Immobilization of Enzymes on Porous Silica for the Micro-Digestion of Oligonucleotides With Analysis by CE.

Yolanda Yvette Davidson
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IMMOBILIZATION OF ENZYMES ON POROUS SILICA FOR THE MICRO-DIGESTION OF OLIGONUCLEOTIDES WITH ANALYSIS
BY CE

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
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in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Chemistry

by

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May 2000

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Abstract

The research in this dissertation has focused on the immobilization of enzymes on silica to perform digestions with DNA and RNA with subsequent analysis by capillary electrophoresis. Solid-phase DNA restriction digest reactors were developed consisting of silica particles modified with a covalently tethered restriction enzyme. This solid-phase restriction reactor enables digestion and separation of minute quantities of DNA with minimal reagent consumption. The restriction enzymes, HaeIII, PstI, and HindIII, were successfully immobilized via glutaraldehyde linkages to porous silica micro-particles. Studies were done to examine the impact of immobilization on enzymatic activity. Digestions of φX174-RF DNA phage and SV40 Viral DNA were performed with the immobilized enzymes by placing the silica particles in solution with the target DNA. The digests were analyzed off-line using capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection. Timed studies were performed to establish optimal conditions for complete digestion. Digests utilizing immobilized HaeIII and HindIII were similar in composition to homogeneous, free solution digests. PstI showed no evidence of activity upon immobilization. The immobilized restriction enzymes could also be used for multiple rounds of digestion; however, longer incubation times were required for successive runs probably due to partial heat denaturation of the restriction enzyme. Digests were
prepared and isolated by use of a simple micro-spin column consisting of a layer of immobilized enzyme-coated silica on a molecular weight cut-off filter.

A method was developed for the digestion of RNA using silica-immobilized enzymes. Additionally, a capillary electrophoresis (CE) method was evaluated for the separation of the resulting oligonucleotides. Ribonuclease A (RNase A) and Ribonuclease T₁ (RNase T₁) enzymes were successfully immobilized via glutaraldehyde linkages to porous silica particles. Studies were performed to establish the minimum time necessary to achieve complete digestion using immobilized enzymes and were compared to similar studies of free solution enzyme digests. Experiments were performed to determine conditions to achieve the best separation and resolution of RNA oligonucleotide digestion products. The quality of the separation in the electrophoresis buffer was pH dependent with the most efficient separation occurring at lower pH. Multiple digests could be performed over several days.
Chapter 1

Capillary Electrophoresis (CE)

1.1 Introduction to Capillary Electrophoresis

Separating individual components in a sample is an important task for the scientist. Electrophoresis is a method that is used to separate charged molecules based on their movement through a fluid in an applied electric field. Electrophoresis is a powerful mechanism for separating proteins and other macromolecules such as DNA and RNA. Tiselius\textsuperscript{1} first introduced this technique in 1937 when he showed that serum proteins, albumin, and $\alpha$-, $\beta$-, $\gamma$-globins could be separated by “moving boundary electrophoresis” and provided the first suggestion of the potential use of electrophoretic analysis of biologically-active molecules. The term electrophoresis was given to this separation technique when Michaelis\textsuperscript{2} separated proteins based on their isoelectric points. Electrophoresis carried out in gels suppresses convective currents introduced by temperature gradients and offer molecular sieves that enhance separation. Slab-gel electrophoresis provides mechanical stability for separation and reduces solute dispersion from convection and diffusion. It is typical for DNA electrophoresis to be performed in slab gels. Preparing gels in the form of thin slabs has several advantages, such as large samples can be loaded into each sample well allowing
the gels to be used for preparative electrophoresis. Once the samples are electrophoresed, the bands can be cut and removed from the gel and used further. Finally, many samples can be run in parallel (≥ 20). It is important to be able to compare samples side by side for techniques such as DNA sequencing, DNA footprinting, and DNA structural polymorphisms. Additionally, Southern blotting can be performed on gels by binding DNA fragments from the gel onto a nylon fiber, which can then be probed for the desired DNA sequence.

Disadvantages of using slab gel systems include speed of separation, which is limited by Joule heating, time consuming steps such as casting of the gel, sample preparation and loading of samples. If the gel is run at too high a current and not sufficiently and uniformly cooled, significant Joule heating can occur and temperature gradients form in the gel. If this happens, the gel can be cooler near the edges than in the center causing the analytes near the edge of the gel to run more slowly than those in the center which can cause "smiling" effects. These "smiling" effects would cause problems with side-by-side comparison of the mobilities of related samples. If a substantial amount of Joule heating is formed, the gel will melt and the samples can be destroyed. Using narrow-diameter capillaries (≤ 100 μm) allows enough heat dissipation to employ high voltage to enhance the separation. Separations are much faster using high field strengths in capillaries than those used in slab gels.

Capillary electrophoresis (CE) has become a powerful separation tool that combines speed, quantitation, peak efficiency and automation and allows for the
separation of similar compounds from complex matrices. In addition, the high surface area-to-volume ratio provides rapid dissipation of Joule heat and allows high electric fields to be used without substantial temperature increase.

1.2 Instrumentation for CE

An attractive feature of CE is the simplicity of the instrumentation. Figure 1.1 shows a schematic of an apparatus for conducting electrophoresis experiments. Essential in all systems is a high voltage power supply, a polyimide coated capillary, two buffer reservoirs, electrodes, and a detector. To perform an electrophoretic separation, the fused silica capillary is filled with the buffer solution. The sample is introduced into the capillary by placing one of the capillary ends into the sample and applying voltage or pressure. Then, with both the inlet and outlet in the buffer solutions, a voltage is applied to the system and the separation is performed. The species in the sample migrate through the capillary to the detector where data is collected, stored and analyzed.

1.3 Detection

Detection in CE for DNA analysis is accomplished primarily by either UV absorption or fluorescence detection. UV is the most widely used detection method and diode arrays offer much spectral information. Almost all commercial CE instruments have UV detectors. High quality fused silica capillaries are used which have a UV cut-off of 170 nm. One of the primary advantages of using UV absorbance detection of nucleic acids is that the detection is based on the intrinsic UV absorbance of DNA and no intercalating dyes or fluorophores are needed,
Figure 1.1. Basic Schematic of a capillary electrophoresis instrument.
thus the DNA molecule is not perturbed. All DNA bases absorb approximately the same amount of UV radiation at 260 nm and the signal intensity increases linearly with the number of DNA base pairs in the electrophoretic band.

Laser-induced fluorescence (LIF) detection is more sensitive and selective than absorption, and very low concentrations of samples can be analyzed. DNA separations are typically performed with LIF for low-level detection using intercalating dyes. If highly efficient fluorophores are used, the sensitivity of LIF detection can be up to six orders of magnitude greater than UV detection. Both glass and fused silica capillaries may be used for fluorescence detection, but fused silica is preferred because its background luminescence levels are lower. Although LIF detection systems are more expensive and complicated to build, the sensitivity makes it more conducive for DNA detection. In addition, high sensitivity allows the use of narrow diameter capillaries whereby higher field strengths can be used to further enhance resolution. Other detection methods that are used for DNA analysis include native fluorescence and radio-isotopes, but these are used less frequently.

1.4 Electrophoretic Process

1.4.1 Electrophoresis

The actual separation that occurs in electrophoresis is based on the velocity of the solutes in the electric field. A charged particle in solution will become mobile when placed in an electric field, and its mobility in the fluid solution is governed by its charge to size ratio. The velocity, \( v_i \) (cm/s), of the
solute under an applied voltage $V \,(V)\), is the product of the apparent solute mobility, $\mu_{\text{app}} \,(cm^2/V \cdot s)\) and the applied field $E \,(V/cm)\), $E = V/L\), where $L \,(cm)\) is the length of the capillary):

$$
\nu_i = \mu_{\text{app}} E. \tag{1}
$$

The mobility of the solute is unique to that molecule and is directly proportional to the electric force ($F_e$) that the solute experiences and inversely proportional to its frictional drag ($F_F$, for a spherical ion) through the medium.

The electrical force is given by

$$
F_e = qE, \tag{2}
$$

and the frictional force by Stokes's Law,

$$
F_F = 6\pi\eta r \nu, \tag{3}
$$

where

$q = \text{charge on ion}$

$\eta = \text{viscosity of solution} \,(N \cdot s/cm^2)$

$r = \text{solute radius} \,(cm)$

$\nu = \text{solute velocity} \,(cm/s)$. 

During steady-state electrophoresis, the frictional force and electric force are equal but opposite and the proportionality constant, $f$, is called the translational friction coefficient, which is equal to $6\pi\eta r$,

$$
qE = 6\pi\eta r \nu \text{ or } qE = f \nu. \tag{4}
$$
Solving for velocity (4) and substituting into (1) yields an equation in terms of the mobility of the analyte \( cm^2/V \cdot s \),

\[
\mu_e = \frac{q}{f}.
\]  

(5)

From this equation, the mobility of the analyte is a property of both the charge \( q \) and the frictional coefficient \( f \). Small, highly charged molecules will migrate rapidly and large, minimally charged molecules will migrate slowly. For a DNA molecule, the total net charge on the molecule is directly proportional to its size (2 charges per base pair) and therefore,

\[
q \sim N,
\]  

(6)

where \( N \) is the number of units (base pairs) in the DNA chain. DNA exists as a free draining coil and its units contribute equally to the overall drag of the chain

\[
f \sim N
\]  

(7)

Substituting equations 6 and 7 into equation 5, the electrophoretic mobility is no longer a function of molecular size,

\[
\mu = \frac{q}{f} \sim \frac{N}{N} = N^0,
\]  

(8)

and is constant with changes in \( N \). Therefore, because the charge and frictional drag are proportional to molecular size, free solution electrophoretic separations of DNA are impossible. To accomplish DNA separations using electrophoresis, DNA must travel through a set of porous polymer networks.
1.4.2 Electroosmotic Flow (EOF)

Helmholtz identified electroosmotic flow (EOF) in the 1800s while he was conducting experiments involving the application of an electrical field to a glass tube containing an aqueous salt solution. Electroosmosis is the bulk flow of a liquid within a capillary due to the movement of counter-ions adjacent to the negatively charged wall, and coupled with the solute's electrophoretic mobility, controls the amount of time solutes remain in the capillary. Because the wall of a fused-silica capillary possesses negatively charged silanols in the pH range of ~2 to 2.5 (See Figure 1.2), positively charged ions in the buffer solution build near the wall forming what is called the electrical double layer. The potential difference is often referred to as the zeta (ζ) potential. As the electric field is applied across the capillary, the positively charged layer is attracted toward the negative electrode (cathode), which results in the bulk flow of the liquid toward that electrode. When the electrophoretic migration occurs, the analytes are swept towards the detector by the bulk flow of the solution in normal polarity where the sample is electrophoresed from the positive electrode (anode) of the capillary to the cathode only when the electrophoretic mobility is smaller than the electroosmotic flow in the case of negatively charged solutes.

EOF can be expressed by the equation:

\[ u_{EOF} = \frac{\varepsilon \zeta}{\eta} E, \]  

or

\[ \eta 

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Figure 1.2. Representation of electrical double layer versus distance from the column wall.
\[
\mu_{\text{EOF}} = \frac{\varepsilon \zeta}{\eta}
\]  

(11)

where \( \nu_{\text{EOF}} \) = velocity of EOF (cm/s)

\[ \mu_{\text{EOF}} = \text{EOF "mobility"} \ (cm^2/V \cdot s) \]

\( \zeta = \text{zeta potential} \)

\( \varepsilon = \text{dielectric constant} \ (C^2/J \cdot cm). \)

The EOF is highly dependent on solution and experimental parameters, such as pH, temperature, voltage, and buffer viscosity and can be manipulated for specific types of separations. Unless EOF is controlled or eliminated, obtaining reproducible separations will be difficult.4,5

1.4.3 Band broadening

In capillary zone electrophoresis, the voltage applied across the capillary determines the separation efficiency, not the length of the capillary itself. The capillary length is limited only indirectly, in that enough capillary surface area must be maintained to dissipate the heat generated by passage of current through the capillary. If this condition is met, a 100 cm capillary with a 10 kV applied potential and a 50 cm capillary with a 10 kV applied potential will give the same separation efficiency.6

On the other hand, the migration time in capillary electrophoresis is a function of the capillary length squared.7 For example, reducing the capillary
length by half, while holding all other factors constant, will reduce the migration time by a factor of 4 with the same separation efficiency. A short capillary can be used to give very short migration times if the applied voltage is maintained and will result in no loss of separation efficiency.

Separation efficiency (\(N\)) and migration time (\(t\)) can be explained from the following equations

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

\[
t = \frac{L^2}{\mu V}
\]

where \(N\) is the separation efficiency expressed as the number of theoretical plates, \(D (\text{cm}^2/\text{s})\) is the diffusion coefficient of the solute, \(L (\text{cm})\) is the capillary length, \(V (\text{V})\) is the voltage applied across the capillary, \(\mu\) is the “effective” electrophoretic mobility (\(\text{cm}^2/\text{V} \cdot \text{s}\)), and \(t\) is time (s) for the solute to migrate the length of the capillary. The electrophoretic mobility of the solute is given by

\[
\mu = \mu_{EL} + \mu_{EOF}
\]

where \(\mu_{EL}\) is the electrophoretic mobility of the analyte and \(\mu_{EOF}\) is the electroosmotic flow mobility. For an analysis, \(\mu_{EOF}\) does not change and can be measured by a neutral analyte for which \(\mu_{EL}\) is zero, but \(\mu_{EL}\) is different for each analyte and is calculated from the migration time of the analyte and the \(\mu_{EOF}\).
From equation 12, one can see that separation efficiency is not a function of capillary length. It is important, however, that there is sufficient capillary surface area to dissipate heat generated by the current applied to the capillary. This can be accomplished by decreasing the capillary inner diameter and/or the electrolyte concentration.

From equation 13, one can see that migration time is a function of $L^2$. Decreasing the capillary length will greatly reduce the migration time. Short capillary lengths will give very short migration times if the applied voltage is maintained at the same level with no loss in separation efficiency. These equations (12 & 13) assume that the entire length of the capillary is used. If the entire capillary is not used due for detection, modifications must be made to account for the distance from the point of injection to the point of detection. The voltage used for separation is only a fraction of the voltage between the point of injection and detection. The equation

$$V_1 = V(I/L)$$  \hspace{1cm} (15)

where $V_1$ is the fraction of $V$ (V) actually used, $L$ (cm) is the total length of the capillary and $l$ (cm) is the length of the capillary from injection to detection gives this relationship. Substitution of equation (15) into equation (12) gives

$$N_{\text{actual}} = \frac{\mu V_1}{2D} = \frac{\mu V}{2D} \frac{l}{L},$$  \hspace{1cm} (16)

and the migration time is modified to

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where \( t_1 \) is the migration time between injection and detection. From these equations, one can see that CE analysis can be done in very short times with good separation efficiency.

### 1.4.4 Resolution and Efficiency

In capillary electrophoresis, the ability to resolve various components in a mixture is important and is usually judged in terms of column efficiency or peak resolution. Resolution \((R)\) or separation of sample components is important in CE. One equation that defines resolution is

\[
R = \frac{2(t_2 - t_1)}{w_1 + w_2} = \frac{t_2 - t_1}{4\sigma} \tag{18}
\]

where
- \( t = \) migration time (solute 1 and solute 2, sec)
- \( w = \) width (in time, s) at baseline
- \( \sigma = \) temporal standard deviation

Because all processes contributing to variance (band broadening) are additive, the total peak variance \((\sigma_{tot}^2)\) is due to variances of the column \((\sigma_{col}^2)\), injection step \((\sigma_{inj}^2)\), and detector or detection event \((\sigma_{det}^2)\). The resolution can also be expressed in terms of mobilities and is defined as:

\[
R = \frac{1}{4} \frac{\Delta\mu_{ep}}{\mu_{ep} + \mu_{eo}} \sqrt{N} \tag{19}
\]
where \( \Delta \mu_{ep} \) = difference in mobility of the two solutes \((cm^2/V\cdot s)\)

\( N \) = the number of theoretical plates

\( \mu_{ep} \) = average mobility of the two species \((cm^2/V\cdot s)\)

Proper control or adjustment of \( \Delta \mu_{ep} \) will aid in achieving the best resolution and the resolution between peaks is dependent on the square root of the number of theoretical plates.

1.4.5 Buffer pH effects

The electroosmotic flow in a capillary is highly pH dependent. The \( \zeta \) potential (See Figure 1.2.) is determined by the surface charge on the capillary. At higher buffer pHs, the silanol groups along the wall are fully ionized yielding a strong \( \zeta \) potential and a very dense double layer. When the electric field is applied, a very strong bulk flow is generated. When the pH is low, the surface of the capillary is predominately protonated and the EOF is lower.

Problems with EOF reproducibility occur most often in a pH range of 4-6. It is important to realize that EOF is difficult to eliminate in fused silica capillaries.

1.5 Controlling Electroosmotic Flow

Control of the electroosmotic flow is essential to achieve the optimal separation in the capillary. If the electroosmotic flow is not controlled, it will be difficult to obtain reproducible migration times and resolution. A number of methods have been employed to reduce the electroosmotic flow by controlling the charge density on the capillary wall or the viscosity of the solution adjacent to the
wall. Buffer additives such as cetyltrimethylammonium bromide have been used
to adsorb small cations to neutralize the wall's surface charge.8 This modification
often reverses the direction of the electroosmotic flow. Changing the viscosity of
the buffer near the capillary wall also aids in controlling the EOF.9 Smith and El
Rassi10 modified the inner wall of fused silica capillaries to have a surface
containing silanols, positively charged quaternary ammonium groups, and a
hydrophilic layer of polyether. The direction of the EOF could be controlled by
manipulating the pH and the polyether chains served to shield the charged groups
from solutes to prevent interactions with the wall. Reduction in the electric field
strength can also lower the EOF. Reducing the electric field strength, however,
increases the analysis time and reduces efficiency and resolution. Careful
attention must be paid to the solutes that are to be separated because at low pH,
molecules become protonated and at high pH molecules become deprotonated.
Other possibilities that can aid in controlling the EOF include changing the
buffer's concentration and ionic strength.

1.6 Joule-Heating

The conduction of electric current through an electrolytic solution results
in the production of heat because of frictional collisions between mobile ions and
buffer molecules. Heat dissipation is important to speed and resolution in
electrophoretic separations. Joule heating occurs as a result of the electric current
passing through the electrophoresis buffer and depends on the capillary diameter,
the field strength and the buffer concentration. The rate of heat production inside the capillary can be estimated by

$$\frac{dH}{dt} = \frac{IV}{LA}$$  \hspace{1cm} (20)

where $L$ is the capillary length (cm) and $A$ is the cross-sectional area (cm$^2$).

Using the equation $I = \frac{V}{R}$, where $R$ ($\Omega$) is the resistance and $k$ is the conductivity, $R = \frac{L}{kA}$ and substituting into equation 20 gives

$$\frac{dH}{dt} = \frac{kV^2}{L^2}.$$  \hspace{1cm} (21)

The amount of heat that must be removed from the capillary is proportional to the conductivity of the buffer and to the square of the field strength.

If Joule heating is not controlled, temperature changes as well as thermal gradients can result across the capillary. As the temperature of the separation buffer increases, the integrity of the sample will be compromised. The high surface-to-volume ratio of capillaries used in CE aids in efficient dissipation even at very high electric fields. The easiest way to reduce Joule heating associated with high ionic strength buffers is to lengthen the capillary or reduce the cross-sectional area of the capillary to reduce heating by decreasing the current density.

In order to determine the electric field strength at which the system cannot dissipate Joule heat, the applied voltage is plotted against the current (Ohm's Law

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The point at which this plot deviates from linearity is the conventional method for determining the optimum separation voltage in CE.

1.7 Separation

1.7.1 Mobility and migration time

Migration time is the time necessary for a solute to migrate from injection to detection. The mobility represents the distance a solute molecule travels in a given time period for a given field strength. The apparent mobility ($\mu_a$) includes a contribution of EOF. Using the following equation, the apparent solute mobility can be calculated to be

$$\mu_a = \frac{l}{tE} = \frac{IL}{tV}. \quad (22)$$

The effective mobility can be obtained using the following equation,

$$\mu_e = \mu_e + \mu_{EOF} \quad (cm^2/V \cdot s), \text{ where} \quad (23)$$

- $V$ = the applied voltage ($V$)
- $l$ = effective capillary length (to detector, $cm$)
- $L$ = total capillary length ($cm$)
- $t$ = migration time ($s$)
- $E$ = electric field ($V/cm$).

The effective mobility ($\mu_{EOF}$) is measured by using a neutral marker such as mesityl oxide that migrates at the same velocity as the EOF.$^{12}$
1.7.2 Solute-Wall Interactions

If the solute interacts with the capillary wall as it migrates through the capillary, it can adversely affect the separation. Adsorption to the wall will result in poor separation due to the interaction between the negatively charged wall and positively charged solutes. Peak tailing will be the result of this interaction.

1.7.3 Capillaries

A fused silica capillary is most often used in CE because it has qualities such as chemically inertness, precise dimension, a low electrical conductivity, high thermal conductivity, and high optical transmission to a wide spectrum of light (190 nm to 900 nm). The polyimide coating that protects the capillary can be easily removed to provide an optical window. This removal can be accomplished by dropping hot concentrated sulfuric acid on the area of the capillary where the detection window is desired. Capillaries with an inner diameter of 25-100 μm and an outer diameter of 350 μm to 400 μm are typical. To reduce analysis time, capillaries are generally made as short as possible. The effective length describes the length of the capillary from the injection end to the detection window and generally makes up the longest part of the capillary.

1.7.4 Separation Media for DNA

DNA fragments cannot be separated by size when they are electrophoresed in free solution. DNA fragments larger than 10 base pairs (bp) have essentially the same electrophoretic mobility due to the constant charge-to-size ratio. The size of the molecule is based on the molecular weight, the three-
dimensional structure, and the degree of solvation (See section 1.4.1). Because of this, a sieving matrix is needed to separate DNA fragments by molecular size. Slab gels use agarose and polyacrylamide gels, but in CE, capillaries are filled with polyacrylamide gels or viscous polymer solutions. This separation medium will be discussed in detail in a following chapter.

1.7.4.1 Gel-filled capillaries

Agarose and polyacrylamide gels were used initially in capillary electrophoresis for separating DNAs. Although some of the best work for resolution of oligonucleotides and double-stranded DNA has been done using these gels, the use of these gel-filled capillaries has suffered numerous problems including poor gel-to-gel reproducibility, bubble formation under electrophoretic conditions, gel matrix collapse under high electric fields and gel preparation difficulties using cross-linked polyacrylamide gels. In addition, high molecular weight DNA is retained and successive runs increase the chance of anomalous peaks. These gel-filled capillaries also have a short shelf life that limits their use. These capillaries fail due to bubble formation inside the capillary. Pressure injections cannot be used with these capillaries, as the gel will be pushed from the capillary. Therefore, efforts are made to limit the use of gels for routine analyses.

1.7.4.2 Entangled Polymer Solutions

A number of researchers have demonstrated that low-viscosity polymer solutions could be used to separate DNA fragments. Concentrated polymer
strands become entangled and form a removable gel inside the capillary instead of a permanent one produced by the use of the cross-linked polyacrylamide. The polymer solution can be pumped into and out of the capillary before each analysis. These solutions are inexpensive, easy to prepare, and prolong the life of the capillary.

Common polymers used for sieving matrices include hydroxyethyl cellulose (HEC), hydroxypropylmethyl cellulose (HPMC), methyl cellulose (MC) and other cellulose derivatives. A wide variety of DNA fragment sizes can be separated using these polymer networks. Some of these entangled polymers suffer from poor resolution. Nevertheless, these polymer solutions produce a system that is more reproducible, replaceable, and robust, which is better than its gel-filled counterpart.

1.8 Detection of DNA Fragments

Although these narrow diameter capillaries improve resolution, they limit the amount of sample that can be introduced into the capillary. Therefore, detection of the fragments must be extremely sensitive. Detection of DNA fragments has been successful using UV absorbance and laser-induced fluorescence (LIF).

1.8.1 LIF Detection

Because of high sensitivity and specificity associated with laser-induced fluorescence (LIF), CE using LIF detection is the method of choice for DNA analysis. LIF detection methods often involve the use of fluorescent intercalators.
that can be added to the separation buffer, which interact specifically with the DNA molecule. Monomeric and dimeric intercalators have been used with laser detection with good resolution.20-22

1.8.2 UV Absorbance

DNA detection at 254 nm is most often accomplished by use of UV absorbance detection. No derivatization or buffer additives are necessary to detect DNA fragments using UV absorbance. In addition, UV detectors are relatively inexpensive as compared to LIF detectors. The small internal diameter of the capillary, however, limits the amount of sample that can be detected by the UV detector. When extremely small amounts of DNA must be detected, detection by laser-induced fluorescence must be performed.

1.9 References


2.1 Introduction to DNA Capillary Electrophoresis

Analysis of DNA molecules has opened the door for many advances in molecular biology. The ability to analyze DNA fragments has led to direct detection and quantification of viruses, identification of individuals in forensic applications, and aid in mapping the human genome. Slab gel electrophoresis was initially the primary technique used to analyze DNA. Because of the time and labor needed to do the analysis, potentially hazardous conditions with carcinogenic intercalating dyes, and many manual steps that are not amenable to automation, researchers have focused on the automated technique, capillary electrophoresis (CE) as an alternative procedure. Through the introduction and use of laser-induced fluorescence (LIF) detection with increased sensitivity for low-level detection, CE has become an attractive alternative to slab-gel for separation and quantitation of nucleic acids. DNA restriction fragments can be separated within twenty minutes or less by capillary gel electrophoresis. High-speed and high resolution separation of DNA restriction fragments can be performed using entangled polymer solutions. DNA analysis has also shown
very good potential as a powerful tool for gene diagnosis, such as single-strand conformation polymorphism (SSCP).\textsuperscript{11-13}

2.2 Theory of Entangled Polymer Solutions

By using dilute, low-viscosity polymer solutions as a separation medium, high-resolution separations of DNA mixtures can be accomplished. Dissolving the polymer material in the aqueous buffer makes these porous gels. The pore size of the gel is determined by the concentration of the polymeric reagent. The most common polymers include poly(ethylene oxide),\textsuperscript{14} polyacrylamide,\textsuperscript{15} dextrans, poly(ethylene oxide) and cellulose derivatives (See Figure 2.1) such as hydroxyethyl cellulose (HEC),\textsuperscript{16} methyl cellulose (MC),\textsuperscript{17} hydroxypropyl cellulose (HPC),\textsuperscript{16} and hydroxypropylmethyl cellulose (HPMC).\textsuperscript{18} These cellulose polymers can also produce a dynamic coating of the capillary to suppress the EOF, which helps to extend column lifetime and can be used for over a thousand runs with minimal loss in resolution. HEC is the most studied, is available in a wide range of molecular weights and is the polymer used in these studies.

2.2.1 Overlap or Entanglement Threshold

Hjerten and coworkers were one of the first groups to report on the separation of low molecular weight compounds and proteins in the presence of polymers and neutral surfactants.\textsuperscript{19} Following this work, Grossman and Soane found that the pore size in these polymer networks depends on the polymer concentration and the radius of the mesh-forming polymer chain.\textsuperscript{16} An important difference exists between polymer solutions that are dilute, where the polymer
Figure 2.1. Structures of cellulose derivatives, each glucose unit has three sites for substitution, \( (R_1, R_2, R_3) \).
chains are hydrodynamically isolated from one another, and more concentrated solutions where the chains overlap and interact. At a certain concentration of the dissolved polymer (entanglement or overlap threshold, \( \Phi^* \)) the polymer strands begin to interact with one another. This volume fraction is defined as,

\[
\Phi = C \rho_p
\]  

where \( C \) (mole/cm\(^3\)) is the polymer concentration and \( \rho_p \) (kg/cm\(^3\)) is the density of the polymer. At a concentration greater than this, the solution becomes entangled (See Figure 2.2). The overlap threshold (\( \Phi^* \)) can be estimated from,

\[
\Phi^* = N^{-\alpha}
\]  

which is a function of polymer size, where \( N \) is the number of polymer segments.\(^{20} \) For any given polymer, \( \Phi^* \) has a unique dependence on molecular weight. At concentrations greater than \( \Phi^* \), the polymer chains entangle to form a mesh with transient pores. Hydroxyethyl cellulose (HEC) is a hydrophilic cellulose derivative, synthesized commercially by reacting alkali cellulose with ethylene oxide at high temperatures. The entanglement threshold (\( \Phi^* \)) for HEC is found to scale to \( N^{-1.2} \), where \( N \) is the number of HEC monomer segments in the polymer chain.\(^7 \) This measurement is not in agreement with classical scaling arguments. It is determined that the relationship to predict the entanglement threshold for any HEC solution is,
Figure 2.2. Schematic representation of the entanglement process where $\Phi$ is the volume fraction of the polymer and $\Phi^*$ is the entanglement threshold.
where $\Phi^*$ is measured as $[(g \text{ HEC})/(g \text{ solvent})]$, $M_n$ is the number-average molecular weight and $M_0$ is the average monomer molecular weight. The monomer molecular weight will vary with the molar substitutions (M. S.), which can be expressed in terms of the moles of ethylene oxide per anhydroglucose unit. On average, HEC has a M. S. of 2.5 giving an average monomer molecular weight of 272 g/mol. Barron and coworkers\textsuperscript{21} showed that HEC is stiff and extended in solution and shows effects of entanglement coupling to a larger degree than the highly flexible, random coil-like polymers for which the entanglement theories were derived.

2.2.2 Mesh Size

An entangled solution is characterized by an average mesh size for the network, $\xi$ (Figure 2.3). In 1979, de Gennes\textsuperscript{20} derived an approximate relationship relating $\xi$ to the polymer volume fraction, $\Phi$, with the assumption that $\Phi > \Phi^*$. The equation used to relate the mesh size to the polymer concentration is,

$$\xi(\Phi) \approx a\Phi^{-3/4},$$

(4)

where $\Phi$ is the polymer volume fraction and $a$ is the length of one repeat unit along the polymer chain. This equation assumes that the polymer is dissolved in an athermal solvent. For effective use of entangled polymer solutions, it is
important to be able to vary the mesh size in the solution. From the above equation, it can be seen that to have a small mesh size, the polymer concentration must be high which would lead to increased viscosity of the solution. To reduce the viscosity of the solution, one must keep the concentration near $\Phi^*$. Substitution of equation 2 into equation 4 yields,

$$\xi(\Phi^*) \approx aN^{0.6}.$$  \hspace{1cm} (5)

Therefore, in order to create a smaller mesh with minimum viscosity, one should use a shorter polymer. To create a larger mesh with minimum viscosity, one should use a longer polymer while operating near the entanglement threshold for that particular polymer. The size (viscosity) and the concentration of the polymer are the most important parameters to manipulate the migration time and resolution. These parameters depend primarily on the size of the DNA fragments being separated. High concentration polymers are best at separating small fragments of DNA and can be used to resolve fragments that differ by 5-10 bp. Often it is difficult to load these viscous solutions into a capillary. Lower concentration polymer solutions have less resolving power than high concentration solutions but have the ability to separate a wider molecular weight range of fragments (100 bp to 10 kbp).\(^6\)

2.2.3 Electrophoresis in a polymer network

Once the network structure of the polymer solution has been established, one can then do electrophoresis. Three main theories describe the migration of a
Figure 2.3. Schematic illustration of the entanglement mesh, where $\xi$ indicates the size of the mesh.
flexible macromolecule through a polymer network: (1) The Ogston model, (2) the reptation model and (3) transient entanglement coupling.

2.2.3.1 Ogston Model

The Ogston model treats the polymer network as a molecular sieve. For this model, migrating DNA molecules behave as an unperturbed sphere. The smaller DNA molecules migrate faster because they have access to a larger fraction of the available pores than the larger DNA molecules. The gel is treated as a distribution of fixed pores with an average pore size. The size of the pore depends on the gel composition, the concentration of the polymer and the degree of cross-linking in the matrix. The theory predicts that the electrophoretic mobility of the migrating solute, with a particle radius of $R_g$, through the porous structure is equal to its free solution mobility, $\mu_0$, multiplied by the probability that the solute will meet a pore large enough to allow the solute to pass. The equation that expresses this mobility is,

$$\mu = \mu_0 P(\xi \geq R_g),$$

where $\xi$ is the radius of the pore or mesh where the molecule is located, and $P(\xi \geq R_g)$ is the probability that a given pore has a radius greater than or equal to the radius of the migrating particle. The Ogston model of the pore size predicts that, in a random network of linear polymers, the fraction of pores large enough to accommodate a spherical particle of radius $R_g$, sometimes referred to as the radius of gyration is,
Figure 2.4. Schematic diagram of the Ogston model.
where \( n \) is the average number of polymer strands per unit volume, \( l \) is the average length of the polymer strands and \( r \) is the thickness of the strands. The model also assumes that the product \( n \cdot l \) is proportional to the concentration of the gel-forming polymer, \( C \). Therefore,

\[
P(\xi \geq R_g) = \exp\left(-\pi nl(r + R_g)^2\right),
\]

(7)

where \( K \) is a constant of proportionality. The retardation coefficient, \( K_r \), is equal to \( -K(r + R_g)^2 \) which is characteristic of a given molecular species in a particular polymer system. Combining equations 6 and 8 we get an expression that describes the migration of a solute through a polymer network according to the Ogston Model,

\[
\mu = \mu_0 \exp(-KC(r + R_g)^2),
\]

(9)

where \( K \) is a constant of proportionality, \( C \) is the concentration of the gel forming polymer, \( r \) is the thickness of the strands, and \( R_g \) is the radius of gyration.

2.2.3.2 Reptation Model

The Ogston model does not take into account the fact that the migrating molecule might deform in order to "squeeze" through a pore. When the size of the DNA increases to a size larger than the mesh size of the sieving matrix \((R_g > \xi)\), the mobility of the migrating molecule will rapidly approach zero. At this point, the large DNA molecules can no longer enter the small pores.
When the DNA fragment is too large to fit into a single gel pore, the reptation theory applies (Figure 2.5). The DNA molecule is considered too large to move from pore to pore without deforming slightly. The DNA molecule migrates "head first" or "snake-like" through the pores. The term reptation comes from the "reptile-like" nature of the motion. The mobility of the molecule by this process is inversely proportional to its molecular size,

\[ \mu \approx N^{-1}, \]

where \( N \) is the size of the molecule. The size of the DNA molecule is measured in bases or base pairs. When high electric fields are applied, the DNA molecule elongates and becomes more rod-like orientating itself in the direction of the field (Figure 2.6). When the electric field increases or the DNA size lengthens, the mobility of the molecule no longer depends on its size and the resolution between the small and large DNA molecules cannot be seen. In essence, as the molecule becomes more elongated, the mobility is no longer related to the molecular length.

Slater and Noolandi\(^22\) proposed the biased reptation model to account for this process. Lumpkin \textit{et al.}\(^23\) developed an expression that explains this process;

\[ \mu \approx K \left( \frac{1}{N} \right) + bE^2 \]

where \( N \) is the DNA molecular weight, \( K \) is a constant, \( b \) is a function of the mesh size of the polymer network as well as the charge and segment length of the migrating solute and \( E \) is the electric field. At low field strengths, the first term in the equation dominates and is dependent on the molecular size of the
Figure 2.5. Schematic diagram of the reptation model.
Figure 2.6. A DNA molecule migrating by the reptation mechanism can be elongated by the electric field (E).
migrating molecule. At high field strengths, the second term in the equation dominates and is basically independent of the molecular weight of the molecule.

A plot of $\log \mu$ versus $C$ should give a straight line with a slope proportional to $R_x^2$. This plot is referred to as a Ferguson plot. Ferguson plots are used to assess true size separation. Ferguson plots can be used to estimate the effective pore size of gels by determining the gel concentration at which the mobility of a given DNA fragment is equal to one half its mobility at zero gel concentration. At this concentration, assuming a Gaussian distribution of pore sizes, a macromolecule with a size equal to the median pore radius of the gel should be able to access half the available volume in the gel. At this gel concentration the radius of the migrating macromolecule should be equal to the median pore radius of the gel.$^{24,25}$

2.2.3.3 Transient Entanglement Coupling

In 1994, Barron and coworkers$^{21}$ proposed a new model for the separation of DNA in polymer solutions. The basis of this separation model was with consideration of the properties of HEC and DNA as well as for polymers in general. HEC, which is branched, contains bulky ethylene oxide side chains, and terminates with hydroxyl groups. In aqueous media, the conformation of HEC is extended and stiff. Double-stranded DNA is stiffer and more extended in solution than extended polymers (i.e., HEC). Therefore, DNA experiences some degree of
entanglement coupling with HEC molecules in solution, and this entanglement coupling could alter the frictional characteristics of DNA molecules moving under the influence of the electric field. It is important to note that extended polymers experience the effects of entanglement less than random-coil polymers such as DNA.

2.3 DNA Intercalation

DNA binding agents tend to interact non-covalently with the host molecule through two general modes: (1) in a groove-bound fashion stabilized by a mixture of hydrophobic, electrostatic, and hydrogen-bonding interactions and (2) through an intercalative association in which a planar, heteratomic moiety slides between the DNA base pairs. This insertion process, known as intercalation, is used by researchers doing DNA separations to improve the resolution of DNA fragments and often as a means of on-column fluorescent derivatization. The intercalation induces changes in the physico-chemical properties of the intercalated DNA as well as well as the intercalators themselves.

2.3.1 Intercalation Model

The intercalator (dye) inserts itself between two stacked base pairs on the DNA double helix and increases the distance from 3.4 Å to ~7-8 Å (Figure 2.7). The double helix unwinds, extends, and stiffens to reduce the stress caused by the intercalator, which ultimately improves the ability of the DNA to interact directly with the polymer network. As a result of its insertion of the intercalator between the bases, (1) the mass of the intercalator-DNA complex to increase, (2)
Figure 2.7. Intercalation on DNA by planar molecules
the overall charge on the molecule to decrease as the DNA phosphates are neutralized, and (3) the electrophoretic behavior of the DNA to change.

Cationic dyes (Figure 2.8) which have planar aromatic or heteroaromatic rings and exhibit enhancements in their fluorescence emission upon complexation with dsDNA include the mono-intercalating dyes ethidium bromide (EtBr),\textsuperscript{27} thiazole orange (TO)\textsuperscript{28} and oxazole yellow (YO)\textsuperscript{28} or bisintercalating dyes oxazole yellow homodimer (YOYO)\textsuperscript{29} and thiazole orange homodimer (TOTO).\textsuperscript{29} Mono-intercalating dyes bind dsDNA with one dye per two base pairs and bis-intercalating dyes bind one dye per 4 base pairs.

The binding constants of mono-intercalators (one chromophore) to dsDNA are $10^5$-$10^6$ M$^{-1}$ in low salt buffer and bisintercalators (two chromophores) binding constants are $10^{10}$-$10^{12}$ M$^{-1}$.\textsuperscript{29} Monomeric dyes are favored generally because in terms of fluorescence yield and resolution. These dyes can be added directly to the buffer and there is no need for pre-column staining. Because the binding constants for the dimeric dyes are much higher, the DNA is typically prestained with the dye and it is not added to the running buffer during electrophoresis. The limit of detection using the dimeric dyes is typically improved compared to that obtained using the monomers, but the dimeric dyes suffer from poor resolution due to the multiple binding modes of the dye.\textsuperscript{30} Choosing the correct dye concentration in the buffer is determined by taking into account resolution, background fluorescence and reproducibility of migration time and peak area.
Figure 2.8. Fluorescent intercalating dyes used for capillary electrophoresis.

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2.3.2 UV Absorbance and Fluorescence Intercalators

Upon intercalating with the DNA, many intercalators experience large fluorescence quantum yields. The resulting DNA-dye complex can have up to 1000 times enhanced fluorescence as compared to UV absorbance. Binding of the dye to DNA constrains the intercalator to a planar conformation which places it in a hydrophobic environment and makes it more fluorescent. Many fluorescent dyes are available commercially and can be used for DNA applications. Ethidium bromide is probably the most common UV absorbance dye used for DNA detection. A number of fluorescent intercalating dyes have been used which include the ones mentioned in the above section.

2.4 References


3.1 Introduction

The study of enzymes is important because life depends on a complex network of chemical reactions brought about by specific enzymes. Since their discovery, enzymes have become invaluable in research especially with use in the analyses of DNA molecules, elucidation of cDNA structure, construction of plasmids, and more recently to clone genes. Without enzymes, most biochemical reactions would be too slow to even carry out life processes. Enzymes do not alter reaction equilibria, but accelerate the forward and reverse reaction rate by factors of at least one million. The most striking characteristics of enzymes are their catalytic power and specificity. These molecules are highly specific both in the reaction they catalyze and their choice of substrates.

Specificity has become a very important area of enzyme studies, as there must be a close fit between the enzyme and substrate. Restriction endonucleases are interesting due to their site-specific recognition and digestion of DNA molecules. If enzymes remain in solution with the reactants and/or products, it is difficult to separate the enzymes from the desired product. Therefore, if enzymes can be attached to a support in some way, they can be used again after the
products have been removed. The term “immobilized” means unable to move or stationary. When the enzyme is trapped in such a distinct phase, it is separated from the bulk phase where the substrate is dispersed, allowing for interaction with the substrate of the enzyme. An enzyme can be physically attached to a solid support over which a substrate is passed and converted to product.

3.2 Enzyme Activity and Stability

The first attempt to purify an enzyme, urease, was done in 1926 by Sumner. Although highly purified enzymes are available, they are rarely used in biotechnology because enzymes have a tendency to steadily lose activity in aqueous solutions. The activity of the enzyme is very dependent on the proper orientation of the “active site” where the enzyme and substrate make contact. Measurement of the enzyme’s activity can be a useful probe of the structural integrity of some parts of the enzyme. To preserve the activity of an enzyme, the isolation, concentration and immobilization procedures must be selected properly and carefully. Changes in the activity of the enzymes due to immobilization have not been studied extensively. Occasionally, there is a decrease in activity of an enzyme when it is immobilized because of the enzyme denaturing. Therefore, care must be taken to avoid conformational disruptions by a variety of environments that the enzyme encounters.
3.3 **Factors Influencing Enzymatic Reactions**

The chief factors that determine the initial rate of a particular reaction are enzyme concentration, substrate concentration, pH, temperature, and the presence of activators and inhibitors. All of these factors will be discussed below.

### 3.3.1 Enzyme Substrate Concentration

Under given conditions, the catalytic power of enzymes comes from the interaction between the enzyme and the substrate. At a constant concentration of enzyme, $V$ (number of moles of product formed per second) is linearly proportional to the substrate concentration, $[S]$, when $[S]$ is small. At high concentrations of the substrate, $V$ is independent of $[S]$ (Figure 3.1). The model proposed that accounts for the kinetic properties of many enzymes, is

$$
E + S \overset{k_1}{\rightleftharpoons} ES \overset{k_2}{\rightarrow} E + P.
$$

(1)

The enzyme, $E$, reacts with the substrate, $S$, to form an enzyme-substrate, ES, complex with a rate of $k_1$. The ES complex can dissociate to $E$ and $S$ with a rate constant of $k_2$ or can proceed to product $P$ with a rate constant of $k_3$. To relate the rate of catalysis to the concentration of substrate and enzyme and the rates of the individual steps, one can start with an expression that the catalytic rate is equal to the product of the concentration of the ES complex and $k_3$ as

$$
V = k_3[ES].
$$

(2)

The formation and breakdown of ES can be given by:

Rate of formation of $ES = k_1[E][S]$ and

(3)
Rate of breakdown of $ES = (k_2 + k_3)[ES]$.  \hspace{1cm} \text{(4)}

Under steady-state conditions, the concentrations of intermediates stay the same while the concentrations of the starting materials and products are changing. This is true when the rates of formation and breakdown of the ES complex are equal,

\[ k_1[E][S] = (k_2 + k_3)[ES]. \hspace{1cm} \text{(5)} \]

This equation can be written as

\[ [ES] = \frac{[E][S]}{(k_2 + k_3)/k_1}. \hspace{1cm} \text{(6)} \]

Substituting a new constant, $K_M$, called the Michaelis constant:

\[ K_M = \frac{k_2 + k_3}{k_1} \hspace{1cm} \text{(7)} \]

into equation (6) gives,

\[ [ES] = \frac{[E][S]}{K_M} \hspace{1cm} \text{(8)} \]

The concentration of the uncombined enzyme $[E]$ is equal to the total enzyme concentration $[E_r]$ minus the concentration of the ES complex.

\[ [E] = [E_r] - [ES] \hspace{1cm} \text{(9)} \]

Substitution into equation 8 gives

\[ [ES] = \frac{([E_r] - [ES])S}{K_M} \hspace{1cm} \text{(10)} \]

and solving for $[ES]$ gives
Substituting this equation into equation 2 for \([ES]\), one gets

\[
[E_S] = [E_T] \frac{[S]/K_M}{1 + [S]/K_M} \quad \text{or}\quad [E_S] = [E_T] \frac{[S]}{[S]+K_M} \tag{11}
\]

The maximal rate \(V_{max}\) is reached when \([S]\) is much greater than \(K_M\) so that \([S]/[S]+K_M\) approaches 1. Therefore,

\[V_{max} = k_1[E_T] \tag{13}\]

Substitution equation 13 into equation 11 gives the Michaelis-Menten relation

\[V = V_{max} \frac{[S]}{[S]+K_M} \tag{14}\]

The kinetic data for this equation can be seen in Figure 3.1. When \([S]\) is much less than \(K_M\), \(V = [S]V_{max}/K_M\) where the rate is directly proportional to \([S]\).

When \([S]\) is much greater than \(K_M\), \(V = V_{max}\) and the rate is maximal and independent of the substrate concentration. When \([S] = K_M\), then \(V = V_{max}/2\) and \(K_M\) is equal to the substrate concentration at which the reaction rate is half the maximal value.

3.3.2 Effect of pH change

Enzymes are very sensitive to changes in pH. Each enzyme has an optimum range for pH where it will be most active. Changes in pH will affect the
binding of the enzyme to the substrate, the catalytic activity of the enzyme, the ionization of the substrate, and in the protein structure of the enzyme.

Figure 3.1. A plot of the reaction rate, $V$, as a function of substrate concentration, $[S]$, for an enzyme that obeys Michaelis-Menten kinetics ($V_{\text{max}}$ is the maximal rate and $K_M$ is the Michaelis constant). Adapted from reference 3.
3.3.3 Effect of Temperature Change

As the temperature increases the rate of reaction also increases. This rise in temperature, however, can affect the stability of the enzyme, which decreases as a result of thermal degradation. An increase of 1 °C in temperature may enhance the reaction rate by more than 10% until the optimum, and after that the enzyme is inactivated. The standard temperature for the measurement of enzyme activity is 30 °C, although 25 °C and 37 °C have been used. Most enzymes, if kept at a high temperature for extended periods of time, will denature.

3.4 Immobilization of Enzymes

When immobilizing an enzyme to a surface, it is most important to choose a method of attachment that will avoid loss of enzyme activity by not changing the chemical nature or reactive groups in the binding site of the enzyme. The surface on which the enzyme is immobilized is responsible for retaining the structure in the enzyme usually through hydrogen bonding. It is known that the main advantage of immobilized enzymes over native dissolved enzymes is the potential for repeated use, and increase in their stability and the simplicity of separating enzymes bound to carriers from the substrates and reaction products. Other advantages include the ability to stop the reaction rapidly by removing the enzyme from the reaction solution, the product does not become contaminated with the enzyme, decay rates are predictable and the elimination of some reagent preparation.
Enzyme preparations that are free of nonspecific nucleases are expensive. Therefore, a procedure that would provide the ability to separate the immobilized endonuclease from substrate and reaction products is important. Stabilization of proteins against irreversible thermal inactivation and thermal denaturation can be accomplished by immobilization. A vast variety of materials have been used for supports such as gels, sepharose, carbonaceous materials, and glass and silica beads. For the work presented here, silica particles have been selected because these particles have a vast range of pore sizes, a large surface area, are inert and relatively inexpensive.

Enzymes can be immobilized by several methods: (1) carrier-binding in which enzymes are attached to water-insoluble carriers (i.e., physical adsorption, ionic binding, and covalent binding), (2) cross-linking, where enzymes are cross-linked by bi-functional or multi-functional reagents, and (3) entrapment, where enzymes are incorporated into the lattices of a semi-permeable gel or enclosed in a semi-permeable polymer membrane (i.e., interfacial polymerization method, liquid drying, phase separation).

3.5 Methods of Immobilization

3.5.1 Carrier-Binding Method

This method is one of the oldest methods for immobilizing enzymes. The amount of enzyme bound to the carrier and the activity after the immobilization depends on the nature of the carrier (Figure 3.2). The carrier is selected based on the particle size, surface area, molar ratio of hydrophilic to hydrophobic groups.
Figure 3.2. Figure of the enzyme bound to the carrier.
and chemical composition. Increasing the ratio of hydrophilic groups and the concentration of the bound enzymes yields a higher activity of the immobilized enzymes. Some commonly used carriers for enzyme immobilization are polysaccharide derivatives such as cellulose, dextran, agarose, and polyacrylamide.

3.5.1.1 Physical adsorption Mode

Immobilization of an enzyme based on the physical adsorption of the protein on the surface of water-insoluble carriers is involved in the physical adsorption mode. There is little conformation change of the enzyme or destruction of its active site. A disadvantage to this method is that the adsorbed enzyme may leak from the carrier due to a weak binding force between the enzyme and the carrier. The major advantage of adsorption for immobilizing enzymes is that usually no reagents and only a minimum of activation steps are required. Adsorption tends to be less disruptive to the enzyme than chemical attachment of the protein because the binding is mainly through hydrogen bonds, multiple salt linkages, and/or Van der Waal’s forces. Because of these weak bonds, desorption of the enzyme can result from changes in temperature, pH, ionic strength or the substrate. Finally, because this type of binding is not specific, other proteins and substances, in addition to the enzyme, can be immobilized which can affect the properties of the enzyme.
3.5.1.2 Ionic Binding Mode

Ionic binding of the enzyme to water-insoluble carriers containing ion-exchange residues is referred to as the ionic binding mode. Generally, polysaccharides and synthetic polymers that have ion-exchange centers are used as carriers. The binding of the enzyme to the carrier is carried out under conditions that are much milder than those needed for the covalent attachment and generally yields high activity for the immobilized enzymes. This type of binding is much stronger than physical adsorption.

3.5.1.3 Covalent Binding Mode

The most utilized method of immobilization is covalent attachment between the enzyme and the solid support matrix. For covalent attachment of the enzyme to the solid support to be effective: (1) the binding reaction conditions must be such that it does not cause loss of enzymatic activity and (2) the active site of the enzyme must not be affected by the reagents used. The functional groups that may be a part of the covalent linkage are an amino, hydroxyl, thiol, carboxyl, imidazole, threonine, sulphydryl, phenolic, or indol group. The conditions under which covalent binding occurs is more complicated than the aforementioned binding modes. Covalent binding of a protein may alter the conformational structure and/or active center of the enzyme, which can result in loss of activity. The covalent attachment of the enzyme to the support should provide stable, insoluble enzyme derivatives that do not leach enzyme into the surrounding solution. For the enzyme to remain active, attachment to the support
must involve functional groups of the enzyme that are not essential for activity. A
number of binding reactions and insoluble carriers are known, and proteins can be
covalently attached even if little is known about the protein structure or active site
of the enzyme.

3.5.2 Entrapping Enzymes

The entrapment method for immobilization of the enzyme is based on the
inclusion of an enzyme within the lattice of a polymer matrix or membrane
(Figure 3.3.). This immobilization is done in such a way as to maintain the
structure of the protein but allowing penetration of the substrate(s). The enzyme
goes into the gel or membrane.

There are two basic types of entrapment, lattice and micro-encapsulation.
In the case of the lattice, the entrapment involves entrapping enzymes within the
interstitial spaces of a cross-linked water-insoluble polymer such as
polyacrylamide and polyvinylalcohol. When enzymes are encapsulated within the
semi-permeable polymer, conditions must be well controlled.

3.5.2.1 Interfacial Polymerization

This procedure involves the enclosure of the enzymes in semi-permeable
membranes of polymers. An aqueous mixture of the enzyme and hydrophilic
monomer are emulsified in a water-immiscible organic solvent. Additionally, the
same monomer is added to the organic solvent by stirring. Polymerization of the
monomers occurs at the interface between the aqueous and organic solvent phases
Figure 3.3. Figure that shows the entrapment method of immobilization where the enzyme is entrapped in a matrix and in droplets.
in the emulsion. The enzyme will be enclosed in the aqueous phase in the membrane of the polymer.

3.5.2.2 Liquid Drying

In this procedure, a polymer is dissolved in a water-immiscible organic solvent, which has a boiling point lower than that of water. An aqueous solution of enzyme is dispersed in the organic phase to form a first emulsion of water-in-oil type. The first emulsion containing aqueous micro droplets is then dispersed in an aqueous phase containing protective colloidal substances such as gelatin, surfactants, and a secondary emulsion is prepared. The organic solvent is removed by warming, and a polymer membrane is produced to give enzyme micro-capsules.

3.5.2.3 Phase Separation

In the phase separation procedure, the polymer is purified by dissolving it in an organic solvent and re-precipitating it. This is achieved by the addition of another organic solvent which is miscible in the first but that does not dissolve the polymer.

3.6 References


Chapter 4

Immobilization of the Restriction Enzymes *HaeIII* and *HindIII* on Porous Silica Particles via a Glutaraldehyde Linkage for the Micro-Digestion of dsDNA with Analysis by Capillary Electrophoresis

4.1 Introduction to Restriction Enzymes

Restriction endonucleases, which cleave deoxyribonucleic acids (DNAs) at specific base sequences, play critical roles in a variety of bioanalytical applications. Products generated from enzymatic digestion of DNA can serve as a fingerprint to the parent molecule. Restriction enzymes recognize a specific DNA base sequence and cleave the DNA at a fixed distance from or within this recognition sequence.\(^1\) Enzymatic cleavage of DNA is important in many areas, such as forensic analysis for human identification,\(^2\) genomic mapping,\(^3\) and providing information about nucleic acid structure.\(^4\)

Enzymatic digests involving DNA are normally performed in solutions incorporating a homogeneous mixture of the enzyme and the target DNA. The steps involved in a restriction analysis can be labor intensive and costly, thereby limiting their effective usage. The assay format typically involves: (1)
addition of the intact DNA to a buffered solution with the appropriate enzyme, (2) incubation of the homogeneous solution at the appropriate pH, temperature, and time most suitable for that particular enzyme, and (3) off-line analysis of the enzymatic digests. Analysis of digests can be performed by techniques such as high-performance liquid chromatography (HPLC), slab gel electrophoresis with agarose or cross-linked polyacrylamide matrices and capillary electrophoresis (CE) with an entangled polymer solution.

There are several disadvantages associated with homogeneous enzymatic digestions performed on DNA using restriction enzymes. Because the catalytic activity of restriction enzymes may decrease rapidly, the digestion conditions must be carefully monitored to ensure reproducibility. Goldberg and St. John determined the effective lifetimes for a wide range of enzymes, and found that half-lives of 0.2 hours to 15 hours are typical. Most restriction enzyme assays were developed for macro-scale separation platforms such as agarose or polyacrylamide slab gel electrophoresis. As such, assays often require large quantities of sample and reagents that are eventually discarded after one reaction, ultimately increasing the cost of the assay. For example, in the CE analysis of restriction digests, only nanoliter to picoliter injection sizes are utilized. Assays utilizing homogenous enzymatic digestions are performed in discrete steps and often require sample handling and operator intervention that can increase the potential for sample contamination and mishandling. In
addition, homogeneous digestion of DNA using restriction enzymes limits the
use of the enzyme to a single assay because the enzyme is not easily recovered
from the reaction mixture. The presence of this enzyme (protein) can be
problematic when implementing CE separations, as it can affect the separation
and/or electrokinetic sample injection.

With the emergence of immobilization technologies, efforts have been
made to attach enzymes to various supports. This approach offers
unique advantages compared to conventional free-solution enzymatic
reactions. Mosbach and coworkers have demonstrated that immobilizing
enzymes onto water-soluble matrices can enhance their stability. Cobb and
Novotny immobilized trypsin in agarose gels for β-casein digestions, and
Kuhr immobilized trypsin onto the surface of aminoalkylsilane-treated fused-
silica capillaries for β-casein digestions. These studies showed that enzymatic
digestion could be performed in-line with the micro-separation, reducing assay
complexity. Additionally, the use of immobilized enzymes has allowed the
opportunity to reduce the volumes of these types of digestions to the nanoliter
and picoliter scale making them more commensurate with the micro-scale
separation techniques, such as CE. Other reported advantages afforded by
enzyme immobilization strategies include reuse of the enzyme, increased
stability, simplicity of separation of enzymes from reaction products and the
potential for on-line processing. El Rassi and coworkers have described
capillary enzyme micro-reactors utilizing surface-immobilized RNA-modifying enzymes for the digestion of RNA. Immobilized RNA-modifying enzymes displayed similar enzymatic activity compared to free solution digestions and were usable for several RNA samples. Thus, the immobilized enzyme exhibited increased chemical and thermal stability under typical digestion conditions.

The focus of this research was to develop a technique for the immobilization of restriction enzymes onto porous silica micro-particles for the digestion of double-stranded DNA samples. It was hoped that this approach would preserve the kinetic properties of the enzyme and thus permit reuse, as well as facilitate digestion in a volume scale more commensurate with micro-analytical separation platforms. In previous work, restriction endonucleases have been immobilized by different linkage chemistries on low temperature-melting agarose, polyacrylamides and sepharoses; these supports are more appropriate for macro-scale digestion formats. In this work, we will demonstrate the ability to use micro-porous silica particles as a support medium for immobilizing restriction endonucleases. HaeIII was selected for these studies and was chosen because of its ability to act on the \( \Phi X174-RF \) DNA producing fragments that are well characterized and easy to monitor via CE. \( HindIII \) and \( PstI \) were studied as well to evaluate the applicability of the immobilization process to other endonucleases. Optimal conditions for
digestion of the target DNA phage with surface-immobilized enzymes were determined.

4.2 Materials and Methods

4.2.1 Reagents

Sodium chloride, 30 % Albumin, Bovine (BSA), sodium phosphate (dibasic), potassium phosphate (monobasic),[tris(hydroxymethly)-aminomethane] hydrogen chloride (Trizma), ethylenediaminetetraacetic acid (disodium salt), and N-2-hydroxyethyl-piperazine-N’-2-ethanesulfonic acid (HEPES) were purchased from Sigma Chemical Company (St. Louis, MO). φX174-RF DNA, SV40 Viral DNA, HindIII, and HaeIII restriction enzymes were purchased from Life Technologies, Inc. (Gaithersburg, MD). Nucleosil silica (4000Å, 10μm diameter pore) was supplied by Macherey-Nagel (Southboro, MA). 3-aminopropylmethylethoxysilane (3-APS) was purchased from United Chemical Technologies, Inc. (Bristol, PA). Glutaric dialdehyde (GA) (50%-w/v) and hydroxyethyl cellulose were obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI). Sulfuric acid and toluene were purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Dithiothreitol (DTT) was purchased from Calbiochem Corporation (La Jolla, CA). HPLC-grade water (18 MΩ) was prepared using a Millipore Milli-Q purification system from Millipore Corporation (Bedford, MA) and was used for the preparation of all buffers and washes. The DNA staining dye, Yo-Pro-1.
iodide (Figure 4.1), was purchased from Molecular Probes, Inc. (Eugene, OR). Polyacrylamide (10%; MW 700,000-1,000,000) was purchased from Polysciences, Inc. (Warrington, PA). All chemicals were used as received without further purification.

4.2.2 Buffers

The electrophoresis buffer consisted of either a 1% (w/v) hydroxyethylcellulose (HEC), 40 mM Tris, 20 mM sodium acetate, 2 mM EDTA (pH 7.5) containing 1 μM Yo-Pro-1 or a 1% HEC (Figure 4.2) in 100 mM Tris Borate EDTA (TBE, pH 8.3) containing 0.8 μM Yo-Pro-1. The electrophoresis buffers were prepared by dissolving HEC in the buffer at the appropriate concentration and at a temperature of ~80 °C with constant stirring. The solution was allowed to cool with stirring at room temperature for ~3 hours or on an ice water bath for ~ 2 hours. The solution was used without filtering. The buffers used for the enzymatic digestions were a 50 mM Tris-HCl, 10 mM MgCl₂, 50 mM NaCl (REact2, pH 8.0) or a 50 mM HEPES, 50 mM NaCl, 10 mM MgCl₂ (pH 7.5). The buffer used to wash the silica was 50 mM sodium phosphate (monobasic or dibasic) buffer (pH 7.0). The immobilized enzyme storage solution was a 30% BSA solution.
4.3 Column Maintenance

4.3.1 Washes

Prior to each day’s run, the capillary was flushed using pressure at 20 psi for ten minutes with de-ionized water. In addition, prior to each electrophoretic run, the capillary was washed with water for one minute and filled with the polymer run buffer for two minutes under pressure. A wait step was done prior to sample injection in de-ionized water to clean the end of the capillary. Proper maintenance of the capillary is essential to the capillary’s lifetime. It was found that if proper maintenance was not taken using the aforementioned steps, capillary columns would last for only a few weeks.

4.3.2 Storage

At the end of the day, the capillary was rinsed again for ten minutes with de-ionized water and stored overnight with both ends in water to prevent drying of capillary. Generally, this rinse was done without the removal of the capillary from the instrument.

4.3.3 Electrokinetic Injection

An electric potential of 10 kV (field strength of 370 V/cm) was applied to the DNA samples generally for 5 seconds regardless of sample concentration for electrokinetic injections. Electrokinetic injection is
Figure 4.1. Structure of Yo-Pro-1 Iodide.

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performed by placing the injection end of the capillary in the sample vial and applying a voltage. The field strength is usually kept below the field strength

Figure 4.2. Schematic diagram of the repeating unit in hydroxyethyl cellulose (HEC).
used for the separation. When the voltage is applied, the analyte enters the capillary.

4.4 Instrumentation

The main components of the P/ACE 5000 Series instrument (Figure 4.3) include an autosampler (two rotating trays) that holds vials of sample and other solutions, a cartridge holder and interface, a high-voltage power supply and electrodes, an optics module and detector, temperature control hardware, a sample injection mechanism and a controller with front panel displays and function switches. A Beckman P/ACE 5010 capillary electrophoresis (CE) with a Laser Module 488-argon ion laser from Beckman Instruments (Fullerton, CA) was the system used in these studies. A band-pass filter was used to enable laser-induced fluorescence (LIF) at 520 nm. The UV Absorbance analysis was also done using this instrument at 254 nm.

4.5 Data Processing Software

For each electropherogram, the migration time of each peak was recorded in addition to the area and height of the peak. Beckman P/ACE Windows software (version 1.0) was used to process the data. The data collection and analysis package enables electropherograms to be collected, analyzed, and reported simultaneously. Integration parameters, calibrations, report format, and post-run tasks can be programmed prior to data collection.
Figure 4.3. Figure of the CE system. From left to right are the argon laser, the CE instrument, and the data processing unit.
4.6 Columns

The columns used in these studies were a 50 μm i.d., 363 μm o.d., C-Elect-N (Supelco, Bellefonte, PA) or a 50 μm i.d., 363 μm o.d. DB-17 J&W Scientific (Folsom, CA). The neutrally coated Supelco capillary has virtually no electroosmotic flow (EOF). The DB-17 is a (50%-phenyl)-methylpolysiloxane bonded and cross-linked coated capillary according to the manufacturer (Figure 4.4). These capillaries were chosen to prevent interactions with the capillary wall during the electrophoresis. The length used in these studies was 27 cm with an effective length of 20 cm.

Using a method similar to Bocek and Chrambach,26 an optical window was etched in a section of the polyimide coating by placing a drop of concentrated sulfuric acid solution on a hot CE-μEZ unit, which was purchased from J&W Scientific (Folsom, CA). After the coating was removed, the optical window was cleaned carefully with methanol to prevent breakage. The capillary was filled with de-ionized water using a syringe prior to this process. Once inside the Beckman cartridge, the capillary was kept at a constant temperature of 25 °C through liquid cooling. Separations were performed in reversed polarity with the cathode on the injection end at this same temperature.
Figure 4.4. Figure of the derivatized capillary wall of a DB-17 coated capillary. The capillary consists of a randomly distributed (50%-phenyl)-methylpolysiloxane bonded and cross-linked coated capillary.
4.7 Separation Conditions

Constant voltage was applied during the separation analysis. The separation voltage that was applied was 8 kV (296 V/cm with a 27 cm capillary) which generated a current of ~31 μA with the Supelco capillary and ~10 μA of current with the J&W Scientific capillary.

4.8 Operation of the Instrument

A method is programmed into the Windows 95 driven computer software that controls the autosampler and labeling of the electropherograms produced. The electrophoresis samples were pipetted into a Beckman sample vial (similar to an eppendorf tube) and placed into the sample holder where a silicone rubber cap was screwed on. The sample was then loaded into the sample tray. The names of the samples were placed in the program prior to the run. The method parameters are shown in Figure 4.5. The capillary temperature was preset to 25 °C with LIF detector conditions \( \lambda_{ex} = 488 \text{ nm}, \lambda_{em} = 520 \text{ nm} \). The capillary was rinsed for 1 minute with de-ionized water from inlet vial 33 to the outlet waste vial 10. This rinse was followed by a 2-minute rinse to fill the capillary with the run buffer from inlet vial 34 to the waste vial 10. The sample was then injected electrokinetically for 3 or 5 seconds at the inlet with the outlet in run buffer vial position 1. The sample was then electrophoresed at -10kV for 10 minutes from the two buffer positions on the inlet (#34) and outlet (#10). A final rinse with de-ionized
water was completed from vial #11 to waste vial #10 before the program ended.

4.9 Derivatization Protocol for Silica

The procedure for derivatizing the surface of the silica prior to immobilization of the enzyme was similar to that described by Locascio-Brown et al. with slight modifications (Scheme 4.1).27 Approximately 0.1 g of spherically shaped porous silica particles (10 μm diameter, 4000Å pore) was placed in a snap-cap vial to which a 10% solution of 3-APS in anhydrous toluene was added. This silica was chosen because of the loading level of the enzyme as well as for its ability to accommodate the DNA as it passes into and out of the pores. The anhydrous toluene was prepared by boiling the solution for approximately 5 minutes or until the volume had been reduced by ~5%. The silica slurry was mixed on a vortex mixer and incubated at 45-50 °C overnight in a water bath. The contents of the tube were centrifuged, and the supernatant fluid was decanted. The excess 3-APS was removed from the silica by washing the silica three times with 200 μL aliquots of each of the following solutions: fresh toluene, absolute ethanol, and de-ionized water. The silica was then reacted with a 5% (v/v) aqueous solution of glutaraldehyde (GA) and the mixture was shaken continuously for 4 hours at room temperature. The GA-derivatized silica had a characteristic pink color. The supernatant was discarded and residual glutaraldehyde reagent was removed.
Figure 4.5. Schematic representation of the program that is used for the Beckman CE system.
Scheme 4.1. Schematic diagram of the attachment of the restriction enzyme to the porous silica particles.
from the silica by extensive water washes. The silica was stored in de-ionized water at 4 °C until used.

For attachment of the restriction enzyme to the GA derivatized silica particles, approximately 9 μL (~0.00944 g) of the silica slurry was added to a snap-cap vial using a Gilson pipette. The reaction buffer (63 μL) was added to the slurry followed by the HaeIII or HindIII enzyme solution (18 μL, 900 U). The reaction mixture was kept on ice for about three hours with occasional mixing by gentle tapping. The reaction vessel was then incubated in a 37 °C water bath for approximately 4 hours with occasional mixing and then placed on an ice water bath overnight. The following day, the vial was centrifuged and the supernatant fluid was removed. The resultant immobilized enzyme was washed three times with 200 μL aliquots of the phosphate buffer and one time with 500 μL of water and stored in a 30% BSA solution until used. This BSA solution was found to preserve the activity of the enzyme until required for use.

4.10 Immobilization of Restriction Enzymes

The work presented here involves the chemical modification of porous silica particles to covalently bind restriction and RNase enzymes. The resulting preparations were studied to determine activity of the bound enzymes as well as the optimal reaction conditions. Porous silica, because of its well-
defined pore diameter, offers many advantages as a support material. The
glass surface, however, has some undesirable qualities such as adsorption of
proteins and some anions. For the immobilization of enzymes, several support
parameters play an important role such as stability, pore diameter surface area
and particle size.

There are a number of ways to covalently attach an enzyme to the
surface of a solid support (Table 4.1). Surface derivatization using silanes is a
common method to impart a particular chemical character or reactivity to solid
substrates, which have free silanols groups on their surfaces. These silane-
coupling agents have one end that reacts with the silanols on the silica surface,
and leaves a new primary amine in our immobilization scheme. Silanizing
reagents have up to three groups that can react with glass silanols. Silanizing
reagents can produce a monolayer thick, cross-linked polymeric film. This
primary amine can be reacted with glutaraldehyde to leave a free aldehyde
functionality. This aldehyde group can then react with a primary amine on the
enzyme to tether it to the surface of the silica. Attaching enzymes to the silica
surface did not prove to be a simple process.

4.11 Silanization for RNase Enzymes

For the RNase enzymes, the silanization with 3-aminopropyltriethoxy
silane (3-APTS) was performed based on a modification of the procedure of
Nashabeh and El Rassi28 with slight modifications. The dry silica was placed
Table 4.1. Covalent Bonding reactions with enzymes to the solid support.

<table>
<thead>
<tr>
<th>Covalent Binding</th>
<th>Support Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diazotization</td>
<td>SUPPORT—N=N—Enzyme</td>
</tr>
<tr>
<td>Amide bond formation</td>
<td>SUPPORT—CO-NH—Enzyme</td>
</tr>
<tr>
<td>Alkylation and Arylation</td>
<td>SUPPORT—CH₂-NH—Enzyme</td>
</tr>
<tr>
<td></td>
<td>SUPPORT—CH₂-S—Enzyme</td>
</tr>
<tr>
<td>Schiff’s base formation</td>
<td>SUPPORT—CH=N—Enzyme</td>
</tr>
<tr>
<td>Amidation reaction</td>
<td>SUPPORT—CNH-NH—Enzyme</td>
</tr>
<tr>
<td>Thiol-Disulfide interchange</td>
<td>SUPPORT—S-S—Enzyme</td>
</tr>
<tr>
<td>Carrier binding with bi-functional reagents</td>
<td>SUPPORT—O(CH₂)₂N=CH(CH₂)₃CH=N—Enzyme</td>
</tr>
</tbody>
</table>
in 10% solution of the 3-APTS in acetone and was reacted at 45 °C overnight (>12 hours). The unreacted silane was washed with fresh acetone and deionized water. The silica was allowed to dry at room temperature. See Chapter 5 for more specific reaction details.

4.12 Aldehyde Linkage and Immobilization

The silanized silica was reacted with a 5% aqueous glutaraldehyde solution for approximately 3 hours. The unreacted aldehyde was washed thoroughly with distilled water. The enzymatic buffer solution was added and the reaction was allowed to proceed at 4 °C with intermittent stirring.

4.13 Study of Immobilization

Initially, problems occurred with the DNA digests of the immobilized restriction enzymes. No DNA was detected in the CE separation sample. It was concluded that the DNA was “sticking” to the silica. To determine the step where the DNA stuck, a DNA sample was added after each step (silanization, aldehyde linkage, and enzyme attachment) in the process and allowed to incubate. It was determined that the DNA “stuck” after the silanization step. It was then believed that the ethoxy-groups on the silanizing agent posed the problem when digestion attempts with DNA took place. To prove this, hexylmethyldisilizane (HMDS) was reacted after attachment of silane. Once the ethoxy-groups were removed, the remaining attachments were the same. When the DNA was then digested, electrophoresis revealed the
DNA with the proper fragmentation pattern. As an alternative to the HMDS step, 3-APS was chosen and worked as an excellent silanizing agent, and the 3-APS could be used without further derivatization.\textsuperscript{23,29,30} The work presented in this dissertation will demonstrate the derivatization of the surface of porous silica and PMMA based on the above procedures.

4.14 Derivatization of PMMA for Enzyme attachment

A method similar to Karandikar\textsuperscript{31} et al. was used to derivatize the poly(methyl methacrylate) (PMMA) leaving a free primary amine (Scheme 4.2). The method used to attach the glutaraldehyde and enzyme was performed as described in the above section.

4.15 Digestion Conditions

The $\phi X174-RF$ DNA or SV40 Viral DNA (100 $\mu$L; 0.025 $\mu$g/$\mu$L) in the reaction buffer was added to 3 $\mu$L of the silica bound and incubated at 37 °C with occasional gentle tapping to mix the reaction. The mixture was then centrifuged and aliquots of the supernatant fluid were removed for analysis by electrophoresis. After the DNA digest was removed, the immobilized enzyme particles were washed three times with 50 mM phosphate buffer and one time with de-ionized water. A drop of 30% BSA solution was added to the reaction vessel and the silica was stored for reuse in the dark at 0 °C. Prior to reusing

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Scheme 4.2. Schematic for the derivatization of the PMMA to attach the \textit{HindIII} enzyme.
the enzyme silica particles, the BSA solution was removed and the silica was washed three times with the phosphate buffer and one time with water.

4.16 Silica-Immobilized Reaction Products Purification

A DNA fragment isolation purification protocol was based on a process developed by Ruiz-Martinez et al. for removal of the small molecules from a sample. Digestion of the DNA by the immobilized HaeIII enzyme was performed in a centrifugal device for ultrafiltration (spin column) with a molecular weight cutoff (MWCO) filter of 300,000 (Pall Filtron, Northborough, MA). To suppress nonspecific binding, the membranes were pre-treated with an aqueous solution of 0.005% (w/v) linear polyacrylamide (LPA; average molecular weight 700,000-1,000,000). A 500 μL aliquot of the LPA solution was placed on the spin column and centrifuged for ~45 minutes at 3000 rpm. The solution was discarded and a 500 μL aliquot of de-ionized water was added to the device and centrifuged at the same speed. The φX174-RF DNA solution with the enzyme-immobilized silica was placed in the ultrafiltration centrifugal device and the chamber was shaken in a Microincubator M-36 (Taitech Instruments USA, Inc., San Jose, CA) at 37 °C.

4.17 Results and Discussion

The HaeIII restriction enzyme recognizes a four base sequence in double-stranded DNA and cuts the strands between G (guanidine) and C (cytosine) within this sequence (5'-GG↓CC-3', 3'-CC↑GG-5'; the arrow
indicates cut site). The HindIII restriction enzyme recognizes a six base sequence on the DNA and cuts the strands between two adenosine residues (A) embedded within the recognition sequence (5'-A↑AGCTT-3', 3'-TTCGATA↑A-5'). Digestion of the circular, double-stranded DNA, φX174-RF phage (5386 bp), with HaeIII produces 11 fragments and is a commonly used standard for size determinations of linear, double stranded DNA from 72 to 1353 bp in length. To evaluate whether coupling of the restriction endonuclease to the silica altered their catalytic properties, the digestion fragments produced were identified by their sizes using CE. Individual assignments to each band in the electropherogram were based upon the reported separations of the φX174-RF/HaeIII restriction fragments. Digestion of the circular, double-stranded SV40 DNA with HindIII produces six fragments ranging from 1046 to 5171 bp. For the experiments presented here, only five fragments were separated for both the free and immobilized digests of this particular DNA, with the 4002 bp and 5171 bp fragments co-migrating. Figures 4.6-4.9 show examples of the fragmentation patterns that will result from these digests. The buffer chosen for the immobilized digestions identically matched the buffer used for the free solution digest and was that recommended by the manufacturer. No detectable differences were observed in the DNA digestion patterns for the free solution or immobilized enzymes suggesting that enzyme immobilization had no direct effect on the initial recognition sequence of the enzyme (Figures 4.6, 4.7, 4.8,
Figure 4.6. Electropherogram of the PMMA immobilized digest of \( \phi X174 \) DNA/HaeIII restriction fragments (0.025 \( \mu g/\mu L \)) with LIF detection. *Capillary:* J&W Scientific, 50 \( \mu m \) i.d. x 27 cm (20 cm to detector). The dye concentration (500 ng/mL) was added to the run buffer composed of 1% HEC in 100 mM TBE (pH 8.3). The sample was injected electrokinetically for 3 s at -10 kV onto the column. The electric field strength used for the separation was 296 V/cm. The digest was complete in \(~240\) minutes.
Figure 4.7. Electropherograms of SV40/DNA HindIII restriction fragments (0.025 mg/mL) with LIF detection free solution and timed study. Capillary: J&W Scientific, 50 μm i.d. x 27 cm (20 cm to detector) Buffer: 1% HEC in 40 mM Tris, 20 mM NaAcetate, 2 mM EDTA (pH 7.5), 1μM YO-PRO-1. Injection: 3s; -10 kV. Separation: -8kV, 10 μA. Electric field strength used for the separation was 296 V/cm. A) Free solution (1 hour); silica immobilized digest: B) 30 min C) 120 min D) 180 min E) 240 min after start of digestion.
Figure 4.8. Electropherogram of ϕX174 DNA/HaeIII restriction fragments (0.025 μg/μL) with LIF detection. *Capillary:* J&W Scientific, 50 μm i.d. x 27 cm (20 cm to detector). The dye concentration (500 ng/mL) was added to the run buffer composed of 1% HEC in 100 mM TBE (pH 8.3). The sample was injected electrokinetically for 3 s at −10 kV onto the column. The electric field strength used for the separation was 296 V/cm. A) Free solution digest B) silica-immobilized enzyme digest sampled after 30 minutes. The numbers above the peaks indicate the DNA fragment size (bp).
Figure 4.9. Electropherogram of φX174-RF DNA/HaeIII restriction fragments (0.025 mg/mL) with LIF detection. Capillary: Supelco CElect-N 50 mm o.d. x 27 cm (20 cm to detector) Buffer: 1% HEC in 40 mM Tris, 20 mM NaAcetate, 2 mM EDTA pH 7.5, 1 μM Yo-Pro-1. A) 30 min B) 60 min C) 90 min D) 120 min
and 4.9). It was observed that the migration rates for the enzyme-immobilized restriction-enzyme digest were consistently faster than the homogeneous solution digest [Figures 4.6, 4.7 (A, E) and 4.8]. In the solution digest case, buffer components such as BSA, glycerol and DTT are present in the reaction solution but absent in the immobilization digest case. The presence of these components may in some way retard the migration of the fragments and/or alter the amount of sample injected into the CE column. The increased viscosity of the free solution digest most likely accounts for the longer migration times observed for this sample. In the electropherogram for the free solution digest (Figure 4.8A), a broad peak was observed under the last four fragments, which could possibly be attributed to the presence of the enzyme used for the restriction digest or other digest components. This peak was present to a lesser extent in the immobilized digest electropherogram, indicating that the peak could have originated from the restriction enzyme, which binds some of the staining dye producing a fluorescence signal. Since this band co-migrates with the high base pair restriction fragments, elimination of this band in the electropherogram may be important when low-level analysis is required, as its presence may mask the components of interest.

To investigate the kinetics of the surface-immobilized restriction digestion of \( \Phi X 174-RF \) using HaeIII, the time-course of the digestion was followed by sampling the reaction mixture every 30 minutes and analyzing the
sample using capillary electrophoresis (Figure 4.8). There are distinct
differences in the digestion pattern at the beginning of the reaction (Figure
4.8A). As can be seen from Figure 4.8, the restriction pattern does not
resemble the free solution case until approximately 2 hours (Figure 4.8C) after
the initiation of the reaction. The manufacturer, in the free solution case,
recommends one hour of digestion. As the reaction solution was mixed by
only occasional tapping of the reaction vessel, the apparent slow kinetics of the
reaction could be ascribed to the fact that diffusional effects play an important
role in determining the rate of the enzymatic reaction or that the enzyme had
gradually denatured (i.e., lower activity). For the digestion reaction to occur,
the DNA must diffuse to the surface of the silica particles or PMMA before it
can be effectively digested. In the free solution case, this diffusional
restriction is not present and the reaction proceeds at a rate which is
determined solely by the kinetics of the restriction enzyme. In the surface-
immobilized case, the reaction proceeded slower because the silica particles
settled to the bottom of the snap-cap vial and the DNA must therefore diffuse
to the bottom of the vial before digestion can commence. The immobilization
of the enzyme on the surface is a random process whereby some of the enzyme
molecules may be inactive due to this orientation. For digestion to occur
properly, the active site of the enzyme must be oriented for proper access to the
DNA molecules. This orientation may account for some of the efficiency of digestion of the enzyme.

To evaluate whether diffusion to the surface for the surface-immobilized case was rate limiting, the reaction vessel was continuously agitated at ~1200 oscillations per minute to keep the silica particles dispersed throughout the reaction solution. The results of this investigation are depicted in Figure 4.8, which show that complete digestion of the φX174-RF with the HaeIII immobilized enzyme could be achieved in 30 minutes as the pattern and the peak intensity did not change after the first CE sample was taken. The free solution digestion of the φX174-RF was monitored and was found to occur in less than 15 minutes (results not shown). It was found, however, that the speed at which the immobilized-enzyme reaction mixture was shaken was critical. When shaken vigorously, the enzyme was rapidly denatured as evidenced by the lack of the appearance of the distinctive CE pattern generated by the HaeIII digest of φX174 DNA. Therefore, the speed of agitation was carefully adjusted to prevent deactivation of the enzyme as well as to keep the silica particles fairly well dispersed in the reaction solution. The observed results may be a consequence of conformational changes associated with the immobilization chemistry used to tether the enzyme to the silica particles or to partial denaturation of the protein due to the forced convection associated with the shaking action. The glutaraldehyde chemistry used to attach the protein to
the silica reacts non-selectively with primary amine sites either on amino acid residues with primary amines not involved in the amide bonds or the free amine end of the protein. If the tethering disrupts the active site of the protein, then the digestion will not occur irrespective of agitation of the reaction solution. In the non-agitated experiments or those agitated at intermediate speeds, digestion of the DNA was observed indicating that for this particular enzyme, the linkage chemistry did not deactivate the enzyme completely. Most likely, when the shaking speed was high, denaturing conformational changes are encountered by the enzyme, distorting its active site. Therefore, to minimize this effect and at the same time enhance the enzyme kinetic rate by increasing diffusion, special attention must be paid to the convection speed.

We also examined the ability to reuse the immobilized enzyme by subjecting it to multiple rounds of digestion with fresh substrate solution. On the second round of digestion with gentle agitation, a characteristic CE digest pattern resulted, but the digestion required 5 hours or longer to complete. We have attributed this degradation in performance to partial heat denaturation of the immobilized protein when exposed to 37 °C conditions required for the first round of digestion.

Additional experiments were performed to study enzyme activity as a function of digestion buffer conditions and buffer compositions. Various amounts (0.5-10 mM) of dithiothreitol (DTT) and β-mercaptoethanol were
added to the digestion buffer. These reagents are known to reduce disulfide bridges that may form between two polypeptide chains of the protein. At low concentrations, DTT stabilizes enzymes that possess free sulfhydryl groups and restores activity lost by oxidation of these groups in vitro. With the addition of DTT to the reaction buffer, complete digestion was accomplished in 30-120 minutes depending on the concentration of the reducing agent added to the solution cocktail. In each case, however, the DNA fragmentation pattern was identical to those shown in Figure 4.7. When the concentration of the reducing agent was high (10 mM), the reaction was found to be complete in ~30 minutes. However, because of loss of activity, the enzyme could not be reused for subsequent digestions. At lower concentrations (0.5 mM), complete digestion was accomplished in ~60 minutes and one additional digest using the same immobilized enzyme sample was found to require 180 minutes. HEPES was also investigated as a possible digestion buffer alternative in an effort to preserve the activity of the enzyme following digestion. The initial digestion time with the HEPES buffer system was much longer (180 minutes) than with the manufacturer recommended buffer. Reuse of the enzyme was possible, but subsequent digests took longer than 7 hours to complete (results not shown).

A simple process utilizing ultra centrifugation (Figure 4.10) was devised for DNA digestion and isolation. The derivatized silica particles were placed as a thin layer on a molecular weight cut-off filter of an ultra-
Figure 4.10. Schematic diagram of the ultra-centrifugation device used to separate the DNA from the reaction mixture.
centrifugation device and the reaction mixture containing DTT, reaction buffer and target DNA were added followed by slight agitation of the reaction vessel. After the desired reaction time, the centrifugation device was spun and aliquots (10 μL) were taken from the incubated mixture and then analyzed by CE. Complete digestions required ~240 minutes. Because of the construction of the ultra centrifugation device, it was difficult to visualize the rate at which the silica slurry was mixed, which could have accounted for the extended reaction time. As with the previous immobilized enzyme experiments, the migration times of the fragments occurred at slightly faster rates compared to the free solution digestion. Again, this was attributed to the absence of extra digestion buffer components in the reaction mix.

To investigate the general applicability of this surface-immobilization approach for other restriction enzymes, experiments were carried out using PstI and HindIII. Attempts to surface immobilize the PstI enzyme to silica particles using the glutaraldehyde linkage chemistry were found to be unsuccessful. At the time these experiments were done, it was not known that PstI could not be immobilized through this linkage. This result, however, is in agreement with those of Reddy et al., who immobilized this particular enzyme to CNBr-activated sepharose and observed that binding of PstI through its primary amino groups resulted in the complete loss of activity.\textsuperscript{35} This suggests that immobilization of the amino groups causes structural changes resulting in
loss of catalytic activity of the enzyme, and that it may be possible to retain activity by linkage via the carboxylate or other functional groups. We also made a preliminary investigation of the immobilization of \textit{HindIII} to silica particles using the glutaraldehyde tethering system as well as tethering to PMMA. Figure 4.6 shows the result of the \textit{\phi}X174RF digest of the PMMA immobilized \textit{HaeIII} and Figure 4.7 shows the results for the digest of SV40 DNA with the surface-immobilized \textit{HindIII}. In these examples, the digestions required \(\sim\)240 minutes; however, reaction conditions such as the choice of the buffer system and/or the addition of reducing agents were not optimized. It is likely that reaction conditions must be optimized for each immobilized restriction endonuclease as well as for each surface that is used.

4.18 Derivatization of the silica with 3-aminopropyltriethoxysilane

Initial experiments to attach the restriction enzymes to the silica involved the use of 3-aminopropyltriethoxysilane (APTS), which has been used frequently in the past, and were similar to that done by Amanakwa and Khur.\textsuperscript{16} After the derivatization was complete, a digestion of the enzyme-immobilized silica with the \textit{\phi}X174-RF phage was performed. When the electrophoresis was performed, no DNA fragments were detected. It appeared that the DNA was "sticking" or somehow binding to the silica. Methanol and 0.1 M NaOH precipitations were attempted, without success, to remove the DNA.
The immobilization procedure was redone and at each step in the derivatization, DNA was added to the silica to see where the “sticking” occurred. It was determined that the DNA was “sticking” at the step where the APTS was attached. It appears that there is hydrogen bonding reactions between the DNA and the ethoxy groups on the APTS and/or the free silanols groups on the silica’s surface. To prevent DNA binding, after attachment of the APTS, the silica was reacted with a 10% hexamethyldisilazane in acetone solution overnight (Scheme 4.3). The remaining steps of the enzyme attachment reaction were done as described in Section 4.10. When the digestion with the DNA was then performed, the fragments were detected in the CE analysis.

4.19 Conclusions

In this work, we demonstrate for the first time the ability to immobilize the restriction enzymes on porous silica micro-particles as well as poly(methyl methacrylate) via a glutaric dialdehyde linkage. This tethering system attached moieties to solid supports through their primary amine functionalities, and, for HaeIII and HindIII, the enzymes were found to remain active upon immobilization. No activity was observed for PstI after immobilization. It has also been shown that the HaeIII enzyme can be reused for repeated digestions, but at a slower rate due to partial deactivation. The ability to immobilize these important enzymes to solid-supports offers some unique advantages compared
Scheme 4.3. Schematic diagram of the derivatization reaction of hexylmethyldisilazane to the silica.
to free solution digestion, namely, the possibility of assay cost reduction through reduced usage of enzyme, minimization of contamination due to sample handling, lack of interference from added reagents (BSA, etc.), implementation with on-line assays, and continuous processing for high throughput applications. For CE analyses of restriction fragments, this immobilization strategy potentially can eliminate some of the reagents present in the non-immobilized enzyme solution, which can co-migrate with the components of interest masking their presence. In addition, the removal of the enzyme can eliminate inhibitory effects produced by the residual enzyme when the target DNA is subjected to additional digestions by other restriction enzymes. We have shown that the addition of reducing agents at some concentrations can aid in stabilization of the system. Investigations are also being conducted to determine ways to miniaturize and integrate this process directly into micro-fabricated electrophoresis systems for the on-line and rapid restriction analysis of DNA.

4.20 References


Chapter 5

Immobilization of Enzymes on Solid Supports for the Micro-Digestion of RNA with Capillary Electrophoresis Analysis

5.1 Introduction to RNase Enzymes

Enzymes play an important role in cleaving deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) into specific products that are more readily analyzed than the parent molecule. Enzymes used on large DNA and RNA molecules often produce products that serve as a fingerprint of the parent molecule. Enzymatic cleavage of DNA products is known to be important in areas such as forensic analysis\(^1\) and genomic-mapping.\(^2\) Enzymatic analysis of RNA has been used as a general method for the determination of organismal relationships\(^3\), RNA structure\(^4,5\), and sequence placement of structurally important modified nucleosides.\(^6,7\) In addition, RNA profiling has been useful for the identification of microorganisms such as retroviruses and bacteria\(^8\) and detection of gene expression using RNA hybridization techniques.\(^9\)

The aim of this work was to immobilize RNases T\(_1\) and RNaseA via a glutaraldehyde linkage onto 10 μm porous fused silica particles for the rapid digestion of RNA using minute quantities of sample.
5.2 Experimental

5.2.1 Reagents

Isoaccepting tRNA$_1^{\text{Tyr}}$ from *E. coli*, polyoxyethylenesorbitan monolaurate (Tween 20), 5% Bovine Serum Albumin (BSA), sodium chloride, anhydrous sodium phosphate, \{tris(hydroxymethyl)aminomethane\} hydrogen chloride (Trizma), and ethylenediaminetetraacetic acid (disodium salt) were purchased from Sigma Chemical Company (St. Louis, MO). 16S and 23S ribosomal RNA was purchased from Boehringer (Mannheim, Germany) and Nucleosil 4000-5 silica was purchased from Macherey-Nagel (Germany). Ribonuclease T$_1$ (RNase T$_1$) and Ribonuclease A (RNase A) were purchased from Ambion (Austin, TX). Potassium phosphate (monobasic), 3-aminopropyltriethoxysilane (3-APS), and (50%) glutaric dialdehyde were obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI). Potassium chloride was purchased from Fisher Scientific (Fairlawn, NJ) and sulfuric acid and acetone were purchased from Mallinckrodt Chemical, Inc. (Paris, KY). Ammonium chloride was obtained from J. T. Baker (Phillipsburg, NJ). HPLC-grade water (18 MΩ), prepared using a Millipore Milli-Q purification system from Millipore Corporation (Bedford, MA), was used for preparation of all buffers. All chemicals were used as received without further purification.
5.2.2 Buffers

The immobilization wash buffer was a phosphate buffered saline (PBS-T)\textsuperscript{10} containing 1.5 mM KH\textsubscript{2}PO\textsubscript{4}, 8 mM Na\textsubscript{2}HPO\textsubscript{4}, 135 mM NaCl, 3 mM KCl and 0.1% Tween 20. The presence of the non-ionic detergent in the buffer prevented aggregation of the silica micro particles. The electrophoresis running buffer (Buffer A) consisted of 18 mM acetic acid, 2 mM ammonium acetate, pH 3.9, and was used for all separations except where noted.\textsuperscript{11} Buffer B (10 mM Tris-HCl) or Buffer C (50 mM Tris-HCl, 1 mM EDTA) was used to perform all free solution and some silica immobilized enzymatic digests unless otherwise noted in the figure caption and the text.

5.2.3 Preparation of the Silica for Enzyme Immobilization

The procedure for derivatizing the silica was similar to that described by Nashabeh and El Rassi\textsuperscript{12} with slight modifications (Scheme 5.1). A small amount of the irregular shaped silica particles was placed in a snap-cap vial to which a 10% solution of 3-aminopropyltriethoxysilane (3-APTS) in acetone was added. The mixture was incubated at 45°C overnight in a water bath. The mixture was centrifuged and the supernatant fluid was removed. The silica was washed three times with 200 µL aliquots each of fresh acetone and de-ionized water and was allowed to air-dry at room temperature. A 1% volume fraction of glutaric dialdehyde in 50 mM phosphate buffer pH 7.0 was then added to the silica and placed on an orbital rotor for continuous mixing for three hours at room...
Scheme 5.1. A schematic diagram showing the binding via glutaraldehyde of an enzyme onto the surface of porous silica particles.
temperature. The silica was centrifuged and the supernatant removed revealing a
color change of the silica from white to pink over the course of the reaction. To
remove any unreacted glutaric dialdehyde, the derivatized silica particles were
washed three times with the PBS-T immobilization buffer and stored in this buffer
until used. For the enzyme immobilization, approximately 2 μL of the silica
slurry was added to a snap-cap vial. Buffer B (29 μL) was added to the silica
followed by RNase T₁ (1 μL, 1000U) or RNase A (1 μL, 1 mg/mL). The reaction
mixture was incubated in a 37 °C water bath for 1.5 hours with occasional mixing.
The solution was then centrifuged, the supernatant removed, and the silica washed
three times with 200 μL aliquots of PBS-T. A 5% BSA solution (200 μL) was
then added to the silica to react with any residual free aldehyde groups at 37 °C
for 1.5 hours. The BSA solution was removed and the silica was washed three
times with 200 μL aliquots of PBS-T. For the off-line digestion, tRNA₁<sub>Tyr</sub> (0.25
or 0.50 μg/μL) in Buffer A or B was added to the silica for reaction at required
time and temperature. After centrifugation, the supernatant was electrophoresed
for analysis of the digestion products.

5.2.4 Capillary columns and packed bed capillaries

CElect<sup>TM</sup>-N neutrally coated capillary columns of 50 or 75 μm ID and 363
μm OD were purchased from Supelco (Bellefonte, PA). The total capillary length
used for the electrophoresis studies was 27 cm with an effective length of 20 cm.
Windows were made by placing a hot sulfuric acid solution on a CE-μEZ unit supplied by J&W Scientific (Folsom, CA).

5.2.5 CE Instrumentation

A Beckman P/ACE 5010 CE instrument from Beckman Instruments (Palo Alto, CA) was used in reversed polarity for all separations. Samples were injected electrokinetically at 370 V/cm for 10 s and separation was performed at an electrical field strength of 370 V/cm unless otherwise stated. Oligonucleotides were detected at 254 nm using a UV detector with the capillary temperature set at 25 °C.

5.3 Results and Discussion

Ribonuclease T₁ (RNase T₁) cleaves the phosphodiester bond between the 3' of a guanine (G) residue and the 5'-hydroxyl group of the adjacent nucleotidyl residue, and RNase A cleaves 3' of uracil (U) and cytosine (C) residues of single stranded RNA. The objective of this study was to investigate the digestion and separation parameters for silica-immobilized and free solution RNase A and RNase T₁ digests of tRNA₁⁰⁰. Digestion of tRNA₁⁰⁰ with RNase T₁ should lead to the formation of the 24 oligonucleotides shown in Table 1. Complete separation of unique fragments should lead to 15 distinct peaks in the electropherogram. Digestion of tRNA₁⁰⁰ with RNase A leads to the formation of the 43 oligonucleotides shown in Table 2. Complete separation of the fragments would lead to 19 distinct peaks in the electropherogram.
5.4 Optimization of CE separation conditions

The effect of the CE running buffer on the separation was first assessed to determine which conditions would produce the best resolution of the digestion products described above. The pH of the separation buffer (Buffer A) was varied using a 50% aqueous ammonium hydroxide solution to determine the best pH for the separation of the oligonucleotides produced from the enzymatic digest. Buffer A was chosen because it provides a means of separating the digestion products without the use of a sieving matrix and is compatible with mass spectrometric detection (work in progress). Figure 5.1 shows the separation of free solution digestion products performed at selected pHs over the range from 3.9 to 7. It was discovered that the best separation and resolution for the largest population of oligonucleotides for this digest occurred in the lower pH range. The overall pattern in the electropherograms at various pHs differed significantly. The number of distinct peaks found in the electropherogram in Figure 5.1A matches remarkably well with the value of 15 peaks expected for this digest. Figure 5.1B also shows peaks matching very closely to the number expected. Because figure 5.1A appeared to match more closely, all subsequent separations in this report were performed using Buffer A at pH 3.9.

5.5 Activities of RNase enzymes in free solution and immobilized to silica

The amount of time required to achieve complete digestion of the \( tRNA_{1}^{Tyr} \) with the immobilized RNase \( T_1 \) and RNase A enzymes was determined.
Figure 5.1. Free solution study of the digest of E. Coli tRNA^Tyr with RNase T1 in 10 mM Tris-HCl pH 7.0; Electrophoresis done in acetic acid/ammonium acetate buffer at various pH with injection at -10 kV for 10 seconds A) pH 3.9 B) pH 4.5 C) pH 5.0 D) pH 6.0 E) pH 7.0

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Table 5.1. Fragments produced by the digestion of tRNA^Tyr^ with RNase T₁.

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</tr>
<tr>
<td>AGp</td>
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<td>1</td>
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</tr>
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</tr>
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</tr>
<tr>
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A= adenosine, G=guanosine, c=cytidine, U=uridine, T=thymine, #=2'-0'-methylguanosine, *=2-methylthio-N6-isopentyladenosine, 4=4-thiouridine (s^4U), Q=queuosine, ψ=pseudouridine

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Table 5.2. Fragments produced by the digestion of RNase A with tRNA^{Tyr}

<table>
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<td>GCp</td>
<td>1</td>
</tr>
<tr>
<td>QUp</td>
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</tr>
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<td>1</td>
</tr>
<tr>
<td>GACp</td>
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</tr>
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<td>#GCp</td>
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<td>1</td>
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<td>AGACp</td>
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</tr>
<tr>
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<tr>
<td>AAAGGGGAGCp</td>
<td>1</td>
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</table>

A= adenosine, G=guanosine, C=cytidine, U=uridine, T=thymine, #=2'-0-methylguanosine, *=2-methylthio-N6-isopentyladenosine, 4=4-thiouridine (s^4U), Q=queuosine, ψ=pseudouridine

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Free solution experiments showed that complete digestion of *E. coli* tRNA$_{\text{Tyr}}$ with either enzyme occurs in less than 20 minutes under the conditions suggested by the manufacturer. Complete digestion of tRNA$_{\text{Tyr}}$ using the immobilized enzyme was accomplished after 60 minutes using the silica-immobilized RNase T$_1$ (Figure 5.2). The free solution and immobilized digests are qualitatively similar indicating the immobilization of the enzyme did not have any significant effect on the activity and specificity of the enzymes (Figures 5.1A and 5.2C). Digests for the immobilized RNase A were completed in less than thirty minutes, suggesting that the catalytic activity of the immobilized RNase A is greater than that of the immobilized RNase T$_1$ (Figure 5.3). The manufacturer reports that for free solution digests, the RNase T$_1$ is significantly more active than RNase A. From this data, it appears that the immobilization has a negative effect on the enzyme kinetics for RNase T$_1$, whereas these negative effects are not so pronounced for RNase A.

The above results suggest that changing either enzyme's micro-environment, for example immobilization to a solid support, did not appear to have any significantly adverse effect on the ability of the enzyme to cleave tRNA$_{\text{Tyr}}$. Digests could be performed using the same enzyme-immobilized silica up to 5 days later using RNase T$_1$ and produced an identical fragmentation pattern to the earlier digest (data not shown). These experiments show that the RNase
Figure 5.2. Electropherograms of silica immobilized RNase T1 time study of the digest with *E. Coli* tRNA\(^{Tyr}\) in 10 mM Tris-HCl pH 7.0; electrophoresed in 18 mM acetic acid, 2 mM ammonium acetate pH 3.8. A) 30 min B) 60 min C) 90 min.
Figure 5.3. Silica-immobilized RNase A time study of *E. Coli* tRNA$^{\text{Yr}}$ in 10 mM Tris-HCl pH 7.0; Electrophoresed in 18 mM acetic acid, 2 mM ammonia acetate pH 3.9 at -10 kV~3.9 $\mu$A with injection -10 kV for 10 seconds A) 30 min B) 60 min C) 90 min.

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enzymes demonstrate the re-use and stability of the enzyme. Through surface immobilization, this procedure can be used for routine separation of RNA digestion reaction products. The products from this digest could not be positively identified and mass spectrometry is needed for further identification.

5.6 Studies of 16S- and 23S- ribosomal RNA

Once digests were successful using tRNA$^{\text{Tyr}}$, experiments were performed to demonstrate the efficacy of silica-immobilized enzymes for micro-digestion of high molecular weight RNA molecules. Digests of 16S and 23S rRNA were performed in free solution and with immobilized enzymes, using conditions optimized for tRNA$^{\text{Tyr}}$ digestion. The results illustrated in Figure 5.4 show electropherograms that are comparable indicating the use of silica-immobilized enzymes can be applied to a range of RNA molecules without loss of the catalytic efficiency or specificity. Further advantages of this technology include rapid sample analysis, simplicity of operation, straightforward separation of reaction products from enzyme, and the ability to perform multiple digests with the same enzyme preparation.

5.7 On-line digestion studies

Attempts were made to perform digestion and separation on-line with some success. Silica that had been derivatized with glutaraldehyde was
Figure 5.4. Electropherograms of 16S- and 23S ribosomal RNA with RNase T₁ in 10 mM Tris-HCl pH 7.0; Electrophoresed in acetic acid/ammonium acetate buffer pH 3.8 with injection at -10 kV for 10 seconds A) Free solution digest B) Immobilized enzyme digest
packed into the front end of the separation capillary. The enzyme was loaded using pressure injection to allow for covalent attachment of the enzyme to the silica. Preliminary results indicate that the digestion and separation can be performed within the same capillary. Figure 5.5 shows various injection times where the tRNA Tyr was allowed to digest on the packed bed for 1.5 hours. The system has not been optimized due to packing problems with the capillaries as well as the system to analyze the samples.

5.8 Conclusion

This work demonstrates the ability to covalently attach the enzymes RNase T1 and RNase A onto porous silica particles via a glutaric dialdehyde linkage. The digestion buffer conditions as well as the separation buffer conditions have been optimized for these enzymes. Further, the immobilization of the RNases has provided long-term stability to the enzymes, as digestion with the same enzyme preparation over several days (more than three) has been accomplished. This work also demonstrates the potential to use packed-beds to do on-line digestion and separation in one step. The ability to reuse the same population of enzyme for repetitive digests will aid in reducing the cost of enzymatic analysis as compared to free solution digests, reproducibility, and separation of reactants and products.
Figure 5.5. Electropherograms for the on-line digestion studies of E. Coli tRNA\textsuperscript{Tyr} with immobilized RNase T\textsubscript{1}. Electrophoresed at -10 kV~3.9 μA. A) injection 30 seconds, B) injection 45 seconds C) injection 30 seconds.
5.9 References


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Conclusion and Future Work

The focus of this document has been the immobilization of enzymes on silica to perform digestions of DNA and RNA with subsequent analysis by capillary electrophoresis. The motivation for this research was to provide a format that would allow eventual application to micro-chip technology.

The principles of capillary electrophoresis were outlined with the focus on the advantages of performing analyses in this area. The important aspects that make capillary electrophoresis more attractive are: (1) speed, (2) quantitation, (3) peak efficiency, and (4) automation. In addition, the high surface area-to-volume ratio of the capillary provides rapid dissipation of Joule heat and allows high electric fields to be used without a substantial temperature increase. This heat dissipation allows for separation to be performed in the capillary without temperature changes due to ineffective heat dissipation and development of thermal gradients across the capillary.

DNA separations in CE were outlined. The ability to analyze DNA fragments has led to interesting information that can aid in mapping the human genome. Slab-gel electrophoresis has provided a way to analyze DNA fragments for years, but the need to automate these separations led to capillary electrophoresis as an alternative procedure. When using capillary
electrophoresis, low-viscosity polymer solutions must be used to accomplish high-resolution separations of DNA mixtures. HEC is the most studied and used polymer used for these separations. The polymer must be used in the proper concentration to achieve the best separation. The migration models that macromolecules follow in the polymer network were described. Intercalating dyes were discussed and the effect that they have on the DNA molecules as well as the CE separation process.

The importance of enzymes and their role in biology were outlined. Restriction endonucleases are interesting due to their site-specific recognition and digestion of DNA molecules. If enzymes are in solution with the reactants and/or products, it is difficult to separate the enzymes from the desired product. The activity and stability of enzymes was discussed. Additionally, the factors influencing enzymatic reactions, such as enzyme concentration, substrate concentration, pH, and temperature, were discussed. The advantages of immobilizing an enzyme onto a surface and precautions that must be taken were discussed. Immobilization methods such as carrier-binding, cross-linking and entrapment were discussed. Finally, the immobilization that was performed in this work was discussed. This immobilization involved the attachment of a restriction enzyme to porous silica particles through a covalent bond.

Solid-phase DNA restriction digest reactors have been developed consisting of silica particles modified with a covalently tethered restriction
enzyme. This solid-phase restriction reactor enables digestion and separation of minute quantities of DNA with minimal reagent consumption. The restriction enzymes, *HaeIII*, *PstI* and *HindIII*, were successfully immobilized via glutaraldehyde linkages to porous silica micro-particles and PMMA. Studies were carried out to examine the impact of immobilization on enzymatic activity. Digestions of φX174-RF DNA phage and SV40 Viral DNA were performed with the immobilized enzymes by placing the solid particles in solution with the target DNA. The digests were analyzed off-line using capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection. Digests also were prepared and isolated by use of a simple micro-spin column consisting of a layer of immobilized enzyme-coated silica on a molecular weight cut-off filter.

A method was developed for the digestion of RNA using silica-immobilized enzymes. In addition, a capillary electrophoresis (CE) method was developed for the separation of the resulting oligonucleotides. Ribonuclease A (RNase A) and Ribonuclease T1 (RNase T1) enzymes were successfully immobilized via glutaraldehyde linkages to porous silica particles. Conditions were optimized to achieve the best separation and resolution of RNA oligonucleotide digestion products. Particles were packed into the front end of the capillary, where on-line digestion and separation were achieved. Multiple digests could be performed over several days.
Future work will be to determine which component(s) in the digestion buffer is contributing to the retardation of the DNA fragments. One experiment that needs to be done that would provide some additional important information would be slab gel experiment. This experiment is important because it would confirm the molecular weights of the fragments produced from the immobilization digest. A standard φX174-RF/HaeIII digest can be electrophoresed simultaneously with the immobilized enzyme digest. This experiment would also provide some additional information to confirm the presence of extra buffer components that could be retarding the migration of the DNA fragments.

In addition, work will be done to determine ways to renature the enzyme after the initial digest. Experiments should be done which involve the addition of components such as glycerol, EDTA, and BSA to the storage buffer to see if these components can assist in keeping the enzyme active. Other experiments can be done to determine if digestion conditions other than 37 °C are favorable for keeping the enzyme active. These immobilized systems can be integrated micro-chip technology. This can possibly be accomplished by placing immobilized enzymes in wells on the chip where digestion can occur. Once the digestion occurs the separation can be accomplished on the chip also. This technology would provide for separation and analysis to be done in one format with absolute minimal sample handling which would lead to sample
contamination. Upon completion of these experiments, these enzymes will then be potentially useful for commercialization.
Vita

Yolanda Yvette Davidson was born in Starkville, Mississippi, on February 5, 1966. She is the daughter of John Dera and Vallie Davidson, and has an older brother John Dera. She attended West Point High School in West Point, Mississippi, from 1980-1984 and graduated valedictorian of her class. There she was a member of numerous clubs including the Honor's Society and Chemistry Club and was selected as Star Student.

In the summer of 1984, she entered Jackson State University in Jackson, Mississippi, on an academic scholarship. While at Jackson State University, she was a member of the MBRS Program, MARC Program, Honor's Division, Alpha Lambda Delta, Chemistry Club, and. She served as president of Delta Sigma Theta Sorority, Delta Pi Chapter. She had numerous internships, which included DOW Chemical Company, Midland, Michigan, and the University of Iowa SURF program. She received a bachelor's degree in chemistry in May 1988.

She received a master's degree from Duquesne University in inorganic chemistry in 1995. In 1994, she received a scholarship from the National Institute of Standards and Technology, Gaithersburg, Maryland, to pursue a doctorate in chemistry at Louisiana State University, Baton Rouge, Louisiana. She is currently a candidate for the degree of Doctor of Philosophy in analytical chemistry. Upon graduation, she plans to continue her career as a research chemist in the Analytical Chemistry Division at NIST in Gaithersburg, Maryland.
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Title of Dissertation: Immobilization of Enzymes on Porous Silica For the Micro-Digestion of Oligonucleotides with Analysis by CE

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