% absorb somewhere in the UV-Vis spectrum.\textsuperscript{13} Therefore, a wide range of compounds should be detectable by UV-Vis absorbance.

Some disadvantages of UV-Vis absorbance include that a large number of materials absorb light in the UV-Vis spectrum and minimizing the background during a separation can be challenging. Additionally, the sensitivity of UV-Vis detection is dependent upon the path length of the detection cell. As a result, the detection limits in
CE applications when employing UV-Vis detection tend to suffer when compared to other detection schemes.13

1.2.4 Electrochemical detection methods

There are two types of electrochemical detectors that have been coupled to CE. The first is conductivity and the second is amperometry. Due to the high electric fields used for CE separations a common problem has arisen when seeking to perform electrochemical detection on a CE system. The resulting electroosmotic currents can be up to six orders of magnitude greater than the faradaic currents measured by an electrochemical detector.22 Isolating the detector electrodes from the high electric field used in CE separations is necessary.1 A method for isolating the electrochemical detectors from the currents supplied by the CE separation involves a porous glass or graphite joint between the end of the capillary and a second capillary that contains the detector electrodes.29–31

1.2.5 Mass spectrometry (MS)

It is possible to couple a capillary electrophoresis instrument directly to a mass spectrometry (MS) ionization source.32,33 Electrospray ionization (ESI) is the most common ionization source that is used when coupling CE to MS. Our group has spent several years developing an interface to improve the performance of CE-ESI-MS.34–37 Other ionization sources have been coupled to CE and they include fast atom bombardment,38 matrix-assisted laser desorption-ionization (MALDI),39 and inductively coupled plasma mass spectrometry (ICPMS).1,21,40 Regardless of the ionization technique
it is important that a volatile background electrolyte be used since the sample migrating through the capillary will need to be vaporized before it can be detected by the mass analyzers. Additionally, it is important to evaluate the background electrolyte that will be used to make sure no components will dirty the mass spectrometer or interfere with the mass range of interest.

In MS experiments there are two modes in which mass spectra can be obtained. The first mode is to scan the entire mass range with the MS. The second mode is to monitor a single ion’s mass as that analyte exits the capillary. Single-ion monitoring (SIM) is faster and provides lower detection limits when compared to scanning the entire mass range. However, SIM is not useful for the characterization of completely unknown samples.
CHAPTER 2:
CAPILLARY ELECTROPHORESIS WITH ONLINE SURFACE-ENHANCED RAMAN SCATTERING (SERS) DETECTOR

2.1 Introduction

The ability to identify and characterize molecules purified through separation lies at the heart of chemical analysis. For column-based separations, common methods of detection include UV-visible absorption, laser-induced fluorescence (LIF), and mass spectrometry. Despite its low cost and flexibility, on-column UV-visible absorption suffers from poor molecular specificity and a lack of sensitivity.\textsuperscript{41,42} On the other hand, LIF offers a high degree of sensitivity but requires fluorescent labels.\textsuperscript{43–45} Since structure determination by migration times alone requires extensive knowledge of the samples beforehand, the use of these two methods is limited for explicit analyte characterization. Mass spectrometry provides exquisite analyte identification for many samples. However, many classes of molecules, such as structural isomers and other molecules with the same mass (isobars), are still challenging to characterize. The cost of high-resolution mass spectrometers necessary for characterizing similar compounds

\textsuperscript{1} A version of this chapter is available as: Negri, P., Flaherty, R. J., Dada, O. O. & Schultz, Z. D. Ultrasensitive online SERS detection of structural isomers separated by capillary zone electrophoresis. \textit{Chem. Commun.} \textbf{50}, 2707–10 (2014) DOI: 10.1039/c3cc49030k.
limits the utility of this technique for routine characterization.\textsuperscript{46,47} As a result, there is a need for new detection techniques capable of providing structural information with high sensitivity and selectivity for chemical analysis.

Here I demonstrate surface-enhanced Raman scattering (SERS) for characterization of three rhodamine isomers separated by capillary zone electrophoresis (CZE). CZE is a powerful analytical technique for separation of charged analytes\textsuperscript{48,49} and has been incorporated into microfluidic devices for high efficiency separations.\textsuperscript{50–52} SERS provides extensive structural and quantitative information about a variety of molecules based on their vibrational transitions\textsuperscript{53} and can be readily performed in solution to facilitate detection in-line with chemical separations.\textsuperscript{54} Given these attributes, SERS has the potential to provide chemical identity of solutes following CZE separation.

There have been previous attempts to couple SERS to CZE. In these studies, CZE-SERS was accomplished by interfacing detection directly on-column or at-line. Direct on-column SERS detection has been achieved using running buffers containing silver colloidal solutions and by laser-induced growth of silver particles at the end of the capillary.\textsuperscript{55,56} The use of colloidal particles has produced detection limits in the nM or pM range; however, memory effects commonly prevent the regeneration of the detection window and limit these configurations to a one-time-use only. Planar SERS substrates in CZE suffer an additional challenge; a metal in an electric field will form a bipolar electrode and cause electrochemical formation of bubbles and degradation of the sample.\textsuperscript{57} In-line CZE-SERS with planar substrates has been limited to mM limits of detection.\textsuperscript{56} An at-line approach to CZE-SERS deposits the effluent onto a moving
Drying the sample adsorbs molecules to the surface and avoids challenges associated with mass transport. This approach also avoids challenges associated with the formation of a bipolar electrode across the SERS substrate; however, designing an interface that guarantees maintenance of the electrical current during the deposition onto the substrate is not trivial. In this chapter, I report an online SERS detector used with a capillary electrophoresis instrument.

2.2 Experimental methods

2.2.1 Materials and reagents

Rhodamine 6G (R6G, ~99%), rhodamine B (RB, ~99%), 5-carboxytetramethylrhodamine (5-TAMRA, ~99%), and sodium tetraborate decahydrate (>99.5) were purchased from Sigma-Aldrich (St. Louis, MO; USA). Ultrapure water (18.2 MΩ cm) was obtained from a Barnstead Nanopure filtration system. All other chemicals were of analytical grade and used without any further purification.

2.2.2 Substrate preparation

SERS-active substrates were fabricated by a previously reported thermal evaporation procedure. These substrates were incorporated into a custom-built flow cell by affixing individual substrates onto a standard microscope slide with two 3 mm diameter holes predrilled 35 mm apart along the center of the slide. Prior to its use, the SERS substrate on the glass slide was soaked overnight in 0.1 M NaOH (Sigma-Aldrich, 99.99%) to dissolve the anodized aluminum oxide (AAO) filter. The resulting SERS-active
substrate was thoroughly rinsed with ethanol (Sigma-Aldrich, 99.5%) followed by a final rinse with ultrapure water.

2.2.3 Raman measurements

Raman measurements were performed using a previously described home-built system.\(^5\) The sample was illuminated through a 40X water-immersion objective (Olympus, NA= 0.8), resulting in a spot size of approximately 0.4 \(\mu\)m\(^2\). The power of the 632.8 nm HeNe laser was \(\sim1.2\) mW, as measured at the sample. Raman back-scattering signal was collected into the same objective lens and directed to the spectrograph and electron multiplying charge coupled device (EMCCD) camera (Newton 970, Andor). The spectral resolution of the Raman measurement was about 3 cm\(^{-1}\) based on the grating (600 gr/mm), entrance slit (25 \(\mu\)m), monochromator path length (320 mm), and CCD pixel size.

2.2.4 CZE-SERS setup

Figure 2-1 shows a schematic diagram of the CZE-SERS experimental setup. The homebuilt flow cell consists of a FEP plastic base plate, a SERS substrate, and a 250 \(\mu\)m thick silicone gasket with a 2 mm slit to define the flow channel, and a stainless steel top plate. The end of a 50 cm bare fused silica capillary (Polymicro Technologies, Phoenix, AZ; USA) with 72 \(\mu\)m i.d., 143 \(\mu\)m o.d. was tightly clamped in between the gasket and the substrate to deliver the sample into the detection region. The capillary dimensions were chosen so that the ratio of the o.d. to i.d. was as close to one as possible. Under these conditions, the distance between the molecules eluting from the capillary and the
SERS active substrate is minimized, which increases detection sensitivity.\textsuperscript{60} The sample injection was pressure driven through the capillary at a flow rate of 1 μL/min using a custom-made injection block.\textsuperscript{61} Hydrodynamic focusing of the

Figure 2-1: The schematic diagrams the experimental CZE-SERS setup including the Raman microscope, the syringe pump, the waste reservoir, the nitrogen gas tank, the high voltage power supply, and the Pt electrode embedded in the custom-built injection block used to transport the sample through the fused silica capillary to the sheath-flow SERS detector. The inset shows the different components of the flow cell. The inlet and outlet ports located on the base of the flow cell are used to accommodate the sheath flow (A). Two holes are drilled in a microscope slide defining the sheath flow path and matching the dimensions of the ports. A sample capillary is pinned on a SERS-active substrate mounted in the center of the glass slide (B). A silicone gasket is cut defining the flow channel between the inlet and outlet ports (C). The top of the flow cell is sealed with a glass coverslip held in place by the top plate (D).
sample stream inside the flow chamber was achieved by pumping the sheath liquid (15 mM sodium tetraborate buffer, pH 9.4) continuously at a flow rate of 10 μL/min through the flow chamber via the inlet port located on the base plate. The sheath liquid flow rate was controlled using a syringe pump (Model NE-500 OEM, New Era Pump Systems Inc., Farmingdale, NY; USA) controlled by LabVIEW (National Instruments, Austin, TX; USA). The liquid was drained out of the flow chamber via the outlet channel connected to the waste reservoir. The flow channel was sealed with a standard cover glass, pressed by the top plate, and secured using four tensioning screws. The system was grounded directly from the SERS substrate during the CZE separations. 6000 spectra were recorded in kinetic series with 50 ms acquisition times.

The sheath flow SERS detector was coupled online to a CZE system. The CZE system is similar to the one previously reported except for the detection module.\textsuperscript{24,61} CZE separation was performed in positive mode on a 50 cm bare fused silica capillary (Polymicro Technologies, Phoenix, AZ; USA) with 72 μm i.d. and 143 μm o.d.

A constant potential of 300 V cm\(^{-1}\) was supplied by a Spellman, CZE 1000R power supply (Spellman High Voltage Electronics Corp., Hauppauge, NY; USA).\textsuperscript{61} The sample, containing 10\(^{-8}\) M rhodamine 6G (R6G), 10\(^{-10}\) M rhodamine B (RB), and 10\(^{-7}\) M 5-carboxytetramethyl-rhodamine (5-TAMRA), was prepared in 15 mM sodium tetraborate buffer (pH 9.4). The CZE separation was performed using a 2.0 s pressure injection, which injects 34 nL of sample. After injection, the capillary was placed in 15 mM sodium tetraborate buffer solution (pH 9.4) and 15 kV (\(~\)40 mA) was applied to the Pt electrode at the sample end of the capillary. SERS measurements were performed in kinetic series.
with 50 ms acquisition times and by using a sheath flow rate of 10 mL min\(^{-1}\) (a sheath flow to capillary flow rate ratio of 100:1). The Raman spectrometer used in this study has been previously described.\(^{59}\) Raman scattering was detected from a 633 nm laser, away from the absorption band of the rhodamine dyes and thus without the benefit of resonance enhancement.

2.2.5 LIF measurements

A high dynamic range LIF detector was used in this experiment that has been described elsewhere.\(^{25}\) Briefly, fluorescent molecules were excited in a sheath flow cuvette using a 25 mW CW 532 nm diode-pumped laser (CrystalLaser, Model CL532-025) and fluorescence emission was collected through a 600 nm DF 40 bandpass filter at a 90\(^\circ\) angle relative to the incident laser beam. Fluorescence was detected using cascaded single-photon counting avalanche photodiode modules (Perkin Elmer, Montreal, PC; Canada). The dye concentrations used for the CZE-LIF experiments were identical to those used in the CZE-SERS experiments to provide a direct comparison.

2.3 Results and discussion

By incorporating our recently demonstrated sheath flow SERS detector,\(^{60}\) I am able to circumvent the challenges noted above and achieve online detection in CZE separations. In particular, the potential drop (bipolar electrode formation) across the SERS substrate is minimized by the increased volume of the sheath flow and confined sample near the electrical ground. Changes observed in the silver oxide background signals suggest a small electrochemical potential is still present. However, I have
successfully used the same SERS substrate in CE applications for up to three days without significant signal degradation. The sheath flow SERS detector enables sequential and high throughput detection of the separated dyes at nanomolar concentrations (attomole–femtomole injections) using a 50 ms acquisition without significant “memory effect” or fouling of the SERS substrate.

Figure 2-2(A) shows the heatmap of the SERS intensity as a function of Raman shift and migration time following the electrophoretic separation of three rhodamine isomers (R6G, RB, and 5-TAMRA). The Raman spectrum observed indicates that R6G migrates at $t_m = 180 \pm 13$ s, RB at $t_m = 220 \pm 19$ s, and finally 5-TAMRA at $t_m = 290 \pm 15$ s. The SERS signal for each peak persists for about 1–2 s or less at these low concentrations. The short duration of the SERS signal is more clearly observed in the 2 s zooms shown in Figure 2-2(B), which illustrate the difference in width of each migration peak.

Figure 2-2(C) shows the SERS electropherogram constructed from the SERS intensity at 1357 cm$^{-1}$ as a function of migration time. This band is attributed to the combined aromatic C–C and C=N stretching modes of rhodamine compounds.$^{62-66}$ The intensity profile at 1357 cm$^{-1}$ provides a convenient signal to characterize the separation efficiency with SERS detection. The spectrally resolved SERS electropherogram of the three rhodamine dyes is characterized by a low and constant background.
Figure 2-2: (A) Heatmap of the observed SERS intensity at each Raman shift as a function of migration time for the electrophoretic separation of R6G, RB, and 5-TAMRA. (B) Zoom in on the dashed vertical rectangles in (A) show 2 s windows corresponding to the detected analytes. (C) SERS intensity profile of the Raman band at 1357 cm$^{-1}$ is plotted against migration time, extracted from the red rectangles shown in (A). This band is attributed to the combined aromatic C–C and C=N stretching modes of rhodamine compounds. The dashed vertical rectangles in (A) highlight the detection of each analyte.
Analysis of the SERS electropherogram (Figure 2-2(C)) shows a peak for R6G at $t = 180.25$ s with a full width at half max (FWHM) of 1.25 s, which suggests a separation efficiency of $N = 115,000 \pm 35,000$ theoretical plates. The SERS electropherogram peak for RB at $t = 219.75$ s shows a more symmetric peak with a FWHM of $0.55$ s. This corresponds to $N = 898,000 \pm 115,000$ theoretical plates. The electropherogram peak for 5-TAMRA at $t = 290.60$ s has a FWHM of $0.40$ s, which corresponds to a separation efficiency of $N = 2,900,000 \pm 620,000$ theoretical plates. Because our analytes fluoresce when excited at shorter wavelengths, I performed laser-induced fluorescence (LIF) to compare the migration times and separation efficiency. Figure 2-3 shows the electropherogram of the same three analyte mixture using LIF detection. The analyte concentrations and separation conditions were kept identical to those used in the optimized SERS experiments to provide a direct comparison. The LIF electropherogram shows three bands associated with the elution of R6G, RB, and 5-TAMRA with a separation efficiency $N = 1,000 – 6,000$ theoretical plates (analyte dependent), which is low for a CZE separation with LIF detection. The poor separation efficiencies are the result of the large injection volume and the high concentration of analytes used for the CZE-LIF experiments. However, CZE-SERS and CZE-LIF generated identical elution order and equivalent migration times under identical separation conditions.

The difference in observed number of theoretical plates provides insight into the mechanism of SERS detection. Only molecules located within a close proximity to the
Figure 2-3: Electropherogram of the three analyte mixture resulting from the CZE separation and detection by LIF. The attenuated signal presented here was extracted from the third avalanche photodiode (APD) on a high dynamic range system equipped with a total of five APDs in series. The signal was treated to account for photon counter dead time (50 ns), background corrected, and a three-point median filter was applied to remove spikes resulting from particulates passing through the detection volume. The experimental conditions (injection time, analyte concentrations, and CZE separation) were kept identical to those used for the CZE-SERS experiments to provide a direct comparison. The LIF electropherogram shows (A) the full scale of the 5-TAMRA peak and (B) all three peaks associated with the elution of R6G (t=187 ± 17 s), RB (t=229 ± 26 s), and 5-TAMRA (t=296 ± 23 s), respectively. The separation efficiencies were calculated from the first APD (no attenuation) using the peak width at baseline and were determined to be 2,630 ± 400 theoretical plates for R6G, 6,200 ± 600 theoretical plates for RB, and 900 ± 25 theoretical plates for 5-TAMRA. The poor column efficiencies are attributed to the large injection volume and the high concentration of analytes.

SERS substrate surface can be detected. Our previous work suggests the observed signal arises from adsorbed molecules. However, it is known that Langmuir behavior inhibits analyte adsorption at low concentrations, typically below 1 nM. I have successfully detected RB at a concentration below this in Figure 2-2. This suggests that hydrodynamic confinement may provide a transiently increased concentration at the surface, such that the SERS detection is only obtained from the highest concentration.
portion of the migrating analyte band. This is in contrast to LIF, where the greater sensitivity enables detection of the width of the entire eluting sample. In Figure 2-4 and Figure 2-5, I show the SERS results from a longer injection and a higher concentration of analytes. The apparent efficiency with SERS detection decreases to a level comparable to LIF due to molecules remaining on the surface for longer periods.

Figure 2-4: (A) Heatmap of the observed SERS intensity of each Raman shift as a function of migration time for the electrophoretic separation of a 102 nL (6 s injection) sample mixture containing $10^{-7}$ M R6G, $10^{-8}$ M RB, and $10^{-6}$ M 5-TAMRA. (B) SERS intensity profile of the Raman band at 1357 cm$^{-1}$ as a function of migration time extracted from the red rectangle shown in (A). This band is attributed to the combined aromatic C–C and C=N stretching modes in rhodamine compounds. The dashed vertical rectangles in (A) highlight the detection of each analyte.
Figure 2-5: Single 50 ms SERS spectrum of (a) R6G (10^{-7} M) extracted from Figure 2-4(A) at \( t = 135 \) s, (b) RB (10^{-8} M) extracted from Figure 2-4(A) at \( t = 165 \) s, and (c) 5-TAMRA (10^{-6} M) extracted from Figure 2-4(A) at \( t = 225 \) s. Averaged SERS spectrum of (d) R6G (10^{-7} M) extracted from Figure 2-4(A) between \( t = 133 \) and 145 s, (e) RB (10^{-8} M) extracted from Figure 2-4(A) between \( t = 160 \) and 168 s, and (f) 5-TAMRA (10^{-6} M) extracted from Figure 2-4(A) between \( t = 220 \) and 226 s. Asterisks indicate the bands intrinsic to each analyte.

The analytes are assigned based on the observed Raman spectrum, indicating that R6G migrates at \( t = 135 \pm 13 \) s, RB at \( t = 160 \pm 15 \) s, and finally 5-TAMRA at \( t = 220 \pm 10 \) s. Clearly, the spectrally resolved SERS electropherogram of the three rhodamine dyes is characterized by a low and constant background. Figure 2-4(B) shows the SERS electropherogram constructed from the SERS intensity at 1357 cm\(^{-1}\) as a function of migration time. The electropherogram peak for R6G shows a broad feature at \( t = 135 \) s with a full width at half max (FWHM) of 12 s, which corresponds to a separation efficiency \( N = 700 \pm 160 \) theoretical plates. The SERS electropherogram peak for RB at \( t = 160 \) s shows a more symmetric peak with a FWHM of 6 s. This corresponds to \( N = 3,940 \pm 1,200 \) theoretical plates. The electropherogram peak for 5-TAMRA at \( t = 220 \) s
has a FWHM of 4 s and a separation efficiency $N = 16,760 \pm 3,600$ plates. Similarly poor numbers of theoretical plates were observed with CE-LIF and correspond to the long initial injection time and the high concentration of the sample into the capillary. The strong adsorption of R6G to the SERS substrate is clearly evident by the significant tailing noted in this measurement. This is in contrast with the sharper migration peaks and resulting higher column efficiencies observed for RB and 5-TAMRA, which suggests a faster desorption mechanism.

The role of adsorption is further evident in the width of the R6G peak relative to the widths observed for RB and 5-TAMRA. Increased adsorption of R6G to the surface results in a longer observed peak width in the SERS electropherogram (Figure 2-2(C)); suggesting R6G has a stronger binding affinity for silver surfaces than RB and 5-TAMRA. The CZE-SERS efficiency appears to correlate to the sample desorption rate from the substrate. In these results, the longer desorption rate observed for R6G can be directly attributed to the difference in structure of the three rhodamine dyes (Figure 2-6). R6G is the only dye out of the three containing a secondary amine group. The $pK_a$ of this amine group has a value of 6.13. When dissolved in borate buffer ($pH 9.4$), the basic form of R6G predominates ($pH > pK_a$). As a result, the secondary amine group is deprotonated and more electron rich. Under these conditions, the nitrogen atom on the R6G molecules is more likely to adsorb to the silver SERS substrate than the other amine groups in RB and 5-TAMRA. These properties explain the higher affinity of R6G for the silver SERS substrate and the resulting slower desorption mechanism observed for this
rhodamine dye. Despite these variations, it is worth noting that baseline resolution is achieved between each analyte, demonstrating no memory effects.

![Molecular structures and corresponding molecular weights of the three rhodamine dyes used in this study.](image)

**Figure 2-6**: Molecular structures and corresponding molecular weights of the three rhodamine dyes used in this study. Of note, rhodamine 6G and rhodamine B have identical molecular weights. Only rhodamine 6G has a secondary amine, to which I attribute its strong affinity for the silver SERS substrate.

In our earlier publication, our SERS detector demonstrated a linear response from nM to mM concentrations of R6G, indicating that quantitation is possible for trace analyte detection. Our previous work further showed that chemical effects alter the desorption rate, which I am investigating to understand their impact on the observed separation.

The main advantage of using SERS over conventional detection techniques (UV and LIF) is that it can provide chemical information to identify and characterize analytes beyond migration times. Figure 2-7(a) shows a single 50 ms SERS spectrum of R6G (10⁻⁸ M) from the electrophoretic separation of the three dye mixture extracted from Figure 2-2(A) at tₘ = 180.25 s. The main features of the R6G spectrum are the bands at 1175, 1306, 1357, 1506, and 1648 cm⁻¹. These bands are associated with the characteristic
strectching modes of the C–H band, C=N, and aromatic C–C stretching vibrations of R6G. Figure 2-7(b) shows a single 50 ms SERS spectrum of RB (10^{-10} M) extracted from Figure 2-2(A) at t_m = 219.75 s. The RB bands are assigned to the aromatic C–H bending (1197 cm^{-1}), the C–C bridge-bands stretching (1276 cm^{-1}), and the aromatic C–H bending vibrations (1357 cm^{-1}, 1506 cm^{-1}, and 1645 cm^{-1}). Finally, Figure 2-7(c) shows a single 50 ms SERS spectrum of 5-TAMRA (10^{-7} M) extracted from Figure 2-2(A) at t_m = 290.60 s. The main features of the 5-TAMRA spectrum are the bands at 1197, 1276, 1354, 1506, and 1643 cm^{-1}. These bands are assigned to the aromatic C–H bending, C–C bridge-band stretching, and aromatic C–C stretching modes of 5-TAMRA.

Figure 2-7: Single 50 ms SERS spectrum of (a) R6G (10^{-8} M) extracted from Figure 2-2(A) at t = 180.25 s, (b) RB (10^{-10} M) extracted at t = 219.75 s, and (c) 5-TAMRA (10^{-7} M) extracted at t = 290.60 s. The average SERS spectrum of (d) R6G (10^{-8} M) extracted from Figure 2-2(A) between t = 179.65 and 180.05 s, (e) RB (10^{-10} M) extracted between t = 220.45 and 221.90 s, and (f) 5-TAMRA (10^{-7} M) extracted between t = 290.45 and 290.95 s are shown. Asterisks indicate the bands intrinsic to each analyte.
Averaging the SERS signal over the duration of the electropherogram peak yields spectra with a S/N ratio ≥ 25 for all three analytes. Figure 2-7(d) shows the average SERS spectrum of R6G extracted from Figure 2-2(A) between \( t = 179.65 \) and \( 180.05 \) s. The averaged SERS spectrum of RB extracted between \( t = 220.45 \) and \( 221.90 \) s is shown in Figure 2-7(e). Of note, the SERS spectrum of RB was acquired from the injection of a few attomoles (\( \sim 100,000 \) molecules). Figure 2-7(f) shows the averaged spectrum of 5-TAMRA extracted from Figure 2-2(A) between \( t = 290.45 \) and \( 290.80 \) s. While all three dyes show similar spectra, as expected based on their structures, the differences observed enable identification of the analytes.

2.4 Conclusions

The SERS response from the substrate is highly reproducible; however, the signal is also a function of where the laser is focused in relation to where the sheath flow confines the molecules in the detection area. I have found it is important to collect signal from this optimum point in the sheath flow.\(^{60}\)

I have demonstrated highly sensitive and ultrafast online SERS detection of structural isomers of rhodamine separated by CZE. SERS spectra of the analytes provided direct spectral signatures associated with the subtle structural differences of the three rhodamine dyes. The limit of detection for SERS reported here is more than 1000X better when compared to the best previously reported LOD using a planar substrate.\(^{56}\) The observed Raman scattering allowed differentiation of two isobaric compounds (R6B and RB, M.W. = 479.02 g mol\(^{-1}\)) at nanomolar concentrations, which is
not achievable by mass spectrometry. The SERS flow detector should be readily incorporated into any liquid separation, such as liquid chromatography. The implementation of this robust and sensitive online SERS flow detector suggests an alternative for the characterization of pharmaceuticals, metabolites, and other analytes.

2.5 Acknowledgments

The University of Notre Dame, NIH Award R21 GM107893, and the Cottrell Scholar Award from the Research Corporation for Science Advancement supported this work.
CHAPTER 3:

NICKED-SLEEVE INTERFACE FOR TWO-DIMENSIONAL CAPILLARY ELECTROPHORESIS

3.1 Introduction

Capillary electrophoresis (CE) is a powerful tool for the separation of complex mixtures.\(^{71-79}\) When very complex samples, such as cell homogenates, are investigated, a single separation is incapable of resolving all of the components present. For complex mixtures it is often beneficial to couple additional separation methods to improve resolution.\(^{80}\) Jorgenson's group demonstrated the coupling of liquid chromatography to capillary electrophoresis in the 1980s.\(^{81}\) In these experiments, fractions were periodically transferred to a fused silica capillary for separation in the second dimension by capillary electrophoresis. Those systems were unable to halt flow in the first dimension. The vast majority of analyte that was separated by the liquid chromatography column would flow to waste and only a small fraction was transferred to the second dimension for electrophoretic separation.

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Michels et al. demonstrated the first example of a two-dimensional CE (2D-CE) separation. In this experiment, proteins from a cell lysate were separated by submicellar concentrations of sodium dodecyl sulfate (SDS); the first dimension background electrolyte had a pH of 7.5 and the second dimension background electrolyte had a pH of 11.1. It was easy to adjust the voltage across the first capillary such that flow of analyte from the first dimension is halted while separation is still being performed in the second dimension. 2D-CE experiments use some combination of capillary zone electrophoresis, capillary sieving electrophoresis, micellar electrokinetic capillary chromatography, and isoelectric focusing. For example, Chen et al. used CSE-MEKC to analyze the proteins in a mouse tumor cell homogenate. The authors investigated the effects of CSE buffer components on separation efficiency and were able to resolve about 60 spots. Additionally, variations of 2D-CE have been used for diagonal CE and on-column digestion of proteins.

The key to the performance of a 2D separation is the design and construction of an efficient, leak free interface that connects the two dimensions. Most systems are designed to immobilize the two capillaries independently. The separation capillaries are manually aligned within a chamber to maximize coaxial overlap. This chamber is then filled with the second dimension background electrolyte and connected to a high voltage power supply to control sample flow. Construction and alignment of these interfaces require exceptional dexterity.

In this chapter, I report the use of a micro-dicing saw to nick a sleeve capillary that is then used as an aligning tool. This nicked sleeve capillary facilitates rapid coaxial
alignment of the separation capillaries used in 2D-CE separations. As I show, this system produces more efficient fraction transfers and improves precision with respect to peak area.

3.2 Experimental

3.2.1 Materials

All capillaries were obtained from Polymicro Technologies (Phoenix, AZ; USA). Buffer reagents were purchased from Sigma-Aldrich (St. Louis, MO; USA).

3.2.1.1 Interface fabrication and sleeve capillary nicking

Interfaces were made from Plexiglas microscope slides. Each microscope slide yielded two Plexiglas chips by cutting the slide in half along its short axis. The Plexiglas chip and sleeve capillaries were first immobilized by use of two types of specialty tape from Semiconductor Equipment Corp (Moorpark, CA; USA). The first, medium tack tape was placed on the underside of a film frame from Perfection Products (Lebanon, IN; USA) with the adhesive side facing upward. Vacuum holds the film frame/medium tack tape in place. Once the medium tack tape is attached to the film frame and all air bubbles are removed, a ~2 × 5 cm piece of heat-release tape (P/N: 3195MS) is put on the sticky side of the medium tack tape, with the heat-release tape's adhesive side facing upward. The material that will be cut by the saw is then placed onto the heat release tape and a second piece of heat release tape is placed, adhesive side down, on top of the material. When the adhesive sides of the heat release tape come into
contact, the adhesive holds until it is heated to its release temperature. After cutting, a Milwaukee Model MHT3300 heat gun was used to inactivate the heat release tape and release the components.

The interfaces were fabricated from 2 mm thick Plexiglas microscope slides from Exakt Technologies Inc. (Oklahoma City, OK; USA) using a micro-dicing saw (Micro Automation Model 1100) with 500 μm thick diamond blades from Thermocarbon Inc. (Casselberry, FL; USA). This micro-dicing saw was used to cut two perpendicular channels in a Plexiglas chip. The width and depth of the two channels differed slightly. The channel that contained the buffer line and waste was cut to be 1 mm wide and 1 mm deep to hold the 1.0 mm OD, 0.50 mm ID borosilicate glass tubes. This buffer plumbing delivers separation buffer for interface flushing and electrical contact. The channel that was perpendicular to the buffer channel holds the sleeve and separation capillaries. This channel was cut to be approximately 700 μm in width and 700 μm in depth. After assembly, the interfaces are sealed by placing a cover slide on top of the Plexiglas chip and applying the UV light curing resin to the perimeter of the cover slide. The low viscosity of the resin allows it to intercalate into open spaces. The entire interface is then exposed to UV light to cure the resin.

Traditional interfaces use separate sleeve capillaries for each separation capillary, as shown in Figure 3-1. Fabrication of a traditional interface requires exceptional dexterity. The alignment of the separation capillaries and ultimately the performance of the traditional interface heavily depend on the skill of the constructor of the interface.
The nicked sleeve capillary interface used a sleeve capillary (152 μm ID, 650 μm OD) that was cut approximately halfway through the inner diameter, resulting in a nick that exposed the inner diameter of the sleeve capillary, while maintaining an adequate amount of supporting capillary under the nick (Figure 3-2). The sleeve capillary was nicked using the micro-dicing saw and diamond blades.

Nicking the capillary halfway through the inner diameter is straightforward when using the micro-dicing saw. The chuck is first “zeroed” by bringing the diamond blade into contact with the chuck, and the computer then stores the zeroed coordinate as zero elevation above the chuck. All subsequent cuts are made by telling the saw how far above that zero elevation the desired cut should be (Figure 3-10 and Figure 3-11). Since the thicknesses of the tapes being used and the capillary are accurately known, nicking of the sleeve capillary becomes trivial by programming the saw to cut an appropriate distance above the chuck.

Figure 3-1: Top view diagram of a traditional 2D-CE interface.
All components in both interfaces were immobilized using an intercalating, ultraviolet light curing resin (KOA 300 Adhesive, Kemexert Corp., York, PA; USA). The polyimide coating was removed from both ends of the separation capillaries with a gentle flame.

Coating removal eliminates interference with injections that could result from excess polyimide left over from cleaving the capillary. Removal of the polyimide coating also simplifies threading of the separation capillary into the sleeve capillary. Separation capillaries were secured in the sleeve capillaries by a drop of epoxy (5 Minute Epoxy, ITW Devcon, Danvers, MA; USA) applied to the point where the separation capillaries exited the sleeve capillary.

3.2.1.2 Fluorescence detector

The laser-induced fluorescence detector that was used for these experiments has been described elsewhere. Briefly, fluorescence was excited in a sheath flow cuvette using a CW 532 nm diode-pumped laser (CrystaLaser Model CL532-025) and fluorescence was collected at right angles from the incident laser beam. Fluorescence
was detected using a single-photon counting avalanche photodiode module (PerkinElmer, Montreal, PQ; Canada).

3.2.1.3 Transfer efficiency evaluation

One-dimensional capillary electrophoresis (1D-CE) was performed in a single, intact fused silica capillary (40.0 cm long, 50 μm ID, 150 μm OD). The peak area from the 1D-CE data set defined 100% transfer efficiency. Two-dimensional capillary electrophoresis (2D-CE) was performed using a traditional interface (Figure 3-3), and the nicked sleeve interface (Figure 3-4).

![Sleeve Capillaries](image)

![Separation Capillaries](image)

Figure 3-3: Traditional two-dimensional interface.

Six interfaces were evaluated, three traditional and three nicked sleeve. The separation capillaries for 2D-CE interfaces were each 40.0 cm long, 50 μm ID, 150 μm OD fused silica capillaries for a total separation capillary length of 80 cm. The interfaces were subjected to two types of transfer efficiency evaluations. The first was the operation of
the 2D interface in pseudo-1D mode, where potential was continuously applied across both capillaries and the same electric field strength was applied across both capillaries.

Transfer efficiency was also evaluated in 2D mode. In this experiment, plugs were transferred from the first capillary to the second capillary. Upon the transfer of a plug into the second dimension, separation in the first dimension was halted by applying the same voltage at the injection block and the interface. In all experiments, the sheath flow cuvette was held at ground potential. An experimental diagram is shown in Figure 3-5.
3.2.1.4 Electrophoresis

A 15 mM sodium tetraborate buffer was used as background electrolyte (pH 9.4) for the experiment. High voltage power supplies (Spellman, Newark, NJ; USA) provided voltages for the separations. For the 1D-CE experiment, a potential of 10 kV was applied. For the 2D-CE experiments, electrical contact was made with the interface by attaching Tygon® R-3603 tubing to the buffer line (Figure 3-1), which was then placed in an Eppendorf tube with a platinum electrode that was connected to the second power supply. During pseudo-1D experiments, the injection block and the interface were held at 20 kV and 10 kV, respectively, producing uniform electric field in the capillaries. In 2D separations, a voltage program was used that held the interface at a constant potential.
of 10 kV, while the voltage at the injection block was varied as shown in Table 3-1. The sample used was 5-carboxytetramethylrhodamine succinimidyl ester (TAMRA) at a concentration of 100 pM (AnaSpec, Inc.; Fremont, CA; USA). All sample injections were performed at 5 kV for 5 seconds; during injection in 2D experiments the interface was held at ground potential.

<table>
<thead>
<tr>
<th>TABLE 3-1</th>
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<tr>
<td>VOLTAGE PROGRAM FOR 2D SEPARATIONS</td>
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</table>

<table>
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<tr>
<th>Injection Block Voltage (kV)</th>
<th>Interface Voltage (kV)</th>
<th>Time (seconds)</th>
</tr>
</thead>
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<td>10</td>
</tr>
<tr>
<td>Separation</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

3.2.1.5 Data processing

Data processing was performed with Matlab on a PC. Data were treated with a three-point median filter to remove spikes generated by particulates passing through the laser beam. The two-dimensional data was reconstructed from the one-dimensional raw data as described earlier.\textsuperscript{78}
3.3 Results and discussion

3.3.1 Transfer efficiency

Peak area was used to compare transfer efficiencies of the traditional and nicked sleeve interfaces. Triplicate data sets were obtained for all experiments using a total of six interfaces, three traditional and three nicked sleeve. As a comparison between 1D-CE and 2D-CE, Figure 3-6 shows normalized peak height and migration time for a 1D and pseudo-1D run. The efficiency of the 1D and pseudo-1D run were 200,000 and 250,000 theoretical plates, respectively. The minor increase in the pseudo-1D operation likely is a result of the larger column volume, which reduces the contribution of injection volume as a source of extra-column band broadening. A representative 2D-CE electropherogram is shown in Figure 3-7.

Figure 3-6: Normalized migration time and peak height to show the difference between the TAMRA peak from 1D and pseudo 1D experiments.
Figure 3-7: (a) Representative 2D electropherogram from the nicked sleeve capillary interface and (b) a close up of the 5-TAMRA peak.

The raw data that was collected for 2D experiments, Figure 3-7, was reconstructed into a two-dimensional image, Figure 3-8. The area of the spot on the image was calculated by simply summing the counts in the region of the spot and then subtracting the counts from the same size background.

The results for each experiment were compared to the results of a 1D-CE experiment that used a single, intact capillary. The peak area from the 1D-CE experiment served as the threshold for 100% transfer efficiency. Table 3-2 and Figure 3-9 show results for all experiments. A marked improvement in transfer efficiency and precision was observed for the nicked sleeve capillary interface compared with the traditional interface. The nicked sleeve capillary interface produced a transfer efficiency greater than 90% for both pseudo-1D and 2D modes with an error of 2.1% and 2.5%,
respectively. On the other hand, the traditional interface leaves much to be desired with transfer efficiencies of 43 ± 11% and 68 ± 7.3% for pseudo-1D and 2D modes, respectively.

Figure 3-8: Representative 2D image plot of 2D nicked sleeve capillary data.

This enhanced performance likely stems from the improved alignment of the separation capillaries when using a nicked sleeve capillary. The traditional interface requires exceptional dexterity to achieve lateral alignment of the separation capillaries in the plane of the interface; however alignment out of the plane of the interface is not easily controlled. Using a nicked sleeve capillary maximizes the coaxial overlap of the separation capillaries and removes the need for manual alignment. The farthest out of
alignment the separation capillaries are permitted to be with the use of the nicked sleeve capillary cannot exceed the difference between the sleeve inner diameter and the separation capillary outer diameter (2 μm in this experiment). In addition to providing improved transfer efficiency and easier assembly, the nicked sleeve capillary interface also improved the precision of data collected. Interestingly, the transfer efficiency of the traditional interface being operated in 2D mode was greater than the transfer efficiency of the same interface being operated in pseudo-1D mode. The increase in efficiency is likely a result of an imperfect balance of electric fields during these experiments, which could result in hydrodynamic backflow that would minimize diffusional loss of analyte from the distal end of the first capillary during the second dimension separation.

Figure 3-9: Transfer efficiencies of the various experiments performed in this investigation.
### TABLE 3-2

**PEAK AREAS AND STATISTICS**

<table>
<thead>
<tr>
<th></th>
<th>Peak Area (Photon Counts)</th>
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<td>1D</td>
<td>1D Traditional Pseudo-1D</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td>1,132,000</td>
</tr>
<tr>
<td><strong>Standard Error of Mean</strong></td>
<td>4,000</td>
</tr>
<tr>
<td><strong>Transfer Efficiency (%)</strong></td>
<td>100</td>
</tr>
<tr>
<td><strong>Coefficient of Variation for Transfer Efficiency (%)</strong></td>
<td>0.35</td>
</tr>
</tbody>
</table>

**NOTE:** data from triplicate runs on three different interfaces for each type of interface

3.3.2 Acknowledgments

This work was supported by a grant from the National Institutes of Health (R21GM103462). I also gratefully acknowledge donation of the film frame by John Vickery at Perfection Products.
3.4 Supplemental information

Figure 3-10: Micro Automation Model 1100 micro-dicing saw.
Figure 3-11: Close-up of the micro-dicing saw. The diamond saw blade is held by compression in the saw blade flange. When cuts are made the blade assembly remains fixed while the X, Y, Z, and rotational translation chuck performs the necessary movements to accomplish the desired cuts.
CHAPTER 4:
CAPILLARY ELECTROPHORESIS COUPLED WITH AUTOMATED FRACTION COLLECTION

4.1 Introduction

Capillary electrophoresis is a useful tool for the analysis of biological samples such as polypeptides, lipids, DNA sequencing fragments, and metabolites.\textsuperscript{26,35,61,90–95} Capillary electrophoresis instruments typically employ absorbance, laser-induced fluorescence, or mass spectrometry for on-line detection and analysis. While on-line detection is essential for most analyses, sample fractionation and recovery are necessary for use of capillary electrophoresis for preparative separations.

There have been few reports of capillary electrophoresis for preparative separations. Gannaro and Salas-Solano reported the use of a commercial capillary electrophoresis instrument that deposits fractions in a 96 well microtiter plate for characterization of deaminated peptide variants.\textsuperscript{96} The technique first measures the migration time of a target molecule. In subsequent runs, the separation voltage is set to zero at the migration time of a target compound; the distal end of the capillary is then

\textsuperscript{3} A version of this chapter is available as: Huge, B. J., Flaherty, R. J., Dada, O. O. & Dovichi, N. J. Capillary electrophoresis coupled with Automated fraction collection. \textit{Talanta} \textbf{130}, 288–293 (2014) DOI: 10.1016/j.talanta.2014.07.018.
placed into a well of a microtiter plate that contains running buffer. The voltage is reapplied for a period of time corresponding to the analyte peak width to deposit the target molecule within the well. Potential is again set to zero, and the capillary tip is returned to the normal outlet buffer. Timing of subsequent fraction collection is reported to be difficult, and a separate run appears to be typically used for each fraction that is collected. This protocol requires a number of capillary manipulations, requires knowledge of the migration time of the components to be collected, and appears to allow collection of only one fraction from an injection.

It would be desirable to deposit fractions in succession into the wells of a microtiter plate without stopping the separation or careful calibration of migration time of analyte. In 1985 Hjertén and Zhu demonstrated the first fraction collection using capillary electrophoresis (CE) as the separation method. Since then, fraction collection has primarily been used to couple CE with matrix assisted laser desorption/ ionization (MALDI) mass spectrometry. Owing to the difficulty of performing on-line CE-MALDI-MS, off-line coupling systems are preferred. Several approaches have been reported for off-line preparative separation and fractionation that mainly differ in the delivery of analyte to the MALDI plate.

I reported a CE-MALDI interface design that employs a drop-on-demand matrix sheath flow controlled by a high-speed inkjet printer valve. That instrument placed the distal end of the separation capillary within an ink-jet nozzle, which was held at ground potential. MALDI matrix solution was introduced through a Tee fitting attached to the nozzle; the MALDI solution was pumped by nitrogen pressure. A high-speed
miniaturized valve controlled deposition to the MALDI plate. In this chapter, I modified our MALDI plate spotter for use as a 96 well fraction collector in preparative capillary zone electrophoresis. I demonstrate the fraction collector for analysis of a dye using a fluorescent plate reader, for analysis of an oligonucleotide using real-time PCR, and for generation of aptamers using CE-SELEX.\textsuperscript{104,105}

4.2 Materials and methods

4.2.1 Materials and reagents

Fused silica capillary (50 μm ID and 150 μm OD) was purchased from Polymicro Technologies (Phoenix, AZ; USA). The fluorescent standard 5-carboxytetramethylrhodamine SE (TAMRA) was purchased from AnaSpec (San Jose, CA; USA). Other reagents were analytical grade and purchased from Sigma-Aldrich (St. Louis, MO; USA). All solutions were prepared from deionized-distilled water obtained from a Barnstead Nanopure System (Thermo-Fisher Scientific, Waltham, MA; USA).

4.2.2 Laser-induced fluorescence detection

Two systems were employed. The first, described in this section, employed laser-induced fluorescence detection and was used to provide a reference separation. The instrument was similar to others reported by our group.\textsuperscript{23,24,106} Briefly, fluorescence was excited using a CW 532 nm diode-pumped laser (CrystaLaser Model CL532-025), which was focused into the sample stream at the center of a sheath-flow cuvette. Fluorescence was collected at right angles and detected by a cascade of single-photon
counting avalanche photo diode modules (PerkinElmer, Montreal, PQ; Canada) with a sampling frequency of 50 Hz.\textsuperscript{25,67}

A bare fused silica capillary (50 μm ID, 150 μm OD, 40 cm length) was used for analysis. The first sample was 1 mM TAMRA prepared in a 15 mM sodium tetraborate running buffer. The sample was injected for 5 s at 5 kV and electrophoresis was performed at 10 kV, supplied by a Spellman High Voltage power supply (CZE 1000R, Newark, NJ; USA). Samples were analyzed in triplicate.

A second sample consisted of a random single-stranded DNA library (Sigma-Aldrich, St. Louis, MO; USA). The 80 base sequence, 5’-AGCAGCAGAGGTACAGATG-N(40)-CCTATCGGTGCTACCGTGAA-3’, was designed with priming regions for PCR amplification at both the 5’- and 3’-ends flanking a random region allowing up to 4\textsuperscript{40} sequences within the pool. A TAMRA fluorescent tag was incorporated at the 5’-end for laser-induced fluorescence detection. The library was injected for 5 s at 5 kV onto a preconditioned 45 cm capillary. The separation running buffer was 10 mM sodium tetraborate/HEPES (Sigma-Aldrich, St. Louis, MO; USA), and the separation was performed at 15 kV.

A third sample consisted of a 10 μL aliquot of the 100 μM stock oligonucleotide library solution added to 10 μL of binding buffer (50 mM TRIS, 100 mM NaCl, 1 mM CaCl\textsubscript{2}). The mixture was heated to 94°C to destroy secondary structures that may have formed during storage. The solution was cooled by 0.5°C/second to a final temperature of 20°C in a thermal cycler (PTC-100, MJ Research). The heat-treated 10 μM random ssDNA library solution was incubated at room temperature with 1 mg/mL human α-
thrombin protein (Hematologic Technologies, Inc., Vermont; USA) for a minimum of 15 min to allow binding.

4.2.3 Fraction collector

A second system employed fraction collection and is diagramed in Figure 4-1. The distal end of the separation capillary was threaded through a Tee fitting using a capillary sleeve and ferrule from Upchurch Scientific (Oak Harbor, WA; USA). The valve, tee, nozzle, and inline filter were the same as used in Vannatta et al.\textsuperscript{103} and were from The Lee Company (Westbrook, CT; USA).

The capillary tip was positioned at the exit of the nozzle to avoid contamination within the spotting apparatus. The capillary and nozzle were secured 2 mm above the surface of the plate to ensure proper sample delivery. The metal nozzle was held at ground potential. The deposition buffer was held under nitrogen pressure at 5 psi. The collection plate was positioned below the instrument on a Prior Scientific microscope stage (Rockland, MA; USA). The stage was mounted to an aluminum breadboard by a Plexiglas block. Instrument control was programmed in LabVIEW (National Instruments, Austin, TX; USA).

The motion of the stage was matched to a 96 well microtiter plate with 9 mm well spacing in the X and Y dimensions in a 12 × 8 pattern. The stage was moved in the X direction in odd rows and the -X direction in even rows, creating a serpentine pattern. Fraction width, which controls time between depositions, was determined from
reference data obtained by the fluorescence detector. Under typical conditions, each
droplet consists of 10 μL sheath liquid and a few picoliters of solution from the capillary.

Figure 4-1: Schematic of the CE-high efficiency fraction-collection instrument. The
schematic illustrates the distal end of the capillary threaded through a tee and resting at
the tip of the nozzle. Buffer flow is controlled at the dispensing valve and washes the
fraction exiting the capillary into a plate well. The 96-well plate is fixed to a motorized
microscope X-Y stage.

4.2.4 TAMRA fraction collection

The separation conditions and the capillary length were identical to those used
for laser-induced fluorescence detection. Due to the dilution of analyte in the fraction
collector and the poor sensitivity of the fluorescent plate reader, I used a very high
concentration TAMRA solution for this characterization experiment. A 1 mM TAMRA
solution was prepared in the 15 mM sodium tetraborate separation buffer and injected
for 5 s at 5 kV. The capillary was preconditioned for 5 min with NaOH, ddH₂O, and
separation buffer before each analysis. Following each preconditioning step, the valve was primed for 2 s with either ddH₂O or separation buffer in the valve reservoir.

Half-area, black with clear bottom, 96-well plates (Corning, Corning, NY; USA) were used for collection. A fraction width of 4 s was determined from the reference data obtained by capillary electrophoresis with fluorescence detection. The valve pulse width was set to 0.05 s, dispensing 15 mM sodium tetraborate buffer through the valve. Fraction collection and electrophoresis began simultaneously. Fluorescence intensity within each of the 96 wells was measured with a SpectraMax M5 Microplate Reader (Molecular Devices, Sunnyvale, CA; USA) using SoftMax Pro software (Table 4-1).

4.2.5 Oligonucleotide fraction collection

A random oligonucleotide library (Sigma-Aldrich, St. Louis, MO; USA) served as an ssDNA pool. The 80 base sequence, 5′-AGCAGCACAGGTCAGATG-N(40)-CCTATGCGTGCTACCGTGAA-3′, was designed with priming regions for PCR amplification at both the 5′- and 3′- ends flanking a random region allowing up to 4⁴⁰ sequences within the pool. No fluorescent tag was attached to this DNA pool.

The library was injected for 5 s at 5 kV onto a preconditioned 45 cm capillary. The separation running buffer was 10 mM sodium tetraborate/HEPES (Sigma-Aldrich, St. Louis, MO; USA), and the separation was performed at 15 kV.

Hard-shell, white well, 96-well PCR plates (Bio-Rad) were used for fraction collection. A fraction width of 7 s was determined by reference data obtained by laser-
TABLE 4-1

SETTINGS APPLIED BY SOFTMAX PRO SOFTWARE FOR TAMRA FLUORESCENCE READINGS ACROSS A 96-WELL PLATE EMPLOYING A SPECTRAMAX M5 MICROPLATE READER

<table>
<thead>
<tr>
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<td>Settling time</td>
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</table>

induced fluorescence detection. The valve pulse width was set to 0.05 s, precisely dispensing valve buffer. To limit sample handling and sample loss, the valve buffer reservoir contained all necessary real-time PCR reagents per the manufacturers protocol; reagents and final concentrations for 10 μL reactions were iTaq Universal SYBR Green supermix (1X) and forward and reverse primers (300 nM each). I deviated from the recommended protocol and diluted the PCR reagents in separation buffer instead of nuclease free water to achieve uninterrupted separation. Fraction collection and electrophoresis began simultaneously.
After fraction collection, the 96-well PCR plate was sealed and centrifuged, bringing the deposited fractions to the bottom of the wells for amplification. The PCR protocol was designed to optimize the reaction based on the annealing temperatures of the forward (5’-AGCAGCACAGAGGTCAGATG-3’) and reverse (5’-TTCACGGTAGCAGCATAG-3’) primers and the pre-mixed components of iTaq Universal SYBR Green Supermix (Bio-rad); 95°C for 3 min followed by 40 cycles of 95°C for 30 s (denature), 56.7°C for 30 s (anneal), and 72°C for 30 s (extend). Following each extension, real-time fluorescence was measured in each well using a CFX96 Touch Real-Time PCR Detection System (Bio-rad).

4.2.6 Aptamer generation

A 10 μL aliquot of the 100 μM stock oligonucleotide library solution was added to 10 μL of binding buffer (50 mM TRIS, 100 mM NaCl, 1 mM CaCl₂). The mixture was heated to 94°C to destroy secondary structures that may have formed during storage. The solution was cooled by 0.5°C/s to a final temperature of 20°C in a thermal cycler (PTC-100, MJ Research). The heat-treated 10 μM random ssDNA library solution was incubated at room temperature with 1 mg/mL human α-thrombin protein (Hematologic Technologies, Inc., Vermont; USA) for a minimum of 15 min to allow binding.

Fractions were collected and amplified as in Section 2.5. Post amplification, pooled wells containing the target were submitted to the Genomics & Bioinformatics Core Facility at the University of Notre Dame (Notre Dame, IN; USA). In preparation for deep sequencing analysis, an Illumina library was constructed on the submitted sample.
Sample quality was verified via a bioanalyzer trace; the majority of the material present was 214 bp. I use Illumina's TruSeq Universal Adapter 5’-

AATGATACGGCGACCAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT and TruSeq Adapter, Index 6 5’-

GATCGGAAGAGCACACGTCTGAACTCCAGTCACGCCAATATCTCGTATGCCGTCTTCTGCTTG.

Sequencing was performed using a MiSeq nano flow cell for a single 140 bp read, and the sample was spiked with 25% PhiX control to generate library diversity. The MiSeq run generated over 1 million high quality reads. The sequences were compared with the established thrombin-binding aptamer sequence.

4.3 Results and discussion

4.3.1 Evaluation of the fraction collector using a fluorescent plate reader to detect TAMRA and its impurities separated by capillary zone electrophoresis

I first evaluated the system by separating TAMRA from trace level impurities, Figure 4-2. I employed an ultrasensitive laser-induced fluorescence detector, which was sampled at 50 Hz (blue trace). In a separate experiment, the same capillary was coupled to our fraction collector for separation of a much higher concentration (1 mM) sample; fractions were collected in a 96 well microtiter plate at four second intervals and fluorescence intensity was measured off-line using a fluorescent plate reader (green trace). High TAMRA concentration was required because of the low sensitivity plate reader used in this experiment, and saturation of the detector limited its dynamic range.
Figure 4-2: Electropherograms produced by the plate reader (green) and laser-induced fluorescence (blue) for the analysis of TAMRA solutions. Insert shows the full-scale electropherograms. A two-point algorithm was used to bring the laser-induced fluorescence electropherogram into alignment with the plate-reader electropherogram (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

A simple two-point algorithm was used to bring the traces into alignment\textsuperscript{107}; the offset between the traces was minor and likely reflects electric field distortions caused by the high concentration TAMRA peak.

The electropherograms consist of a strong peak generated by TAMRA and two much lower intensity peaks due to fluorescent impurities present in the dye. The signal generated from the microtiter plate reader nicely matches that of the laser-induced fluorescence signal.
I fit a Gaussian function to the TAMRA peak according to Eq. 4-1

\[ \text{Gaussian}(t) = a \times e^{-0.5(t-t_0)/\text{sigma}^2} \]  

where \( t \) is time, \( a \) is peak amplitude, \( t_0 \) is the migration time, and \( \text{sigma} \) is the peak width. The fraction collector was reasonably reproducible; the relative standard deviation in peak amplitude was 20% and the relative standard deviation in migration time was 1% (\( N = 3 \)), which are typical values in capillary zone electrophoresis using manual sample injection. The mean and standard deviation of the tetramethylrhodamine peak width was 5 ± 1 s and was likely limited by our 4-s spot deposition period.

4.3.2 Evaluation of the system using real time PCR to detect oligonucleotides

I injected a random pool of oligonucleotides into the CZE system and captured fractions in a microtiter plate. I placed enzyme and primers in the sheath fluid of the fraction collector, which eliminated the need for subsequent addition of the real-time PCR reagents to the microtiter plate. Real-time PCR was used to quantify the amount of oligonucleotide deposited in each well, Figure 4-3.

Figure 4-3 presents two sets of traces. The first set consists of the converted CT values, where the signal = \( 1/2^{\text{CT}} \), and provides an estimate of the total amount of DNA deposited in each well. The traces for the oligonucleotide-thrombin mixture and the control sample are quite similar. This similarity is expected because only a small fraction of the oligonucleotide pool will complex with thrombin and generate a mobility shift.
Figure 4-3: Electropherograms from real-time PCR analysis of an 80-mer oligonucleotide mixture that contains a random 40-mer insert (blue), and the 80-mer oligonucleotide mixture pre-incubated with thrombin to promote aptamer binding (green). Left panel: The $C_T$ values reconstruct the electropherogram and should map the DNA content. $C_T$ values are reported for each well that contains amplified template greater than the software-determined threshold. This threshold can cause wells containing only trace amounts of amplified template to go unreported. Right panel: The endpoint signal reports the relative fluorescence signal (Endpoint RFU Signal Intensity) after 40 cycles of PCR amplification. The endpoint signal is a powerful test for the presence of any of the oligonucleotide mixture, irrespective of concentration (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

A much more powerful tool for identifying the presence of DNA-thrombin complexes employs the endpoint signal after 40 cycles; this amount of amplification is sufficient to detect a very small number of molecules of the oligonucleotide pool. Real-time PCR signals reach an endpoint signal of reasonable intensity if any amplifiable DNA is present.

Figure 4-3 results in two important observations. First, the essentially zero signal before 6 min in the oligonucleotide-thrombin mixture, and before 7.5 min in the control
mixture demonstrates the extraordinarily low background signal generated by this interface, which is essentially contamination free.

Second, there is a ~1-minute window between 6.5 and 7.5 min that generates appreciable endpoint signal for the oligonucleotide-thrombin mixture but no signal for the oligonucleotide pool itself. It is these oligonucleotides that are expected to contain aptamers that bind to the target molecule.

4.3.3 Thrombin aptamers generated using CE-SELEX.

Electropherograms were generated by capillary electrophoresis and laser-induced fluorescence detection of both the fluorescently labeled ssDNA library (blue trace) and the library bound to thrombin (green trace), Figure 4-4. A two-point algorithm was used to bring the traces in alignment. The uncomplexed library begins to migrate at 8 min. The binding of an aptamer to human α-thrombin protein molecule produces a migration shift that deviates from the unbound library sequences; the most prominent display of this effect occurs at 7 min.

I repeated the separation of the DNA-thrombin mixture using an unlabeled library and fraction collection in a 96-well plate. To simplify subsequent analysis, PCR reagents were employed as the sheath liquid. After fraction collection, the plate was sealed and centrifuged before placing it into the real-time PCR instrument. Figure 4-3 is a reconstruction of the real-time PCR data. In contrast to our control data (blue trace), I observe by end-point fluorescence signal that trace amounts of sequences have shifted in migration in the DNA-thrombin mixture (green trace). Based on the real-time
Figure 4-4: Capillary electrophoresis-laser-induced fluorescence detection of unbound library and library bound to thrombin. The thrombin-bound library shows several new peaks migrating in the 7–8 min region corresponding to CE-SELEX aptamers bound to thrombin. Green trace is the thrombin bound library; blue trace is the unbound library. The bar at the top of the trace indicates the collection window used for sample collection in the CE-SELEX experiment (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

PCR data combined with the CE-LIF data of Figure 4-4, I pooled the contents of the wells spanning 1 min prior to the uncomplexed library peak; the pooled contents were then submitted for deep sequencing.

4.3.4 Aptamer sequencing and validation

Preprocessing extracted roughly 800,000 reads from the original data set of 1,082,975 sequences. Using Perl program code, the ligated adapter sequences were trimmed, followed by the priming regions. The desired sequences of lengths 38–42 bases, representing the random region, were selected for analysis; this subset encompasses approximately 98% of the high quality reads. I assume 4 or more instances
of neighboring guanines constitute an exact or near-exact match based on the three-dimensional structure of the thrombin-binding DNA aptamer\textsuperscript{108}; 48,000 sequences contain four or more ‘GG’. The data were transformed into FASTA format and mapped against the established 15-mer\textsuperscript{109} and 29-mer\textsuperscript{110} thrombin-binding aptamer (TBA) sequences utilizing the Burrows-Wheeler alignment tool (BWA).\textsuperscript{111} BWA fastmap analysis returned 10,534 sequence matches to the 15-mer TBA and 17,606 matches to the 29-merTBA, omitting duplicate matches found on a single read. MEME motif discovery tool (version 4.9.1) was also used to detect enriched sequences/motifs to confirm results.\textsuperscript{112}

Roughly 6% of the sequences are consistent with known thrombin-binding aptamers. Higher stringency fraction collection, additional rounds of PCR amplification, and additional selection cycles would undoubtedly lead to a larger fraction of target binding aptamers generated.

4.4 Conclusions

I report a flexible and low-cost fraction collector for preparative capillary zone electrophoresis. I demonstrated its application to the isolation of putative aptamers against thrombin. The fraction collector provides reasonable separation efficiency and modest sample carryover. However, this fraction collector has two obvious limitations. First, sample is subjected to over 1000-fold dilution during fraction generation. Second, the use of a 96 well microtiter plate limits the number of fractions that can be collected and the temporal resolution of fraction collection. Fortunately, it is trivial to adjust the
spotter to match the footprint of higher density microtiter plates, such as those containing 384 or 1536 wells.

4.5 Acknowledgments

I gratefully acknowledge support from the National Institutes of Health (5R01GM096767). I thank the University of Notre Dame's Genomics Core Facility for sequencing services and Dr. Jun Li in the Department of Applied and Computational Mathematics and Statistics for assistance with data preprocessing. This material is based upon work supported by the National Science Foundation Graduate Research Fellowship under Grant no. DGE-1313583.
5.1 Introduction

Capillary-zone electrophoresis – electrospray ionization – mass spectrometry (CZE – ESI – MS) is attracting renewed interest. This interest is stimulated by the development of high-sensitivity interfaces for coupling CZE to MS. In 2007 Moini developed a sheathless interface that employed a porous capillary tip as the nanospray emitter. Several research groups have used Moini’s sheathless interface for bottom-up and top-down proteomics. For example, the Yates group coupled CZE to an Orbitrap Elite mass spectrometer through a sheathless CZE-ESI interface for top-down profiling of the *Pyrococcus furiosus* proteome.

Other interfaces have been reported for coupling CZE with mass spectrometry. In 2010, Chen’s group developed a flow-through microvial interface that has been used for metabolite, glycan, and intact protein analysis. Tang’s group developed a sheathless CZE-MS interface that utilized a large inner diameter separation capillary and a detachable smaller inner diameter porous ESI emitter. This design significantly
improved the concentration detection limit for peptides by producing a large sample loading volume and stable nanoESI operation.

Our group has described three generations of electrokinetically pumped sheath-flow nanospray interfaces. These interfaces employ electroosmotic flow inside of the glass emitter to drive sheath fluid at nanoliter/minute rates, generating stable and robust electrospray.

A common issue is present in all CZE electrospray interfaces. One power supply is connected to the proximal end of the separation capillary and another is applied at the electrospray interface. The separation is driven by the difference in potential between these power supplies. The power supply connected to the electrospray interface has two roles. One role is to drive electrospray towards the (usually) grounded mass spectrometer inlet, and the other role is to complete the circuit for electrophoresis.

Conventional high voltage power supplies used for electrospray are sources of current but are unable to sink current. This inability to sink current leads to limitations on experimental parameters such as capillary length, buffer ionic strength, separation voltage, and spray voltage. Under low current conditions, the power supply responsible for the spray voltage operates as a current source and that supply’s control circuit holds the electrospray emitter at the desired voltage. However, when the capillary electrophoresis system is operated under relatively high current conditions (high separation electric field, high ionic strength separation buffer, short capillary length, and large inner diameter capillary), a situation can arise wherein the current flowing through the capillary is greater than the current generated by the power supply responsible for
the spray voltage. In this case, the electrospray voltage is not controlled by the power supply responsible for the spray voltage and instead floats to a higher value, where the capillary and electrospray act as voltage dividers.

As explained by Hau et al.\textsuperscript{127}, the CZE circuit can approach tens of microamperes of current, while the electrospray current leaving the sprayer is typically hundreds of nanoamperes.\textsuperscript{128} The current provided by the CZE circuit can cause the sprayer voltage to deviate from its programmed value. The liquid-filled capillary forms a voltage divider that leads from the CE power supply to the ESI power supply and ultimately to ground. In general, the observed sprayer potential depends primarily on the ratio of the electrical resistance between the capillaries, which is, in turn, a function of the conductivity of the electrolyte solution and of the length and inner diameter of the capillaries.

When the electrospray voltage is not accurately controlled, the sensitivity, reproducibility, and detection limit of the instrumentation suffers. To avoid the current-sinking challenge our group has performed separations with smaller inner diameter capillaries (≤ 30 μm i.d.), long capillaries (45 – 100 cm), lower ionic strength separation buffers (e.g. 0.1% formic acid), and reduced separation voltage. If the current-sinking challenge is removed, then the researcher is given greater freedom in experimental conditions. Shorter capillaries with larger inner diameters may be employed while using a higher conductivity separation buffer, which enables faster separations and larger loading amounts.
The current-sinking challenge was identified two decades ago and persists to this day. Two practical solutions for this problem have been identified. The first is to float the CE circuitry on the electrospray voltage, which would result in the effective capillary voltage no longer depending on the electrospray voltage. However, this first approach would increase the cost of the setup due to the need for additional safety precautions, such as insulated electronics on the CE instrument. The second approach requires that the electrospray power supply act not only as a current source but also as a current sink. This second approach is not without its own challenges. I are not aware of a commercially available, off the shelf high voltage power supply that is capable of sinking the current generated by capillary electrophoresis. Therefore, some fabrication is required to safely assemble an electrospray power supply capable of sinking current. I report one such electrospray power supply and demonstrate its capabilities compared to a Spellman CZE-1000R, which is the most common electrophoresis power supply employed by research groups in the field.

5.2 Experimental section

5.2.1 Materials and reagents

Acetic acid and hydrofluoric acid (HF) were purchased from Fisher Scientific (Pittsburgh, PA; USA). Bovine serum albumin (BSA), angiotensin II (human, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe), and formic acid (FA) were purchased from Sigma-Aldrich (St. Louis, MO; USA). Methanol and water were purchased from Honeywell Burdick & Jackson (Wicklow, Ireland). Fused silica capillary was purchased from Polymicro Technologies
(Phoenix, AZ; USA). Electrospray emitters were borosilicate glass capillary (1.0 mm o.d., 0.75 mm i.d., and 10 cm in length, Item #: B100-75-10) from Sutter Instrument (Novato, CA; USA) that was pulled with a Sutter Instrument P-1000 flaming/brown micropipette puller.

5.2.2 Methods

5.2.2.1 General instrumentation configuration

An LTQ-XL mass spectrometer (Thermo Fisher Scientific) was used for all experiments. Only MS1 spectra were acquired. The scan range of the ion trap mass analyzer was m/z 350 – 1800. The separation power supply was a Spellman CZE-1000R (Hauppauge, NY; USA). Our group has traditionally used an additional Spellman CZE-1000R as the electrospray power supply. The Spellman used as the electrospray power supply will be referred to as the Spellman power supply. An alternative electrospray power supply was fabricated in-house using an HVM Technologies CHV0017 high voltage amplifier (New Braunfels, TX; USA). The HVM amplifier used as an electrospray power supply will be referred to as the HVM amplifier.

5.2.2.2 Initial power supply evaluations

A 60 cm bare fused silica capillary (50 μm i.d., 150 μm o.d.) was used to initially test the capabilities of the HVM amplifier and the Spellman power supply. The distal end of this separation capillary was not etched by HF. The electrospray emitter used in this initial experiment had an emitter opening of 10 μm. The sheath buffer used was 0.1%
(v/v) FA in water containing 10% (v/v) methanol. Three different separation buffers were used to evaluate the capabilities of the HVM amplifier and the Spellman power supply. The first separation buffer used was 0.1% (v/v) FA, the second separation buffer used was 5% (v/v) acetic acid, and the third separation buffer used was 0.5% (v/v) FA.

Electrophoresis was controlled by LabVIEW software. The injection end of the separation capillary and an electrode were fixed in an injection block. The electrode provided high voltage for CE separation. A gas line was connected to the injection block to provide pressure for capillary flushing and sample injection; no pressure was used during separation. A gas regulator was used to control the gas pressure. The base plate of the injection block contains a recessed compartment for a trimmed PCR tube. No sample was injected during the initial power supply evaluations.

To test the ability of the HVM amplifier and the Spellman power supply to sink current, a constant spray voltage was applied while gradually increasing the separation voltage applied at the injection block. This procedure was done for each separation buffer listed above. Spray voltage was measured in the vial that supplied the sheath buffer using a Fluke 80K-40 HV Probe (Everett, WA; USA). Prior to applying the separation voltage, a spray voltage was set and measured with the high voltage probe. Once the spray voltage was set, the separation voltage was applied and the spray voltage was recorded. The spray voltage was set at 1.4 kV throughout the initial power supply evaluations while the separation voltage was gradually increased from 0 kV to 30 kV in 2 kV increments.
5.2.2.3 BSA sample preparation

BSA in 100 mM NH₄HCO₃ (pH 8.0) containing 8 M urea was denatured at 37°C for 30 min, followed by standard reduction and alkylation with DTT and IAA. After dilution with 100 mM NH₄HCO₃ (pH 8.0) to reduce the urea concentration below 2 M, protein digestion was performed at 37°C with trypsin at a trypsin/protein ration of 1/30 (w/w) for 12 h. After acidification with FA, the protein digest was desalted with a C18-SepPak column (Waters, Milford, MA; USA) and then was lyophilized with a vacuum concentrator (Thermo Fisher Scientific, Marietta, OH; USA). The dried protein digest was stored at −20°C before use.

5.2.2.4 20 μm i.d. capillary experimental conditions

The separation capillary had a length of 31 cm, an i.d. of 20 μm, and an o.d. of 150 μm. Approximately 5 mm of the distal end of the separation capillary was etched using HF to an o.d. of ~45 μm. Details of capillary etching with HF were previously reported. The separation capillary was etched to decrease the distance between the distal end of the capillary and the emitter opening, increasing sensitivity. The electrospray emitter used in this experiment had an emitter opening of 20 μm. The sheath buffer used in the electrospray emitter was 0.1% (v/v) FA in water containing 10% (v/v) methanol. The separation buffer was 5% (v/v) acetic acid. The separation voltage was 27 kV and the spray voltage was 1.6 kV.
5.2.2.4.1 Injection conditions

Injection conditions were calculated using Eq. 5-1:

\[ P \times S = \frac{3200 \times L \times C}{D^2} \]

Eq. 5-1

where \( P \) is the pressure in mbar (1 mbar \( \approx \) 0.015 psi), \( S \) is the injection time in seconds, \( L \) is the injection length in mm, \( C \) is the capillary length in cm, and \( D \) is the capillary inner diameter in \( \mu \)m. The injection conditions used were 2.0 seconds at 10 psi, which gave an injection length of 5.4 mm. The first sample used was a BSA digest diluted in 0.1% (v/v) FA to a concentration of 0.5 mg/mL. The second sample used was angiotensin II diluted in 0.1% (v/v) FA to concentrations of 20, 10, 5, and 2 \( \mu \)M. Data was collected in triplicate.

5.2.2.5 50 \( \mu \)m i.d. capillary experimental conditions

The separation capillary had a length of 31 cm, an i.d. of 50 \( \mu \)m, and an o.d. of 150 \( \mu \)m. Approximately 10 mm of the distal end of the second capillary was etched using HF to an o.d. of \( \sim \)65 \( \mu \)m. The separation capillary was etched to decrease the distance between the distal end of the capillary and the emitter opening. The electrospray emitter, sheath buffer, and separation buffer were identical to those used in the 20 \( \mu \)m i.d. capillary experiment.

An initial test was done with each electrospray power supply. The initial test sought to determine the maximum separation voltage that could be applied to the separation capillary before the spray voltage was no longer controlled by the electrospray power supply. The spray voltage was set to 1.7 kV. The maximum voltage
applied by the separation power supply that would not alter the spray voltage for the HVM amplifier and the Spellman power supply was 19 kV and 11 kV, respectively.

Three experimental conditions were determined as a result of the initial test. These conditions are outlined below in Table 5-1. In each of the conditions the spray voltage was set to 1.7 kV prior to applying a separation voltage. The first condition used the HVM amplifier with a separation voltage of 19 kV. The second condition used the Spellman power supply with a separation voltage of 11 kV. These first two conditions demonstrate the capabilities of each of the electrospray power supplies at the maximum voltage applied by the separation power supply that would not alter the spray voltage. The third condition used the Spellman power supply with a separation voltage of 19 kV and provides a comparison of the ability of the two electrospray power supplies to sink current.

**TABLE 5-1**

**SEPARATION AND ELECTROSPRAY VOLTAGES FOR 50 µM I.D. CAPILLARY**

<table>
<thead>
<tr>
<th>Electrospray Power Supply Used</th>
<th>Separation Voltage (kV)</th>
<th>Spray Voltage (kV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HVM Amplifier</td>
<td>19</td>
<td>1.7</td>
</tr>
<tr>
<td>Spellman Power Supply</td>
<td>11</td>
<td>1.7</td>
</tr>
<tr>
<td>Spellman Power Supply</td>
<td>19</td>
<td>1.7</td>
</tr>
</tbody>
</table>
5.2.2.5.1 Injection conditions

Injection conditions were calculated using Equation 1 to maintain a constant injection length of 5.4 mm. The injection conditions used were 0.8 seconds at 4 psi. The samples used for the 50 μm i.d. capillary experiment were identical to those used for the 20 μm i.d. capillary experiment. The first sample used was a BSA digest diluted in 0.1% (v/v) FA to a concentration of 0.5 mg/mL. The second sample used was angiotensin II diluted in 0.1% (v/v) FA to concentrations of 20, 10, 5, and 2 μM. Data was collected in triplicate.

5.3 Results and discussion

5.3.1 Initial power supply evaluations

I first evaluated the performance of the two electrospray power supplies with separation buffers of varying conductivity while maintaining a constant sheath buffer composition. The separation buffers, in order of increasing conductivity, were 0.1% (v/v) FA, 5% (v/v) acetic acid, and 0.5% (v/v) FA. The sheath buffer was 0.1% (v/v) FA in water containing 10% (v/v) methanol throughout the evaluation. A spray voltage of 1.4 kV was applied to and measured in the sheath buffer reservoir using a high voltage probe prior to applying a separation voltage. The spray voltage setting was not changed while increasing the applied separation voltage. The ability of the HVM amplifier and Spellman power supply to sink current was evaluated by measuring the spray voltage present in the sheath buffer reservoir while gradually increasing the separation voltage. The results are summarized in Figure 5-1. A positive deviation from 1.4 kV indicated that the
electrospray power supply failed to sink the current resulting from the separation parameters.

Figure 5-1: Spray voltage measured versus separation voltage applied for 0.1% FA (a), 5% acetic acid (b), and 0.5% FA (c). The solid (blue) trace corresponds to the Spellman power supply and the dashed (red) trace corresponds to the HVM amplifier.

The HVM amplifier is capable of sinking more current than the Spellman power supply. Figure 5-1 clearly illustrates that the HVM amplifier (red, dashed line) maintains the applied spray voltage of 1.4 kV throughout the 0.1% FA and 5% acetic acid evaluations. Only when the highest conductivity buffer, 0.5% FA, is used does the HVM amplifier fail to maintain the 1.4 kV spray voltage throughout the evaluation. Even though the HVM amplifier fails to sink the current throughout the 0.5% FA evaluation, it maintains the 1.4 kV spray voltage for an additional 10 kV of separation voltage past where the Spellman power supply (solid, blue line) fails.
5.3.2 20 µm i.d. capillary experiment

Following the initial evaluation of the electrospray power supplies I compared electropherograms generated using the HMV amplifier and Spellman power supply. The first sample that was tested was a BSA digest at a concentration of 0.5 mg/mL. The base peak electropherograms for the HVM amplifier and Spellman power supply are shown in Figure 5-2. The data from each electropherogram was smoothed with a 5-point Gaussian convolution. While the base peaks for the two power supplies differ, the overall profile of the BSA digest electropherograms is similar.

![Figure 5-2: Base peak electropherograms of 0.5 mg/mL BSA digest with the HVM amplifier (a) and the Spellman power supply (b).](image)

The next sample was angiotensin II at concentrations of 20, 10, 5, and 2 µM. Electropherograms of 2 µM angiotensin II are presented in Figure 5-3 for the HVM amplifier (Fig. 3a) and the Spellman power supply (Fig. 3b). The data from each
electropherogram was smoothed with a 5-point Gaussian convolution. The peak intensities and migration times are very similar between the two power supplies.

![Electropherograms](image)

Figure 5-3: Electropherograms from 2 µM angiotensin II with the HVM amplifier (a) and the Spellman power supply (b).

Unweighted least squares plots were linear for both the HVM amplifier (slope = $1.799 \times 10^6$, $R = 0.9920$) and the Spellman power supply (slope = $2.088 \times 10^6$, $R = 0.9940$). The $y$-intercepts for the HVM amplifier and the Spellman power supply were equal to zero within experimental error.

5.3.3 50 µm i.d. capillary experiment

After the 20 µm i.d. capillary experiment, I tested the limits of the HVM amplifier and the Spellman power supply by repeating the experiment with a 50 µm i.d. capillary and an additional voltage condition. The increased inner diameter of the separation capillary provides greater than a six-fold increase in capillary cross-section, which results in a proportional increase in current flow through the capillary.
In the initial test of the 50 µm i.d. capillary, the Spellman power supply was not able to sink current above a separation voltage of 11 kV. The difference between the HVM amplifier and the Spellman power supply is illustrated most dramatically when comparing the electropherograms from the 2 µM angiotensin II runs at the three voltage conditions in this experiment (Figure 5-4).

![Electropherograms](image)

**Figure 5-4:** Electropherograms of 2 µM angiotensin II with the HVM amplifier and a separation voltage of 19 kV (a), the Spellman power supply and a separation voltage of 11 kV (b), and the Spellman power supply and a separation voltage of 19 kV (c).

The electropherogram generated by the HVM amplifier and a separation voltage of 19 kV (Fig. 4a) results in a migration time just over two minutes and a base peak intensity of roughly 800,000. The electropherogram generated by the Spellman power supply and a separation voltage of 11 kV (Fig. 4b) results in a significantly later migration time (~ six minutes) with similar base peak intensity. The electropherogram generated by the Spellman power supply and a separation voltage of 19 kV (Fig. 4c) results in a higher background and a lower base peak intensity of approximately 525,000. In Fig. 4c...
the migration time of angiotensin II is identical to that of Fig. 4a where the HVM amplifier was used under exactly the same conditions. However, the base peak intensity in Fig. 4a is approximately 50% greater and the background is roughly one third of what is seen in Fig. 4c.

Unweighted least squares calibration curves were linear for the HVM amplifier (slope = 1.4 x 10^6, R = 0.9945), the Spellman power supply with a separation voltage of 11 kV (slope = 1.5 x 10^6, R = 0.9962), and the Spellman power supply with a separation voltage of 19 kV (slope = 6.12 x 10^5, R = 0.9967). The y-intercepts for all three plots were equal to zero within experimental error.

5.4 Conclusion

Spellman CZE-1000R power supplies are used widely for capillary electrophoresis separations and have been used extensively by our group as an electrospray power supply. However, as illustrated in this work, the Spellman power supplies are not well suited for fast separations that employ high electric fields with high ionic strength separation buffers. Our group identified this limitation in a publication that characterized our third-generation electrokinetically pumped sheath-flow nanospray interface. The HVM amplifier is clearly capable of sinking current and is marketed as such. I did find the limit of where the HVM amplifier fails to sink the current resulting from the separation voltage (Fig. 1c). Overall, the HVM amplifier is well suited for fast separations that employ high electric fields and high ionic strength separation buffers.
5.5 Acknowledgements

I thank Dr. William Boggess in the Notre Dame Mass Spectrometry and Proteomics Facility for his help with this project. This project was supported by a grant from the National Institutes of Health (R01GM096767).
5.6 Supplemental information

Figure 5-5: Wiring schematic of the fabricated HVM amplifier.
6.1 Introduction

Antibodies are an important part of the immune system. They are produced by lymphocytes to recognize and bind specific target molecules called antigens, which are usually of foreign origin. When an antigen is identified, antibodies are raised to a unique site on the molecule known as an epitope. Antibodies are roughly Y-shaped proteins that consist of two heavy chains and two light chains that are held together by multiple disulfide bonds. Within the structure of antibodies there are two major domains, the variable domain and the constant domain (Figure 6-1). Antibodies accomplish target (antigen) binding through variations in the variable domains. The variable domains have three complementarity-determining regions that are responsible for antibody binding mediation.\textsuperscript{130}

Over the past three decades, medical research has been transformed by the use of monoclonal antibodies in the diagnosis and treatment of human disease.\textsuperscript{131,132} The first monoclonal antibody to be approved by the Food and Drug Administration (FDA) for use in humans was an antirejection agent for organ transplant patients, Orthoclone
Figure 6-1: Representation of Immunoglobulin G (IgG), the most abundant antibody in the human body.

OKT3. Since the approval of Orthoclone OKT3 in 1986 dozens of monoclonal antibodies have been approved for use on diseases such as infections, autoimmunity, and cancer. However, some inherent properties of antibodies have proven to be significant disadvantages in clinical applications. These disadvantages include: aggregation at high concentration, immunogenicity, toxicity, high cost associated with mass production, batch-to-batch variability, instability at elevated temperatures and the target molecule must elicit an immune response.

In the 1980s, while studying HIV and adenovirus, researchers discovered that nucleic acids were capable of joining with proteins as a form of molecular recognition. The nucleic acids were termed “aptamers”, from the Latin word aptus, meaning “fitting” and the Greek word meros meaning “particle”. Further characterization would show that the binding affinities of aptamers are comparable to those observed for antibodies, both being in the low nanomolar to picomolar range. However, aptamers do not share any of the previously mentioned draw-backs described for antibodies. Aptamers are
capable of recovering their native conformation after a temperature insult and can maintain affinity for their target(s) upon re-annealing; production of aptamers is remarkably reproducible and extremely cost effective due to the synthesis methods being purely chemical in nature; there has been little to no evidence of immunogenicity from aptamers; and finally, aptamers can be generated against any target, regardless of the immunogenicity of the species.

Aptamers can be either ribonucleic acids (RNA) or deoxyribonucleic acids (DNA) and are typically single stranded (ssRNA or ssDNA). Generally, “RNA offers the possibility of intracellular expression, whereas DNA is more stable”\textsuperscript{135} Aptamers accomplish target binding by forming stable three-dimensional (3D) structures. These 3D structures have been described and include hairpins\textsuperscript{136}, pseudoknots\textsuperscript{137}, or the G-quadruplex\textsuperscript{138} to name a few (Figure 6-2).

The first method for generating aptamers against a target molecule was described in 1990 and is known as SELEX, or Systematic Evolution of Ligands by Exponential Enrichment. SELEX is a combinatorial chemistry method of in vitro selection for functional molecules (e.g. proteins). The SELEX process begins with an initial library, which consists of oligonucleotides of a specific length. These oligonucleotides contain a random region of nucleotides that is flanked on either side by priming regions of known sequence. The random region gives rise to a theoretical library diversity of $4^N$, where $N$ is the number of nucleotides in the random region. After the library is generated it is introduced to the target(s). Oligonucleotides with little to no affinity for the target(s) are out competed by those of higher affinity. The library-target mixture is then subjected to
Figure 6-2: Schematic secondary structures of: (A) hairpin, (B) pseudoknot, and (C) G-quadruplex. Adapted from Radom et al., 2013.\textsuperscript{135}

Figure 6-3: SELEX begins with a randomized nucleic acid library. The library undergoes an iterative process of binding, elution, and amplification.\textsuperscript{139}
a separation step. Unbound oligonucleotides are discarded. The bound oligonucleotides are collected, amplified, and reintroduced to the target(s) for another round of SELEX (Figure 6-3). The iterative process is repeated until only the highest affinity oligonucleotides remain.

The iterative nature of the original SELEX process was time consuming and labor intensive. A promising improvement upon the SELEX method has emerged with the coupling of capillary electrophoresis to SELEX (CE-SELEX). In CE-SELEX, an initial library is still used and introduced to target(s) as described above. The separation of bound and unbound oligonucleotides is performed using capillary zone electrophoresis (CZE), which separates analytes based on their size-to-charge ratio. This is a major improvement upon the original SELEX methodology that used nitrocellulose filter paper to immobilize target molecule(s) and required several manual washing steps to remove unbound oligonucleotides. In CZE oligonucleotides migrate as a relatively tight band regardless of sequence length. Therefore, any peaks that exhibit a shift from the bulk library peak would be indicative of oligonucleotides that are bound to a target molecule and could be targeted for further rounds of SELEX or characterization. Additional benefits of CE-SELEX are that CE can be easily automated, separation of bound and unbound aptamers is rapid and efficient, and CE-SELEX is capable of generating high affinity aptamers in 1 to 3 rounds of selection, whereas traditional SELEX often requires 10 or more rounds of selection. While CE-SELEX was a drastic improvement on the traditional SELEX methodology, CE-SELEX remains a serial process, which prevents the method from being used for high through-put applications.
To improve upon the accomplishments of CE-SELEX, I designed an instrument to overcome the serial nature of the SELEX process. Briefly, a mixture of proteins can be separated. Fractions of the separated proteins can be mixed with an oligonucleotide pool and CE-SELEX can be performed on the fractions to generate aptamers against a set of proteins in a single run. Our approach involved fabricating an interface that brings together three capillaries. A schematic of the interface is shown in Figure 6-4.

![Schematic of the interface](image)

Figure 6-4: Schematic of the 2D-CE-SELEX interface. Shown is the nicked sleeve capillary, which has been nicked twice. Each nick lines up with a buffer inlet tube that can be flowed to waste between runs.

The first capillary separates proteins or other target molecules, the second capillary is a short 1 cm section that serves as a mixing chamber where targets are mixed with the oligonucleotide pool, and the third capillary separates bound aptamer sequences from the bulk unbound nucleic acid library. I have termed this process 2D-CE-SELEX. Upon exiting the distal end of the third capillary effluent is deposited into a collection vessel, such as microtiter plate via a fraction collector I developed[^142], maintaining the separation accomplished by CE.
6.2 Materials and methods

6.2.1 Materials and reagents

Fused silica capillary (50 μm ID, 150 μm OD; and 152 μm ID, 650 μm OD) was purchased from Polymicro Technologies (Phoenix, AZ; USA). Reagents were analytical grade and purchased from Sigma-Aldrich (St. Louis, MO; USA). All solutions were prepared from deionized-distilled water obtained from a Barnstead Nanopure System (Thermo-Fisher Scientific, Waltham, MA; USA). The separation buffer was 10 mM sodium tetraborate, 10 mM HEPES.

6.2.2 Laser-induced fluorescence detection

The detection scheme was similar to other I have reported. Briefly, fluorescence was excited using a CW 532 nm diode-pumped laser (CrystaLaser Model CL532-025), which was focused into the sample stream at the center of a sheath-flow cuvette. Fluorescence was collected at right angles and detected by a single-photon counting avalanche photo diode (PerkinElmer, Montreal, PQ; Canada) with a sampling frequency of 50 Hz.

6.2.3 Electrophoresis with fluorescently labeled thrombin-binding aptamer

Initially, the operation of the 2D-CE-SELEX interface was performed with a 10 nM solution of fluorescently labeled thrombin-binding aptamer (TBA). In this initial test no target was present for binding. The thrombin-binding aptamer used was the 15-mer (TBA-15) that has the sequence GGTTGGTGTGGTTGG. The purpose of this initial test was
to evaluate the operation of the 2D-CE-SELEX interface and optimize conditions for online binding of TBA-15 to its target, human α-thrombin.

Interface fabrication and assembly was improved further after the nicked-sleeve interface that was discussed earlier. The nicked-sleeve interfaces used a large sleeve capillary that had been nicked roughly halfway through its diameter as an aligning tool for the separation capillaries. The separation capillaries were then immobilized by placing a drop of epoxy (5 Minute Epoxy, ITW Devcon, Danvers, MA; USA) at the point where the separation capillaries exited the sleeve capillary.

A sleeve capillary (153 μm ID, 657 μm OD) that was approximately 4.0 cm in length was nicked as before and the polyimide coating was removed by a flame. An intact 80.0 cm separation capillary was inserted into and centered along the length of the sleeve capillary. Next, the UV light curing resin (KOA 300 Adhesive, Kemexert Corp., York, PA; USA) was applied at the sleeve capillary nick and the low viscosity of the liquid resin allowed it to fill the space between the separation capillary outer surface and the sleeve capillary inner surface. After the UV light curing resin filled the length of the sleeve capillary the assembly was placed under UV light to cure. Once cured the entire assembly was taken to the micro-dicing saw used previously and nicked using a diamond blade (thickness = 68 μm; Catalog Number 2.187-2.5A-22RU-3, Thermocarbon, Casselberry, FL; USA) twice with a distance between the nicks of 1.0 cm. The nicks in the capillary assembly were halfway through the inner diameter of the separation capillary, creating an aperture for electrical contact once the interface was assembled. A Plexiglas slide had been cut to receive two buffer lines, two waste lines, and the capillary
assembly. The dimensions of the channels in this Plexiglas slide were identical to those reported previously.\textsuperscript{143} Once the 2D-CE-SELEX interface was fully assembled, the separation capillary was trimmed on either side so that the distance between either nick and the proximate end of the separation capillary was 30.0 cm. The assembled 2D-CE-SELEX interface is diagramed in Figure 6-4 and does not show a difference between the sleeve and separation capillary for simplicity. The distance between the centers of the channels that were perpendicular to the capillary assembly was 1.0 cm to match the distance between the nicks in the capillary assembly. The 1.0 cm section between the two nicks served as a mixing chamber or incubator.

An instrumental scheme is shown in Figure 6-5 that shows how the power supplies were connected and where different buffer reservoirs were located with relation to the capillary assembly.

A solution of TBA-15 was introduced to the buffer line at Buffer 2 (Figure 6-5) by applying a negative pressure on the waste line opposite the buffer line at Buffer 2 while the end of the buffer line was submerged in a 10 nM solution of TBA-15. The buffer line then contained an abundance of TBA-15 for the experiment. The 2D-CE-SELEX interface was then primed for the experiment and a voltage program was used that filled the incubator with TBA-15 and successively transferred plugs from Capillary 1 to the Incubator and from the Incubator to Capillary 2. Table 6-1 shows the voltage program used in this experiment. The length of the separation step was chosen to allow adequate time for any bound target to migrate from the bulk aptamer band.
Figure 6-5: Instrumental scheme for performing 2D-CE-SELEX.

<table>
<thead>
<tr>
<th>Power Supply 1 (kV)</th>
<th>Power Supply 2 (kV)</th>
<th>Power Supply 3 (kV)</th>
<th>Time (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transfer</td>
<td>16.5</td>
<td>10.5</td>
<td>10.0</td>
</tr>
<tr>
<td>Separation</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
</tr>
</tbody>
</table>

6.2.4 Electrophoresis with fluorescently labeled thrombin-binding aptamer and thrombin

After initially testing the 2D-CE-SELEX interface with the 10 nM solution of fluorescently labeled thrombin-binding aptamer 15-mer (TBA-15), human α-thrombin
(thrombin) was introduced to the interface by constant infusion into the first separation capillary (Capillary 1). The fluorescently labeled TBA-15 was introduced at the first nick via the first buffer line. Thrombin was used at a concentration of 220 µg/mL and the TBA-15 was used at 100 nM. The increase in TBA-15 concentration was in an effort to ensure binding and excess TBA-15 in a given separation window to demonstrate the migration of bound TBA-15 away from the remaining, unbound TBA-15.

Using Figure 6-5 as a reference, Capillary 1 was filled with 220 µg/mL thrombin diluted in the separation buffer (10 mM sodium tetraborate, 10 mM HEPES); Buffer 2 contained 100 nM TBA-15 diluted in separation buffer; the Incubator was filled with unbound TBA-15 that awaited transfer of thrombin into the Incubator; and Capillary 2 separated bound TBA-15 from unbound TBA-15.

The voltage program used was identical to that used in Table 6-1. Due to the high concentration of TBA-15 in this experiment, a neutral density filter (NDUV10B, ThorLabs Inc., Newton, NJ; USA) was used to reduce the laser power by a factor of 10. Reducing the laser power effectively reduced the fluorescence to a range that was acceptable for the avalanche photodiode detector.

6.2.5 Data processing

Data processing was performed with Matlab on a PC. Data were treated with a three-point median filter to remove spikes generated by particulates passing through the laser beam.
6.3 Results and discussion

6.3.1 Electrophoresis with fluorescently labeled thrombin-binding aptamer

In this initial test, successful operation of the 2D-CE-SELEX interface was marked by periodic peaks in an electropherogram that corresponded to the fluorescently labeled TBA-15 with a low background signal between the peaks (Figure 6-6). The time between peaks, five minutes, was chosen to ensure adequate time for subsequent peaks that would migrate away from the unbound TBA-15 once the target (thrombin) was introduced to the interface.

Figure 6-6: 2D-CE-SELEX electropherogram with fluorescently labeled TBA-15.

The amount of time to allow between peaks can be determined by performing one-dimensional separations (CE-SELEX)\textsuperscript{104,141} on an aptamer that has been incubated with the intended target. Each five minute window in Figure 6-6 represents a single
“separation window” and the time should be chosen such that any bound peaks would occur in the same separation window as the parent, unbound aptamer peak from which they originate.

6.3.2 Electrophoresis with fluorescently labeled thrombin-binding aptamer and thrombin

When thrombin was introduced to the 2D-CE-SELEX interface an additional peak grew into prominence that was attributed to TBA-15 bound to thrombin (Figure 6-7). The separation mode used in this chapter was capillary zone electrophoresis (CZE). In CZE, a change in a molecule's size-to-charge ratio will result in a mobility shift. Since the TBA-15 is fluorescently labeled, the peaks that are seen in the electropherograms of this chapter must have been from the TBA-15. Upon binding to thrombin a drastic change to the size-to-charge ratio of TBA-15 would occur and therefore a shift in the migration time (mobility) of any TBA-15 bound to thrombin.

The results displayed in Figure 6-7 were promising and investigated further. Ultimately, the additional peak that grows into prominence was attributable to an artifact of the separation I believe was the result of using more than one Spellman CZE 1000R at a time. The Spellman power supplies are incapable of sinking any current but are sources of current. Having multiple current sources (power supplies) and no isolation between the power supplies resulted in a lack of control over the output voltages of the power supplies. This current sinking challenge is not as prominent when the length of the capillary is substantial (tens of cm) because the fluctuation in voltage is
spread over the entire length. For example, a 500 V fluctuation on a 30 cm capillary when the applied voltage is 10 kV would result in an electric field of about 350 V/cm rather than the intended 333 V/cm, a 5% difference. Since the incubator was only 1.0 cm in length, any deviation from the applied voltage would be detrimental to the control of sample flow through the interface. To use the same example as above, a 500 V fluctuation in this experiment would have doubled the intended electric field across the incubator and, depending which power supply was deviating most, could reverse the flow of sample or transfer additional sample into the second separation capillary unintentionally during a separation.

With an abundance of fluorescent TBA-15 present in the 2D-CE-SELEX interface, imprecise control over the applied voltages would result in unintentional “leaking” of
fluorescent TBA-15 into the second separation capillary, Capillary 2, from the Incubator.

At the time of this experiment, I was unaware of the availability of other options for outputting high voltages in a way that could also sink current. Since this experiment was conducted I have become aware of current sinking high voltage amplifiers that could provide more precise control of applied voltages on the 2D-CE-SELEX interface. One such high voltage amplifier was used in mass spectrometry experiments outlined and discussed in a previous chapter (Chapter 5).
CHAPTER 7:
FUTURE WORK / CONCLUSIONS

Several of the chapters of this dissertation can be found as journal articles in the scientific literature. Since the publication of these journal articles, work has continued and resulted in further use of the methods and instrumentation I have developed for capillary electrophoresis.

The online SERS detector for capillary electrophoresis separations has been utilized for the separation and identification of the 20 proteogenic L-amino acids,\textsuperscript{144} the separation and characterization of eight biologically-active peptides,\textsuperscript{145} a modified version was used for combined SERS and electrochemical detection,\textsuperscript{146} and an improved version was developed for increased SERS detection efficiency for characterizing rare events in flow.\textsuperscript{147}

The nicked-sleeve interface has been used to detect peptide phosphorylation stoichiometry in diagonal capillary electrophoresis\textsuperscript{148} and for analyzing phosphopeptides with immobilized alkaline phosphatase.\textsuperscript{149}

Finally, the automated fraction collector for capillary electrophoresis separations is in use for the separation and collection of bacteria. The majority of bacteria that are known (~99%) cannot be cultured in a laboratory. This work seeks to separate unculturable microbial populations and collect isolated, enriched bacteria populations.
After separation and collection the isolated bacteria will be sequenced for characterization and evaluation. Ideally, understanding these microbial populations will lead to new approaches in alternative energy and pharmaceuticals.


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