2013

# Super-aptamer bio-imprinted hydrogels : an investigation into the optimization and characterization of cross-linked polymeric materials displaying macromolecular amplified responses

Nicholas Alexander Gariano
*Louisiana State University and Agricultural and Mechanical College*, hokietiger9803@gmail.com

# SUPER-APTAMER BIO-IMPRINTED HYDROGELS: AN INVESTIGATION INTO THE OPTIMIZATION AND CHARACTERIZATION OF CROSS-LINKED POLYMERIC MATERIALS DISPLAYING MACROMOLECULAR AMPLIFIED RESPONSES

A Dissertation

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

in

The Department of Chemistry

by
Nicholas A. Gariano
B.A., B.S., Virginia Tech, 2003
August 2013

To my family and friends who, through their continued love and support, have made it possible for me to pursue my dreams.

## ACKNOWLEDGEMENTS

My family has always been a source of support, encouragement, love, and perpetual understanding. In particular, my parents have always done whatever was possible or necessary to help guide me and make me the person I am today. I would not be in this position if it weren't for their influence and the importance of education they have instilled in me. This dissertation represents the lifetime pursuit of my goals and interests; none of which would have been possible without them.

My friends, many of whom are like family to me, have been the daily support in my life. They have been an ear when I needed to talk, a distraction when frustrated, and have helped support and encourage me throughout the winding path I've taken to get to where I am today.

I have also had many advisors in my college career. They have been teachers in the truest definition providing me both with opportunities to grow and learn and examples of what it means to be a professional. In particular my graduate advisor, Dr. Spivak, has always been helpful, understanding, and a willing guide and mentor.

# TABLE OF CONTENTS

## ABSTRACT

It is becoming more important to detect ultra-low concentrations of analytes for biomedical, environmental, and national security applications. Equally important is that new methods should be easy to use, inexpensive, portable, and if possible allow detection using the naked eye. Detection of low concentrations of analytes generally cannot be achieved directly, but requires signal amplification by catalysts, macromolecules, metal surfaces or supramolecular aggregates. The rapidly progressing field of macromolecular signal amplification has been advanced using conjugated polymers, chirality in polymers, solvating polymers and polymerization/depolymerization strategies.

The use of molecularly imprinted polymers is ideal for creation of novel sensors to meet the demands of bioanalytical fields. Often referred to as plastic antibodies, molecularly imprinted polymers can often approach the activity and specificity of antibodies.[1] Additionally, they are cheap to produce relative to traditional bioassays, exhibit greater stability to pH, heat, enzymatic degradation, and are reusable over long periods of time. The ever expanding field of molecularly imprinted polymers generally focuses of new techniques and materials aimed at increasing the selectivity of artificial binding sites based of improved shape, size, or functional selectivity both as a function of bulk polymer matrix and functional receptor molecules located in the active site.

Here, a new type of aptamer-based hydrogel with specific response to target proteins demonstrates an additional category of macromolecular signal amplification. This super-aptamer assembly provides the first example of using protein-specific aptamers to create volume changing hydrogels with amplified response to the target protein. A remarkable aspect of these

super-aptamer hydrogels is that volume shrinking is visible to the naked eye down to femtomolar concentrations of protein. This extraordinary macromolecular amplification is attributed to a complex interplay between protein-aptamer crosslinks and the structure of the hydrogel network surrounding it.

Additionally, the further investigation of the role played by N, O-bismethacrylethanolamine toward the recognition of a pair of enantiomers was explored. Previous work showed its utility as a one monomer moleculary imprinted polymer material capable of enantiomeric separation. Its further characterization was explored here concerning its ability to be used as a binding receptor in concert with its role in reducing polymer matrix non-selective binding. The deconvolution of this duality could lead to the design of better monomers capable of greater specificity and selectivity in monolithic molecularly imprinted polymers.

# CHAPTER 1: INTRODUCTION TO PROTEIN IMPRINTING

## 1.1 Historical Development and Direction of Protein Imprinting

The detection, recognition, and identification of specific DNA strands, proteins, and other biomacromolecules have become increasingly important and researched topics in recent years. In particular, immuno-assays, sensors, and other manners of analytical devices have become popular and necessary for increasingly efficient and sensitive detection of biomarkers for a myriad of diseases.[2-7] The general direction research is geared toward earlier detection of disease by creating devices that are capable of detecting increasingly smaller concentrations of biomarkers. Since Mosbach first published the use of antibody mimicking molecularly imprinted polymers (MIPs) as a replacement for antibody assays, many researchers have been seeking to create a synthetic imprinted system that could be used as a receptor for larger biomolecules.[8]

The issues of selectivity and specificity are at the core of the problem in detecting biomarkers when using MIPs. Traditional bulk polymer imprinting does not begin to approach detection limits for in vivo concentrations of some biomarkers or the selectivity necessary to distinguish between different kinds of biomolecules.[9,10] Biological receptors such as antibodies or aptamers, while able to detect biomacromolecules much more specifically, are already widely applied in many biosensors. In order to create a better detection system, the synergistic combination of highly sensitive bioreceptors capable of higher selectivity towards biomarkers will be incorporated with synthetic molecular imprinted technology.

### 1.2 Design of Molecularly Imprinted Polymers

Classical molecular imprinting involves the pre-organization of a complex between functional monomers and a target template. The complex is then added to a solution containing a large concentration of cross-linker and the mixture undergoes bulk polymerization forming a solid polymer around the template. After polymerization the monolithic polymer contains the pre-organized functional monomers locked in their orientation around the template molecule. After removal of the template, the material can be used for recognition of the imprinted molecule.

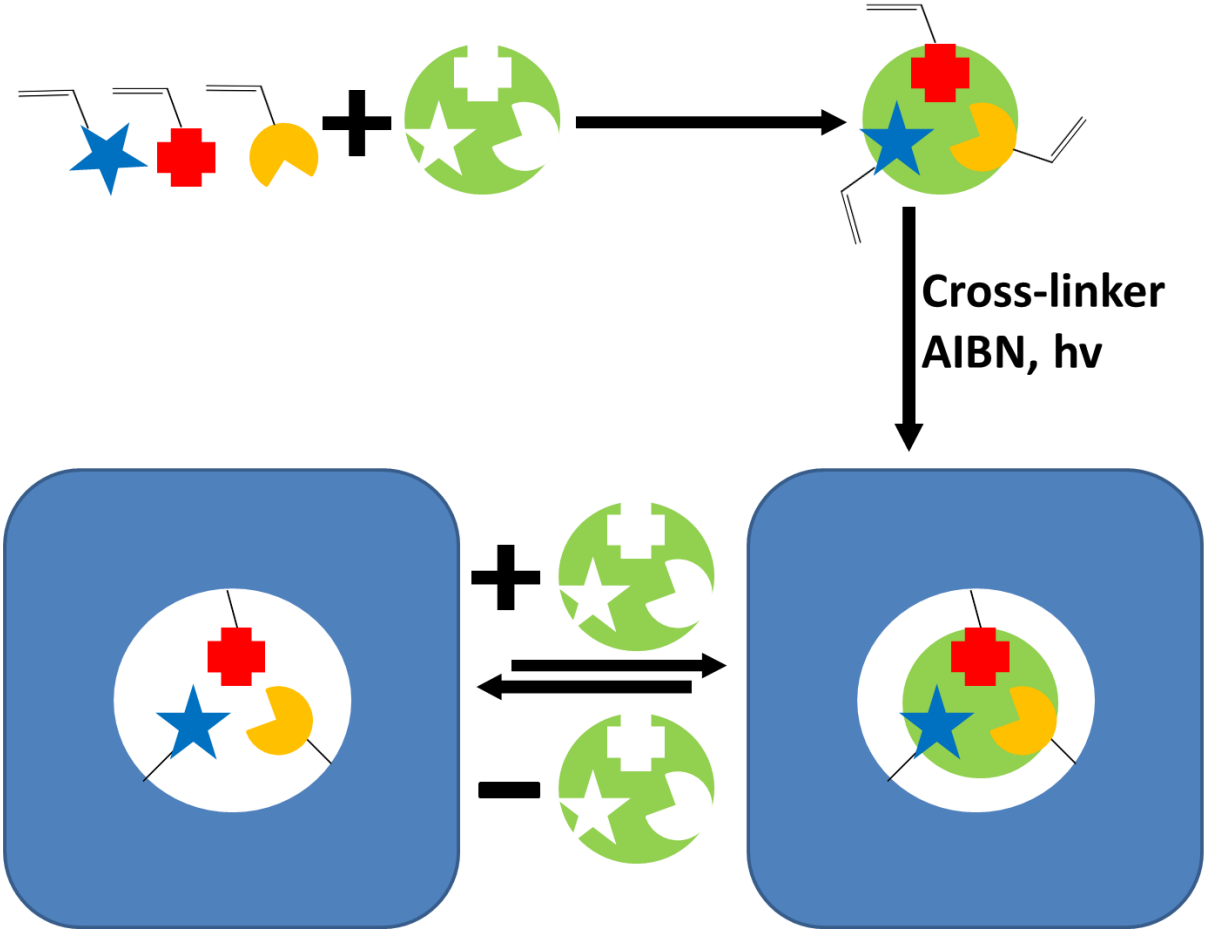


Figure 1: Schematic representation of molecular imprinting.

### 1.3 The Efficacy of using MIPs for Protein Recognition

MIPs have been shown to be very effective for the recognition of small organic molecules with low molecular weights. One of the main problems in creating MIPs with biomacromolecular templates is that traditional MIPs have high degree of cross-linking due to the large concentration of cross-linker in their formulations. The high degree of cross-linker is necessary in these systems to make size and shape selectivity towards the target molecule.[11,12] This creates a problem when dealing with larger molecules. While imprinting biomacromolecules is feasible and would create pores that would likely be good receptors for recognition, the high cross-link density negates the utility of such materials because the diffusion of large macromolecules through the matrix would be kinetically unfavorable if not impossible. Conversely, decreasing the degree of cross-linking would result in a lower selectivity toward an imprinted template because of a decrease in rigidity and a consequential loss of size and shape selectivity.

MIPs have been found to create binding constants similar to biological receptors and are comparably resistant to harsh conditions such as temperature, pH, solvents, and pressure.[9,10] For these reasons, the use of MIPs could be advantageous compared to other means of biomacromolecular detection. In order to create useful analytical systems by imprinting biomacromolecules, it is necessary to create novel materials where diffusion across a cross-linked matrix is not a factor while still maintaining very specialized shapes and binding interactions between the target and the receptor material.

## 1.4 Techniques for Molecular Recognition of Proteins

### 1.4.1 Traditional approaches to molecular imprinting of proteins

Several approaches have been explored for the synthesis of advanced recognition materials based on MIPs. MIPs have been made using functional monomer and functional cross-linker technology similar to traditional bulk imprinting. Most of these systems have resulted in polymers that exhibit low specificity for the target protein even though binding constants for the target proteins have been on the order of $10^5$.[10] Another popular approach has been the synthesis of MIPs inside of particle pores and by suspension polymerization so that diffusion for sites at or near the surface would be more accessible to removal and rebinding of protein templates.[13] Additionally, lower degrees of crosslinking can also make transport of protein more facile. The pre-organization of functional pre-polymer cross-linkers, referred to as assistant recognition polymer chains (ARPCs), with the template protein has also been shown. These approaches also resulted in formation of non-homogeneous binding sites that showed inefficient selectivity for the target protein when in the presence of a cell extract cocktail. The polymer beads formed were found to bind to many proteins with varied affinities.[14]

One of the drawbacks shared by most classical imprinting techniques for bioimprinting is the set of conditions used during polymerization. MIPs are typically polymerized in organic solvents and use functional monomers that rely on intermolecular interactions with a template to form imprinted sites. Biomacromolecules often have very complex tertiary and quaternary structures that are only stable in aqueous environments as opposed to combinations of organic phases. Therefore an effective model for molecular imprinting of biomacromolecules would need to be stable, effective, and applicable in an aqueous environment.

Perhaps the most successful use of MIPs for recognition of proteins has been the use of epitope imprinting where small pieces of proteins called epitopes are polymerized with monomer, cross-linker, initiator and porogen to create small imprinted cavities where functional segments on the exterior of proteins can be imprinted. This partially alleviates the drawback of using proteins in non-aqueous systems because short segments of protein are less likely to greatly deform and result in bad size and shape selectivity in the polymer. This type of MIP has been found to be able to selectively bind protein analytes at μg/L concentrations.[15-17]

### 1.4.2 Using bioreceptors to improve protein recognition in MIPs

Another major inherent problem for imprinting of biomarkers using this type of system is that the level of detection and specific binding of targets is not adequate for biological concentrations. In addition to having low concentrations under biological conditions, other biomacromolecules in blood or other bodily fluids are often nonspecifically bound to receptors giving a false response which can be problematic for applications toward the early detection of diseases.[5] Bulk imprinting methodologies typically use monomers containing functional groups such as amines, amides carboxylic acids, or non-polar polar groups to give entropically favorable interactions. These functional groups predominantly take advantage of intermolecular interactions such as hydrogen bonding, dipole interactions, and, to a lesser degree, Van der Waals forces as the driving forces to create molecular recognition. Simple combinations of these types of molecular interactions, while effective for recognition of small molecules, are not capable of creating the degree of specificity or high binding affinity required for low concentration detection of proteins found in vivo.

These types of molecularly imprinted systems have been used in attempting to make MIPs that selectively identify the presence of different protein analytes in different solutions.

However, in these cases the specificity of imprinted polymers using simple functional monomers is not able to selectively bind proteins by more than one or two orders of magnitude in comparison to non-imprinted analytes.[10] Additionally, these monomers cannot adequately bind to a target molecule in aqueous environments where most protein detection would be useful. The strong hydrogen bonding nature of water often interrupts or weakens the imprinting interactions that would be used for detection of native biomacromolecular targets.

Biological and artificial biological receptors are not only capable, but designed, to work under aqueous in vivo conditions. The specificity and binding constants of these molecules also often far exceeds the capabilities of bulk imprinted polymers.[10] One reason that bioreceptors are able to recognize targets with much higher specificity is because they contain a set of multivalent interactions that are geometrically designed to create entropically favorable interactions between the receptor and the target molecule which are known to increase the affinity of a receptor its target analyte[18,19] While bulk imprinting can mimic this behavior in various imprinted systems, it does not create homogenous receptors that bind with comparable affinities.

In more recent research, the use of receptors having higher binding constants and binding specificity than traditional functional monomer systems have been used in order to imprint macromolecules. In these cases the need for highly cross-linked systems is less important to impart good receptor formation because the receptors inherently have a stronger interaction with a template. By using bioreceptors and artificial bioreceptors, the need for controlling overall shape and size are replaced by the ability of a receptor to bind very specifically to a smaller region of a protein where multivalent interactions and shape recognition imparted by the bioreceptors can allow for binding of specific proteins.

A wide array of biological receptors is available for biochemical recognition of proteins in MIP systems including enzymes, antibodies, DNA and ligands.[20-23] While the use of these types of receptors has been shown extensively in solution and surface sensor systems they are only starting to be utilized in MIPs. Miyata has shown the use of both antibodies and ligands in preparing MIP hydrogels that can detect protein, antibodies, and sugar molecules in nanomolar concentrations.[24-30] Other groups have also successfully explored protein detection using hydrogels with differing functional monomers, but in order to obtain lower levels of detection and better selectivity the use of bioreceptors is necessary.[31-40]

Of the available choices of bioreceptors capable of high specificity and strong binging to target biomarkers, aptamers are perhaps the most advantageous. Aptamers are oligonucleotides, either DNA or RNA, that have high affinity and selectivity for a specific target compound that can range from a small molecule[41] such as a drug[42,43] to complex biomacromolecules such as proteins or DNA[44,45]. Their inherent selectivity and specificity is rooted in the procedure of aptamer discovery where the SELEX process[46] is used to identify a particular nucleotide sequence that has high affinity toward a target from a library of other polynucleotides of equivalent size. The selectivity and specificity of aptamers are comparable to those of antibodies[47], having binding constants on the order of $10^{-9}$. One of the major advantages that aptamers have over antibodies is that it's cheaper to synthesize an aptamer nucleotide sequence while using animals or sustained cell cultures to produce antibodies can be expensive and time consuming in addition to requiring more purification to isolate the desired receptor. Additionally, the structures of aptamers, particularly DNA aptamers, are more stable than the protein structure of antibodies.

### 1.4.3 Using aptamers as receptors in molecularly imprinted polymers

Proximity ligation is the first type of supramolecular recognition assay that involves the assembly of aptamers with their target to increase affinity toward a protein target. The idea of proximity immobilization was pioneered by Fredriksson and Gustafsdottir and since has been used in a variety of systems utilizing both aptamers and antibodies for recognition of target proteins.[48-51] Proximity ligation works by attaching specific sequences of nucleotides to receptor molecules. These nucleotide sequences are capable of ligation in the presence of another common connecting nucleotide sequence. In the presence of target protein, a complex will form between the two receptors and the target where the connecting nucleotide ligates to both receptors creating a supramolecular structure with a binding affinity toward the target that is higher than using either receptor separately. There are several drawbacks to this technique though. Because proximity ligation is a solution phase assay, it can't be used for real time monitoring of binding, binding constants, and binding kinetics that are possible on solid supports such as surface plasmon resonance (SPR). A solid support with corresponding feedback also obviates the use of fluorescent probe as in cases with "molecular pincer" technology that uses ligation as a trigger for fluorescence detection of binding events and formation of a supramolecular structure in solution.[52] Additionally, the alignment of nucleotides so that the 5' end of one chain lines up with the 3' end of the other is not always guaranteed or possible with all systems. Lastly, at low concentrations used for formation of the supramolecular complex, the dissociation constant for each aptamer to the target is never more than 100 pM, which means that the presence of two aptamer chains on each protein in solution is highly unlikely and unfavorable at lower concentrations of protein.[48]

Another popular method of making an imprinted polymer receptor for proteins is by using surface immobilized proteins to make a surface imprinted polymer. In order to make imprinted surface receptor sites, protein is immobilized on a surface and placed face down in a solution of monomer and cross-linker. The solution is then sandwiched between another plate that has alkene functionality. Polymerization of the mixture results in collection of surface receptor sites once the protein function plate is delaminated, extracting the protein from the polymer. Like traditional MIP techniques though problems arise regarding the native structure of the protein. While imprinting using this method has shown promise, the protein is often denatured either in the deposition process or the high concentration monomer/cross-linker solution that is necessary to obtain high crosslink density for good shape recognition of the polymers.[10,53,54]

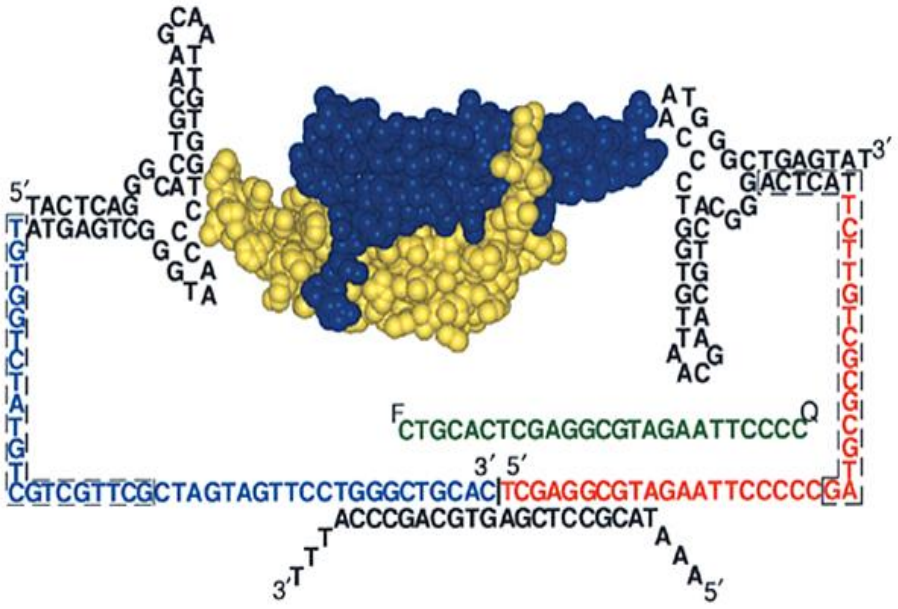


Figure 2: Proximity Ligation of PDGF-ββ using ligated homodimeric aptamers for recognition.

In recent work by the Spivak group[55], aptamer receptors complimentary to mutually exclusive binding sites on α-human thrombin were placed on a surface using proximity immobilization where mixed monolayers on gold SPR chips were functionalized with aptamers

that were tethered with varied linking groups. It was shown that the synergistic binding of thrombin using both aptamers increased the binding constant by three orders of magnitude compared to either individual aptamer. The increase in SPR response and binding constant can be specifically attributed to the proximity of aptamers on the surface of the SPR chip. Similar to the favorable entropic association between a multivalent MIP receptor and a target molecule, the 2-D ordering of multiple bioreceptors containing high specificity binding to a target validated the premise that imprinting could be used to determine pM concentrations and changes in concentration for possible biomedical devices toward the early detection of disorders and diseases where the expression of protein is affected.

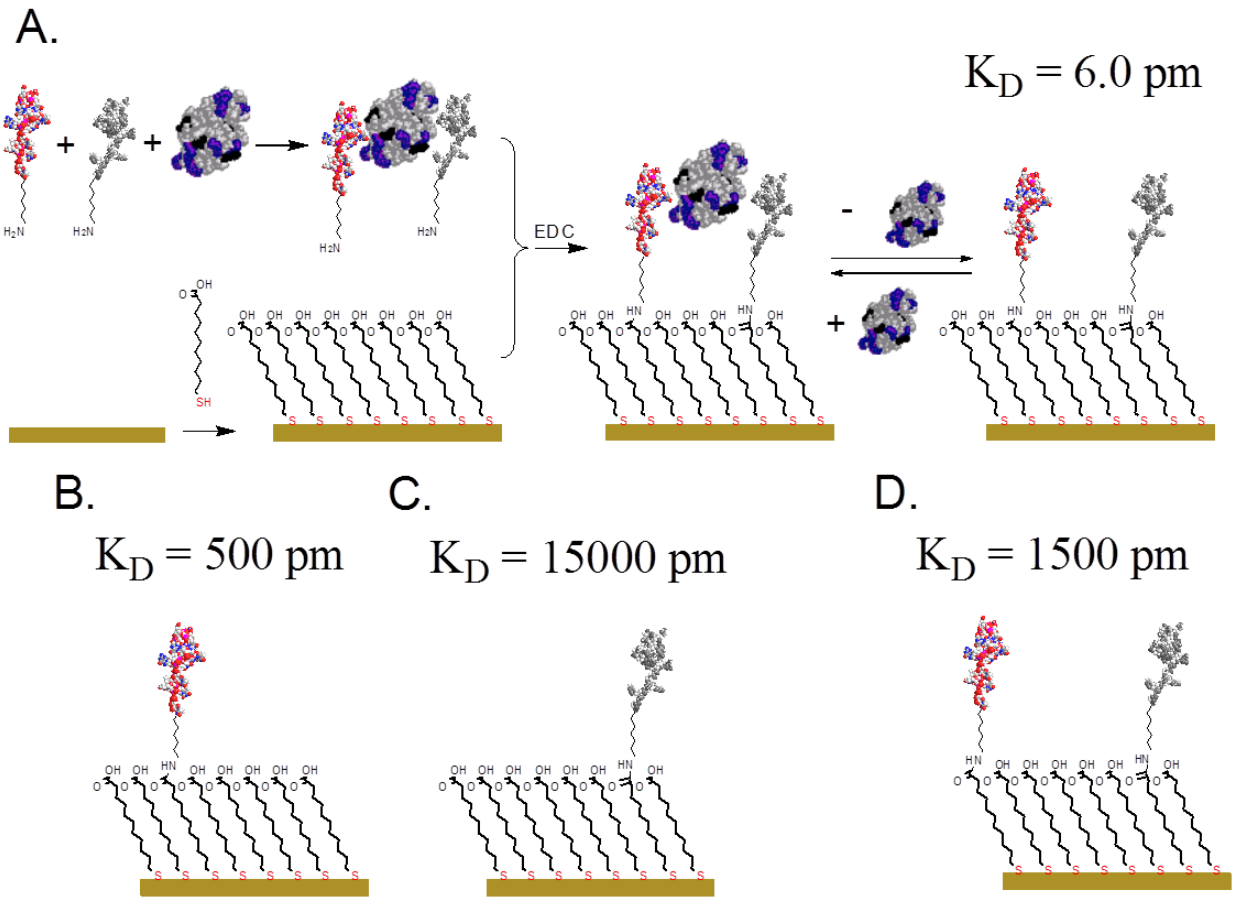


Figure 3: Proximity immobilization scheme for multivalent aptamer binding to thrombin. The proximity immobilization technique (A.) shows the use of multiple receptors on a surface acting

in concert to provide a greater binding constant and specific molecular recognition compared to the use of each receptor used individually (B. and C.) or when the two receptors are not spatially oriented on the surface in a pre-organized manner (D.)

# CHAPTER 2: CREATION AND CHARACTERIZATION OF THROMBIN IMPRINTED HYDROGELS DISPLAYING AMPLIFIED RESPONSE USING APTAMERS AS RECOGNITION AGENTS

## 2.1 Introduction to Protein Imprinting

The origins of molecularly imprinted polymer technology can be traced back to the desire to create biological mimics consisting of synthetic polymeric materials designed to duplicate the active site-substrate binding interactions of antibodies and enzymes for a host of applications. Because of this inherent design, MIPs, at their most basic classification, are generally useful for separations and sensors. Towards that end it highly desirable to create materials that are cheap, rapidly responsive, selective, sensitive, and relatively simple to use in the detection of desired analytes.[56] The development of methodology that permits the naked eye detection of a target molecule in a complex mixture without the need for sophisticated instrumentation is an important challenge for improving sensors and assays and would the best case scenario in developing a sensor for molecular recognition.[57]

The detection of larger biomacromolecules such as proteins by classical MIPs with high cross-link densities is limited because of problems diffusion of template molecules in and out of the polymer matrix,[58] and systems that do exist are often not useful at lower concentrations often seen in biological systems. Reaching such low detection limits is necessary for MIPs to ultimately be useful for detection of biomarkers for medical diagnostics, environmental toxins or compounds of interest for national security. The characterization and quantification of MIPs is often limited by the insoluble nature of heavily cross-linked polymeric materials which can also lead to problems with decreased porosity when detecting larger analytes. Predominantly, methods for characterization of MIPs have been limited to techniques such as solid state NMR[59]

and FT-IR[60] to look at chemical and morphological properties Likewise, chromatographic or batch rebinding techniques are the main methods for observation of analyte binding by HPLC or similar methods and UV-vis detection of residual unbound analytes in contrast to incubated concentrations. Using these methods, identification and detection of low concentration binding events can sometimes be difficult to quantify.

Methods of detection for low levels of analytes rely heavily on signal amplification, and can be achieved in a variety of ways including catalysts, macromolecules, metal surfaces and supramolecular aggregates.[57] Focusing on macromolecular amplification, one of the most successful methods is the "molecular-wire" approach used for fluorescent chemosensors.[61,62] A related approach is the use of polymer aggregation or folding in the presence of a target molecule to affect visible or fluorescent probes.[63-66] The 'sergeants-and-soldiers principle' makes use of molecular chirality to influence macromolecular chirality such as helix formation[67-69] or orientation of liquid crystals.[68] Last, polymerization[70] or depolymerization[71,72] responses have been used as macromolecular detectors of target molecules.

In developing a methodology that involves simple measurements detectable by the human eye, a less cross-linked polymer would be ideal. Changes in its morphology could results in macroscopic changes. One type of material that could be useful for such a task is a hydrogel. Hydrogels are insoluble polymer networks that are extensively hydrated and provide elastic, semi-wet, 3D environments suitable for material-to-biology communication.[73,74] Hydrogels have been shown to be useful as responsive materials because the volume of water held by a certain mass of dry polymer can be greatly affected by small changes in the microstructure of the hydrogel network.

Hydrogels provide many of the factors necessary for creating a protein imprinted polymer. They are relatively cheap to make because the majority of the polymer is typically comprised of acrylamide. Their porosity is much greater than monolithic imprinted polymers making them useful for a system where protein diffusion is necessary. Additionally, they provide a visibly amplified response to a wide variety of stimuli. Responsive hydrogels are an important component of the field of "Smart Materials", characterized by change in morphology, volume, or color in response to external stimuli such as temperature, pH, analytes and electric or magnetic fields.[24,75-80] This is of particular interest for MIPs because, of all possible communication pathways, interaction at the molecular level is the most specific for triggering material response to a biological target.

In recent years the use of hydrogels to imprint larger molecules using the bulk molecular imprinting technique has been performed.[31-35,37,38,40,81,82] Because these materials have a lower cross-link density compared to monolithic imprinted polymers they lose rigidity in the polymer matrix effectively decreasing the shape and size recognition component of imprinted sites. To make up for the decrease in size and shape recognition, increasing the functional interaction with the template protein is necessary for creating highly selective binding sites. In most cases there is a charge component necessary to achieve selectivity of binding in addition to hydrogen bonding character of the largely acrylamide polymer backbone.[33,34] Such MIPs have been useful for the detection of protein,[40,82] viruses,[31] cells, sugars,[35] and other biomacromolecular analytes.[32] While this technique has been useful for detection using polymeric beads for HPLC analysis it utilizes relatively high cross-link densities relative to most responsive hydrogels. This means that even though the material is able to selectively identify an imprinted analyte by chromatographic methods it will be unusable for the evolution of easily discernible volume responses necessary in a simple sensor for direct detection.

While lowering the cross-link density of polymeric materials helps to make them more porous for the diffusion of biomacromolecules in and out of imprinted sites it also decreases the size and shape recognition of those imprinted sites. Consequently, creation of highly specific and selective MIPs for biomacromolecules requires the use of greater functional recognition. Instead of trying to copy nature by making artificial binding sites, the direct incorporation of bioreceptors such as antibodies, ligands, and proteins into imprinted hydrogels could greatly increase the strength of binding and lower levels of detection by increasing the affinity of binding between receptors and targets. They would also inherently increase the specificity for a particular target and the selectivity in a non-purified mixture of macromolecules in biological media.

A non-templated hydrogel has recently demonstrated that biological molecules, i.e. DNA hybrids, can be used as reversible cross-linkers that exhibit a volume change response in the material.[83] Hydrogels with response mechanisms more closely related to those in the study presented here have been demonstrated by Miyata et. al.[24,27,30] These examples utilized the method of biomolecular imprinting where template proteins are pre-complexed to polymerizable bioreceptors which are subsequently copolymerized with hydrogel monomers. Removal of the imprinted protein reversibly dissolves crosslinks resulting in a swelling response of the hydrogel. The template protein in a typical example provided approximately 0.24 mole percent of the overall amount of crosslinks in the reported polymers, and the macromolecular response was detected in the narrow range of approximately 2.0 to 6.0 × $10^{-7}$ M protein concentration.[30] The aptamer-based hydrogel materials presented herein use a much lower concentration of target protein as part of the pre-polymer complex, now on the order of 1.0 × $10^{-3}$ mole percent of the overall crosslinking density. The resulting hydrogels were responsive to protein concentrations ranging over several orders of magnitude, in the $10^{-14}$ to $10^{-6}$ M range. A surprising result is that although the super-

aptamer hydrogels described here have three orders of magnitude lower percentage of reversible crosslinks, the volume change is equal to or larger than previous bioimprinted hydrogels.[27,30] The high response to low concentrations of target protein as a result of disrupted reversible crosslinks indicates the response is significantly amplified for the super-aptamer hydrogels described here.

The study presented herein investigates an original aptamer-based hydrogel system with volume response to ultra-low levels of protein analytes, with a different mechanism behind the signal amplification than the four known types listed earlier. Aptamers are an exciting new class of synthetic bioreceptors evolved in-vitro by the SELEX[84,85] method to give artificial receptors that can often rival the binding of antibodies.[86-88] The term aptamer refers to any synthetic DNA, RNA, or protein receptor whose efficacy of binding is identified through a library selection and amplification process and typically involves the utilization of secondary and tertiary superstructures of polynucleic acids and polyamino acids in recognition of particular targets[89]. They have been used in a variety of molecular recognition systems including fluorescent molecular beacons,[90-92] gene therapy,[93] directed drug delivery,[94] column chromatography,[95] and molecular sensors of a wide array of targets such as DNA, RNA,[96] proteins,[97-99] ions,[100-104] cells,[105-107] and a host of small molecules. Aptamers are advantageous in comparison to the other types of bioreceptors mentioned previously because they are cheaper to synthesize, are easily functionalized for a multitude of applications, are generally more stable for longer periods of time in a wider range of conditions, and are relatively small biomacromolecules compared to antibodies and ligands.

In the interest of detection at low concentrations it is necessary to increase the binding affinity of an imprinted complex toward its target analyte. One method to accomplish this is to create a multireceptor system for template imprinting and recognition. This technique was found

to increase the binding constant of thrombin to an imprinted surface where two aptamer receptors were attached in a pre-organized manner as opposed to random binding of the receptors.[108-110] There are three known protein targets, VEGF,[111,112] PDGF,[113-116] and thrombin,[89,117-125] which have two specific binding sites on the protein surface which have had aptamers evolved for specific recognition. Of those three, VEGF and PDGF are homodimeric proteins having two identical binding sites while thrombin has two distinct binding sites. The formation of a more specific preformed imprinted complex by targeting two explicit binding domains should be advantageous in creating a more specific imprinted site.

The term "super-aptamer" used here describes the cooperative use of aptamers in creating a synergistic binding response and illustrates a general approach to developing responsive materials toward any biomarker. The high level of macroscopic amplification by these super-aptamer hydrogels makes it competitive with other analytical methods of detection for ultra-low concentrations of target analytes. The ability to see the response of the super-aptamer hydrogels by the naked eye makes this a useful assay for low-cost, facile, and portable detection of molecules of interest for biomedical, environmental, and warfare analysis.

## 2.2 Design and Polymerization of Hydrogels for Thrombin Imprinting

### 2.2.1 Aptamer functional monomers and the preformed aptamer-thrombin complex

The aptamers, A1 (GGT-TGG-TGT-GGT-TTG) and A2 (AGT-CCG-TGG-TAG-GGC-AGG-TTG-GGG-TGA-CT), are identified in the literature as those oligonucleotides that have a high affinity for exosite one and two respectively on the outer surface of thrombin.[126,127] In order to incorporate the aptamers into the hydrogel matrix the aptamers need to be functionalized with a polymerizable methacrylamide group. These functionalized aptamers, F1 and F2, shown below in Figure 1 were purchased from Integrated DNA Technologies. Their internal structures

are designed with a sequence of six ethoxylate groups and five thymidine residues as spacers between the polymerizable acrylamide group and the aptamer. These internal modifications were previously designed for the use of creating imprinted self-assembled monolayers and allow for flexibility and optimization of the reformation of the imprinted complex.[55] The presence of the phosphate groups are an artifact of the synthetic scheme used to make the functionalized aptamers.

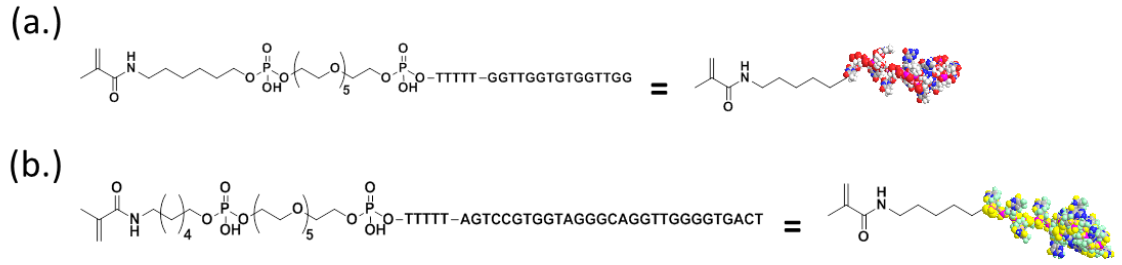


Figure 4: Structure of functionalized aptamers. The structures of functional aptamers F1 (a.) and F2 (b.) are shown. The two structures are correspondingly named because they contain the A1 and A2 aptamers respectively.

The first step in creating thrombin-imprinted hydrogels is making the preformed aptamer-protein complex. This is achieved by conjugation of equimolar amounts of F2 and thrombin for a period of 30 min with periodic agitation. Because the volume of aptamer and thrombin are generally less than 20 μL, 100 mM phosphate buffer solution (PBS) (5 mM KCl, 1mM $MgCl_2$, and 150 mM NaCl at a pH of 7.2) is typically added to allow for good mixing and to create a volume that can be easily agitated. Once A2 has coupled with thrombin, F1 is added to the Th-F2 complex in PBS and incubated for an additional 30 minutes under periodic agitation to form the F1-Th-F2 pre-imprinted complex. The order of addition matters because A2 has a strong and selective affinity for exosite 2 on thrombin while A1 has the ability to bind to both exosite one

and two. However, the affinity of A1 towards exosite two is not as strong as the affinity of A2 towards exocite two.

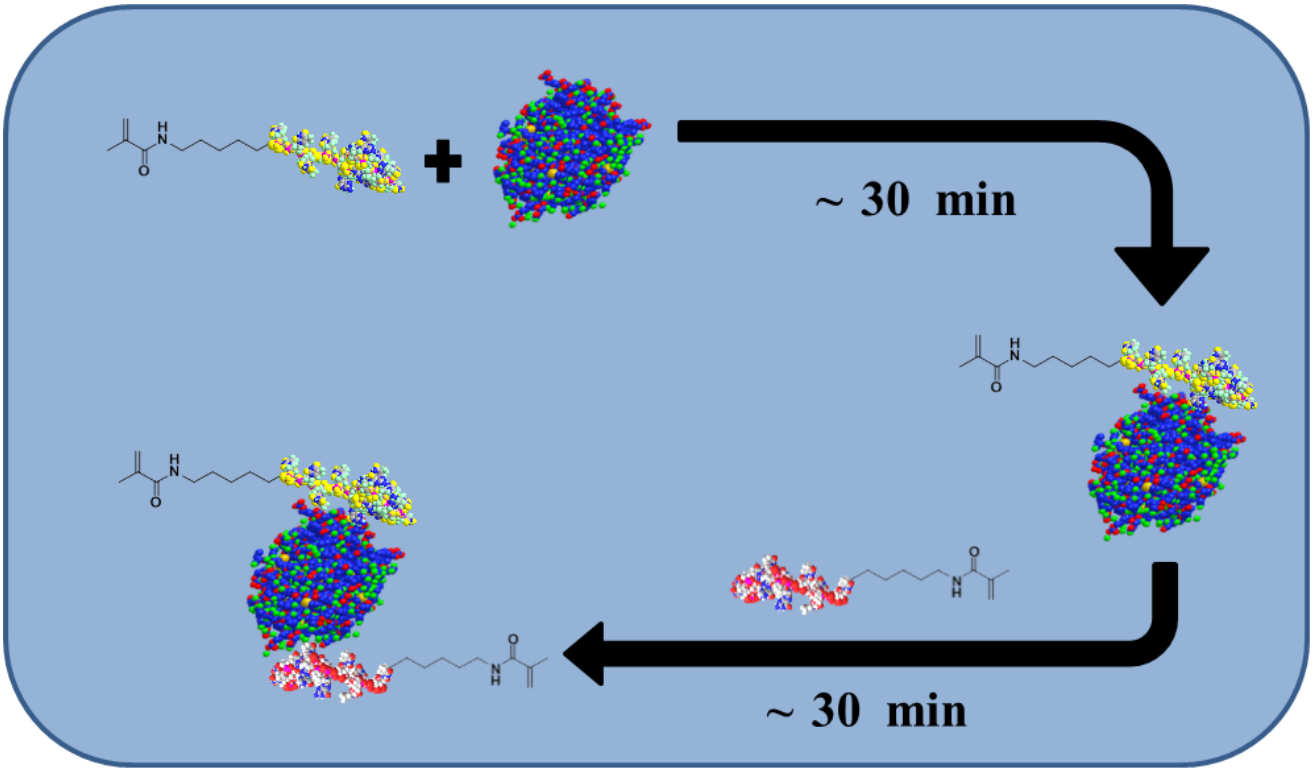


Figure 5: Scheme for formation of the pre-formed aptamer protein complex.

**2.2.2 Procedure for the polymerization of hydrogels**

The complex to be imprinted is added to the mixture of acrylamide (AAm), *N*,*N*'-Methylenebisacrylamide (MBAM), and 5 µL of 10 weight percent ammonium persulfate (APS). PBS is then added in variable amounts to bring the final volume of the polymerization solution to 100 µL. The solutions are then degassed with nitrogen for approximately 5 min. When ready to polymerize, 0.5 µL of tetramethylethylenediamine (TEMED) was added and the solution was quickly vortexed and loaded into either a 1.7 mm inner diameter capillary tube or a disc gel mold. Gelation usually occurs within one minute, however, this can vary depending on concentration of monomers and the polymerizations are undisturbed for 3 hours to ensure complete polymerization. After 3 hours gels are put into 100 mM PBS (pH=7.4) for subsequent

washing steps to remove unpolymerized residual monomer components and to equilibrate the hydrogels to their fully swollen state in PBS.

### 2.2.3 Polymerization of hydrogels into disc forms

For these studies, hydrogels were molded into two main forms: discs and capillaries. Both versions have been prepared previously in the literature and have advantages and disadvantages for collecting data.[24-30,128,129] Both types of hydrogels are prepared the same way with the exception of the last step where polymerization solutions are loaded into different molds.

Hydrogel discs were synthesized by loading the polymerization solution between 2 glass plates separated by two 1 mm thick microscope slide spacers. In this way, hydrogels were polymerized to a constant thickness of 1 mm and had a generally circular shape. In cases where the shape needed to be very circular with definite dimensions, the hydrogels were cut with a circular cork borer. Diameters of either 8.7 or 10.3 mm were used depending on the measuring technique and the swelling ability of the gel over the course of experiments.

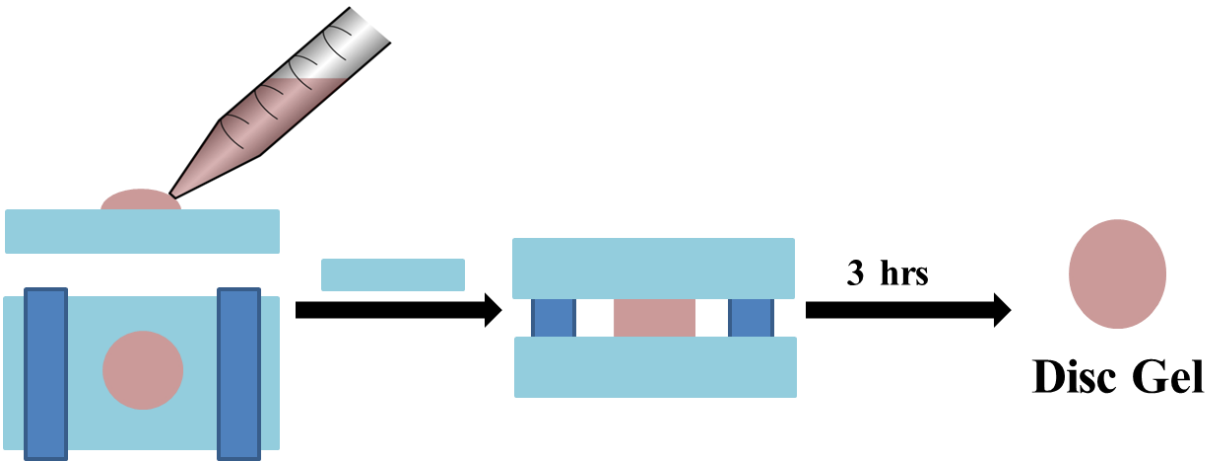


Figure 6: Representation the process used to make disc gels. Typically, hydrogel discs were between 1 and 1.5 cm in diameter and 1mm in height.

### 2.2.4 Polymerization of hydrogels inside of capillary tubes

The second type of hydrogel used in the study is the capillary hydrogels. These are molded by drawing the polymerization mixture into a capillary once the TEMED is added. This step must be done quickly because the viscosity of the solution increases rapidly. Ideally the solution should be as fluid as possible when loading in order to form a well-defined meniscus on either side of the gel. The formation of well-defined meniscuses is necessary for obtaining consistent measurements of the gel lengths and the response of the gels toward thrombin.

The amount of solution drawn into the capillary, and by extension the length of the hydrogel, is important in making gels that will behave well during shrinking and swelling caused by solution changes seen in later steps. This is a function of how deep the polymerization solution in the vial is and the inner diameter of the capillary tube. Because the polymerization solutions are typically made to be 100 µL, most of the variation in hydrogel length comes from the diameter of the capillary tube and the reproducibility of loading capillaries quickly as the solution begins to polymerize. Ideally, it has been found that hydrogels should be approximately 6 mm long initially so that addition of excess PBS will swell the gels to a final volume where the length of the gel in the capillary tube is roughly 10 mm and fill roughly 50 percent of the capillary. If gels are shorter it typically becomes hard to see significant volume changes and when they become too long it takes excessively long periods of time to equilibrate the hydrogels from one solution to another. When they are longer it also limits the amount of solution that can be added to the tube which will also increase equilibration times.

Figure 7: Illustration of solution changing in a capillary tube. Solutions in capillary tubes are changed by using a micro-syringe. All of the previous solution is removed so that the meniscus of the gel has no excess liquid on it. Then, once the gel length has been measured a fresh solution is injected. This procedure is repeated daily until a gel's length equilibrates in a given solution.

Capillary tubes need to be a minimum of 0.7 mm in diameter to accommodate syringe needles for removal and addition of solutions. It was found that gels of optimal size were obtained when the inner diameter of the capillary tube was 1.7 mm. Tubes with smaller diameters exhibit a larger capillary force resulting in longer gels that fill too much of the tube. Conversely, larger diameter capillary tubes create very short gels because of a decreased capillary force comparatively.

### 2.3 Formulation of Hydrogels that are Capable of Discernible Volume Change

The initial formulation of hydrogels used 11.3 mg (.16 moles) of AAm as the major component of hydrogels. Besides the AAm content, the other initial constant used was to make the total volume of each polymerization mixture 100 µL. This volume was chosen because it represented a volume of material that would be adequate for making hydrogels made by either the capillary or disc method and would also be small enough that incorporation of functional monomers later would be cost effective. All other quantities were based on mole ratios with respect to the initial AAm concentration.

The next component that needed to be optimized was the inclusion of a cross-linker to form a matrix that would hold its form upon exposure to excess PBS. After scanning the literature, MBAM was found to be the most common cross-linker used in aqueous gel

formulations.[24-30] The initial formulation of hydrogels was derived from a series of papers in the literature based on the ratio of AAm to MBAM.[25-28,30] The ratio of AAm to MBAM is important because it governs the cross-link density of the hydrogel as well as average molecular weight between cross-links. The pertinent physical manifestations of these properties are the swelling ability of the hydrogel in PBS after polymerization and the modulus of the hydrogel as it pertains to the ability to mechanically handle the hydrogels during normal measurements.

The modulus of a hydrogel is not solely determined by the mole percent of cross-linker. It will also vary greatly with respect to the concentration of monomer in the initial polymerization solution. Two gels can contain the same weight of solid material and not necessarily swell to the same volume in PBS after polymerization is complete. This is because the gelled matrix will not be the same if the hydrogels are polymerized at different concentrations. Therefore, a two-dimensional study was done using disc gels to show the effect of both the concentration of monomers in the polymerization solution and the mole percent of MBAM relative to AAm in those polymerization solutions.

### 2.3.1 Varying MBAM content to obtain hydrogels usable for regular measurements

A series of hydrogels having varied MBAM content and reaction volume was created. The MBAM quantities were 0.125, 0.25, 0.5, 1.0, and 1.5 mole percent of the total monomer concentration and were chosen in reference to other systems where hydrogels were used as sensors and responsive materials. The total volume of each polymerization solution was 0.5, 1.0, or 1.5 mL. The AAm content was held constant at 56.5 mg per reaction so that the total polymer in each hydrogel would remain relatively normalized because the AAm is the major molar component of the each polymerization mixture. The solutions of AAm, MBAM, APS, and

TEMED were gelled into discs as described earlier and once polymerized the hydrogels were evaluated based on the rough approximation of their modulus and their swell ability.

For this initial study the modulus of the hydrogels is crudely quantified by determining whether or not hydrogels were solid enough to handle once polymerized. During the course of weeks of measurements disc gels are handled several times a day with forceps as solutions are changed and measurements are taken. While the wear and tear on capillary hydrogels is significantly less, needles are often pushed right up against the edge of the hydrogels as solutions are changed and can cause damage to the meniscus if the material is too unstable causing error that can increase over the course of time.

The amounts of MBAM and PBS used resulted in hydrogels that ranged, depending on their composition, from very solid with little elastic property to oozing material that does not easily hold their form. The qualitative results described are summarized in Table 1. The desired utility of the gels and the properties required will vary depending on the method of polymerization and the application of gel measurement performed. Disc gels need to be more solid than capillary gels because they are handled with forceps and the capillary gels will equilibrate faster if they are more elastic. While there was some degree of variance in the properties of each gel, the assignments and determinations were made based on what concentrations could be used for handling disc gels.

Table 1: Physical characterization of hydrogels with varying MBAM and PBS composition.

| | **MBAM (mole percent)** | | | | |
|---|---|---|---|---|---|
| **Total Volume (mL)** | **0.0125** | **0.025** | **0.5** | **1.0** | **1.5** |
| **0.5** | bad | bad | good | good | good |
| **1.0** | bad | bad | bad | good | good |
| **2.0** | bad | bad | bad | Bad | good |

Table 1 shows a rough approximation of how much PBS and MBAM are required to create gels that will be able to handle the rigors of testing. The determination of whether gels were suitable for long term testing was subjective. The disc gels were qualitatively assigned as either "good" or "bad" based on their capacity for long term use in multiple gel measurements. The gels labeled as "good" could be handled multiple times without breaking and "bad" gels were too fragile for repeated handling. In most cases gels that were borderline bad gels were still completely gelled; they were just not solid enough to be handled with tweezers routinely without breaking. However, there is some likelihood that they could be used for studies in capillary tubes where there is less wear and tear from measurements.

The gels containing 1.5 mole percent MBAM were all very rigid and did not noticeably absorb any PBS after polymerization and they were deemed unuseful for studies where volume changes were expected and necessary. Conversely, gels containing 0.125 mole percent MBAM formed gels at most concentrations, but even when they did they were generally too fragile for repeated handling. The gels with 1.0 mL PBS showed a very pronounced transition at a MBAM concentration of 1.0 mole percent and there was not as much PBS absorbed after polymerization as the same MBAM concentration with 0.5 mL PBS. Overall, the gels with 0.5 mL PBS showed a reasonably linear increase in their mechanical properties with increasing amounts of MBAM and were deemed the most viable gels for the allotted concentration of AAm.

### 2.3.2 Varying MBAM composition to maximize the swelling ability of hydrogels

Because the overall goal of optimizing hydrogel composition is to create responsive gels that will change volume with very little change in the crosslink density, it was necessary to find a formulation that would allow for additional swelling upon addition of excess PBS. By balancing

the swelling ability of gels with their durability, an optimized gel recipe can be reached. As the gels with 0.5 mL PBS showed the greatest tunability with respect to modulus in Table 1, further testing was done to quantify their swelling ability at different MBAM concentrations relative to the amount of AAm.

The swelling ability of disc hydrogels was measured as function of the polymer and water content as shown in Equation 1, where $W_{polymerized}$ is the weight of the hydrogel after 3 hours of polymerization, and $W_{PBS}$ is the weight of the hydrogel after it has fully swelled in PBS for 2 days. By taking the difference of the two and dividing by the fully swollen weight we can get a normalized percent swelling which can be compared to swell ability measurements of other gels.

**(Equation 1)** $$Percent\ Swelling = \frac{W_{PBS} - W_{Polymerized}}{W_{Polymerized}} \text{ x } 100\%$$

Additionally, the percent solids content, $W_{solids}$, is a commonly measured quantity that is a measure of the polymer content of a hydrogel and is expressed as the percentage of weight of the xerogel, $W_{xerogel}$, compared to the fully swelled hydrogel weight. A xerogel is a solid formed from a gel by drying with unhindered shrinkage.

**(Equation 2)** $$W_{solids} = \frac{W_{PBS} - W_{xerogel}}{W_{PBS}} \text{ x } 100\%$$

The percent swelling of the gel will show how able the gel is to swell after polymerization and the percent solids will similarly show the total amount of water a hydrogel holds when fully swollen in solution is relative to its dry weight. More importantly, the percent swelling of the gel will be related to the gels ability to respond to differing solution. If the gels are highly cross-linked they will not be able to change greatly with respect to differing solutions as a function of osmotic pressure or in response to stimuli, which in this case is an imprinted

protein. While the percent solids measurement is similar, it does not take into account the intermediate swollen state of the hydrogel after polymerization which will be a function of the concentration of monomers in the polymerization solution.

A series of hydrogels containing 0.125, 0.25, 0.5, 0.75, 1.0, and 1.5 mole percent MBAM was created with two replicates of each gel formulation. In addition to MBAM, 11.3 mg AAm was polymerized using APS and TEMED in a reaction volume of 100 µL where the excess volume of the solution was composed of PBS. The gels solutions were initiated, put into disc molds, and allowed to polymerize for a period of three hours. Once polymerized the weight of each gel was measured and then the gels were placed into PBS for 2 days to swell to their final equilibrated volumes and the weights were again measured. Lastly, the gels were put into an oven at 40 ºC for 2 days so that all water would be evaporated from the gels. The weight of the xerogel was recorded and equations 1 and 2 were applied to plot the swelling ability and percent solids versus the mole percent of MBAM below.

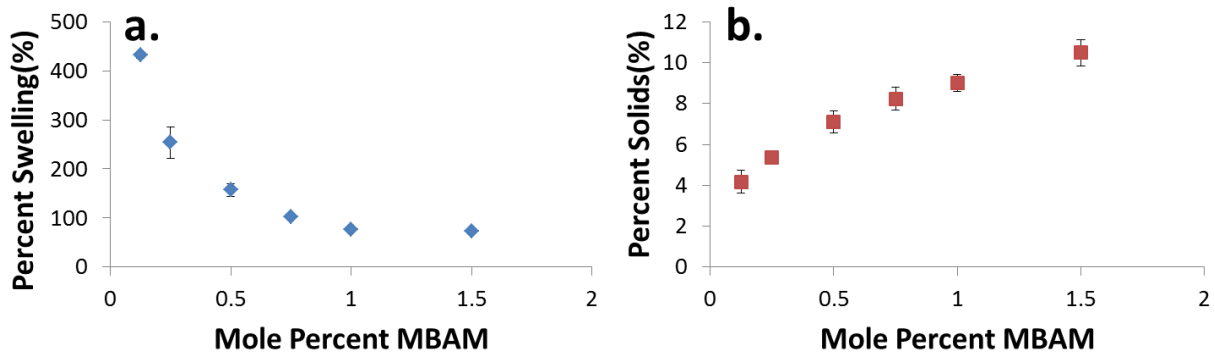


Figure 8: Effect of MBAM concentration on the water content and swelling ability of hydrogels. a.) The swelling ability of hydrogels is measured with respect to the mole percent of MBAM as determined by Equation 1. b.) The percent solids content of the same hydrogels is also measured with respect to the mole percent of MBAM by Equation 2.

The percent swelling of the gels predictably increases as the mole percent MBAM relative to AAm decreases because as the crosslink density of the gels decreases and the gels are able to further expand to a more fully hydrated state. Similarly, the percent solids decreases as the mole percent MBAM increases. The results show that the gels become more constricted at higher crosslink densities they are less able to swell in PBS. The mechanical properties of hydrogels limit the potential range of MBAM content from 0.25 percent to 1.0 percent for the current system. While a maximum percent swelling would obviously occur at the lowest MBAM content, disc gels are not very easy handled at 0.125 or 0.25 mole percent. At 0.5 mole percent MBAM the swelling ability in PBS is still good and the hydrogel chains are still cross-linked to a low enough degree that they should be able to give good responses to protein in both disc and capillary imprinted systems.

### 2.3.3 Quantifying the inclusion of aptamer-thrombin complex into imprinted hydrogels

The last component of the imprinted hydrogels is the inclusion of the imprinted complex. The mole percent of binding receptors used in similar systems in the literature varies greatly and does not always use equimolar amounts of each receptor when multi-receptor systems are used.[25-27,30] Based on responses observed with these other systems, it was determined that a ratio of ~10,000:1 would be a good starting value for the ratio of each component of the imprinted complex relative to AAm as described in Table 2.

Table 2: Initial recipe for thrombin imprinted hydrogels. The reagents listed in the table are ratios used for a 100 microliter batch where PBS is the solvent for each stock solution as well as the polymerization solution.

| **Reagent** | **MW (g/mol)** | **Mass of reagent (mg)** | **Reagent concentration (M)** | **Molar equivalents of reagents** |
|---|---|---|---|---|
| thrombin | 36700 | $6.07\times10^{-2}$ | $1.65\times10^{-5}$ | 1.0 |
| F1 | 6838 | $1.13\times10^{-2}$ | $1.65\times10^{-5}$ | 1.0 |
| F2 | 11198 | $1.86\times10^{-2}$ | $1.65\times10^{-5}$ | 1.0 |
| Acrylamide (AAm) | 71 | 11.3 | 1.59 | $9.64\times10^{4}$ |
| methylene bisacrylamide(MBAM) | 154 | 0.123 | $7.97\times10^{-3}$ | $4.83\times10^{2}$ |

While the reagents are reported in Table 2 are in relation to the number of moles of the imprinted complex, most studies hereafter will relate to the number of molar equivalents relative to AAm because the concentration of polymer in the hydrogel is a function of the AAm content as greater than ninety-nine mole percent of each gel is AAm. All hydrogels reported here, unless otherwise stated, have a constant concentration of AAm 1.59M. This is important for being able to compare hydrogel responses based on changes in other reagents. Even though the AAm concentration is the only value that will remain constant, the total concentration of monomer in the polymerization solutions will remain relatively constant because all of the other monomers are in much lower concentrations.

### 2.4 Measuring Bio-Imprinted Hydrogel Responses as a Function of Water Content

After the initial gels were created for roughly approximating the mechanical properties, new gels containing imprinted complex were made to measure and quantify the responsive nature of protein bio-imprinting. Because the response of removing or rebinding protein to an

imprinted complex involves the addition or removal of crosslinks in the hydrogel, it logically follows that the main manifestation of such an event would be the respective shrinking or swelling of the hydrogel in the presence of excess PBS as shown in Figure 9.

There are two main methods for physically measuring volume changes for both the disc and capillary molded hydrogels. Because 90 percent of the weight of a hydrogel is typically water, volume changes should be able to be measured directly by the change in weight. Any loss or gain in weight will be due to a respective loss or gain in the volume of water in the polymer network upon addition or loss of thrombin. This should hold true as long as PBS is used as the solvent, otherwise volume changes of the polymer could be due to changes in solubility or salinity. The second method for observing hydrogel response is to directly measure the dimensions of the hydrogels in order to calculate volume changes. Volume changes were approximated by measuring one dimension and observing the percent change as reversible crosslinks were formed and broken. Unless otherwise specified, measurements of capillary gels were performed by holding all but one dimension constant for easier and more accurate measurement of volume changes. This was simply done by leaving the gels inside the capillary tube after polymerization so that the hydrogel could only swell in length but not diameter.

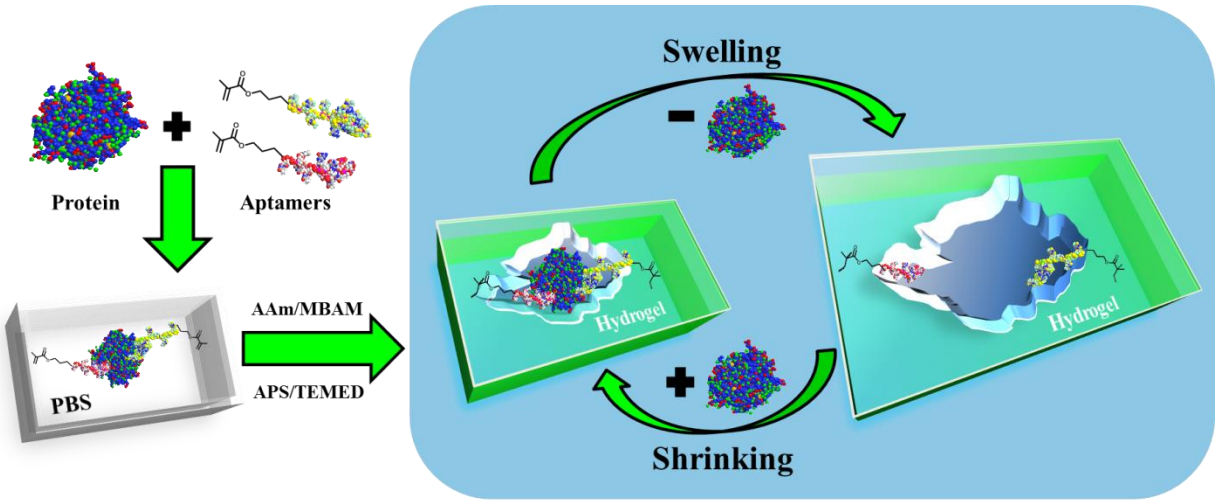


Figure 9: Outline of the biomolecular imprinting scheme used to create "super-aptamer" thrombin responsive hydrogels.

### 2.4.1 Measuring the imprinting effect using length changes of hydrogels in capillary tubes

The first imprinted hydrogels were polymerized in capillary. This method, previously seen in the literature, is a simple means of monitoring volume changes in responsive hydrogels. Because the gels are measured in capillary tubes, the volume changes are limited to one dimension. The lengths of hydrogels are measured using a magnifying glass and a reticle having 0.1 mm intervals. Length measurements are recorded as the distance between meniscuses on either side of the capillary tube or in cases where the meniscus is not clear the shortest distance between the two edges of the gel. Measurements are normalized by calculating percent change in length which is proportional to the overall volume change (equation 1).

**(Equation 3)** $$\text{Percent Shrinkage} = \frac{d_0 - d}{d_0} \times 100\%$$

Where:

d = length of the gel in the presence of protein.

$d_0$ = length of the gel in the absence of protein.

The length, $d_0$, is always reported as the length of gels re-equilibrated in PBS after regeneration. It should also be noted that in cases where weight or other hydrogel dimensions are measured, the same equation can be used where the percent shrinkage is the difference between initial and final states divided by the initial measurement.

#### 2.4.1.2 Quantitative validation of the proposed change in hydrogel morphology

A set of three hydrogels was polymerized according to the recipe in Table 2. The polymerization solutions were loaded into capillary tubes and polymerized for three hours. Following polymerization, the gels were equilibrated in PBS to remove residual monomer and allow for complete swelling of the gels. They were then regenerated using a 4.3 M guanidine hydrochloride / 1.4 M sodium chloride (GuCl/NaCl), re-equilibrated in PBS, and then equilibrated in 1 μM thrombin in PBS. Solutions in the capillary tubes were changed daily and equilibration in each solution was determined as the point where the gel length did not change for 2-3 days after the expected change occurred and the rate of change began to decrease.

Our initial results for response of an imprinted hydrogel showed that imprinted hydrogels swell upon removal of thrombin and shrink 5-12% when rebound with thrombin in PBS (Figure 10). Non-imprinted gels containing only AAm and MBAM showed no increase in volume when "regenerated" and would not shrink upon being exposed to the thrombin solution. In fact, in some cases the AAm control gels swelled slightly upon addition of thrombin solution to the capillary tube.

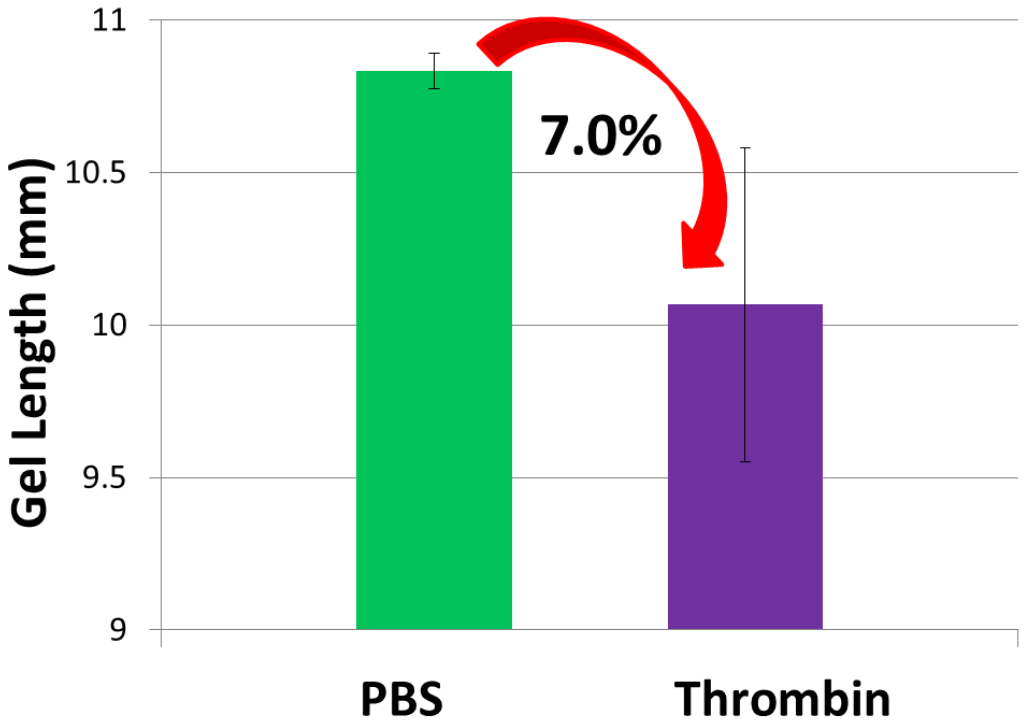


Figure 10: Initial results for response of imprinted hydrogels to thrombin. Three thrombin imprinted hydrogels were polymerized via the recipe in Table 2 then regenerated with GuCl / NaCl, re-equilibrated in PBS and rebound with 1 µM thrombin. The percent shrinkage represents the change as describes in Equation 3 between the regenerated length in PBS and the equilibrated length in thrombin solution.

While this was a promising result, determining the final volume change required 4 to 7 weeks to complete one cycle of regeneration and rebinding depending on the length of gel in the capillary tube. In addition to the undesirable time required for volume changes, gels would also occasionally exhibit some type of growth over such a long period if there was any dust contamination. While gels were found to be stable for many months, continued poking of the meniscus of the gel at the hydrogel-solution interface sometimes resulted in damage to the gels and could deteriorate the reproducibility of measurements over time.

### 2.4.2 Increasing imprinted response times by measuring the change in weight

In order to speed up the response times of the hydrogels it was hypothesized that an increase in the surface area to volume ratio would expedite the diffusion of protein and other

solution analytes in and out of the gel. Toward that end, imprinted and plain AAm disc gels were prepared for comparison of the volume change due to thrombin recognition. Once the hydrogels were polymerized they were equilibrated in successive regeneration, buffer, and rebinding solutions as was done with the capillary gels previously. During this time, solutions were initially changed once a day. Every time the solution was changed the gels were measured three times each to get an average value and a standard deviation. Between measurements the gels were left in solution for five minutes to prevent them from drying out.

The gels all swelled approximately twenty percent in regenerating solution because it was a saturated salt solution as opposed to the more dilute PBS used for polymerization and rebinding. When gels were re-equilibrated in PBS and later rebinding solution there was no real appreciable difference in the weight compared to their initial state prior to regeneration. This was not necessarily a negative result for an imprinted response. The differences that should have been seen by removing and adding thrombin were masked by the inherent error in the experiments. Because the gels were relatively small at about 70-100 mg, a 5-7 percent change was within one 4-5mg standard deviation seen in both AAm and imprinted disc measurements. The major reason for the error was the excess PBS on the gels when taken out of solution. Attempts at removing excess water with kim-wipes or paper towels often gave larger deviations because they would absorb water from the gels as well as from the surface.

As there was a larger change seen in regeneration solution, the equilibration procedure could be optimized. It took between 5 and 7 days to equilibrate the gels in regenerating solution when solutions were changed once a day. In order to increase the equilibration time, solutions were changed more frequently each day. As before, multiple measurements were taken at each solution change and it was determined that disc gels will equilibrate within two days if the

solutions are changed 5 times a day with at least 1-1.5 hours between solution changes. When time was shorter than one hour between solution changes the gels didn't show a very appreciable difference and even when more solution changes were performed in a single day it still took more than 24 hours to fully equilibrate. As expected, equilibration experiments showed that gel size or weight gradually reached a maximum with respect to equilibration time and number of solution changes. Also, as the maximum value was approached the maximum value, the change with respect to time decreased. Having multiple solution changes beyond those outlined would facilitate faster equilibration at first but had less of an impact as the gel approached equilibration.

### 2.4.3 Measuring hydrogel surface area to quantify an imprinted response

Because the change in hydrogel volume could not be deconvoluted from error by weighing, it was decided that a direct change in volume could be determined more accurately by measuring the diameter because it was the larger dimension of volume change and easier to measure mechanically than the thickness. Towards that end, imprinted disc gels were made as before, but it quickly became obvious that measuring the diameter of a disc gel was not a very reproducible endeavor. Cutting hydrogels into discs very rarely results in very clean cut edges and as such, disc gels are never perfectly circular, making it difficult to reproducibly measure the diameter. Therefore, instead of directly measuring the diameter, integration using paper weighing methods was employed by scanning the gels, printing out the representative scan, then cutting out and weighing the paper representations of the discs.[130]

The averages and standard deviations suggest that even with multiple samples, enlarged pictures, and duplicate pictures of each sample, any response, if there was one, was not distinguishable from the error propagated by the measurement and data collection processes. Generally speaking, the gels exhibited a standard deviation of 2-4% in most cases and never

showed a response greater than 4 to 5 percent. The error in these samples can be most likely attributed to the method itself and human error. In addition, when scanning images, the gels often cast shadows and made it difficult to determine the actual surface edge of gels in pictures which in turn increased the error in averaged measurements. Also, the photos, while larger and not quite as likely to propagate error by a slight miss-cut, also became less detailed around their edges when blown up. As a result the edges were more pixelated, further convoluting the edges of the gel pictures.

### 2.4.4 Following volume changes of capillary gels removed from the tube

As it was becoming more apparent that using disc gels to measure volume changes was not to be possible; an alternative method was necessary if a faster equilibration method could be found. Toward that end, capillary gels were polymerized inside of a tube and, after polymerization was complete, were removed from the tube for subsequent measurement. To do so, gels were first dehydrated slightly so that they could be pushed out of the tube without smashing the gels or deforming the ends. Once gels were removed, the edges of the gels, which form a meniscus in the capillary tubes, are cut off so that measurement of the gel length can be measured at a definitive edge. Solutions were changed similarly to previous methods weighing measurements showed similar results to those seen with the disc gels with responses in the order of 3 to 5 percent in the presence of rebinding solution with an error of about 3 to 4 percent indicating that the difference between imprinted gels and controls gels was difficult to determine,. Surprisingly, tracking the gel lengths didn't result in a discernible difference between the response and the error either. Because the error of the measurements was one percent or less for most samples several, there must be another underlying reason why no response can be tracked by this method. Either the gels are not showing any response outside of

the capillary tubes or the swelling with respect to length is less than the swelling with respect to diameter when the gels are unhindered by a capillary tube. Tracking volume changes of the capillary gels inside a tube gave results which were smaller than the error. It is possible that the constraints of the capillary tube dimensions forces larger changes in length because there is no other dimension in which to swell. That may also be the reason for the longer gel swelling and relaxation times in each of the solutions as equilibration occurs at a faster rate outside of the tubes as compared to the gel equilibration times inside the tubes.

Thus, measuring volume changes inside the capillary was the most effective means of observing the presence or absence of thrombin in an imprinted gel, even if the time to complete a cycle of rebinding was greater than desired. It was evident that gels would equilibrate faster if solutions were changed more often, especially within the first several days after a different solution was added to a capillary tube. It is also important to note that imprinted hydrogels would exhibit an imprinted rebinding response commensurate with previous results if the gels did not fully equilibrate in regenerating solution. The equilibration of the hydrogel appears to require more time than the dissociation of the protein from aptamers. The increased equilibration time in a capillary is likely due to a greater relaxation time required because of the spatial constraints of the glass capillary tube.

From this point on, solutions in capillary gels were utilized, and changed 3-5 times a day to facilitate faster equilibration times. Also, it was observed over the course of several experiments that regeneration of aptamers actually takes place faster than the equilibration time of the gel because gels exhibited the same shrinkage when rebound if the equilibration step during regeneration was not fully completed but instead cut short by several days when the change in length was 0.1mm or less per day. Assuming that the concentration of regenerating

solution is at its maximum throughout the gel, this shows that the capillary gels do not need to reach their complete equilibration lengths in regenerating solutions. Doing so requires waiting for long relaxation times associated with swelling approximately twenty percent and also the additional time required to shrink when regeneration solution is removed in favor of PBS. For future experiments, the regeneration of imprinted sites will be considered complete as long as the gels have gone through the shrinking due to the change in osmotic pressure after the initial change to regeneration solution and the subsequent swelling as the salt concentration increases. When the rate of swelling reaches 0.1 mm per day or less the regeneration of imprinted sites within the hydrogel were be considered finished. Typically, gels have already swollen greater than 10% by the time that rate of length increase occurs and the solution is already mostly equilibrated as evidenced by the fact that there is not a visible change in refractive index of the solution in and around the gel upon further solution changes.

One additional change that could also help decrease the response times of gels inside of a capillary tube is to decrease the size of the gels. Typically, in previous tests it was desired to make gels as long as possible to maximize the response of imprinted gels compared to AAm gels and the error. Gels can only be polymerized to lengths of about 15 mm at most because of practical limitations of polymerizing in capillary tubes, but once swollen completely in PBS and regenerated these same gels can be up to 20 or 25 mm. This can increase the equilibration time as discussed before because longer gel lengths require more time to relax and also swell and shrink completely in response to solution changes. Additionally, these gels are polymerized in tubes that are 25-30 mm in length and if the capillaries fill up ninety percent or more of the tube then the amount of solution that can be equilibrated with each solution change is greatly reduced. If the gels polymerized are limited to 5-10 mm when polymerized the time is greatly reduced for

equilibration in each solution both because gels themselves are shorter and also because the solution removed and inserted with each solution change will be greater facilitating a more rapid equilibration.

### 2.5 Regeneration of Imprinted Sites within Hydrogels by Removal of Thrombin

As illustrated, the shrinking and swelling of the gels is a direct reflection of the presence or absence of thrombin. In order to remove thrombin from imprinted hydrogels it is necessary to either denature the protein bound to the gel, temporarily denature the aptamer structure, or interrupt the conjugation between the two. In the first case, enzymatic degradation can be utilized because the protein is not needed after regeneration. In the latter two cases the addition of salts that act as chaotropic agents towards intermolecular and intramolecular associations of protein and DNA are used so that the structure of the aptamers can be conserved for later rebinding of additional thrombin.

#### 2.5.1 Regeneration using guanidine hydrochloride and sodium chloride

Guanidine hydrochloride was the regenerating solution used for the initial studies mentioned above. In these cases, a saturated salt solution containing 4.3M GuCl/1.4M NaCl was used for the removal of thrombin. The gels initially exhibit a slight shrinkage in length because of the difference in osmotic pressure observed when going from a lower salt concentration to a higher concentration. The gels then swell to approximately 120 percent of their original size once equilibration was reached. As the gels approach equilibrium, the increased salt concentration acts to increase the polarity of the solvent and destabilize the hydrogen bonding of g-quartet secondary structure of the aptamers that is responsible for conjugation with thrombin.[131,132] As shown in Figure 10, the use of GuCl/NaCl solutions typically resulted in the removal

of enough thrombin from imprinted sites to facilitate shrinkage between four and twelve percent upon addition of thrombin to imprinted gels that were re-equilibrated in PBS after regeneration.

### 2.5.2 Regeneration using sodium perchlorate

Sodium perchlorate was another chaotropic salt found to remove thrombin from an affinity chromatography assay where the A1 aptamer to thrombin was immobilized on the column.[133] The column was found to specifically bind thrombin as opposed to other common proteins, but the thrombin stuck to the column and didn't elute under continued buffer flow. 2 M sodium perchlorate was found to destabilize the aptamer-thrombin interaction and the thrombin eluted from the column.[134]

The use of sodium perchlorate as a regeneration agent was tested for regeneration of imprinted gels as an alternative to using GuCl/NaCl regenerating solution. Two imprinted capillary gels were polymerized using the recipe in Table 2. The gels were regenerated in 4.3M GuCl/1.4M NaCl, re-equilibrated in PBS, and then rebound with 1 μM thrombin in PBS. The gels were then regenerated using 2 M sodium perchlorate, re-equilibrated in PBS, and again rebound with 1 μM thrombin in PBS.

The gels exhibited 4.3 percent shrinkage upon rebinding with thrombin after having been regenerated with GuCl/NaCl. In contrast, the gels showed a response of less than 1% when rebound with thrombin after being regenerated with the sodium perchlorate. While the amount of thrombin that eluted from the capillary tube was never directly measured, it can be deduced from the experiment that the GuCl/NaCl solution was a more effective solution for interrupting the aptamer protein interaction because the response seen upon rebinding was greater. The other

possible reason for the increased response could be that the sodium perchlorate solution is only effective for regenerating the A1 aptamer from thrombin.

### 2.5.3 Regeneration using a trypsin digests

Trypsin was tried as a means of protein removal from the DNA aptamers without the use of a chaotropic agent. Trypsin is a serine protease which breaks amid bonds in proteins where the carboxyl side of the amino bond is a lysine or arginine, but only in cases where the next residue on the amine side of the lysine or arginine is not proline.[135] It is commonly used to degrade proteins into smaller subunits which are more easily sequenced for determination of the primary structure of proteins. Ideally, by enzymatically degrading thrombin there will no longer be a complete binding receptor site with which to bind either aptamer. The residual protein pieces will be able to diffuse out of the gel quicker than they would as a full globular protein. As a consequence, there should be a shorter equilibration period. The use of a protein in buffer as a regeneration solution should also decrease the equilibration time of the regeneration step because there isn't a large change in the salt concentration as compared to the chaotropic agents mentioned earlier.

A set of three imprinted capillary gels were polymerized using the recipe in Table 2. The gels were then regenerated using 4.3M GuCl/1.4M NaCl solution, re-equilibrated in PBS, then rebound with 1 μM thrombin. All of the gels were equilibrated in PBS to remove excess thrombin and then injected with a 12 μg/mL trypsin solution in PBS. The solutions were changed several times a day in subsequent days. After being left overnight to incubate at room temperature the solutions surrounding the gels in the capillary tubes all began to appear opaque and exhibit growth of some sort. The turbidity made it difficult to accurately judge the edges of the gel meniscuses and determine the equilibration length of the gel. The problem worsened in

subsequent days of treatment because the turbidity also became prevalent in the gel as opposed to just the solution surrounding the gel in the capillary tube. Within 2-3 days the gels were no longer usable and it was determined that this was not a very viable method for removing thrombin from the gels.

### 2.6 Altering the Hydrogel Formulation to Optimize the Imprinting Effect

Because the imprinting effect has been shown to be reproducible in multiple different gels, it's important to optimize the imprinting effect with respect to the recipe in Table 2. Doing so will allow for a greater signal to error response to thrombin in comparing results for non-specific binding of non-imprinted proteins. The increase in signal to error will also help increase the imprinted response compared to controls and allow for a better representation of the effect of thrombin in imprinted hydrogels in order to confirm or identify deviations from the super-aptamer binding scheme put forth in Figure 9.

#### 2.6.1 Maximizing imprinting effect by optimizing MBAM concentration

A set of three hydrogels were polymerized containing recipes similar to the one showed in Table 2 varying only in the content of MBAM. The three gels contained 0.25, 0.5, and 1.0 mole percent MBAM relative to the moles of AAm in the gels. Table 5 shows the response of each gel after it had been regenerated with 4.3M GuCl/1.4M NaCl, re-equilibrated in PBS, and then rebound using 1 μM thrombin in PBS.

The values reported in Table 3 for percent shrinkage show that the optimum amount of MBAM required to maximize the imprinted response toward thrombin was 0.5 mole percent MBAM with respect to AAM. The gel containing twice as much MBAM showed a lower response because, having a higher cross-link density, it was not able to swell as much in response

to thrombin and not necessarily because the MBAM content altered the proximity of aptamers or the ability of thrombin to rebind and reform the imprinted complex. The gel containing less MBAM also showed a lower response to thrombin compared to gel containing 0.5 mole percent. In this case the aptamers are not as proximal to each other in the gel to fully rebind. When thrombin was added back to the gel, pre-organized pairs of aptamers were no longer close enough in the gel to overcome the increased relaxation observed as compared to the 0.5 mole percent MBAM gel. Because the cross-link density was lower there was less rigidity in the hydrogel matrix and the imprinted site was not as completely conserved.

Table 3: Optimization of imprinted response with respect to MBAM cross-linker content.

| **Entry** | **MBAM (mg)** | **Equivalents** | **Mole Percent** | **% Change in volume** |
|---|---|---|---|---|
| 1 | 0.123 | 1 | 0.5 | 5.46 ± 0.95 |
| 2 | 0.0613 | 0.5 | 0.25 | 4.37 ± 2.3 |
| 3 | 0.246 | 2 | 1.0 | 2.59 ± 2.2 |

**2.6.2 Maximizing the imprinting effect by optimizing the amount of preformed complex.**

A similar study was done to optimize the imprinting effect by finding the most advantageous concentration of preformed aptamer complex. Three gels were polymerized using the recipe in Table 2 and varied only by the aptamer complex concentrations having $1.65x10^{-5}$, $0.83x10^{-5}$, and $4.95x10^{-5}$ M. These concentrations, relative to the recipe in Table 2, correspond to 1.0, 0.5, and 3.0 equivalents of imprinted complex respectively. The response of each imprinted gel is shown in Table 4.

The gel containing one equivalent of complex was found to shrink the most in 1 μM thrombin after being regenerated with 4.3M GuCl/1.4M NaCl and re-equilibrated in PBS. The

gel containing 0.5 equivalents of complex showed a lower shrinkage which would be predicted in reference to the gel with one equivalent because, having less complex, it should show less of a response to the imprinted protein. The gel with 3 equivalents also showed a lower response though. This may be due to an overabundance of complex in the gel; meaning that the imprinted complex is not correctly reformed. Because the hydrogels have relatively low crosslink densities, the arrangement of the polymer around thrombin is not as well defined. If protein were to rebind to the A1 in one imprinted site and the A2 of a different site then the reformation of the original volume observed before regeneration may not be obtained. As a consequence, such a binding event may decrease the overall amount of thrombin which can be rebound because the presence of thrombin across two sites would likely negate the addition of another thrombin molecule to the two remaining aptamers. The increased excluded volume created when the first molecule of thrombin is bound would likely preclude the addition of a second.

Table 4: Optimization of imprinted response with respect to imprinted complex content.

| Entry | A1-Thrombin-A2 pre-polymer complex (M) | Equivalents | % Change in volume |
|---|---|---|---|
| 1 | $1.65\times10^{-5}$ | 1.0 | 5.46 ± 0.95 |
| 2 | $0.83\times10^{-5}$ | 0.5 | 2.93 ± 1.45 |
| 3 | $4.95\times10^{-5}$ | 3.0 | 4.83 ± 0.05 |

### 2.6.3 The hydrogel recipe for optimal response to the imprinted thrombin target.

After performing the two studies to determine the optimal concentrations of MBAM and imprinted aptamer-thrombin complex, it was found that $7.97\text{x}10^{-3}$ M and $1.65\text{x}10^{-5}$ M, as shown in Table 2, were the best ratios of cross-linker and complex to AAm for obtaining a maximal

response to thrombin. There are several other ratios not immediately visible in the table that should be pointed out. First, the ratio of AAm to MBAM in the hydrogel is roughly 200:1. This ratio is fairly consistent with other responsive hydrogels in the literature and is an integral property to the hydrogel. However, this ratio is not the only information needed to determine the performance of the hydrogel. The initial concentration is integral in determining the eventual percent solids of the gel after swelling fully following polymerization. For this reason the fact that this ratio of monomers is dissolved in 100 μL was kept constant to ensure comparable results for future experiments. Also, the cross-link density due to imprinted complex, assuming that both complex and MBAM equally affect the cross-link density, is roughly 200:1 in favor of the MBAM. Because very small amounts of complex apparently have a much greater effect on the response of the imprinted complex as opposed to changes in concentration of MBAM, it can be hypothesized that the two types of cross-linkers do not equally participate in or affect the cross-link density of the hydrogel.

**2.7 Confirmation of the Incorporation of Imprinted Complex into Hydrogels.**

The presence of the thrombin aptamer complex in the polymerization solution has shown to result in gels that give consistent results when rebound with thrombin solutions. Thus far, it has been presumed that the functionalized aptamers and thrombin were polymerized along with AAm and MBAM upon initiation of the polymerization solution. In order to show that the imprinted response seen is a function of the presence of the complex in the gel it is important to confirm that both the functional aptamers and thrombin are incorporated into the polymer structure.

### 2.7.1 Measuring residual thrombin and aptamer concentration in imprinted hydrogels.

The easiest and perhaps most direct way to measure whether the complex is incorporated is to rinse an imprinted hydrogel with PBS after it finishes polymerizing and see if residual functional aptamer or thrombin is dissolved in the eluent. It is well known that both DNA and protein absorb in the UV region of the spectrum at 260 nm and 280 nm respectively. In order to avoid a convoluted overlap, the absorbance due to DNA and thrombin was shown independently by making gels containing aptamers with and without thrombin present in the polymerization mixture.

A set of disc gels were polymerized by the recipe in Table 2; with two imprinted gels containing the full thrombin aptamer complex, two non-imprinted gels containing only the functional aptamers, and two AAm gels containing neither the aptamers nor the thrombin. Once the gels were polymerized, they were placed into 0.5 to 1.0 mL PBS to rinse out any residual monomer and thrombin. The solution was changed five times a day for two days and all aliquots were saved for UV analysis. These rinse solutions were measured by using a Varian Cary 50 Spectrophotometer with a 1 cm path length in a quartz cuvette from 400 nm to 200 nm. Calibration curves were created both for thrombin and the functional aptamers to quantify their concentrations in the PBS rinse.

The $\lambda_{max}$ observed for the calibrations curve of the functional monomers F1 and F2 was 257 and the regression line obtained according to Beer's Law resulted in good linear correlation of 0.999. As expected, there was not a peak obtained for the presence of protein at the usual $\lambda_{max}$ of 280 though. There was, however a large peak at 205 nm correlating to the amide bonds in the protein backbone. When the wash solutions of all six gels were compared they all showed a large peak at 205 nm and the peak has a prolonged shoulder that went through the range for

DNA at 260 nm. Because all wash solutions contained a large peak at 205 nm, while not all had protein to wash out, it was evident that residual acrylamide washed from samples was present and protein concentration could not be adequately measured by UV at either 205 nm or 280 nm.

Table 5: Quantifying of the incorporation of F1 and F2 into the gel structure by UV detection. A series of two imprinted and two non-imprinted gels were polymerized. After polymerization the gels were rinsed several times with PBS and the rinsing solutions were saved for UV detection of residual aptamer functional monomers.

| | **Non-Imp wash** | **Deviation** | **Imp wash** | **Deviation** |
|---|---|---|---|---|
| Abs at 257 nm | 0.06 | ± 0.01 | 0.07 | ± 0.00 |
| Concentration (μg/mL) | 3.10 | ± 0.28 | 3.55 | ± 0.07 |
| Total mass of DNA (μg) | 15.45 | ± 1.63 | 17.55 | ± 0.35 |
| Abs 260 nm | 0.05 | ± 0.01 | 0.06 | ± 0.00 |
| Abs 280 nm | 0.06 | ± 0.01 | 0.07 | ± 0.00 |
| Abs 260/280 | 1.21 | ± 0.00 | 1.12 | ± 0.05 |

The concentrations of DNA in the wash solutions were determined based on their absorbance at 257 nm. Using the calibration curve, the absorbance values correlated to approximately 15 μg of total aptamer as shown in Table 3. That quantity of DNA equates to roughly 50 percent of the functional aptamer that was put into the polymerization mixture. This means that only 50 percent of the aptamer was polymerized into the hydrogel. There was some convoluted overlap of the peak when analyzing PBS solutions used to wash residual monomers from the gels. As it was seen that there was relatively no absorbance shown for thrombin at 280 nm, the DNA quantification can be more accurately determined by comparing the ratio of absorbance at 280 nm versus the absorbance at 260 nm because there should be no protein seen in the wash solutions at 280. It is well known that for DNA samples the ratio of absorbance between 260 and 280 is between 1.75 and 1.85. This ratio holds for the absorbance seen in the

DNA standards used for linear regression calculations even though there was some variation seen at lower concentrations as the limit of detection for the instrument is approached.

Table 6: UV absorbance of measured concentrations of functional aptamers. Equimolar amounts of each F1 and F2 were mixed as in polymerization solutions. The concentration of these functional monomer solutions were known and the UV absorbance was measured for comparison to UV measurements of unknown wash samples in Table 3.

| | **Concentration (mg/mL)** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Wavelength (nm)** | **29.76** | **14.88** | **9.92** | **7.44** | **5.95** | **4.96** | **4.25** | **2.98** | **1.98** | **1.49** |
| 280 | 0.642 | 0.332 | 0.212 | 0.156 | 0.122 | 0.096 | 0.078 | 0.052 | 0.036 | 0.024 |
| 260 | 0.456 | 0.241 | 0.152 | 0.111 | 0.087 | 0.067 | 0.052 | 0.033 | 0.024 | 0.013 |
| 260/280 | 1.71 | 1.70 | 1.72 | 1.73 | 1.74 | 1.77 | 1.83 | 1.87 | 1.90 | 0.56 |

The values for the ratio at 260 nm to 280 nm for the wash solutions in Table 3 show drastically lower values compared to those shown in Table 4 for the control solutions. These lower ratios show that there is either an impurity that absorbs at 260 nm, 280 nm, or both. So, while it can be confirmed that at least 50 percent of the functional aptamers are incorporated into the polymer backbone, it is likely that there is much more than 50 percent incorporated in the gels.

### 2.7.2 Using fluorescent probes for detection of bound thrombin in hydrogels

It was shown by Hamaguchi et. al. that the presence of thrombin in solution could be detected using a fluorescence probe having a fluorophore and a quenching agent separated by the A1 sequence and a partial complimentary sequence to A1. The looped structure created by the hybridization resulted in the quenching of the fluorophore, however in the presence of thrombin the protein-aptamer binding was favored over the hybridization that gave rise to the looped structure. When the looped structure arising from the hybridization was displaced the fluorophore was no longer quenched and a fluorescence signal

was observed. The fluorescence observed was also shown as a function of the strength of hybridization, which was controlled by varying the number of hybridized nucleotides. It was found that the optimal response was observed when a five nucleotide sequence was used for hybridization to A1.[136]

A similar set of experiments were performed here in order to observe the amount of thrombin bound to an imprinted complex before and after removal of thrombin. Because it was necessary to measure fluorescence inside of the gel in order to measure the presence or absence of complex in our system, the desired target for a proposed fluorescence probe was the A1 aptamer as opposed to thrombin because only the aptamer portion of the imprinted complex was covalently bound to the hydrogel. The fluorescent probe was designed and purchased from Integrated DNA Technologies having a sequence of: 5'-Fluorescein-CCA-ACG-GTT-GGT-GTG-GTT-GG-Dabcyl-3'. The probe contained a 15 nucleotide sequence on the 3' end which is complimentary to the structure of A1 and another sequence of five nucleotides on the 5' end necessary to form a hybridized loop structure that would give rise to fluorophore quenching. The probe was measured to have relatively no fluorescence response at $5x10^{-7}$M in PBS. Upon addition of 1.65 nmoles of F1 to 1 mL of the previous probe solution a $\lambda_{max}$ was observed at 513.8 nm. The maximum fluorescence intensity seen was relatively similar to values obtained for free fluorescein in PBS at the same concentration.

Initially, capillary hydrogels were polymerized containing 1.65 nmoles F1 in 1 mm PBS to test the probe's ability to identify aptamer incorporated within a hydrogel. Fluorescence measurements were performed on gels that were removed from the capillary tube before and after incubation with $5x10^{-7}$M probe in PBS. There was some variance of the fluorescence intensity between measurements and different samples because of the alignment of the gel within the cuvette was difficult to duplicate in each measurement. However, it was clearly visible that the gels fluoresced upon addition of the probe solution because the fluorescence intensity increased from the level of baseline noise to a significant fluorescence signal comparable to solution measurements of the probe with in the presence of thrombin.

Additionally, imprinted gels containing 1.65 nmoles of preorganized complex in 1 mm of PBS were similarly tested. The gels exhibited a fluorescence response when incubated either before or after the removal of thrombin and it was determined that the F1 in the hydrogel had a higher affinity for the probe than for thrombin. This was confirmed by testing the fluorescence intensity of 1 mL PBS containing 1.65 nmoles of a F1-thrombin complex before and after addition of $5x10^{-7}$M probe.

### 2.8 Confirming the Formation of Imprinted Materials

In addition to being reversibly and consistently responsive to an imprinted analyte, one of the other hallmarks of an imprinted material is its ability to bind specifically and selectively to that target molecule. Ideally, that means that the material should have little or no response to a non-imprinted molecule and that it should be much more sensitive to lower concentrations of the imprinted species as opposed to non-imprinted molecules. Additionally, the efficacy of the imprinted response should be directly determined by the formation and incorporation the imprinted complex. Therefore, it is important to rule out any other alternative structure which might occur as a function of the reactants during the polymerization. .

#### 2.8.1 Confirming that the imprinted response is a function of the pre-organized complex

The presence of a polymerizable pre-organized imprinted complex around a target analyte is a prerequisite for the creation of an imprinted polymer. While previously it has been implied that the formation and presence of a pre-organized aptamer-thrombin complex was responsible for the shrinkage seen in response to thrombin in imprinted hydrogels, it is necessary to rule out any other possible structures or protein absorption as a function of varied incorporation of the various subunits of the presumed complex. Several combinations of each functionalized aptamer A1 and A2 as well as thrombin were polymerized into a series of otherwise non-functional hydrogels to look at the response in comparison to an imprinted

hydrogel as seen earlier. Seven gels were polymerized as labeled in Figure 11 below where: the "imprinted" gel contained the entire pre-organized aptamer thrombin complex previously described, the "Non-imprinted" gel contained both F1 and F2 in no pre-organized complex and without the presence of thrombin, the "F1-Th" and "F2-Th" gels each contained a preformed complex between the respective functional monomer with thrombin yet were missing the complimentary second function aptamer, the "F1-only" and "F2-only" gels which have no pre-organized complex but included one of the two functional monomers into the polymerization solution, and an "AAm" gel having neither thrombin, nor either aptamer available for incorporation into the gel.

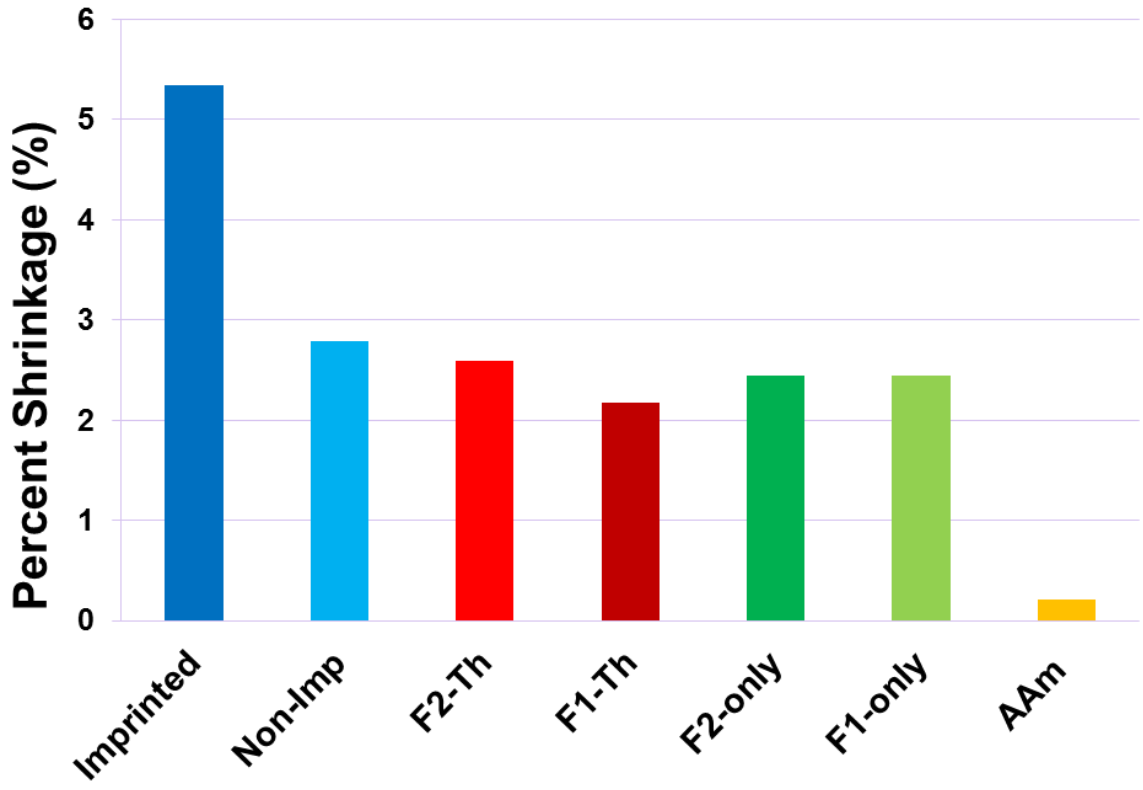


Figure 11: Comparative binding of imprinted gels versus several non-imprinted control gels containing variable components of the preformed aptamer-thrombin complex.

The data in Figure 10 illustrate the importance of the incorporation of a preformed aptamer-thrombin complex to ensure the formation of imprinted sites within the hydrogels. The first, and most important, piece of information in the table is the fact that the imprinted hydrogel in lane 1 from the left shows about double the amount of response in comparison to any of the other controls samples in lanes 2-7. Because the non-imprinted gel in lane two shows no different response from any of the other controls, it can be inferred that the random inclusion of the functional aptamers into the hydrogel does not give a good response to thrombin. This also means that the pre-organization is necessary and that the imprinting effect seen here and previously is a function of the special orientation of the aptamers in the gel as determined by the presence of thrombin during polymerization. Having only one of the functional aptamers and thrombin does not increase the effect observed with the non-imprinted gel. This confirms that the response attributed to the imprinted complex requires the presence of both aptamers. In particular, this is important for the F1-Th hydrogel because, as it was previously mentioned, the A1 aptamer has some affinity for the A2 exocite on the surface of thrombin. The data for these gels in indistinguishable from the response of the gels in lanes 5 and 6 which vary only in the presence of thrombin in a pre-organized complex with either functional aptamer individually. This again confirms that there is no extra "imprinted" response seen when either aptamer is polymerized in conjunction thrombin without the presence of its counterpart. Lastly, the completely non-functionalized hydrogel, AAm, has relatively no response to thrombin upon addition of 1 μM thrombin in PBS. This shows that because each aptamer by itself very selectively and strongly binds to thrombin, as prescribed by the SELEX process, thrombin will bind to any gel that has either aptamer present in it, but that the true imprinted response will not be seen unless the pre-organization of aptamers, as controlled by the presence of thrombin before

polymerization, occurs. Because some response can be seen even though we know that neither individual aptamer nor a non-imprinted random inclusion creates an imprinted response, we know that it is possible that some response in the hydrogel may be due to any presence of thrombin for whatever reason because the AAm gel clearly shows none of the response seen in any of the other control gels. The amount of thrombin bound in that case may not matter though because the non-imprinted gel in lane 2 has twice the total number of moles of aptamer compared to any of the other 4 control gels in lanes 3-6.

### 2.8.2 Reversible rebinding of thrombin into imprinted hydrogels

It has been shown that F1, F2, and thrombin, via the imprinted complex, are incorporated into the hydrogel. It was also shown in Figure 11 that the shrinking response initially seen in imprinted hydrogels was due to the functional imprinting of thrombin into the hydrogel matrix. One of the hallmarks of an imprinted material is its ability to reversibly rebind an imprinted target in repetitive cycles of regeneration and rebinding of that target. In performing such a test, a repeatable response will show that an imprinted site is formed and that the material has some shape, functional, and or size recognized to the intended target.

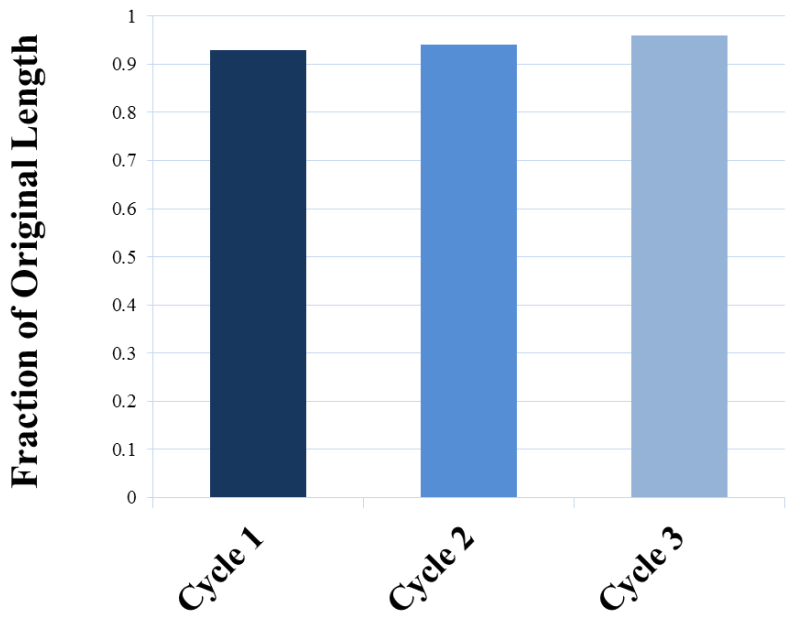


Figure 12: Response of an imprinted gel through multiple cycles of rebinding. Reproducibility of shrinking is shown for three consecutive cycles of volume shrinking by one representative hydrogel. Each cycle began with when thrombin was removed using GuCl / NaCl, followed by a washing step with PBS to remove excess regenerating solution. Rebinding was subsequently evaluated by incubation with 1.0 µM thrombin solution in PBS buffer.

An imprinted polymer was formed using the recipe in Table 2 and put through three successive cycles of regeneration and rebinding. Figure 12 shows that the gel exhibited a relatively constant volume shrinkage from cycle to cycle ranging between four to seven percent. This data shows that the gel response can vary for any given cycle. However, imprinted gels will always respond by shrinking more than a gel that didn't have the imprinted complex in its original formulation. The decrease in response observed over time as the number of cycles increases is an artifact, however, not seen in results for all imprinted gels.

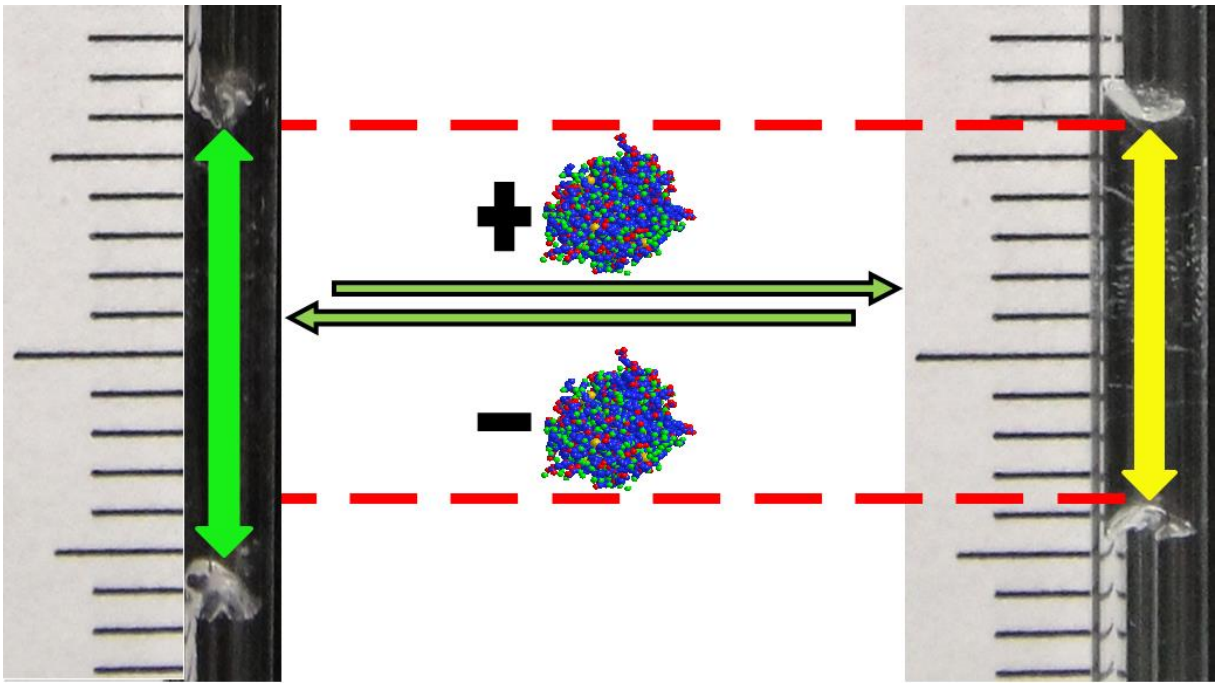

Figure 13: Observed length change seen with respect to thrombin content in imprinted hydrogels. The gel on the left is an imprinted hydrogel regenerated with GuCl / NaCl and then re-equilibrated with PBS while the gel on the right is the same sample that has been equilibrated in 1 μM thrombin.

As was mentioned earlier, the gels are measured using a magnifying glass and a reticle which allows for measurements with a limit of 0.1 mm. By measuring the gels from meniscus to meniscus as seen in Figure 13, the gels exhibit fairly reproducible shrinking over multiple cycles as observed in Figure 12. While in some cases the gel can increase its initial volume in PBS from cycle to cycle, the percent change seems to remain the same. These results have been seen to remain constant for at least 6 months and up to 5 cycles of regeneration and rebinding. This is not necessarily a limit for these materials; there is just not any data past this point. However, if the volume does increase from cycle to cycle there is a tendency for the gel to be more fragile and more prone to damage from the process of daily measurements where a blunt needle is pushed up against the gel meniscus during the process of changing solutions in the capillary tube.

### 2.8.3 Determination of the operational concentration range for imprinted hydrogels

The reproducible rebinding of thrombin to the imprinted complex has already been seen over several studies here. In terms of creating a valued and usable sensor, it is important to determine the range of utility and detection limits for a responsive material. In Figure 12 below, a series of three imprinted hydrogels were polymerized as per the recipe in Table 2, regenerated with 4.3M GuCl/1.4M NaCl, re-equilibrated in PBS, and rebound with various concentrations of thrombin in PBS over several orders of magnitude. The thrombin solutions were injected into capillary tubes on a daily basis until an equilibrated length was reached for each concentration, starting first with the lowest concentration and increasing in concentration with each equilibrated measurement. When changing concentrations the gels were not regenerated or rinsed of the previous solution; just injected with a new concentration continually until a new equilibrated gel length was reached.

The results of the study show that the imprinted hydrogels are responsive to a wide variety of concentrations of thrombin ranging from 1 aM to approximately 1 pM in PBS. The lower side of this spectrum is particularly interesting because the limit of detection is lower than limit of detection for proteins possible for many analytical instruments and far exceeds responsive gels in the literature which have similar concentrations of imprinted biomacromolecules. At that lower limit of detection the percent shrinkage is roughly two percent. This number is analogous to the response seen in the non-imprinted controls in Figure 8. Similarly, the imprinted hydrogels showed some response to concentrations of thrombin lower than those shown measuring about two percent.

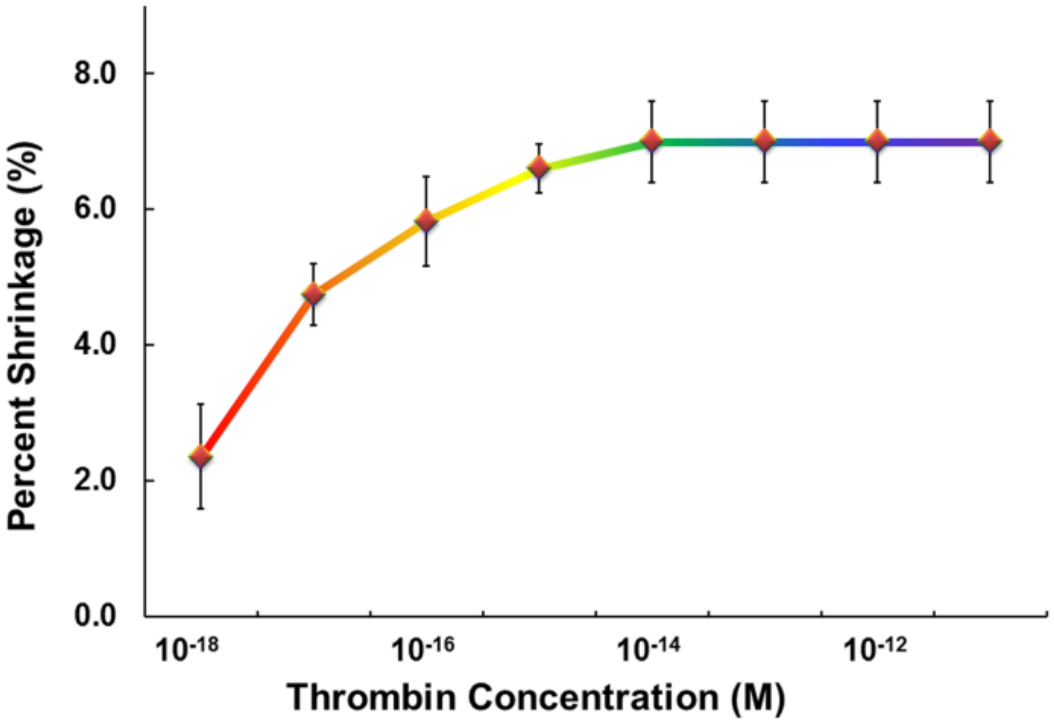


Figure 14: Binding isotherm of percent change in volume of imprinted hydrogels as a function of thrombin concentration.

**2.9 Drying Imprinted Hydrogels to Maximize Rebinding Response**

The responses seen in Figures 12 show that the magnitude of shrinking due to the reformation of the complex upon rebinding of thrombin is fairly consistent from cycle to cycle of rebinding. It does not, however, show that the imprinted complex is completely reformed upon rebinding. When optimizing the MBAM content to maximize rebinding response in Table 3 it was observed that lower cross-linker concentration results in a lower percent shrinkage during rebinding despite the material's ability to shrink and swell more with less cross-linker as shown in Figure 8. It was hypothesized that greater relaxation of the polymer chains due to regeneration and swelling in PBS resulted in a less optimal proximity of the two aptamers at lower MBAM concentrations.

Three imprinted hydrogels were polymerized according to the recipe in Table 2. The gels were regenerated with 4.3M GuCl/1.4M NaCl, re-equilibrated in PBS, and then rebound with 1 nM thrombin. Once the response due to binding was complete the gels were left uncovered overnight so that they could partially dehydrate. The exact extent of dehydration could not be measured because the process tends to deform the meniscuses of the gels making an exact length measurement impossible, however, it was approximated that they were 50 percent of the PBS solution had evaporated. Then the gels were again placed into 1 nM thrombin rebinding solution. The solution was changed several times a day until the gel was again equilibrated in the rebinding solution.

Figure 15 displays that hydrogels initially shrank roughly two percent in thrombin solution before drying. After gels were dried as described above and placed back into the thrombin solution it was found that the gels did not swell completely back to the length seen where they were shrunken 2 percent. In fact, the lengths equilibrated during the $2^{nd}$ rebinding step after drying were smaller than those initially seen before drying and resulted in an additional 5.5 percent change from the initial regenerated state in PBS for a total of 7.5 % shrinkage. This effect was found to be reproducible, both by different gels and in multiple cycles of rebinding. Because the volume of the gel is consistent from cycle to cycle when regenerated, it can be deduced that the structure of the gel is reset to the same state each time thrombin is removed whether the gel had been dried or not. If the gels are not permanently changed by drying then the larger volume change associated with rebinding after drying must be a function of the degree of thrombin rebinding seen within imprinted gels.

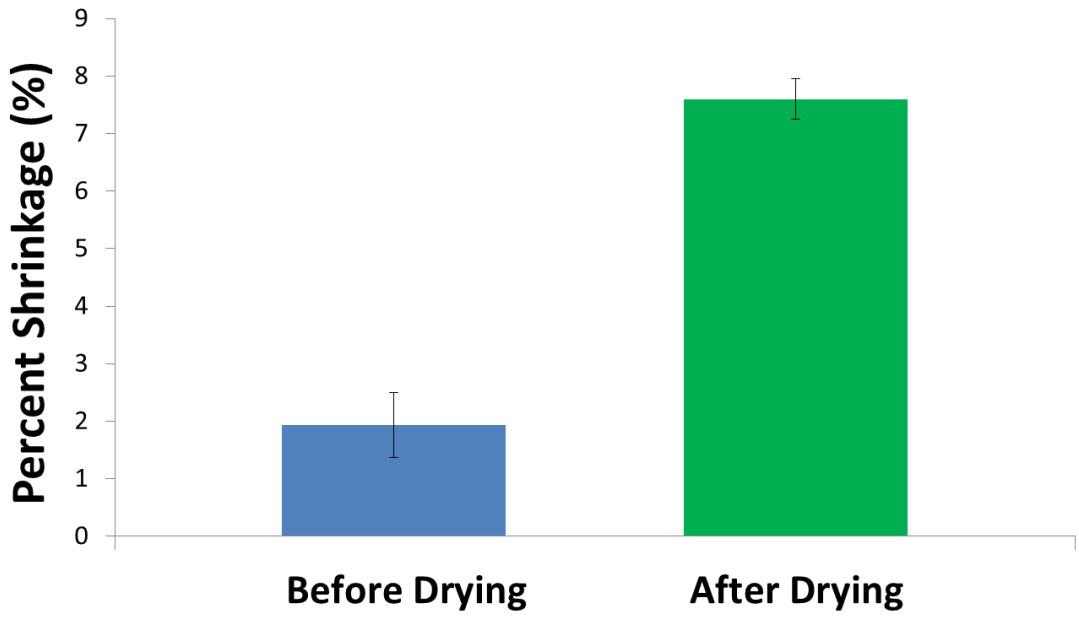


Figure 15: Effect of drying gels on the imprinted response.

The aptamer-thrombin complex, while imprinted into the hydrogel structure, is somewhat distorted upon removal of the protein and the imprinted site is expanded resulting in a decrease in the proximity of the aptamers. It is well known that when making imprinted polymers, not all imprinted sites are equal and that there is a distribution of binding sites based on the random nature of a polymer chain and the statistical incorporation of any of the monomers into the backbone of the polymer. By this reasoning, some imprinted sites within the polymer would be more distorted by the relaxation due to regeneration as opposed to their original swollen state in PBS before regeneration. This relaxation due to the loss of protein is likely also exacerbated by the release of additional tension created when the gels swell in PBS after polymerization. In total, the relaxation may cause some imprinted aptamer pairs to be too far apart for rebinding to be completely efficient. By bringing the aptamers closer together in the partially dried state, as depicted in Figure 16, the reformation of the aptamer complex could be more easily achieved resulting in a larger than previously seen percent shrinkage.

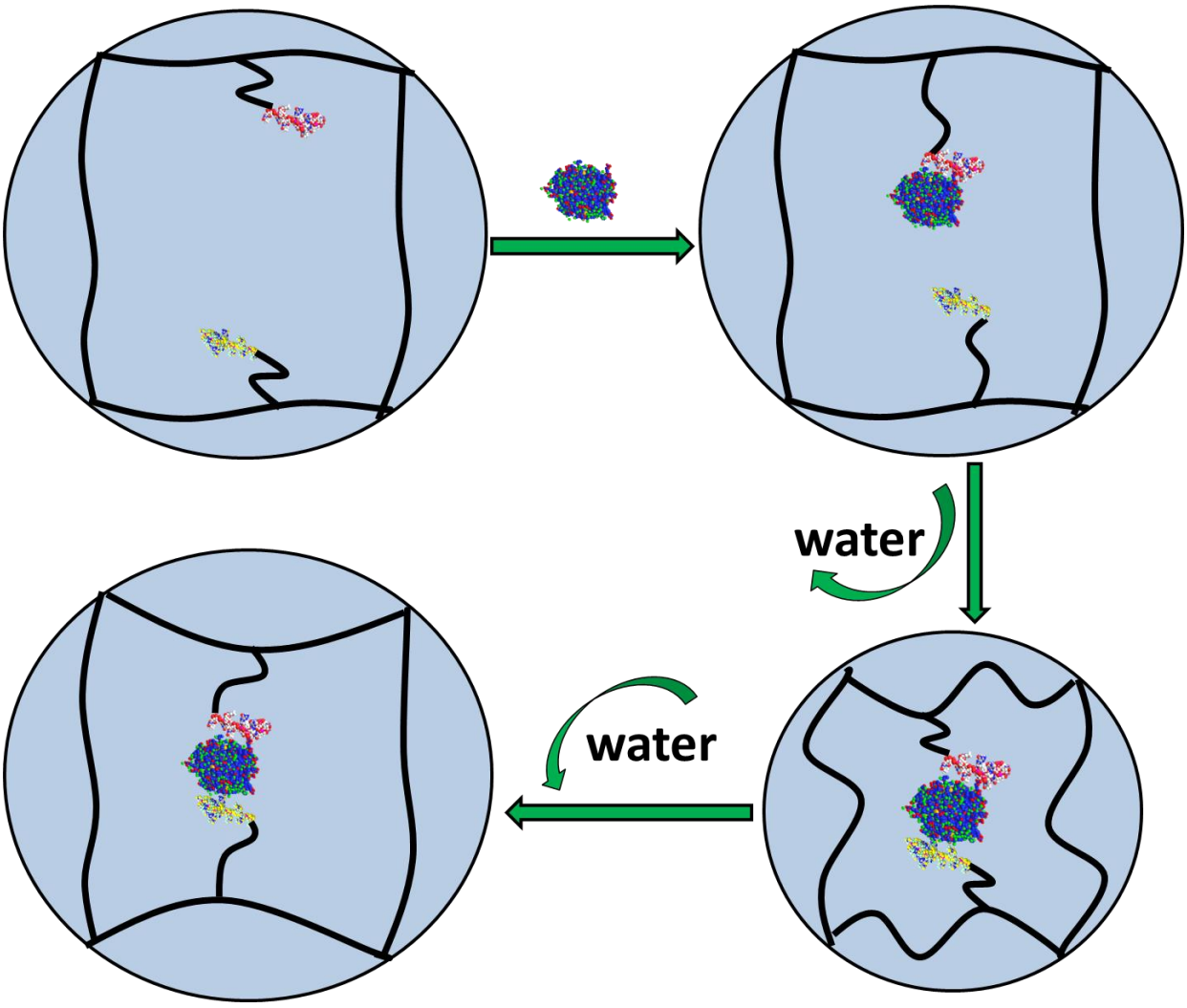


Figure 16: Representation of the hypothesized scheme for increased volume change of rebound gels after drying.

## 2.10 Exploring the Selectivity of Imprinted Gels and Their Utility in other Biological Media

### 2.10.1 Utility of imprinted gels in artificial urine

The imprinted hydrogels have been shown to be responsive to thrombin as compared to non-imprinted hydrogels as well as in repetitive cycles of rebinding. Their utility as an effective sensor is highly dependent on detecting thrombin in biological media while avoiding any purification or isolation steps prior to analysis. The gels would not be effective for in-vivo detection because nucleases are present in bodily fluids such as blood, and cause degradation of

DNA strands such as the aptamers that are responsible for the response seen when rebinding thrombin. In order to be effective as a non-invasive in-vitro sensor for thrombin the gels will need to be both stable and effective in bodily fluids such as urine.

A set of three imprinted capillary hydrogels were prepared using the formulation in Table 2. Following polymerization, the gels were regenerated, re-equilibrated in PBS, and then rebound with 0.1 μM thrombin in PBS. After the gels were equilibrated in the rebinding solution they were allowed to dry approximately 50 percent before being rehydrated with rebinding solution. This was done to see if an exaggerated shrinking response would be obtained when rebinding thrombin to partially dehydrated hydrogels relative to the volume in PBS. Once the gels equilibrated in thrombin again they were regenerated and re-equilibrated; this time in an artificial urine solution[137] instead of PBS. The artificial urine recipe was comprised of 0.1 mg/mL L(+)-lactic acid (80%) 0.4 mg/mL citric acid, 0.49 mg/mL magnesium sulfate 2.1 mg/mL sodium bicarbonate 0.37 mg/mL calcium chloride 10 mg/mL urea, 0.8 mg/mL creatinine, 1.2 mg/mL potassium phosphate (dibasic), 5.2 mg/mL sodium chloride, 3.2 mg/mL sodium sulfate, 1.3 mg/mL ammonium chloride, 0.0012 mg/mL iron sulfate, and 0.95 mg/mL potassium phosphate. Once equilibrated in the urine solution, the gels were then incubated in an equivalent urine solution spiked with 0.1 μM thrombin. As before, the gel was allowed to dry slightly after an initial equilibration so that an increased response to the thrombin upon rehydration could be observed. Incubating solutions used for all equilibration steps were changed several times a day and measurements were taken once a day until there was no change in the length of the gels for two to three days.

Based on the data in Figure 17, it was evident in first cycle of rebinding that the imprinted hydrogel showed a response to thrombin even though the change in volume was not as

great as in previous examples. Additionally, by partially drying and re-equilibrating the gel in rebinding solution increased the response with respect to the initial volume in PBS after regeneration. Likewise, the gel responded to thrombin in the artificial urine solution and gave a similar increased response to the thrombin when partially dried. The response seen due to thrombin in PBS was lower than the response in artificial urine, both when comparing the responses before and after partially drying the gel. This is likely due to the varied salt concentration and composition between the PBS and the artificial urine and appears to be relatively consistent for each of the three gels tested. More importantly, it shows that the imprinted gels are able to respond equally or better in a urine solution than in the experimental PBS conditions. The gels therefore have the potential to be used as ex-vivo sensors for thrombin without the use of complicated test procedures or sample preparation.

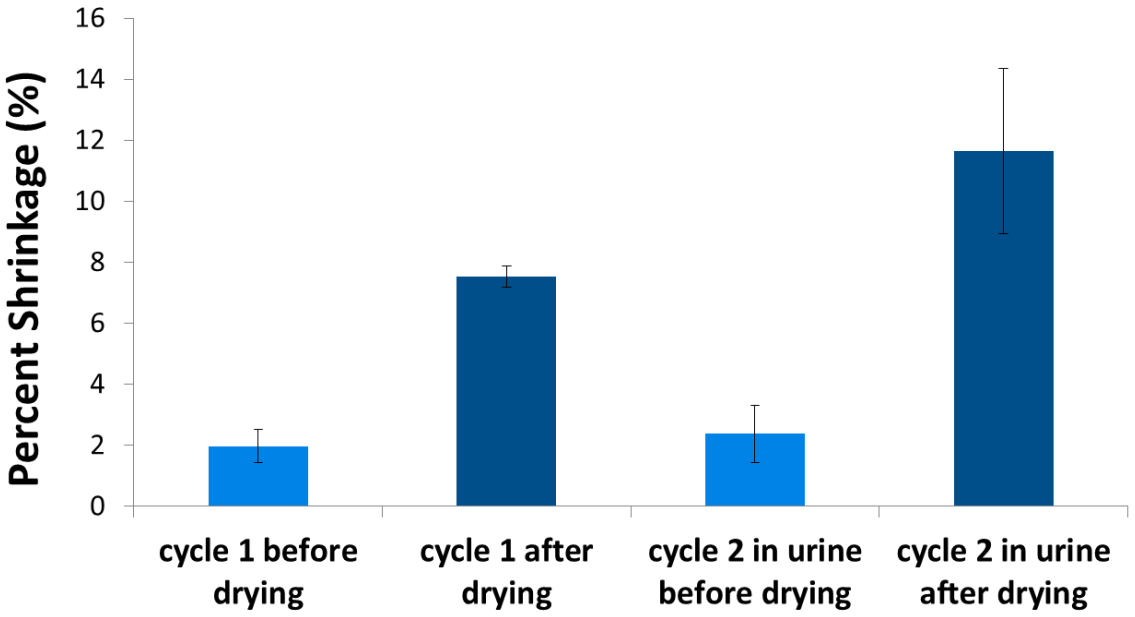


Figure 17: Response of imprinted hydrogels to thrombin in PBS compared to the response of thrombin in artificial urine before and after drying. Hydrogels were tested for sensitivity to thrombin in PBS for cycle 1 and artificial urine in cycle 2. In both cycles the gel response was measured both before and after partial dehydration and rehydration in rebinding solution.

### 2.10.2 Selectivity and specificity of imprinted hydrogels for thrombin

Specificity was evaluated using imprinted hydrogels where thrombin was removed after several cycles of thrombin rebinding. Incubation of the imprinted hydrogels with several alternative proteins including bovine serum albumin (BSA), lysozyme, and cytochrome at concentrations of 1 μM in most cases actually caused the gels to swell up to one percent as opposed to the shrinking that would occur in the presence of thrombin. Additionally, the competitive binding of thrombin with BSA was probed by first regenerating an imprinted gel with 4.3M GuCl/1.4M NaCl then re-equilibrating in PBS before incubating the gel in 1 μM BSA. The gel swelled 0.78 percent at equilibrium and without being regenerated or re-equilibrated in PBS the gel was equilibrated with 1 μM thrombin. It was subsequently shown that the hydrogel was still active toward thrombin after the BSA treatment even when the BSA was not removed first, and the gel displayed shrinking on the same order that is normally found for the thrombin imprinted gels.

## 2.11 Tracking the Source of the Imprinted Response with Respect to Thrombin Content

It has been shown thus far that the functional aptamers F1 and F2 form a complex with thrombin and when incorporated into imprinted hydrogels are responsible for the response seen when rebinding. It was presumed thus far that the mechanism responsible for the response was the loss and reformation of reversible crosslinks throughout the hydrogel network. In order to confirm or disprove this hypothesis the mechanical properties of the gels need to be determined so that a correlation can be made between the gel properties and the presence or absence of thrombin in imprinted gels.

### 2.11.1 DMA determination of modulus as a function of thrombin in imprinted hydrogels

A set of hydrogels was synthesized for DMA analysis where the instrument is capable of very small force controlled gap measurements. A set of disc hydrogels were polymerized for testing: two imprinted hydrogels following the recipe in Table 2, and eight AAm gels containing no complex but having varying amounts of MBAM. The recipe in Table 2 uses 0.5 mole percent MBAM while the control gels contain 0.25, 0.5, 1.0, and 1.5 mole percent MBAM relative to AAm. These MBAM concentrations were chosen to see if there is a large difference in modulus with respect to the change in cross-link density; both of the control gels and the imprinted gels. All gels were polymerized then equilibrated in PBS to fully swell. They were then regenerated with GuCl/NaCl and re-equilibrated in PBS and were finally rebound with 0.1 μM thrombin in PBS. The gels were measured in PBS before and after regeneration and again in thrombin following rebinding. The gels were equilibrated in each solution by changing the solution 5 times a day for 2 days. Static oscillatory compression modulus measurements were taken using a force controlled gap of 0.05 N, an initial load force of 0.001N, and an initial oscillation of 2 μm. Data was not collected for the first minute of measurement so that the materials' strain could normalize with respect to the stress applied then data was collected every 3 seconds for an additional minute. Each disc gel was run twice and the 4 measurements (there were 2 replicates of each gel) were averaged.

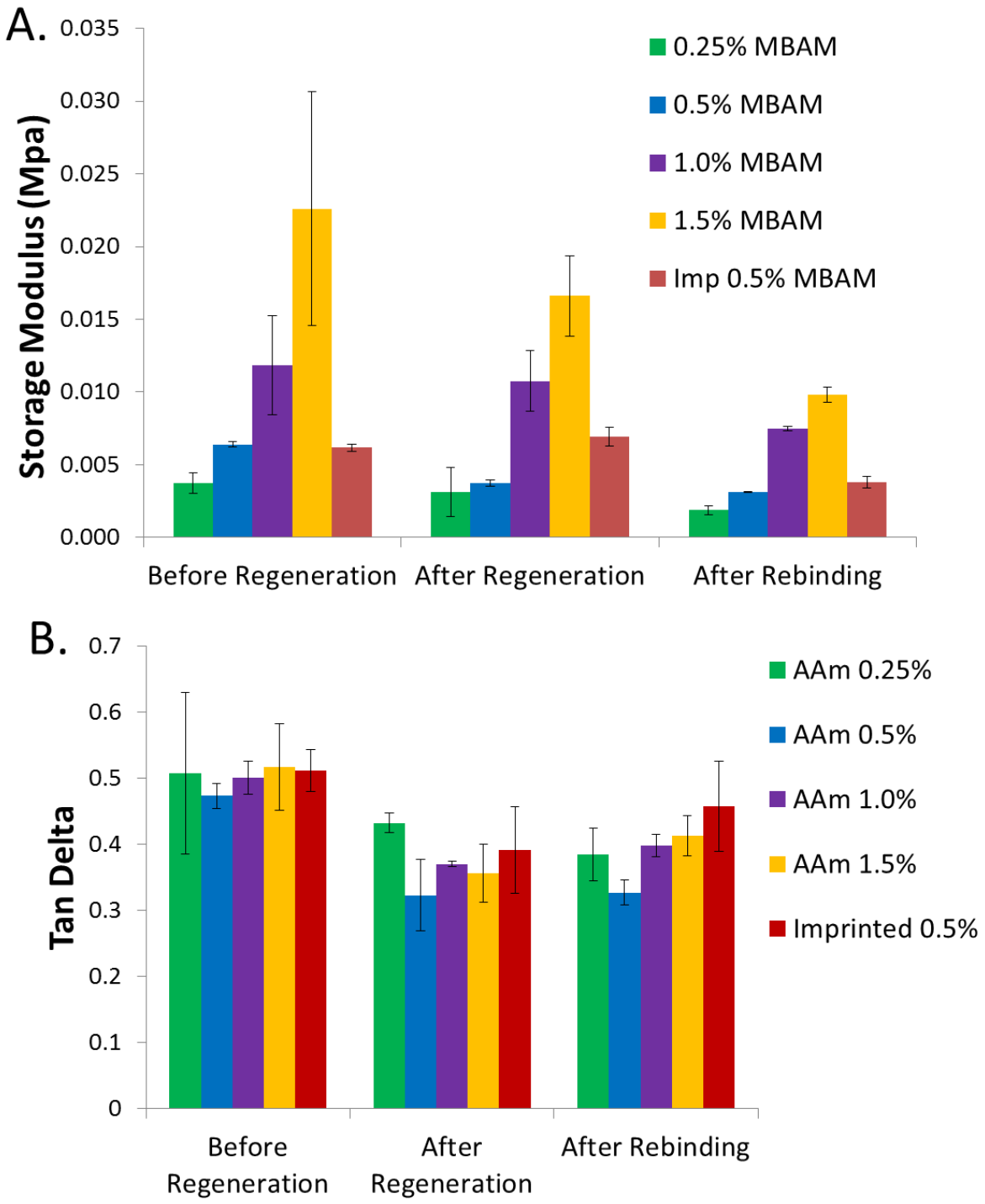


Figure 18: Storage modulus of control AAm gels with varying MBAM content compared to an imprinted hydrogels. The a.) storage modulus and b.) tan delta are shown for a set of gels containing varied amounts of MBAM as compared to imprinted hydrogels over the course of one cycle of rebinding. Only samples labeled as imprinted were imprinted. All others were non-imprinted

### 2.11.2 Determining the cross-linking ability of the imprinted complex

The presence or absence of thrombin in imprinted hydrogels did not appear to change the mechanical properties of the hydrogels by DMA. It was previously hypothesized that the presence or absence of thrombin would result in a higher cross-link density that was directly responsible for the change in the volume observed in imprinted hydrogels. In order to verify the results seen by DMA, it is important to verify the effect that the presence of absence of thrombin plays in creating a more cross-linked and more ordered polymer matrix

In several unsuccessful attempts, disc hydrogels were polymerized having no MBAM and higher concentrations of imprinted complex using the same ratio of AAm to polymerization solution (11.3 mg per 100 μL). Using 1, 2, 5, 10, and 50 equivalents of imprinted complex relative to the values in Table 2 resulted in polymers discs that were not solid enough to handle and in most cases didn't increase the viscosity more than a formulation containing 0 equivalents of MABM. The gel containing 50 equivalents did result in a polymer that had higher viscosity, but did not appear to come close to the gel point. This result is not entirely unexpected though. 50 equivalents of imprinted complex only corresponds to roughly ten percent of the total moles of MBAM cross-linker used in the recipe in Table 2. In order to get a good comparison of this result it needs to be determined how much MBAM is required to gel 11.3 mg of AAm in 100 μL batches.

In hydrogels used thus far the ratio of MBAM to imprinted complex was 483:1, and at this ratio it is hard to deconvolute the relative effect each component plays in the physical properties of an imprinted hydrogel. In order to probe this point, a series of AAm gels were formulated having low MBAM content and no imprinted complex. Imprinted complex was omitted in order to determine the minimal concentration of MBAM required to gel the AAm

polymer so that a physical characterization and comparison could be made. This value can be used to approximate the amount of complex that would be necessary to gel the polymer in the absence of MBAM if both the complex and MBAM were equivalent in their ability to gel the polymerization solution.

Disc gels were polymerized containing the standard 11.3 mg AAm per 100 μL of polymerization solution and varying amounts of MBAM including 0.5, 0.25, and 0.125 equivalents where one equivalent is 0.5 mole percent relative to AAm or the amount of MBAM used in Table 2. All three formulations polymerized and to gelled to varying degrees. The gels containing 0.5 and 0.25 equivalents polymerized into discs, but were very hard to handle, very elastic in nature, and easily broken when handled with forceps. The polymer containing 0.125 equivalents could only loosely be considered a gel as it was not able to be handled but didn't' flow as a polymer solution would. At 0.125 equivalents of MBAM, the gels would still contain sixty times the normal amount of imprinted complex as described in Table 2. In order to get to lower concentrations of MBAM the hydrogel formulation will need to be adjusted.

The properties of hydrogels depend on the concentration of monomer in the polymerization solution.[138] Using that knowledge, the concentration of AAm was increased by a factor of 5 so that 56.5 mg of AAm was dissolved in 100 μL of total solution volume. Using this new formulation, a similar set of hydrogels was produced having 0.25, 0.1, 0.05, 0.01, 0.005, 0.0025, and 0 equivalents of MBAM, again relative to the value in Table 2. The relative amounts of MBAM, AAm, and the corresponding theoretical molar equivalents of MBAM to complex are shown in Table 7. Upon polymerization, all of the solutions formed solid polymer, including the formulation containing no MBAM, even though some samples took much longer to solidify in the mold. Because the polymer containing no MBAM did not gel, having no cross-

linker, but still formed a solid product, all of the polymers were evaluated by putting the resultant discs into PBS to fully swell for a period of one day. The gels containing 0, 0.0025, and 0.005 equivalents of MBAM dissolved, created viscous solutions and were deemed to have not reached the gel point. The gel containing 0.01 equivalents swelled to a volume of roughly four times its original shape but was solid enough to be handled using forceps. At this concentration, the amount of MBAM used would be equal to roughly 5 times the amount of imprinted complex used in Table 2.

Table 7: Relative ratios of cross-linkers. The values for equivalents of each monomer are relative to each individual monomer amount described in Table 2 where one equivalent of MBAM is 0.123 mg and one equivalent of AAm is 11.3 mg. There was no imprinted complex included in the gels. The ratio is reported in the table to illustrate how much MBAM is included relative to the normal amount of complex in an imprinted hydrogel (1.65 nmoles).

| **Equivalents MBAM** | **Mole Ratio of Imprinted Complex to MBAM** | **Equivalents AAm** | **Ratio of MBAM to AAm** |
|---|---|---|---|
| 1 | 483.0 | 1 | 200 |
| 0.25 | 120.8 | 5 | 4000 |
| 0.1 | 48.3 | 5 | 10000 |
| 0.05 | 24.2 | 5 | 20000 |
| 0.01 | 4.8 | 5 | 100000 |
| 0.005 | 2.4 | 5 | 200000 |
| 0.0025 | 1.2 | 5 | 400000 |

A series of polymers were made to probe the efficacy of the imprinted complex in creating a cross-linked gel as compared to the MBAM containing gels containing a higher concentration of AAm. The smallest amount of MBAM shown to cross-link gels with increased AAm was 0.01 equivalents. A gel with an equivalent number of moles of cross-linker, as shown in Table 7, was created having five equivalents of imprinted complex. The resulting polymer disc was put into PBS to swell and after one day the polymer completely dissolved creating a

viscous solution in PBS. In a similar experiment, 0.005 equivalents of MBAM were polymerized with 56.5 mg AAm and five equivalents of imprinted complex. This polymer, like the previous two, did not gel and dissolved in the PBS it was placed in following polymerization.

While an end point was not found where the imprinted complex would gel the polymerization solution, it is evident that the imprinted complex plays a relatively small role in the stiffness of the imprinted gels in the presence of thrombin. The MBAM appears to be responsible for most, if not all, of the mechanical properties of the hydrogels. Even still, the reformation of the imprinted complex shown in previous successful studies does affect a noticeable and very important response in the hydrogel volume that is related to the completion of the imprinted complex cross-link within the polymer backbone.

**2.12 Conclusions**

The development and optimization of bioimprinted hydrogels incorporating a protein imprinted complex consisting of two polymerizable aptamers and a removable thrombin protein were described. The presence of the specific imprinted complex resulted in a hydrogel which was able to respond to the addition or removal of thrombin by shrinking and swelling in volume between 5-6 percent on average and up to 10 percent under certain conditions. This response compares favorably with other reports using biomacromolecular receptors such as antibodies, proteins, and glycoproteins and in addition to providing response that is discernible from error, the imprinted hydrogels are very sensitive to a wide variety of protein concentrations. In fact, this system consisting of a multireceptor recognition mechanism provides response to proteins that far exceeds other imprinted hydrogel sensors and provides responses below the detection limit of many conventional methods of protein identification to reproducibly sense proteins at concentrations as low attomolar and femtomolar levels over approximately six orders of magnitude.

The responses seen by these materials represent a perceived change in the number of cross-links by 0.2 percent of the overall number of cross-links within the hydrogel. The fact that a change in this very small ratio of the total cross-links provides a 5-10 percent volume response hints that there is some amplified response beyond the simple explanation of the change in the cross-link density. The inability to track the cross-linking ability of the imprinted complex leads to a possible revised yet still uncertain view of the fundamental source of the volume response. A more complex situation appears to occur where the removable cross-links that have been shown to exist and be in some way responsible for the volume change are amplified by other characteristics of the imprinted hydrogel structure. It can be certain that this change is not a simple function of a change in osmotic pressure or the loss and gain of weight due to the protein analyte. There is likely a change in the microscopic assembly of the hydrogel structure that manifests itself on the macroscopic scale.

Despite the evolving understanding of the mechanism of response, the results of these protein imprinted sensors cannot be ignored. This hydrogel system negates that need for trained personnel, expensive equipment, or complex detectors, making them ideal for portable or point-of-care applications. Reproducible measurements only require the use of a magnifying glass, a ruler, and measurements taken by eye to outperform much more complex and expensive sensors and instrumentation. Thus, these materials bring to light new avenues for the design of improved materials for molecule-specific responsive polymers and in addition to helping to provide greater understanding of the origins of macromolecular amplification they can ultimately be used for biosensors, drug delivery, and responsive microdevices.

# CHAPTER 3: PROBING THE EFFICIENCY OF NOBE AS A SUCCESSFUL OMNiMIP

## 3.1 Introduction

### 3.1.1 MIP cross-linkers and their effect on imprinting efficacy

In traditional MIPs the cross-linker is relatively useless in terms of creating a functionally imprinted site within the polymer matrix. Generally they are considered inert and do not influence the interaction of functional monomers with the imprinted template. Their primary function is in creating a highly cross-linked polymer matrix in order to increase the rigidity of the polymer. The rigidity of the polymer matrix, while useless for functional recognition, aids in the formation of shape recognition within MIPs.

A wide variety of cross-linkers have been utilized in the creation of MIPs. Traditional cross-linkers containing two polymerizable double bonds such as divinyl benzene (DVB) and ethylene glycol dimethacrylate (EGDMA) have been explored at length and even compared in some situations leading to a higher frequency of use for EGDMA due to a slightly higher imprinting factor in in several situations.[139,140] The structure of many other cross-linkers such as trimethylolpropane trimethacrylate (TRIM), pentaerythritol triacrylate (PETRA), pentaerythritol tetraacrylate (PETEA), and triethanolamine trimethacrylate have been altered from previous cross-linkers and designed largely for the increase in cross-link density by the increasing the number of polymerizable functional groups.[141-146] Additionally, cross-linkers have been designed with the aim of increasing functionality for possible improved recognition by synthesizing monomers which are derived from amino acids[147] or by adding increased intermolecular interactions.[148] While these too, like those with higher degrees of unsaturation, in

certain situations can lead to a slightly increased imprinting factor they still remain functionally inert and do not greatly affect the imprinting factor.

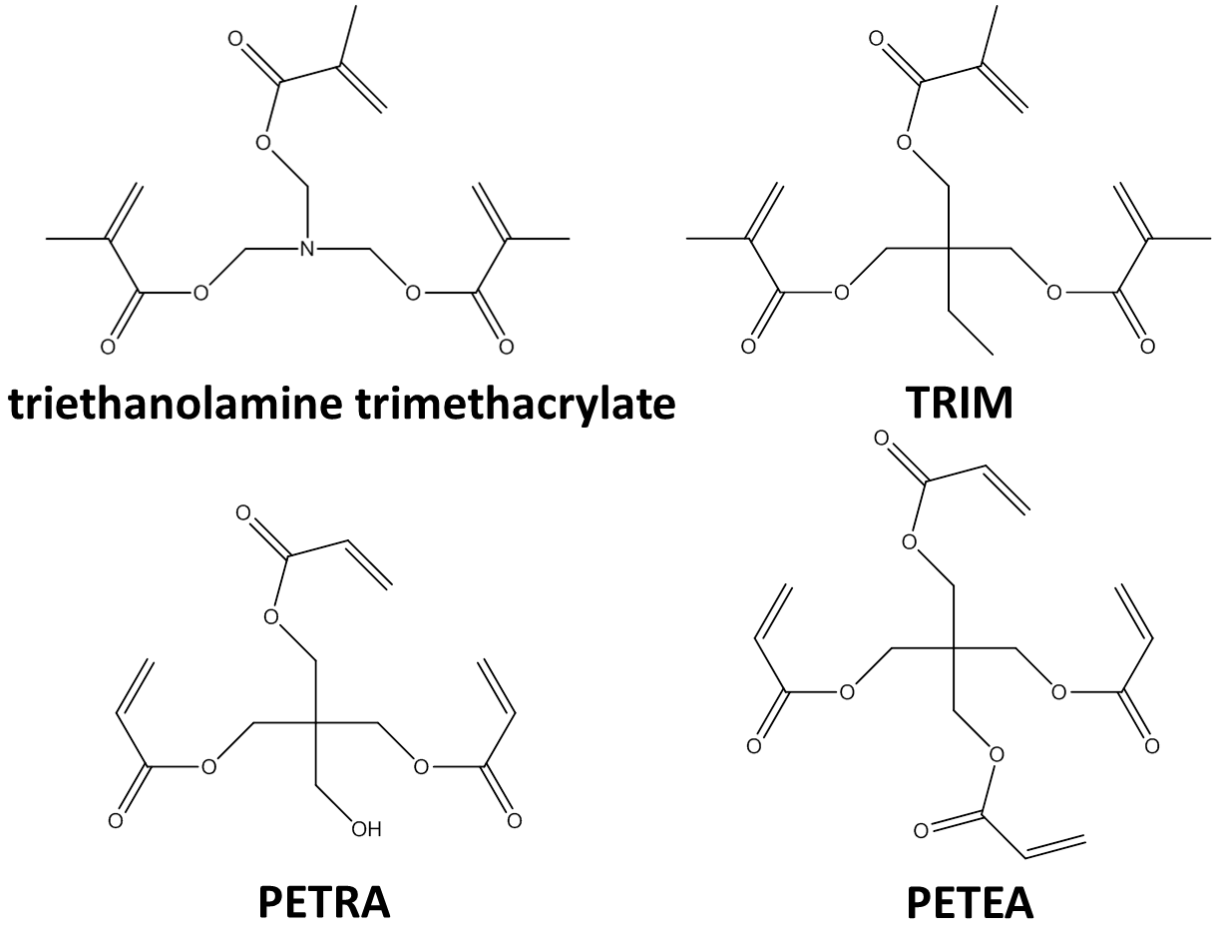


Figure 19: MIP cross-linkers having more than two double bonds.

### 3.1.2 History of OMNiMIPs

In the interest of creating cross-linkers which could aid in functional imprinting in addition to creating of shape selectivity, there have been several innovations in cross-linker design to create cross-linkers similar in structure to EGDMA while increasing the intermolecular interactions. N,N'-ethylenedimethacrylamide (EDAM) was explored by the Shea research group. It differs from EGDMA, having methacrylamide groups instead of methacrylates and gives the

cross-linker two functional moieties capable of hydrogen bonding.[149] While the monomer was found to be insoluble in organic solvents, which are necessary for non-covalent MIP interactions, the inclusion of hydrogen bonding into the cross-linking structure was still a sound theory.

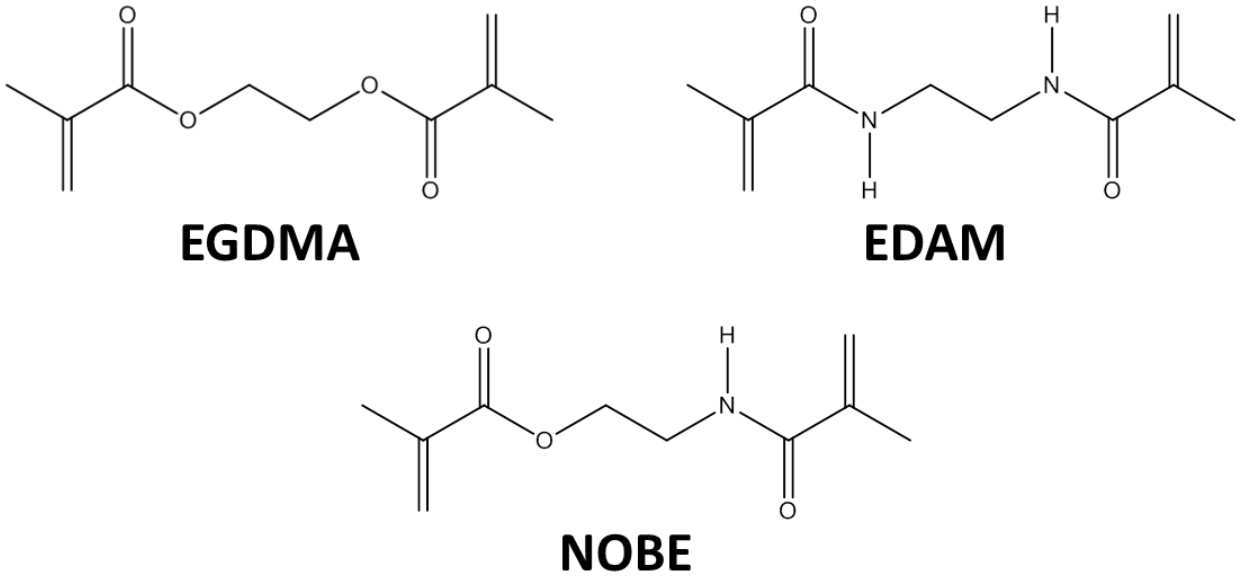


Figure 20: Cross-linkers for the design of hydrogen bonding OMNiMIPs.

The Spivak research group has also developed novel cross-linkers to improve the imprinting factor for templates. N, O-bismethacryolethanolamine (NOBE) is a cross-linker that was designed to overcome the solubility problems previously seen with EDAM. It is a hybrid between the structures of EGDMA and EDAM having one methacrylamide group and one methacrylate group separated by two carbons. It was found to be soluble in a wide variety of organic solvents and resulted in a greater imprinting factor when copolymerized with methacrylic acid (MAA) compared to similar polymers made with EGDMA.[150-152]

It was also later found that NOBE, without the use of functional comonomers such as MAA, was able to outperform copolymers which did contain MAA.[153] This new class of cross-linker capable of forming imprinted sites within monolithic polymer particles without the use of

non-cross-linking functional comonomers was named one monomer molecularly imprinted polymers (OMNiMIPs). By eliminating functional monomers, it was easier to imprint templates capable of hydrogen bonding because it was no longer necessary to formulate new copolymer recipes for each new template, choose between combinations of functional monomers and cross-linkers, or to worry about decreasing the cross-link density of copolymers because the inclusion of non-cross-linking functional monomers would decrease the molar ratio of cross-linker in the formulation.[154,155]

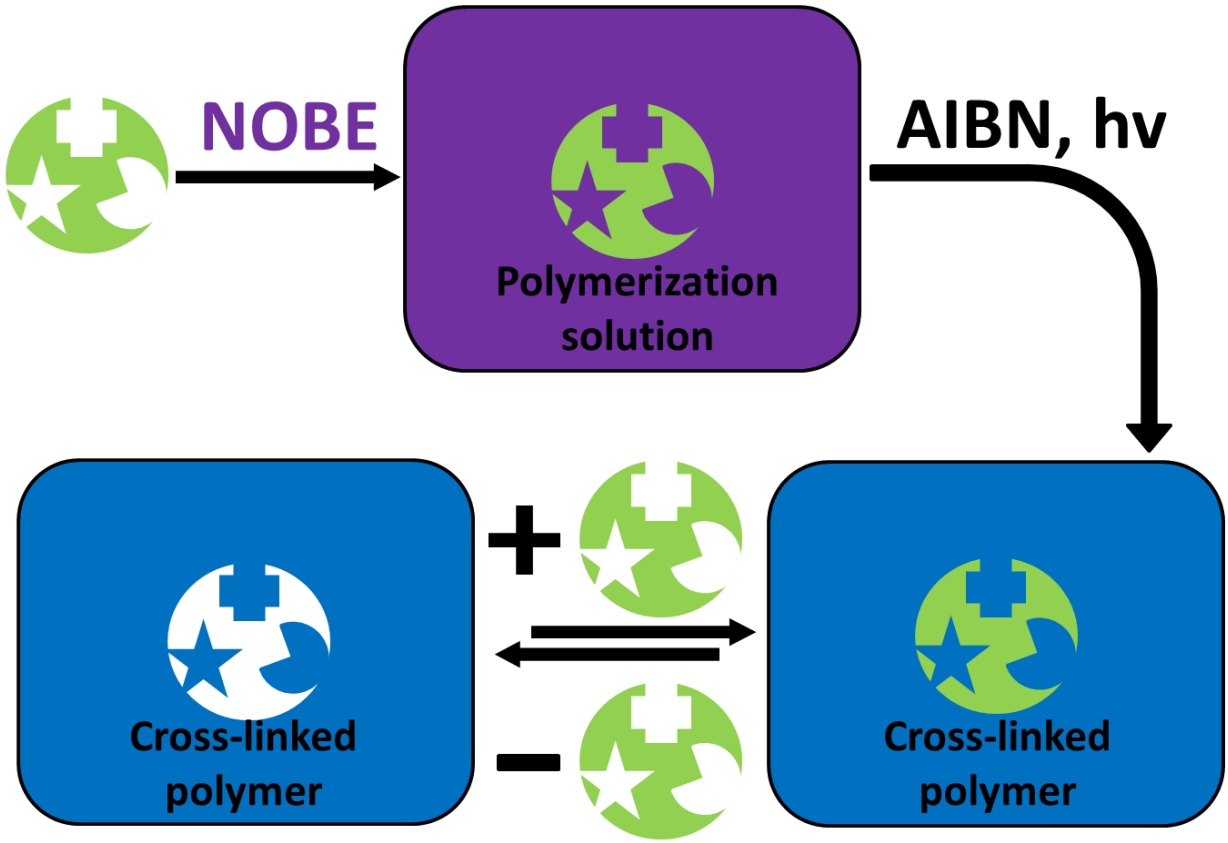


Figure 21: Scheme for synthesis, regeneration, and rebinding of OMNiMIPs. The scheme for OMNiMIP synthesis first involves the preorganization of functional cross-linker with template followed by photopolymerization to for monolithic cross-linked polymer around the template molecule. The reversible binding of template to and from the polymer product is also shown where a shape and functional imprinted cavity is conserved following polymerization around the template.

### 3.1.3 Measuring the enantiomeric selectivity of imprinted binding sites

Kinetic measurements of the imprinting effect are measured by HPLC as a function of retention time of an injected analyte versus the void time of the column. A sample HPLC chromatogram is displayed in Figure 22 as an example of a racemic binding experiment where the column is injected with a mixture of L and D enantiomers of analyte. This theoretical chromatogram represents a polymer imprinted with the L enantiomer. The non-imprinted D enantiomer has some affinity for the column, but it is much smaller than the affinity of the imprinted L enantiomer. The peak broadening seen for the L enantiomer shows that within the polymer the imprinted sites are not equivalent and therefore the retention time will vary depending on the varied strength of binding.

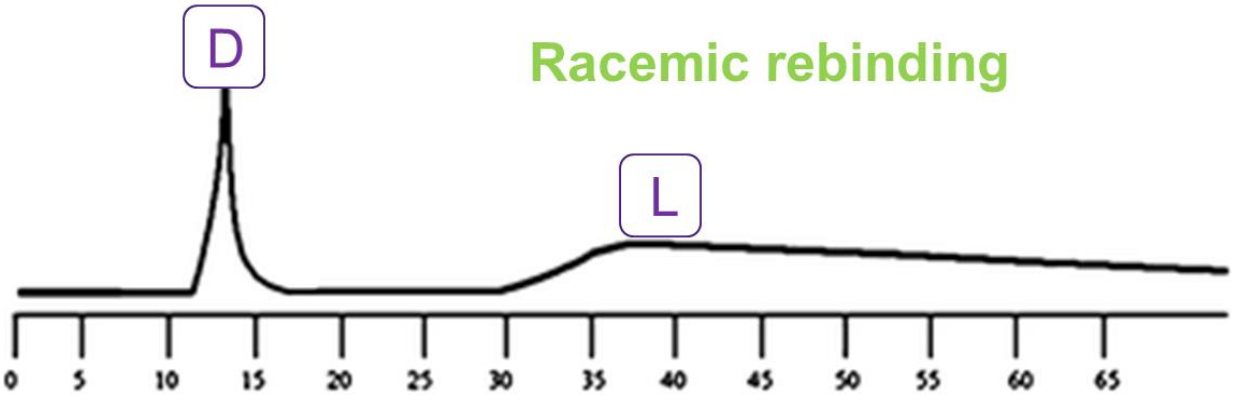


Figure 22: Theoretical HPLC chromatogram for racemic binding of an L molecularly imprinted polymer.

The imprinting factor α for a given polymer is obtained by comparing competitive binding of two enantiomers using HPLC where the polymer is imprinted with one of the two enantiomers. A racemic mixture is injected into a column packed with the polymer and the selective binding of each enantiomer gives rise to an α value using Equations 6. The elution times of each analyte, $V_{Lr}$ and $V_{Dr,}$ are compared to a standard void time, $t_0$. The void time is

determined by adding a small fraction of solvent, such as acetone, to an injection that will pass through the column as fast as the running solvent but will show up in the chromatogram where the running solvent will not. The factors $k_L$ (Equation 4) and $k_D$ (Equation 5) represent the standardized elution time of each enantiomer. The imprinting factor, α, is the ratio of the two, and represents the relative affinity of the imprinted polymer for the imprinted enantiomer compared to its affinity for the non-imprinted enantiomer.

**(Equation 4)** $$k_L = \frac{V_{Lr} - V_0}{V_0}$$

**(Equation 5)** $$k_D = \frac{V_{Dr} - V_0}{V_0}$$

**(Equation 6)** $$\alpha = \frac{k_L}{k_D}$$

Similarly, the imprinting factor α' is determined under non-competative binding conditions. It is obtained using HPLC by injecting independent successive solutions of each enantiomer to a column packed with a polymer imprinted to one specific enantiomer. Equations 7 and 8, like Equations 4 and 5, compare the retention times of each analyte compared to the void time. The imprinting factor, α', is calculated in Equation 6 by comparing the standardized retention time factors $k'_L$ and $k'_D$.

**(Equation 7)** $$k'_L = \frac{V_L - V_0}{V_0}$$

**(Equation 8)** $$k'_D = \frac{V_D - V_0}{V_0}$$

**(Equation 9)** $$\alpha' = \frac{k'_L}{k'_D}$$

### 3.1.4 Previous results of NOBE's use as an OMNiMIP

As would be expected by its structural design, the efficacy of NOBE as an OMNiMIP is closely related to its ability to hydrogen bonding to a template under polymerization and rebinding conditions. The imprinting effect of NOBE polymers was found to be highly dependent on the use of a non-polar solvent so that solvent interactions do not disturb the hydrogen bonding of NOBE to template. Additionally, the role in hydrogen bonding of creating a more defined matrix was also studied by varying the concentrations of NOBE and template and the resulting strength of hydrogen bonding as seen by IR. It was shown that NOBE creates a network of hydrogen bonding throughout the matrix as opposed to dimers or other small aggregations.[156]

While the role of NOBE is known as a hydrogen bonding functional OMNiMIP monomer, it is less certain how the inclusion of NOBE directly affects the recognition of a template molecule relative to its enantiomer. It is uncertain whether the increased imprinting factor seen with NOBE as an OMNiMIP is a result of better functional recognition, hydrogen binding within the polymer matrix, or a combination of the two. In order to clarify NOBE's role, copolymers were made here with EGDMA at varying concentrations with NOBE to look at the change in imprinting factor as a function of NOBE concentration.

## 3.2 Experimental

### 3.2.1 Synthesis of N, O-bismethacrylethanolamine (NOBE)

200 mL dichloromethane (DCM) was added to a 500 mL round bottom flask. 1 equivalent of ethanolamine (4 g), 0.2 equivalents 4-dimethylaminopyridine (DMAP) (1.6 g), and 2.8 equivalents of methacrylic acid (15.8 g) were added to the flask and it was allowed to cool to 0 ºC. Next, 2 equivalents of N,N'-dicyclohexylcarbodiimide (DCC) (27.0 g) were dissolved into

50 mL of DCM and the solution was added drop-wise to the round bottom flask. The mixture was kept under nitrogen and allowed to stir at room temperature for 48 hours. The resulting solution was filtered to remove the N, N'-Dicyclohexylurea (DCU) and extracted four times with a 1 M solution of HCL and eight times with a saturated sodium carbonate solution. The organic layer was dried with anhydrous magnesium sulfate, concentrated to 10 mL, and further purified by column chromatography using a silica 50/50 hexane/ethylacetate. The resulting purified NOBE product weighed 9.7 g for a 75 percent yield. The purified product was confirmed by $H^1$NMR characterization.

### 3.2.2 Synthesis of NOBE/EGDME copolymers

Polymerization mixtures were made in 13 x 100 mm test tubes. Initially, 0.2 g Boc-L-tyrosine (seven mole percent relative to monomer) was added to 3 mL acetonitrile and the mixture was sonicated. To that solution .016 g AIBN (one percent relative to monomer) and a 2 g mixture of EDGMA and NOBE was added. The solution was purged with nitrogen for five minutes then capped and sealed with teflon tape and parafilm. The sample was put into a photochemical reactor immersed in a temperature controlled water/ethylene glycol bath. A UV light source (medium pressure 450 W mercury arc lamp) jacketed in a borosilicate double-walled immersion well was placed at the center of the polymer mixtures. The polymerization was initiated at 20°C and the temperature was maintained by cooling the jacket surrounding the lamp and circulating the apparatus solution. The polymerization was allowed to proceed for 8 h during which time the temperature of the water/ethylene glycol bath remained relatively constant. This procedure was repeated for mixtures of NOBE and EDGMA containing 10, 20, 30, 40, 50, 65, and 80 mole percent NOBE relative to EGDMA.

### 3.2.3 Regeneration of MIP and analysis of imprinting factor by HPLC

The boc-L-tyrosine template was removed by Soxhlet extraction with methanol for 48 hours. The polymers were ground using a mortar and pestle and the fraction of particles measuring between 25 and 37 μm were isolated using U.S.A. Standard Testing Sieves. The selected particles were slurry packed into 100 mm long 2.1 mm inner diameter stainless steel columns for HPLC characterization. The polymers were washed on line using 99:1 acetonitrile: acetic acid at a flow rate of 0.1 mL/min to remove residual template and equilibrate the column. The same flow rate and solvent was used for all HPLC experiments. The efficacy of imprinting was determined by injecting 0.1 mM solutions of boc-L-tyrosine, boc-D-tyrosine, or a racemic mixture of the two. Additionally, the void volume for the column was measured by injecting 99:1 acetonitrile: acetic acid spiked with acetone. A UV detector set to 260 nM was used to measure the absorbance of the analytes as they eluted from the column.

## 3.3 Results and Conclusions

### 3.3.1 Interpretation of α' as a function of NOBE concentration

The values of α' were determined, using Equation 6, by measuring the elution volumes of boc-L-tyrosine and boc-D-tyrosine using HPLC. Figure 23 shows the varied imprinting effect within a group of polymers having differing ratios of NOBE to EGDMA. It can be observed that the imprinted sites in the polymer particles are selective towards the imprinted L enantiomer as opposed to the non-imprinted D enantiomer because the value of alpha' is greater than one. Also, the imprinting factor α' increases as the concentration of NOBE in the imprinted polymer increases illustrating that NOBE is playing a direct role in the imprinting of the template.

Notably, the α' curve is non-linear with respect to the fraction of NOBE in the copolymers. This means that there is not a simple relationship between the imprinting factor and

the NOBE content. The incorporation of more functional cross-linker results in better and more complex binding sites, however there appears to be several transitions in the curve which suggests that there are critical concentrations for NOBE at which its role in imprinting may change. As such, there appears to be several mechanisms by which NOBE impacts the efficacy of imprinting.

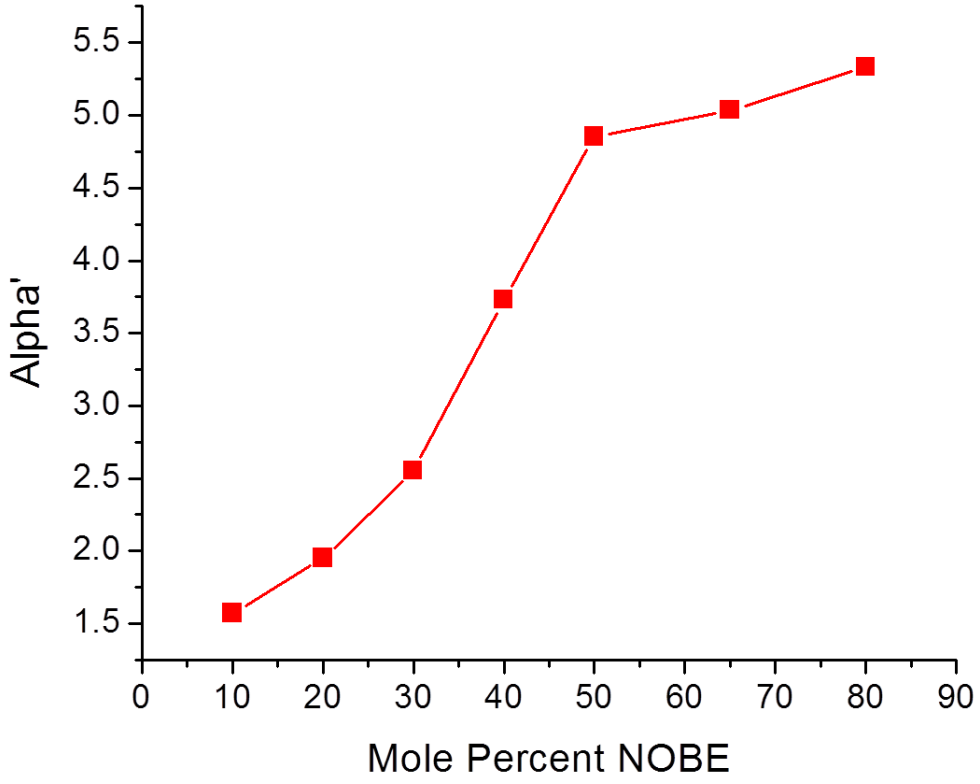


Figure 23: Alpha and alpha' as a function of the mole percent of NOBE in a copolymer.

### 3.3.2 Comparing the response to L and D enantiomers to explain the effect of NOBE

While the exact effect of NOBE content can't be explained solely by observing the values of α', the graph of $k'_L$ and $k'_D$ versus NOBE content can help describe the contributions of NOBE to the imprinting factor. In Figure 24 it can be seen that the values of $k'_L$ are relatively

equivalent to $k'_D$ until the NOBE content increases to greater than 20 percent. It was previously seen that it is necessary to have at least two moles of functional cross-linker for each mole of template in order to create shape selectivity in imprinted sites.[156,157] It was further seen that three moles of functional cross-linker per mole of template was required to create imprinted sites with the highest affinity possible for an imprinted template.[158] In polymers containing 20 percent NOBE there is roughly three moles of NOBE for each mole of imprinted template.

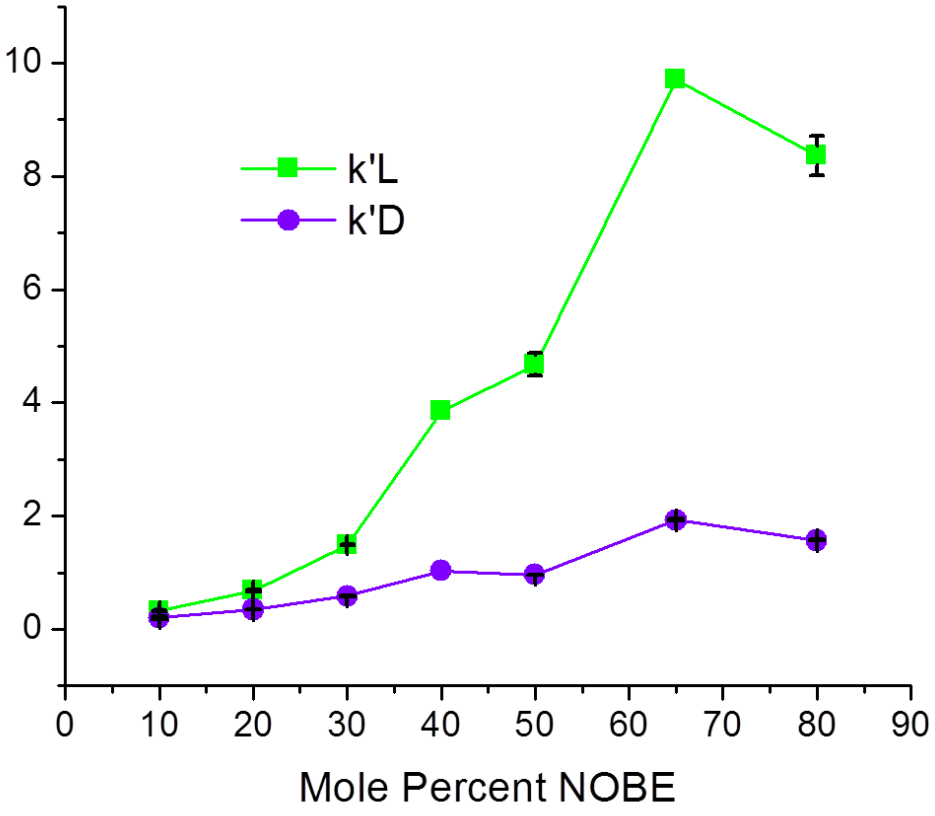


Figure 24: k'L and k'D values as a function of mole percent NOBE in the copolymer.

At comonomer compositions where NOBE is greater than 20 percent the imprinting factor becomes increasingly greater than one and continues to increase until roughly 65 percent NOBE. As the number of moles of NOBE increases past the useful concentration for template

recognition the increase in imprinting factor is likely due to a polymer matrix effect that increases the relative selectivity for the imprinted enantiomer with respect to the non-imprinted enantiomer. This can be accomplished either as a function of increasing the binding strength of the imprinted enantiomer or by decreasing the strength of binding of the non-imprinted enantiomer. Because maximum binding is reached at relatively low concentrations where NOBE to template ratios are approximately three to one, higher concentrations of NOBE can lead to excess hydrogen bonding at which a more well-defined polymer matrix is formed. Excess hydrogen bonding between free NOBE molecules can lead to a stronger physical crosslinking in the polymer matrix compared to the cross-link structure obtained solely with EGDMA. The presence of excess hydrogen bonding could limit the formation or voids in the polymer particles that can give rise to higher non-specific binding of the non-imprinted enantiomer. The increase in α attributed to matrix hydrogen bonding reaches a maximum at roughly 65 percent NOBE in the copolymer. At higher concentrations of NOBE there is little to no increase in the imprinting factor.

### 3.4 Conclusions

By observing the change in the imprinting factor with respect to the NOBE content of NOBE/EGDME copolymers the source of NOBE's efficacy as an OMNiMIP monomer was obviated. At NOBE concentrations below 20% the hydrogen bonding of the functional cross-linker was primarily involved in the creation of binding sites for the template. At higher NOBE concentrations the excess hydrogen bonding gave rise to a better defined polymer matrix capable of decreasing non-specific binding of the non-imprinted D enantiomer. At 70 % NOBE, the α value reaches a maximum of 7.5 which was roundly double the value of 3.9 seen in previous experiments where 100 % NOBE was used.[156] This shows that there is a point when inclusion of

more NOBE does not increase the imprinting factor. While the recipe varies slightly, with these polymers containing 7 % template as opposed to 5 %, it is unlikely that the difference would double the α value comparatively. Further studies will need to be done to confirm this result however, and to better define the decrease in the $k_L$ and $k'_L$ seen at highers NOBE concentrations. Presently it can only be confirmed that the α value reaches a maximum when 70 % of the copolymer composition is comprised of NOBE.

# CHAPTER 4: CONCLUDING REMARKS

There is an ever increasing drive toward to detection, understanding, and manipulation of the complex biochemical molecules and processes involved in living systems. As that proliferation occurs, so too does the need to advance the field of bioanalytical technology geared toward the recognition of bioanalytes. There is, and will continue to be, an increasing need to improve upon existing materials and technologies to create devices that are capable of specific, rapid, and facile detection of biomacromolecules such as proteins, DNA, RNA, and sugars.

The use of MIPs for sensors and separations has been widely explored. Creation of novel MIPs for the development and improvement of new technologies for recognition and separation of biomacromolecules has been a promising avenue in the advancement of bioanalytics. The incorporation of new matrix materials and receptors for decreasing the limit of detection and increasing the selectivity of MIPs for specific biomarkers has led to new MIPs that rival traditional bioanalytical devices.

Here it was shown that the incorporation of aptamer receptors imprinted into a hydrogel matrix gave rise to a MIP material capable of extremely low levels of detection in the femtomolar and attomolar range for a target protein. Furthermore, detection of the protein was realized by the facile measurement of the hydrogel using only a ruler, a magnifying glass, and observation using the naked eye. Additionally, the hydrogels were reusable and capable of selectively binding the imprinted protein both in the presence of other protein and in biological media such as artificial urine.

At the heart of this study is the observed amplified response which facilitated the low level of detection and the macroscopic response observed in relation to extremely low

concentrations of protein. Despite the evolving understanding of the mechanism of response, the results of these protein imprinted sensors cannot be overstated. The use of multibioreceptor imprinting in collaboration with the volume responsive nature of hydrogels has increased ease of biomarker detection and offers an alternative to traditional analytical techniques that require sophisticated instrumentation and highly trained personnel. This hydrogel system negates that need for trained personnel, expensive equipment, or complex detectors, making them ideal for portable or point-of-care applications. Thus, these materials bring to light new avenues for the design of improved materials for molecule-specific responsive polymers and in addition to helping to provide greater understanding of the origins of macromolecular amplification.

# REFERENCES


(1) Wulff, G.; Gross, T.; Schonfeld, R. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 1962-1964.
(2) Collett, J. R., 2006.
(3) Kim, S. C.; (Genoprot Co., Ltd., S. Korea). Application: KR

KR, 2007, p No pp given.
(4) Medley, C. D.; Smith, J. E.; Tang, Z.; Wu, Y.; Bamrungsap, S.; Tan, W. *Anal. Chem. (Washington, DC, U. S.)* **2008**, *80*, 1067-1072.
(5) Melker, R. J.; Dennis, D. M.; Martin, C. R.; Stewart, J. D.; (USA). Application: US

US, 2006, p 17 pp , Cont -in-part of U S Ser No 744,789.
(6) Strehlitz, B.; Mann, D.; Reinemann, C.; Stoltenburg, R.; (Ufz-Umweltforschungszentrum Leipzig-Halle GmbH, Germany). Application: DE

DE, 2007, p 12pp.
(7) Zhou, C.; Jiang, Y.; Hou, S.; Ma, B.; Fang, X.; Li, M. *Anal Bioanal Chem* **2006**, *384*, 1175-1180.
(8) Vlatakis, G.; Andersson, L. I.; Mueller, R.; Mosbach, K. *Nature (London)* **1993**, *361*, 645-647.
(9) Bossi, A.; Bonini, F.; Turner, A. P. F.; Piletsky, S. A. **2007**, *22*, 1131-1137.
(10) Takeuchi, T.; Hishiya, T. **2008**, *6*, 2459-2467.
(11) Simon, R.; Collins, M. E.; Spivak, D. A. *Anal. Chim. Acta* **2007**, *591*, 7-16.
(12) Spivak, D. A.; Simon, R.; Campbell, J. *Anal. Chim. Acta* **2004**, *504*, 23-30.
(13) Long, Y.; Xing, X.; Han, R.; Sun, Y.; Wang, Y.; Zhao, Z.; Mi, H. **2008**, *380*, 268-275.
(14) Haupt, K. *Nature Biotechnology* **2002**, *20*, 884-885.
(15) Rachkov, A.; Minoura, N. *J. Chromatogr., A* **2000**, *889*, 111-118.
(16) Rachkov, A.; Minoura, N. *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol.* **2001**, *1544*, 255-266.
(17) Nishino, H.; Huang, C.-S.; Shea, K. J. *Angew. Chem., Int. Ed.* **2006**, *45*, 2392-2396.
(18) Mammen, M.; Chio, S.-K.; Whitesides, G. M. *Angew. Chem., Int. Ed.* **1998**, *37*, 2755-2794.
(19) Mulder, A.; Huskens, J.; Reinhoudt, D. N. *Organic & Biomolecular Chemistry* **2004**, *2*, 3409-3424.
(20) Whitcombe, M. J.; Chianella, I.; Larcombe, L.; Piletsky, S. A.; Noble, J.; Porter, R.; Horgan, A. *Chem. Soc. Rev.* **2011**, *40*, 1547-1571.
(21) Subrahmanyam, S.; Piletsky, S. A.; Turner, A. P. F. *Anal. Chem.* **2002**, *74*, 3942-3951.
(22) Borrebaeck, C. A. K.; Wingren, C. *J. Proteomics* **2009**, *72*, 928-935.
(23) Tothill, I. E. *Semin. Cell Dev. Biol.* **2009**, *20*, 55-62.
(24) Miyata, T. *Polym. J. (Tokyo, Jpn.)* **2010**, *42*, 277-289.
(25) Miyata, T.; Asami, N.; Okawa, K.; Uragami, T. *Polym. Adv. Technol.* **2006**, *17*, 794-797.
(26) Miyata, T.; Asami, N.; Uragami, T. *Nature (London)* **1999**, *399*, 766-769.
(27) Miyata, T.; Asami, N.; Uragami, T. *Macromolecules* **1999**, *32*, 2082-2084.
(28) Miyata, T.; Asami, N.; Uragami, T. *J. Polym. Sci., Part B Polym. Phys.* **2009**, *47*, 2144-2157.
(29) Miyata, T.; Hayashi, T.; Kuriu, Y.; Uragami, T. *Journal of Molecular Recognition* **2012**, *25*, 336-343.
(30) Miyata, T.; Jige, M.; Nakaminami, T.; Uragami, T. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 1190-1193.
(31) Bolisay, L. D.; Culver, J. N.; Kofinas, P. *Biomaterials* **2006**, *27*, 4165-4168.
(32) Casey, B. J.; Kofinas, P. *J. Biomed. Mater. Res., Part A* **2008**, *87A*, 359-363.
(33) Janiak, D. S.; Ayyub, O. B.; Kofinas, P. *Macromolecules (Washington, DC, U. S.)* **2009**, *42*, 1703-1709.
(34) Janiak, D. S.; Ayyub, O. B.; Kofinas, P. *Polymer* **2010**, *51*, 665-670.

(35) Parmpi, P.; Kofinas, P. *Biomaterials* **2004**, *25*, 1969-1973.
(36) Ghasemzadeh, N.; Nyberg, F.; Hjerten, S. *J. Sep. Sci.* **2008**, *31*, 3954-3958.
(37) Ghasemzadeh, N.; Nyberg, F.; Hjerten, S. *J. Sep. Sci.* **2008**, *31*, 3945-3953.
(38) Hjerten, S.; Liao, J. L.; Nakazato, K.; Wang, Y.; Zamaratskaia, G.; Zhang, H. X. *Chromatographia* **1997**, *44*, 227-234.
(39) Rezeli, M.; Kilar, F.; Hjerten, S. *J. Chromatogr., A* **2006**, *1109*, 100-102.
(40) Tong, D.; Hetenyi, C.; Bikadi, Z.; Gao, J. P.; Hjerten, S. *Chromatographia* **2001**, *54*, 7-14.
(41) Guthrie, J. W.; Hamula, C. L. A.; Zhang, H.; Le, X. C. **2006**, *38*, 324-330.
(42) Burke, J. M.; Berzal-Herranz, A. *Faseb J.* **1993**, *7*, 106-112.
(43) Patel, D. J.; Suri, A. K. *Rev. Mol. Biotechnol.* **2000**, *74*, 39-60.
(44) Perlette, J.; Li, J.; Fang, X.; Schuster, S.; Lou, J.; Tan, W. *Rev. Anal. Chem.* **2002**, *21*, 1-14.
(45) Rajendran, M.; Ellington, A. D. *Opt. Biosens.* **2002**, 369-396.
(46) Tuerk, C.; Gold, L. *Science* **1990**, *249*, 505-510.
(47) Ellington, A. D. *Curr. Biol.* **1994**, *4*, 427-429.
(48) Fredriksson, S.; Gullberg, M.; Jarvius, J.; Olsson, C.; Pietras, K.; Gustafsdottir, S. M.; Oestman, A.; Landegren, U. **2002**, *20*, 473-477.
(49) Gustafsdottir, S. M.; Nordengrahn, A.; Fredriksson, S.; Wallgren, P.; Rivera, E.; Schallmeiner, E.; Merza, M.; Landegren, U. **2006**, *52*, 1152-1160.
(50) Gustafsdottir, S. M.; Schallmeiner, E.; Fredriksson, S.; Gullberg, M.; Soederberg, O.; Jarvius, M.; Jarvius, J.; Howell, M.; Landegren, U. **2005**, *345*, 2-9.
(51) Gullberg, M.; Gustafsdottir, S. M.; Schallmeiner, E.; Jarvius, J.; Bjarnegard, M.; Betsholtz, C.; Landegren, U.; Fredriksson, S. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 8420-8424.
(52) Heyduk, E.; Heyduk, T. *Anal. Chem.* **2005**, *77*, 1147-1156.
(53) Shiomi, T.; Matsui, M.; Mizukami, F.; Sakaguchi, K. *Biomaterials* **2005**, *26*, 5564-5571.
(54) Lin, H.-Y.; Hsu, C.-Y.; Thomas, J. L.; Wang, S.-E.; Chen, H.-C.; Chou, T.-C. *Biosensors & Bioelectronics* **2006**, *22*, 534-543.
(55) Balamurugan, S.; Obubuafo, A.; Soper, S. A.; McCarley, R. L.; Spivak, D. A. *Langmuir* **2006**, *22*, 6446-6453.
(56) Li, J.; Wei, G.; Zhang, Y.; Elsevier Ltd.: 2012, p 35-55.
(57) Scrimin, P.; Prins, L. J. *Chem. Soc. Rev.* **2011**, *40*, 4488-4505.
(58) Dickert, F. L.; Hayden, O. *Fresenius' J. Anal. Chem.* **1999**, *364*, 506-511.
(59) Annamma, K. M.; Mathew, B. *Mater. Sci. Appl.* **2011**, *2*, 131-140.
(60) Wulff, G.; Sarhan, A. *Angew. Chem., Int. Ed. Engl.* **1972**, *11*, 341.
(61) Zhou, Q.; Swager, T. M. *J. Am. Chem. Soc.* **1995**, *117*, 12593-12602.
(62) Zhou, Q.; Swager, T. M. *J. Am. Chem. Soc.* **1995**, *117*, 7017-7018.
(63) Du, Y.; Li, B.; Wang, E. *Acc. Chem. Res.*, Ahead of Print.
(64) Garcia, J. M.; Garcia, F. C.; Serna, F.; de la Pena, J. L. *Polym. Rev. (Philadelphia, PA, U. S.)* **2011**, *51*, 341-390.
(65) Kim, H. N.; Guo, Z.; Zhu, W.; Yoon, J.; Tian, H. *Chem. Soc. Rev.* **2011**, *40*, 79-93.
(66) Dore, K.; Dubus, S.; Ho, H.-A.; Levesque, I.; Brunette, M.; Corbeil, G.; Boissinot, M.; Boivin, G.; Bergeron, M. G.; Boudreau, D.; Leclerc, M. *J. Am. Chem. Soc.* **2004**, *126*, 4240-4244.
(67) Green, M. M.; Reidy, M. P.; Johnson, R. D.; Darling, G.; O'Leary, D. J.; Willson, G. *J. Am. Chem. Soc.* **1989**, *111*, 6452-6454.
(68) Green, M. M.; Cheon, K.-S.; Yang, S.-Y.; Park, J.-W.; Swansburg, S.; Liu, W. *Acc. Chem. Res.* **2001**, *34*, 672-680.
(69) Yashima, E.; Maeda, K.; Nishimura, T. *Chem.--Eur. J.* **2004**, *10*, 42-51.
(70) Sikes, H. D.; Hansen, R. R.; Johnson, L. M.; Jenison, R.; Birks, J. W.; Rowlen, K. L.; Bowman, C. N. *Nat. Mater.* **2008**, *7*, 52-56.
(71) Sella, E.; Lubelski, A.; Klafter, J.; Shabat, D. *J. Am. Chem. Soc.* **2010**, *132*, 3945-3952.
(72) Peterson, G. I.; Larsen, M. B.; Boydston, A. J. *Macromolecules (Washington, DC, U. S.)* **2012**, *45*, 7317-7328.

(73) Ulijn, R. V.; Bibi, N.; Jayawarna, V.; Thornton, P. D.; Todd, S. J.; Mart, R. J.; Smith, A. M.; Gough, J. E. *Mater. Today (Oxford, U. K.)* **2007**, *10*, 40-48.
(74) Wischerhoff, E.; Badi, N.; Lutz, J.-F.; Laschewsky, A. *Soft Matter* **2010**, *6*, 705-713.
(75) Mano, J. F. *Adv. Eng. Mater.* **2008**, *10*, 515-527.
(76) Liu, J.; Liu, H.; Kang, H.; Donovan, M.; Zhu, Z.; Tan, W. *Anal. Bioanal. Chem.* **2012**, *402*, 187-194.
(77) Lyon, L. A.; Meng, Z.; Singh, N.; Sorrell, C. D.; St, J. A. *Chem. Soc. Rev.* **2009**, *38*, 865-874.
(78) Tokarev, I.; Minko, S. *Adv. Mater. (Weinheim, Ger.)* **2010**, *22*, 3446-3462.
(79) Hernandez, R.; Sacristan, J.; Asin, L.; Torres, T. E.; Ibarra, M. R.; Goya, G. F.; Mijangos, C. *J. Phys. Chem. B* **2010**, *114*, 12002-12007.
(80) Jeong, B.; Gutowska, A. *Trends in Biotechnology* **2002**, *20*, 360-360.
(81) Takatsy, A.; Kilar, A.; Kilar, F.; Hjerten, S. *J. Sep. Sci.* **2006**, *29*, 2802-2809.
(82) Zayats, M.; Kanwar, M.; Ostermeier, M.; Searson, P. C. *Macromolecules (Washington, DC, U. S.)* **2011**, *44*, 3966-3972.
(83) Peng, L.; You, M.; Yuan, Q.; Wu, C.; Han, D.; Chen, Y.; Zhong, Z.; Xue, J.; Tan, W. *J. Am. Chem. Soc.* **2012**, *134*, 12302-12307.
(84) Polisky, B.; Academic Press: 1998, p 161-177.
(85) Toole, J. J.; Griffin, L. C.; Bock, L. C.; Latham, J. A.; Muenchau, D. D.; Krawczyk, S.; Gilead Sciences, Inc., USA . 1992, p 83 pp.
(86) Ellington, A. D.; Szostak, J. W. *Nature (London)* **1990**, *346*, 818-822.
(87) Tuerk, C.; Gold, L. *Science (Washington, D. C., 1883-)* **1990**, *249*, 505-510.
(88) Tombelli, S.; Minunni, M.; Mascini, M. *Biosens. Bioelectron.* **2005**, *20*, 2424-2434.
(89) Russo, K. I.; Merlino, A.; Giancola, C.; Randazzo, A.; Mazzarella, L.; Sica, F. *Nucleic Acids Res.* **2011**, *39*, 7858-7867.
(90) Abe, T.; Yamazaki, S.; Nakamura, Y.; Shi Emu Shi Shuppan: 2007, p 54-64.
(91) Goulko, A. A.; Li, F.; Chris, L. X. *TrAC, Trends Anal. Chem.* **2009**, *28*, 878-892.
(92) Liu, F.-W.; Lan, X.-P. *Zhongguo Shengwu Huaxue Yu Fenzi Shengwu Xuebao* **2006**, *22*, 787-793.
(93) Zhou, J.; Rossi, J. J. *BioDrugs* **2012**, *26*, 393-400.
(94) Tan, Y.; Zhang, X.-m. *Shengwu Huaxue Yu Shengwu Wuli Jinzhan* **2012**, *39*, 410-415.
(95) Zhao, Q.; Wu, M.; Chris, L. X.; Li, X.-F. *TrAC, Trends Anal. Chem.* **2012**, *41*, 46-57.
(96) Barik, S.; Bitko, V.; Wiley-Blackwell: 2012; Vol. 2, p 845-883.
(97) Famulok, M.; Mayer, G. *Acc. Chem. Res.* **2011**, *44*, 1349-1358.
(98) Spiridonova, V. A. *Biomed. Khim.* **2010**, *56*, 639-656.
(99) Zheng, J.; He, P.; Fang, Y. *Huaxue Jinzhan* **2009**, *21*, 732-738.
(100) Chang, H.-T.; Li, C.-L.; American Chemical Society: 2012, p ENVR-39.
(101) Chung, E.; Gao, R.; Ko, J.; Choi, N.; Lim, D. W.; Lee, E. K.; Chang, S.-I.; Choo, J. *Lab Chip* **2013**, *13*, 260-266.
(102) Li, L.; Huang, X.; Yi, X.; Lv, Y.; Beijing University of Aeronautics & Astronautics, Peop. Rep. China . 2013, p 9pp.
(103) Wang, F.; Wu, Y.; Zhan, S.; He, L.; Zhi, W.; Zhou, X.; Zhou, P. *Aust. J. Chem.* **2013**, *66*, 113-118.
(104) Woo, H. Y.; Sim, H. G.; Kim, B. R.; Kang, M. J.; Jung, I. H.; Pusan National University, Industry-University Cooperation Foundation, S. Korea . 2013, p 23pp.
(105) Lassalle, H.-P.; Marchal, S.; Guillemin, F.; Reinhard, A.; Bezdetnaya, L. *Curr. Drug Metab.* **2012**, *13*, 1130-1144.
(106) Sun, X.-l.; Zhang, F.; Zhang, Y.-z. *Shipin Yu Shengwu Jishu Xuebao* **2012**, *31*, 449-454.
(107) Dharmasiri, U.; Balamurugan, S.; Adams, A. A.; Okagbare, P. I.; Obubuafo, A.; Soper, S. A. *Electrophoresis* **2009**, *30*, 3289-3300.
(108) Balamurugan, S.; Obubuafo, A.; McCarley, R. L.; Soper, S. A.; Spivak, D. A. *Anal. Chem. (Washington, DC, U. S.)* **2008**, *80*, 9630-9634.

(109) Balamurugan, S.; Obubuafo, A.; Soper, S. A.; McCarley, R. L.; Spivak, D. A. *Langmuir* **2006**, *22*, 6446-6453.
(110) Balamurugan, S.; Obubuafo, A.; Soper, S. A.; Spivak, D. A. *Anal. Bioanal. Chem.* **2008**, *390*, 1009-1021.
(111) Bridonneau, P.; Bunch, S.; Tengler, R.; Hill, K.; Carter, J.; Pieken, W.; Tinnermeier, D.; Lehrman, R.; Drolet, D. W. *J Chromatogr B Biomed Sci Appl* **1999**, *726*, 237-247.
(112) Potty, A. S. R.; Kourentzi, K.; Fang, H.; Jackson, G. W.; Zhang, X.; Legge, G. B.; Willson, R. C. *Biopolymers* **2009**, *91*, 145-156.
(113) Floege, J.; Ostendorf, T.; Janssen, U.; Burg, M.; Radeke, H. H.; Vargeese, C.; Gill, S. C.; Green, L. S.; Janjic, N. *Am. J. Pathol.* **1999**, *154*, 169-179.
(114) Ostendorf, T.; Kunter, U.; Grone, H. J.; Bahlmann, F.; Kawachi, H.; Shimizu, F.; Koch, K. M.; Janjic, N.; Floege, J. *J. Am. Soc. Nephrol.* **2001**, *12*, 909-918.
(115) Ruslinda, A. R.; Tajima, S.; Ishii, Y.; Ishiyama, Y.; Edgington, R.; Kawarada, H. *Biosens Bioelectron* **2010**, *26*, 1599-1604.
(116) Hasegawa, H.; Taira, K. I.; Sode, K.; Ikebukuro, K. *Sensors* **2008**, *8*, 1090-1098.
(117) Griffin, L.; Albrecht, G.; Latham, J.; Leung, L.; Vermaas, E.; Toole, J. J.; Gilead Sciences, Inc., USA . 1998, p 115 pp., Cont. of U. S. Ser. No. 934,387, abandoned.
(118) Bracht, F.; Schror, K. *Biochem Biophys Res Commun* **1994**, *200*, 933-937.
(119) Griffin, L. C.; Tidmarsh, G. F.; Bock, L. C.; Toole, J. J.; Leung, L. L. *Blood* **1993**, *81*, 3271-3276.
(120) Macaya, R. F.; Schultze, P.; Smith, F. W.; Roe, J. A.; Feigon, J. *Proc Natl Acad Sci U S A* **1993**, *90*, 3745-3749.
(121) Paborsky, L. R.; McCurdy, S. N.; Griffin, L. C.; Toole, J. J.; Leung, L. L. *J Biol Chem* **1993**, *268*, 20808-20811.
(122) Padmanabhan, K.; Padmanabhan, K. P.; Ferrara, J. D.; Sadler, J. E.; Tulinsky, A. *J Biol Chem* **1993**, *268*, 17651-17654.
(123) Tasset, D. M.; Kubik, M. F.; Steiner, W. *J Mol Biol* **1997**, *272*, 688-698.
(124) Wang, K. Y.; Krawczyk, S. H.; Bischofberger, N.; Swaminathan, S.; Bolton, P. H. *Biochemistry* **1993**, *32*, 11285-11292.
(125) Wang, K. Y.; McCurdy, S.; Shea, R. G.; Swaminathan, S.; Bolton, P. H. *Biochemistry* **1993**, *32*, 1899-1904.
(126) Bates, P. J.; Laber, D. A.; Miller, D. M.; Thomas, S. D.; Trent, J. O. *Exp. Mol. Pathol.* **2009**, *86*, 151-164.
(127) Gatto, B.; Palumbo, M.; Sissi, C. *Curr. Med. Chem.* **2009**, *16*, 1248-1265.
(128) Markowitz, M. A.; Turner, D. C.; Martin, B. D.; Gaber, B. P. *Appl. Biochem. Biotechnol.* **1997**, *68*, 57-68.
(129) Omidian, H.; Rocca, J. G.; Park, K. *J. Controlled Release* **2005**, *102*, 3-12.
(130) Dyson, N. A. *Chromatography integration methods*; Royal Society of Chemistry: Cambridge, 1990.
(131) Tucker, W. O.; Shum, K. T.; Tanner, J. A. *Curr. Pharm. Des.* **2012**, *18*, 2014-2026.
(132) Collins, K. D. *Biophys. J.* **1997**, *72*, 65-76.
(133) Zhao, Q.; Li, X.-F.; Shao, Y.; Le, X. C. *Anal Chem* **2008**, *80*, 7586-7593.
(134) Zhao, Q.; Li, X.-F.; Shao, Y.; Le, X. C. *Anal. Chem. (Washington, DC, U. S.)* **2008**, *80*, 7586-7593.
(135) Pavlov, P. V.; Leonova, A. G.; Smirnova, M. V. *Zh Mikrobiol Epidemiol Immunobiol* **1960**, *31*, 65-69.
(136) Hamaguchi, N.; Ellington, A.; Stanton, M. *Anal. Biochem.* **2001**, *294*, 126-131.
(137) Fabregat, A.; Pozo, O. J.; Marcos, J.; Segura, J.; Ventura, R. *Drug Test. Anal.* **2010**, *2*, 630-636.
(138) Lawyer, T.; McIntosh, K.; Clavijo, C.; Potekhina, L.; Mann, B. K. *Int. J. Cell Biol.* **2012**, 737421, 737429 pp.
(139) Wulff, G.; Akelah, A. *Makromol. Chem.* **1978**, *179*, 2647-2651.

(140) Wulff, G.; Vietmeier, J.; Poll, H. G. *Makromol. Chem.* **1987**, *188*, 731-740.
(141) Ye, L.; Weiss, R.; Mosbach, K. *Macromolecules* **2000**, *33*, 8239-8245.
(142) Glad, M.; Reinholdsson, P.; Mosbach, K. *React. Polym.* **1995**, *25*, 47-54.
(143) Kempe, M.; Mosbach, K. *Tetrahedron Lett.* **1995**, *36*, 3563-3566.
(144) Kempe, M. *Anal. Chem.* **1996**, *68*, 1948-1953.
(145) Dong, X.; Sun, H.; Lue, X.; Wang, H.; Liu, S.; Wang, N. *Analyst (Cambridge, U. K.)* **2002**, *127*, 1427-1432.
(146) Piletskii, S. A.; Kukhar, V. P.; Fedoryak, D. M. *Ukr. Khim. Zh. (Russ. Ed.)* **1989**, *55*, 872-875.
(147) Sellergren, B.; Ekberg, B.; Mosbach, K. *J. Chromatogr.* **1985**, *347*, 1-10.
(148) Wulff, G.; Vietmeier, J. *Makromol. Chem.* **1989**, *190*, 1717-1726.
(149) Shea, K. J.; Stoddard, G. J.; Shavelle, D. M.; Wakui, F.; Choate, R. M. *Macromolecules* **1990**, *23*, 4497-4507.
(150) Spivak, D. A.; Sibrian-Vazquez, M. *Mater. Res. Soc. Symp. Proc.* **2002**, *723*, 5-10.
(151) Sibrian-Vazquez, M.; Spivak, D. A. *Macromolecules* **2003**, *36*, 5105-5113.
(152) Spivak, D. A.; Kim, H. *Polym. Prepr. (Am. Chem. Soc., Div. Polym. Chem.)* **2003**, *44*, 661-662.
(153) Sibrian-Vazquez, M.; Spivak, D. A. *J. Am. Chem. Soc.* **2004**, *126*, 7827-7833.
(154) Sibrian-Vazquez, M.; Spivak, D. A. *J. Am. Chem. Soc.* **2004**, *126*, 7827-7833.
(155) Spivak, D. A.; Sibrian-Vazquez, M.; Houck, S. *Polym. Prepr. (Am. Chem. Soc., Div. Polym. Chem.)* **2005**, *46*, 1103-1104.
(156) Lejeune, J. P. Dissertation, Louisiana State University, 2009.
(157) Simon, R.; Collins, M. E.; Spivak, D. A. *Anal. Chim. Acta* **2007**, *591*, 7-16.
(158) Meng, A. C.; LeJeune, J.; Spivak, D. A. *J Mol Recognit* **2009**, *22*, 121-128.

# VITA

Nicholas Alexander Gariano, son Anne and Patrick Gariano was born in New Orleans in 1980. He earned his bachelor's degrees in chemistry and biochemistry at Virginia Tech in 2003. As an undergraduate he participated in undergraduate research in several polymer science research groups and gained an appreciation for the subject matter. After graduation he worked as a research associate in the polymer science department at the University of Southern Mississsippi for 3 years during which he decided to pursue a research career in polymer science.

In the fall of 2006 he began graduate school at Louisiana State University and the following spring he joined the research group of Dr. David Spivak. Using his previous interest in chemistry, biochemistry, and polymer science, he worked on several projects involving recognition of biomacromolecules. During that time he was awarded several Coates travel awards and the Timethy S. Evenson Award for Excellence in Macromolecular Science. He was also an active student leader holding positions as president, vice president, and coordinator of community outreach and fundraising as a member of the macromolecular studies graduate student association over 5 years. He participated in several national and international conferences to present his work while developing his skills as a polymer chemist. Nick will be receiving the degree of Doctor of Philosophy on May 16, 2013 at the LSU spring commencement.