CAPILLARY ELECTROPHORESIS SEPARATION OF SINGLE STRANDED DNA OLIGONUCLEOTIDES WITH AN UNCOATED BARE FUSED SILICA CAPILLARY

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ABSTRACT

This thesis research was based on a novel Capillary Zone Electrophoresis (CZE) technique developed by Mangano et al. for separating ssDNA oligonucleotides differing in only one base pair at low pH with a DB-Wax coated capillary. This thesis research employed similar methodology, but used an uncoated bare-fused silica capillary to explore if similar results could be obtained. An uncoated bare-fused silica capillary is an economical alternative to the coated capillary.

When analyzing the ssDNA oligonucleotides with the uncoated capillary using the same phosphate running buffer as the coated capillary method, the sample was detected as two partially separated peaks. When using a potassium phosphate monobasic running buffer to reduce the EOF, but not coat the capillary, the two peaks were present with greater resolution. To further investigate why two peaks were present, a diethylenetriamine (DIEN) phosphate running buffer was used to dynamically coat the capillary walls to prevent sample interactions with the fused silica. When analyzing the sample with this running buffer the ssDNA was detected as one peak. In addition, an impurity analysis was performed with size exclusion chromatography. No major impurities or degradation products were detected.

With an uncoated bare-fused silica capillary the silica bound to the capillary wall is exposed. It is likely that ssDNA oligonucleotides interact with the fused silica to become cleaved during separation. When the ssDNA oligonucleotide becomes cleaved the more positive ions from the ssDNA separates from the more negatively charged ions. As a result, two peaks are observed. Because the entirety of the ssDNA molecule is not maintained during the separation, the determination of composition based on migration time can not occur. Likewise, the method can not be used to quantify ssDNA. In order to preserve the ssDNA molecule throughout the separation a reagent that masks or coats the fused silica wall should be employed.
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DEDICATION

I would like to dedicate this thesis to my sons, Gabriel and Ethan Garman, for whom I cherish and love. I pray that they will be inspired by my accomplishments, and that they will excel in everything they will do.
INTRODUCTION

Capillary electrophoresis (CE) is a rapidly maturing technique for basic pharmaceutical analysis (polar compounds), ion analysis, chiral pharmaceutical analysis, glycoprotein analysis, and genetic analysis for which CE can guarantee the Safety, Quality, Identity, Purity and Potency (SQIPP) of a pharmaceutical product. One analysis CE is widely used for is the determination of SQIPP for synthetic oligonucleotide based medicine. The typical technique for oligonucleotide analysis is performed by using capillary gel electrophoresis (CGE) at high pH under denaturing conditions. This type of oligonucleotide separation is mainly based on size differences. Although this analytical technique is widely used, the analysis time may be long and the reagents and capillaries can be expensive. However, another approach to analyzing oligonucleotides was made possible at low pH using capillary zone electrophoresis (CZE). This new technique is a more economical and efficient one. However, unlike CGE, little is known about the effects of nucleobase composition on separation.

The CE instrumentation is practically a hybrid of gel electrophoresis and HPLC. The general components that make up the CE instrumentation are a fused-silica capillary with an optical viewing window, a controllable high voltage power supply, two electrode assemblies, two buffer reservoirs, and typically an ultraviolet (UV) detector (see Figure 1). After a capillary is filled with buffer, and the ends are placed in the buffer reservoirs, a voltage is introduced across the capillary. Separation of the analytes injected will then occur based on several factors.
Figure 1: Diagram of a Capillary Electrophoresis System

The most influential process that drives CE is electroosmosis. Electroosmosis is a consequence of surface charge on the wall of the capillary, which is related to the zeta potential. Electroosmosis is generated in a capillary when an electric field, is transmitted by the drag of ions acting in a thin sheath of charged fluid passing across the silica bound to the capillary wall, moving towards cathode. This movement is called electroosmotic flow (EOF), and is a result of an imbalance between positive and negative ions in the bulk solution that are trying to balance the fixed negative charge on the silica wall. This balancing act of charge at the capillary wall is called the zeta potential.

Equation 1: Zeta Potential

\[ \zeta = \frac{4\pi \eta \mu_{eo}}{\varepsilon} \]

\[ \varepsilon = \text{dielectric constant of buffer, } \zeta = \text{zeta potential, } \eta = \text{viscosity, } \mu_{eo} = \text{coefficient for electroosmotic flow} \]

The region between the capillary wall and the electrophoretic buffer, which is responsible for the EOF, consists of three layers: the negatively charged silica surface, the immobile layer (Stern layer or inner Helmholtz plane), and the diffuse layer of cations. These layers are illustrated in Figure 2. The isoelectric point (pI) of fused silica is about 1.5, (pK= 6.3, [24]) and the degree of ionization of the silica is controlled mainly
by the pH of the buffer. At neutral to alkaline pH the EOF is significantly stronger than the electrophoretic migration. A strong EOF will unfavorably sweep the analyte towards the negative electrode resulting in little or no separation. The point of zero EOF has been found to occur precisely at pH 2.3. [23]

Figure 2: Effect of pH on the Capillary Wall

The time required for an analyte to migrate through the capillary to the optic window is defined as its migration time. Migration time is a function of the analyte’s electrophoretic mobility and the mobility component of the EOF (see Equations 2 & 3).

Equation 2: Migration Time

\[ t_m = \frac{L_d}{(\mu_{ep} + \mu_{eo})E} \]

\( t_m \) = migration time, \( L_d \) = capillary length, \( \mu_{ep} \) = electrophoretic mobility, \( \mu_{eo} \) = electroosmotic mobility, \( E \) = electric field strength

Equation 3: Electrophoretic Mobility

\[ \mu_{ep} = \frac{q}{6\pi\eta R} \]

\( \mu_{ep} \) = electrophoretic mobility \( (m^2 V^{-1} sec^{-1}) \), \( q \) = net charge, \( R \) = stokes radius, \( \eta \) = viscosity

There are currently many techniques that utilize CE to test oligonucleotides and similar biomolecules. All of these CE techniques generally rely on optimizing increased selectivity, reduction of EOF, and a reduction of adsorption of the biomolecules to the capillary wall [1, 2, 8, 10, 11, 16, 19, 21, 32]. For example, a CZE study was performed to analyze the effect of diethylenetriamine (DIEN) phosphate to act as a dynamic coating
reagent with the bare-fused silica capillary to prevent protein adsorption to the capillary wall. [21] Another example of a study performed using CZE was the use of a single stranded DNA binding protein in the running buffer (electrolyte) to capture all of the ssDNA in a racemic mixture of dsDNA and ssDNA. The increased weight added to the ssDNA would allow its electrophoretic mobility to change so that it would resolve from the dsDNA. [11]

Traditionally, electrophoretic separation of oligonucleotides is performed using capillary gel electrophoresis (CGE) in a basic buffer after the oligonucleotide has been denatured. At higher pH, the EOF becomes increasingly strong so the use of a sieving media, typically a gel, is required to reduce the rate of migration. Because of the mechanics of sieving media different species are mainly separated by size differences alone. However, an alternative method of oligonucleotide separation was developed by Mangano et al. using capillary zone electrophoresis (CZE) without the need of sample pre-treatment. [5]

Capillary zone electrophoresis, also known as free solution capillary electrophoresis (FSCE), is the most commonly used CE technique. With this method the sample is introduced into the capillary as a plug near the inlet, or anode (+). By applying an electric field the ions in the plug will separate into zones of a homogenous composition, each with a characteristic electrophoretic mobility (EOM). The zones will then migrate toward the outlet, or cathode (-), as a result of the electro-osmotic flow (EOF) created by the electric field. Often, a UV/Vis detector detects the arrival of each zone to the optical window near the outlet. The order of separation that occurs in CZE is illustrated in Figure 3. [22] A cation of smaller size will migrate toward the cathode
faster than a larger sized cation with the same charge. Also, a smaller sized anion will migrate slower than an anion of a larger size. Larger analytes are influenced more by friction than smaller analytes. In the case of larger anions, friction prevents the analyte from being repulsed by the “like” charge of the cathode.

Figure 3: Order of Migration based on Mass and Charge

Of the many factors that contribute to CZE separation, the running buffer, or electrolyte, can have dramatic effects on the separation efficiency. The electrolyte solution provides a conductive medium through which charged analyte molecules can migrate towards the cathode. An effective running buffer would be one that is of higher ionic strength than the sample slug being injected into the capillary. For CZE, the sample is often injected as a slug containing a low ionic strength media, typically water. Because of the ion concentration differences between the electrolyte and sample slug, the sample ions experience a lower electric field and decrease in velocity. This will allow the sample to concentrate into zones, and migrate through the capillary based on their electrophoretic mobilities. For this research, the sample was either hydrodynamically or electrokinetically injected into the capillary. Hydrodynamic injections are pressure driven and allow for known volumes of sample to be injected into the capillary. Electrokinetic injections are performed by applying an electric field directly to the sample and allowing the sample ions to migrate into the capillary. This method is typically applied to coated capillaries because it preserves the coating, which may otherwise be
compromised by an applied pressure. However, quantitative analysis is difficult with the electrokinetic injection because of the many variables associated with the electrophoretic migration of sample ions. Both hydrodynamic and electrokinetic injection techniques were used with all of the buffers for this thesis research.

One of the main advantages of CZE is that there is no need for a pressure-driven flow, unlike HPLC. A pressure-driven flow results in a parabolic flow profile and thus band broadening. Since capillaries are of small inner diameter, and the migration of ions result in a laminar flow profile, the major factor contributing to band broadening is longitudinal diffusion. Assuming that the only contribution to band broadening is longitudinal diffusion, the variance of the migrating zone width ($\sigma^2$) can be written as:

\[
\sigma = 2Dt = \frac{2DL^2}{\mu_{ep}V}
\]

Where $D$ is the diffusion coefficient of the solute, $L$ is the capillary length, $\mu_{ep}$ is the electrophoretic mobility, $V$ is the voltage applied across the capillary, and $t$ is the migration time. [22]

For this thesis research three different running buffers were used. They are the phosphate buffer, potassium phosphate monobasic buffer, and diethylenetriamine phosphate running buffers. The phosphate buffer was chosen at the same concentration as was used by Mangano et al. with the coated capillary. The other buffer was the potassium phosphate monobasic buffer. An aspect of the cation component for this buffer is its ability to decrease the EOF. [25] The third buffer chosen for this thesis research was diethylenetriamine phosphate, which was shown to act as a dynamic coating reagent with the bare fused silica capillary to decrease sample adsorption to the capillary
Each buffer that was chosen has specific properties and benefits, including the buffer's ability to conduct an electric current. If a buffer’s conductivity is high (i.e. 10 mS/cm) it would require a lower applied voltage to conduct a high current. For capillary electrophoresis a high current will typically improve resolution and reduce migration times. Below are the measured conductivities of each buffer at varying concentrations and pH.

Table 1: Conductivities of the Potassium Phosphate Monobasic, Diethylenetriamine Phosphate, and Phosphate Running Buffers at Different pH and Concentrations at 20°C.

<table>
<thead>
<tr>
<th>Running Buffer ID</th>
<th>Concentration</th>
<th>Conductivity (mS/cm) @ pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water titrated with phosphoric acid to pH</td>
<td></td>
<td>pH 2.3</td>
</tr>
<tr>
<td>Water titrated with phosphoric acid to pH</td>
<td>1.89</td>
<td>1.22</td>
</tr>
<tr>
<td>5 mM</td>
<td>3.12</td>
<td>2.22</td>
</tr>
<tr>
<td>10 mM</td>
<td>3.76</td>
<td>2.64</td>
</tr>
<tr>
<td>15 mM</td>
<td>4.58</td>
<td>3.25</td>
</tr>
<tr>
<td>20 mM</td>
<td>4.92</td>
<td>3.89</td>
</tr>
<tr>
<td>Potassium Phosphate Monobasic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mM</td>
<td>4.35</td>
<td>3.23</td>
</tr>
<tr>
<td>30 mM</td>
<td>8.05</td>
<td>6.43</td>
</tr>
<tr>
<td>75 mM</td>
<td>11.67</td>
<td>10.58</td>
</tr>
<tr>
<td>Diethylenetriamine Phosphate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mM</td>
<td>4.35</td>
<td>3.23</td>
</tr>
<tr>
<td>30 mM</td>
<td>8.05</td>
<td>6.43</td>
</tr>
<tr>
<td>75 mM</td>
<td>11.67</td>
<td>10.58</td>
</tr>
</tbody>
</table>
Predicting migration times by mass/charge ratio differences at low pH

An oligonucleotide is a short nucleic acid polymer consisting of less than 50 base pairs. For single stranded DNA oligonucleotides, each of the four types of nucleotides are connected together by a phosphate backbone. The degree of protonation at low pH, pKa, and charge for each of the four types of nucleobases, plus the phosphate backbone are illustrated in Figures 4 and 6. [31] The hydrogen donor and acceptor sites for each nucleobase are illustrated in Figure 5.

Figure 4: Degree of Nucleobase and Phosphate Backbone Protonation vs. pH

![Fig 4](image)

Figure 5: Hydrogen Donor and Acceptor Sites on Each Nucleobase

![Fig 5](image)
Several authors have correlated electrophoretic mobility to the charge and dimensions of small peptides to establish their relationships [28, 29, 30]. Two models in particular establish a correlation between positive charges and molecular weight. The two models are the RSN [29], and CWZ [30]. The coefficients of a, b, and c are based on a linear correlation between the specified variables in the equations. The variable $n_{(aa)}\text{p}$ is the number of positive charges, $M$ is molecular weight, $Z$ is the molar fraction (or net charge), and $\mu$ is the EOM of the analyte.
Equation 5: CWZ and RSN Models

\[
\begin{align*}
\text{CWZ Model} & \quad \mu = \frac{a_{\text{molecule}}}{M^{1/2}} + c \\
\text{RSN Model} & \quad \mu = \frac{aZ}{M^b} + c
\end{align*}
\]

For peptides, there is always some degree of positive charge at low pH, but this is not the case for single stranded DNA oligonucleotides. For any given ssDNA oligonucleotide at low pH, the net charge will be negative. For the ssDNA composition model that was by Mangano et al., a correlation between migration time and \( R = (A+C)/(G+T) \) was established (see Figure 5). Where A is Adenine, C is Cytosine, G is Guanine, and T is Thymine. In addition, the R ratio represents the number of positively charged nucleobases divided by the number of neutral nucleobases.

Figure 7: R Model- Migration Time vs. Oligonucleotide Composition (Data Extrapolated from the Literature, [5])

As observed in Figure 5, there is a correlation, but different migration times can be achieved for the same ratio value. This scatter occurs because varying amounts of adenine and thymine are not discriminated by the composition ratio value. This means separation is not dependent upon the R ratio, but is more specifically dependent on other factors. Where, size and charge differences are likely candidates. The charge is dependent on the pH (see Equation 6). [23] When comparing oligonucleotides of similar
length, the molecular weight, or mass of the ssDNA molecule may be correlated to its size.

Equation 6: Electrophoretic Mobility as a Function of pH

\[ \mu = AZ = A \left( \sum_i 10^{(pH-pK_a(i))} + \sum_j \frac{1}{1+10^{(pH-pK_a(j))}} \right) \]

Where \( A = 1/6\pi \eta r_s \)

The correlation related to the CWZ model is based on positively charged peptides, and will not work for ssDNA (See Equation 7). Single stranded DNA is negatively charged at low pH. Therefore, a modification to the CWZ model could lead to potential benefits in more accurate migration time predictions for the negatively charged ssDNA. Such a modification is seen below in Figure 6.

Figure 8: Mass/Charge Ratio Model: Migration Time vs. Mass/Charge Ratio (Data Extrapolated from the Literature [5])

Equation 7: CWZ Model of Peptide Migration Time and EOM Predictions at Low pH

\[ t_m = m_0 + m_1 \frac{M^{0.5}}{n_{(aa)p}} \]

Where \( n_{(aa)p} \) is the number of amino acids that at acidic pH value can assume a positive charge, \( m_0 \) is the extrapolated migration time of a species with M equal to 0, \( m_1 \)
is equal to $6\pi \eta b L / E$, where $b$ is a factor of proportionality between the Stokes radius and $M^{0.5}$.

Single stranded DNA oligonucleotide composition alone is not sufficient in accurately describing the separation mechanics in free solution capillary electrophoresis, and a model incorporating mass and charge is necessary. If migration time can be correlated to the mass and charge of an oligonucleotide a semiempirical approach to the prediction of electrophoretic mobility can be made based on knowing the oligonucleotide composition alone.
METHODOLOGY

Below are the original parameters established by Mangano et al. These original parameters were used as a starting point for the development of a method that used an uncoated capillary at low pH to separate ssDNA oligonucleotides by mass/charge ratio differences.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capillary</td>
<td>J&amp;W, DB-Wax (coated)</td>
</tr>
<tr>
<td>Capillary ID</td>
<td>50 µm</td>
</tr>
<tr>
<td>Capillary Effective Length</td>
<td>50 cm</td>
</tr>
<tr>
<td>Running Buffer</td>
<td>Water titrated to pH with H3PO4</td>
</tr>
<tr>
<td>Running Buffer, pH</td>
<td>2.3, 2.5, 2.8, 3.0, and 3.3</td>
</tr>
<tr>
<td>Sample Concentration</td>
<td>4 µM in Water</td>
</tr>
<tr>
<td>Injection</td>
<td>20 psi*sec.</td>
</tr>
<tr>
<td>Temperature</td>
<td>20°C</td>
</tr>
<tr>
<td>Separation Voltage</td>
<td>10 kV</td>
</tr>
<tr>
<td>Wavelength</td>
<td>260 nm</td>
</tr>
</tbody>
</table>

All chemicals and reagents were of ACS or AR grade, and were purchased through a commercial vendor. All small volumetric transfers were performed with a set of calibrated micro auto-pipettors with sterile tips. All glassware that was used was class A. MilliQ-H2O (18 MΩ) Millipore water was used for all of the experiments. The CZE equipment is a calibrated P/ACE Beckman Capillary Electrophoresis System. The sample housing and the capillary are temperature controlled. All separations were performed at 20 °C. Acquisition of the data for the CZE was acquired through P/ACE System MDQ Version 2.2 and by Thermo Atlas Version 8.2.2.

The uncoated bare-fused silica capillary was manufactured by Agilent Technologies, Part No. 190-0131 0.050 mm × 1 mm with a pre-burned window, and was cut to the desired length. A new capillary was conditioned for each of the running buffers that were used. To condition the capillary it was rinsed with MilliQ-H2O at 40
psi for 5 minutes, then 0.05 N HCl for 10 minutes, MilliQ-H₂O again for 5 minutes, and then running buffer for 15 minutes. After rinsing, a current was allowed to pass over the capillary for 15 minutes followed by another rinse with the running buffer for 5 minutes. After each injection the capillary was rinsed with the running buffer at 40 psi for 5 minutes.

Preparation of 0.05 N HCl:

5 mL of Reagent Grade 1 N HCl, J.T. Baker, into a 100 mL volumetric flask containing ~ 20 mL of MilliQ-H₂O. Filled to volume with MilliQ-H₂O and mixed well.

At the beginning of each experiment the suitability of the system (capillary and instrument) was verified with multiple injections prior to, and during the analysis, with 0.3 mg/mL niacin. Niacin was purchased from Calbiochem (Cat# 481918) at 99.9% purity by acidometry. To verify that the migration times did not vary from injection to injection, the %RSD from six injections of niacin was calculated. Likewise, every 10 or less sample injections were bracketed with an injection of niacin. The migration times of the bracketing niacin injections were also compared to the migration times of the original six consecutive injections.

Niacin, or nicotinic acid, is a small aromatic compound with a pKa of 4.85 and MW of 123.11 g/mol, which at low pH has a positive charge. [38] See the below figure for a structure of niacin.
Figure 9: Chemical Structure of Niacin

Niacin was favored for determining system suitability because it is a small stable structure that absorbs at the same wavelength as oligonucleotides, 260 nm. The optimal working concentration of niacin was determined by analyzing varying concentrations with a stand alone UV/Vis spectrophotometer, using a 0.100 mm quartz cell, and MilliQ-H₂O as the blank.

*Preparation of 0.3 mg/mL Niacin:*

\[
0.3 \text{ mg/mL Niacin} = \frac{24 \text{ mg Nicotinic Acid}}{80 \text{ mL}}
\]

The electrolytes chosen for this research are the phosphoric acid solution, potassium phosphate monobasic buffer, and diethylenetriamine phosphate buffer. Throughout the development of the CZE method, each electrolyte's concentration was adjusted, along with certain instrument parameters, until the desired separation would occur. The pH of each electrolyte was measured with a pH meter calibrated using NIST traceable pH 1.09 and pH 4.01 buffers that were purchased from Orion. The preparations of the electrolytes at their optimal, working, concentration are as follows.

*Preparation of the Phosphoric Acid Solution, pH 2.5:*

200 mL of MilliQ-H₂O was titrated to a pH of 2.5 with concentrated (86%) phosphoric acid (ACS Grade, J.T. Baker) with a calibrated pH meter.
Preparation of the 10 mM Potassium Phosphate, Monobasic Buffer, pH 2.5:

Transferred 680 mg of Potassium Phosphate, Monobasic (ACS Grade, J.T. Baker, MW 136.09 g/mol) into a beaker. Dissolved in ~ 475 mL of MilliQ-H₂O then titrated the solution to a pH of 2.5 with concentrated (86%) phosphoric acid (ACS Grade, J.T.Baker) with a calibrated pH meter. Transferred the solution to a 500 mL volumetric flask and filled to volume with MilliQ-H₂O. Mixed well. See below for the calculated concentration.

\[
10 \text{mM} \text{KH}_2\text{PO}_4 = \frac{0.68 \text{g}}{0.5 \text{L}} \times \frac{\text{mol}}{136.09 \text{g}} \times \frac{1000 \text{mM}}{M}
\]

Preparation of the 75 mM Diethylenetriamine Phosphate Buffer, pH 2.5:

Tared a beaker containing ~225 mL of MilliQ-H₂O on a calibrated top loader balance with a resolution of two decimal places. Weighed in 1.93g of diethylenetriamine, (min. 95% DIEN, Strem Chemicals, Cat# 111-40-0, MW 103.17 g/mol) and mixed well. Titrated the solution to a pH of 2.5 with concentrated (86%) phosphoric acid (ACS Grade, J.T.Baker) with a calibrated pH meter. Transferred the solution to an amber 250 mL volumetric flask and filled to volume with MilliQ-H₂O. Mixed well. See below for the calculated concentration.

\[
75 \text{mM DIEN} = \frac{1.93 \text{g}}{0.25 \text{L}} \times \frac{\text{mol}}{103.17 \text{g}} \times \frac{1000 \text{mM}}{M}
\]

All single stranded oligonucleotides were custom synthesized, and desalted, by Sigma-Aldrich. A certificate of analysis was obtained for each custom ssDNA oligonucleotide. The samples were purchased at a concentration of 100 µM in purified H₂O. Each oligonucleotide was identified based on their R value (A+C)/(G+T), and were
stored at -20 °C until use. The ssDNA oligonucleotide compositions that were used for this thesis research are listed in Table 2.

Table 2: Single Stranded Oligonucleotides that were used for this Thesis Research.

<table>
<thead>
<tr>
<th>ID</th>
<th>Sequence</th>
<th>MW</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-1.22</td>
<td>CCCATGCCACAATGGTTAGG</td>
<td>6102</td>
</tr>
<tr>
<td>R-0.54</td>
<td>CTCATGGAGGAGTATTGATGGGC</td>
<td>6188</td>
</tr>
<tr>
<td>R-2.33</td>
<td>CCCTGCCACTCTCATCAATC</td>
<td>5933</td>
</tr>
<tr>
<td>R-1.86</td>
<td>CCATCAATACTCCATGAGCG</td>
<td>6046</td>
</tr>
</tbody>
</table>

Dilutions of the samples were performed in MilliQ-H₂O (18 MΩ Water) to the desired concentration. Below is the dilution scheme used to achieve a sample concentration of 4 µM.

**Preparation of the 4 µM Oligonucleotide Solution:**

The stock oligonucleotide was allowed to thaw at room temperature then briefly centrifuged. 8 µL of the 100 µM sample was transferred into 192 µL of MilliQ-H₂O and mixed gently with a pipette tip.

The solubility of the oligonucleotides in the running buffer was tested prior to CZE analysis so as to ensure that the capillary would not become clogged. This was accomplished by transferring 20 µL of a 100 µM sample into a small volume 1 cm quartz cell, which was then filled to volume with the desired running buffer. After mixing the quartz cell, it was held up to the light to observe if precipitation occurred. Neither of the running buffers used for this research caused the ssDNA oligonucleotide to fall out of solution.

During an investigation to detect impurities that the ssDNA oligonucleotides might have, HPLC- Size Exclusion Chromatography (SEC) was performed. The HPLC-SEC analysis was performed with an Agilent HP 100 HPLC system using two TSK
Bioscience gel G3000SW-XL 7.8 x 30 cm gel columns with a TSK gel guard column of 7 μm 6.0 x 4.0 cm.

SEC Mobile Phase Preparation:

Mixed 4.4 g of sodium phosphate monobasic, dihydrate (J.T.Baker) and 25.8 g of sodium phosphate, dibasic, 12-hydrate, crystal (J.T.Baker). The mobile phase was vacuum filtered through a 0.45 μm glass fiber filter (Whatman) before use.

A 50 μM sample solution of R-1.22 was analyzed with a flow rate of 0.5 mL/min, run time of 90 minutes, and wavelength set to 260 nm. Also, a 25 mM R-1.22 solution was prepared by mixing 25 μL of 100 μM R-1.22 with 75 μL of pH 2.5 phosphate buffer. The solution was allowed to stand for several hours before being injected. In addition, differing oligonucleotide compositions were analyzed with a 50 mM mixture of R-1.22 and R-0.54 oligonucleotides made at 20 μL. See the below figure for the chromatogram of this injection.

Figure 10: Impurity Analysis of a Mixture of R-1.22 and R-0.54 with SEC Overlaid with a MilliQ-H2O Injection and Individual Injections of both the R-1.22 and R-0.54 Oligonucleotides.

Because there is not enough resolution between the two different oligonucleotides it is likely that it will not separate oligonucleotides differing in one base pair. However, the
method should be able to detect impurities that are different in size from oligonucleotides.

The development of the methods for use with the uncoated capillary was based on the original parameters established by Mangano et al. The following are the instrument parameters that were established through needed modifications to optimize the separation. Each of the parameters is identified based on the electrolyte that was used. Each of the methods’ efficiencies is discussed in the “Results and Discussions” section of this report.

Table 3: Instrument Parameters for the Developed Methods

<table>
<thead>
<tr>
<th>Electrolyte</th>
<th>Capillary Dimensions</th>
<th>ssDNA Conc.</th>
<th>Injection</th>
<th>Sample and Cartridge Temp.</th>
<th>Voltage Setting</th>
<th>Detector Setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphoric Acid Solution, pH 2.5</td>
<td>50 µm ID x 50 cm effective length</td>
<td>20 µM</td>
<td>120 psi*sec.</td>
<td>20°C</td>
<td>10 kV, 30 µA max.</td>
<td>260nm (High Sensitivity)</td>
</tr>
<tr>
<td>10 mM Potassium Phosphate, Monobasic Buffer, pH 2.5</td>
<td>50 µm ID x 50 cm effective length</td>
<td>20 µM</td>
<td>120 psi*sec.</td>
<td>20°C</td>
<td>10 kV, 30 µA max.</td>
<td>260nm (High Sensitivity)</td>
</tr>
<tr>
<td>75 mM Diethylenetriamine Phosphate Buffer, pH 2.5</td>
<td>50 µm ID x 50 cm effective length</td>
<td>100 µM</td>
<td>10 kV for 60 sec.</td>
<td>20°C</td>
<td>5 kV, 30 µA max.</td>
<td>260nm (High Sensitivity)</td>
</tr>
</tbody>
</table>

For the development of each method, the CZE parameters and their impact on separation were considered based on the diagram in Table 3.
Table 4: Influential Parameters that Affect the CZE Separation Efficiency. [34]

<table>
<thead>
<tr>
<th>pH</th>
<th>Temperature</th>
<th>Injection Volume</th>
<th>Viscosity</th>
<th>Electroosmotic Flow</th>
<th>Sensitivity/Resolution</th>
<th>Diffusion</th>
<th>Current</th>
<th>Ionic Strength</th>
<th>Electrophoretic Mobility</th>
<th>Capillary Surface Characteristics</th>
<th>Molecular Charge</th>
<th>Analyte-Wall Interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>↑ ↓</td>
<td>↑ ↓ ↑ ↑</td>
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<tr>
<td>Analyte-Wall Interactions</td>
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<td>Cap. Inner Surf. Negative Charge</td>
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<tr>
<td>Molecular Charge</td>
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<tr>
<td>Electrophoretic Mobility</td>
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</table>

An increase in the parameters listed on the y-axis will result in an increase (↑) or decrease (↓) in the effects of the parameters listed on the x-axis. For example, an increase in the pH will result in an increase in the electroosmotic flow. If (↕) is listed then depending upon the capillary, buffer, and analyte an increase or decrease in that parameter will occur when the respective parameter in the y-axis is increased.
RESULTS AND DISCUSSIONS

There are only a few research studies that correlate electrophoretic mobilities of peptides and proteins to their physiochemical properties. This is due in part to the lack of a well developed theory for the expected relationships, and to the difficulty involved with the estimation of the many variables related to the CZE technique.

One of the physiochemical challenges influencing the electrophoretic mobility of single stranded DNA’s is that at low pH it has a net negative charge. Also, at low pH, portions of the ssDNA molecule carry a positive charge on the amino groups. Because capillary electrophoresis separates analytes based on size and charge differences, conflicts may arise due to the dual charged nature of ssDNA. Also, ssDNA is highly flexible, and unlike double stranded DNA it will change its spatial configuration rapidly. These characteristics of ssDNA must be considered when describing how the biomolecules migrate through the uncoated bare fused silica capillary at low pH.

The pKa of the fused silica capillary wall is 6.3. [24] This means at a pH of 2.3-2.5 the capillary wall will mostly be negatively charged (see Figure 2). When the electric field is applied, cations at the capillary wall will move towards the cathode, and create the electroosmotic flow (EOF). The more cations that move to the cathode the greater the EOF. At high pH the fused silica wall is essentially saturated with cations, which results in higher EOF. However, if the fused silica is masked, or coated, the EOF can be reduced or eliminated. If the EOF is eliminated differences in electrophoretic mobilities can be leveraged for separation. This is why the reduction of EOF is typically favored. Another advantage to coating the capillary is to prevent sample adsorption. By blocking
the reactive sites of the silica, the adsorption of the analyte to the capillary wall can be avoided.

Because the coated capillary method established by Mangano et al. successfully separated oligonucleotides differing in one base pair, it will be used as the “control” for assessing the uncoated capillary methods’ efficiency. The representative electropherogram for the established method is illustrated in figure 9.

Figure 11: Representative Electropherogram, using the Method Established by Mangano et al. of a Mixture Including Two Synthetic 49-mers Differing for a Single Base at Position 31 using a Coated Capillary with a Phosphate Buffer Titrated to a pH of 2.5 with Phosphoric Acid. [12]

Each experiment in this thesis research was executed with a system that was verified by assessing migration time changes with multiple injections of niacin. If the migration times varied significantly, then the capillary was equilibrated longer.

Below is a representative electropherogram for niacin with the Phosphate Buffer at pH 2.5.
The analysis of a 4 µM R-1.22 sample was performed at the instrumental conditions specified for the coated capillary. However, a peak was not detected. Therefore, the injection volume and sample concentration was increased in increments until an injection volume of 120 psi*sec and sample concentration of 20 µM, at which two partially separated peaks were observed. The presence of these two peaks was confirmed by making multiple injections. After the peaks were confirmed, the effect of the running buffer pH on the peak profile was explored. The R-1.22 sample was analyzed with a running buffer titrated to a pH of 1.9, 2.1, 2.5, and 2.7. The pH that produced two well defined peaks was at a pH of 2.5. See the below representative electropherograms for pH 2.1, 2.5, and 2.7. No peaks were detected at a pH of 1.9.
Figure 13: Electropherogram of a 20 μM Sample (R-1.22) with the Phosphate Running Buffer at pH 2.1

Figure 14: Electropherogram of a 20 μM Sample (R-1.22) with the Phosphate Running Buffer at pH 2.5

Figure 15: Electropherogram of a 20 μM Sample (R-1.22) with the Phosphate Running Buffer at pH 2.7
For a solution containing one type of oligonucleotide, one peak should be present. Two separate peaks for a one component solution means that an impurity is present, there are two different oligonucleotides in solution, or the oligonucleotides undergo a chemical reaction to form two oligonucleotide products. See Figure 14 for an illustration of the four possibilities for a single oligonucleotide to result in two peaks being detected.

Figure 16: Illustration of the Four Possibilities for a Single Oligonucleotide to Result in Two Peaks being Detected.

1) Oligonucleotide + Impurity $\rightarrow$ Oligonucleotide + Impurity

2) Oligonucleotide $\xrightarrow{\text{Reaction}}$ Oligonucleotide + Degradant

3) Oligonucleotide $\xrightarrow{\text{Reaction}}$ Oligonucleotide$_1$ + Oligonucleotide$_2$

4) Oligonucleotide$_1$ + Oligonucleotide$_2$ $\rightarrow$ Oligonucleotide$_1$ + Oligonucleotide$_2$

The two partially separated peaks were originally thought to be from ssDNA that failed “capping” during synthesis. If, during synthesis, the ssDNA failed “capping” strands of ssDNA will continue through additional cycles of synthesis and result in a sample solution containing ssDNA of varying base pair compositions. Regardless, an impurity analysis of the ssDNA needed to be performed.

Size Exclusion Chromatography (SEC) was used to perform the impurity analysis. SEC relies on high performance liquid chromatography and a column with a meshed gel to separate analytes based on size. The larger analytes elute before the smaller analytes due to the lag time for smaller analytes while they move through the cavities of the meshed gel. The chromatogram obtained when analyzing 80 µL of the R-1.22 sample with HPLC-SEC is seen below.
Figure 17: Chromatogram of a 50 μM Sample (R-1.22) using Size Exclusion Chromatography.

Only one major peak, with smaller tailing peaks, is present for the impurity analysis of the R-1.22 ssDNA. If there existed an impurity at the same capacity to produce a peak response approximately equal to that of the oligonucleotide, as was observed for the CZE analysis, it would have been detected equally so in this SEC analysis. In addition, if an oligonucleotide that failed the capping portion of synthesis were present in the sample solution it would have been detected with a greater peak response than what is seen in Figure 15.

In another experiment, the influence of the low pH running buffer on degradation was tested. The sample was diluted in the pH 2.5 phosphate buffer and allowed to sit for 2 hours before being analyzed. The chromatogram that resulted from this experiment is seen below.
No degradation products were detected as a result of being exposed to the low pH buffer. Because there were no major impurities, degradation products, or additional oligonucleotides detected in the sample solution it is likely that the oligonucleotide is undergoing a reaction within the capillary. It is also likely that because these two peaks are not observed with the coated capillary method, as performed by Mangano et al., it is related to the exposed silica associated with the uncoated bare-fused silica capillary.

With the CZE method, another running buffer that didn’t coat the capillary was explored. By using potassium phosphate monobasic as the running buffer the ionic strength of the system was changed. With the addition of the positively charged cation, or potassium ion, in the running buffer, the rates at which positive and negative ions migrate through the capillary were changed. [22] As it was performed with the method using the phosphate buffer, injections of 0.3 mg/mL niacin were made to verify the suitability of the instrument. See the below electropherogram for the 0.3 mg/mL niacin using the potassium phosphate buffer at pH 2.5.
Figure 19: Electropherogram of 0.3 mg/mL Niacin using 10 mM Potassium Phosphate Monobasic Buffer at pH 2.5

The migration time of the niacin peak was increased by ~3 minutes from that of the analysis performed using the phosphate running buffer. The R-1.22 sample was injected at a pH of 2.0, 2.3, 2.5, 2.8, and 3.1. For representative electropherograms of the R-1.22 and R-2.33 samples injected with the 20 mM potassium phosphate buffer at pH 2.5, see Figures 18-19. In addition to this analysis a mixture of R-1.22 and R-2.33 was injected, see Figure 20. A summary of the migration times for each solution can be located in Table 4.

Table 5: Summary of Migration Times for 20 µM R-1.22, R-2.33, and Mixed Solution (R-1.22+R-2.33) using 10 mM Potassium Phosphate Monobasic Buffer at pH 2.5.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Migration Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak 1 (Unk.)</td>
</tr>
<tr>
<td>R-1.22</td>
<td>7.6</td>
</tr>
<tr>
<td>R-2.33</td>
<td>7.9</td>
</tr>
<tr>
<td>Mix (R-1.22+2.33)</td>
<td>8.2</td>
</tr>
</tbody>
</table>
Figure 20: Electropherogram of 20 μM R-1.22 Injected at 120 psi*sec. using 10 mM Potassium Phosphate Buffer at pH 2.5.

Figure 21: Electropherogram of 20 μM R-2.33 Injected at 120 psi*sec. using 10 mM Potassium Phosphate Monobasic Buffer at pH 2.5.
Table 6: Migration Times for R-1.22 and R-2.33 using 10 mM Potassium Phosphate Monobasic Running Buffer at pH 2.0, 2.3, 2.5, 2.8, and 3.1.

<table>
<thead>
<tr>
<th>pH</th>
<th>Sample ID</th>
<th>Peak 1 (min)</th>
<th>Peak 2 (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>R-1.22</td>
<td>None Detected</td>
<td>None Detected</td>
</tr>
<tr>
<td></td>
<td>R-2.33</td>
<td>None Detected</td>
<td>None Detected</td>
</tr>
<tr>
<td>2.3</td>
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<td>17.1</td>
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<td>10.9</td>
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<tr>
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<td>R-2.33</td>
<td>7.1</td>
<td>11.0</td>
</tr>
</tbody>
</table>

Figure 22: Electropherogram of a 20 μM R-1.22 and 2.33 Mixed Solution Injected at 120 psi*sec. using 10 mM Potassium Phosphate Monobasic Running Buffer at pH 2.5.

The peaks in an electropherogram, represent the various zones formed in the separation process, which are characterized by electrophoretic mobilities. The migration time is the time required for the peak maximum to reach the detector. Because the zones do not travel at the same velocity, the time required for them to pass the detector differs.
The total variance of a zone, once corrections have been applied for zone velocity and finite detector width, is given by the below equation.

Equation 8: Total Variance of a Zone.

\[ \sigma_{\text{tot}}^2 = \sigma_{\text{diff}}^2 + \sigma_{\text{inj}}^2 + \sigma_{\text{int}}^2 \]

Where \( \sigma_{\text{diff}}^2 \) is the variance due to diffusion, \( \sigma_{\text{inj}}^2 \) is the variance due to the injection plug length, and \( \sigma_{\text{int}}^2 \) represents zone-broadening variance arising from interactions between the analyte and the walls of the capillary. [35] Therefore, the presence of two peaks is not related to the variance in a zone, but is instead two different analytes with varying electrophoretic mobilities.

It is well understood that the bare-fused silica capillary may adsorb biomolecules due to electrostatic interactions. Adsorption is the process whereby molecules adhere to solid surfaces. Adsorption to the capillary wall results in either loss of analyte or band broadening. Still, little is known about how single stranded DNA oligonucleotides interact with the bare fused silica capillary. However, there are several physiochemical properties that can be used for explaining the interactions that take place with the ssDNA and the capillary wall during CZE separation. These are diffusion, thermal convection, and coupled transport, such as the combination of convection and diffusion. Also, variables such as concentration, velocity, molecular size, and molecular configuration are important considerations for determining how the ssDNA will react with the capillary wall. In the case of diffusion, the following equation may be applied.

Equation 9: Simple Diffusion

\[ \frac{\delta C}{\delta t} = D \frac{\delta^2 C}{\delta x^2} \]
Where $C$ is concentration, $D$ is the diffusion coefficient, and $x$ is distance. If the rate of adsorption is equal to the rate of diffusion within a distance then the following equation may be considered.

**Equation 10: Rate of Surface Concentration with Respect to the Diffusion Coefficient.**

$$\frac{dn}{dt} = C_0 \left( \frac{D}{\pi t} \right)^{1/2}$$

Where $n$ is the surface concentration of protein, $C_0$ is the bulk concentration of protein, and $t$ is time. This equation shows that a high diffusion coefficient results in a larger number of molecules arriving at the capillary surface and potentially binding with it. [37]

The properties that affect interactions with biomolecules and surfaces are summarized below:

**Table 7: Summary of the Properties that Affect Interactions with Surfaces.**

<table>
<thead>
<tr>
<th>Property</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>Larger molecules have larger surface area, and are more likely to interact with the wall</td>
</tr>
<tr>
<td>Structure Stability</td>
<td>Less stable biomolecules, can unfold to a greater extent and form contact points with the surface.</td>
</tr>
<tr>
<td>Charge</td>
<td>Molecules near the isoelectric point of the surface generally adsorb more readily.</td>
</tr>
<tr>
<td>Unfolding Rate</td>
<td>Molecules that rapidly unfold or change confirmation are more likely to contact the surface.</td>
</tr>
</tbody>
</table>

Adsorption may not always result in band broadening, but other factors related to the analyte may cause a reaction with the fused silica. For example, ion exchange with the capillary wall could result in cleaving portions of the ssDNA. Also, because the ssDNA contains both positive and negative charges at low pH, the separation of charges may be causing a physical separation of the compound, wherein the more positively charged ions of the ssDNA molecule separate from the more negatively charged ions.
The diffusive properties of single stranded DNA also provide evidence that this molecule will have a higher probability of interacting with the fused silica wall. Research by another group determined the diffusion coefficients of ssDNA. “The diffusion coefficients of ssDNA oligonucleotides containing 20 nucleotides are approximately 50% larger than those of double-stranded DNA oligonucleotides of the same size, reflecting the greater flexibility of ssDNA molecules.” [36] With a coated capillary sample adsorption to the wall is essentially eliminated by blocking the fused silica, and thus preventing the sample from interacting with it.

Depending on the charged nature of the single stranded DNA, oligonucleotide interactions with the capillary wall may include forces additional to those of electrostatic origin, resulting in significant variations in the electrophoretic behavior. However, these interactions with the capillary can be resolved by modifying the inner wall of the capillary. If the fused silica is coated, or prevented from interacting with the sample, one peak should be observed instead of two. This may be performed by using a chemical compound to mask the activity of silanol groups so they are not accessible to interactions with the ssDNA. For this thesis research project, the chemical compound used to dynamically coat the capillary is diethylenetriamine, or DIEN.

Diethylenetriamine is a linear ethyleneamine containing two primary and one secondary nitrogens, and sufficient transparency at low UV wavelengths. Because the amine groups are protonated at low pH they will form hydrogen bonds with the negatively charged silica wall. After allowing this reagent to the coat the capillary wall the ssDNA oligonucleotides was analyzed. Figure 21 provides a representative electropherogram of the DIEN method.
Figure 23: Representative Electropherogram of the Developed Capillary Zone Electrophoresis Method for Single Stranded DNA Oligonucleotide Separation using a Bare-Fused Silica Capillary with Diethylenetriamine Phosphate Running Buffer at pH 2.5

Figure 24 illustrates that, unlike the uncoated method, a variety of injection volumes will result in the detection of the oligonucleotide.

Figure 24: Electropherogram of Varying Sample Injection Volumes with 75 mM DIEN Phosphate Buffer at pH 2.5.

The DIEN coated capillary method offers a more robust analysis than the uncoated capillary method. However, the EOF was significantly greater than the EOM of the sample. Because of this the sample migrated through the capillary at the speed of the EOF. This resulted in no separation between oligonucleotides of differing base pair compositions. However, because of the high EOM, if the oligonucleotide had become cleaved the portions of cleaved ssDNA can not be separated. Because of this it may be inferred that the second peak detected in the uncoated capillary method is an
oligonucleotide. Otherwise, it is likely that the DIEN coating prevents the ssDNA from interacting with the capillary walls, and results in one peak being detected. Without coating the capillary, the ssDNA is exposed to the fused silica wall, which may result in separating the oligonucleotide into two components of differing EOM. This phenomenon is likely to be enhanced by the high diffusion coefficient associated with ssDNA.
CONCLUSION

Separating single stranded DNA (ssDNA) oligonucleotides differing in base pair compositions with capillary zone electrophoresis (CZE) at low pH using an uncoated bare-fused silica capillary analysis was compared to a similar method that used a coated DB-Wax capillary. In addition, the correlation between migration time and base pair composition was further explained for how ssDNA separation occurs at low pH with CZE when using the DB-Wax coated capillary.

Experiments for ssDNA oligonucleotide detection at low pH with CZE using a DB-Wax coated capillary was conducted using a variety of single stranded DNA oligonucleotides of varying base pair compositions based on a A+C/G+T (adenine + cytosine/ guanine + thymine) ratio (R). The described method was able to distinguish between two oligonucleotides differing in one base pair. [6] However, because the R ratio composition model does not fully explain the contribution of every nucleobase with relation to migration time, a revised model was developed. The developed model incorporates the mass/charge ratio contributions of each nucleobase and correlates these values with migration time. This correlation offers better understanding of ssDNA’s EOM, where the migration time of any given oligonucleotide can be predicted when using a DB-Wax coated capillary at low pH. Specifically, the correlation developed is migration time vs. log(MW/Z^2).

It was also the intent of this thesis research to develop a CZE method similar to that of using the DB-Wax coated capillary for analyzing single stranded DNA oligonucleotides. The developed method adopts similar methodology except an uncoated bare-fused silica capillary was used. The uncoated capillary method is an economical
alternative to the coated capillary method. With an uncoated bare-fused silica capillary the silica bound to the capillary wall is exposed. It is likely that the ssDNA interacts with the fused silica to become cleaved during separation. As a result, two peaks are observed. Because the entirety of the ssDNA molecule is not maintained throughout the separation, the determination of composition based on migration time can not occur. Likewise, the method can not be used to quantify ssDNA. In order to preserve the ssDNA molecule throughout the separation a reagent that masks or coats the fused silica wall should be employed.

When the capillary walls were coated with the diethylenetriamine (DIEN) phosphate buffer only one peak was observed. However, because the EOF was greater than the EOM, oligonucleotides of different compositions could not be separated. If a portion of the oligonucleotide was cleaved it would not be separated from other cleaved portions. The results from the DIEN phosphate buffer method either confirms that the second peak found in the uncoated capillary method is a cleaved portion of the ssDNA, or that the coating prevents interactions of the ssDNA with the fused silica wall. Regardless, the coated capillary method is a more robust method of detecting ssDNA oligonucleotides than the uncoated capillary method. Further development of the DIEN method may result in being able to separate oligonucleotides differing in one base pair. Recommended pathways of development would be to include a sieving media, increase the capillary length, and/or to reverse the separation mode.
REFERENCES


[34] Landers, James P, Handbook of Capillary Electrophoresis, 1997, CRC Press LLC.

