AN ABSTRACT OF THE DISSERTATION OF

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Title: RNA and DNA Aptamers as Affinity Stationary Phases For Liquid Chromatography and Capillary Electrochromatography

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Vincent T. Remcho

Due to a number of disadvantages associated with the use of antibodies as affinity stationary phases, researchers have recently began to explore the use of RNA and DNA aptamers for use as affinity stationary phases. These molecules have been shown to be a viable choice for applications in many methods relying on molecular recognition. In this work, the use of aptamers as affinity stationary phases in several chromatographic formats and the effect of different mobile phase compositions on aptamer-target interactions were explored.

Our first work with aptamers involved the use of them in open-tubular liquid chromatography (OTLC) and open-tubular capillary electrochromatography (OTCEC). Different immobilization schemes were employed in order to use aptamers in both of these formats. The effect that mobile phase compositions exerted on the chromatographic retention factor (k') of an RNA aptamer for its target molecules was explored.

We next moved onto applying an aptamer stationary phase in a packed bed format. We found that immobilizing an amine-modified DNA aptamer onto

azlactone/bis-acrylamide copolymer particles was an easy one-step method. The resulting stationary phase was capable of selectively retaining the protein target of the DNA aptamer, α-thrombin. We found the interaction between the aptamer and its target to be largely dependent upon ionic strength.

Our final study of this research project involved creating a monolithic porous polymer aptamer-derivatized stationary phase. Monolithic stationary phases have been found to offer enhanced efficiency when compared to more traditional packed bed stationary phases. Within this research group it was desirable to create a monolithic stationary phases for their compatibility with a microchip format. We created a porous polymer containing a functional group on the surface of the pores which was capable of reacting with the amine-modified DNA aptamer. The result was the DNA aptamer immobilized onto the surface of the monolith. This stationary phase was found to be capable of retaining α-thrombin (the aptamer target). The monolith was also characterized as to the dissociation constant ($K_D$) of the aptamer-thrombin complex, and the total number of active binding sites ($B_t$) immobilized on the monolith surface.

# RNA and DNA Aptamers as Affinity Stationary Phases For Liquid Chromatography and Capillary Electrochromatography

by
Stacey L. Clark

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Stacey L. Clark, Author

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# CONTRIBUTION OF AUTHORS

My research advisor, Vincent T. Remcho, assisted in writing all chapters of this dissertation. His name appears on all published and submitted work contained herein.

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## Chapter 1: Introduction

### 1.1 INTRODUCTION

Affinity separations based on molecular recognition commonly use biological macromolecules as stationary phases. Historically, the most common macromolecules employed have been antibodies. The use of antibodies as affinity sorbents has many disadvantages including: i) the large size of antibodies which greatly limits the amount of antibody loaded onto the stationary phase, ii) the instability of antibodies which greatly limits mobile phase selection in affinity chromatography, and iii) the limitation of molecules for which antibodies can be generated. These disadvantages have led to research efforts which focus on finding alternative stationary phases capable of selective retention of the target molecule(s). Among these alternatives are aptamers.

Aptamers are oligonucleotides, usually single stranded, which are isolated and amplified based on their affinity for a target molecule [1, 2]. These molecules are generated by a technique known as *systematic evolution of ligands by exponential enrichment* (SELEX). These molecules have recently been used for their ability to selectively bind their target(s) in many analytical techniques. This work describes the creation and use of RNA and DNA aptamer stationary phases for open tubular

capillary electrochromatography (OTCEC); and liquid chromatography in open-tubular, packed and monolithic formats.

This dissertation is organized as follows. In the introduction chapter, relevant chromatographic theory is discussed, followed by a short perspective on affinity chromatography. Chapter two of this work discusses the generation of RNA and DNA aptamers, and analytical applications which have used the molecular recognition capabilities of these molecules. Chapters 3 through 6 describe the results of my research using RNA and DNA aptamers as stationary phases.

The main thrust of this work has been to create stationary phases containing aptamers, with the end goal being the use of aptamer stationary phases in a miniaturized format. The miniaturization of HPLC columns has been brought about largely because of the desire to enhance efficiency and resolution. In this work, the desire for miniaturization is partly driven by this reason, but also because of the thrust within this research group to develop microfabricated analytical devices, and eventually to create total micro analytical systems. The development of these devices is dependent upon the ability to create stationary phases *in situ* in a format which is compatible with these miniature devices.

The first experiments described herein involved the creation of an aptamer stationary phase in an open-tubular format. The open-tubular format was used for both open-tubular liquid chromatography and open-tubular capillary electrochromatography (OTCEC) separations. The aptamer used in these initial studies was an RNA aptamer which was isolated and amplified based on its

recognition of flavin mononucleotide (FMN) [3]. The RNA aptamer also recognizes the flavin moiety in flavin dinucleotide (FAD). In the chromatographic studies, the interaction between the RNA aptamer and FMN was found to depend largely upon solvent conditions. FMN was separated from a nontarget molecule, and the retention of FMN was found to change drastically when an organic solvent was added to the mobile phase. These studies are detailed in Chapter 3.

The immobilization method used in the initial chromatographic study was found to be inappropriate for use in electrochromatographic studies. The immobilization method used in initial studies involves a degree of cross-linking among the initial reagent molecules. The resulting gel coating the inner walls of the fused silica capillaries could be seen under 40 X magnification. The chromatographic performance of these capillaries was irreproducible, which we believe to be due to a limited measure of control over the thickness of the gel coated onto the capillaries. This encouraged us to modify the immobilization procedure before electrochromatographic separations were studied. The modified immobilization method did not result in a gel, but, we believe, in a monolayer of aptamer molecules bound to the inner wall of fused silica capillaries. These capillaries were found to be appropriate for use in capillary electrochromatography (CEC), and gave reproducible results. The resulting OTCEC studies on the aptamer stationary phase (chapter 4) used this modified immobilization method.

CEC involves applying an electric field across the length of a capillary, resulting in the generation of electroosmotic flow (EOF). This EOF is the bulk

transport mechanism present in these electrically-driven separations. The transport of a charged analyte in OTCEC depends upon the size and charge of the analyte, and the partitioning of the analyte in the stationary phase. Even though theory does exist to explain how these two different parameters affect the retention of charged analytes in CEC, it was found that theory was insufficient when applied to the two negatively-charged analytes, FMN and FAD.

A method which makes possible the characterization of these capillary columns based on the partitioning of FMN and FAD into the aptamer stationary phase is described in chapter 4. This method involves adjusting the net EOF inside the capillaries to zero by the use of opposing pressure. Using this method, the interaction between the RNA aptamer and its targets was characterized under different mobile phase conditions. Specifically, the dependence of the interaction on the presence of the magnesium ion was explored since it is known that the presence of $Mg^{2+}$ stabilizes the three-dimensional structure of many oligonucleotides [4].

The next phase of this research focused on the creation of an aptamer stationary phase for use in a traditional HPLC format, namely, the generation of a packed bed for HPLC separations. This packed format is capable of a higher loading capacity and often greater resolution in liquid chromatographic separations. For this phase of the project, a DNA aptamer specific for the human protein thrombin was chosen [5]. This phase of the project used particles which are capable of reacting with a nucleophile in one rapid step so that the generation of a packed bed containing an

aptamer stationary phase is fast and simple. Chapter 5 details this step of the research project.

Once the stationary phase was generated, the particles were packed into HPLC columns. These columns were shown to be capable of specifically retaining the aptamer's protein target. This interaction was studied using the HPLC columns resulting from the immobilization of the DNA aptamer specific for α-thrombin. The interaction between thrombin and the DNA aptamer involves a charge association between negatively–charged regions of the aptamer and positively-charged regions of α-thrombin. It was found, as expected, that the ionic strength of the solvent had a large effect on the interaction.

The next step was important in the application of an aptamer stationary phase in a microdevice so that the stationary phase is ready to apply in a microchip format. This step involved casting a porous polymer inside capillary columns *in situ*. After a quick and easy immobilization method to anchor the DNA aptamer inside the capillaries, an aptamer stationary phase was created. These studies are discussed in chapter 6.

The casting of the monolith in the capillaries involves the polymerization of monomers in the presence of a porogenic solvent. Contained in the monomer solution is the functional monomer, 2-vinyl-4,4-dimethylazlactone, which is the functionality responsible for reacting with the aptamer, resulting in the generation of an aptamer stationary phase. Therefore, this monomer must be contained on the surface of the pores created by the porogenic solvent.

In order to characterize the monolithic aptamer stationary phase, frontal chromatography was used. This technique involves the application of a constant concentration of target (α-thrombin) and measuring the volume needed to elute this concentration. This study resulted in the extraction of the dissociation constant ($K_D$) of the aptamer-thrombin complex, and the number of total active binding sites ($B_t$) which were immobilized onto the monolithic stationary phase. This is the first report we know of which accomplished the immobilization of an aptamer onto a monolithic stationary phase. As a direct result of this study, it is feasible to cast this monolith inside microchip devices in order to achieve specific recognition, which is one goal within this research group.

## 1.2 THEORETICAL BACKGROUND

The term chromatography refers to a separation technique relying on the partitioning of analytes into or sorption onto a stationary phase. The analytes are driven past the chromatographic sorbent by a mobile phase, and the differing amounts of time each analyte spends in the stationary phase as compared to the mobile phase is responsible for the separation. There are many different types of chromatography which are divided based on the mechanism of the interaction between analytes and the stationary phase. The type of chromatography used for the experiments in this work is limited to affinity chromatography, which will be discussed later in this chapter. The following is a discussion of chromatographic theory. The studies discussed in this work were conducted using liquid chromatography (LC) and capillary

electrochromatography (CEC). The discussion which follows will be limited to LC and CEC as appropriate.

### 1.2.1 *Resolution in Liquid Chromatography*

Resolution ($R_s$) is unit less and refers to how well two peaks are separated during a chromatographic experiment. This depends on two factors: the difference in elution times of the two peaks and width of the two peaks as described by equation 1.1:

$$R_s = \frac{\Delta t_r}{\frac{1}{2}\left(W_{b,1} + W_{b,2}\right)} \tag{1.1}$$

where $\Delta t_r$ is the difference in elution times of two peaks with the widths of the peaks at the base equal to $W_{b,1}$ and $W_{b,2}$, respectively. The master resolution equation explains this parameter in terms of the retention factor (k'), selectivity ($\alpha$) and number of theoretical plates (N) and is given by equation 1.2.

$$R_s = \left(\frac{k'}{k'+1}\right)\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{\sqrt{N}}{4}\right) \tag{1.2}$$

The retention factor is a ratio of the number of moles of analyte in the stationary phase relative to the number of moles of analyte in the mobile phase. In a chromatography experiment, the analyte is being carried through the column by the mobile phase, and its partitioning out of the mobile phase and into the stationary phase is responsible for retention of the analyte. Therefore we can say that the retention factor is also equal to a ratio of the amount of time the analyte spends in the stationary

phase relative to the amount of time the analyte spends in the mobile phase. The value k' can be experimentally determined by the equation:

$$k' = \frac{t_r - t_0}{t_0} = \frac{t'_r}{t_0} \tag{1.3}$$

where $t_r$ is the retention time of a retained analyte, $t_0$ is the retention time of an unretained analyte, and $t'_r$ is called the adjusted retention time.

The term α refers to the selectivity between two neighboring peaks. The term can be described in terms of the retention times of the peaks and retention factor of the two peaks:

$$\alpha = \frac{t'_{r2}}{t'_{r1}} = \frac{k'_2}{k'_1} \tag{1.4}$$

where the subscript two refers to the later-eluting peak. Both k' and α are dependent upon the thermodynamics of the analyte-stationary phase interaction.

The number of theoretical plates of a chromatographic column is a measure of the rate of band broadening. This is also referred to as efficiency. This number depends on the retention time of the analyte ($t_r$) and the width of the peak (w) and is given by equation 1.5.

$$N = \frac{16 t_r^2}{w^2} \tag{1.5}$$

This parameter is a carryover from distillation theory and depends on physical rather than chemical phenomena. This variable is dependent upon the rate of diffusion of the analyte, as well as the kinetic properties of the analyte-stationary phase interaction.

Another way to represent the number of theoretical plates is by the quantity H which represents the height equivalent of a theoretical plate:

$$H = \frac{N}{\sigma^2} = \frac{L}{N} \tag{1.6}$$

where $\sigma^2$ is the variance of the peak in units of time and L is the length of the chromatographic column.

As can be seen above the resolution of a chromatographic separation is a function of both the time between elution of peaks as well as the width of the peaks. While the thermodynamic properties of the analyte-stationary phase interaction are controllable by changing the mobile phase or stationary phase only, the peak width is a property which can be controlled (within limits) by the physical properties of the chromatographic column. To take a closer look at the quantity H, see equation 1.7:

$$H = A + \frac{B}{\mu} + \mu(C_s + C_m) \tag{1.7}$$

where the A term represents the contribution of eddy diffusion to band broadening; the B term refers to molecular diffusion; the C term refers to the contribution of mass transfer, with $C_s$ and $C_m$ referring to the mass transfer of the analyte in the stationary phase and mobile phase respectively; and $\mu$ is the linear velocity of the mobile phase. All of these terms will be discussed in more detail below, as will factors in the chromatographic column which contribute to these terms.

The A term, or eddy diffusion term, is present only in packed columns. This term refers to the band broadening of the peak due to multiple paths available to the analyte. This term is proportional to the diameter of the packing material as well as a

quantity called the packing factor. The packing factor refers to the uniformity of the packed bed, and is a factor that is difficult to control. In order to minimize this contribution, the smallest possible particles should be used. While there is a limit to the size of particles used in LC due to high back pressure considerations, it is possible to use much smaller particles in CEC. This is because the flow of mobile phase through the column in CEC is due to EOF, and not applied pressure. Particles less than one micrometer in diameter have been applied in CEC separations and there has been an increase in efficiency (reduced H) seen as a result of the smaller particles [6, 7].

The molecular diffusion term (B) refers to axial or longitudinal molecular diffusion of the analyte within the chromatographic column. This term is proportional to the diffusion coefficient of the analyte in the mobile phase. Therefore, this term is inversely proportional to the linear velocity of the mobile phase, since slower velocity gives the analyte more time to diffuse. In LC this term is negligible compared to gas chromatography (GC) since the diffusion coefficients of liquid analytes is much smaller than that of gases.

The C term refers to the rate of band broadening due to slow mass transfer of the analyte. The $C_s$ term refers to the mass transfer kinetics of the analyte in the stationary phase. This term is dependent upon the thickness of the stationary phase. Stationary phases for LC are commonly present as a bonded monolayer on a solid support. Since this makes the layer as thin as possible, this term is generally accepted as being minimal.

The $C_m$ term refers to slow mass transfer in the mobile phase. Due to the packing, as well as the laminar flow profile in pressure-driven separations, there is a difference in the velocity of the mobile phase as a function of position in the column. In a laminar flow system, the velocity of molecules traveling inside the column is a function of their distance from the closest surface defining the flow path (particle surface or column wall). Those closer to the center of the flow path will move faster. This feature contributes to broadening of the chromatographic peak. The electroosmotic flow profile, the flow profile inside CEC capillaries due to the application of an electric field, is a flat, plug-like profile. This velocity gradient typical of laminar flow is not present, instead all molecules travel through the capillary column at the same nominal velocity. Therefore, this portion of the $C_m$ contribution to band broadening is minimal in CEC separations. This effect will be discussed in more detail in chapter 4.

Another $C_m$ contribution to band broadening present in packed columns which use porous particles is the presence of stagnant zones of mobile phase inside the pores of the stationary phase particles. Analytes which diffuse into these zones will fall well behind those analytes traveling at a velocity near the average velocity inside the column. This effect is present in LC and CEC columns packed with porous particles. The only mechanism by which analyte can move into and out of these zones is by diffusion, so this contribution is inversely proportional to the diffusion constant of the analyte. This effect is exacerbated by high mobile phase velocities and larger particles.

*1.2.2 Affinity Chromatography*

The method of affinity chromatography is based on the principle of binding a biologically active molecule using a specific and reversible interaction. The principle dates back to 1910 [8]. However, the first report of the use of an affinity ligand physically attached to the stationary phase was in 1951 [9]. The affinity stationary phase was used to purify antibodies with the antigen used as the physically attached stationary phase. Today, affinity chromatography stationary phases include a solid support with the ligand physically attached to the support through a linker molecule. Linker molecule chemistry was pioneered by Cuatrecasas et al., who successfully solved the problem of steric accessibility of the stationary phase [10].

There are several choices as to the solid support for the affinity chromatographic stationary phases. The earliest studies were conducted on biopolymers which were functionalized with the appropriate ligand. Agarose, dextran, cellulose, and starch are among the biopolymers which have been used as a support for covalently bonding a ligand for use in affinity chromatography. Inorganic materials, such as silica-based particles, have also been used. The use of these materials developed as immobilization techniques for bonding affinity ligands developed. There have also been reports of synthetic polymers for use as the support material in affinity separations. A detailed description and review of materials for use as support for affinity separations is beyond the scope of this treatment. Several sources go into depth about the creation of affinity chromatographic stationary phases and the different support material available [11, 12].

The types of ligands that have been used as affinity sorbents are numerous, and include lectins, enzymes and their substrates, receptors, nucleic acids, and antibodies. The most widely-used affinity chromatographic stationary phases are antibodies. Therefore, the use of antibodies will be discussed at length. These are also the most appropriate to compare with aptamers, the topic of this dissertation, since they are produced to be specific for a certain analyte in affinity chromatography.

Almost any substance, including a protein, can produce an immunological response as long as the molecule is foreign to the host. This response produces antibodies, which can then be used in diagnostic assays and affinity separations. Antibodies are secreted by B cells in the event of an immunological response, or after they have been stimulated by the presence of an antigen. The use of these molecules for their molecular recognition capabilities has been popular since the 1950s [13]. By the 1970s, the use of antibodies for affinity separations was widespread. At that time, antibodies were produced by exposing animals to a certain antibody, followed by isolation of the antibody from the animal's blood. These antibodies were produced by a process of "bleeding and boosting" in which the animal is exposed to the antibody initially, followed by a cycle of bleeding the animal to analyze the blood for the antibody and boosting the animal by inoculating the animal with more antigen [14]. This cycle was repeated until it was found that the concentration of the antibody in the animal's blood did not increase by further boosting.

Antibodies are large molecules, typically 160 to 250 kDa. These molecules have binding constants for their corresponding antigen of about $10^6$ to $10^{12}$ $M^{-1}$.

Antibodies can be polyclonal or monoclonal. Polyclonal antibodies exhibit affinity for several sites on the antigen molecule, whereas monoclonal antibodies exhibit affinity for one determinant site of the antigen molecule.

The use of antibodies as affinity sorbents generally involves two steps. The sample is first added to the stationary phase, and then eluted in a different step. The elution step involves lowering the pH of mobile phase or adding a high concentration of salt to the mobile phase. The elution step must involve addition of a substance harsh enough to disrupt the binding of the antibody to the antigen, but gentle enough so that neither the antibody or the target is denatured. Strategies have been employed which bypass the elution step, and higher efficiency has been accomplished [15]. This type of affinity chromatography, dubbed weak affinity chromatography, involves relatively "weak" monoclonal antibodies with association constants less than $10^{-4}$ M.

Although the use of antibodies has greatly benefited immunology research, there are limitations. These limitations include: 1) the generation of antibodies is difficult with molecules that are extremely toxic, (2) the identification and production of a large number of antibodies is expensive, (3) the identification of antibodies begins within an animal, therefore, isolation of antibodies that recognize a target under conditions other than physiological conditions is not possible, and (4) kinetic parameters of antibody-antigen interactions are uncontrollable These disadvantages have caused researchers to look for other options when in need of molecular recognition for analytical applications.

The use of RNA and DNA aptamers, alternative agents for molecular recognition, as affinity stationary phases is a growing field. In the next chapter, the generation and use of these molecules in analytical techniques is discussed. This discussion is limited to the applications of aptamer which led to the development of stationary phases which incorporated aptamers.

# 2. Aptamers as Analytical Reagents

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## 2.1 INTRODUCTION

Many important analytical methods are based on molecular recognition. Aptamers are oligonucleotides that exhibit molecular recognition; they are capable of specifically binding a target molecule, and have exhibited affinity for several classes of molecules. The use of aptamers as tools in analytical chemistry is on the rise due to the development of the "systematic evolution of ligands by exponential enrichment" (SELEX) procedure. This technique allows high-affinity aptamers to be isolated and amplified when starting from a large pool of oligonucleotide sequences. These molecules have been used in flow cytometry, biosensors, affinity probe electrophoresis, capillary electrochromatography, and affinity chromatography. In this paper we will discuss applications of aptamers which have led to the development of aptamers as chromatographic stationary phases and applications of these stationary phases; and look towards future work which may benefit from the use of aptamers as stationary phases.

With the development of the SELEX method (systematic evolution of ligands by exponential enrichment), it is possible to quickly isolate and amplify oligonucleotide sequences that are capable of high affinity binding to a target molecule [1, 2]. This class of oligonucleotides is known as aptamers. It has long been known that certain RNA and DNA sequences exhibit recognition for certain molecules in biological systems. However, due to the small diversity of interactions with nucleic acids, it was thought that this affinity was weak at best. More recently, it has been shown that aptamers exhibit affinities for molecules that rival those typical of

antibodies [16], and that a combinatorial library made up of random oligonucleotide sequences contains more molecular diversity than a similar library of peptides [17].

The high affinity exhibited by aptamers is attributable to their ability to "incorporate" small molecules into their nucleic acid structure, and to integrate into the structure of large molecules such as peptides [18]. Aptamers, which are largely unstructured in solution, fold upon binding their target molecule [18]. This, largely, is the reason that aptamers have shown high affinity and specificity for target molecules. Aptamers have shown affinity for both small molecules and macromolecules. Table 2.1 lists some aptamers and their targets.

**Table 2.1**. Some aptamers and their target molecules.

| Target | Oligonucleotide | Reference |
|---|---|---|
| T4 DNA polymerase | RNA | [1] |
| organic Dyes | ssDNA | [2] |
| L-arginine | RNA | [37] |
| FAD, FMN, $NAD^+$, $NMN^+$ | RNA | [3] |
| riboflavin, NMN | RNA | [38] |
| human IgE (protein) | RNA, ssDNA | [31] |
| HIV type I Rev (residues 34-50) | RNA | [39] |
| L-selectin (protein) | ssDNA | [34] |
| SelB (E. coli protein) | RNA | [40] |
| S-adenosyl methionine | RNA | [41] |
| Ras binding-domain of Raf-1 (residues 51-131) | RNA | [42] |
| S-adenosyl homocysteine | RNA | [43] |
| human oncostatin M (glycoprotein) | RNA | [36] |
| Ff gene 5 protein | ssDNA | [44] |

## 2.2 SELEX

Systematic evolution of ligands by exponential enrichment, SELEX, is a procedure which involves repeating cycles of a selection round followed by an amplification round (Figure 2.1). The first accounts of SELEX involved isolation of RNA that showed affinity for a protein [1] and several organic dyes [2]. Since that time, however, SELEX has been used to isolate aptamers capable of binding many different molecules (Table 2.1). The first step of this procedure involves generating a large pool of random oligonucleotide sequences. It has been reported that roughly one in $10^{10}$ random RNA sequences will exhibit specific binding with a small molecule [2]. Therefore, the starting pool must be quite large to generate a high probability of producing an "active" aptamer.

Following the generation of a sufficiently large pool of random sequences, the sequences are exposed to a target molecule. Usually the target is bound to a supporting medium, sometimes in the form of an affinity chromatographic column. Those sequences that do not show affinity for the target molecule elute early, while those which bind the target must be washed from the column. Those sequences exhibiting affinity for the target molecule are amplified by polymerase chain reaction (PCR). This is followed by several more cycles of the same treatment. The end result is a final oligonucleotide population that is dominated by those sequences which bind the target best.

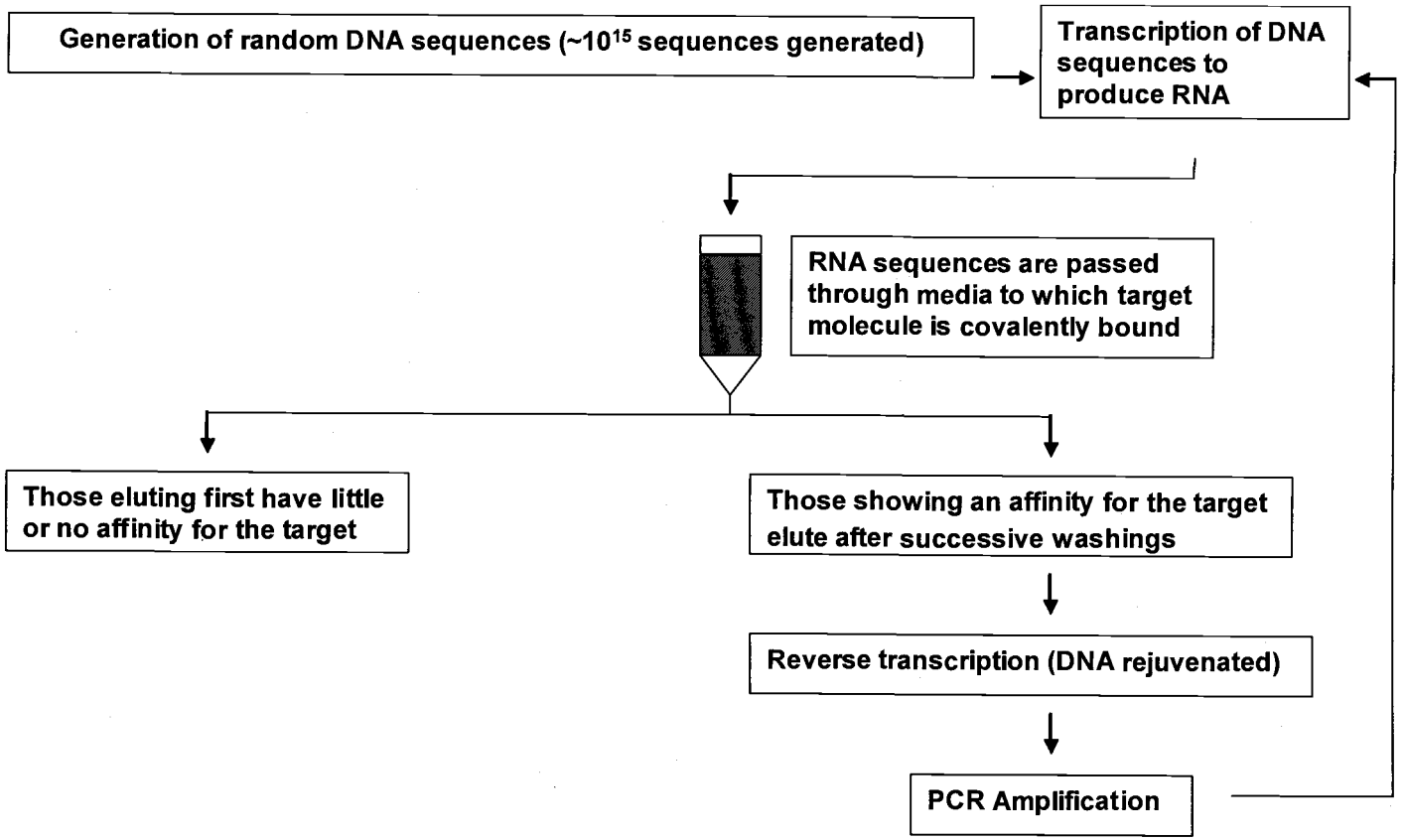


**Figure 2.1.** Schematic diagram of SELEX. RNA aptamers which bind a target molecule are selected.

The first studies using SELEX generated RNA aptamers [1, 2]. The amplification step involved reverse transcription of the RNA sequences, followed by PCR to generate a large population of DNA encoding the desired aptamer. The double stranded DNA was then subjected to transcription which regenerated the original RNA in large quantity.

Generally, several rounds are required to generate aptamers with the desired binding ability. Given that one round of the SELEX method involves preparing and purifying a large population of RNA sequences, isolating those sequences which bind to the target, reverse transcription and PCR amplification of the binding sequences, one SELEX experiment could last weeks to months. However, with the advent of "automated SELEX," a SELEX experiment can be conducted in days [19].

SELEX can result in sequences which are capable of highly specific recognition. An RNA aptamer which binds L-arginine 12000 times better than D-arginine has been isolated [20], as well as an RNA aptamer which binds theophylline 10000 times better than it binds caffeine [21].

## 2.3 APPLICATIONS IN ANALYTICAL CHEMISTRY

Aptamers hold much promise as molecular recognition tools for incorporation into analytical devices. Aptamers have been used in flow cytometry [22, 23], biosensors [24, 25], affinity probe capillary electrophoresis [26], capillary electrochromatography [27, 28], and affinity chromatography [29, 30]. However, this discussion has been limited to those examples which led to the incorporation of

aptamers as stationary phases and those in which aptameric stationary phases were utilized in electrophoretic and chromatographic separations.

### *2.3.1 Biosensors*

Biosensors depend on the power of a molecular recognition element. Therefore, it seems logical to explore the use of aptamers as the molecular recognition element in biosensors. Biosensors specific for the amino acid L-adenosine [24] and for the human protein thrombin [25] have been developed using aptamers.

The biosensor designed by Kleinjung et. al. [24] was designed to be specific for L-adenosine. It incorporated fluorescently labeled L-adenosine and the specific RNA aptamer was bound to an optical fiber core. This was accomplished by first derivatizing the aptamer by adding biotin to the 3' end. The surface of the optical fiber was derivatized with avidin, after which the biotinylated RNA aptamers were added. Using the biosensor and monitoring by fluorescence the total internal reflection, they were able to obtain the association and dissociation rates and detected L-adenosine in the submicromolar range.

The association and dissociation rates of the aptamer/L-adenosine complex were obtained by monitoring the total fluorescence with time. The concentration of L-adenosine was obtained through competitive binding. Non-labeled L-adenosine was added to the reaction mixture to compete with the fluorescently labeled L-adenosine. Figure 2.2 shows the calibration curve for L-adenosine using the aptamer-based biosensor.

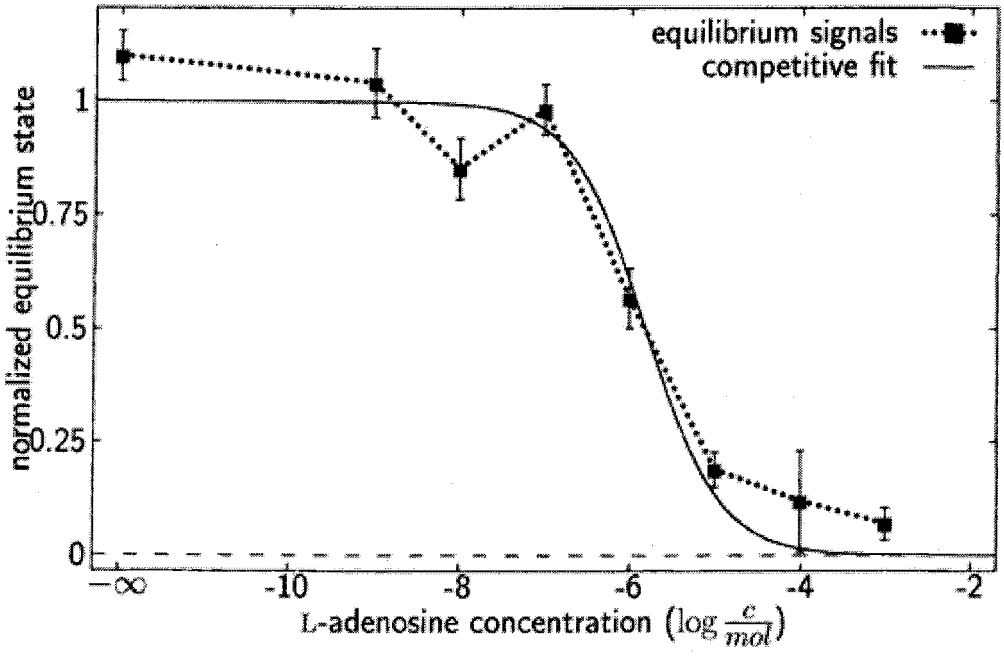


**Figure 2.2**. Competitive L-adenosine assay. The normalized equilibrium states for the binding of the aptamer to fluorescently labeled L-adenosine vs. the unlabeled L-adenosine concentration in logarithmic scale.

■ equilibrium signals — competitive fit Reprinted from [24] with permission. Copyright 1998 American Chemical Society.

Potyrailo et. al. [25] designed a biosensor for human thrombin. The ssDNA aptamer was first derivatized with an amino group on the 3' or 5' end and then was fluorescently labeled. The silanol surface of the glass support was converted to a diol by attaching (glycidoxypropyl)trimethoxy silane (GOPS) under acidic conditions. The surface was then activated by adding 1, 1'-carbonyldiimidazole. The imidizole group on the surface was then reacted with the amino-modified aptamer. The detection scheme employed is shown in Figure 2.3. This scheme permitted the detection of as

little as 0.7 amol of thrombin with an RSD of 4%. Evanescent-wave-induced fluorescence anisotropy of the aptamer was monitored.

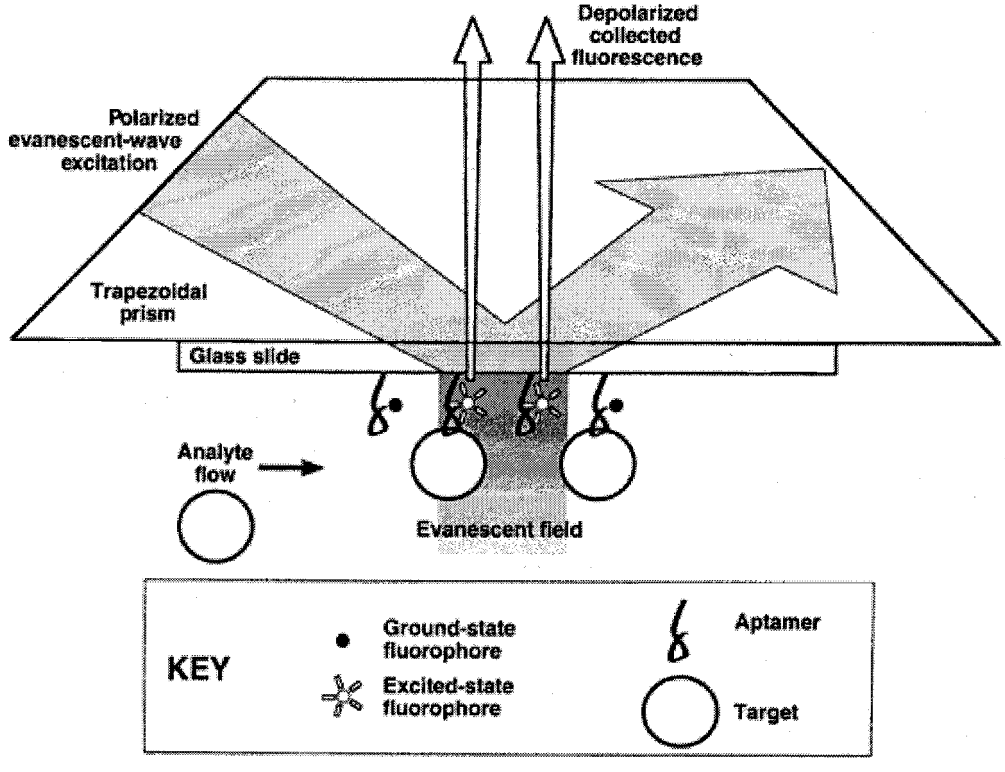


**Figure 2.3.** Detection scheme for an aptamer-based biosensor. The polarization components of the aptamer in an unbound state and in a bound state are monitored to indicate changes in the fluorescence anisotropy. Reprinted from [25] with permission. Copyright 1998 American Chemical Society.

Fluorescently labeling the aptamer allows for a reduction in sample preparation time, and does not result in a loss of function. Fluorescence anisotropy changes as the rotational diffusion rate of the fluorescent complex changes. The binding of the target to the aptamer results in a change in size of the complex, resulting in a change in the rotational diffusion rate. Therefore, the measured fluorescence anisotropy also changes. The change of anisotropy as a function of thrombin concentration is shown in Figure 2.4.

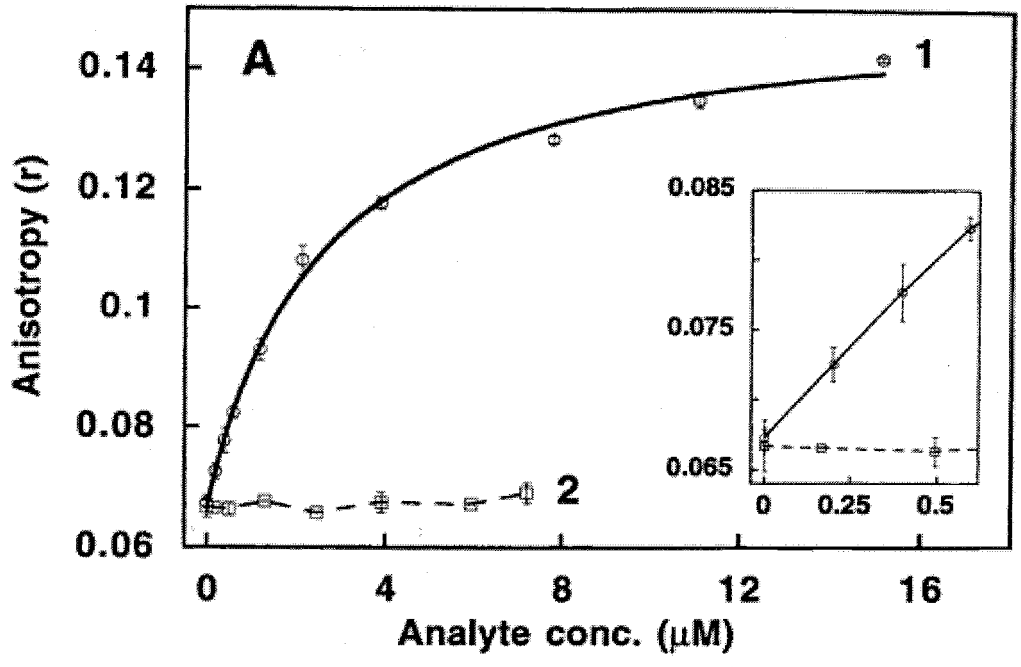


**Figure 2.4.** Fluorescently labeled thrombin-aptamer displays specificity for thrombin. Fluorescence anisotropy of labeled aptamer as a function of thrombin concentration (1) and elastase concentration (2). The inset is an expanded view for low analyte concentrations. Error bars represent on standard deviation. Reprinted from [25] with permission. Copyright 1998 American Chemical Society.

*2.3.2 Capillary Electrophoresis and Capillary Electrochromatography*

Capillary electrophoresis (CE) is a separation technique traditionally used to separate analytes based on differences in their size and/or charge. Affinity probe capillary electrophoresis and capillary electrochromatography, in contrast, are capable of separating uncharged species. In affinity probe capillary electrophoresis, high-

affinity binding is used in a CE scheme to determine analytes. Capillary electrochromatography incorporates stationary phase reagents to separate analytes.

German et. al. [26] incorporated a DNA aptamer into an affinity probe electrophoresis experiment to quantify human immunoglobulin E (IgE). IgE is a protein that triggers allergic reactions. This ssDNA aptamer had previously been isolated based on its affinity for IgE [31]. The detection limit for IgE using this technique was 46 pM with a linear dynamic range of $10^5$.

In this experiment, the DNA aptamer was fluorescently labeled. Samples consisting of aptamer only and aptamer and IgE were injected onto a capillary. The capillary had previously been coated with polyacrylamide to reduce IgE adsorption on the silica wall of the capillary. The peak area for labeled aptamer was used for the quantification of IgE. Figure 2.5 shows the calibration curve for IgE.

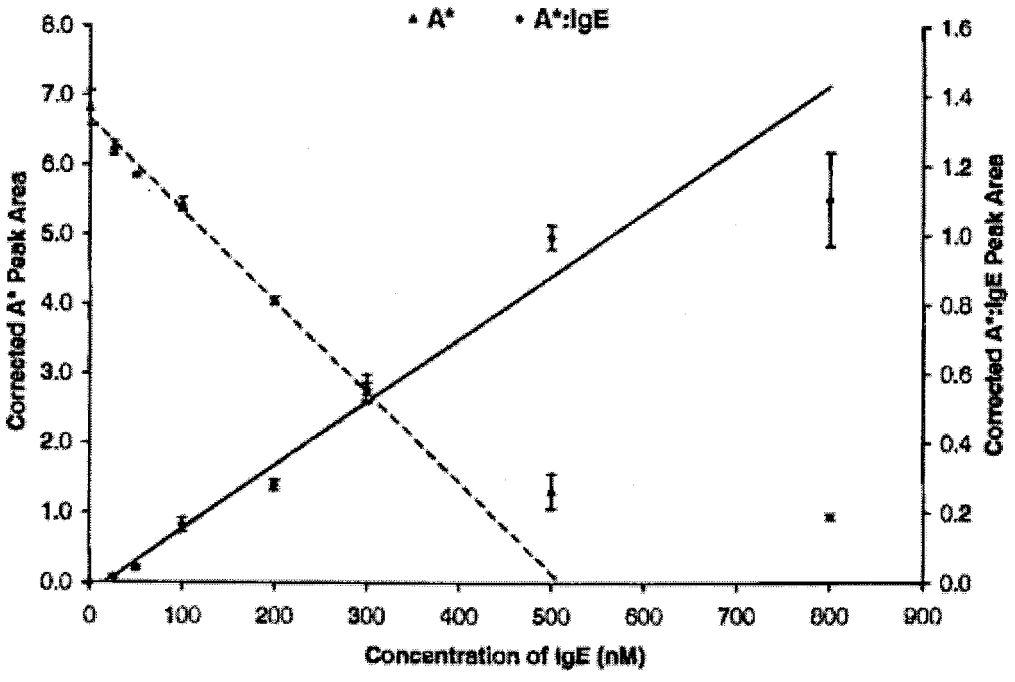


**Figure 2.5.** Calibration curve using samples containing 300 nM of labeled aptamer (A*) with various concentrations of IgE. Line is best fit from linear regression for concentrations from 0 to 300 nM IgE. Error bars represent one standard deviation. Reprinted from [26] with permission. Copyright 1998 American Chemical Society.

Kotia et. al. [27] used two ssDNA aptamers as stationary phases in capillary electrochromatography experiments to separate nontarget molecules. One of the aptamers was earlier isolated based on its affinity for thrombin [5], and the second was a similar oligonucleotide sequence. The aptamers were covalently bound to fused silica capillaries using several immobilization schemes. The scheme listed above [25] was but one of several used. In another immobilization method [32], the aptamer was

thiol-modified by derivatizing it with a disulfide, and then reducing the disulfide. The silanol surface was converted to an amino group linked to the surface through a propyl group, which was then treated with a heterobifunctional cross-linking agent to incorporate an iodoacetamido group. This surface was then treated with the thiol-modified aptamer. The last immobilization method [33] also used a thiol-modified aptamer which was added to an amino-modified silanol surface. However, in this case the silanol surface was modified with this group via a UV-initialized polymerization reaction. Capillaries derivatized with aptamer using each immobilization scheme were used to successfully separate a mixture. Mixtures of amino acids, enantiomers, and polycyclic aromatic hydrocarbons were separated. Figure 2.6 shows the resultant electropherogram for a mixture of tryptophan enantiomers (D- and L-trp) injected onto a capillary using a DNA aptamer as the stationary phase and the immobilization scheme described in reference [25].

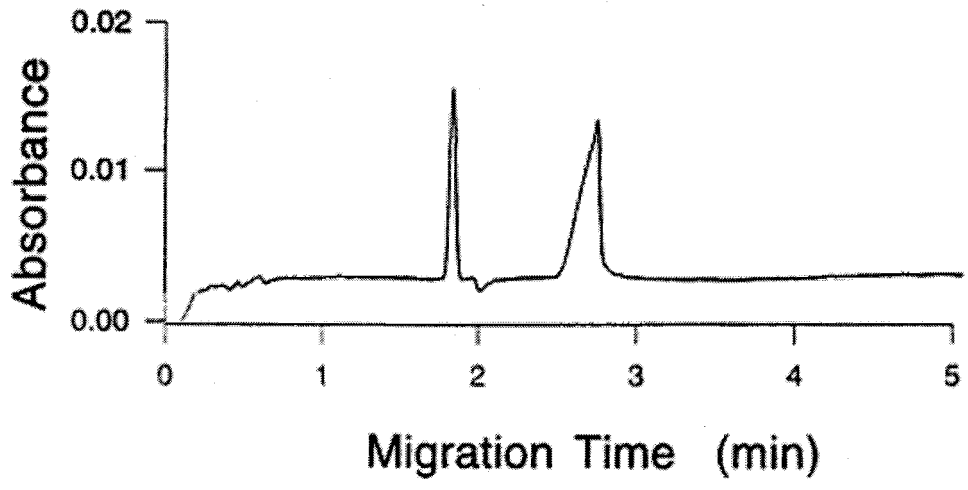


**Figure 2.6.** CEC separation of enantiomers using capillary coated with aptamer. Separation of D-trp and L-trp. Concentration is 0.5 mM for each enantiomer. Reprinted from [27] with permission. Copyright 2000 American Chemical Society.

Rehder and McGown also used aptamer-derivatized capillaries to separate nontarget molecules in CEC. The aptamer used was generated for its stability, and was based on the structure of the aptamer specific for human thrombin mentioned above [5]. The aptamer was coated to the wall of a fused silica capillary via the method used by O'Donnell et al. [32], but with the aptamer linked to the wall of the fused silica capillary through sulphosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (S-SMCC) rather than a propyl group. The capillaries were used to separate two forms of β-lactoglobulin which differ by only two amino

acid residues (LgA and LgB). However, the separation was also accomplished using capillaries coated with only the organic linker molecule S-SMCC. The separation occurring with the linker-coated capillaries resulted in a much greater retention time. This led the authors to the conclusion that the aptamer-coated capillaries achieved a much "gentler" and less denaturing separation than the hydrocarbon stationary phase, which may be important when considering the separation of proteins and peptides. Figure 2.7 shows the separation conducted on aptamer-coated capillaries.

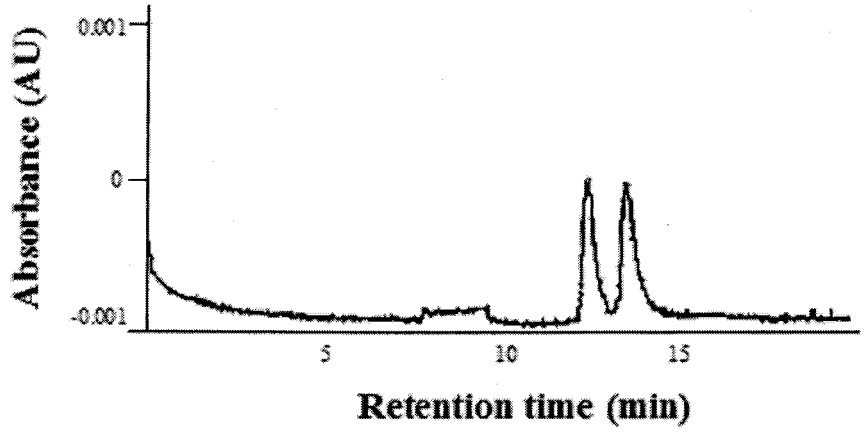


**Figure 2.7.** CEC separation of LgA and LgB using aptamer coated capillary. Concentration is 50 μM for each protein. Reprinted from [28] with permission. Copyright 2001 Wiley-VCH publishers, Inc.

### *2.3.3 Affinity Chromatography*

Affinity chromatography is based on molecular recognition of the stationary phase for the analyte. Traditionally, antibodies have been incorporated into the stationary phase to provide molecular recognition. Aptamers are very promising in this field because they are capable of molecular recognition, and theoretically, they can be generated to be specific for any molecule.

Romig et al. [29] incorporated an aptamer specific for L-selectin [34] into an affinity chromatography column. The aptamer was biotinylated at the 5' end and then incubated with a streptavidin-linked resin. The resin was then packed into a chromatographic column, and the resulting column was used to purify human L-selectin-Ig fusion protein produced in Chinese hamster ovary cells transfected to express the protein. When the aptamer column was used in the initial purification step, a 1500-fold purification with an 83% single step recovery was the result. Figure 2.8 shows the chromatogram when 220 μg of partially purified protein was eluted with a linear EDTA gradient.

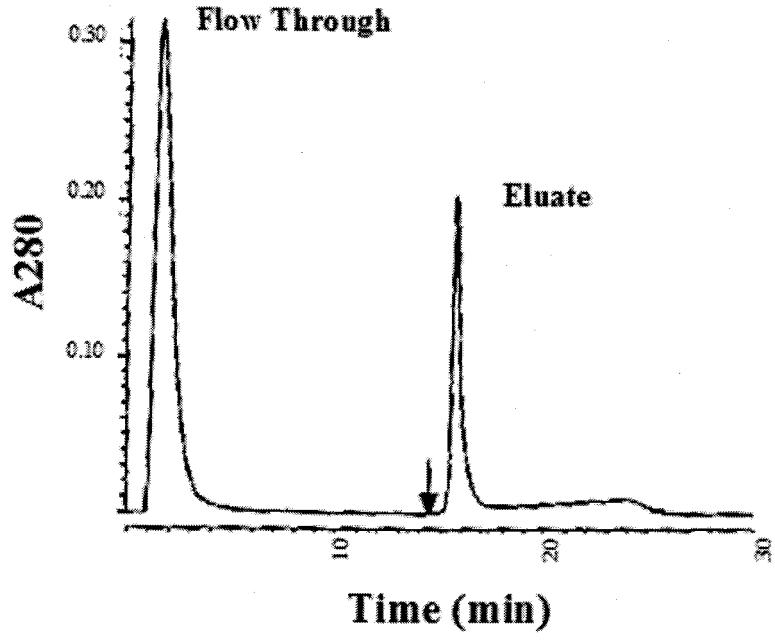


**Figure 2.8.** Chromatogram of a partially purified LS-Rg on aptamer affinity column. Approximately 220 μg of total protein injected onto column. A linear gradient of NaCl (150 mM to 1.15 M in 10 minutes) was initiated at arrow. Reprinted from [29] with permission. Copyright 1999 Elsevier

Deng et al. [30] used an aptameric stationary phase to separate cyclic-AMP, $NAD^+$, AMP, ADP, ATP, and adenosine. The aptamer used had earlier been isolated due to its ability to bind adenosine/ATP [32]. A biotin label was attached to the aptamer through a 15-carbon linker on the 3' end. The biotinylated aptamer was then incubated with POROS streptavidin media (polystyrene porous particles) or streptavidin porous glass beads. The particles were then packed in fused-silica

capillaries, resulting in affinity chromatography capillaries. The resulting particles contained 20 nmol binding sites per 100 μL of media. This value is 3.3 times larger than that reported for antibodies on similar media.

Due to the relatively weak affinity this aptamer exhibits for the adenosine analogues, several mobile phase conditions were studied to optimize the separation. It was found that ionic strength had a strong effect on the retention of the analytes, while pH and buffer composition had a lesser effect. Figure 2.9 shows a chromatogram of adenosine and adenosine analogues on the aptamer affinity column. The columns were also used in conjunction with frontal chromatography to obtain dissociation constants for the anchored aptamer with adenosine and its analogues. The resulting $K_d$ value for the aptamer-adenosine complex was comparable to the value formerly reported for the complex in solution [32]. Therefore, immobilizing the aptamer to a surface does not appear to alter its activity.

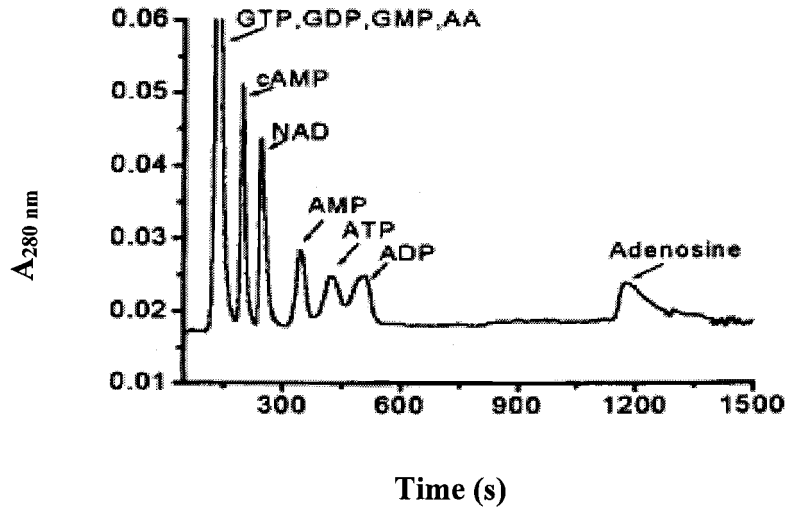


**Figure 2.9.** Separation of adenosine and analogues on aptamer affinity chromatographic column. Mobile phase 20 mM $KH_2PO_4$, 20 mM NaCl, 10 mM $MgCl_2$ at pH 6.6. Reprinted from [30] with permission. Copyright 2001 American Chemical Society.

## 2.4 FUTURE WORK

Despite the promise that aptamers show as molecular recognition tools in analytical chemistry, there have been relatively few examples of the use of aptamers in analysis. This may be, in part, due to the variable stability of aptamers. RNA aptamers are especially vulnerable to ribonucleases due to the 2' hydroxyl on the ribose sugar; however, Rhodes et al. [36] found that substituting a more stable functional group into this position on the ribose sugar did not alter the affinity of their

aptamer for its target. They isolated an RNA aptamer specific for the protein OSM (oncostatin M), and then substituted all pyrimidine positions with 2' fluorine and 14 of the 18 purine positions with 2' O-methyl. They found that the aptamer retained complete affinity for OSM.

With the knowledge that it is possible to make RNA aptamers, in particular, more stable without loss of affinity, the use of aptamers may increase. The handling of ssDNA aptamers is not as much a concern since they are not as susceptible to nucleases. Future work with aptamers holds promise in any analytical field which relies on molecular recognition. More specifically, aptamers may find successful application as stationary phases for chromatographic and electrophoretic separations and extractions.

# 3. Open Tubular Liquid Chromatographic Separations Using an Aptamer Stationary Phase

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c/o Wiley-VCH, 111 River St., Hoboken, NJ 07030
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## 3.1 ABSTRACT

Aptamers are oligonucleotides which are isolated *in vitro* based on their affinity for a target molecule. These molecules hold promise for use in analytical methods which rely on molecular recognition. In this study, we report the immobilization of an RNA aptamer on the inner walls of fused silica capillaries. Due to the small size of these molecules, the resulting surface coverage of these capillaries made them suitable for use in open tubular liquid chromatographic studies. These capillaries also exhibited stability when an organic modifier was added to the mobile phase. These characteristics of aptamers make them desirable over traditional affinity chromatography stationary phases.

## 3.2 INTRODUCTION

Aptamers are oligonucleotides which are isolated *in vitro* based on their affinity for a target molecule by a technique known as systematic evolution of ligands by exponential enrichment (SELEX). The first reports of this technique involved isolating RNA which showed affinity for a protein [1] and ssDNA sequences exhibiting affinity for several organic dyes [2]. Since that time, aptamers have been isolated based on affinity for many different classes of molecules including small molecules [3, 35, 37, 41], peptide sequences [39, 42], and other oligonucleotides [45]. These molecules have exhibited affinities which rival those typical of antibodies [16], and have shown enantioselectivity [20].

Due to their molecular recognition capabilities, these molecules are promising in any analytical application relying on high specificity. More specifically, aptamers have found use in biosensors [24, 25, 46], capillary electrochromatography [27, 28], and affinity chromatography [29, 30]. The use of these molecules as molecular recognition agents in analytical devices is relatively new, and has been reviewed [47]. These oligonucleotides show many advantages over other selective agents traditionally used in such applications. Aptamers are small in size, easily and reproducibly synthesized, and easily engineered. The size of these molecules allows for more complete surface coverage and a simpler three dimensional structure which makes them more rugged than larger molecules. In this study, we used a 35 base RNA aptamer which was isolated based on its affinity for the flavin portion of the small biological cofactors flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) [3]. FMN and FAD are biological cofactors capable of electron transfer. FAD is found in its free form, and participates in the citric acid cycle where it is reduced to $FADH_2$. FMN is also capable of electron transfer, and is found in some flavoproteins.

## 3.3 EXPERIMENTAL SECTION

### *3.3.1 Materials*

Tris(hydroxymethyl)aminomethane (Tris), thiourea, (glycidoxypropyl)trimethoxysilane (GOPS), 1, 1'-carbonyldiimidazole (CDI), phosphate buffered saline (PBS), FAD, FMN and anthracene were purchased from Sigma (St. Louis, MO, USA). Hexylamine was purchased from Aldrich (Milwaukee, WI, USA). Acetone and ethyl ether were purchased from Mallinckrodt (St. Louis, MO, USA). The 5' amine modified RNA aptamer was purchased from Oligos, Etc. (Wilsonville, OR, USA). HPLC grade acetonitrile (ACN) was purchased from Fisher Scientific (Pittsburgh, PA, USA), and was dried over 4-A molecular sieves prior to use. All other chemicals were used as received. All fused silica capillary tubing was purchased from Polymicro Technologies (Phoenix, AZ, USA).

### *3.3.2 Capillary Preparation*

The 35 base RNA aptamer was covalently bound to the inner walls of fused silica capillaries 50 μm in inner diameter using a method developed in-house, modified from a previously reported method [48]. The first step of the immobilization process involved simultaneously modifying the silanol surface of the capillary with (glycidoxypropyl)trimethoxysilane (GOPS) and converting the epoxide ring of GOPS into a diol. The capillaries were filled with a solution consisting of 10% GOPS in aqueous HCl (pH 3.5). This reaction took place at 90° C for 5 hours. The capillaries were then flushed with several volumes each of water, acetone, and ether; then dried

overnight at 90° C. Next, 0.5 M 1, 1'-carbonyl diimidazole (CDI) in dry ACN was added at ambient temperature and allowed to react for 2.5 hours. This accomplished the addition of an imidazole ring to the capillary surface which acts as a leaving group. The capillaries were rinsed by flushing with acetonitrile and dried under argon. Finally, the amine-modified RNA aptamer was added in 50 mM sodium phosphate (200 μM at pH 8). For capillaries modified with the linker only, hexylamine at the same concentration and pH was added to the capillaries.

After the immobilization was complete, the capillaries were immediately dried under argon and stored at 4° C until use. Immediately before use, each capillary was flushed with 0.1 M PBS for several minutes to remove any unreacted aptamer. Figure 3.1 illustrates the modification procedure, showing the surface of the fused silica capillaries after each step of the immobilization process. As can be seen, cross-linking along the inner wall of the fused silica surface is one desirable trait. This allows for more surface area to be available for the immobilization of the aptamer.

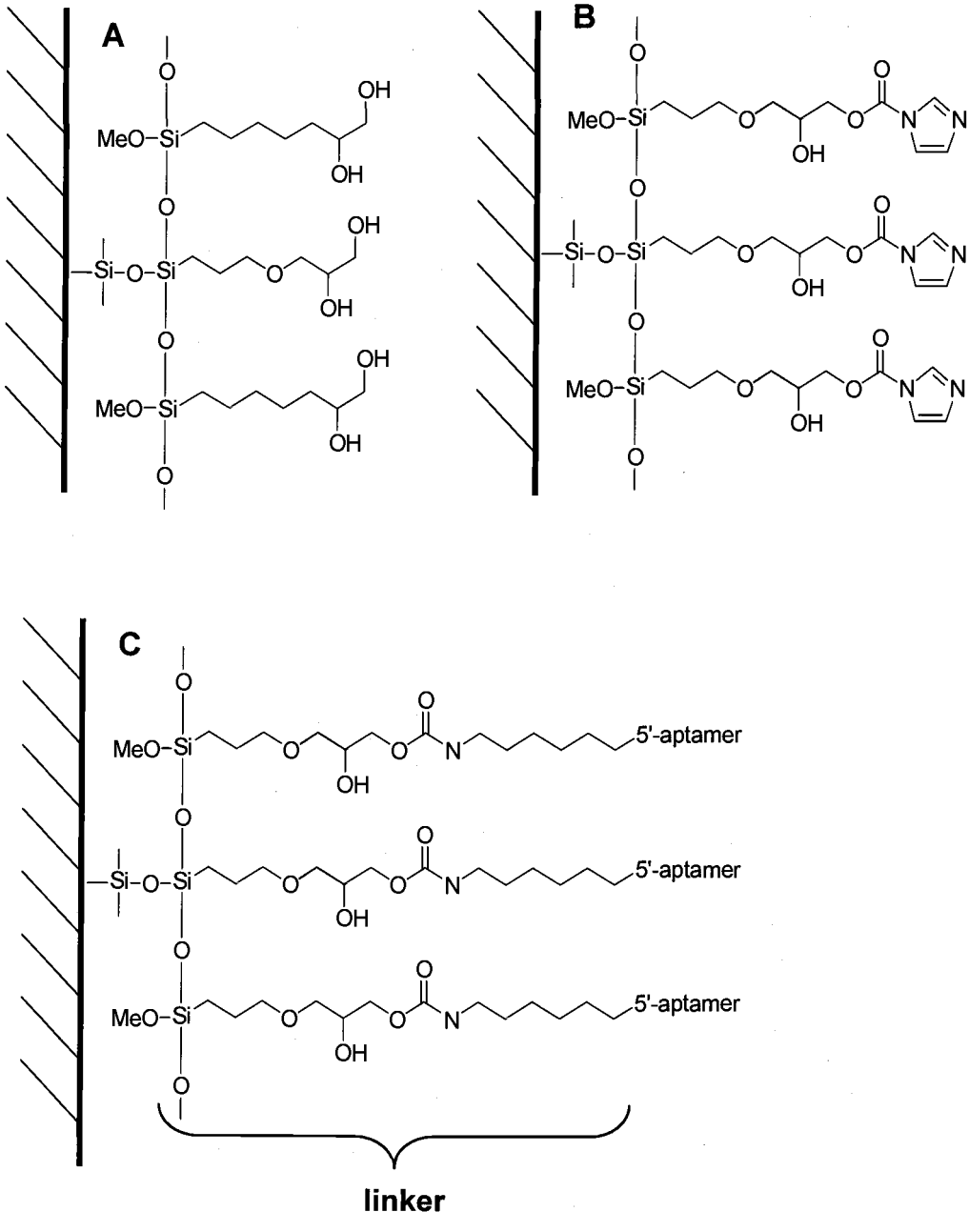


**Figure 3.1.** The chemical composition of the inner surface of the fused silica capillary after each step of the immobilization method. (A) GOPS is added to the fused silica surface and the epoxide is simultaneously opened to form a diol. (B) An imidazole group is added to the surface as a leaving group. (C) The 5' amine modified RNA aptamer is added.

*3.3.3 Instrumentation*

All separations reported were conducted on a Hewlett Packard $^{3D}$CE equipped with a diode array detector. All separations took place at a temperature of 20° C on capillaries of 35 cm total length, with a length to detection of 26.5 cm. Separations were achieved by applying 50 mbar constant head pressure to the inlet vial. Detection was achieved online by burning away the polyimide coating of the capillary to create a detection window. Data was collected at a wavelength of 200 nm.

## 3.4 RESULTS/DISCUSSION

The 50 μm inner diameter capillaries, while large for open tubular LC, provided an easily manipulated format for initial studies. In addition, success with a capillary of this inner diameter would require and indicate a high degree of linker and aptamer immobilization.

Although the aptamer derivatized capillaries proved unable to discriminate between FMN and FAD, they were able to recognize either target over other molecules which do not contain the flavin moiety recognized by the aptamer. Due to the slightly larger $K_A$ reported for FMN, it was used in all studies [3]. All separations reported were accomplished by applying 50 mbar constant head pressure to the inlet vial. The mobile phase was 50 mM tris/HCl (pH 7.6) with varying amounts of ACN added in some cases.

Figure 3.2 shows the separation of FMN and anthracene on a capillary derivatized with the RNA aptamer. In order to investigate any effect of the C6 group

on the separation, the experiment was also conducted on a capillary modified with the C6 linker only (Figure 3.3). These studies indicate that the retention of FMN on the aptamer-derivatized capillaries was the result of the molecular recognition exhibited by the aptamer. On the capillaries modified with the linker anthracene is slightly retained, likely due to a reverse-phase mechanism. The C6 group on the inner surface which is responsible for the reverse-phase retention of anthracene was largely inaccessible to the analytes in the aptamer-derivatized capillaries because of the presence of the aptamer.

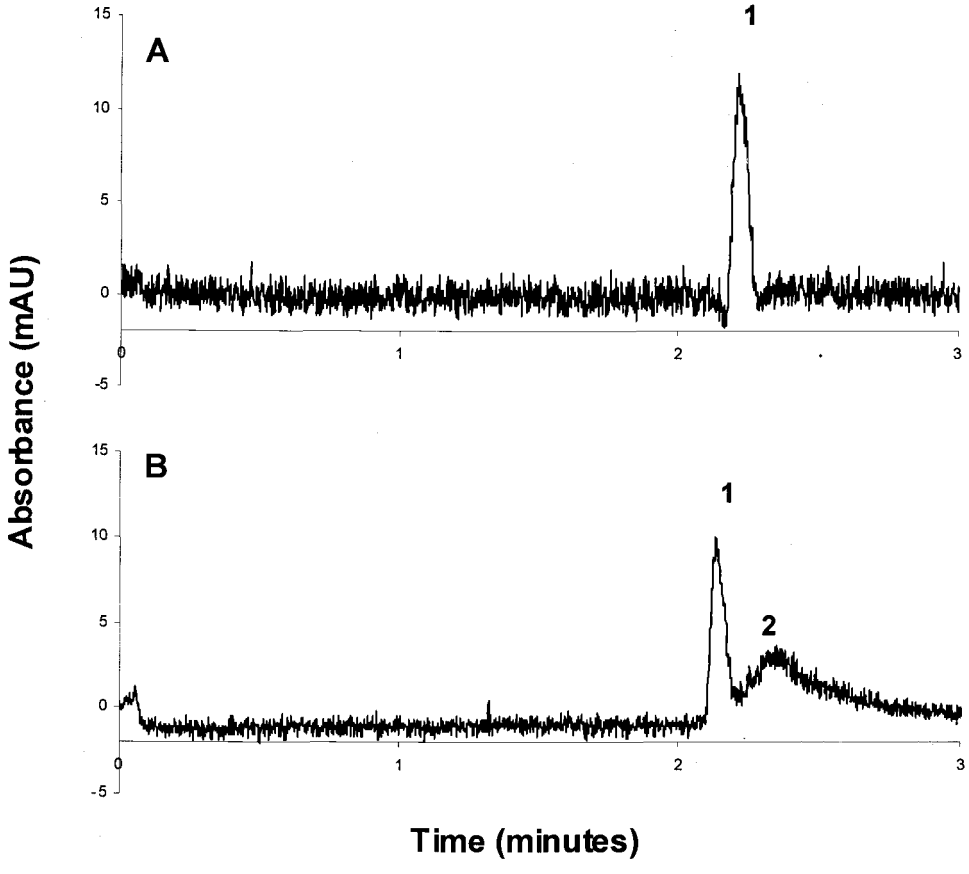


**Figure 3.2.** Aptamer capillary recognizes FMN. Chromatograms showing anthracene only (A) and a mixture of anthracene and FMN (B) on an aptamer-derivatized capillary. Separations were conducted by applying 50 mbar constant head pressure at the inlet, the mobile phase is 50 mM tris/HCl (pH 7.6). Peak (1) is anthracene and peak (2) is FMN.

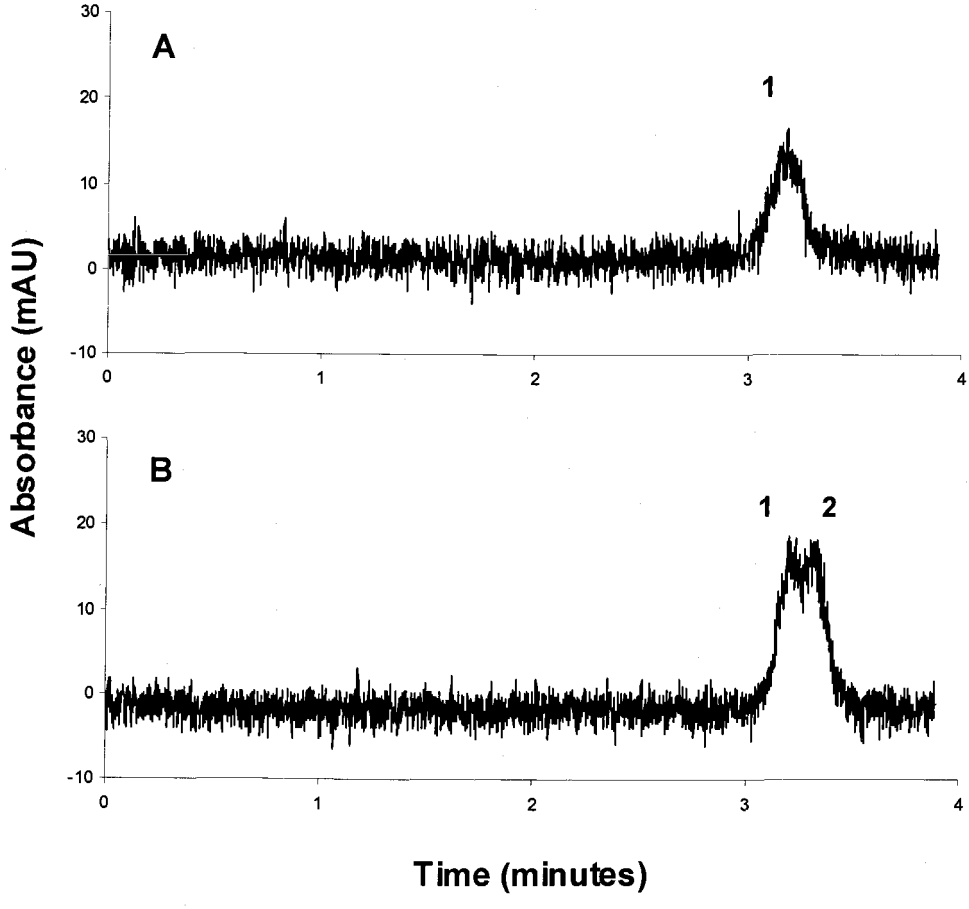


**Figure 3.3.** Separation of FMN and anthracene on capillary modified only with the linker molecule. Chromatograms of FMN only (A) and FMN and anthracene (B) on a capillary modified only with the linker molecule. Conditions the same as in Figure 2. Peak (1) is FMN and peak (2) is anthracene

The immobilized aptamer also sorbed molecules unrelated to the target. One such molecule was thiourea. Under the above conditions, the aptamer seemed to retain thiourea more strongly than FMN. Figure 3.4 shows the separation of a sample of FMN and thiourea. By spiking the sample with thiourea, it was clear that thiourea was the more retained analyte. Different mobile phase compositions were investigated to explore and optimize the specificity and ruggedness of the aptamer stationary phase. The selectivity of the stationary phase is altered by the addition of organic solvent in the mobile phase (Figure 3.5). It is clear from Figure 3.5 that the preferentially retained analyte changed from thiourea to FMN as the component of ACN was increased in the mobile phase. There was no separation of FMN and thiourea observed when capillaries modified with the linker molecule only were tested under the same conditions.

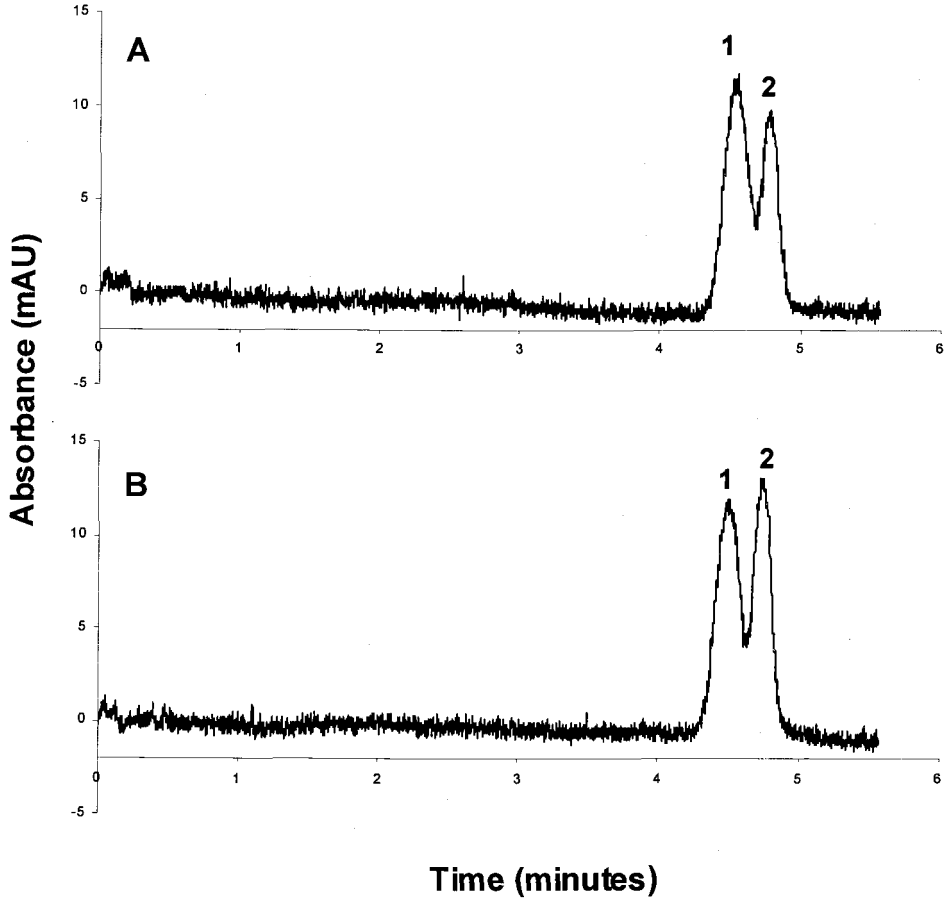


**Figure 3.4.** Aptamer retains molecule unrelated to target molecules. The separation of FMN (1) and thiourea (2) on an aptamer-derivatized capillary before (A) and after (B) the sample is spiked with thiourea. Conditions the same as in Figure 2.

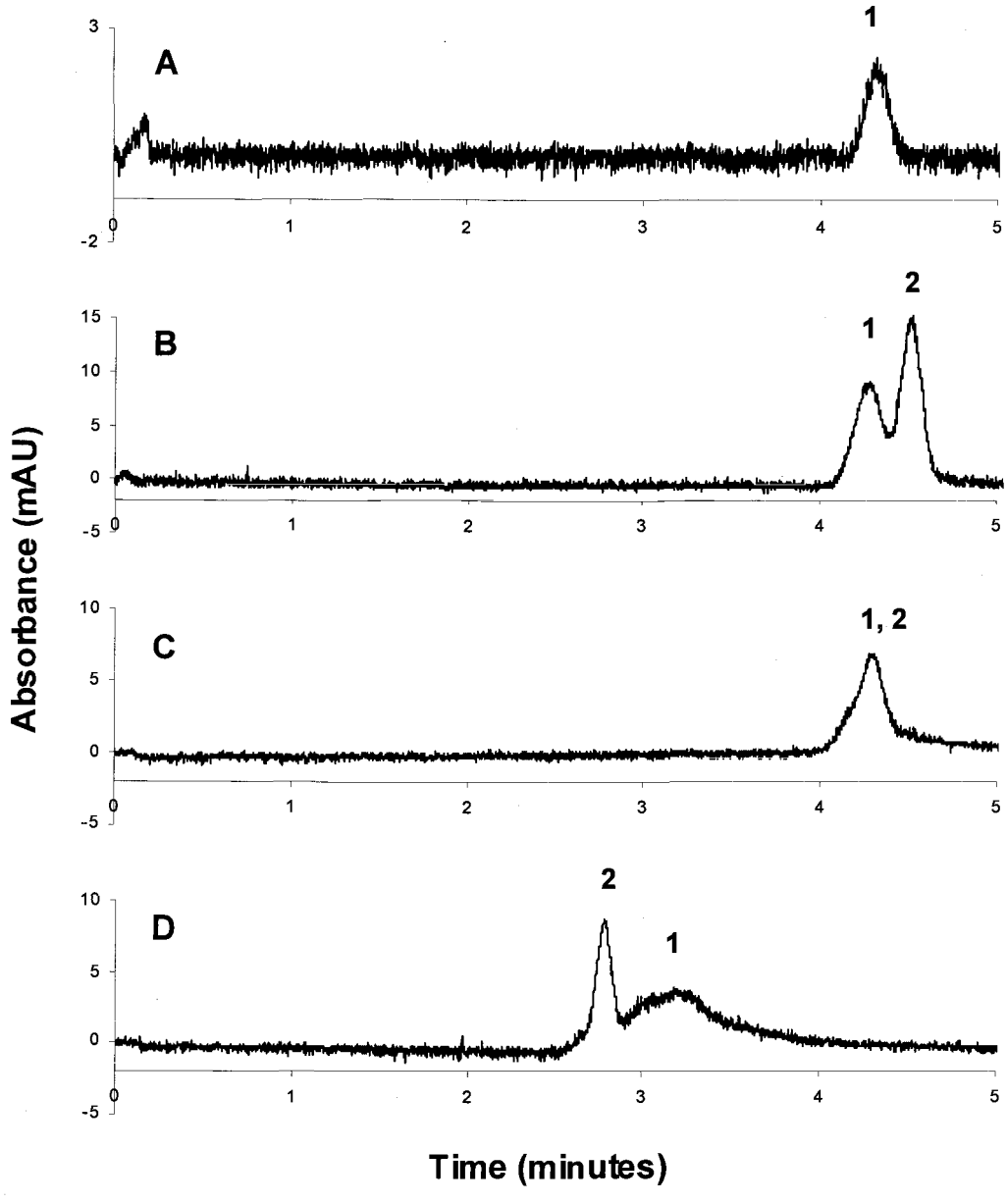


**Figure 3.5.** The effect of mobile phase composition. The separation of FMN (1) and thiourea (2) using an aptamer stationary phase. Chromatograms are shown of FMN in 50 mM tris/HCl at pH 7.6 (A), FMN and thiourea in 50 mM tris/HCl at pH 7.6 (B), the same sample (FMN and thiourea) when mobile phase consists of 50% ACN (C) and 80% ACN (D) by volume.

The aptamer stationary phase showed a noticeable degradation over time when ACN was present in the mobile phase. However, the stationary phase was easily regenerated by flushing with a buffer containing 10 mM $MgCl_2$. Divalent cations are known to stabilize oligonucleotides due to their interaction with base $\pi$ systems within the oligonucleotide [4], and these conditions were found to regenerate the aptamer sorbent. Again, no separation was observed on capillaries modified only with the linker

## 3.5 CONCLUSION

Aptamers show promise as specific stationary phases. The cross-linking which occurs under the immobilization conditions allows more surface to be available to the aptamer. The increased amount of aptamer available to the analyte proved suitable for open-tubular LC in capillaries with an inner diameter of 50 μm. The aptamer proved to be stable when an organic solvent was added to the mobile phase; this resulted in an enhancement in the specific component of retention relative to the non-specific retention component of the aptamer. In the future, these characteristics may allow aptamers to find broader use as affinity chromatography stationary phases.

The immobilization method presented in this chapter held several disadvantages for use in electrochomatographic separations. The first step of the immobilization method involves adding GOPS to the capillary in aqueous solution at an acidic pH. This results in the cross-linking of the GOPS reactant as can be seen in figure 3.1. This cross-linking results in the formation of a gel coating on the inner

walls of fused silica capillaries, which can be seen under magnification. We found this reaction to be very uncontrollable, and thus irreproducible. We also found that the coating of this gel masked the silanol groups on the inside of the capillaries to such an extent that electroosmotic flow (EOF) was negligible, and thus electrically-driven separations were impossible. In the next chapter of this thesis, the immobilization method is modified so that the result is not a gel-like coating on the inner walls of fused silica capillaries but instead a monolayer of the aptamer so that EOF is achievable. These capillaries were suitable for electrically-driven separations. This work is described in the next chapter.

# 4. Electrochromatographic Retention Studies on a Flavin-Binding RNA Aptamer Sorbent

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*Analytical Chemistry*
1155 16th St., NW, Washington, DC 20036
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## 4.1 ABSTRACT

Aptamers are oligonucleotides which are isolated and amplified based on their recognition of a target molecule. In this study, an RNA aptamer isolated and amplified based on its affinity for flavin mononucleotide (FMN) was covalently bound to the inner walls of fused silica capillaries. This aptamer recognizes the flavin moiety of both FMN and flavin adenine dinucleotide (FAD). When an attempt was made to evaluate these capillaries according to existing theory, the theory proved to be insufficient. We describe a new method to evaluate capillaries for use in OTCEC of charged analytes which combines open-tubular capillary electrochromatography (OTCEC) and flow counterbalanced capillary electrophoresis (FCCE). This method enabled us to extract k' and evaluate $k_{CEC}$ values for these capillaries, and the dependence of these values on $Mg^{2+}$ concentration was explored. The k' values for these capillaries ranged from 0.0951 to 0.2530, and from 0.0255 to 0.1118 for FMN and FAD respectively.

## 4.2 INTRODUCTION

Aptamers are single-stranded oligonucleotides which are isolated and amplified based on their affinity for a target molecule by "systematic evolution of ligands by exponential enrichment" (SELEX) [1, 2]. Aptamers have been isolated based on their affinity for a diverse collection of target molecules including small molecules [35, 37], peptide sequences [39, 41, 42], proteins [5, 49], and other oligonucleotides [45]. The large number of possible oligonucleotide sequences and

their molecular diversity make possible the isolation of aptamers that show affinity for a large variety of molecules [17].

The use of these molecules in analyses requiring molecular recognition shows promise and has been reviewed [47]. Aptamers have found use in such techniques as flow cytometry [22, 23], sensors and biosensors [24, 25, 50, 51], affinity chromatography [29, 30, 52], affinity capillary electrophoresis [26, 53], and capillary electrochromatography [27, 54]. These molecules show advantages over other molecules traditionally used in such applications, including their small size and their easy and reproducible synthesis. The small size of these molecules offers two potential advantages for the use of these molecules in specific separations: an increase in achievable surface coverage, and stability resulting from the simple three-dimensional structure of these molecules.

Capillary electrochromatography (CEC) is a technique which combines capillary electrophoresis (CE) with micro-scale liquid chromatography (micro-LC). By combining the selectivity of chromatography and the efficiency of CE, more resolving power can be achieved. In CE, a potential difference is applied across the length of fused silica capillary tubing. The application of this potential causes the formation of an electric double layer along the inner wall of the capillary. Figure 4.1 is a diagram showing the electric double layer inside a fused silica capillary. Silanol groups on the inside of the capillary (4.1A) give rise to the negative charge of the surface. The electric double layer forms along the inner walls of the capillary (4.1B). The electric double layer consists of two layers: the Stern and Gouy layers. The Stern

layer contains ions of opposite charge to the charged surface which are adsorbed to the surface through electrostatic attraction. This layer is immobile. The Gouy layer contains a high concentration of ions of opposite charge to the surface (relative to bulk solution concentrations), but there are also counterions which are present in the solution in this layer. By the end of this layer, concentrations of ions are that of the bulk solution. During electrophoretic separations, the positively charged ions within the Gouy layer migrate towards the cathode, and by viscous drag all solution components are "pulled" towards the cathode. This flow is referred to as electroosmotic flow (EOF). In a pure CE separation, all neutral analytes present migrate at the velocity of this flow; and it is the profile of this flow which is responsible for the enhanced efficiency of electrophoretic separation. Figure 4.2 shows the profiles of EOF and laminar flow (which is the result of applied pressure).

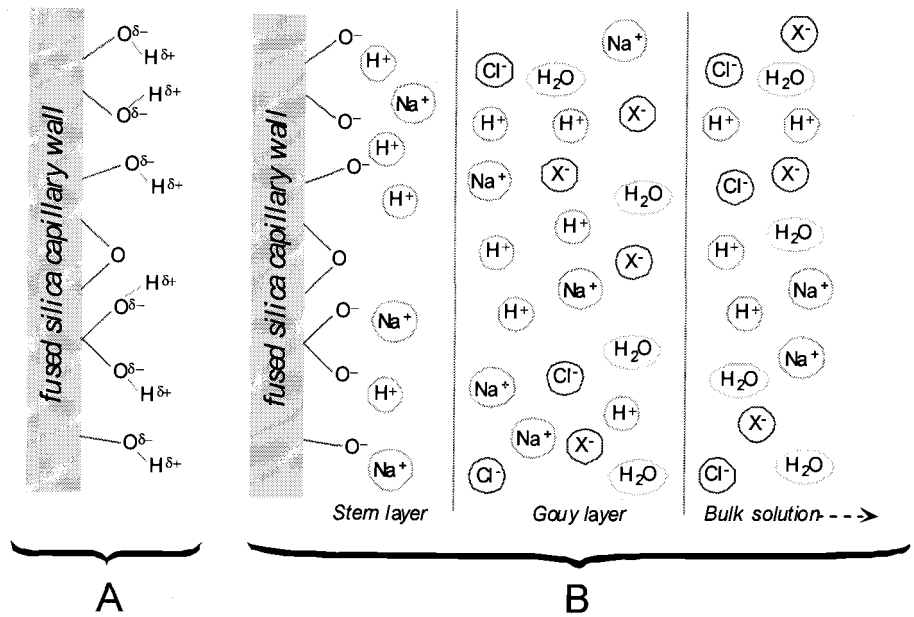


**Figure 4.1.** The electric double layer. The silanol groups present on the surface of fused silica capillaries (A) are responsible for the negative charge present at the surface. When an electric field is applied across the length of the capillary, the result is the formation of the electric double layer (B).


**Figure 4.2.** Flat profile of EOF (A) and parabolic profile of pressure-driven flow (B).

CEC is a hybrid technique which combines the advantages of both electrophoretic and chromatographic techniques. In CEC, a stationary phase is present. However, bulk transport in CEC separations is due to EOF. This means that the technique combines the selectivity of liquid chromatography by the preferential partitioning of analytes into the stationary phase, and the efficiency of CE because of the flat EOF profile present. For these reasons, we chose to pursue open tubular CEC (OTCEC) studies of the aptamer stationary phase.

Flow counterbalanced capillary electrophoresis (FCCE) is a technique which was originally devised to increase the resolving power of capillary electrophoresis [55]. This technique involves using pressure in the presence of an electric field to closely control the electrokinetic migration of analytes through a capillary. In the original studies, the migration of the analytes was reversed by application of pressure in order to move the analytes back and forth across the detection window so that the analytes remained in the separation field until separation was achieved.

In the study presented here, we used a modified version of FCCE in order to adjust the net electroosmotic flow (EOF) to zero inside both aptamer-modified and bare fused silica capillaries. An RNA aptamer consisting of 35 bases, isolated based on its affinity for the flavin moiety of the small biological cofactors FMN and FAD [3], was immobilized inside the fused silica capillaries used for OTCEC. The RNA aptamer and its targets are shown in Figure 4.3. Using flow counterbalanced open tubular capillary electrochromatography (FC-OTCEC), the method described herein,

the chromatographic value k' for the aptamer stationary phase was experimentally determined for different mobile phase conditions.

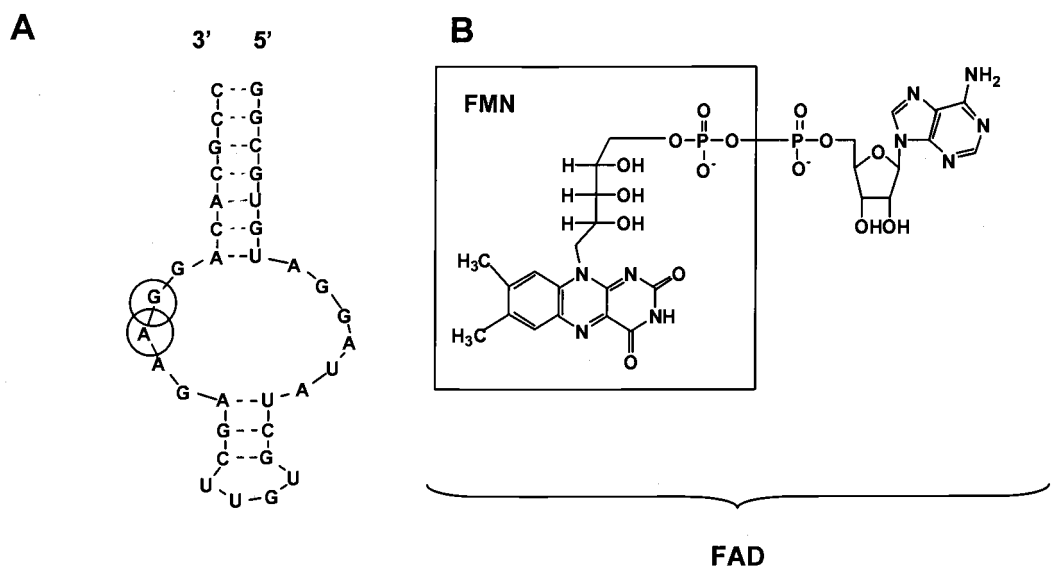


**Figure 4.3.** The RNA aptamer (A) and its targets (B). The positions A26 and G27 are circled as referred to in the text.

## 4.3 EXPERIMENTAL SECTION

### *4.3.1 Materials*

Tris(hydroxymethyl)aminomethane (Tris), thiourea, (glycidoxypropyl)trimethoxysilane (GOPS), 1,1'-carbonyldiimidazole (CDI), phoshpate buffered saline (PBS), sodium phosphate, FAD, and FMN were purchased from Sigma (St. Louis, MO, USA). Acetone, ethyl ether and magnesium chloride were purchased from Mallinckrodt (St. Louis, MO, USA). The 5'-amine modified RNA aptamer was purchased from Oligos, Etc. (Wilsonville, OR, USA). HPLC grade acetonitrile (ACN) and xylene were purchased from Fisher Scientific (Pittsburgh, PA, USA). The ACN was dried over 4 Å molecular sieve prior to use. All other chemicals were used as received. Fused silica capillary tubing was purchased from Polymicro Technologies (Phoenix, AZ, USA).

### *4.3.2 Capillary Preparation*

The 35-base RNA aptamer was covalently bound to the inner walls of fused silica capillary tubing using a modified version of a previously published method [48]. First, the capillaries were filled with a solution consisting of 75% xylene, 25% GOPS, and 0.1% Hünig's base by volume and placed at 80° C overnight. Capillaries were then flushed with xylene, dried by flushing with argon, and filled with dilute HCl (pH 3.5) and placed at 80° C for five hours. Capillaries were then flushed with several volumes each of water, acetone, ethyl ether, and argon to dry. Each was then maintained at 100° C for at least ten hours to ensure dryness. Next, the capillaries

were filled with a saturated solution of CDI in ACN and allowed to react at room temperature for 5 hours. Capillaries were flushed with ACN and dried with argon before being filled with the 5'-amine modified RNA aptamer (220 μM) in 50 mM sodium phosphate (pH 8) . The capillaries were left at room temperature for three hours, then dried with argon and stored at 4° C. To prepare them for use, the capillaries were flushed with 0.1 M PBS for several minutes to remove any unreacted aptamer. Figure 4.4 shows the complete reaction resulting in the immobilization of the RNA aptamer. All capillaries used in the study were cut to 35.0 cm with a detection window burned 8.5 cm from the outlet.

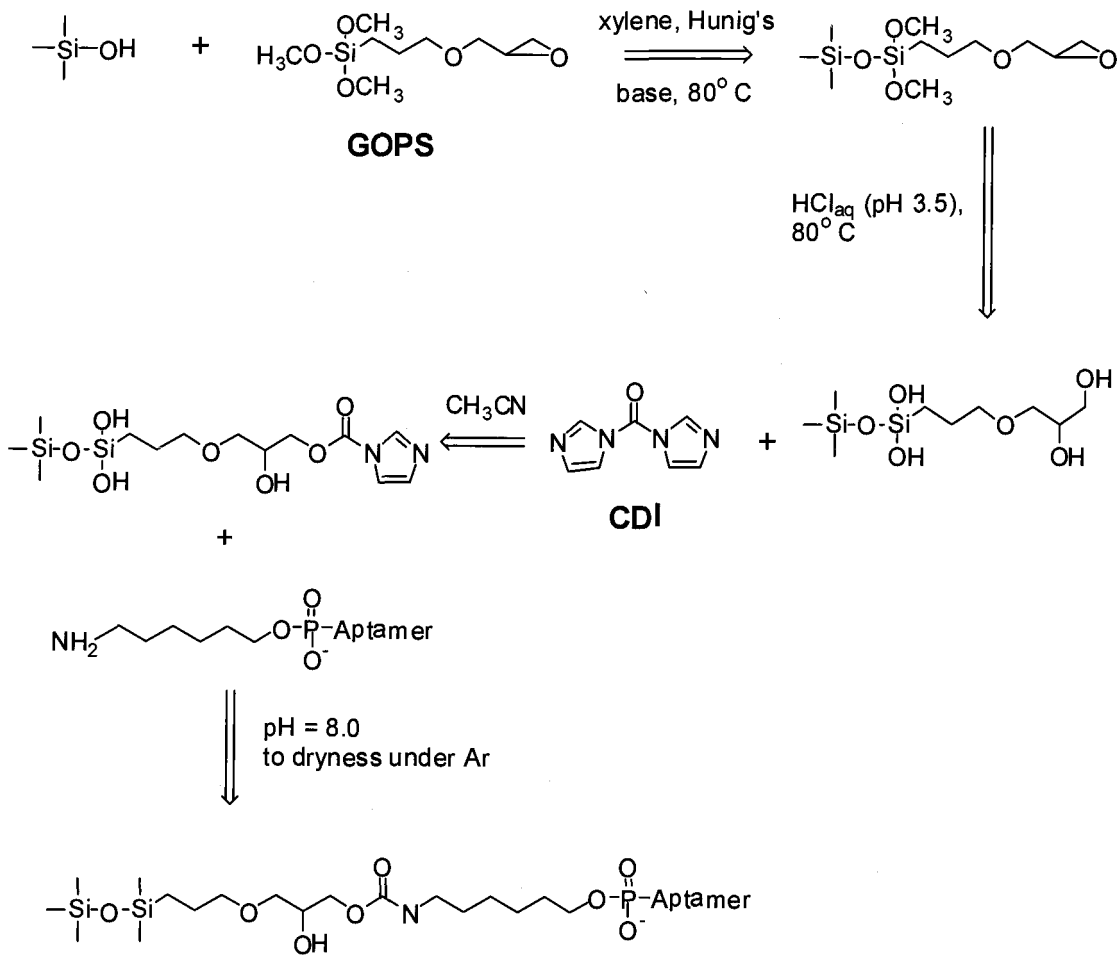


**Figure 4.4.** Scheme resulting in covalent bonding of an RNA aptamer onto the inner walls of a fused silica capillary.

*4.3.3 Instrumentation*

All separations were conducted on a Hewlett Packard $^{3D}$CE equipped with a diode array detector. The capillary was kept at 20° C during all separations. Detection was achieved online by burning the polyimide coating of the capillary to create a detection window, and data was collected at 254 nm.

*4.3.4 OTCEC*

Open tubular CEC studies were performed on capillaries of 50 μm inner diameter. During each run, -30 kV was applied across capillaries 35.0 cm in total length. The run buffer was 20 mM tris/HCl (pH 7.6). As different concentrations of $MgCl_2$ were added to the mobile phase, the ionic strength was kept constant at 20 mM by addition of the run buffer. Therefore, any effect the ionic strength may have on the recognition exhibited by the aptamer for its targets was factored out of the evaluation of the effect of $Mg^{2+}$ concentration.

The negative polarity applied during each run caused the electroosmotic flow (EOF) to be reversed, so that the analytes traveled across 8.5 cm to the detection window. Thiourea was used as the $t_0$ marker in all experiments. The polarity was reversed in these experiments due to the small EOF generated in aptamer-derivatized capillaries which caused excessively long run times. Under the conditions stated, only FAD could be eluted from aptamer-derivatized capillaries. We believe this is due to both the slow EOF in these capillaries and the higher affinity the aptamer exhibits for FMN relative to FAD.

*4.3.5 Flow Counterbalanced-OTCEC*

Capillaries used in the FC-OTCEC experiments were also 35.0 cm in total length and 50 μm in inner diameter. In these experiments, pressure was applied which opposed the direction of EOF. During each run, -5 kV was applied while a constant pressure was applied to the inlet vial. While the direction of EOF was from the anode to the cathode, the negatively charged analytes traveled from the cathode to the anode. The contribution from EOF inside the capillary was zero due to the pressure counterbalance, so that the velocity of the analytes was a function of their size, charge, and extent of retention on the aptamer stationary phase.

The pressure was monitored several ways to confirm that it effectively counterbalanced the EOF. First, the EOF for the capillary was established using thiourea as the $t_0$ marker in the mobile phase under investigation while applying a potential of 5 kV. Next, the capillary was filled with the mobile phase and each end of the capillary was placed in 5 mM tris/HCl. A constant voltage of -5 kV was applied across the capillary while a constant pressure was applied to the inlet vial. If the pressure applied was too high or too low to counterbalance EOF, a decrease in current was observed (Figure 4.5). Once the necessary counter-balancing pressure was established, the $t_0$ marker was injected and flowed through the capillary using the pressure which produced a constant current in the experiments described above, but in the absence of an electric potential. This experiment was undertaken to be certain that the neutral marker eluted at the same time as it did under EOF in the absence of a potential. During runs involving only FMN or FAD, the analyte was injected at the

cathode. A constant pressure was applied at the inlet while -5 kV was applied across the capillary. To make certain that the pressure was successfully offsetting EOF, the pressure was monitored to make sure that the pressure vs. time profile was flat, meaning that there was no net flow through the capillary. FMN and FAD were injected separately into both bare fused silica capillaries and aptamer-derivatized capillaries under a variety of mobile phase conditions. Values given for the migration times and electrophoretic mobilities of each analyte represent an average of at least 12 runs.

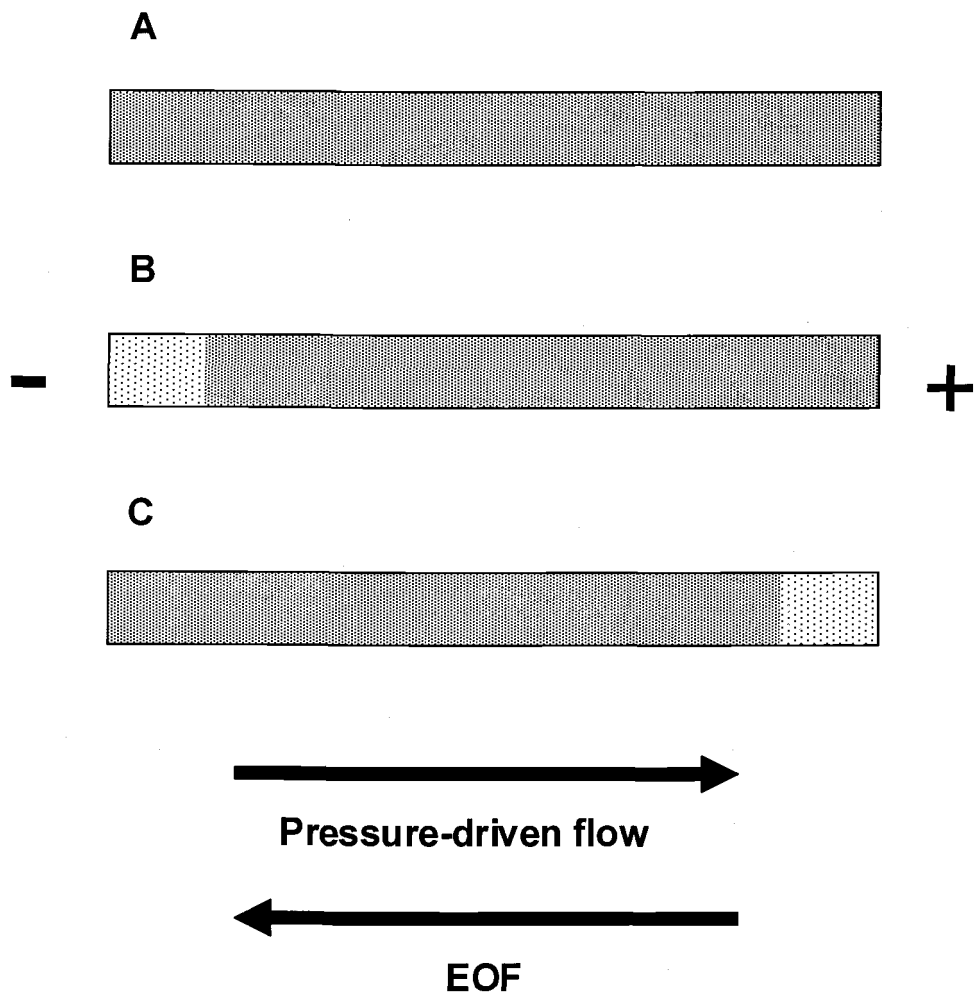


**Figure 4.5.** Schematic of the use of pressure to oppose EOF. Capillary is filled with 20 mM tris/HCl (pH 7.6) while inlet and outlet vials are filled with 5 mM tris/HCl (pH 7.6). (A) Pressure driven flow is exactly equal and opposite to EOF resulting in constant pressure and no net flow. (B) Pressure driven flow is greater than EOF, resulting in the transport of electrolyte in the inlet vial into the capillary, accompanied by a decrease in current. (C) Pressure driven flow is lesser in value than EOF, allowing electrolyte in the outlet vial to enter the capillary and current to decrease.

## 4.4 RESULTS/DISCUSSION

The 3-D structure of oligonucleotides is stabilized by the presence of divalent cations through interactions between the metal ion and $\pi$ electrons present in the oligonucleotide [4]. These interactions have been shown to affect the affinity exhibited by RNA and DNA aptamers toward their target molecules [29, 43]. Therefore, when characterizing these capillaries, different concentrations of $MgCl_2$ were used to study the effect $Mg^{2+}$ might have on the affinity shown by the aptamer-derivatized capillaries for FMN and FAD.

### *4.4.1 OTCEC*

Open-tubular CEC experiments were undertaken in which the aptamer-derivatized capillaries were compared with bare fused silica capillaries under the same conditions. Figure 4.6 shows a comparison of the electroosmotic mobility in a bare fused silica capillary and in a capillary derivatized with the RNA aptamer. Although the aptamer is negatively charged under the separation conditions, if this charge contributes to the electroosmotic mobility generated in the capillaries, the contribution must be minimal since the electroosmotic mobility in the aptamer-derivatized capillaries is smaller than in bare fused silica capillaries.

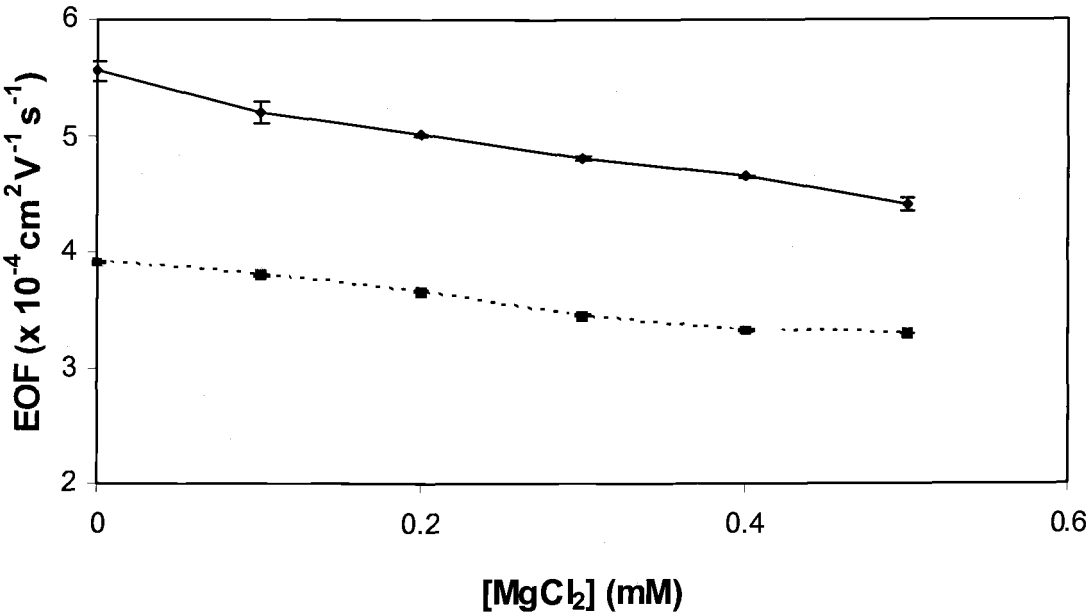


**Figure 4.6.** EOF in bare fused silica and in aptamer-derivatized capillaries. The run buffer was adjusted to a constant ionic strength of 20 mM with the addition of tris/HCl (pH 7.6), the neutral marker was thiourea, the capillaries were 35.0 cm in total length and 26.5 cm to detection, 5 kV was applied, hydrodynamic injection was 25 mbar for 2.5 seconds, error bars represent one standard deviation for $n \geq 7$ runs. —— Bare fused silica capillary ------ aptamer-derivatized capillary

The migration of charged analytes in CEC is a function of both their electrophoretic mobility ($\mu_{ep}$) and chromatographic retention. The $\mu_{ep}$ of a charged analyte is directly proportional to the applied field strength (E) and the charge of the analyte (q), and inversely proportional to the viscosity of the mobile phase and the

analyte's Stokes radius. Experimentally, this value is calculated using the following equation:

$$\mu_{ep} = \frac{L_T \times L_D}{V}\left(\frac{1}{t_A} - \frac{1}{t_0}\right) \tag{4.1}$$

where $L_T$ is the total length of the capillary, $L_D$ is the length to detection, V is the total voltage applied across the capillary, $t_A$ is the migration time of the analyte, and $t_0$ is the migration time of a neutral marker. The extent of chromatographic retention of the analyte is characterized by the retention factor (k'):

$$k' = \frac{t'_r}{t_0} \tag{4.2}$$

where $t'_r$ is the adjusted retention time of the analyte and $t_0$ is the time necessary for an unretained analyte to travel to the point of detection. In CEC experiments, the migration of a charged analyte is given by equation 4.3 [56, 57]:

$$k_{CEC} = k' - \frac{\mu_{ep}}{\mu_{ep} + \mu_{eof}}(k'+1) \tag{4.3}$$

where $k_{CEC}$ is the CEC retention coefficient, $\mu_{eof}$ is the electroosmotic mobility, and the term $\mu_{ep}/(\mu_{ep}+\mu_{eof})$ corresponds to the retention of the analyte in pure CE mode. As k' approaches zero, $k_{CEC}$ approaches $\mu_{ep}/(\mu_{ep}+\mu_{eof})$ and the retention mechanism is completely electrophoretic. That is, there is no chromatographic contribution to the retention of the analyte.

Using equation 4.3, we attempted to evaluate the extent of aptamer-target interaction inside the aptamer-derivatized capillaries. Figure 4.7 shows two comparisons of the two sets of capillaries. In part A, the comparison of $\mu_{ep}$ in bare

fused silica capillaries and in aptamer-derivatized capillaries is shown. It is clear that $\mu_{ep}$ of FAD in aptamer-derivatized capillaries is different from the $\mu_{ep}$ in bare fused silica capillaries; the $\mu_{ep}$ of FAD in aptamer-modified capillaries is less negative than in fused silica capillaries. This means that the analyte is actually moving faster inside aptamer-derivatized capillaries (with respect to EOF) which seems counter-intuitive. Part B shows a comparison of the value $\mu_{ep}/(\mu_{ep}+\mu_{eof})$ in bare and modified capillaries. According to this figure, the k' approaches zero at higher $Mg^{2+}$ concentrations since the value $\mu_{ep}/(\mu_{ep}+\mu_{eof})$ inside aptamer-modified capillaries approaches that value in bare capillaries at higher $[Mg^{2+}]$. Since it was shown that the modification of the capillaries had an effect on $\mu_{eof}$ (Figure 4.6) and the value $\mu_{ep}/(\mu_{ep}+\mu_{eof})$ has a dependency on $\mu_{eof}$, it is unclear whether Equation 4.3 can be used to evaluate these capillaries in an unbiased manner.

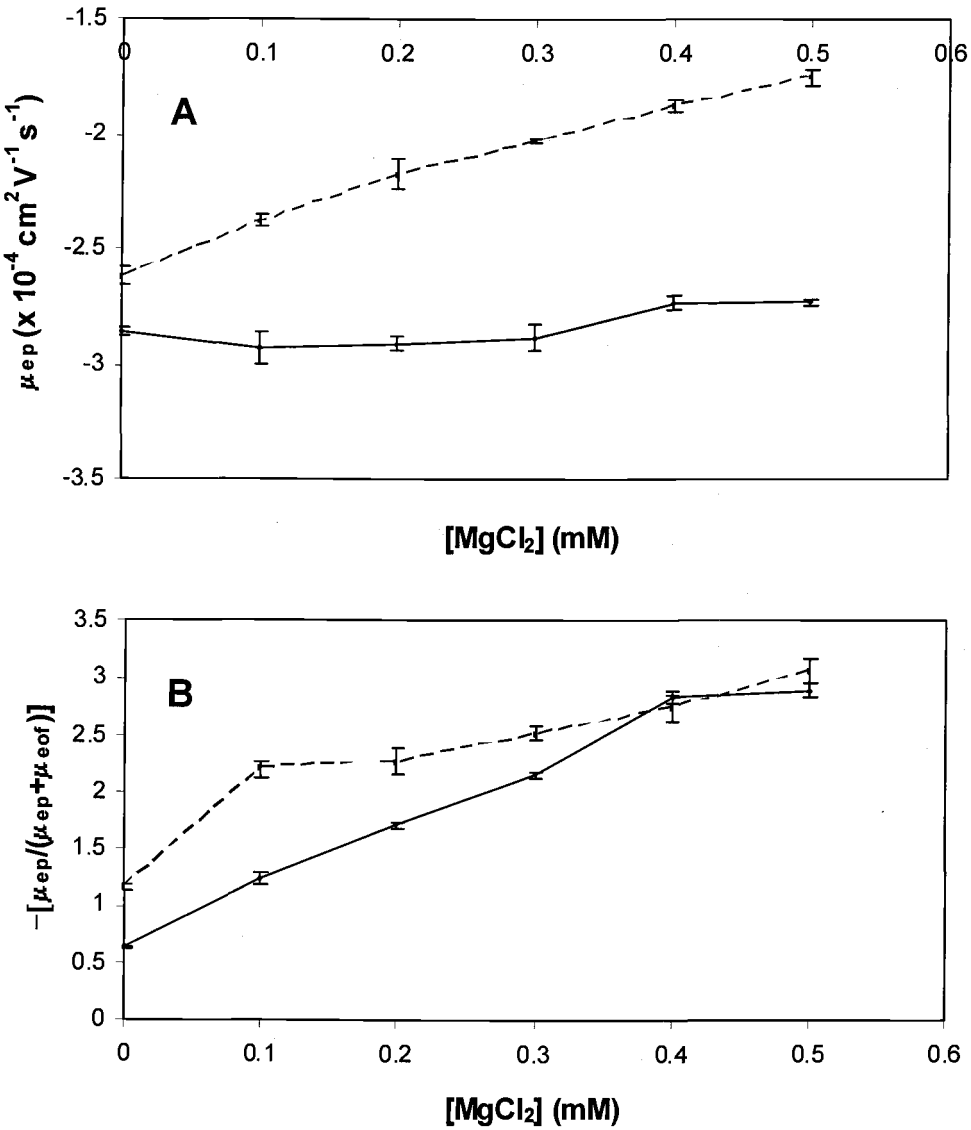


**Figure 4.7.** $\mu_{ep}$ (A) and $-[\mu_{ep}/(\mu_{ep}+\mu_{eof})]$ (B) of FAD in bare fused silica and in aptamer-derivatized capillaries. Capillaries were 35.0 cm in total length, 8.5 cm to detection, -30.0 kV applied, run buffer kept at a constant ionic strength of 20 mM with the addition of tris/HCl (pH 7.60), FAD concentration is 250 μM, hydrodynamic injection was 50 mbar for 1 sec, error bars represent one standard deviation for $n \geq 7$ runs. —— bare fused silica capillary ------ aptamer-derivatized capillary

*4.4.2 FC-OTCEC*

These experiments were undertaken in order to fully explore the chromatographic retention occurring on the aptamer stationary phase. In these experiments, pressure was applied across bare fused silica capillaries and aptamer-derivatized capillaries which effectively counterbalanced the EOF present in the capillaries. Using migration times of the analytes FMN and FAD under these conditions, k' for the aptamer stationary phase was extracted in the following way:

$$k' = \frac{t_{apt} - t_{fs}}{t_{fs}} \qquad (4.4)$$

where $t_{apt}$ is the migration time of the analyte in an aptamer-derivatized capillary and $t_{fs}$ is the migration time of the analyte in a bare fused silica capillary. The net EOF was zero in the experiments, so the migration of the analyte through the capillary was a function of only the size and charge of the analyte and the extent of its interaction with the aptamer. Therefore, if there was no chromatographic retention of the analyte, the migration times on bare and aptamer-modified capillaries should be identical under the same conditions. Table 4.1 shows the k' values for FMN and FAD which were calculated using equation 4.4.

**Table 4.1.** k' values for FMN and FAD in aptamer-derivatized capillaries. Capillaries were 35.0 cm in total length and 26.5 cm to detection, 50 μm in inner diameter, -5 kV was applied while a constant pressure was applied at the inlet to counterbalance the EOF, buffer was kept at a constant ionic strength of 20 mM by the addition of tris/HCl (pH 7.6), FAD and FMN concentration was 250 μM, hydrodynamic injection achieved by applying 50 mbar for 1 sec, reported standard deviation is for $n \geq 12$ data points.

| | **FMN** | | | **FAD** | | |
|---|---|---|---|---|---|---|
| $[MgCl_2]$ (mM) | k' | standard deviation | relative std. dev. | k' | standard deviation | relative std. dev. |
| 0 | 0.0951 | 0.0010 | 1.1% | 0.0255 | 0.0004 | 1.6% |
| 0.1 | 0.1979 | 0.0036 | 1.8% | 0.0534 | 0.0010 | 1.9% |
| 0.2 | 0.2530 | 0.0042 | 1.7% | 0.0535 | 0.0011 | 2.1% |
| 0.3 | 0.1877 | 0.0037 | 2.0% | 0.0645 | 0.0010 | 1.6% |
| 0.4 | 0.1533 | 0.0014 | 0.9% | 0.0688 | 0.0013 | 1.9% |
| 0.5 | 0.1753 | 0.0032 | 1.8% | 0.1118 | 0.0035 | 3.1% |

The values for k' show a dependence on the concentration of $Mg^{2+}$ in the run buffer. For FMN, k' shows a maximum at 0.2 mM, while k' for FAD increases across all concentrations. The $k_d$ values for the aptamer-FMN and -FAD complexes in

solution are 0.5 and 0.7 μM respectively [5]. The recognition exhibited by the RNA aptamer has been studied through NMR spectroscopy and molecular dynamics simulations, and occurs through a base-pair interaction between an adenine in the RNA aptamer (A26 in Figure 4.1) and the uracil-like edge of the flavin portion of FMN [58, 59]. The molecular dynamics simulation studies also reported additional H-bonding between FMN and the RNA aptamer that occurred through the movement of the phosphate group in FMN towards a guanine present in the aptamer (G27) [59]. Although the molecular dynamics simulation studies took the presence of molecular water associated with FMN and the RNA aptamer into account, the presence of $Mg^{2+}$ was not considered. The NMR studies were conducted in the presence of $Mg^{2+}$. We believe that this may be the cause of the observed dependence of k' on [$Mg^{2+}$] for FMN in our studies. The presence of a high concentration of $Mg^{2+}$ may shield the phosphate group of FMN so that this interaction is lessened, whereas the phosphate groups are more effectively shielded in the FAD molecule. Neither set of studies evaluated the aptamer-FAD complex.

According to equation 4.3 and Figure 4.7B, the maximum and minimum values of k' for FAD should be at 0.1 and 0.4 mM [$Mg^{2+}$] respectively. However, according to Table 4.1, k' for FAD increases with increasing [$Mg^{2+}$] through all concentrations evaluated. This result shows that the variation in electroosmotic flow for modified OTCEC capillaries makes it impossible to evaluate $k_{CEC}$ using this equation. The interaction between the aptamer stationary phase and FAD seems to

affect the value $\mu_{ep}$ in OTCEC experiments (Figure 4.7A), and the same pattern is seen in $\mu_{ep}$ as in k' (Table 4.1).

## 4.5 CONCLUSION

Existing theory has proven to be insufficient when evaluating retention of analytes in OTCEC. This is largely due to the variation of electroosmotic flow invoked by modifying the inner walls of fused silica capillaries. We have presented an alternative method for evaluating the extent of interaction between analytes and the stationary phase in OTCEC, and have extracted k' values for these capillaries under conditions in which EOF has no net effect on migration. Using this method, the affinity exhibited by an immobilized RNA aptamer towards its two target molecules was characterized in different [$Mg^{2+}$] concentrations.

Thus far this work has described the use of aptamer stationary phases in an open-tubular format. Due to the relatively slow diffusion of analytes in liquids, this format does not provide desirable resolution, which is why most liquid chromatography is performed using a packed bed format. In order to accomplish the immobilization of an aptamer stationary phase onto particles, a simpler immobilization method was needed. The immobilization method described in chapters 2 and 3 of this work proved to be tedious with too many steps for use to derivatize a packed bed. Also, the main goal of this project was to generate an aptamer stationary phase in a miniaturized format. Therefore, an immobilization method which was simpler to conduct and easy to miniaturize was needed. The next two chapters describe aptamer

stationary phases based on azlactone immobilization chemistry. This functionality is capable of bonding an amine-modified aptamer. This functionality was used in a packed-bed format and a polymeric format. The porous polymer format is described in chapter 6 and is polymerized *in situ* so that it is suitable for use in "lab-on-a-chip devices.

# 5. Immobilization and Evaluation of an Aptamer Stationary Phase Specific for the Human Protein Thrombin

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## 5.1 ABSTRACT

We report in this study the immobilization of a DNA aptamer onto particles for use as a stationary phase in HPLC, and the evaluation of this stationary phase under different mobile phase conditions. The particles used in this study were an acrylamide/azlactone copolymer, in which the azlactone functionality is used to immobilize the amine-modified DNA aptamer. This DNA aptamer was isolated based on its affinity for the human protein α-thrombin. The resulting stationary phase was capable of selectively retaining α-thrombin. The retention of α-thrombin was explored under different mobile phase conditions. The effect of ionic strength and the presence of monovalent and divalent cations were evaluated. Although it is known that the presence of monovalent and divalent cations have a stabilizing effect on the DNA aptamer, the presence of the cations decreased the retention of the protein. We postulate that this could be due to interference of the cations with ion association between the negatively-charged phosphate groups of the DNA aptamer with positively-charged regions of the protein. Since ion association occurs between many aptamers and their targets, this could be a means to optimize and exert greater control over these interactions.

## 5.2 INTRODUCTION

Affinity chromatography is a separation technique which relies on highly specific interactions to yield analyte retention. Traditionally, the interaction between antibodies and antigens has been most commonly utilized in affinity separations. The

main disadvantages of the use of antibodies as stationary phases are their large size, limited stability in various chemical environments, and the limited range of molecules against which antibodies have been raised. The relatively large size of antibodies prevents high surface loading onto stationary phase supports, and also promotes complicated three-dimensional structures. These complex structures are easily degraded and nearly impossible to retrieve once they are lost. This makes the resulting stationary phases extremely sensitive to mobile phase conditions. For these reasons, other molecules capable of selective recognition continue to be explored for use in affinity chromatography.

Aptamers are oligonucleotides which are capable of specific recognition of small molecules or larger molecules such as proteins. Aptamers are produced and isolated by *systematic evolution of ligands by exponential enrichment* (SELEX). This technique isolates and amplifies oligonucleotides based on their recognition of a target molecule and results in the production of some oligonucleotide sequences that bind to a target with high specificity: aptamers [1, 2]. These molecules can be synthesized in a relatively short period of time, tend to be smaller in size than antibodies, and theoretically can be produced to bind any target molecule. In addition, they have been shown to exhibit affinities which rival those of antibodies [16], and have been generated to recognize a large variety of target molecules [47]. These characteristics make aptamers quite viable for use in techniques relying on molecular recognition. They have been used for their ability to selectively bind their target molecule(s) in flow cytometry [22, 23], sensors and biosensors [24, 25, 50, 51], affinity

chromatography [29, 30, 52, 60], affinity capillary electrophoresis [61], affinity capillary electrochromatography [62], and in the separation of enantiomers [63, 64].

In this work, we studied a DNA aptamer isolated and amplified based on its affinity for the human protein α-thrombin [5]. α-thrombin plays an important role in blood coagulation by converting fibrinogen to fibrin. This leads to the formation of a fibrin clot. The 15-base DNA aptamer probe we used was also found to inhibit the cleavage of fibrinogen by thrombin *in vitro* [5]. The three-dimensional structure of this DNA aptamer (Figure 5.1) relies on the formation of G-quartets, and this structure has been shown to have a dependence on the presence of monovalent and/or divalent cations. The cation found most effective at promoting this G-quartet structure is the $K^+$ ion [65].

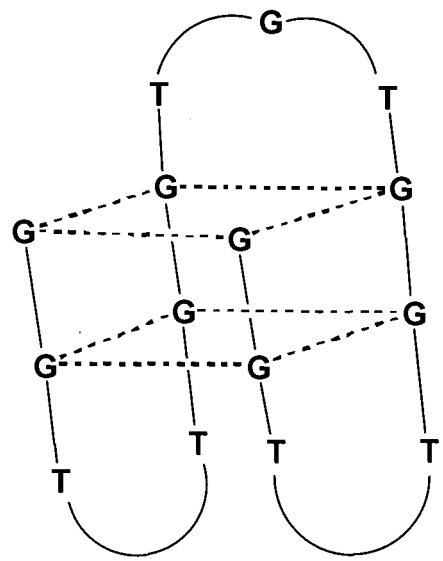


**Figure 5.1.** The DNA aptamer containing two G-quartets.

In order to study the interaction of the DNA aptamer and thrombin, and to explore the dependence of this interaction on the presence of monovalent and divalent cations, we immobilized the DNA aptamer onto bis-acrylamide/azlactone particles. These particles were developed by 3M Company, and have been used for the immobilization of proteins for affinity chromatography [66]. The azlactone functionalities on these particles readily react with nucleophiles, and their chemistry has been reviewed [67]. Studies using aptamers as stationary phases immobilized onto particles, up to now, have used streptavidin-linked particles onto which biotin-modified aptamers were immobilized [29, 30, 60, 63, 64]. The immobilization method presented in this work is a simple one-step method which resulted in an aptamer

stationary phase. The reaction of the azlactone functionality and the amine-modified aptamer is shown in Figure 5.2. Using this stationary phase, the chromatographic retention factor (k') was characterized for the elution of thrombin in different concentrations of the monovalent cations $K^+$ and $Na^+$; as well as the divalent cations $Mg^{2+}$ and $Ca^{2+}$. These cations were components of the buffer used to select this DNA aptamer using SELEX [5]. The aptamer-thrombin complex was found to be affected by the concentration of these cations; however, the greatest effect on the interaction proved to be the ionic strength of the mobile phase. This supports the argument in favor of an ion-association model for retention.

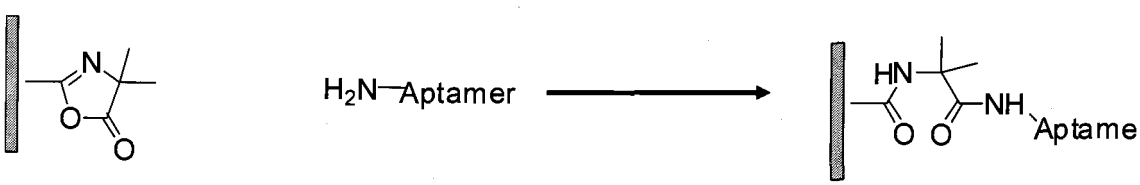


**Figure 5.2.** Immobilization of the amine-modified DNA aptamer onto the stationary phase.

## 5.3 EXPERIMENTAL SECTION

### *5.3.1 Materials*

Tris(hydroxymethyl)aminomethane (tris), sodium phosphate, calcium chloride, ethanolamine, and bovine hemoglobin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Magnesium chloride and potassium chloride were purchased from Mallinckrodt (Paris, KY, USA). Sodium chloride was purchased from Fisher (Fair Lawn, NJ, USA). The 15-base 5' C6 amine-modified DNA aptamer (5'-amine-C6-GGTTGGTGTGGTTGG-3') was purchased from Oligos Etc., Inc. (Wilsonville, OR, USA). Human α-thrombin was purchased from Haematologic Technologies, Inc. (Essex Junction, VT, USA). The bis-acrylamide/azlactone particles were a gift from 3M Company (Minneapolis, MN, USA). All chemicals were used as received.

Mobile phases were made by dissolving the proper concentration of tris salt and adjusting the pH by the addition of acetic acid. Any metal ions present in the buffer were then added at the proper concentration. In order to study the effect of ionic strength on the retention of α-thrombin, the selection buffer used to select the DNA aptamer (20 mM tris/acetate at pH 7.5, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$) was diluted by the reported ratio with distilled, deionized water. The effect of the presence of cations on the retention of α-thrombin was studied by varying the concentration of the cation under study and keeping all other concentrations constant at the dilution of mobile phase which was found to baseline resolve α-thrombin from $t_0$. A 1:1 dilution of the selection buffer was found to result in baseline resolution. This dilution corresponds to the following individual

component concentrations: 10 mM tris/acetate (pH 7.5), 70 mM NaCl, 2.5 mM KCl, 0.5 mM $MgCl_2$, and 0.5 mM $CaCl_2$.

*5.3.2 Stationary Phase Preparation*

The 15-base DNA aptamer was dissolved in 50 mM sodium phosphate (pH 8.0) at a concentration of 10 mg/mL (2.08 mM). The aptamer was allowed to react with the bis-acrylamide/azlactone particles by adding one mL of the above aptamer stock solution to 10 mg of particles. The reaction between the azlactone functionality of the particles and the modified DNA aptamer is shown in Figure 5.2. The mixture was placed in a shaker and allowed to react for 5 hours. The particles were removed from the aptamer solution by centrifugation, and washed several times with 50 mM phosphate (pH 8.0). After the washings, 1 mL of 3 M ethanolamine was added to the particles to quench the reaction and to cover any exposed active sites. After 2 hours, the particles were removed from ethanolamine, and washed several times with 20 mM tris/acetate (pH 7.5). Approximately 20 mg of particles were placed in 1mL of 20 mM tris/acetate. The particles were then packed into a stainless steel HPLC column 10 cm in length and 1 mm in inner diameter using an ISCO (Lincoln, NE, USA) 100 DX syringe pump.

*5.3.3 Chromatography*

All chromatography experiments were completed on an HPLC consisting of a Hitachi (Tokyo, Japan) L-6200 Intelligent pump equipped with an L-4000 UV detector. Injection was 5 μL using a Rheodyne (Rohnert Park, CA, USA) injection valve. Data was collected using PowerChrom (ADInstruments Colorado Springs, CO, USA). All data was collected at a wavelength of 214 nm. All samples were in 20 mM tris/acetate (pH 7.5) and the volumetric flow rate was set to 0.100 mL/min. The stationary phase was evaluated by comparing the retention factor (k') of α-thrombin in different mobile phase conditions. The k' was calculated using the following equation:

$$k' = \frac{t_r - t_0}{t_0} \qquad (1)$$

where $t_r$ is the retention time of the retained analyte, and $t_0$ is the retention time of an unretained analyte.

## 5.4 RESULTS/DISCUSSION

The DNA aptamer was immobilized on acrylamide/azlactone particles. The azlactone group is responsible for bonding the amine group of the modified DNA which is shown in Figure 5.2. The immobilization process proved to be fast and simple. The need for large linker molecules, as are present in streptavidin-linked particles, is bypassed. The resulting stationary phase was capable of selectively retaining α-thrombin.

The protein α-thrombin was found to be retained well beyond $t_0$ in a mobile phase consisting of 10 mM tris/acetate (pH 7.5), 70 mM NaCl, 2.5 mM KCl, 0.5 mM $MgCl_2$ and 0.5 mM $CaCl_2$. The protein is stored in 1:1 (v/v) glycerol/$H_2O$. In order to verify that the glycerol eluted in the void volume, a blank consisting of the same dilution of a 1:1 (v/v) glycerol/$H_2O$ solution as the stock solution of α-thrombin was injected onto the column. Figure 5.3 shows the chromatograms of the blank, thrombin, and hemoglobin; which clearly shows that α-thrombin is the only analyte retained on the aptamer stationary phase.

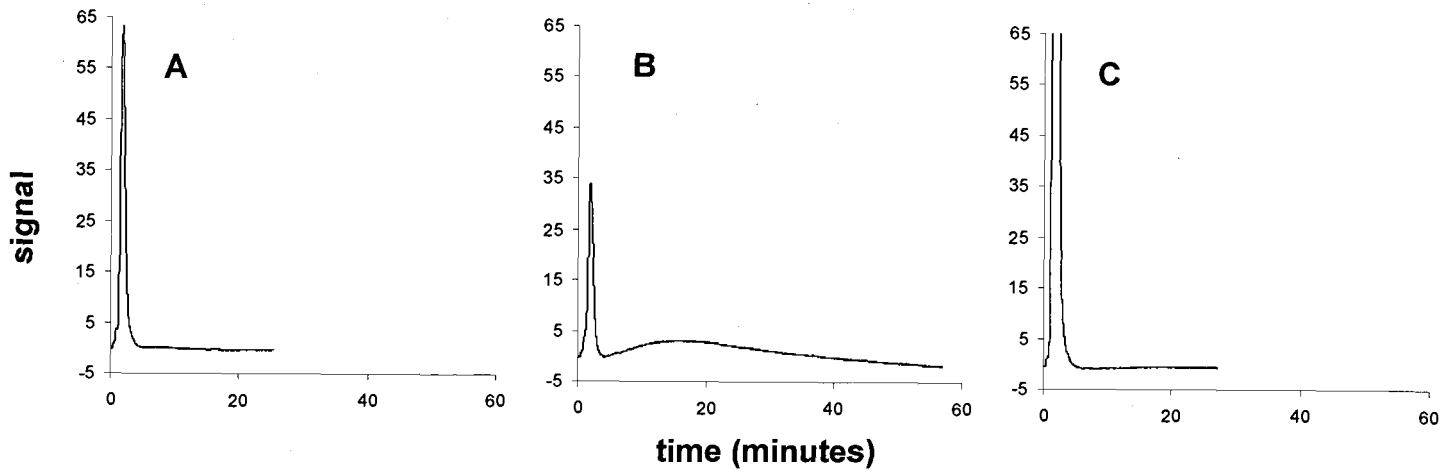


**Figure 5.3.** The retention of α-thrombin on the aptamer stationary phase. A. A blank consisting of glycerol in 20 mM tris/acetate, pH 7.5. B. A dilution of α-thrombin (0.2 mg/mL concentration) from a stock solution which consists of thrombin in a 1:1 mixture (v/v) of glycerol and water. C. A sample containing bovine hemoglobin (0.2 mg/mL).

*5.4.1 Monovalent and Divalent Cations*

The monovalent and divalent cations presented in this study were those present in the selection buffer used for the SELEX method: $K^+$, $Na^+$, $Mg^{2+}$, and $Ca^{2+}$. The various concentrations of the ions were adjusted by addition of the chloride salt of each ion. The concentrations of all species not under study were kept constant at the concentration which yielded retention of α-thrombin well beyond the void volume, as reported above.

In our studies, we found that in the presence of either $K^+$ or $Na^+$, k' is largely decreased. Figure 5.4 shows k' values for α-thrombin as a function of [KCl] (A) and [NaCl] (B). It is clear that both ions have an inhibitory effect on the formation of the aptamer-thrombin complex. When the concentration of $Na^+$ is increased by more than 20 mM, the result is elution of α-thrombin in close proximity to $t_0$, such that the evaluation of k' is impossible. However, the increase in concentration of $K^+$ necessary to demonstrate the same effect is > 50 mM. The presence of NaCl at a concentration less than 60 mM resulted in an increase in the retention of thrombin to the extent that an asymmetric, broad peak was the result. This peak was difficult to detect which made k' impossible to evaluate.

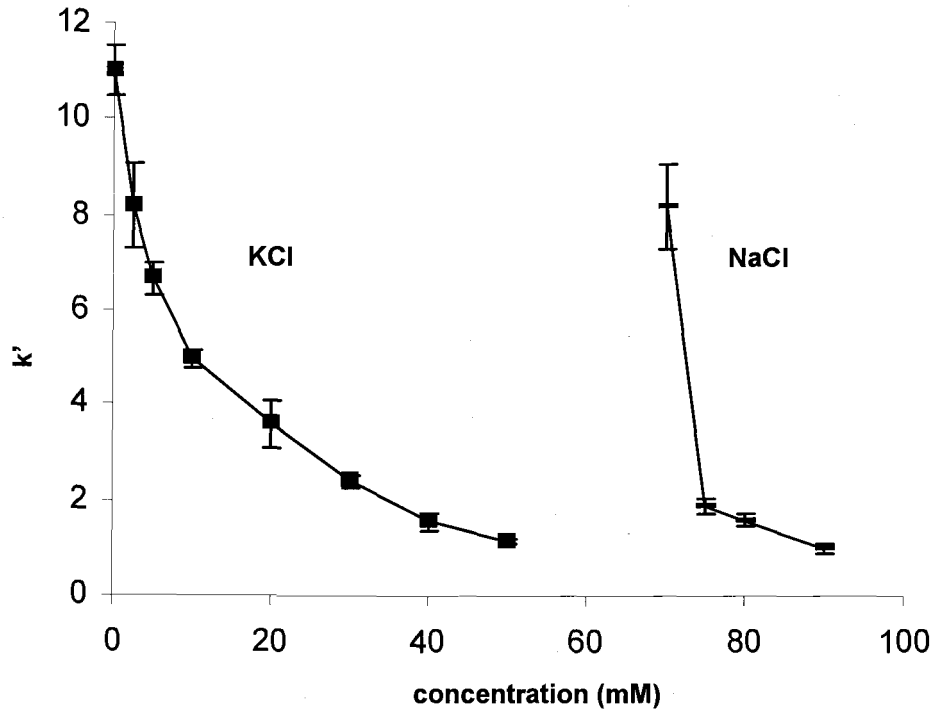


**Figure 5.4.** The effect of monovalent cations on the retention of α-thrombin. Error bars represent 1 standard deviation for $n \geq 3$ runs.

The divalent cations investigated in this study included $Mg^{2+}$ and $Ca^{2+}$. Figure 5.5 shows the resulting k' values when the concentrations of $Mg^{2+}$ and $Ca^{2+}$ were varied. Overall, the effect of each on the retention of α-thrombin was similar. When the concentration of $MgCl_2$ reached 15 mM or greater, thrombin eluted too closely to the void volume to evaluate k'. The effect of $CaCl_2$ was slightly larger, the k' could not be evaluated at a concentration of 10 mM again due to insufficient retention of α-thrombin relative to the void volume.

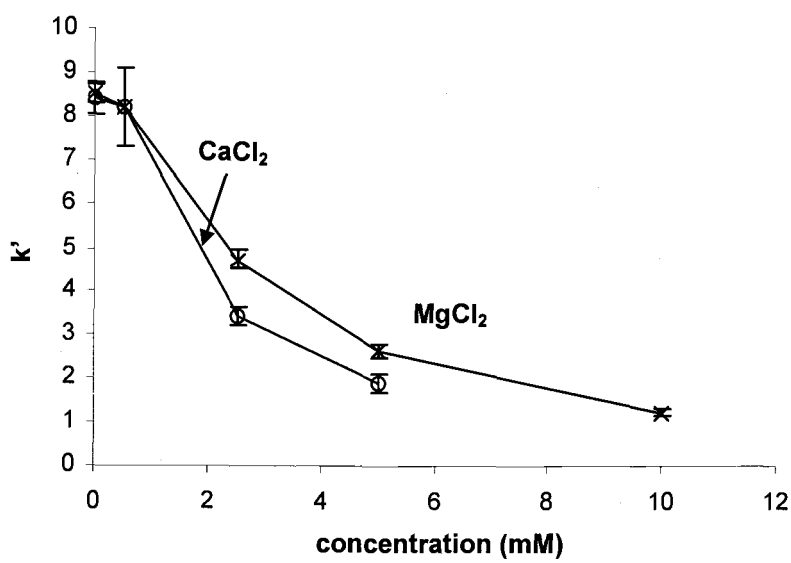


**Figure 5.5.** The effect of divalent cations on the retention of α-thrombin. Error bars represent 1 standard deviation for $n \geq 3$ runs.

*5.4.2 Ionic Strength*

Retention of α-thrombin on the aptamer stationary phase was found be highly dependent upon the ionic strength of the mobile phase. Different eluent ionic strengths were produced by diluting the selection buffer which consisted of 20 mM tris/acetate (pH 7.6), 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$. Figure 5.6 shows the elution of a mixture of hemoglobin and α-thrombin in mobile phases of three different ionic strengths. The first mobile phase shown is the selection buffer, which is also the highest in ionic strength. Using this mobile phase (A), α-thrombin could not be appreciably retained beyond $t_0$. However, a 1:1 volume dilution of this mobile phase (C) resulted in baseline resolution of the protein from $t_0$. Dilutions shown are 1:3 ($H_2O$/selection buffer) (B) and1:1 (C) by volume. A dilution of 3:1 (v/v) resulted in a later elution of α-thrombin, however, the peak was very broad and almost impossible to identify. The salt concentrations of the 1:1 dilution which gave rise to baseline resolution of thrombin are 10 mM tris/acetate (pH 7.5), 70 mM NaCl, 2.5 mM KCl, 0.5 mM $MgCl_2$, and 0.5 mM $CaCl_2$.

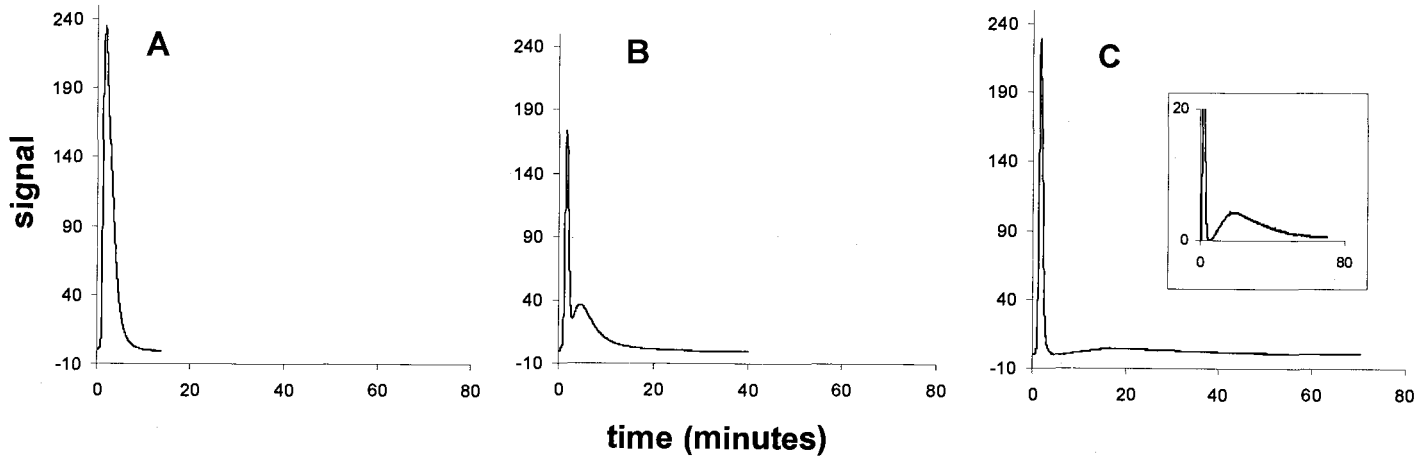


**Figure 5.6.** The effect of ionic strength on the retention of α-thrombin. Samples are a mixture of 0.2 mg/mL each of bovine hemoglobin and α-thrombin. A. Mobile phase is the selection buffer used to select the DNA aptamer using the SELEX method: 20 mM tris/acetate (pH 7.5), 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$. B. The selection buffer was diluted by a ratio of 1:3 v/v ($H_2O$/selection buffer). C. The selection buffer was diluted by 1:1. Inset shows the separation with an expanded y-axis scale so that the α-thrombin peak can be seen more clearly.

In order to more fully understand the effect of ionic strength on the interaction between the aptamer and thrombin, and to separate this effect from the effects of the individual cations on retention, the data resulting from the addition of cations was rearranged as a function of ionic strength. Figure 5.7 shows the data as a function of the change in ionic strength of a 1:1 dilution of the selection buffer (10 mM tris/acetate pH 7.5, 70 mM NaCl, 2.5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$). This representation illustrates the effect of ionic strength and the presence of the various ions on the separation.

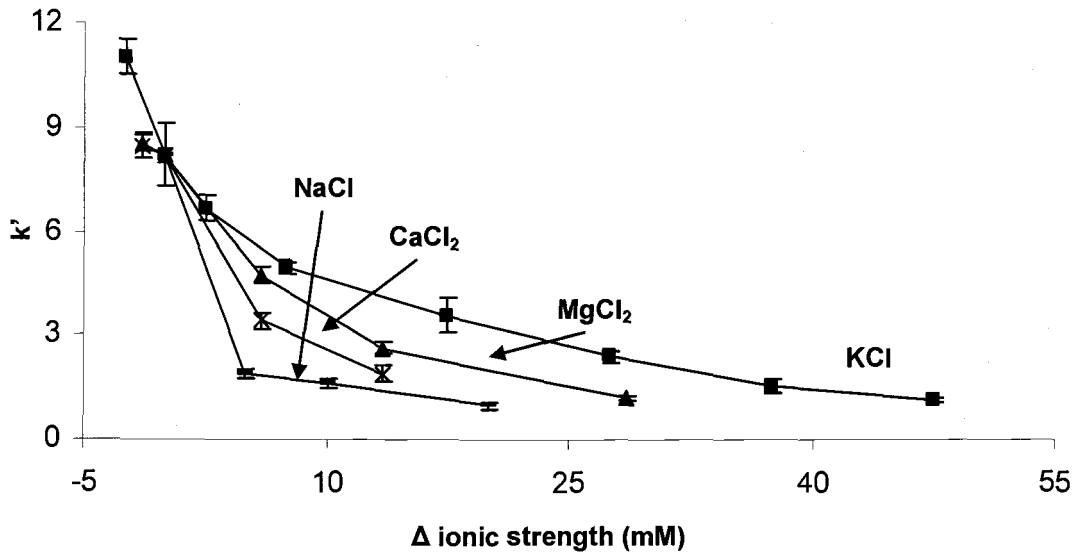


**Figure 5.7.** The effect of each cation is shown as a function of the change in ionic strength produced when the concentration of each cation is added or subtracted from the mobile phase. The change is reported as it relates to the change from 10 mM tris/acetate (pH 7.5), 70 mM NaCl, 2.5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$. Error bars represent one standard deviation for $n \geq 3$ runs.

It has been shown through x-ray crystallography studies that the formation of the DNA aptamer-thrombin complex depends largely upon ionic interactions between the negatively-charged phosphate groups on the aptamer and positively-charged regions present in α-thrombin [68]. All cations studied here proved to have a disruptive effect on this interaction.

There have been studies conducted on the stabilizing effect of cations on the formation of G-quartets within the DNA aptamer. This stabilizing effect has been

attributed to the formation of an intramolecular complex between the DNA aptamer and positively charged ions [65]. The ions associate with the G-quartet structure within the aptamer. The cation with the greatest stabilizing effect is $K^+$; however, $Na^+$, $Mg^{2+}$, and $Ca^{2+}$ are among those that have also been found to stabilize G-quartets. Data from the x-ray crystallography studies [68] suggested that the DNA aptamer was present in its G-quartet form in the aptamer-thrombin complex, but it is unclear whether a cation is present and acting to stabilize these G-quartets. However, it is important to note that this study was conducted with only NaCl present in the mother liquor from which the crystal was grown.

In this study, we found that $K^+$ has the least disruptive influence on the formation of the aptamer-thrombin complex. This may be due to the larger interaction between $K^+$ and the G-quartet in the aptamer molecule relative to its interaction with the negatively charged phosphate groups in the phosphodiester backbone of the aptamer. The presence of $K^+$ has also been found to increase the retention of some nontarget molecules when the DNA aptamer was used as a stationary phase in electrochromatography [27]. Since many aptamer-target interactions may rely at least in part on ionic interactions, we feel that eluent ionic strength could play a major role in regulating these interactions. This could be a useful tool when taking advantage of the molecular recognition capabilities of aptamers in affinity chromatography, as well as in other analytical applications.

## 5.5 CONCLUSION

We have presented an immobilization method for a DNA aptamer which is easily and rapidly implemented. The resulting stationary phase was capable of selectively retaining the target of the DNA aptamer, the human protein α-thrombin. Retention of the target was evaluated under different mobile phase conditions. It was found that the greatest influence on formation of the aptamer-thrombin complex was through eluent ionic strength, with some cations having a larger effect on retention than others. The formation of the aptamer-thrombin complex arises in part through ion association, which occurs between the negatively charged phosphate groups on the DNA molecule and cation regions on the surface of the protein. Although the G-quartet structure of the DNA aptamer is stabilized by the presence of the cations, the cations tend to disrupt the association between the aptamer and its target. This allows control over this interaction, and possibly over many other interactions involving different aptamers and their targets.

The final step of this research project was the casting of a porous polymer in *situ* and its subsequent derivatization with an amine-modified aptamer to produce a miniaturized aptamer stationary phase. Looking back to equation 1.7, it is clear that stationary phases in a miniature format should enjoy an increase in resolution. This is because both the A and $C_m$ term are proportional to the diameter of the packing material. However, in LC separations there is a limit on how small particles can be because of back pressure considerations. This problem has been addressed with the polymerizations of monomers in *situ* which have a large abundance of small pores, so

that the increase in resolution can be realized without the large back pressures. The advantage of this format in this research group has been the recent direction the group has taken in exploring the use of microchips. This format of miniaturized stationary phases is compatible with a microchip separation format.

The next chapter reports the use of an aptamer in a porous polymer monolith stationary phase. This stationary phase is based on the same azlactone functional group for the immobilization of an amine-modified aptamer. The aptamer used was the same 14-base DNA aptamer described in this chapter. The azlactone functionality was present in the form of the monomer 2-vinyl-4,4-dimethylazlactone. The monolithic stationary phase described was capable of the selective retention of α-thrombin. The stationary phase was characterized as to the dissociation constant of the aptamer-thrombin complex ($K_D$), and the number of total active aptamer binding sites ($B_t$) immobilized onto the monolithic stationary phase.

# 6. The Generation of Aptamer Stationary Phases in Packed Bed and Monolithic Formats for Affinity Chromatography

## 6.1 ABSTRACT

We report the generation of aptamer stationary phases in both packed bed and monolithic formats. Both of these chromatographic formats relied on an azlactone functional group on the support, onto which the amine-modified DNA aptamer was anchored. The 15-base DNA aptamer used was isolated based on its affinity for α-thrombin. The affinity the aptamer exhibited for thrombin was characterized under different mobile phase conditions by monitoring the retention factor (k'). It is known that the presence of monovalent and divalent cations stabilize the 3-dimensional structure of the DNA aptamer. We studied the effect these ions exerted on the aptamer-thrombin complex. It was found the greatest effect was due to the ionic strength of the mobile phase. By conducting frontal analysis studies on the monolithic capillaries, we found the dissociation constant ($K_D$) of the aptamer-thrombin complex and the number of active binding sites to be 42 nM and 0.069 μmol of binding sites per milliliter of media, respectively.

## 6.2 INTRODUCTION

In recent years, molecules known as aptamers have been explored for use in affinity separations. These molecules are isolated based on their affinity for a target molecule by a technique known as *systematic evolution of ligands by exponential enrichment* (SELEX) [1, 2]. Traditionally, the most widely applied type of interaction in affinity separations has been antibody-antigen interactions. Though they have proven to be invaluable, these interactions do have several disadvantages including the

large size of antibodies, their limited stability in different solvent systems, and the limited number of targets for antibodies. These limitations have led researchers to explore the applicability of aptamers as stand-ins for antibodies for use in affinity separations. Aptamers have been used in affinity chromatography to retain target molecule(s) in open tubular [62] and packed formats [29, 30, 60, 61]. The use of aptamers in analytical devices to achieve molecular recognition has been reviewed [47].

In recent years, there have been great strides in research to produce *in situ* porous polymer monoliths for use as stationary phases in liquid chromatography. The recent efforts to produce stationary phases in this format came about largely because of the results published by Hjertén et al. [69]. When using cross-linked nonporous agarose for pressure-driven separations, they found that the agarose gel was being compressed, and this resulted in enhanced efficiency and resolution of their separation. Unfortunately, back pressures eventually reached such high levels that the columns were useless. This led researchers to focus on finding a medium which was highly porous, with small, controlled pore diameter. This has been accomplished by *in situ* polymerization of monomers inside capillary tubing [70]. This format has also been found to be compatible for use in micro total analytical systems (μTAS) or microchips [71].

The azlactone functionality as an anchor for affinity sorbents was introduced as bis-acrylamide/azlactone copolymer beads by 3M company. The azlactone functionality readily reacts with the thiol group of proteins to produce affinity sorbents

[66]. In order to create packed bed columns containing the DNA aptamer, we used these beads as the stationary phase support. The monomer 2-vinyl-4,4-dimethylazlactone has been used to generate porous polymers for use as amine scavengers[i] and as a support for the immobilization of trypsin to produce enzymatic microreactors capable of protein digestion [73, 74]. We used this monomer as a component of a monolith to provide a means of functionalization of the monomer with an amine-modified aptamer.

In this study we prepared a porous polymer by the polymerization of the monomers 2-vinyl-4,4-dimethylazlactone, 2-hydroxyethyl methacrylate, and ethylene glycol dimethacrylate in the presence of the initiator 2,2-dimethoxy-2-phenylacetophenone, and decanol as a porogenic solvent *in situ* in fused silica capillary tubing. The polymer was subsequently derivatized with the aforementioned DNA aptamer (isolated based on its ability to recognize α-thrombin) [5]. The resulting stationary phases were found to be capable of the selective retention of α-thrombin. The retention of α-thrombin was explored under different mobile phase conditions by monitoring the chromatographic retention factor, k'. The dissociation constant, $K_D$, of the aptamer-thrombin complex, and also the number of active binding sites were experimentally determined by frontal analysis. When blank capillaries were prepared by immobilization of ethanolamine only onto the azlactone-containing monolith, no retention of α-thrombin was observed.

## 6.3 EXPERIMENTAL SECTION

### *6.3.1 Materials*

Tris(hydroxymethyl)aminomethane (tris), sodium phosphate, calcium chloride, ethanolamine, ethylene glycol dimethacrylate, decanol, 2,2-dimethoxy-2-phenlacetophenone, and bovine hemoglobin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Magnesium chloride and potassium chloride were purchased from Mallinckrodt (Paris, KY, USA). Sodium chloride and glacial acetic acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA). 3-(trimethoxysilyl)-propyl methacrylate and 2-hydroyethyl methacrylate were purchased from Acros Organics (Fair Lawn, NJ, USA) The 15-base 5' C6 amine-modified DNA aptamer (5'-amine-C6-GGTTGGTGTGGTTGG-3') was purchased from Prestige DNA (Woburn, MA, USA). Human α-thrombin was purchased from Haematologic Technologies, Inc. (Essex Junction, VT, USA). The bis-acrylamide/azlactone particles and the monomer 2-vinyl-4,4-dimethylazlactone were a gift from 3M Company (Minneapolis, MN, USA). Teflon-coated fused silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA).

Mobile phases were prepared by dissolving the reported concentration of tris salt and adjusting the pH by the addition of acetic acid. Metal ions present were added at the reported concentration by addition of the chloride salt of each metal. For the ionic strength studies, the buffer used to isolate the DNA aptamer using SELEX (20 mM tris/acetate, pH 7.5; 140 mM NaCl; 5 mM KCl; 1 mM $MgCl_2$; and 1 mM $CaCl_2$) was diluted at the reported ratios. For the monovalent and divalent cation

concentration studies the packed columns were used. In order to conduct these studies, the mobile phase conditions under which α-thrombin was baseline resolved from $t_0$ were established (10 mM tris/acetate, pH 7.5; 70 mM NaCl; 2.5 mM KCl; 0.5 mM $MgCl_2$; 0.5 mM $CaCl_2$). During the study, the concentration of the cation under study was added or subtracted from the mobile phase composition above.

*6.3.2 Packed Bed Stationary Phase Preparation*

The DNA aptamer was dissolved in 50 mM phosphate buffer (pH 8.0) at a concentration of 10 mg/mL (2 mM). 10 mg of the bis-acrylamide/azlactone particles were then added to 1 mL of solution. The particles were reacted with the aptamer for 5 hours while in a shaker, then were removed from solution by centrifugation. They were washed at least 3 times with 50 mM phosphate buffer at pH 8. In order to mask any exposed reactive sites, the particles were reacted with 3 M ethanolamine for 2 hours. The particles were then washed with several volumes of 20 mM tris/acetate (pH 7.5), and packed into an HPLC column 10 cm in length and 1 mm in inner diameter using a concentration of approximately 20 mg of particles per 1 mL of 20 mM tris/acetate. Packing was accomplished using an ISCO (Lincoln, NE, USA) 100 DX syringe pump. The initial pressure applied was 50 psi. In the final stage of the packing procedure, 200 psi was applied until the column was completely packed.

*6.3.3 Monolithic Stationary Phase Preparation*

The inner walls of the Teflon-coated fused silica capillaries were first vinylized according to a published procedure [75]. This step was necessary to attach the monoliths to the inner walls of the capillaries. The monomers were placed in a solution with 1-decanol (as the porogenic solvent) and 2,2-dimethoxy-2-phenylacetophenone (the initiator) at the following concentrations in weight %: ethylene glycol dimethacrylate at 15%, 2-hydroxyethyl methacrylate at 9%, 2-vinyl-4,4-dimethylazlactone at 6%, 1-decanol at 70%, and the initiator 2,2-dimethoxy-2-phenylacetophenone at 1% with respect to the monomers. Teflon-coated fused silica capillaries with an inner diameter of 75 μm were filled with the mixture and exposed to UV light for 6 minutes using a long wave Spectroline (Westbury, NY, USA) X-15A UV lamp (output 330 to 380 nm). In order to create a detection window on-line, a short segment of each capillary was masked with black tape during the UV exposure period. After removal of the capillaries from UV light, they were flushed with methanol for at least 10 hours to remove the porogenic solvent and residual monomer solution. Detection windows were created by burning the teflon coating of the capillaries using a Fujikura (Futtsu, Japan) arc fusion splicer in an open section of the tubing adjacent to the monolith.

After the polymerization process, the capillaries were derivatized with the 15-base DNA aptamer dissolved in 50 mM sodium phosphate (pH 8.0) at a concentration of 10 mg/mL (2 mM). The capillary was first filled with the aptamer solution, then flushed at a volumetric flow rate of 0.1 μL/min for 5 hours. This was accomplished by filling

a 30 μL injection loop with the DNA aptamer solution, then placing the injection valve in the "inject" position. The injection loop was refilled several times during the derivatization process. The capillary was then flushed with 3 M ethanolamine at the same flow (0.1 μL/min) for 3 hours. Figure 6.1 depicts the immobilization reaction and the resulting monolithic sorbent. This structure is identical to that on the azlactone beads.

Blank capillaries were created in packed and monolithic formats by reaction of the azlactone-containing support with ethanolamine, omitting immobilization of the aptamer. All other experimental parameters were identical to those reported above.

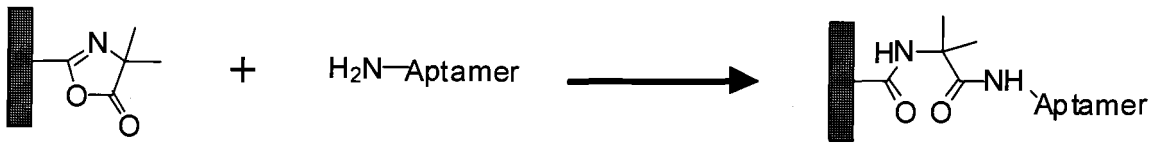


**Figure 6.1.** The reaction between the azlactone functionality and the amine-modified DNA aptamer. The azlactone functionality is present on the surface of the bis-acrylamide/azlactone beads and on the surface of the pores in the monolith.

*6.3.4 Chromatography Using Packed Columns*

Chromatography on packed HPLC columns was accomplished using a Hitachi (Tokyo, Japan) HPLC system which consisted of an L-6200 Intelligent pump with an L-4000 UV detector. Injection was accomplished using a Rheodyne (Rohnert Park, CA, USA) equipped with a 5 μL sample loop. Data was collected at 214 nm using PowerChrom (ADInstruments, Colorado Springs, CO, USA). All samples were dissolved in 20 mM tris/acetate, pH 7.5. Volumetric flow rate for all studies was set to 0.100 mL/min. The stationary phase was evaluated by comparing the retention factor (k') of α-thrombin in different mobile phase compositions using the following equation:

$$k' = \left( \frac{t_r - t_0}{t_0} \right) \qquad (6.1)$$

where $t_r$ is the retention time of the retained analyte, and $t_0$ is the retention time of an unretained analyte.

### *6.3.5 Chromatography Using Monolithic Capillaries*

All capillary chromatography was performed using an Agilent (Newport, DE, USA) 1100 series capillary pump. An Upchurch (Oak Harbor, WA, USA) nanopeak micro valve was used with an injection loop of 50 nL which was held in the inject position for 5 seconds unless otherwise stated. For frontal chromatography studies, the same valve was used equipped with an injection loop of 20 μL, and was held in the inject position for the entire chromatography experiment. Volumetric flow rate was 0.3 μL/min in all studies. Detection was accomplished using the onboard diode array detector of an $HP^{3D}$ CE (Agilent, Newport, DE, USA).

## 6.4 RESULTS/DISCUSSION

Monoliths have been created using the azlactone monomer as an anchoring group in order to anchor trypsin for the creation of enzyme microreactors. It was found that the median pore size increased rapidly when the concentration of porogenic solvent exceeded 55% [74]. In the same study, it was found that 60% decanol yields a porous monolith (49.9% total pore volume), and that polymerization was complete in around 6 minutes. Considering this study, we originally created porous polymers using a monomer mixture containing 60% (wt. %) porogenic solvent. However, we found that under these conditions, the resulting monoliths yielded high back pressures

which exceeded the pressure limitations of our experimental set-up. The monoliths created using 70% porogen concentration proved to be appropriate for use in our studies. SEM images of these monoliths (Figure 6.2) show that they are homogeneous and porous.

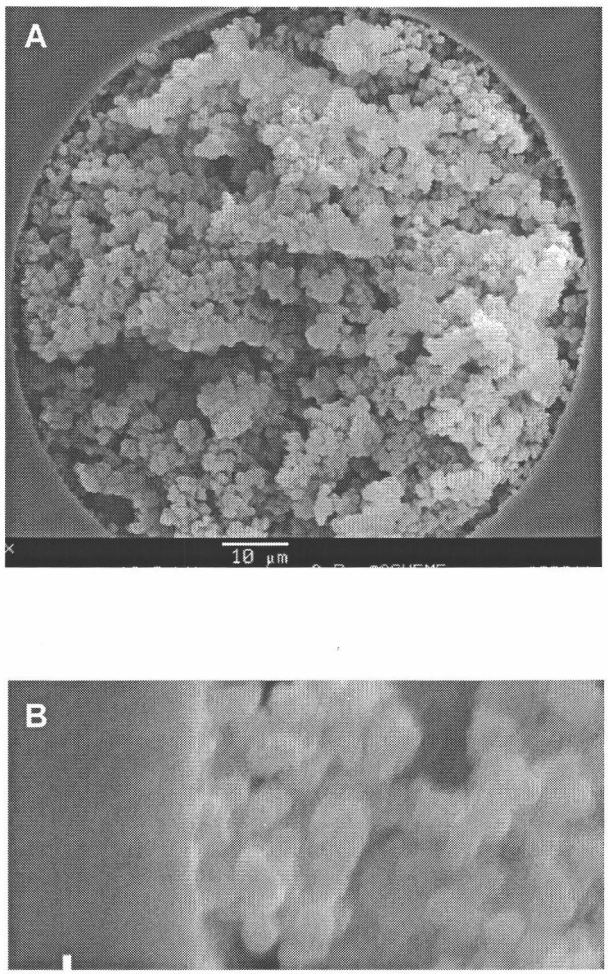


**Figure 6.2.** SEM images of the monolithic stationary phase. The entire capillary (A) is shown at 1000X magnification and the monolith is shown at the capillary wall (B) at 10,000X magnification.

In an earlier publication in which we studied the effect of mobile phase composition on the retention of α-thrombin in the packed bed format, we demonstrated that the protein hemoglobin and glycerol (in which the protein is stored) both eluted in the void volume of the column [76]. In order to make certain there was no retention of either species on the monolithic aptamer stationary phase, we performed the same experiment. Figure 6.3 shows the chromatograms of glycerol, hemoglobin, and a mixture of hemoglobin and thrombin. The glycerol results in a peak at $t_0$ which looked identical to the result when a blank consisting of 20 mM tris/acetate (pH 7.5) was injected using the same mobile phase. The mobile phase consisted of the selection buffer used to select the DNA aptamer using SELEX (20 mM tris/acetate, pH 7.5; 140 mM NaCl; 5 mM KCl; 1 mM $MgCl_2$; and 1 mM $CaCl_2$) . It is clear that only the thrombin is retained on the aptamer stationary phase in the monolithic format. When the same experiment was conducted on blank capillaries (prepared by reacting ethanolamine with the azlactone functionality, and eliminating the immobilization of aptamer), we observed no separation of hemoglobin and α-thrombin – both eluted in the void volume. There was some tailing associated with the aptamer stationary phase. Tailing on these phases has been attributed to slow kinetics of aptamer-target association/dissociation [63]. We believe the tailing seen on this stationary phase is due to this factor as well as the properties of the polymer itself, since we saw a smaller amount of tailing on the blank capillaries.

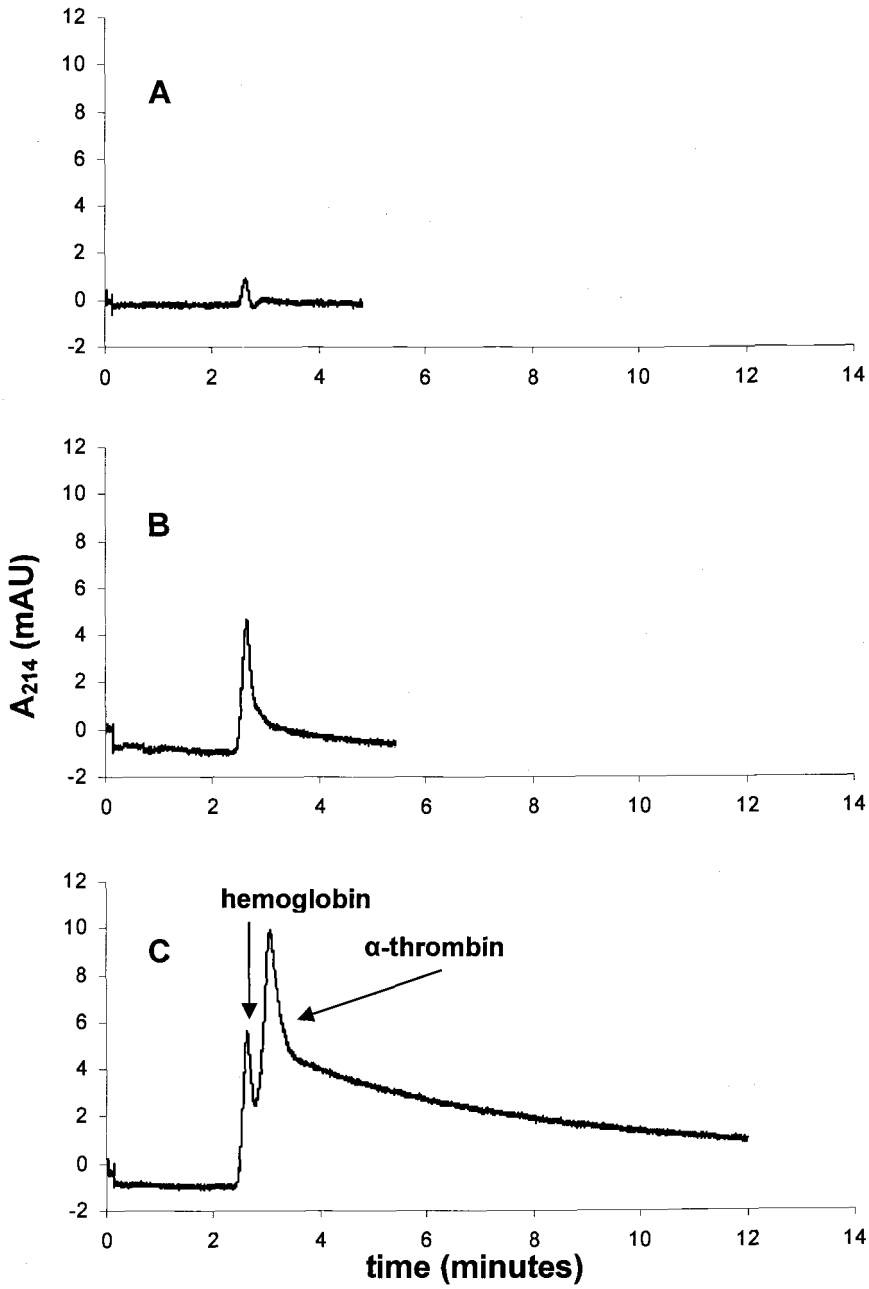


**Figure 6.3.** Chromatograms of glycerol (A), hemoglobin (B), and a mixture of hemoglobin and α-thrombin (C) on the monolithic aptamer stationary phase. Concentrations of both hemoglobin and α-thrombin were 0.2 mg/mL, and mobile phase is the selection buffer used to select the DNA aptamer. All other experimental conditions stated in the experimental section.

*6.4.1 Monovalent and Divalent Cations*

The 3-D structure of the DNA aptamer used in this investigation is known to contain two G-quartets. The G-quartet structure of the aptamer is stabilized by the presence of monovalent and divalent cations, with $K^+$ providing the greatest stabilizing effect [65]. We chose to study the effect of monovalent and divalent cations on aptamer-thrombin complexation. Surprisingly, we found that the presence of cations has an unfavorable effect on the complex. We varied the concentrations of $Na^+$, $K^+$, $Mg^{2+}$, and $Ca^{2+}$ (the cations present in the buffer used to select the DNA aptamer), while we monitored k' of α-thrombin. The results of this study are shown in Figure 6.4. The change in mobile phase conditions is reported as the change from the mobile phase composition which resulted in baseline resolution of α-thrombin from $t_0$. In order to understand these results we concentrated on an x-ray crystallography study of the aptamer-thrombin complex [68]. This study did not find evidence of the presence of a cation in the aptamer-thrombin complex. The study also found evidence of a change in the aptamer's conformation when it is bound to a cation versus when it is bound to thrombin. It is important to note that the only cation present in the x-ray crystallographic study was $Na^+$.

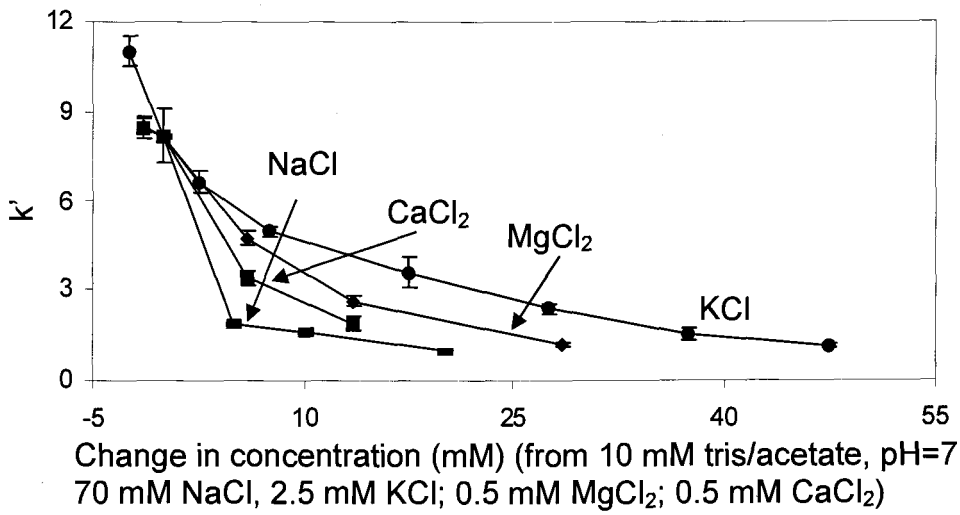


**Figure 6.4.** The effect of ionic strength and individual cations on the retention of α-thrombin on the aptamer stationary phase. The study was conducted on the packed bed stationary phase. Concentrations of α-thrombin and hemoglobin in the sample were 0.2 mg/mL. Error bars represent one standard deviation for $n \geq 3$ runs.

*6.4.2 Ionic Strength*

The effect of eluent ionic strength was studied in both the packed bed and monolithic stationary phases by diluting the selection buffer by the ratios reported in Figure 6.5. While the achievable resolution was different for the two sets of columns, the same trend was seen: increased resolution with decreased cation concentration. Baseline resolution was achieved for the packed columns at a ratio of 1:1, while it was achieved in the monolith capillaries at a ratio of 1:3 (selection buffer:$H_2O$). This is

most likely because of differing amounts of immobilized aptamer on the two different support media. Figure 6.5 shows the resulting chromatograms on both the monolithic stationary phase (left) and the packed bed stationary phase (right) in selection buffer (A), and 1:1 (selection buffer:$H_2O$) (B), and 1:3 (selection buffer:$H_2O$) (C) . Using the mobile phase composition seen in C on the packed bed column resulted in an extremely broad α-thrombin peak which was nearly impossible to detect. It is clear from the data that decreasing the ionic strength results in an enhancement in the interaction between the two molecules. Given the results of the cation study presented above, this conclusion was not unexpected.

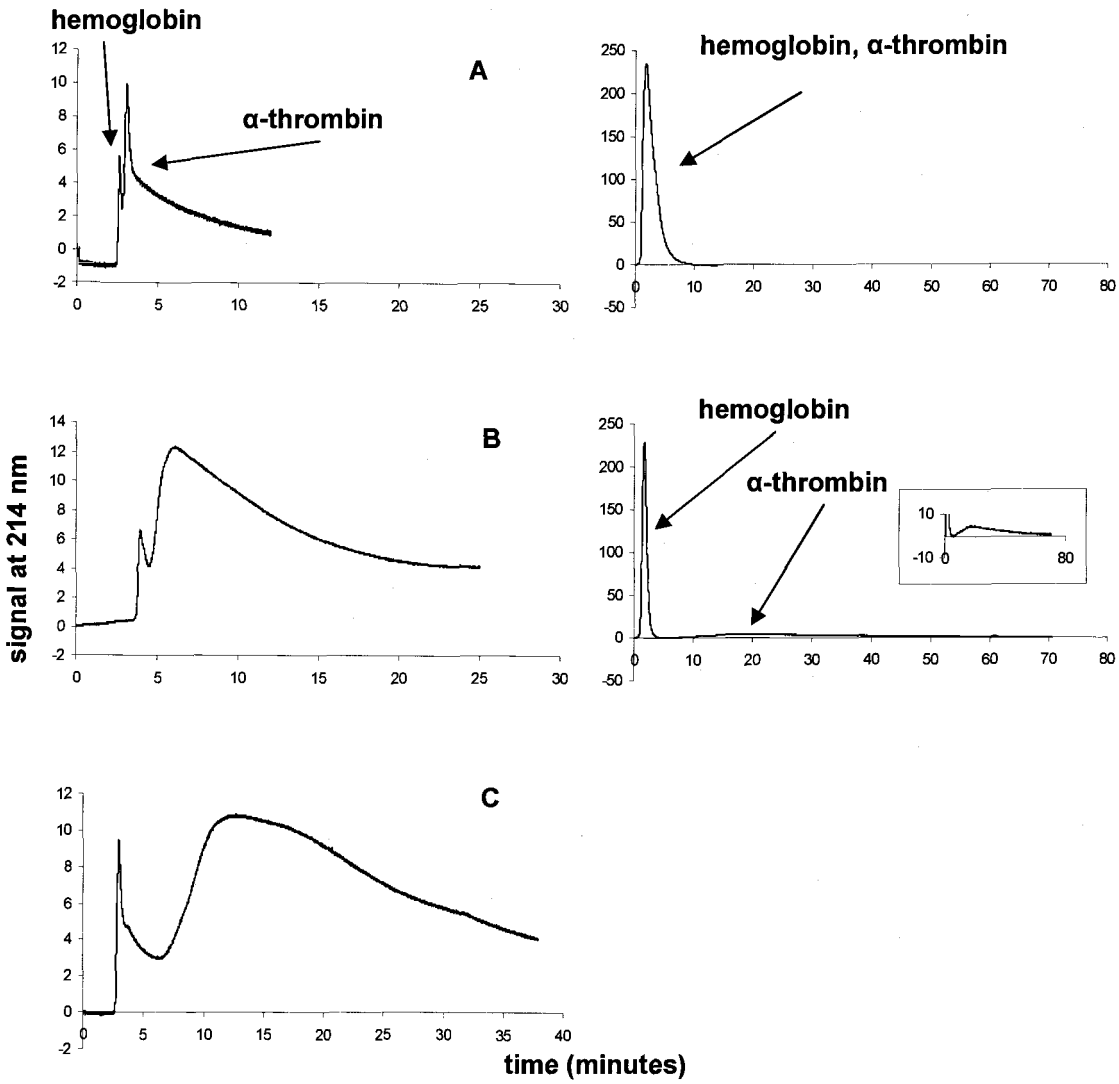


**Figure 6.5.** Comparison of the aptamer stationary phase in monolithic and packed bed formats. Mobile phase compositions were the selection buffer used to select the DNA aptamer using SELEX (20 mM tris/acetate, pH 7.5, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$) (A); a 1:1 dilution of the selection buffer (10 mM tris acetate, pH 7.5, 70 mM NaCl, 2.5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$) (B); and a 3:1 dilution (6.7 mM tris/acetate, pH 7.5, 47 mM NaCl, 1.7 mM KCl, 0.3 mM $MgCl_2$, and 0.3 mM $CaCl_2$) (C). Monolithic stationary phase is on the left and packed bed stationary phase is on the right. The mobile phase conditions of part C when applied to packed bed format resulted in a very broad α-thrombin peak which was almost impossible to detect, and is not shown.

*6.4.3 Characterizing the Monolithic Stationary Phase*

Frontal chromatography was conducted on the monolithic aptamer sorbent in order to characterize the sorbent. The dissociation constant ($K_D$) of the aptamer-thrombin complex and the number of active binding sites ($B_t$) were both determined. Unfortunately, we were unable to similarly characterize the packed bed sorbents due to the limited detection sensitivity of our experimental set-up. The $K_D$ study was conducted in a mobile phase of 1:3 (selection buffer:$H_2O$) since this was the mobile phase which gave the best resolution of α-thrombin from $t_0$ while still enabling easy detection of the α-thrombin peak.

Frontal chromatography has previously been used in order to extract the above experimental values in an affinity chromatography experiment [77, 78]. Parameters in frontal chromatography are related to $K_D$ parameters according to the following equation:

$$K_D = \frac{[A][B]}{[AB]} = \frac{[A]_0\{[B]_0 - [A]_0(V - V_0)/\upsilon\}}{[A]_0(V - V_0)/\upsilon} = \frac{B_t}{V - V_0} - [A]_0 \qquad (6.2)$$

where [A] is the concentration of free target (α-thrombin), [B] is the concentration of free aptamer, [AB] is the concentration of aptamer-thrombin complex, $[A]_0$ is the concentration of continuously applied thrombin that requires a volume of V to elute from the capillary, $[B]_0$ is the concentration of active aptamer anchored onto the monolithic support, $V_0$ is the volume necessary to elute a molecule unretained by the stationary phase, υ is the volume of the monolithic bed, and $B_t$ is the number of active

binding sites of the aptamer immobilized on the stationary phase. Equation 6.2 can be rearranged to:

$$\frac{1}{[A]_0(V-V_0)} = \frac{K_D}{B_t}\frac{1}{[A]_0} + \frac{1}{B_t} \qquad (6.3)$$

Equation 3 yields a linear relationship when $1/\{[A]_0(V\text{-}V_0)\}$ is plotted against $1/[A]_0$ with the slope equal to $K_D/B_t$ and the y-intercept equal to $1/B_t$. The value V is taken as the volume required for the concentration to be equal to $[A]_0/2$.

Figure 6.6 shows an example frontal elution chromatogram and the linear plot which yields $K_D$ and $B_t$ information. We found $K_D$ of the aptamer-thrombin complex under our experimental conditions to be 41 nM. This is close to half of the published dissociation constant [79]. The published $K_D$ was experimentally determined in the selection buffer used to select the DNA aptamer (20 mM tris/acetate, pH 7.5; 140 mM NaCl; 5 mM KCl; 1 mM $MgCl_2$, 1 mM $CaCl_2$). We postulate that if any cations are released upon the binding of the aptamer to α-thrombin, one would expect a shift in the $K_D$ value when the concentration of cations is varied. Our results support this since we realized an increase in k' when the individual concentrations of the cations $Na^+$, $K^+$, $Mg^{2+}$, and $Ca^{2+}$ were decreased. The decrease in $K_D$ varies with ion concentration according to equation 6.4 [80]:

$$\frac{d(\ln K)}{d(a_x)} = \Delta\upsilon_x - \frac{pm_x}{55.6}(\Delta\upsilon_w) \qquad (6.4)$$

where K is the equilibrium constant, $a_x$ is the mean activity, $\Delta\upsilon_x$ is the number of salt ions displaced or bound during binding, and $\Delta\upsilon_w$ is the number of water molecules displaced or bound. We found the total number of binding sites ($B_t$) immobilized on

the monolithic support to be 0.69 pmol. Using the volume of the empty capillary and the dead volume reported for the injection valve to calculate the volume of the monolith present inside the capillary, this corresponds to approximately 0.035 μmol per 1 mL of media. This is close to 20 fold smaller than the loading capacity of protein A onto the bis-acrylamide/azlactone particles reported by 3M company, though this is not unexpected due to the difference in surface area of the two formats. Polymerization conditions and immobilization conditions could be further optimized in order to maximize the loading capacity of the monolithic support.

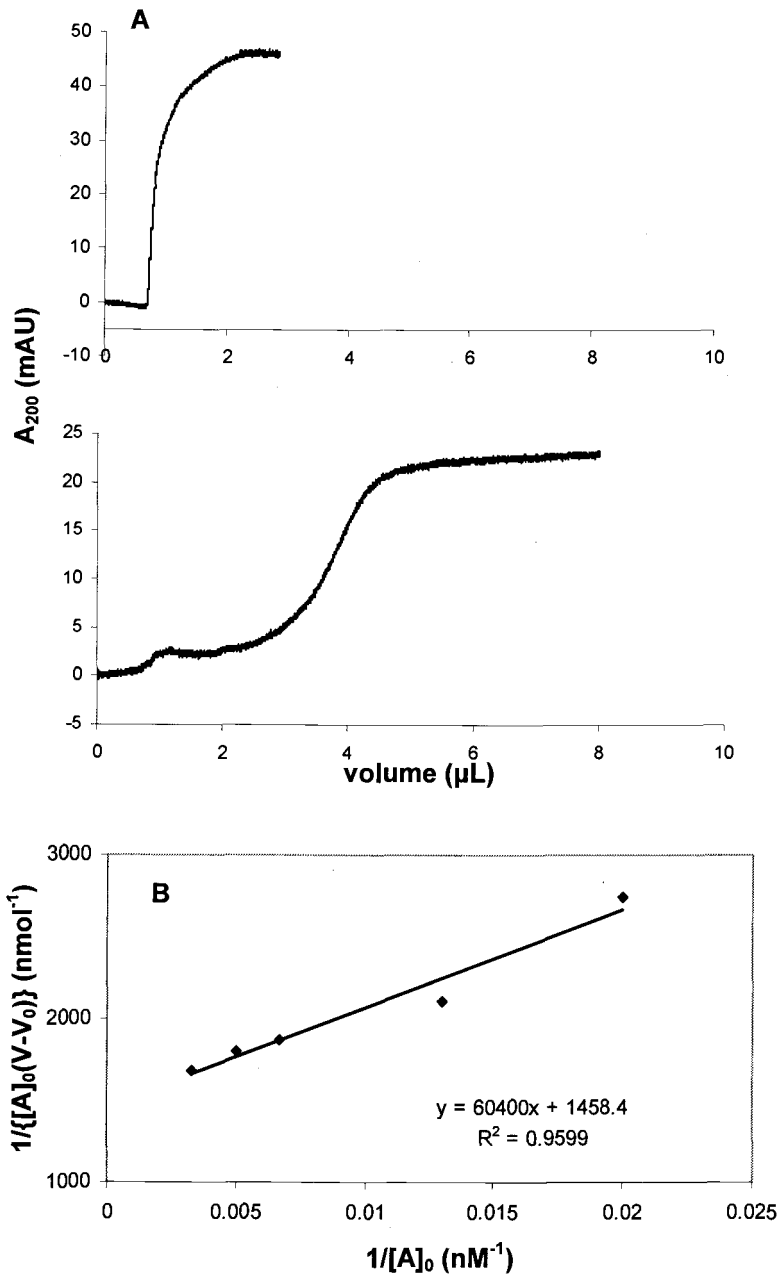


**Figure 6.6.** Characterization of the monolithic aptamer stationary phase. Example of frontal chromatograms using the monolithic aptamer stationary phase (A) using 0.2 mg/mL hemoglobin as the marker of $t_0$ (top) and 200 nM α-thrombin (bottom), and the linear plot of $1/\{[A]_0(V-V_0)\}$ vs $1/[A]_0$ using different concentrations of α-thrombin ($[A]_0$).

## 6.5 CONCLUSIONS

We have shown two chromatographic formats employing an aptamer stationary phase for use in affinity chromatography. To our knowledge, this is the first report of a method for use of an aptamer sorbent in the monolithic format. We have demonstrated that the aptamer retains affinity for its target molecule after being immobilized onto solid supports using azlactone chemistry. We were able to immobilize the DNA aptamer onto the monolithic support at a concentration of 0.069 μmol per 1 mL of media. This is an early report of our success, and we plan to continue the work by further optimizing both the polymerization conditions and aptamer immobilization conditions in order to produce a greater yield of the aptamer present on the surface of the monolithic support.

# Chapter 7: Conclusions

There were two goals of this research effort: (1) explore the applicability of RNA and DNA aptamers as affinity stationary phases, and (2) to immobilize an aptamer onto the surface of a porous polymer so that the stationary phase could be easily applied in a microchip format. In this work, we have realized both of these goals.

Traditionally, antibodies have been the most common affinity sorbent used in affinity chromatography. The use of these molecules has the inherent disadvantages of: (1) large size which prohibits loading large amounts onto stationary phases; (2) the generation of antibodies for molecules which are extremely toxic or not toxic at all is impossible; (4) the generation of large numbers of antibodies is expensive; (5) antibodies are generated to bind a target under physiological conditions, therefore, mobile phase conditions are very limited; and (6) kinetic properties of antibody-antigen interactions are impossible to control. These limitations have led researchers to explore the applicability of other molecules as affinity stationary phases.

Among the other molecules being explored are RNA and DNA aptamers. Aptamers have many advantages which make them desirable as affinity stationary phases. The most important advantage lies in the technique in which aptamers are generated, the SELEX method. This method makes it theoretically possible to isolate

an aptamer which displays affinity for any target molecule. It is because of this method that aptamers are able to be engineered to be active in specific solvent conditions; also it is possible to isolate an aptamer within a range of dissociation constants and kinetic parameters by the SELEX technique. As this method becomes less expensive, an increase in the use of aptamers in analytical devices could be seen.

Our first experience in using aptamers as a specific stationary phase came in the realm of open tubular liquid chromatography experiments. We chose an open-tubular format because of the knowledge this group has obtained in silanol chemistry, so it was the most straightforward approach with which to begin. Our first immobilization method involved creating a covalent bond between the inner wall of fused silica capillaries and an RNA aptamer through a linker molecule created by the linkage of (glycidoxypropyl)trimethoxysilane (GOPS) to the inner wall of fused silica capillaries, followed by the addition of carbonyldiimidazole. Upon the addition of the amine-modified RNA aptamer, the imidazole group acts as a leaving group so that the RNA aptamer is covalently bound through the amine group and a 6-carbon linker. After this was complete, any active sites still present were covered by the addition of ethanolamine.

Our first immobilization involved the initial step (addition of GOPS) occurring under acidic, aqueous conditions in order to add the GOPS to the inner wall, and to simultaneously open the epoxide ring to form a diol. This caused cross linking of the GOPS. Initially, we viewed this as an advantage in that it would maximize the amount of RNA aptamer bound to the surface. These capillaries were used for

chromatographic studies of the RNA aptamer stationary phase. We found that the RNA aptamer was capable of selectively retaining its targets, although the targets themselves (FMN and FAD) could not be separated. We also found that the aptamer retained molecules unrelated to the targets, which was minimized by modifying the mobile phase.

Unfortunately, we found that the immobilization chemistry was very susceptible to external conditions which were difficult to control, so much so that the resulting capillaries were irreproducible. We also found that this stationary phase was inappropriate for use in electrochromatographic studies. When a potential difference was applied across the length of the capillaries, we found that the EOF present was negligible. We attributed this to the masking of silanol groups by the gel present inside the capillaries. Our next step was to create an aptamer stationary phase as a monolayer of aptamer inside fused silica capillaries which we believed would be suitable for electrochromatographic studies.

The immobilization method used to accomplish the immobilization of aptamer in a monolayer inside fused silica capillaries was a modification of the method used for open tubular liquid chromatography. We chose to accomplish the addition of GOPS and the opening of the epoxide ring in two discrete steps to avoid the cross-linking of GOPS inside the capillaries. The first step (the addition of GOPS) was performed under organic conditions. This was followed by the introduction of acidic, aqueous conditions to the capillaries in order to open the epoxide ring and form a diol

which is necessary for the addition of carbonyldiimidazole. We found that the resulting capillaries were suitable for electrochromatography experiments.

We found that the retention behavior of negatively-charged analytes in open-tubular capillary electrochromatography (OTCEC) has not been well studied. When we attempted to characterize the aptamer stationary phase using existing theory, we found that theory was deficient in explaining the retention behavior of negatively-charged analytes. We felt in order to separate the retention of these molecules due to their size and charge from retention due to their interaction with the aptamer stationary phase, it was necessary to adjust the electroosmotic flow (EOF) inside the capillaries to zero. We accomplished this by using opposing pressure during the separations.

We explored the interaction of the targets FMN and FAD with the aptamer stationary phase by monitoring the retention factor (k'). Since the divalent cation $Mg^{2+}$ is known to stabilize the 3-dimensional structure of RNA and DNA, and has been shown to stabilize the interaction of aptamers with their targets, we studied the effect of $Mg^{2+}$ concentration on the retention of FMN and FAD on the aptamer stationary phase. We found that the dependence varied, with k' of FMN being largest at 0.2 mM $Mg^{2+}$ then decreasing at higher concentrations; whereas k' for FAD increased across all concentrations. Chapter 4 discussed these results.

We next chose to create an aptamer stationary phase in a more efficient chromatographic format, namely packed beds. The first immobilization chemistry we attempted was based on the method we used for open tubular separations. We chose to attempt to derivatize silica particles with a DNA aptamer. Using the

immobilization we used for open-tubular capillaries proved to be tedious and irreproducible. This was due to the multitude of steps involved, and the need to keep the particles free of water at certain points in the process. We chose to look for an alternative method.

When looking for an alternative immobilization method, we wanted something simple with one or two steps involved. We found reports of azlactone/bis-acrylamide copolymer particles which were developed by 3M Company for the immobilization of proteins. They were developed to react with the thiol group of proteins, but had been found suitable to react with other nucleophilic groups as well. The immobilization of the amine-modified aptamer onto the azlactone particles consisted of one step. After the immobilization, we covered any remaining active groups with ethanolamine.

The DNA aptamer we chose for study is specific for the protein α-thrombin. The 3-dimensional structure of this aptamer consists of two G-quartets, which are stabilized by the presence of monovalent and divalent cations. We chose to explore the effect monovalent and divalent cations have on the interaction between the DNA aptamer and thrombin. The aptamer is also present in the aptamer-thrombin complex in the G-quartet structure, so we were surprised to find that the presence of all cations in our study resulted in decreased k' values (Figure 5.7). We also found that ionic strength had a large impact upon k' of α-thrombin on the aptamer stationary phase. Many isolated aptamers associate with their target(s) by electrostatic interactions. We feel that ionic strength will prove to be a way to better control these interactions.

The last step of this research project involved immobilizing an aptamer onto a monolithic stationary phase. The main reason for this goal is due to the recent efforts of this research group to create total analysis systems in microchip format (μTAS). This group's efforts involve the casting of polymeric stationary phases inside small channels on microchips to create stationary phases. We wanted a simple format of polymerizing a suitable sorbent, and the subsequent immobilization of an aptamer onto the surface of pores within the polymer for the creation of a miniaturized aptamer stationary phase.

We chose to use the same azlactone-containing monomer responsible for creating the azlactone/bis-acrylamide copolymer beads, 2-vinyl-4,4-dimethylazlactone. The other monomers were ethylene glycol dimethacrylate, and 2-hydroxyethyl methacrylate which were present with the initiator 2,2-dimethoxy-2-phenylacetophenone and the porogenic solvent 1-decanol. The mixture was exposed to UV light for six minutes in order to promote polymerization. The polymer was then flushed with methanol, then with a solution containing the DNA aptamer in order to create the stationary phase. Remaining active groups were covered by flushing with ethanolamine.

The resulting stationary phase was found to be capable of retaining α-thrombin. The stationary phase was characterized according to the dissociation constant of the aptamer-thrombin complex, and the number of active binding sites present. These results were discussed in Chapter 6, and are the first report of an aptamer stationary phase in a monolithic format of which we are aware.

Within this dissertation it has been demonstrated that aptamers are a viable alternative to antibodies for use as affinity stationary phases. We have also been able to apply an aptamer stationary phase in a monolithic format. Other than the advantage of a possible enhancement of efficiency in this format, this format can be easily applied to microchips in order to perform affinity chromatography or extractions in a microchip format.

# BIBLIOGRAPHY


[1] Tuerk, C.; Gold, L. *Science* **1990**, *249*, 505.

[2] Ellington, A.; Szostak, J. *Nature* **1990**, *346*, 818.

[3] Burgstaller, P.; Famulok, M. *Agnew. Chem. Int. Ed. Engl.* **1994**, *33*, 1084.

[4] McFail-Iom, L.; Shui, X.; Williams, L. D. *Biochemistry* **1998**, *37*, 17105.

[5] Bock, L. C.; Griffin, L. C.; Latham, J. A.; Vermaas, E. H.; Toole, J. J. *Nature* **1992**, *355*, 564.

[6] Lödtke, S.; Adam, T.; Unger, K. K. *J. Chrom. A* **1997**, *786*, 229.

[7] Reynolds, K. J.; Colón, L. A. *J. Liq. Chrom. & Rel. Technol.* **2000**, *23*, 161.

[8] Starkenstein, E. *Biochem. Z.* **1910**, *24*, 210.

[9] Campbell, D. H.; Luescher, E.; Lerman, L. S. *Proc. Natl. Acad. Sci. U.S.A.* **1951**, *37*, 575.

[10] Cuatrecasas, P.; Wilchek, M. *Biochem. Biophys. Res. Commun.* **1968**, *33*, 235.

[11] Ngo, T. T. ed. *Molecular Interactions in Bioseparations*, Plenum Press, NY, 1993.

[12] Mohr, P.; Pommerening, K. *Affinity Chromatography: Practical and Theoretical Aspects*, Marcel Dekker, Inc., New York, 1986.

[13] Yalow, R. S, Berson, S. A. *Nature* **1959**, *184*, 1648.

[14] Cuatrecasas, P., Anfinsen, C. B. *Annu. Rev. Biochem.***1971**, *40*, 723.

[15] Ohlson, S., Lundblad, A., Zopf, D. *Anal. Biochem.* **1988**, *169*, 204.

[16] Gold, L., *J. Biol. Chem.* **1995**, *270*, 13581.

[17] Jayasena, S. D. *Clin. Chem.* **1999**, *45*, 1628.

[18] Hermann, T., Patel, D. *Science* **2000**, *287*, 820.

[19] Cox, C. J., Rudolph, P., Ellington, A. D. *Biotechnol. Prog.* **1998**, *14*, 845.

[20] Geiger, A., Burgstaller, P., von der Eltz, H., Roeder, A., Famulok, M., *Nucleic Acids Res.* 1996, *24*, 1029.

[21] Zimmerman, G., Jenison, R., Wick, C., Simorre, J., Pardi, A., *Nature Struct. Biol.* 1997, *4*, 644.

[22] Davis, K. A., Abrams, B., Lin, Y., Jayasena, S. D., *Nucleic Acids Res.* 1996, *24*, 702.

[23] Davis, K. A., Lin, Y., Abrams, B., Jayasena, S. D., *Nucleic Acids Res.* 1998, *26*, 3915.

[24] Kleinjung, F., Klussman, S., Erdmann, V. A., Scheller, F. W., Furste, J. P., Bier, F. F, *Anal. Chem.* 1998, *70*, 328.

[25] Potyrailo, R. A., Conrad, R. C., Ellington, A. D., Hieftje, G. M., *Anal. Chem.* 1998, *70*, 3419.

[26] German, I., Buchanan, D. D., Kennedy, R. T., *Anal. Chem.* 1998, *70*, 4540.

[27] Kotia, R. B., Li, L., McGown, L. B., *Anal. Chem.* 2000, *72*, 827.

[28] Rehder, M. A., McGown, L. B., *Electrophoresis* 2001, *22*, 3759.

[29] Romig, T. S., Bell, C., Drolet, D. L., *J. Chromatogr. B* 1999, *731*, 275.

[30] Deng, Q., German, I., Buchanan, D., Kennedy, R. T., *Anal. Chem.* 2001, *73*, 5415.

[31] Weigand, T. W., Williams, P. B., Dreskin, S. C., Jouvin, M., Kinet, J., Tasset, D., *J. Immunol.* 1996, *157*, 221.

[32] O'Donnell, M. J., Tang, K., Koster, H., Smith, C. L., Cantor, C. R., *Anal. Chem.* 1997, *69*, 2438.

[33] Strother, T., Hamers, R. J., Smith, L. M., *Nucleic Acids. Res.* 2000, *28*, 3535.

[34] Hicke, B. J., Watson, S. R., Koenig, A., Lynott, C. K., Bargatze, R. F., Chang, Y., Ringquist, S., Moon-McDermott, L., Jennings, S., Fitzwater, T., Han, H., Varki, N., Albinana, I., Willis, M. C., Varki, A., Parma, D., *J. Clin. Invest.* 1996, *98*, 2688.

[35] Sassanfar, M., Szostak, J. W., *Nature* 1993, *364*, 550.

[36] Rhodes, A., Deakin, A., Spaull, J., Coomber, B., Aitken, A., Life, P., Rees, S. J., *J. Biol. Chem.* 2000, *275*, 28555.

[37] Famulok, M., *J. Am. Chem. Soc.* 1994, *116*, 1698.

[38] Lauhon, C. T., Szostak, J. W., *J. Am. Chem. Soc.* 1995, *117*, 1246.

[39] Xu, W., Ellington, A. D., *Proc. Natl. Acad. Sci. USA* 1997, *94*, 7475.

[40] Klug, S. J., Huttenhofer, A., Kromayer, M., Famulok, M., *Proc. Natl. Acad. Sci. USA* 1997, *94*, 6676.

[41] Burke, D. H., Gold, L., *Nucleic Acids Res.* 1997, *25*, 2020.

[42] Kimoto, M., Sakamoto, K., Shirouzu, M., Hirao, I., Yokoyama, S., *FEBS Letters* 1998, *441*, 322.

[43] Gebhardt, K., Shokraei, A., Babaie, E., Lindqvist, B. H., *Biochemistry* 2000, *39*, 7255.

[44] Wen, J., Gray, C. W., Gray, D. M., *Biochemistry* 2001, *40*, 9300.

[45] Aldaz-Carroll, L., Tallet, B., Dausse, E., Yurchenko, L., Toulmé, J., *Biochemistry* **2002**, *41*, 5883.

[46] Lee, M., Walt, D. R., *Anal. Biochemistry* **2000**, *282*, 142.

[47] Clark, S. L., Remcho, V. T., *Electrophoresis* **2002**, *23*, 1335.

[48] Maskos, U., Southern, E. M., *Nucleic Acids Res.* **1992**, *20*, 1679.

[49] Cox, C. J.; Hayhurst, A.; Hesselberth, J.; Bayer, T. S.; Georgiou, G.; Ellington, A. D. *Nucleic Acids Res.* **2002**, *30*, 108e.

[50] Stojanovic, M. N.; de Prada, P.; Landry, D. W. *J. Am. Chem. Soc.* **2001**, *123*, 4928.

[51] Liss, M.; Petersen, B.; Wolf, H.; Prohaska, E. *Anal. Chem.* **2002**, *74*, 4488.

[52] Clark, S. L.; Remcho, V. T. *J. of Separation Sci.* **2003**, *26*, 1451.

[53] Pavski, V.; Le, X. C. *Anal. Chem.* **2001**, *73*, 6070.

[54] Rheder, M. A.; McGown, L. B. *Anal. Chem.* **2001**, *22*, 3759.

[55] Culbertson, C. T.; Jorgenson, J. W. *Anal. Chem.* **1994**, *66*, 955.

[56] Walhagen, K.; Unger, K. K. *Anal. Chem.* **2001**, *73*, 4924.

[57] Matyska, M. T.; Pesek, J. J.; Boysen, R. I.; Hearn, M. T. W. *Anal. Chem.* **2001**, *73*, 5116.

[58] Fan, P.; Suri, A. K.; Fiala, R.; Live, D.; Patel, D. J. *J. Mol. Biol.* **1996**, *258*, 480.

[59] Schneider, C.; Sühnel, J. *Biopolymers* **1999**, *50*, 287.

[60] Deng, Q., Watson, C. J., Kennedy, R. T., *J. Chromatogr. A* **2003**, *1005*, 123.

[61] Berezovski, M., Sergey, K., Sergey, N., *J. Am. Chem. Soc.* **2003**, *125*, 13451.

[62] Clark, S. L., Remcho, V. T., *Anal. Chem.* **2003**, *75*, 5692.

[63] Michaud, M., Jordan, E., Ravelet, C., Villet, A., Ravel, A., Grosset, C., Peyrin, E., *J. Am. Chem. Soc.* **2003**, *125*, 8672.

[64] Michaud, M., Jordan, E., Ravelet, C., Villet, A., Ravel, A., Grosset, C., Peyrin, E., *Anal. Chem.* **2004**, *76*, 1015.

[65] Kankia, B. I., Marky, L. A., *J. Am. Chem. Soc.* **2001**, *123*, 10799.

[66] Coleman, P. L., Walker, M. M., Milbrath, D. S., Stauffer, D., M., Rasmussen, J. K., Krepski, L. R., Heilmann, S. M., *J. Chromatogr. B* **1990**, *512*, 345.

[67] Heilmann, S. M., Rasmussen, J. K., Krepski, L. R., *J. Polym. Sci. Part A: Polym. Chem.* **2001**, *39*, 3655.

[68] Padmanabhan, K., Padmanabhan, K. P., Ferrara, J. D., Sadler, J. E., Tulinsky, A., *J. Biol. Chem.* **1993**, *268*, 17651.

[69] Hjertén, S., Konguan, Y., Liao, J. L., *Macromol. Chem., Macromol. Symp.* **1988**, *17*, 349.

[70] Peters, E. C., Petro, M., Svec, F., Fréchet, J. M. J., *Anal. Chem.* **1998**, *70*, 2288.

[71] Ericson, C., Holm, J., Ericson, T., Hjertén, S., *Anal. Chem.* **2000**, *72*, 81-87.

[72] Tripp, J. A., Svec, F., Fréchet, J. M. J., *Comb. Chem.* **2001**, *3*, 216.

[73] Xie, S., Svec, F., Fréchet, J. M. J., *Biotechnol. Bioeng.* **1999**, *62*, 30.

[74] Peterson, D. S., Rohr, T., Svec, F., Fréchet, J. M. J., *Anal. Chem.* **2002**, *74*, 4081.

[75] Schweitz, L., Andersson, A. I., Nilsson, S., *Anal. Chem.* **1997**, *69*, 1179.

[76] Clark, S. L., Remcho, V. T., *J. Chromatogr. A.*, submitted.

[77] Arnold, F. H., Schofield, S. A., Blanch, H. W., *J. Chromatogr.* **1986**, *355*, 1.

[78] Kasia, K., Oda, Y., Nishikata, M., Ishii, S., *J. Chromatogr.* **1986**, *376*, 33.

[79] Macaya, R. F., Waldron, J. A., Beutel, B. A., Gao, H., Goesten, M. E., Yang, M., Patel, R., Bertelsen, A. H., Cook, A. F., *Biochemistry* **1995**, *34*, 4478.

[80] Perkins, T. W., Mak, D. S., Root, T. W.m Lightfoot, E. N., *J.Chromatogr. A* **1997**, *766*, 1.