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2D LAYOUT OF BEAD-BASED THIOAPTAMER/APTAMER SELECTION PLATFORM FOR THERAPEUTICS AND DIAGNOSTICS

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2D LAYOUT OF BEAD-BASED THIOAPTAMER/APTAMER SELECTION PLATFORM FOR THERAPEUTICS AND DIAGNOSTICS

by

Xu Zhao.

THESIS

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As A Matter of Elegancy, Subtlety, Serendipity and How to Articulate

To my beloved maternal grandma, Suzhi Tian…

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2D Layout of Bead Based Thioaptamer/Aptamer Selection Platform for Therapeutics and Diagnostics

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Abstract: Nucleic acid research has expanded the way we can intervene with biological systems. Especially, oligonucleotide agents (ODN or aptamers) are believed to affect cell function via complementary recognition or binding to specific proteins by forming tertiary structure. This opens new ways in therapeutics and diagnostics. The phosphoro- mono-/di- thioate substitutions in the backbone (termed “thioaptamer”) grants ODN nuclease resistance and higher binding affinity.

A bead-based combinatorial library, in which every bead contains a unique species of aptamers, provides a promising platform for selection of aptamers and thioaptamers. To successfully screen the bead-based library, 2D layout of beads in gel and on bead screening model is proposed. To develop the 2D layout of beads and its corresponding functional assays, a model system is first established: NF-kappa B proteins were expressed, purified and characterized. Thioaptamer XBY6, which specifically targets NF-kappa B protein, and its natural origin, I-kappa B were synthesized and verified. Thioaptamer purification using FPLC and HPLC was also investigated, and

several 5'-funtionalized thioaptamers were successfully purified. Electrophoretic mobility shifting assay (EMSA) has been used to verify XBY6 binding, and ELISA assay has been used to verify I-kappa B binding towards human recombinant NF-kappa B protein. Preliminary study of bead in 2D gel showed applicability of bead-based selection and thus on bead functional assays were developed. Both double strand one species library with I-kappa B sequence and a 2^{12}=4096 different species beads library were constructed and verified. The library was then tested using on-bead EMSA like assay and ELISA assay. Both assays showed encouraging results for 2D layout selection and further enhancement of visualization (signal/noise improvement) is discussed.

The project suggested that 2D layout of beads in gel (PAGE) is well suited for parallel high-throughput selection of thioaptamers and aptamers, thus paving a new way for drug discovery and future therapeutics and diagnostics.

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

This chapter provides an introduction to the basic principles of nucleic-acid based therapeutics and diagnostics and the phosphothioate-substituted oligonucleotide and its role in therapeutics and diagnostics. New strategic methods such as bead based selection for nucleic acid therapeutics are emphasized.

1.1 NUCLEIC ACID THERAPEUTICS AND DIAGNOSTICS

With the revelation of many molecular pathways that are involved in important diseases and the detection and quantification of abnormal gene expressions as hallmarks of diseases, we have been provided unprecedented opportunities for developing suitable methods and drugs to promote human health. Especially, the knowledge of the biological role of nucleic acids is always expanding into unexpected new functionalities: the discovery of DNA as the main carrier of genetic information was the beginning of modern molecular biology; RNA has been found as gene transcriptor and recognized as key components in protein synthesis; the notion that nucleic acid ligand could regulate the activity of target proteins was found in the 1980s; the idea of gene expression modification by exogenous nucleic acids was also derived (Paterson, Roberts et al., 1977). RNA interference has been found to be one of the most profound ways that a gene is regulated and became the trigger of nucleic acid therapeutics; the functionality of nucleic acids are constantly expanded by new ideas and experiments. With the maturation of oligonucleotide chemistry, aptamers emerged as a new class of potential drug molecules that may provide a wide range of applications in human disease (Nimjee,

Rusconi et al., 2005), including but not limited to cancer, inflammation, and cardiovascular and infectious diseases. Many chemical modifications are also introduced to either expand the scope of functions of nucleic acids or to remove the barriers that prevent nucleic acids from applications in real clinical scenarios. Thioaptamers are a novel form of nucleic acids which basically present structures that have not been found in nature and give a promising prospect in bringing in new features that help avoid disadvantages normal nucleic acids have exhibited in drug development research.

It is a very natural and conceptually elegant idea to utilized nucleic acids in therapeutics, and it is a widely held view that to reach the ultimate goal of "on demand" regulation of gene expression, the approach with various types of nucleic acid is viable and essential. Numerous strategies are under development (Gewirtz, 2000; Opalinska and Gewirtz, 2002; Nimjee, Rusconi et al., 2005) and most of them show the promise of high specificity and low toxicity, while also in many cases, blocks hold up progress: even though some encouraging results are found, nucleic-acid therapeutics are still in the early stage of development. Also, problems such as how to effectively deliver the molecule to the right target cell or cell organelle, how to select the right DNA/RNA sequence to physically target are the major hurdles. This project has provided one new strategy as an attempt at solving one of the problems that hold back nucleic acid molecules from treating and diagnosing human diseases.

The varieties of proposed mechanisms of nucleic acid function *in vivo* and their potential use in therapeutics and diagnostics are discussed below.

1.1.1 Gene Transcription modulation

The first and well investigated category of nucleic acid therapeutics relies on some type of sequence specific recognition according to the Watson-Crick base-pairing model (Opalinska and Gewirtz, 2002; Kalota, Dondeti et al., 2006). As early as the 1970s, people realized that an ”antisense” short DNA oligodeoxynucleotide could inhibit viral replication in culture (Stephenson and Zamecnik, 1978). The reverse-complementary antisense nucleic acids have been shown to be able to manipulate gene expression both as DNAs to inhibit gene transcription and as RNAs to inhibit gene translation. The mechanisms involved are more complicated than the initial expectations, and our knowledge of gene regulation and its underlying physical-chemical principles have been tremendously increased by investigating these gene expression alteration phenomenon.

At the level of transcription, homologous recombination (Stasiak, 1996), triple-helix-forming oligodeoxynucleotides (TFOs) (Kim and Miller, 1998), and transcription factor binding domain competition (Sharma, Perez et al., 1996) are all possible ways to interfere with gene expression. Homologous recombination is the set of fundamental events that drive evolution. In nature, these events happen during the stage of DNA replication in germ cell lines during the process of meiosis, or in virus infected cells during mitosis. If the target gene’s related sequence is cloned into vectors (like recombinant virus that infect cells you are interested in) and then integrated into cells by vector incorporation process such trans-infection, the target gene is able to be tweaked in a “on demand’ way by the result of the final homologous recombination events. While this is a very powerful tool that has been utilized frequently in cell biology research, it has not been effective in any clinical application. With our knowledge and technology

regarding controlling this process increasing, the method will probably play a more important role in gene regulation manipulation.

TFOs are structures created by the formation of Watson-Crick and Hoogsteen bonds between the double helix and the third strand (Fig1-1). So a form of biologically inert, intermolecular DNA triple helices is formed in which the third strand in fact binds the major groove of the duplex. These complexes are formed in a very sequence-specific manner and provide us with a mechanism for targeting any DNA sequence of interest without interfering with others (Fig1-1) (Luo, Macris et al., 2000; Knauert and Glazer, 2001). Typical TFOs are 10-30 nucleotides in length and have shown the ability to repress transcription of several genes both *in vitro* and *in vivo* (Cooney, Czernuszewicz et al., 1988; Grigoriev, Praseuth et al., 1993). Decoys of double strand DNAs which resemble the sequence of the promoter region or other regulatory elements of genes can act as the competitor of the endogenous DNAs for binding transcription factors and result in reducing the expression level of the target genes or even shutting off them (Paterson, Roberts et al., 1977; Stephenson and Zamecnik, 1978; Sharma, Perez et al., 1996; Morishita, Higaki et al., 1998). Short decoys based on the consensus binding sequence of specific transcription factors like TCF (Seki, Yamamoto et al., 2006) outperformed scrambled sequences in inhibiting a variety of downstream target genes. These processes are limited by nucleosome structure which constrains the accessibility of non-activated genes and the rapid degradation of these decoys by intracellular nucleases. Structural modifications to produce decoys that exhibit high stability are developed and discussed in section 1.2

Figure 1-1: Triple-helix structure. (T-AT C-GC)

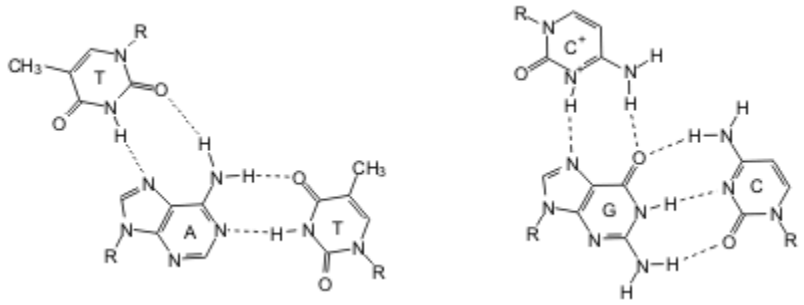


Illustration of the TFOs structure by Watson-Crick bonds and Hoogsteen bonds. The triple helix structure are sequence specific and by carefully designing the third strand, a specific DNA duplex can be targeted in such a way that the gene expression of this gene that bear the targeted duplex is altered.

1.1.2 Gene Translation modulation

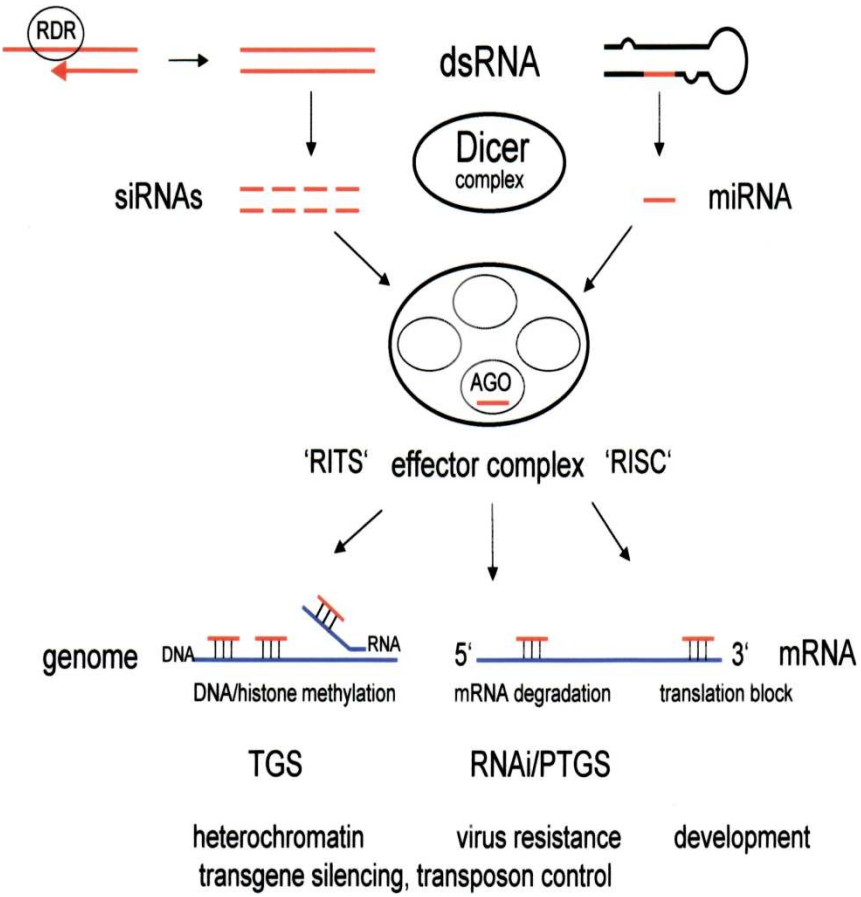
At the level of translation, mRNAs are the major focus. Proteins that RNA encodes are so diverse in their physical-chemical properties, subcellular localization and binding abilities that a systematic method of direct intervention to these targeted proteins are quite impossible. On the other hand, mRNAs are more accessible than its encoded protein as well as the original DNA gene in a sense that it is involved in translation, transcription and transportation between the nucleus and the cytoplasm. It is interesting to note that mRNA stability is an essential part of the gene control machinery and thus, similar to DNA decoys, RNA decoys that could possibly bind to a specific mRNA and affect the stability of it would be an ideal way to manipulate gene expression. Either a vector which contains the form of sequence of the desired RNA are needed to induce antisense RNA molecule or decoys are brought into cells through other means (Agrawal

and Zhao, 1998). An oligonucleotide which could hybridize to the complementary fragment of the endogenously expressed mRNA would prevent it from translation, and almost certainly initiates the degradation processes (Crooke, 1998; Jen and Gewirtz, 2000). A newly discovered mechanism which is far more complicated than simple antisense pairing and more effective in repressing gene expression (the word silence specifically indicates the level of suppression is very high) is called RNA interference (RNAi). People believe RNAi represents one of the most conserved cellular defense strategies against invading pathogens in eukaryotes (Tuschl and Borkhardt, 2002). It has been proposed that dsRNAs are frequently generated by pathogens and that the presence of dsRNA will activate a specific process that would shut down the genes most related to these dsRNAs. Usually a long (500~1000 bp) dsRNA (Elbashir, Martinez et al., 2001) will first be fragmented by the RNase III family of nucleases (called DICER),(Ketting, Fischer et al., 2001). Those 21-25 bp dsRNA (small interference RNAs) would act as a guide to sequence specific degradation of mRNAs which contains the homologous region of these siRNAs (Bernstein, Caudy et al., 2001). Experiments have shown that after transfection of siRNAs into mammalian cells, long-lasting silencing of genes at the post-transcriptional level was observed, as well as sequence-unrelated innate immune response (Stark, Kerr et al., 1998). It is even more striking that the silencing is extraordinary specific: one nucleotide mismatch will often prevent the RNAi function (Brummelkamp, Bernards et al., 2002).

The highly specific feature of siRNAs apparently makes it a prospective novel therapeutic platform. For example, RNAi was used to target the BCR-ABL mRNA which plays essential roles in several types of leukemias and lymphomas (Wilda, Fuchs et al., 2002). Unfortunately, the delivery of siRNAs to the appropriate cell types and subcellular localizations remains uncertain and not fully controllable. Chemical modifications of

siRNAs such as substitutions by phosphorothioate and attachment of lipophilic tails are being considered. My project will try to address several problems like selecting the right modifications that may solve the previously mentioned problems.

Figure 1-2: Proposed RNA interference mechanism



Dicer RNase III will first degrade the long dsRNA into 21-25 nt siRNA duplexes. A RNA-induced silencing complex (RISC) endonuclease is formed and targets mRNAs homologous to the siRNAs. AGO2 may be required for this formation. The RISC complex is the main mediator for RNA degradation as well as histone methylation and

chromatin reorganization and gene transcription suppression (Modified from Marjori A. Matzke, Antonius J. M. Matzke ,2004).

In all possible nucleic acid applications, we see delivery appears again and again as a major issue limiting the efficacy and availability to different cell types. It is well known that nucleic acids are predominantly negatively-charged; crossing the cell membrane passively is definitely not a thermodynamically favorable reaction. The oligonucleotides are usually being absorbed or enter by endocytosis (Yakubov, Deeva et al., 1989; Beltinger, Saragovi et al., 1995). Possible receptors may also be involved. Simple approaches such as direct loading based on physical damage of cell membrane may be useful in research. However, they are not considered an effective vehicle for clinical delivery of ODNs. Successful attempts have been made by utilizing cationic liposomes, cationic porphyrins, fusogenic peptides and artificial virosomes (Jen and Gewirtz, 2000; Kalota, Dondeti et al., 2006). Taking advantage of the natural ability of viruses to enter into the host cell, modified viruses are also employed as deliver vectors (Thomas, Ehrhardt et al., 2003) . The main strategy behind these vectors is to substitute the replication part of viral genes by a gene of interest. The ability of these vectors could be enhanced if these viral particles could be build up by modified ODNs which bring in more safety. A selection strategy of possible ODNs will help accelerate this process.

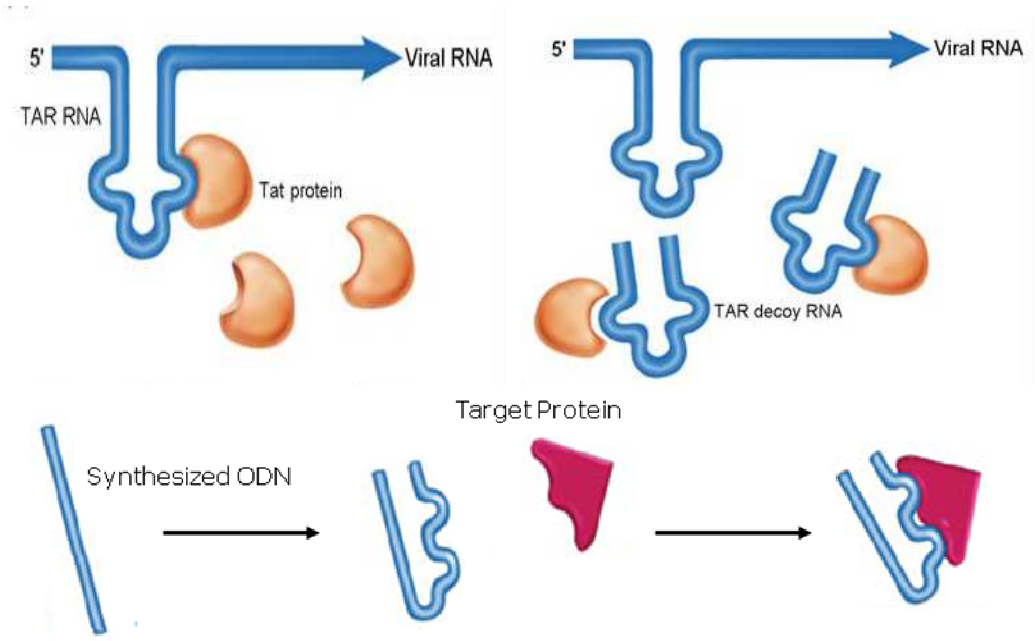
1.2 APTAMERS AND THEIR ROLE IN DRUG DEVELOPMENT AND CLINICAL APPLICATIONS

1.2.1 The origin and concept of aptamers

Different from base-pairing model based “antisense” nucleic acid and siRNA molecules, which gain the specificity solely on information (sequence specific) matching, some DNA and RNA molecules are also able to fold into complex three-dimensional structures and function through the affinity based binding model and can act as a ligand or an enzyme that target specific proteins or other functional molecules(Zhang, Blank et al., 2004; Nimjee, Rusconi et al., 2005). The concept was derived from the discovery of ribozymes and DNAzymes that exhibit catalytic activity towards some sequence-specific cleavage of mRNAs (Eckstein, 1996; Earnshaw and Gait, 1997), as well as viral RNA/DNA interaction with hosts (Cullen and Greene, 1989). Classical studies of HIV and adenoviruses have found that there are small structured RNA encoded by these viruses to bind cellular proteins with very high specificity and affinity so as to inhibit the innate antiviral response of the host cell and to recruit replication apparatus. A classical example is HIV virus. The virus encodes a nonstructural protein called Tat (Trans-activator of Transcription) which helps enhance the reproduction cycle; a short, hairpin like structure in the 3’UTR region called TAR (trans-activation response element) of HIV virus transcript specifically binds to Tat protein and induces viral replication cycles. TAR can also interact with cyclin T1 to affect cell cycles. Adenoviruses also encode a ligand like RNA structure to control interferon-induce response (Burgert, Ruzsics et al., 2002). These phenomena have inspired scientists to find similarly structured DNA/RNAs for therapeutics. A TAR-like oligonucleotide was first demonstrated to inhibit HIV replication. This kind of ODN decoy was then called “aptamers”, derived from the Latin word “aptus” , meaning “to fit” (Ellington and Szostak, 1990), to describe these small

sized (6kDa to 40kDa), complex structured nucleic acids. It is believed that the structure is stabilized by both Watson-Crick bonds and other non-canonical intramolecular interactions, as revealed by the “hammerhead” and “hairpin” structures in the ribozymes (Dahm and Uhlenbeck, 1991; Sawata, Shimayama et al., 1993; Hegg and Fedor, 1995; Hampel, 1998). Aptamers have combined two very important aspects of biological systems: heritable and replicable information and the ability to perform specific tasks by forming complex structures.

Figure 1-3: Function of TAR RNA in HIV replication and the concept of aptamer using TAR decoy to inhibit Tat-induced HIV replication.



Tat protein specially bind to TAR RNA structure to remove this hairpin to initiate the whole gene replication cycle. It is a self-accelerating process and allows HIV to have an explosive response once a threshold amount of Tat is produced. A decoy bearing the same 3D structure of TAR RNA could compete with the very limited amount of Tat

protein in the initial stage of HIV replication cycle. Adapted from (Nimjee, Rusconi et al., 2005).

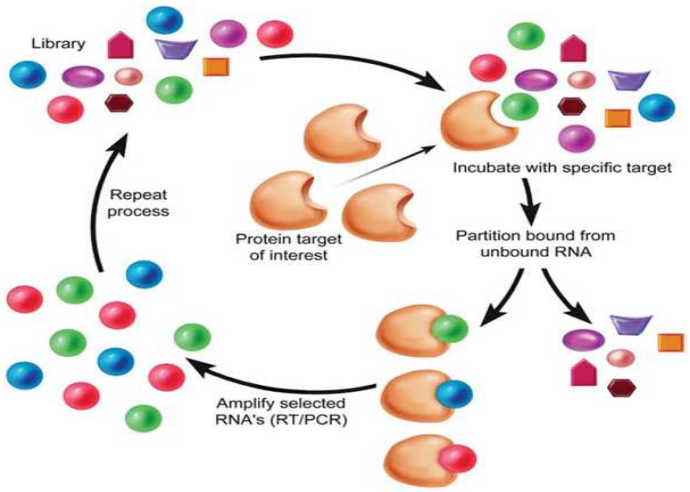
1.2.2. Selection of aptamers

In the 1990s, a revolutionary method of generating interesting aptamers was developed (Ellington and Szostak, 1990; Tuerk and Gold, 1990; Tuerk and MacDougal-Waugh, 1993) and termed SELEX (systematic evolution of ligands by exponential enrichment). Initially, a large number of different molecules (up to 10^{15} at the convenience scale) (Ellington and Szostak, 1992) are generated by combinatorial chemistry, usually in the form of a random, single stranded DNA library with a centralized random region flanked by known sequences at both ends. A typical 40 nucleotide random region can be composed of 10^{24} distinct molecules, so the sequence space containing even 10^{15} molecules still constitutes a tiny part of all possible species of molecule. RNAs are generated by reverse-transcriptase. Secondly, the library will be incubated with the target protein of interest under defined conditions such as specific buffer, pH, etc. Molecules that bound to the target are separated from the unbound or loosely bound molecules, usually using techniques like affinity chromatography. Depending on the nature of the target and the proposed property you want to gain, such as a fast association rate, slow dissociation rate, high affinity, or high specificity against closely related proteins, the methodology can by quite different. The key concept here is the separation of the potentially interesting molecules from those of less importance. It is worth mentioning that this is not a one by one screening method like those processes used in pharmaceutical firms, because 10^{15} species are impractical to be screened conventionally. Since aptamers already contain within themselves the code for

amplification and synthesis, DNA/RNA polymerase can be used to amplify the number of molecules in the pool that contains the selected sequences. Then, after amplification, we have a similar scale library in which the desired molecules are enriched and finally dominate after iterative rounds of selection, normally 6-12 rounds (Jayasena, 1999). The leading aptamers in the final selection can then be cloned and sequenced (James, 2001; King, Bassett et al., 2002; Bassett, Fennewald et al., 2004; Lee, Hesselberth et al., 2004; Zhang, Blank et al., 2004; Proske, Blank et al., 2005).

As mentioned above, due to the limiting scale of the preparations, the proportion of the whole sequence space covered decrease exponentially as the length of the random sequences increases. However, in order to form a structure capable of specific binding, nucleic acids need to be sufficiently long, usually greater than 35 nucleotides. Most researchers believe that perhaps one in 10^{11} different species in the initial library could form the desired binding motif (Burke and Gold, 1997; James, 2001).

Figure 1-4: Illustration of SELEX process.



First, a library of DNA or RNA is generated that contains a large number of distinct structures. Aptamers in the pool that could bind to the target protein and was

partitioned from those didn't bind. An amplified library is generated based on the enriched partition. New rounds of selection repeat these steps providing a smaller library of tightly binding aptamers. Adapted from (Nimjee, Rusconi et al., 2005).

In fact, there is an underlying assumption of SELEX or similar methods for *in vitro* selection: A direct and unique relationship between the sequence and the 3D structure of the aptamer. It resembles Anfinsen's "one sequence one structure" theory behind protein folding. This may be true when we look at some known folding of functional RNAs such as the *Tetrahymena* intron (Amano, 1997; Amano, 2003) or the hammerhead ribozyme (Bassi, Mollegaard et al., 1999), which fold rapidly into the native states. But most aptamers are found to be able to form several distinct conformations, of which only one may be competent at binding with the target. The structural pleiomorphism poses a problem in the process of iterative selection because interested aptamers are in low copy number in early rounds, the copy of one aptamer may not be in the right conformation to bind with the target and may not be selected. This situation will be worse if it happens in the initial round (Zuker, 1989; Mathews, Sabina et al., 1999). Conformational flexibility of aptamers is not uncommon and has been observed in many cases (Xu and Ellington, 1996; Yang, Kochoyan et al., 1996).

These considerations give the early stages of selection great importance. This is sometimes troublesome since in the early stage the whole process is more or less operated in a "blind" fashion. Nevertheless, the selection process *in vitro* has produced very fruitful results and opened the doors of application in therapeutics and diagnostics using aptamers.

1.2.3 Aptamers as drug candidates

It is a little bit surprising that the scope of targets that aptamers can bind is very broad, and up to date, there has not been found any restriction of target type. From small molecules such as metal ions (Ciesiolka, Gorski et al., 1995; Hofmann, Limmer et al., 1997), amino acids (Burgstaller, Kochoyan et al., 1995; Harada and Frankel, 1995), organic molecules(Ellington and Szostak, 1992; Mannironi, Di Nardo et al., 1997), antibiotics (Wallis, Streicher et al., 1997; Wallace and Schroeder, 1998), nucleotides (Sassanfar and Szostak, 1993; Kiga, Futamura et al., 1998), peptides (Nieuwlandt, Wecker et al., 1995; Williams, Liu et al., 1997), to a growing number of therapeutic protein targets such as virus non-structural proteins (Kumar, Machida et al., 1997; Browning, Cagnon et al., 1999), cell adhesion receptors (O'Connell, Koenig et al., 1996; Ulrich, Magdesian et al., 2002), enzymes (Tuerk and Gold, 1990; Kubik, Stephens et al., 1994; Bridonneau, Chang et al., 1998), growth factors and interleukins (Jellinek, Lynott et al., 1993; Kubik, Bell et al., 1997), coagulation factors (Rusconi, Yeh et al., 2000; Rusconi, Scardino et al., 2002), antibodies (Wiegand, Williams et al., 1996) and even the viral particles (Pan, Craven et al., 1995) have all proven successful. The great spectrum of molecules that aptamers can reach draws the comparison with antibodies which also have a broad scope of molecular recognition. Both DNA and RNA aptamers often bind their targets with equilibrium dissociation constants (K_d) in the range of 10^{-12} M to 10^{-9} M. These indicate the aptamer-target binding events are very specific interactions and are comparable to, or even better than, those of antibodies used in therapeutic and diagnostic applications. Besides, no evidence of immunogenicity has been encountered using aptamers (Eyetech Study Group, Group, 2002). The importance of lower immunogenicity should not be underestimated in therapeutics, because this is the major hurdle that prevents antibodies from being used as drugs. Because the aptamer selection process is

only a chemical process without a biological system as animals, a wider range of manipulations, such as a variety of selection conditions like cross-active reduction, high stringency and low stringency binding, kinetic parameters tuning and chemical modifications are possible. The shelf-life of aptamers is also much longer (years) and the denaturation of aptamers can be reversed (Yang and Gorenstein, 2004; Ulrich, Trujillo et al., 2006).

The properties of aptamers make them ideal drug candidates and raise the hope of a new dimension in drug discovery. Enthusiasm for developing aptamers as drug candidates is constantly rising, with many potential applications currently in the drug testing pipeline or in early developmental stages**.** Indeed, in December 2004, the first aptamer to be successfully developed as a therapeutic agent in humans, an anti-VEGF (vascular endothelial growth factor) aptamer from Macugen received FDA approval for treatment of human ocular vascular disease (all types of neurovascular age-related macular degeneration ,AMD).

Aptamers' role in therapeutics is not limited as drug agents. The detection and quantification of molecules play an essential role in diagnostic applications. Similar to antibodies, a two-site binding assay, usually referred as a "sandwich assay", has been effectively tested. Pioneering experiments successfully used a vesicular endothelial growth factor (VEGF) targeted RNA aptamer with a fluorescein tag to detect the monoclonal antibody captured VEGF (Drolet, Moon-McDermott et al., 1996). The sandwich assay can even be carried out on a chip using modified aptamer as detecting agents (thioaptamer, see later section) (Wang, Yang et al., 2006). Biological markers for cancers and cardiovascular disease could be identified and later tested using aptamers as a way to screen patients for selective therapies. The development of quick detection on affinity sensors using aptamers is promising since the ability of aptamers to turn over

(aptamers can be denatured and re-fold much more easily) is well suited for the role of sensors. In one recent work, an aptamer specific to HIV-1 Tat, chemically synthesized biotinylated TAR RNA, was immobilized on the sensor surface. Subsequent binding of fragments of Tat on the aptamer surface resulted in a change in both resonance frequency and motional resistance of the sensor (Minunni, Tombelli et al., 2004). Most of these studies show good specificity and reproducibility. There is still a need to improve the sensitivity to reach the threshold for clinical use, but the use of aptamers as bio-recognition elements is a new direction of clinical diagnostics.

With the possibility of aptamers synthesized at a defined density at precise locations on a solid surface (McGall, Labadie et al., 1996; Gao, LeProust et al., 2001), aptamer arrays can be used in parallel to analyze thousands of targets. A wealth of information in proteomics would be revealed on the robust aptamer microarrays, and in the near future the combination of aptamers with antibodies and other detecting agents will make inroads in the proteomics and other translational research fields.

1.2.4 Chemical modification on Aptamers

Chemical modifications are introduced into aptamers for several reasons. First, the attachment of different groups other than the four naturally occurring nucleotides effectively increases the structural diversity of molecule libraries. For example, aptamers with 5-(1-pentynyl)-2-deoxyuridines have been found to bind human thrombin using a library build up from normal nucleotides as well as modified thymidine nucleotide analogs. Interestingly, aptamers selected from the modified library were strikingly different from those isolated from a normal library both in the primary and the secondary structures (Bock, Griffin et al., 1992; Latham, Johnson et al., 1994). Other examples included uncommon base pairs like IsoG/IsoC, 1,5-diaminopyrimidine/xanthine, etc

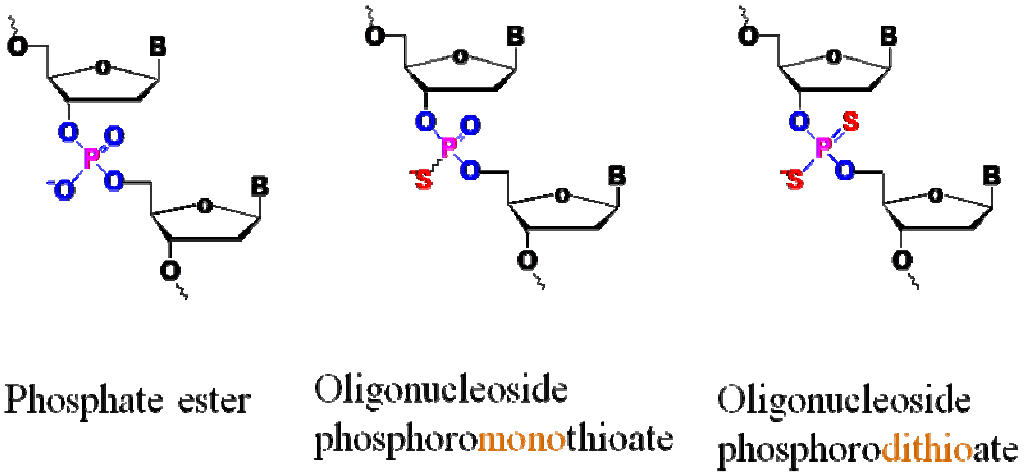
(Piccirilli, Krauch et al., 1990). To find possible ligands to bind to hydrophobic targets, libraries modified with hydrophobic groups are more suitable (Schweitzer and Koo, 1995). Various functional groups, such as 5-bromouracil and 5-iodouracil residues, which can be used to generate photo-cross-linkable aptamers, are incorporated at the end of aptamers to facilitate downstream applications. Amino acids could also be incorporated to create peptide-nucleic acid libraries.

1.3 THIOAPTAMERS

The major concern regarding aptamers as drug candidates is that as natural RNA/DNA, and especially in RNA, the hydroxyl group is very reactive and attacks the neighboring phosphodiester bond to produce a cyclic 2'-3'-phosphate. The nucleic acid backbone could be broken down in the reaction. The degradation of DNA and RNA are catalyzed by many nucleases found ubiquitously in biological systems, both by intracellular and extracellular nucleases. It is estimated that the half-life of RNA molecule in biological samples is in the range of 5-30 minutes. DNA may be stable for hours (Yang and Gorenstein, 2004). Based on the nucleophilic attack theory on ODN degradation, modifications of the phosphorodiester bonds in the ODN backbone are expected to enhance the stability. Though several chemical modifications are introduced by researchers to conquer this problem, only phosphorothioate modifications (phosphoromonothiate and phosphorodithoate) are compatible with polymerases. Gorenstein and Caruthers independently synthesized an important class of sulfur-containing oligonucleotide, dithiophosphate (S2-ODNs) oligonucleotides. The dithioates contain internucleotide phosphodiester groups with sulfur substitutions at both of the

non-bridging phosphoryl oxgens, while monothioates only substitute one oxygen atom. **We denote these as "thioaptamers".**

Figure 1-5: Thioaptamer chemical structure, compared with normal nucleotide phosphordiester bond.

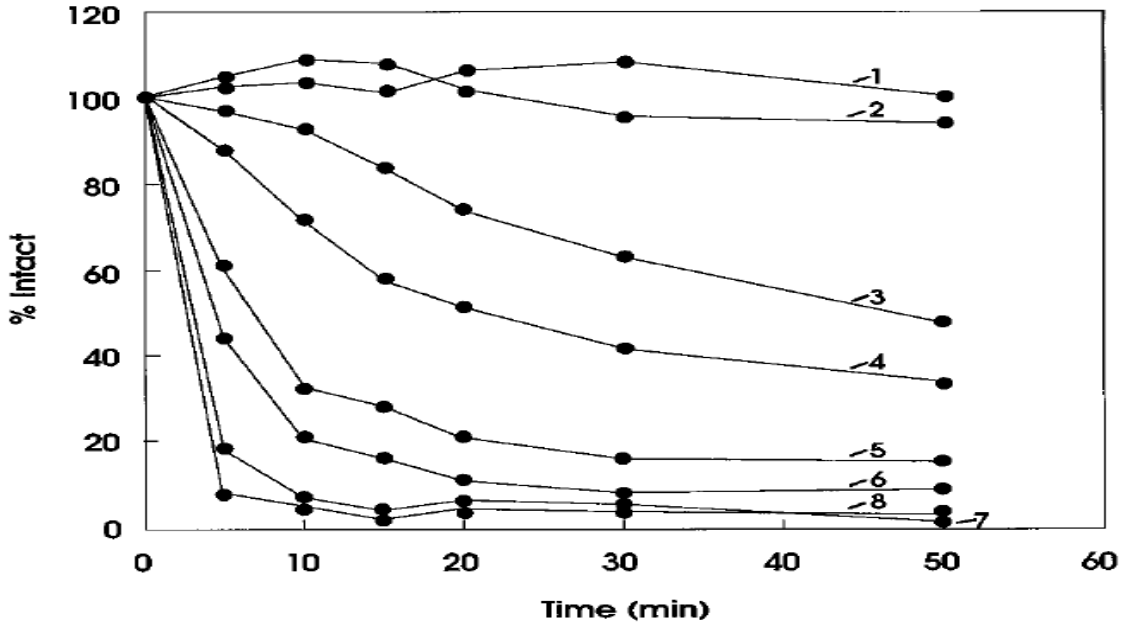


Comparison of phosphorodiester bond, phosphoromonothioate and phosphrodithoate bond.

The introduction of sulfur atoms in oligonucleotides has two major effects. First, the nuclease-resistance is increased with the half-life of S1-ODNs against nucleases increased relative to phosphorodiesters. However, the stability of phosphorothioate oligonucleotides against nucleases *in vitro* varies greatly depending on ODN sequence and the type of nucleases present (Stein, Subasinghe et al., 1988). As a general rule, there is a direct correlation between the number of phosphorothioate replacement linkages and stability (See Fig-1-6). Either alternating phosphorothioate and phosphorodiesters or substituting the nucleotide at the 3' end has been shown to be effective (Hoke, Draper et al., 1991).

Figure 1-6: Nuclease resistance of S1-ODNs against nuclease.

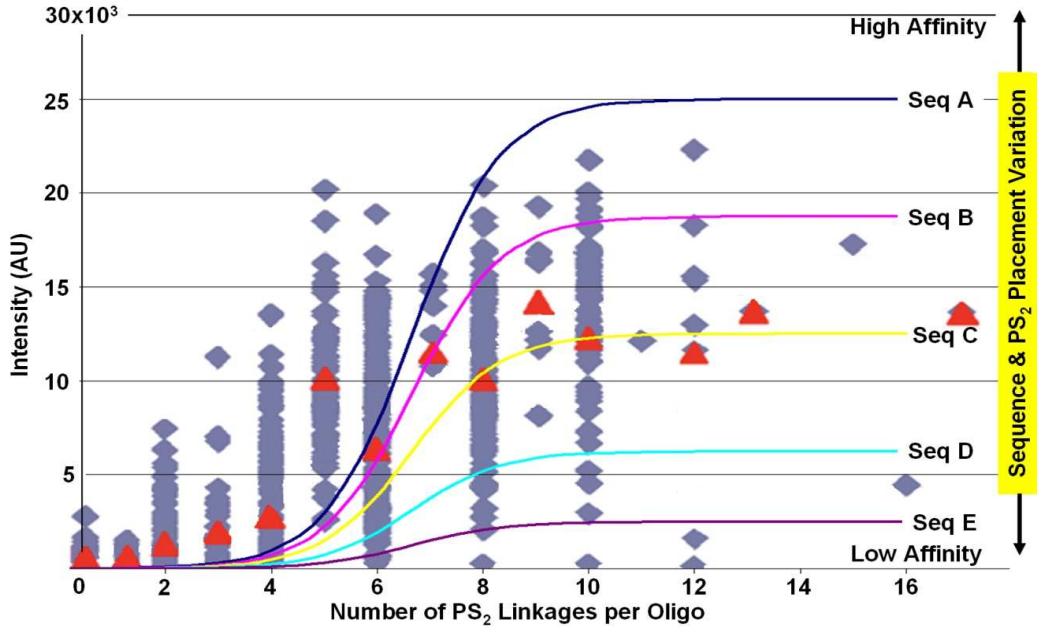
Oligo	Sequence
1	Cs Cs As Cs As Cs Cs Gs As Cs Gs Gs Cs Gs Cs Cs C
2	Cs Cs As Cs As Cs Cs Gs AoCs Gs Gs Cs Gs Cs Cs C
3	Cs Cs As Cs As Cs Cs GoAoCs Gs Gs Cs Gs Cs Cs C
4	Cs Cs As Cs As Cs Cs GoAoCoGs Gs Cs Gs Cs Cs C
5	Cs Cs As Cs As Cs CoGoAoCoGs Gs Cs Gs Cs Cs C
6	Cs Cs As Cs As Cs CoGoAoCoGoGs Cs Gs Cs Cs C
7	Cs Cs As CoAoCoCoGoAoCoGoGoCoGs Cs Cs C
8	CoCoAoCoAoCoCoGoAoCoGoGoCoGoCoCoC
9	CoCs AoCs AoCs CoGs AoCs GoGs CoGs CoCs C



The underlined codes in the table above indicate the position of the numbered ODN contains a normal base. Normal codes indicate monothioate substituted nucleotide. Data are from (Monia, Johnston et al., 1996).

Secondarily, sulfurization of the phosphoryl oxgens of ODNs often leads to enhanced binding affinities. Though the effects can also be non-specific, by limiting the total number of thioated phosphates and carefully selecting the positions of the substitutions one can enhance favorable interactions (King, Ventura et al., 1998; Yang, Fennewald et al., 1999; King, Bassett et al., 2002; Bassett, Fennewald et al., 2004). The effect of enhanced binding is more striking when considering phosphorodithioate ODNs. The inhibitory effect of S2-ODN against HIV-1 reverse transcriptase *in vitro* is reported to be at least 30-fold higher than the corresponding phosphorothioate analog of similar length, and 600 fold greater then normal aptamer(Marshall, Beaton et al., 1992). Both NMR and *ab initio* data (Volk, Power et al., 2002; Volk, Yang et al., 2002) showed it is possibly due to the fact that the soft, bigger sulfur atoms disrupt the ordinary metal ion coordination with the ODN and make it easier to break metal phosphor bond for binding with the target proteins. For example, the sulfur atom in S2-DNA increases the electrostatic repulsion across the narrow minor groove in DNA as a result of the stripping off of the cations from the DNA backbone (Volk, Yang et al., 2002). As mentioned in the previous section, modifications introduce the structural diversity as well as deviation from the normal ODN structure. Simple sulfur replacements into known aptamers generally will not produce the best thioaptamers, because the number of substitutions, the position of substitutions and the specific sequences of the thioaptamers all contribute to their specific structures and, therefore, their specific binding affinities (Fig 1-7).

Figure 1-7: Thiaptamer binding affinity by sequence, number of phosphodithioate linkages and different positions of placement.



Various sequences of ODN were synthesized on chips. Each sequence is synthesized in parallel on these chips in different spots (blue). Each specific spot represents a defined number and positions of phosphorthioate substitutions. Each red triangle represents the average binding affinity of those with same number of substitutions in sequence. (Binding affinity is proportional to the optical intensity in Y axis) Each curve (A to E) represents the fitting curve of binding affinity increase by increasing phosphorodithioate modifications in that specific sequence. Data confirmed the hypothesis that even though the number of phosphorothioate substitution is correlated with binding affinity enhancement, affinity is still correlated with position of substitution as well. So a carefully selected thioaptamer could gain a very high, and specific, binding affinity (Gao and Gorenstein, unpublished).

Taking into account the therapeutics, phosphorodithioate substitution presents another advantage over phosphoromonothioates, since dithioates have an achiral phosphorus center that is also iso-electronic to phosphate (Monia, Johnston et al., 1996). In contrast, phosphoromonothioate substitution creates chiral mixtures. Thus, in the chemical synthesis we can get a mixture of the Rp-diastereomer and the Sp-diastereomer at each modification site. This is troublesome when many modifications are introduced, since you can only get a mixture of many thioaptamers.

Nevertheless, thioaptamers may be a very substantial advance in aptamer therapeutic research,, and indeed, the first FDA approved aptamer drug Vitravene is an S1-ODN. Also, a number of S-ODNs have been tested in clinical trials (Yang and Gorenstein, 2004).

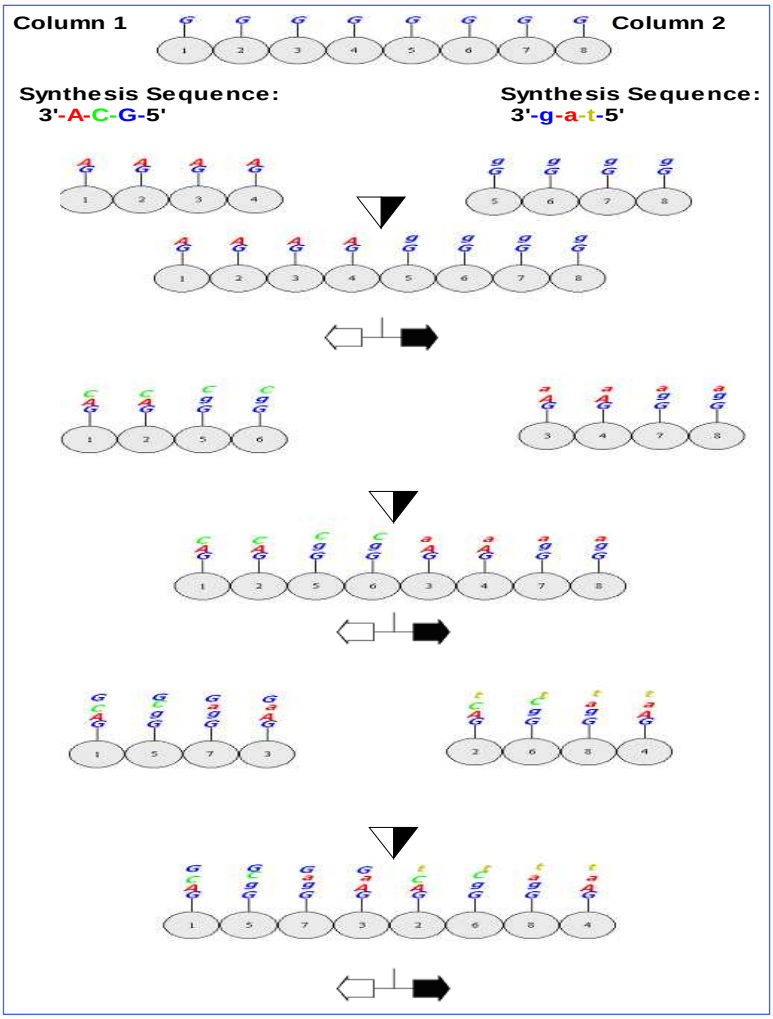
1.4 BEAD BASED SELECTION METHODS FOR DEVELOPMENT OF THIOAPTAMERS AND OTHER NUCLEIC ACID AGENTS

Most methods developed for screening new binding partners involve either using biological systems to generate a selection pool or generating new molecules by combinatorial chemistry. One unique method is derived on the concept of "One-Bead-One-Compound" (Yang, Bassett et al., 2002). If one single unit (say a bead, for example) of the library displays only one type of compound at significant amount, and these units are spatially separable and able to be tested concurrently, then the selection process would focus on distinguishing the unit and identifying the chemical structure on the positively selected unit. In the context of aptamer and thioaptamer, this means building libraries on a solid support **(bead)** for a randomized thioaptamer library which contains a big population of thioaptamers with different structures, some of which may interact

specifically with our target molecule. Especially, there is only one unique type of thioaptamer on one bead, while different beads have different thioaptamers of their own. In this way, we achieve the species diversity in the whole library and the local concentration of a specific thioaptamer is condensed, which makes it available to envision target-candidate binding. The idea also solves another problem with S2-ODNs: the selection process is not limited to substrates accepted by polymerases. Polymerases only accept the Sp-diastereomers as substrates and they only catalyze the formation of the Rp-phophorothioate diester configuration. Any selection that involves using polymerase to produce ODNs cannot produce the Sp-phosphoromothioate or the S2-ODNs (as in the SELEX method).

The key component of bead based selection relies on the "split synthesis" method. The approach is to synthesize several ODNs library concurrently and in between steps, the library is mixed together and then split into aliquots onto different columns of the synthesizer. In the process of beads mixing and splitting into new aliquots, beads from the library will randomly go through different routes into one of several parallel synthesized libraries, different compounds will form. However, all compounds on one bead will share the same reaction route so the bead contains a unique type of compound on it. The split-pool rounds would possibly create M^N species if beads are enough in N rounds of M concurrently synthesized columns.

Figure 1-8: Schematic for the one bead one thioaptamer/aptamer library sythesis



Two different sequences are synthesized on 2 parallel columns (left and right) . After each nucleotide is added to the bead, synthesis is paused and beads from the two columns are mixed (as the reverse triangle shows). The beads are split into two aliquots again to go on to the next step of the synthesis. During this mix and split, some beads from left will join the right and vice visa. After 3 rounds, 8 possible sequences could be created, and each bead has its own unique specific sequence on it.

Yang and Gorenstein first validated this concept in thioaptamer research in his pioneering paper in 2002. Preliminary result showed a small scale of one-bead one-thioaptamer library and some functional assays were tested (Yang, Bassett et al., 2002). A successful strategy must address the following problems: how to identify a large number of assays (10^6~10^8 beads) in a timely and costly fashion; what kind of assays are suitable for on bead binding detection; manual screening of bead libraries under a microscope may be useful to test the concept, but, for example, to select more intense fluorescently stained thioaptamer beads within potentially billions of beads is not practical. Practical concerns need to be addressed too: what kind of solid support is suitable for selection and what effects would the bead have on the proposed functional/binding assays, will the signal be weakened or will the bead induce more noise? More specifically, what quantity of ODNs can be synthesized on one bead (with a specific size) and what are the effects of this quantity? Is that sufficient to be screened out by suitable assays or other techniques? Our group has been focusing on these aspects for several years and this project has tried to establish a strategy to attack several technical problems along the way and this may finally pave the way that leads to the realization of bead based selection of thioaptamers.

Chapter 2: NF-kappaB Ig-kappaB and XBY-6 as the model system for bead based thioaptamer selection

This chapter describes the preparation of NF-kappaB protein and its natural binding sequence decoy Ig-kappaB as well as XBY-6, the leading thioaptamer found to specifically bind to NF-kappa B *in vitro* in order to build up a model system for bead based selection, including a brief introduction of the biological function of NF-kappa B, a focus of recent drug discovery research.

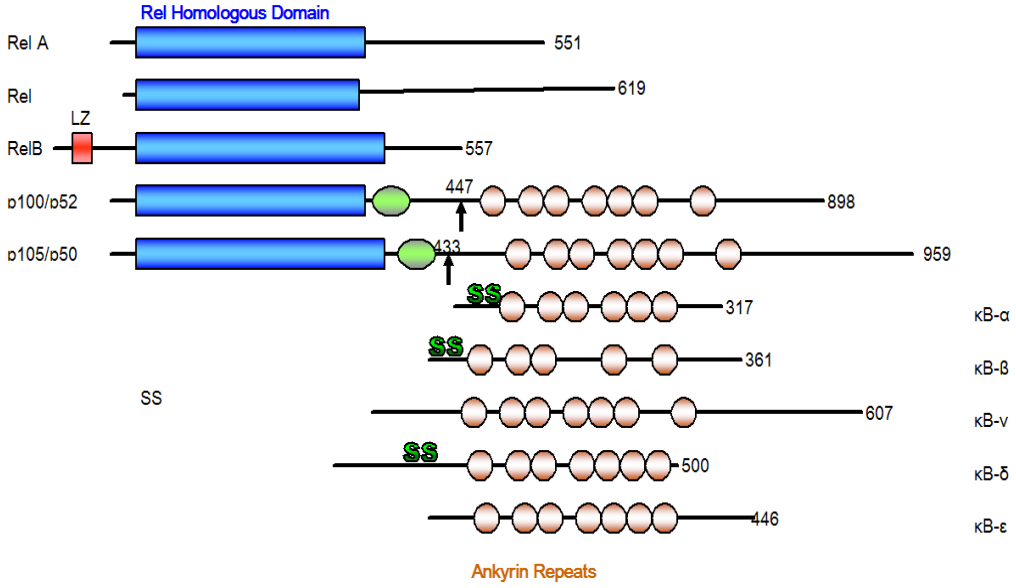
2.1 THE NF-KAPPA B PATHWAY.

NF-kappa B (Nuclear Factor Kappa B) and Rel proteins are called "Evolutionarily conserved mediator of immune responses" (Ghosh, May et al., 1998). Members of the NF-kappa B protein family are a group of transcription factors sharing a Rel homology domain (RHD) : NF-kappaB-1 (p50), NF-kappaB-2 (p52), Rel (c-Rel), RelA (p65 or NF-kappaB3), RelB. They form homodimers or heterodimers such as p50/p105 (precursor of p50), p52/p100 (precursor), $(RelA)_2$, C-Rel/NF-kappaB (Gilmore, 1999). The most common Rel/NF-kappa B dimer in mammals is p50/RelA (p50/p65) heterodimers which is called NF-kappa B specifically. The RHD domain contains several core function sub-domains responsible for dimerization and DNA-binding, regulation, as well as a nuclear-localization sequence (NLS) at the RHD C-terminus. In the inactive form, its inhibitor I-kappa B protein uses the ankyrin repeats (six to seven) region to bind to the RHD domain and thereby NLS is masked and NF-kappa B protein is located in the cytoplasm. A delicate form of self-regulation is the fusion of NF-kappa B with I-kappa B as precursor

p105 and p100, so initially NF-kappa B is self-inhibitory. Processing of precursors by releasing the I-kappa B part would induce the remained p50 dimer to translocate into the nucleus unless bind by other I-kappa B protein. The more common activation is induced by the phosphorylation-induced ubiquitination of the I-kappa B protein. Upon receiving some specific intra- and extra-cellular stimuli such as cytokines, oxidant-free radicals, ultraviolet irradiation, and bacterial or viral products, which are usually an indication of stress or exposure to either bacterial or viral invasion, phosphorylation of I-kappa B happens at key serine residues, and then I-kappa B will be ubiquitinated and undergoes rapid degradation through the proteasome. Once released and translocated into the nucleus, NF-kappa B becomes activated and promotes a variety of target genes (Karin, Yamamoto et al., 2004). The majority of targeted genes are related to the host immune response, which includes inflammatory cytokines and chemokines, immunoreceptors as MHC molecules, antigen presentation molecules and cell adhesion molecules. Therefore, NF-kappa B plays a central role in the human immune response, especially in the acute phase immune response (inflammation). It is not surprising that NF-kappa B and its regulatory pathways have been intensely investigated as a target for drug development. Interestingly, I-kappa B is also a target gene of the NF-kappa B pathway, so a negative feedback and auto-shut down mechanism exists: newly-synthesized I-kappa B could bind to NF-kappa B and lead to transportation back to the cytoplasm and restore the inactive state. Upstream of those target genes, there are about 10 base pair DNA sites which NF-kappa B specifically recognizes. Different dimers have different but overlapping specificities of DNA-binding sites (called kappa B sites), thus provide combinatorial regulations of sets of genes(Kunsch, Ruben et al., 1992; Gilmore, 1999). The consensus sequence of p50 homodimer shows a more symmetric binding motif, GGGGATYCCC (Y means pyrimidine), compared to the p65 homodimer binding motif GGGRNTTTCC

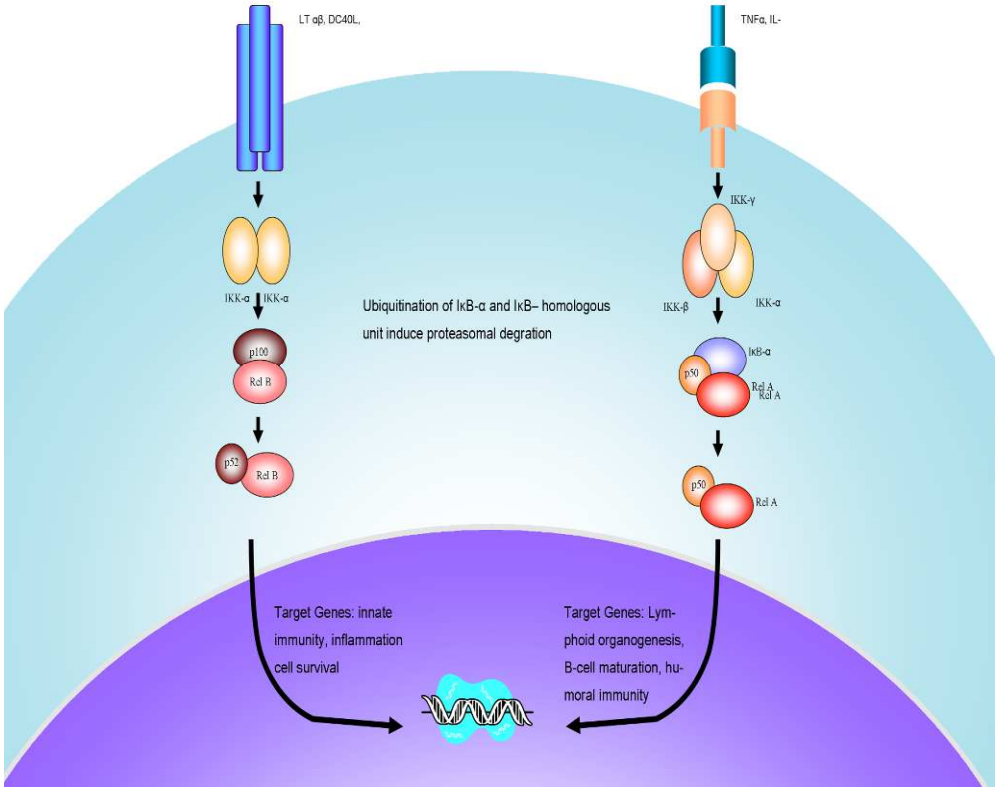
(R is purine and N means any nucleotide). Of c-Rel targeted sequences, a high degree of variation was observed and the current 10nt consensus is NGGRN(A/T)TTCC. If one looks at which half side of the consensus sequence is more conservative, less variation is found in the 5'-half of the p50 consesus sequence site, whereas the 3'-half is less tolerated to variation for p65. This is consistent with the observation that p50/p65 binds to DNA in preference of p50 on the 5' half site and p65 on the 3' half site.

Figure 2-1: NF-kappa B/Rel Family



All proteins in the NF-kappa B family contain the Rel homology domain (RHD, red rectangle). I-kappa B family proteins contains several Ankyrin repeats (in blue circle), which could bind to RHD and inhibit NF-kappa B activity. Both p100 and p105 are precursors containing both RHD and Ankyrin repeats, thus they are self-inhibitory. (Figure is based on Nature Review, Karin, Yamamoto et al., 2004)

Figure 2-2: NF-kappa B signaling pathways.



NF kappa B can be activated through two cell signaling pathways: The first category is activated by TNF-alpha, IL-1, LPS. The IKK three subunit complex will then recruited to phosphorylate I-kappa Bs to induce their degradation. The second category will recruit IKK homodimer to process p100 precursor to release p50 (Figure is based on Nature Review,Karin, Yamamoto et al., 2004).

As a transcript factor that has a specific consensus target sequence, oligonucleotide decoy resembling the binding sequences can be used to compete for protein binding, and thus can be used as drug agents to regulate the NF-kappa B pathway. As mentioned before, the phosphodiesterform of ODNs is only appropriate for *in vitro* studies due to their instability in the presence of nucleases. Thioapatmers with well-designed phosphorothioate and phosphorodithioate internucleoside linkages may be the more promising candidate. Indeed, after carefully design and thorough investigation, XBY-6 , a 14mer dsDNA (Forward strand 5'-CCAGGAGATTCCAC-3', Reverse Strand: 3'-GGTCCTCTAAGGTG-5') with 6 phosphorodithoate substitutions (all of the Ts in the sequence) has been found most effective in terms of binding affinity (Kd ~ picomolar range) as well as in other features like nuclease-resistance and specificity (Volk, Yang et al., 2002). Unlike fully thioated aptamers, which fail to bind specifically to NF-kappa B and generate a smear in whole cell extract pull down experiments, XBY6 does not only show binding affinity *in vitro*, but also is able to specifically pull down only NF-kappa B homodimer protein in nuclear extracts (Yang, Fennewald et al., 1999). This confirms the previous hypothesis that by carefully selecting the right number and positions of modifications, one can gain higher binding affinity without losing specificity.

The high binding affinity of XBY-6 towards p50 homodimer vs. p65 homodimer and their natural binding sequence I-kappa B make a perfect model system for technology development for selecting binding partners of ODNs and proteins. The success of thioaptamer hangs on whether we are able to build a systematic, high-throughput selection strategy that can analyze large scale thioaptamer/aptamer libraries in an applicable fashion. As a starter, p65 and p50 with their natural binding partner and their modified stronger variant, XBY-6, would be a model system for testing functional assays and the feasibility of bead based selection.

2.2 CONSTRUCTION OF THE P65 AND P50 PGEX PLASMID

Recombinant human NF-kappaB subunit p50 and p65 was constructed in pGEX vectors (See Fig 2-5). pGEX is a GST gene fusion system with IPTG inducible *lac* promoter region. Proteins are expressed as fusion proteins with a 26kD glutathio S-transferase (GST). pGEX contains an ATG and ribosome binding site and a stop codon for each reading frame. It allows the removal of GST unit by cleavage with thrombin. The plasmid also provides *lac Iq* repressor for use in *E. Coli.* Anti-ampicilin gene is used for selection.

Figure 2-3: NF-kappa B p50 subunit

```
       1 MAEDDPYLGR PEQMFHLDPS LTHTIFNPEV FQPQMALPTA DGPYLQILEQ PKQRGFRFRY
      61 VCEGPSHGGL PGASSEKNKK SYPQVKICNY VGPAKVIVQL VTNGKNIHLH AHSLVGKHCE
     121 DGICTVTAGP KDMVVGFANL GILHVTKKKV FETLEARMTE ACIRGYNPGL LVHPDLAYLQ
     181 AEGGGDRQLG DREKELIRQA ALQQTKEMDL SVVRLMFTAF LPDSTGSFTR RLEPVVSDAI
     241 YDSKAPNASN LKIVRMDRTA GCVTGGEEIY LLCDKVQKDD IQIRFYEEEE NGGVWEGFGD
     301 FSPTDVHRQF AIVFKTPKYK DINITKPASV FVQLRRKSDL ETSEPKPFLY YPEIKDKEEV
     361 QRKRQKLMPN FSDSFGGGSG AGAGGGGMFG SGGGGGGTGS TGPGYSFPHY GFPTYGGITF
     421 HPGTTKSNAG MKHGTMDTES KKDPEGCDKS DDKNTVNLFG KVIETTEQDQ EPSEATVGNG
     481 EVTLTYATGT KEESAGVQDN LFLEKAMQLA KRHANALFDY AVTGDVKMLL AVQRHLTAVQ
     541 DENGDSVLHL AIIHLHSQLV RDLLEVTSGL ISDDIINMRN DLYQTPLHLA VITKQEDVVE
     601 DLLRAGADLS LLDRLGNSVL HLAAKEGHDK VLSILLKHKK AALLLDHPNG DGLNAIHLAM
     661 MSNSLPCLLL LVAAGADVNA QEQKSGRTAL HLAVEHDNIS LAGCLLLEGD AHVDSTTYDG
     721 TTPLHIAAGR GSTRLAALLK AAGADPLVEN FEPLYDLDDS WENAGEDEGV VPGTTPLDMA
     781 TSWQVFDILN GKPYEPEFTS DDLLAQGDMK QLAEDVKLQL YKLLEIPDPD KNWATLAQKL
     841 GLGILNNAFR LSPAPSKTLM DNYEVSGGTV RELVEALRQM GYTEAIEVIQ AASSPVKTTS
     901 QAHSLPLSPA STRQQIDELR DSDSVCDSGV ETSFRKLSFT ESLTSGASLL TLNKMPHDYG
     961 QEGPLEGKI

Human nuclear factor kappa-B, subunit, NP_003989, encodes a 105 kD protein
which  undergo cotranslational processing by the 26S proteasome to release
the 50 kD protein. The 105 kD protein is a self-inhibitory (also as aRel
protein-specific transcription inhibitor) and the 50 kD protein is the
subunit of activated transcript factor containing a DNA binding subunit of
the NF-kappa-B (NFKB) protein complex.
Data Source: NCBI
```

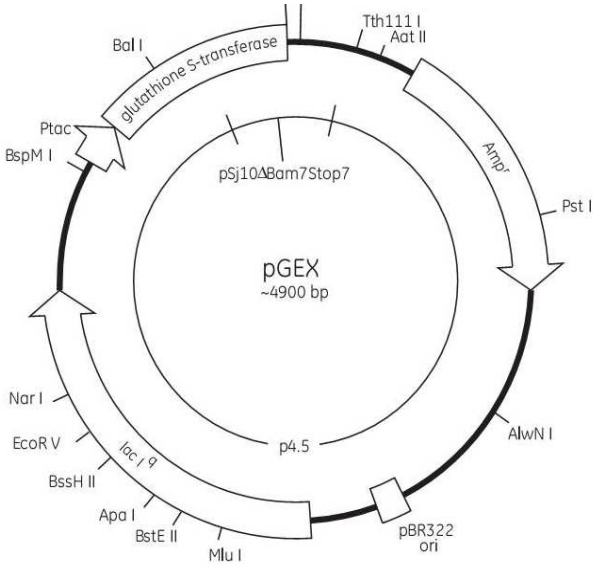
Figure 2-4: NF-kappa B p65 sub unit.

```
      1 MDELFPLIFP AEPAQASGPY VEIIEQPKQR GMRFRYKCEG RSAGSIPGER STDTTKTHPT
     61 IKINGYTGPG TVRISLVTKD PPHRPHPHEL VGKDCRDGFY EAELCPDRCI HSFQNLGIQC
    121 VKKRDLEQAI SQRIQTNNNP FQVPIEEQRG DYDLNAVRLC FQVTVRDPSG RPLRLPPVLS
    181 HPIFDNRAPN TAELKICRVN RNSGSCLGGD EIFLLCDKVQ KEDIEVYFTG PGWEARGSFS
    241 QADVHRQVAI VFRTPPYADP SLQAPVRVSM QLRRPSDREL SEPMEFQYLP DTDDRHRIEE
    301 KRKRTYETFK SIMKKSPFSG PTDPRPPPRR IAVPSRSSAS VPKPAPQPYP FTSSLSTINY
    361 DEFPTMVFPS GQISQASALA PAPPQVLPQA PAPAPAPAMV SALAQAPAPV PVLAPGPPQA
    421 VAPPAPKPTQ AGEGTLSEAL LQLQFDDEDL GALLGNSTDP AVFTDLASVD NSEFQQLLNQ
    481 GIPVAPHTTE PMLMEYPEAI TRLVTGAQRP PDPAPAPLGA PGLPNGLLSG DEDFSSIADM
    541 DFSALLSQIS S

Human nuclear factor kappa-B, p65, Q04206, encodes a 65 kD protein which
interacts with p50 to form heterodimer.p65 shows a weak DNA-binding site.
Data Source: NCBI.
```

p50 is sub-cloned from region 11-400aa. The approximate molecular weight is 46kDa.The region contains Rel homology domain. P65 is sub-cloned from region 12-317aa. The approximate molecular weight is 34 kDa. The plasmids were constructed and provided by the courtesy of Dr. Norbert Herzog.

Figure 2-5: Schematic illustration of pGEX vector



2.3 EXPRESSION AND PURIFICATION OF THE P50 AND P65 PROTEIN

The pGEX-50 and pGEX-65 were transfected into *E.coli* BL21 competent cells. (Invitrogen Inc). Each is cultivated in ampicillin added (100ul/plate, 100ug/ml) LB agar plates. A single colony from each plate was picked up and grown overnight at 37 ^{o}C with shaking (200rpm) in 20 ml LB with 100 ug/ml ampicillin. The overnight cell culture was diluted into 2 liters of LB (1.6% Tryptone, 1.0% yeast extract, 0.5% NaCl) with 100 ug/ml ampicillin and grown at 37 ^{o}C with shaking. A600 (absorbance at 600nm) was monitored every 15 minutes until the cell culture was in the mid-log phase (0.5 of A600). Then isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5mM. The induction continued for 3~4 hours before cells were harvested by centrifugation (2000x g for 45min). Samples at different time points were collected and tested on a 15% SDS-PAGE gel against molecular weight makers to make sure the protein is induced. (Fig 2-6) Cell pellets were collected and immediately stored at -80 ^{o}C.

Figure 2-6: NF-kappa B p65 induction

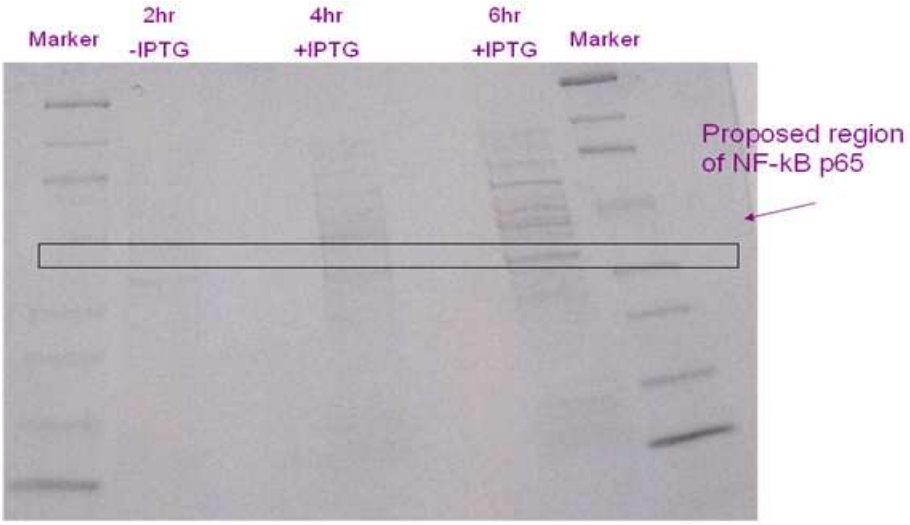
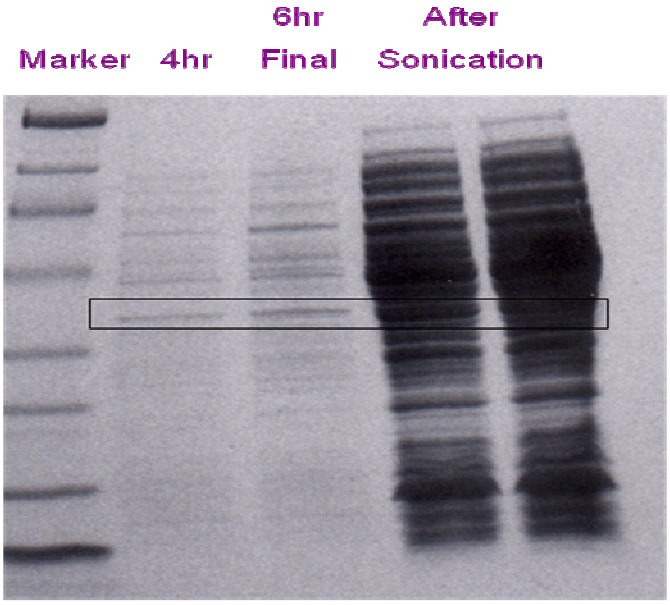


Figure 2-7:NF-kappa B p65 sonication



Recombinant p65 and p50 has a GST fusion and so was purified GST column (Amersham Biosciences). Further purification was done on a mono-Q column.

2.3.1 Glutathione S-Tranferase affinity Column

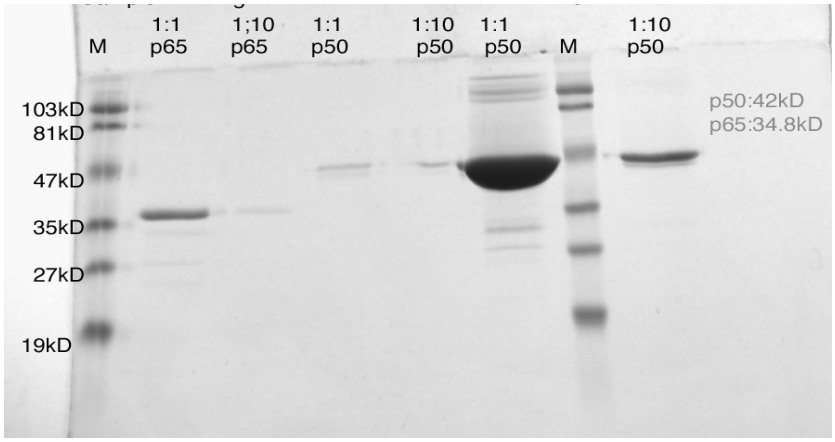
The cell pellets were suspended in suspension buffer (20mM NaH_2PO_4, pH 7, 1M NaCl, 1mM EDTA). Arptinin and phenyl-methylsulfonyl fluoride (PMSF) were also added to a final concentration of 1mg/mL and 1mM respectively. Lysozyme was last added to the suspension to a final concentration of 1 mg/mL. Cells were lysed by repeated sonication on ice. (For 6 times, 30 seconds sonication with 30 seconds interval in between.). The lysates were then clarified by centrifugation at 10,000 g for 45min. Debris in pellets were collected and the soluble fraction was loaded directly on to a glutathione Sepharose affinity column pre-equilibrated with the suspension buffer. High

salt buffer (1M NaCl with suspension buffer) was used to wash the column to remove the possible contamination of bacterial nucleic acids and proteins. Absorbance of A280 and A260 were monitored until a baseline is reached. Low salt suspension buffer (100mM NaCl) was then used to equilibrate the column before thrombin cleavage. 100 units of thrombin (Amersham Biosciences) was then added on to the affinity column and left overnight at 4^{o}C for cleavage completion. At this point, A280 was measured and the protein concentration was estimated using calibrated extinction coefficient. (18,050 $M^{-1}cm^{-1}$ for p65 and 23,755 $M^{-1}cm^{-1}$ for p50, see 2.2)

2.3.2 Mono-Q anion-exchange Column

The cleaved, eluted protein was then concentrated using in 10 ml using spin column (Genotech) by centrifugation. The sample was then loaded onto a Mono-Q anion-exchange column, 25mm x 200cm. The flow contains NF-kappa B since both p50 and p65 PI is 7.42, 6.23 respectively and does not bind to the column. This purification scheme successfully results in soluble protein that is greater than 99% pure as measured by SDS-PAGE using Commassie staining. MALDI TOF mass spectroscopy confirmed the correct molecular weight. All protein fractions were pooled together and DTT was added to a final concentration of 5mM. Glycerol was also added to 20% (v/v) to the final storage solution and then the protein was frozen in liquid nitrogen and stored at -80 ^{o}C.

Figure 2-8: p65 and p50 concentration and molecular weight measured by SDS-PAGE



2.3.3 Determination of Protein Concentrations

During purification, concentrations of NF-kappa B protein and total protein were determined by measuring its absorbance at 280nm. To estimate the concentration, the molar extinction coefficient of p65 and p50 were estimated by standard tools (ProtParam server: http;//au.expasy.og/tools/protparam.html). (Pace, Vajdos et al., 1995) Concentrated p65 or p50 was diluted 100-fold into 6.0 M guanidium hydrochloride, and 150mM NaC1, 50mM Tris, pH 7.4 respectively to compare A280 absorption ratio in native condition versus denatured condition. The estimated molar extinction coefficient is 18,050 $M^{-1}cm^{-1}$ for p65 and 23755 $M^{-1}cm^{-1}$ for p50, respectively. The collected sample of p65 after thrombin cleavage has concentration of about 0.567 mg/ml.

The final protein concentration was measured by a more accurate way, the Bradford assay. The assay is based on the fact that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465nm to 595nm when it binds to protein (Bradford, 1976). Basic and aromatic amino acids residues, especially arginine,

are the primary target of Coomassie blue binding. The anionic form of Commassie Blue is stabilized upon binding by hydrophobic and ionic interactions thus generate the absorbance spectrum change. The extinction coefficient of dye-protein complex is rather constant over a 10-fold concentration range. Thus, by selecting an appropriate ratio of dye and sample concentration, an accurate quantization can be determined applying Beer's law. Gamma immunoglobulin IgG is one of the most used standards.

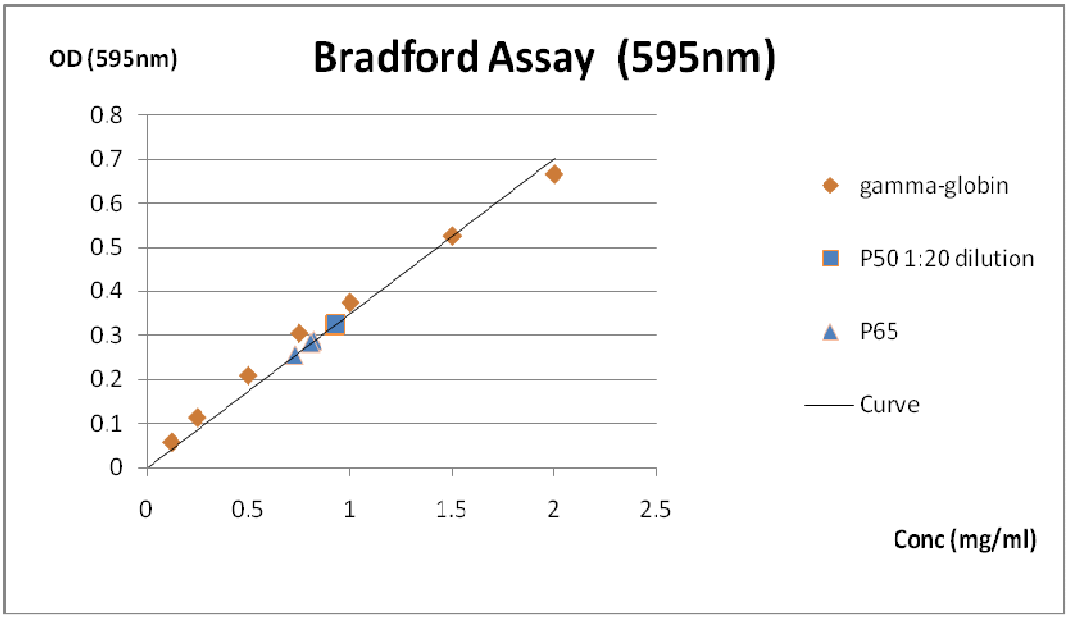
The procedure is as follows: Seven dilutions of protein IgG (0.25 mg/ml, 0.5 mg/ml, 0.75 mg/ml, 1mg/ml, 1.5 mg/ml, 2 mg/ml) are prepared. 10ul of each standard sample and protein samples are put into a test tube (proteins solutions are assayed in triplicates) and 0.5 ml of diluted dye reagent (Bio-Rad) is added and incubated at room temperature for 10mins. Absorbance is measured at 595nm and the concentration of protein is calculated. For protein concentration measured outside of the linear range, the appropriate dilution is calculated and the absorbance is re-measured.

Table 1: Protein concentration measure by Bradford Assay

Sample	OD at 595nm	Conc (mg/ml)	Overange
p50 1:1	5.458	18.2101576	V
p50 1:1	5.669	18.9174992	V
p50 1:10	4.028	13.4163258	V
p50 1:10	3.475	11.5624874	V
p65 1;1	0.793	2.57153872	X
p65 1;1	0.728	2.35363728	X
p65 1:10	0.094	0.22826014	X
p65 1:10	0.045	0.06399598	X

Bradford assay for p50 and p65 measured at 595nm. For OD is greater than 1, (indicated by V in the last column). Protein is diluted accordingly and re-measured. See Fig 2-9

Figure 2-9: Bradford Assay for p50 and p65 final concentration determination (triplicates)



Absorption at 595nm is measured and concentration is calculated using [Concentration]= 0.351 x [Absorption]

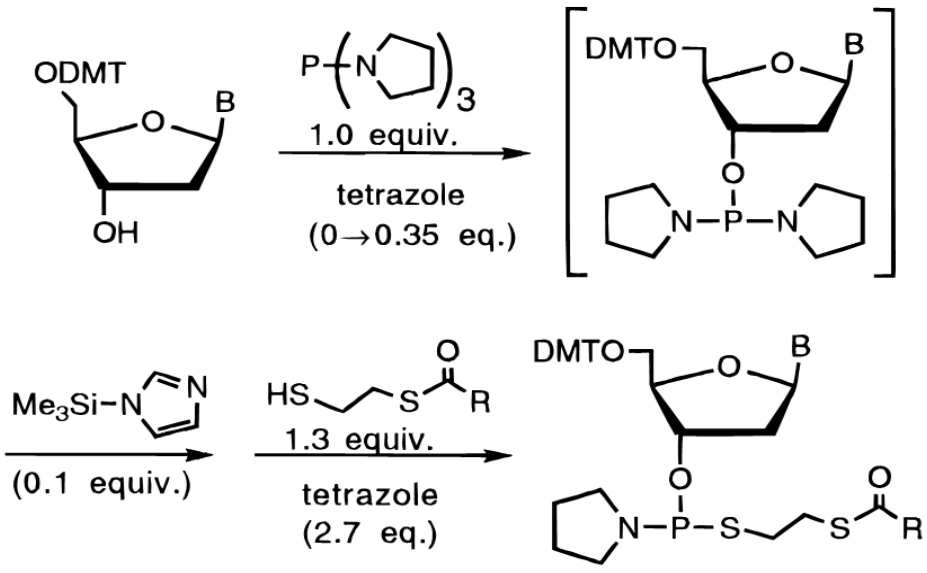
The final stored concentrations of p50 and p65 are 18.4 and 0.78 mg/ml, respectively. (Measured as monomer form).

In conclusion, NF-kappaB protein p50 and p65 have been successfully expressed and purified for further study. NF-kappa B protein will be later used as a model target for bead based selection of thioaptamer and aptamers.

2.4 XBY6 AND OTHER THIOAPTAMERS'S SYNTHESIS AND PURIFICATION

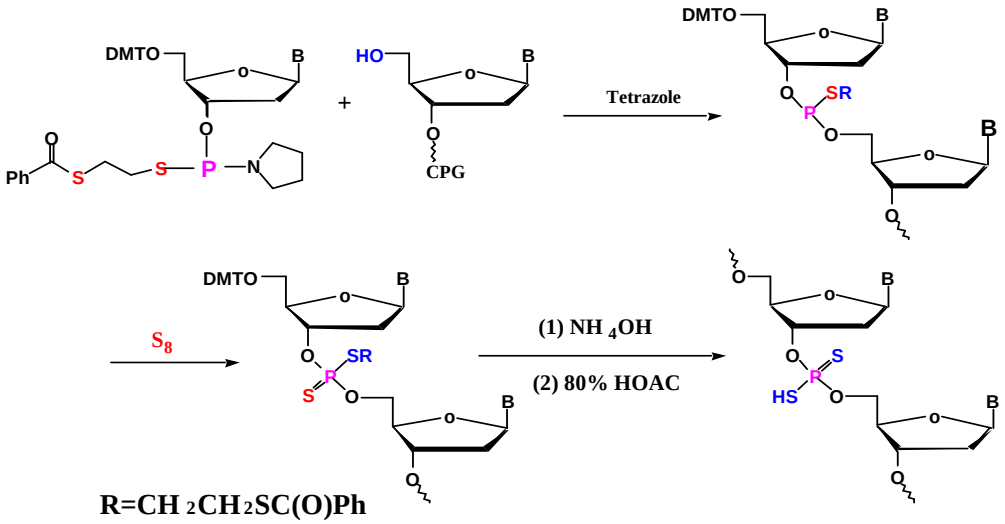
Due to well-behaved features of thioaptamers like relatively high nuclease-resistance and more effective binding to proteins, the process of synthesis, isolation and characterization of thioaptamers have been intensively developed. Up to date, the solid-phase synthesis based on nucleoside phosphorothioamidite chemistry independently developed by Gorenstein's group (Faraschtchi and Gorenstein, 1988) and by Caruthers' group have been shown most successful. The key is to provide active nucleoside phosphorothioamidite building blocks and base deblocking conditions in order to suppress side reactions (Yang, Fennewald et al., 1999). Fig 2-7 shows the phosphorothioamidite developed by Gorenstein and Caruthers and Fig 2-8 shows use of phosphorothioamidite to synthesis phosphorodithioate in solid-support synthesis of ODNs.

Figure 2-10: Synthesis of Oligonucleoside phosphorothioamidite.



(Wiesler and Caruthers, 1996)

Figure 2-11:Synthesis of Oligonucleoside phosphorodithioates



(Wiesler and Caruthers, 1996)

Dr. Xianbin Yang further optimized the synthesis system. Synthetic S2-ODNs have been purified by PAGE, anion exchange column and RP-HPLC (Yang, Fennewald et al., 1999). PAGE can separate S2-ODNs with high purity (>97%), but the manual operation nature makes it extremely troublesome and low loading capacity. RP-HPLC is complicated in purifying S2-ODNs due to the complex chromatogram nature of crude synthetic mixture of S2-ODNs. Strong ion-exchange (Mono-Q) column can separate S2-ODNs based on the number of charges and sulfurs and gain higher than 95% yield when used to purify S2-ODNs from failed, monothioated, or desulfurization products. The retention time of S2-ODNS was demonstrated to be linearly dependent on the number of sulfurs on the strong anion exchange column other than the sequence. The higher the pH, the higher the resolution that can be achieved, but above pH 8, the retention time increases nonlinearly (Yang, Hodge et al., 2002).

Since we are planning to build up a selection strategy for further thioaptamer selection, XBY-6 is biotin labeled at the 5'end for future identification purposes. Attaching a special function group at the end of a nucleic acid is frequently used for downstream purposes for diagnostics and therapeutics. However, because the diverse nature of these modifications changed the behavior of S2-ODNs in anion-exchange affinity columns, the purification of these 5'-functionalized S2-ODNs from a mixture of even more complicated crude sample containing no attachment full-length S2-ODNs, failed sequences or desulfurized S2-ODNs, monothioated ODNs become more challenging. So we tested the FPLC system with strong anion-exchange column followed by RP-HPLC system to purify these 5'-functionalized S2-ODNs.

2.4.1 Purification of several functional group attached thioaptamers using strong anion-exchange chromatography and reverse-phase chromatography in tandem.

To test and record different functional group's effects on the process of purification, seven selected groups such as 5'-amino, 5'-biotin, 5'-phosphate, 5'-thiophosphate, 5'-flurescein phosphoramidite (FAM), 5'- oligonucleotide modifying reagent (OPeC) and 5'-aldehyde (Glen Research , all modifications) were used.

Synthesis and coupling of functional group

Sequences used:

XBY6 forward strand: 5'-CCAGGAGATTCCAC-3' (red indicated as dithioate)

XBY6 reverse strand: 5'-GTGGAATCTCCTG-3'

XBY-S1 forward strand: 5'- CCAGTGACTCAGTG-3'

XBY-S2 reverse strand: 5'- CACTGAGTCACTGG-3'

By courtesy of Dr. Yang, all ODNs were synthesized on the 1 umol scale. The functional group is coupled.

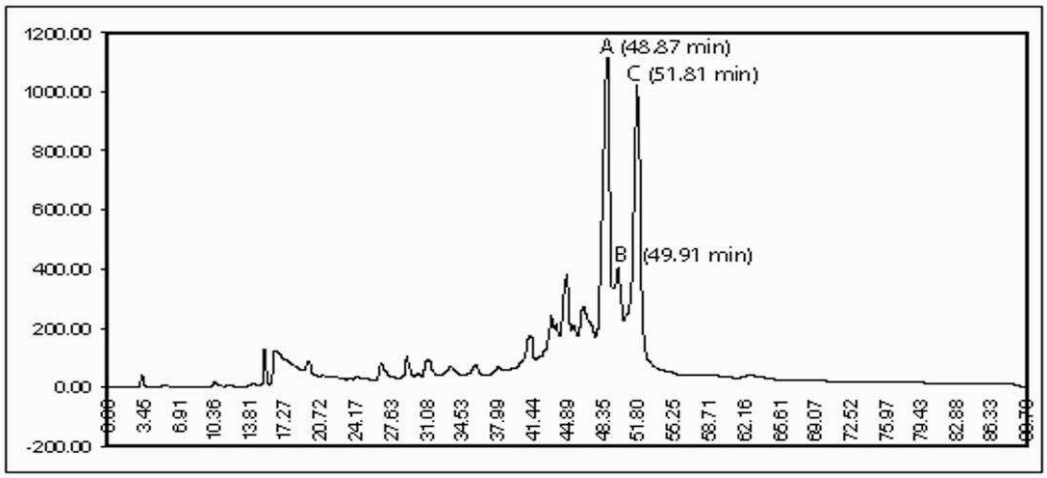
FPLC purification

The system is from Amersham Biosciences, AKTA FPLC P-920 (Unicorn). All samples were loaded on to a MonoQ 5/5 column packed with 10um porous beads (Amersham Biosciences). Mobile phase buffer A (low salt buffer) contains 25mM Tris-Cl, 1mM EDTA pH 8, and mobile phase buffer B (high salt buffer) contains 1M NaCl, 25mM Tris-Cl , 1mM EDTA, pH8. Absorption at 254nm is used to monitor the system. Gradient elution at the rate of 1 ml/min is used to separate ODNs, S2-ODNs and those S2-ODNs with a functional group attached at the 5' end. Flowing profile is setup by unicorn control center as 80 minutes in step I for increasing buffer B from 0% to 100%, 4 minutes in step II for holding 100% buffer B flow, and 1 minute in step III for decreasing buffer B from 100% to 0% and 5 minutes in step IV for holding flow at 0% buffer B.

RP-HPLC purification

The system was purchased from Amersham Biosciences, AKTA HPLC-P-900 (Unicorn). A small column Hamilton PRP-1 10um (150x4.1mm) was used for analytical purposes and a bigger column Hamilton PRP-1 10um (205 x 7mm) was used for purification purposes. Mobile phase buffer (water phase buffer) A contains 100mM NH_4OAc at pH 7 and mobile phase buffer (non-water phase buffer) B contains HPLC grade acetonitrile. Gradient elution at the rate of 1 ml/min for the Hamilton PRP (150 x 4.1mm) column , and 2 ml/min for the Hamilton PRP (305 x 7mm) column was used for the separation of 5'-functionalized S2-ODNs from other impurities and no-functional-group attached S2-ODNs. The gradient flow profile is setup by unicorn control center as 3 steps: step I, 2 minutes flow at 0% buffer B, step II, increase buffer B from 0% to 25% in 48 minutes, step III, decrease buffer B from 25% to 0% in 1 minute.

Figure 2-12: Co-injection of 5'-functionalized-XBY6 forward strand. The time-absorption spectrum at 254nm on Mono Q Column



X axis is the representation of time point of the flow, Y axis represents the absorption.

Table 2: Retention times for various derivatives of thioaptamers (with 5'-functional groups or without)

#	Thioaptamers/Aptamers	Sequence and 5'-functional group	Retention time(min)	
			AEC-MQC	RP-HPLC
1	Reference	5'-CCAGGAGATTCCAC	38.79	17.81
2	XBY6-F	5'-CCAGGAGA$T_{S2}T_{S2}$CCAC-3'	49.06	21.63
3	5'-Amino-XBY6-F	5'-Amino-CCAGGAGA$T_{S2}T_{S2}$CCAC-3'	49.07	29.61
4	5'-Biotin-XBY6-F	5'-Biotin-CCAGGAGA$T_{S2}T_{S2}$CCAC-3'	49.08	26.82
5	5'-FAM-XBY6-F	5'-FAM-CCAGGAGA$T_{S2}T_{S2}$CCAC-3'	49.53	27.35
6	5'-OPeC-XBY6-F	5'-OPeC-CCAGGAGA$T_{S2}T_{S2}$CCAC-3'	51.77	29.31
7	5'-Aldehyde-XBY6-F	5'-Aldehyde-CCAGGAGA$T_{S2}T_{S2}$CCAC-3'	51.57	24.28
8	XBYS2-F	5'-CCAGT_{S2}GACT_{S2}CAGT_{S2}G-3'	56.75	21.73
9	5'-Amino- XBYS2-F	5'-Amino-CCAGT_{S2}GACT_{S2}CAGT_{S2}G-3'	56.92	29.02
10	5'-Biotin- XBYS2-F	5'-Biotin-CCAGT_{S2}GACT_{S2}CAGT_{S2}G-3'	57.01	26.45
11	5'-FAM- XBYS2-F	5'-FAM-CCAGT_{S2}GACT_{S2}CAGT_{S2}G-3'	57.83	28.01
12	5'-OPeC- XBYS2-F	5'-OpeC-CCAGT_{S2}GACT_{S2}CAGT_{S2}G-3'	59.02	29.72
13	XBYS2-R	5'-CACT_{S2}GAGT_{S2}CACT_{S2}GG-3'	57.15	21.61
14	5'-Amino- XBYS2-R	5'-Amino-CACT_{S2}GAGT_{S2}CACT_{S2}GG-3'	56.38	28.90
15	5'-Biotin- XBYS2-R	5'-Biotin-CACT_{S2}GAGT_{S2}CACT_{S2}GG-3'	57.75	26.29
16	5'-FAM- XBYS2-R	5'-FAM-CACT_{S2}GAGT_{S2}CACT_{S2}GG-3'	57.94	28.24

All sequences have been verified via MALDI-TOF mass spectroscopy. "F" means forward strand, "R" means reverse strand. Red characters indicate phosphorodithioate modification on the position.

Since Mono-Q column has been successfully used to analyze and purify thioaptamers with phosphorodithioate modifications, these modified thioaptamers with 5' end functional groups were first tested on it. The co-injection of 5'-amino, 5'-biotin and 5'-phosphate labeled XBY6 forward strand showed somewhat identical retention times to

their unmodified XBY6 forward strand counterpart. 5'-thiophosphate, 5'OPeC and 5'-aldehyde labeled XBY6 forward strand showed only slightly differences. Only 5'FAM-labeled XBY6 forward strand showed a significant difference (See Table 2). Similar observations were found on XBY-S2 thioaptamers. Fig 2-8 A peak represents 5'-amino, 5'-biotin, and 5'phosphate, and B peak represents 5'-thiophosphate, 5'-OPeC and 5'-aldehyde. C peak represents 5'-FAM. RP-HPLC then was used to further purify these collected samples from Mono-Q column. An estimated 4~7% of impurity was found. It is worth mentioning that the retention time of these modified thioaptamers showed significant differences from their unmodified counterparts. For example, 5'-amino-XBY6-F's peak retention time 29.61 min is well resolved from the peak retention time 21.63 min of its precursor, XBY6-F. It is also worth mentioning that the same 5'-end modification has similar effects on retention times of different sequences. This is understandable in that the hydrophobicity/hydrophilicity is the major factor that will affect RP-HPLC performance. So RP-HPLC chromatography is suitable to further purify these thioaptamers and it is a side benefit here that RP-HPLC also decreases the salt in the final purified product.

In conclusion, we have successfully established a method to purify normal thioaptamers and thioaptamers with 5'-end modifications by several functional groups by an FPLC strong anion-exchange column Mono-Q followed by a HPLC PRP Column. 5'-biotin labeled XBY-6 has been collected for further study.

2.4.2 XBY-6

As mentioned in 2.1, the high affinity and specific of XBY6 toward NF-kappB transcription factor make it a good candidate as a model system for study of thioaptamers.

Biotin labeled XBY6 forward stand and its reverse strand were first purified (2.3.1) and then desalted and concentrated. To form the double stand XBY6, 5'-biotin labeled XBY-6 forward strand and its complementary strand are mixed in equal molar quantity in annealing buffer (10mM Tris-C1, 1mM EDTA, 100mM NaCl, pH 7.4), heated to 88 ^{o}C and cooled down slowly to room temperature. The final concentration of stored biotin labeled XBY 6 is 288 ug/ml. The purity was tested on 15% PAGE gel.

2.5 NF-KAPPA B FUNCTIONAL ASSAY

In the discussion of the one-bead-one compound concept, we have mentioned the successful strategy usually depends upon on-bead functional assays. To establish the on-bead functional assay suitable for thioaptamer selection, we will try to tune two of the most common assays in nucleic acid research: Electrophoretic Mobility Shift Assay (EMSA) and Enzyme-Linked ImmunoSorbent Assay (ELISA). Both assays are based on specific recognition of DNA/RNA protein binding or protein-protein binding and serve as natural choice for our on bead specific binding assay. These assays are first verified on the NF-kappaB~XBY6 system and NF-kappB~Ig-kappaB system, so later these assays will be legitimately tested on our bead based assays. The parameter of the binding experiments was also measured for the reference of our later study.

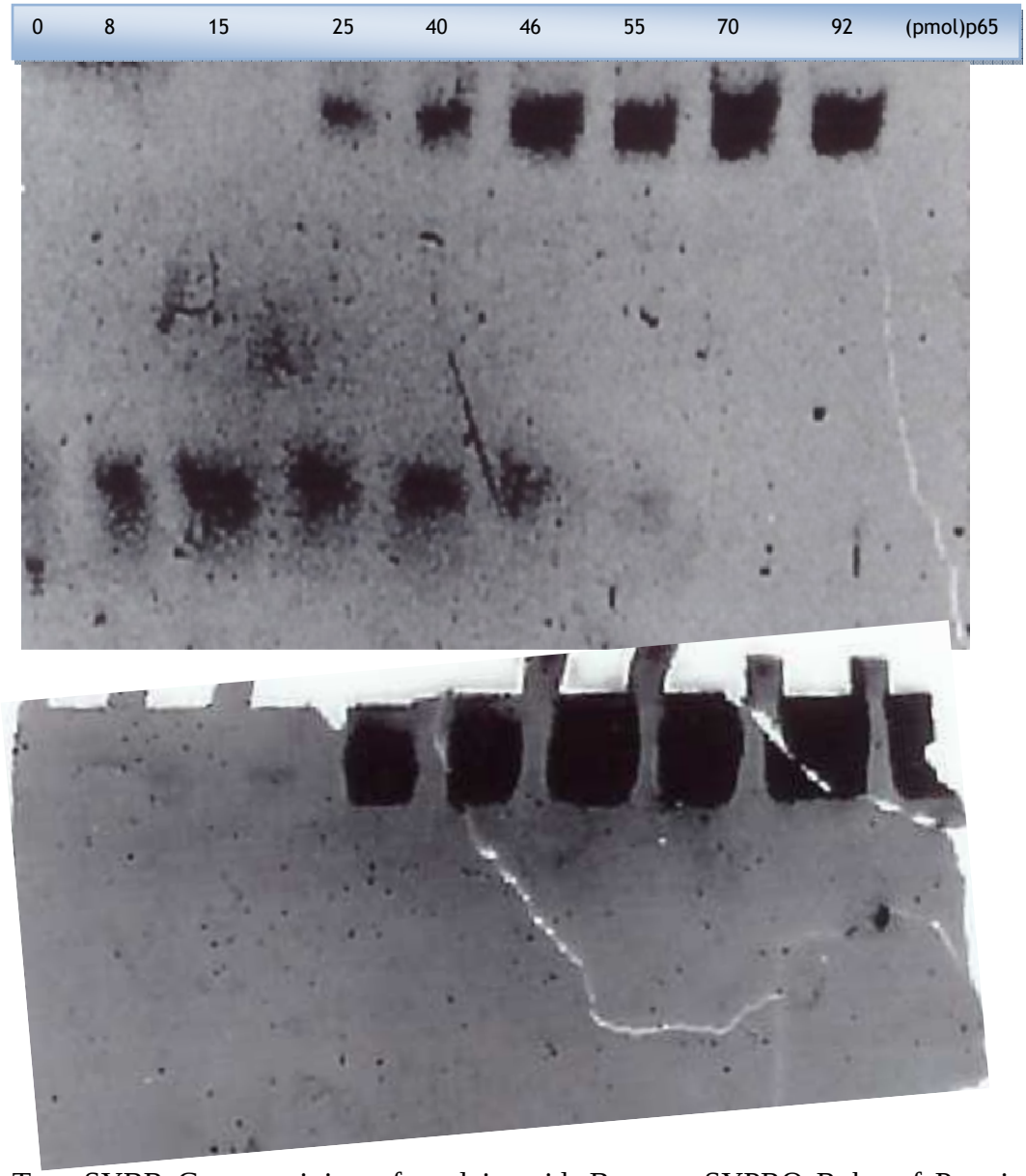
2.5.1 EMSA assay

The EMSA technique is based on the fact that DNA-protein binding complex will migrate slower than free DNA in agarose gel or native polycacrylamide (PAGE) gel. The "shift" means the migration difference between bound DNA and unbound counterpart. The assay can be realized by radio labeling of DNA, chemical modification of DNA with reporter group, or most recently, specific nucleic acid dyes and protein dyes. Two fluorescent dyes were used in this experiment: SYBR Green, which is a nucleic acid specific staining dye maximally excited at 495nm and has a secondary excitation peak at 255nm.The florescence emission of SYBR Green dye bound to nucleic acid is centered at 520nm. SYPRO Ruby is a protein specific staining dye, maximally excited at 450nm which has a secondary excitation peak at 280nm. The fluorescence emission of SYPRO Ruby stain bound to protein is centered at 610nm.

Electrophoretic mobility shift assays

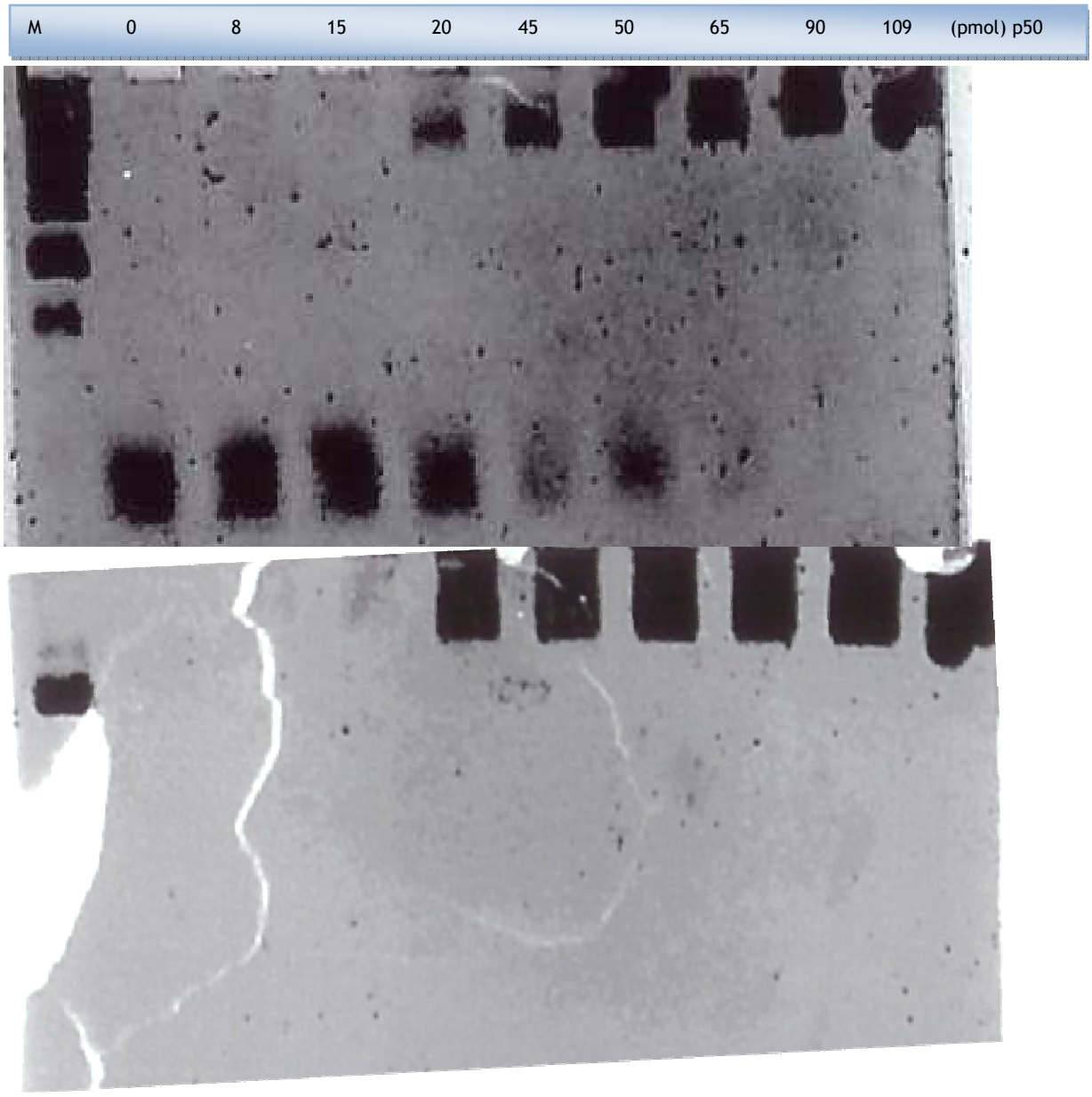
A fixed amount of XYB-6 (15pmol) was incubated with serial dilutions of purified recombinant NF-kappB RelA (p65) or p50 in a 20 ul reaction under standard reaction conditions (20mM Hepes, PH 7.5, 50mM KCl, 2.5 mM MgCl2 20mM DTT, 10% glycerol, poly I-C can interfere with SYBR Green and is removed thereafter).After 1 hour, the reaction was loaded onto a standard 6% polyacrylamide gel in 0.25 x TBE. Following electrophoresis, the oligonucleotides were stained with SYBR Green for 20 mins and the subsequence protein staining use SYPRO Ruby overnight.

Figure 2-13:EMSAs assay of p65 homodimer binding XBY-6.



Top: SYBR Green staining of nucleic acid. Bottom: SYPRO Ruby of Protein. All reaction wells are containing 15pmol XBY-6. P65 is titrated from 15pmol to 92pmol (monomer concentration).

Figure 2-14:EMSAs assay of p50 homodimer binding XBY-6.



Top: SYBR Green staining of nucleic acid. Bottom: SYPRO Ruby of Protein. All reaction wells are containing 15pmol XBY-6. P50 is titrated from 8pmol to 109pmol (monomer concentration).

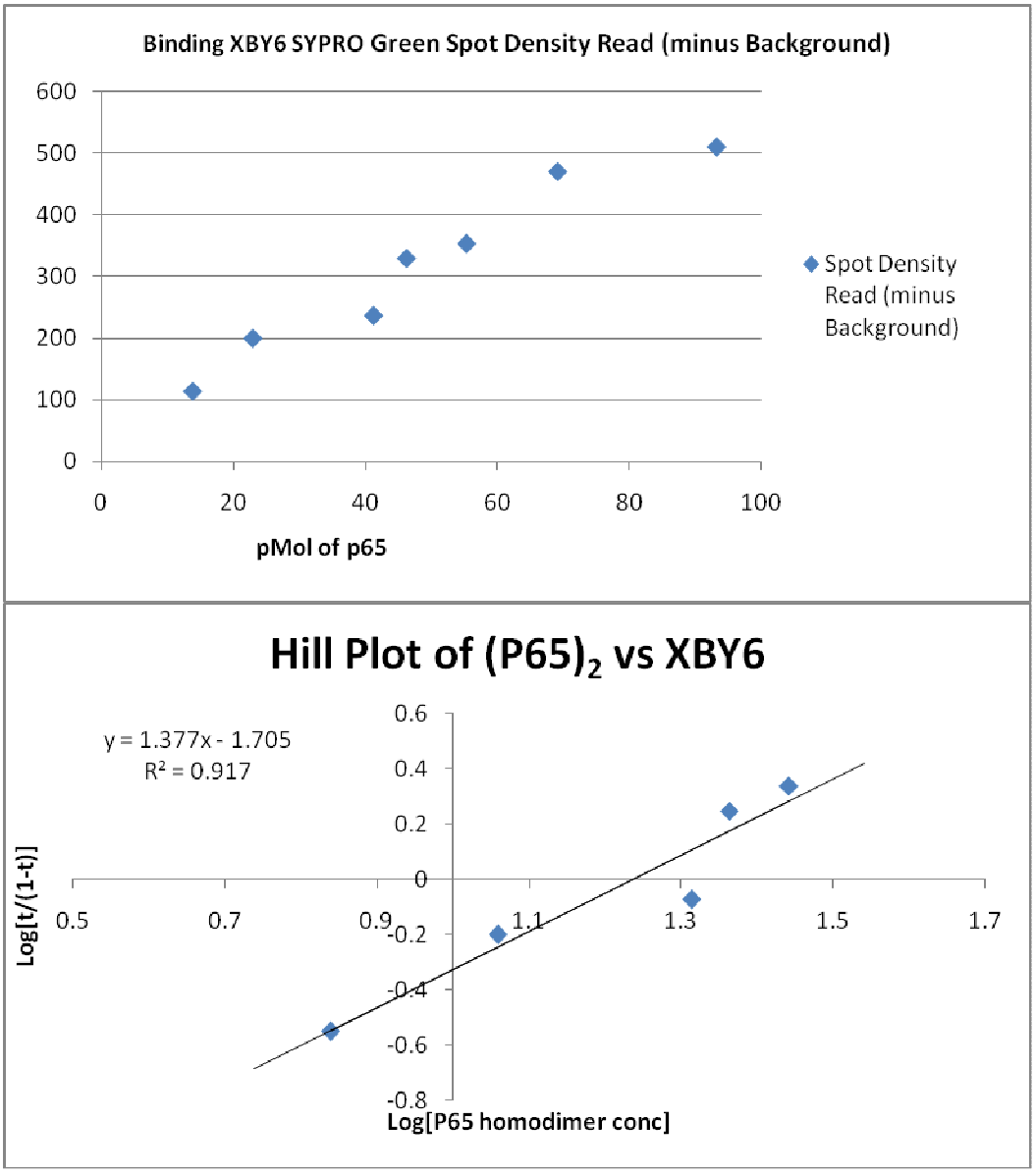
XBY-6 is successfully shifted by p65 under the standard condition. (Fig 2-9) The binding affinity is under the subnanomolar Kd range. The molar ratio at 50% binding of XBY6 vs p65 homo**dimer** is 1:0.75~1:1.5, which indicates a simple non-cooperatively binding model of XBY-6 binding homo**dimer** RelA (p65). Which confirmed previous results, while the Kd is in the 0.4 to 0.5 nM range , which is significant tighter binding than previously measured by PAGE (Kd = 1.44 nM, D. King, 2002).

A similar setting was also performed for homodimer p50 and the result showed a very similar binding pattern. (Fig-2-10) The molar ratio at 50% binding of XBY6 vs p65 **dimer** is 1:0.75~1:1.7, which also indicates a simple non-cooperatively binding model of XBY-6 binding homo**dimer** p50. The Kd is in the 0.4 to 0.5 nM range .

Hill plot analysis is further used to measure the binding affinity and cooperatively. The signal is read by image density of each plot on the gel. The data reading contains manual selection of the plot so a human bias may exist. Hill plot analysis revealed similar results: The cooperativity of binding (p65 homodimer vs XBY-6) is 1.38, which indicated the binding is primarily a single site, non-cooperative binding. The Kd is even smaller, approximately at 0.17nM by the data (the maximum is estimated by the highest concentration of p65, so in the hill plot the data point is not used) (Fig 2-11). The lower binding affinity is further confirmed by my colleagues (0.15 nM measured using surface plasma resonance by S. Sarkar and D. Volk, unpublished results). The result is encouraging that XBY6 has shown a very high binding affinity, which means it is very suitable for pioneering research since it can give a very good signal to noise ratio, given the high binding affinity of it towards NF-kappa B. The availability of bead based selection rely on local concentration of thioaptamer to be high enough to show a distinguishing signal when it binds its partner. The higher binding affinity of XBY 6

provides a robust model binding experiment to test on a variety of assays for bead based selection.

Figure 2-15:Data analysis of the EMSA binding curve of P65 homodimer vs XBY6.



Data is collected by Image Reader; the plot density is measured against background (Above). Hill plot revealed the cooperatively number and Kd (Bottom).

2.5.2 ELISA assay

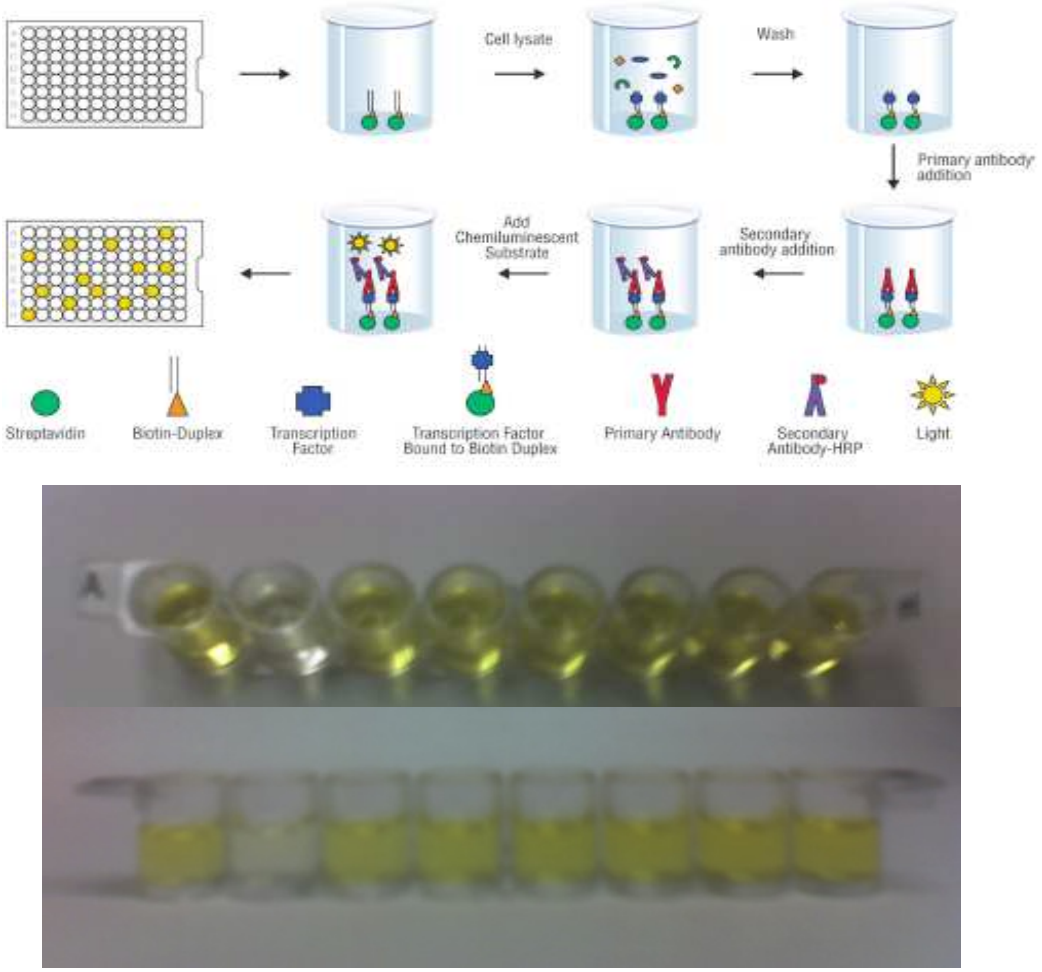
ELISA assay is traditionally used to detect protein-protein interaction. It involves two layers of specific recognition. One is the antibody that is specific to the antigen of interest and the second is an "anti-anti body" which recognized the antibody conserved region and this second antibody is usually linked to an enzyme that catalyzes chromogenic or fluorogenic changes in the presence of corresponding substrates. (Fig 2-12 above). Since NF-kappa B specifically recognizes DNA sequence pattern, by immobilizing the specific double strand DNA on the 96 well, similar techniques can be applied to NF-kappa B too.

ELISA assay

Reagents were all purchased from Rockland Inc. Immobilized Ig-kappaB sequence (Forward strand: 5'GGGACTTTCC-3') was attached on a 96 well plate. The 96 well plate was equilibrated to room temperature prior to opening. Complete transcription factor buffer (CFTB) was prepared as in the manufacturers' manual on the same day of the experiment. Positive control is LPS stimulated nuclear extract. All samples and controls were added with 100ul of 1xCFTB. After incubation overnight at 4 ^{o}C and washing, anti-NF-kappaB (p50) (Rabbit) was added at 1:100 dilution. The addition of HRP Goat anti-Rabbit conjugated secondary antibody at 1:100 dilution was done after 1h room incubation of the plate. To develop the colors, developing solution is added into the plate and then the plate was incubated with gentle agitation without light for 15 to 45

minutes. Stop solution is added when wells turned to dark blue and the absorbance at 450 nm and several other wave lengths were recorded.

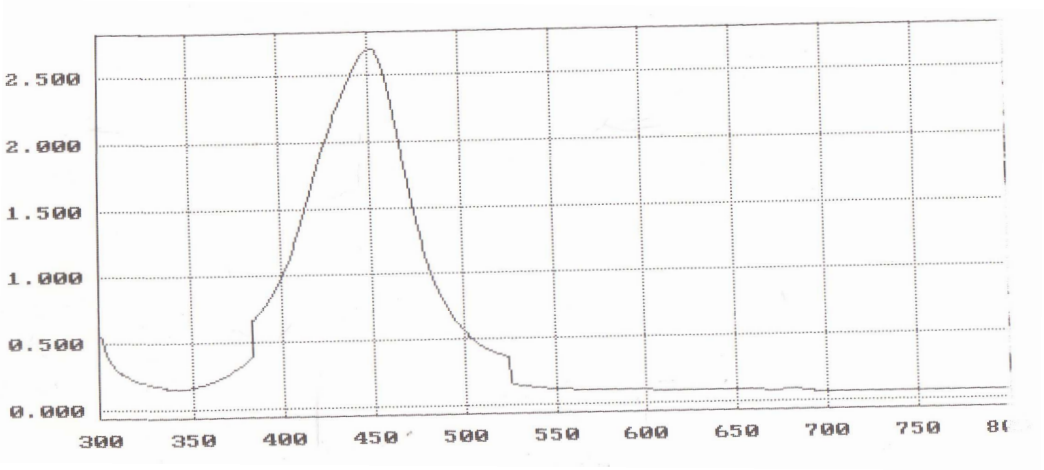
Figure 2-16: Schematic illustration of ELISA assays of NF-kappa B (p50) (bind to Ig-kappa B sequence on immobile well surface)



Above: Each well is coated with a consensus ds-Ig-kappa B sequence. Bottom: A Positive control, B: Blank, C to H: p50 serial dilutions (1:20000, 1:10000,1:5000,1;2000,1:1000,1:500, respectively)

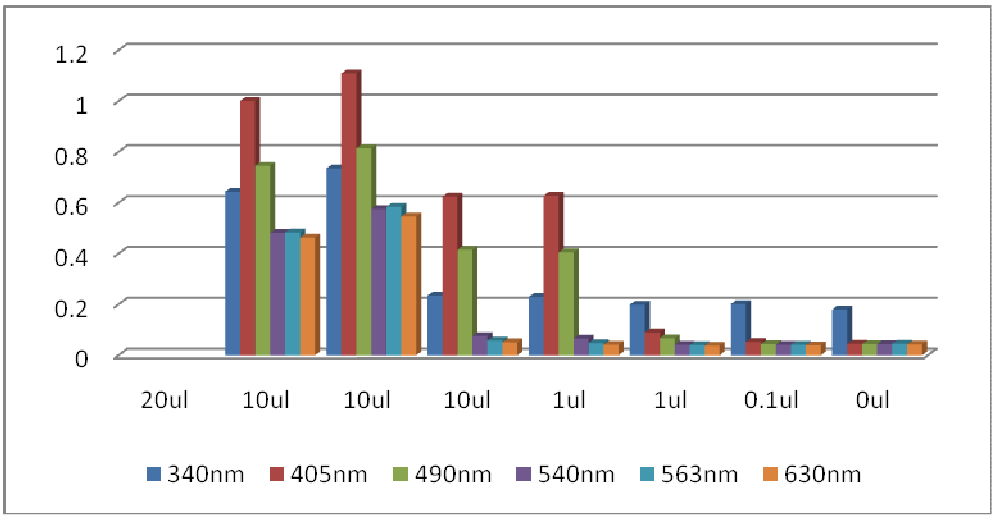
The experiments confirmed p50 activity. All tests showed very high signal, indicating the 96-well is saturated; it is understandable that due to the fact that in the physiological condition, the concentration of p50 is extremely low. Based on literature, dilution at 1:1000000 of our stored protein is in the appropriate range of 450 nm readout. A full spectrum scan and absorption at various wavelengths confirmed this result.

Figure 2-12:Full spectrum scan of ELISA assay colorimetric reaction



The reaction is saturated using 20 ul sample; the scan was from 300-800 nm using 1:5 dilution of the reaction mixture.

Figure 2-17: Absorption at various wavelengths of ELISA colorimetric reaction



In conclusion, the NF-kappa B p50 protein was well behaved and the ELISA assay scheme is appropriate to detect the reaction and is thus suitable for our further investigation for use with bead functional assays.

Chapter 3: 2D gel layout for bead based selection

This chapter describes the strategy of the 2D layout of thioaptamer bead library as a parallel selection platform and preliminary studies on the availability of 2D PAGE gel as the selection platform.

3.1 2D LAYOUT OF BEADS LIBRARY

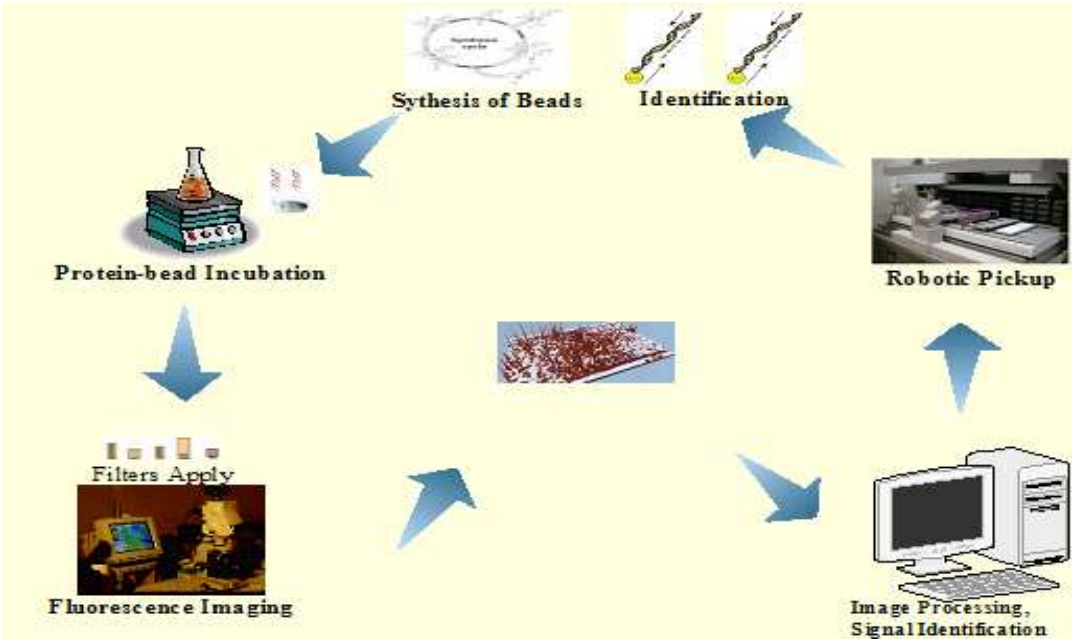
As discussed in 1.4, one bead one compound library synthesized based on "split and pool" methods is attractive an alternative method to SELEX method *in vitro*. The one-bead one oligonucleotide library, specifically a thioaptamer combinatorial library, has been first demonstrated by Yang (Yang, Bassett et al., 2002). Yang, et al. further verified that on bead sequence can be read out by using normal PCR techniques. This laid down the foundation for bead-based selection of thioaptamers. But a big question remains: as a normal constructed library potentially contains 10^8 beads, how can we effectively screen that many beads in a timely fashion. Our laboratory Researchers first tried flow cytometry as a sorting method, basically inspired by the idea that cells are also on a scale similar to the beads used in library construction. Preliminary data has been reported (Yang, Li et al., 2003), but this method faces current technological limitations. It is somewhat understandable since flow-cytometry was initially designed for cell sorting, which means the major task is to separate several major portions and each portion of the whole cell contains a great number of sorted cells. In the bead based selection scenario, the task has been changed to find one or two hundrends in 10^6-10^8 beads, so flow-cytometry in its current form requires very high speed sorting of rare beads. While this is possible for smaller beads, it is quite difficult for bigger beads (65um in diameter bead,

for example. Currently, longer ODN synthesis on beads (greater than 60 bases) is limited to the 65um beads)

A simple but elegant idea is proposed to solve this screening dilemma: if beads are spread out in a 2D layout to the extent that each bead is identifiable and we can carry out the functional assay on the whole layout, the requirement of 10^6-10^9 assays and simple screening is fulfilled, especially if we can use advanced image processing algorithms developed in the IT industry (Fig 3-1).

To investigate the possibility of a 2D layout for screening a bead library, polyacrylamide gel (PAGE) is used and some preliminary studies using simple fluorescein attached single sequence library is carried out to examine possible signal strength in terms of bead identification.

Figure 3-1: Schematic illustration of bead based selection of thioaptamers in 2D layout in Gel method



3.2 TEST OF SINGAL/NOISE RATIO ON FLUORESCEIN-LABELED BEADS IN 2D PAGE GELS

In fact, to successfully build a one-bead one compound library, the bead (the solid support material in essence) is the first question to address. As for on-bead screening we are discussing here, the compounds are always covalently attached to the solid support during the selection process. Since most biological assays requires physiological conditions, in other term, aqueous media, the solid support and its linkers must be water compatible. Besides, this covalent link should survive in all the steps during synthesis. Another requirement when constructing a bead library is the uniformity of both size and substitution. And of course the aggregation or the stickiness of beads toward each other should be avoided, too. Taking these into account, very few commercially available solid supports are suitable for our need. For example, long chain alkylamine controlled pore glass (LCA-CPG) is usually the choice for normal DNA/RNA synthesis. But it lacks some important features that are required for a bead library: first, during the ammonia deprotection step, the covalent link will be cleaved; second, these kinds of supports are designed for commercial synthesis of DNA/RNA, so the homogeneity and size are not of concerns (Yang, Bassett et al., 2002). ChemGenes Corp. first developed a customized polystyrene bead of uniform sizes with a non-cleavable hexaethyleneglycol linker, which demonstrated the ability to retain the covalent link through all processes. Two kinds are available: 20 um in diameter and 65 um in diameter. For the purpose of initial technological development for thioaptamer selection, the 65 um beads were used.

Fluorescein labeled beads

The synthesis procedure is similar to the one mentioned in section 2.3, except in the last step of deprotection: After 30% ammonia treatment at 37^{o}C for 21hrs, beads are

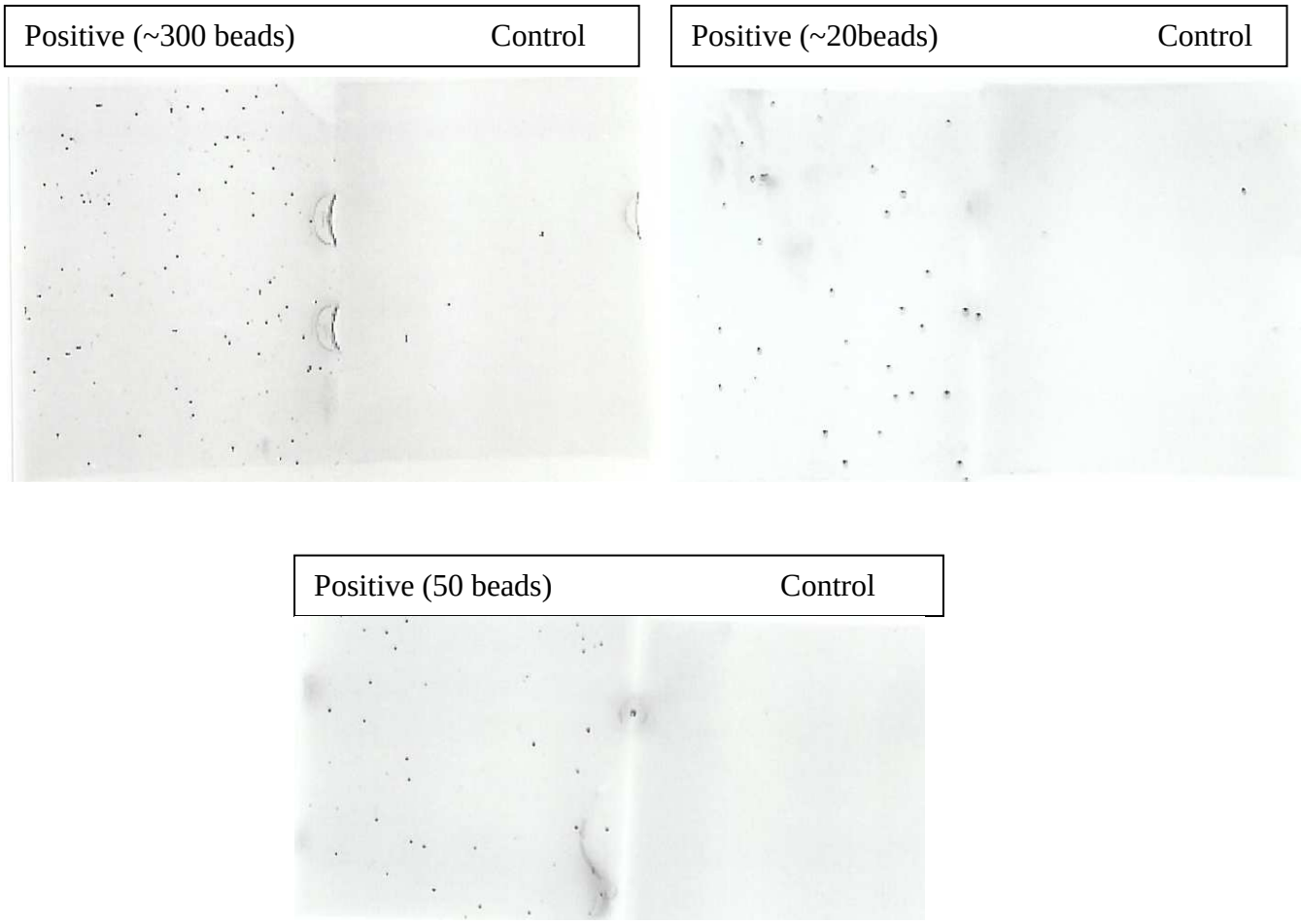
collected and washed. The beads are attached with 42bp single strand DNA with flourescein at the 5' end.

Beads in the PAGE gel

Beads are injected into 15% PAGE gel by mixing beads with soluble PAGE mixture before adding the last catalyst. (Either 10% APS or TEMED as the last one). Upon adding the final catalyst, solution is immediately vortexed to homogenize the bead distribution in solution and the solution is injected into a Bio-Rad mini-gel (8.6 x 6.8 cm x 0.5mm). The gel is then examined with a FluorChem 8800 system, excited by UV at 304nm. (Fig 3-2) Various conditions are tested or alternated to minimize the noise.

The result (Fig 3-2) showed the feasibility of 2D layout to identify beads. For the first time, we clearly identified beads in a 2D layout. (Previous attempts have been made by colleagues in our lab, but showed considerable noise). Different loading of beads successfully showed different numbers of spots as expected and verifed the idea that beads could be screened by reading a 2D image of a gel containing a bead library. Further experiments can be developed on the bead, and as long as we can get a positive signal on a similar signal/noise ratio of these fluorescein labeled beads (the real cutoff of signal vs noise could be much lower, given the strong signal these experiments have presented).

Figure 3-2: Fluorescein attached beads in PAGE gel.



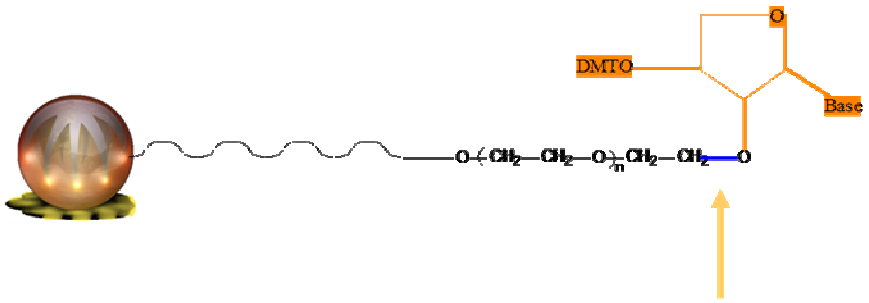
Chapter 4: Functional assays on beads library

This chapter describes the development of selection assays on the 2D gel layout of bead based thioaptamer/aptamer libraries. Two major functional assays, EMSA assay and ELISA-like sandwich assay, were tested on the beads library. Results are discussed.

4.1 BEAD LIBRARY CONSTRUCTION

As mentioned in section 3.1, polystyrene beads with non-cleavable hexaethyl-eneglycol bonds have been shown to be the most promising solid support (Fig 4-1) for bead based selection. To finally establish a strategy that can distinguish thioaptamers that bind to their target from those with very weak binding affinity, several bead libraries, including a one species library as the initial model for optimizing parameters, are discussed below. All beads were purchased from ChemGene Corp. It is estimated that 1 mg of beads contains approximately 4.7×10^4 beads, so that the 1 umol scale of ODN attachable linkers needs 28.6 mg of beads (1.3×10^6 beads). One bead can have 0.77 pmol of an ODN molecule synthesized on it (4.6×10^{11} molecules).

Figure 4-1: Polystyrene bead with non-cleavable linker



4.1.1 Ig-kappa B double stand bead library

Ig-kappa B sequence is the natural target of NF-kappa B in the heterodimer p50/p65 form. Homodimers of p50 and p65 were also shown to bind this sequence *in vitro* (Kunsch, Ruben et al., 1992). So it is convenient for us to test our bead library based on these well investigated binding partners.

Ig-kappa B forward strand synthesis (on bead)

Two strategies were used: one with the forward Ig-kappa B sequence in the middle, flanked by two 15mer primers for later identification purposes. The other strategy is to directly link the Ig-kappa B forward sequence onto the bead, but use only a 10bp spacer on the 3' end. Although the bead is sphere-like, it is far from being a smooth sphere and the attachment spots may lie in deep pockets on these spheres that may exclude protein binding by spatial hindrance. A 10 bp spacer could help mitigate this problem. The chemical procedure is basically the same as the "split synthesis" method (see 4.2) but no "split and pool" strategy is employed.

Forward strand with primers:

5'-CCT ACT CGC GAA TTC AGT TGA GGG GAC TTC CCC AGG CGG ATC CGG TGG TCT G'-3 (red indicates the Ig-kappa B sequence)

Forward strand with spacer:

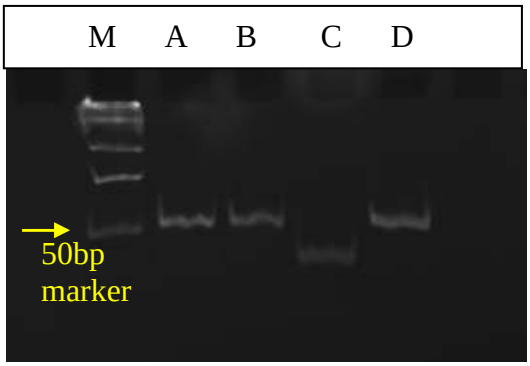
5'-AGT TGA GGG GAC TTC CCC AGG CTT TAC TTA TT-3'

The synthesis results were confirmed by PCR for the beads containing the forward strand and primers, and the beads with the forward strand linked with a spacer were confirmed by later EMSA gel and the yield of the synthesis.

Complementary strand synthesis

(For the beads with forward strand Ig-kappa B flanking with primers) Klenow fragment is used to fill the 5' overhang. 0.01 umol primer (5'- CAG ACC ACC GGA T-3') was mixed with 1/1000 loading of totally synthesized beads in 1x PCR buffer ($MgCl_2$ is added to 2mM as final concentration). 36 units of Klenow, 40 umol of each dNTP, were added and the reaction was incubated at 37 ^{o}C with mild shaking (100 rpm) for either 1 or 24 hrs. Beads were then washed by 1x PBS and centrifuged twice.

Figure 4-2: PAGE of Ig-kappa B bead based PCR (Ig-kappa B forward stand with primers)



A: beads with second strand synthesis by Klenow 3hrs. B: single strand bead. C: control. D: beads with second strand synthesis by Klenow 1hr.

Complementary strand annealing

For the beads with the forward strand Ig-kappa B sequence flanked on one side by a spacer, a total of 48.4 OD (0.21 umole, 1442 ug) of the reverse strand of Ig-kappa B (5'-GCC TGG GAA AGT CCC CTC AAC T-3' , purchased from Midland, Inc.) was dissolved in 1000 ul of in 1x annealing buffer (10 mM Tris-Cl 1 mM EDTA 100 mM NaCl, pH7.4), 0.025 umol of reverse strand was mixed with 1/32 of the total number of synthesized beads The mixture was occasionally shaken, and then was incubated in a 88 ^{o}C water bath with occasional shaking, after which the bath was allowed to slowly cool down to room temperature. Samples of the mixtures were collected at both the beginning and the end of the annealing reaction, and concentrations in solution (unbound complimentary ODN) were measured by UV absorbance at 260 nm (280 nm was also monitored)

Table 3: Absorption before and after the annealing reaction of Ig-kappa B forward strand bead with the reverse strand Ig-kappa B in solution

	Sample I		Sample II	
	A280	A260	A280	A260
Before	0.3524	0.5361	0.3533	0.5389
After	0.2471	0.4275	0.2488	0.4307

From the difference in absorbance values before and after annealing (Table 3), it was estimated that 20% of the reverse strand has been annealed with ODN on the beads. Based on the molarities calculated, the loading of beads is 1/32 total beads (Initially 28.5mg 65um beads is loaded., this was counted as 1 umol; The yield is about 72.6% based on DMT data. (Initial trytyl data: $1.68x10^{6}$, end trytyl data: $1.22x10^{6}$) .The loading

of reverse strand is 0.025 umol. So it was estimated (0.025 x 20%) / (1/32x1x72.6%) = 22% of DNA on beads is annealed with the second strand

Nevertheless, this is currently the most definitive evidence that the second strand is bound to the first ODN stand on the bead. With a successfully annealed double strand bead library, it was then possible to test functional assays in further study.

4.1.2 A randomized beads library

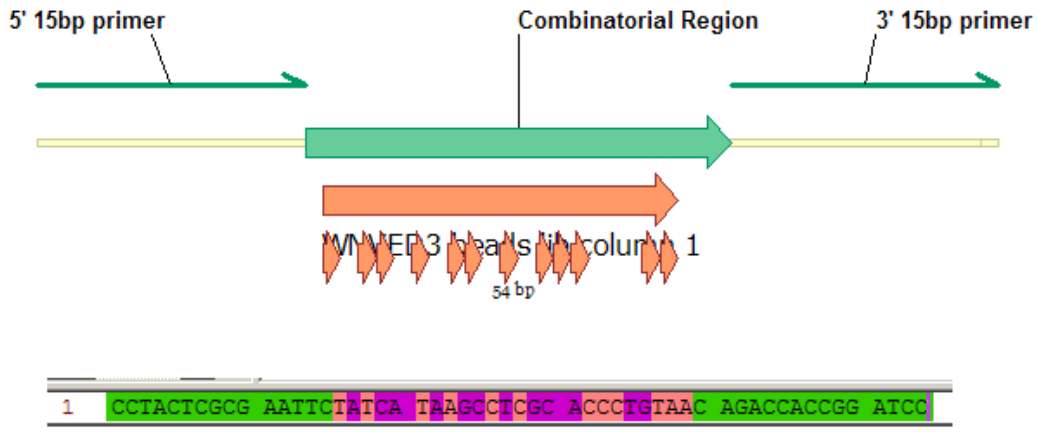
The long term goal of this project is to create an efficient platform for therapeutic thioaptamer selections. A bead library containing randomized sequences of aptamers and thioaptamers will serve as the major reservoir for future drug agents. A small scale library was constructed in order to verify the idea and could serve as the initial selection pool for 2D layout selection.

Chemical synthesis of thioaptamer and aptamer on beads

Polystyrene beads (60~70 um in diameter) with non-cleavable hexaethyleneglycol linkers are used as solid support for library synthesis. Phosphoramidite and thiophosphoramidte are used as precursors for phosphate, phosphorothioate and phosphorodithioate nucleosides, respectively. Two selected sequences were synthesized at the 1 umol scale on an Expedite 8909 System. They were synthesized in parallel on two columns. Upstream and down stream primers were synthesized using normal phosphoramidite chemistry. The “split and pool” strategy was performed by manual operation as follows: At each of the combinatorial sites (see Fig 4-3 for design diagram), the reaction is stopped, the beads were then mixed and re-aliquoted into two columns and the reaction was continued. Where sulfur substitution is needed, the Beaucage reagent is used instead of the normal oxidizing agent. The S2-ODNs required the use of

thiophosphoramidites as well as the Beaucage regent. The fully synthesized S-ODN and S2-ODN was deprotected with saturated ammonia (30%) at 37 ^{o}C with mild shaking (150 rpm) for 21 hrs and then washed with distilled water.

Figure 4-3: Sequence design of a 2^{12} = 4096 kinds of one-bead one aptamer library



Each bead in the library contains ODNs that are 55 base pairs long. The 15bp primer is first synthesized, followed by the combinatorial region. All pink spots indicate combinatorial sites at which a "split and pool" operation is utilized. After the combinatorial region, another 15 bp primer sequence is added.

The two column sequences synthesized are as follows:

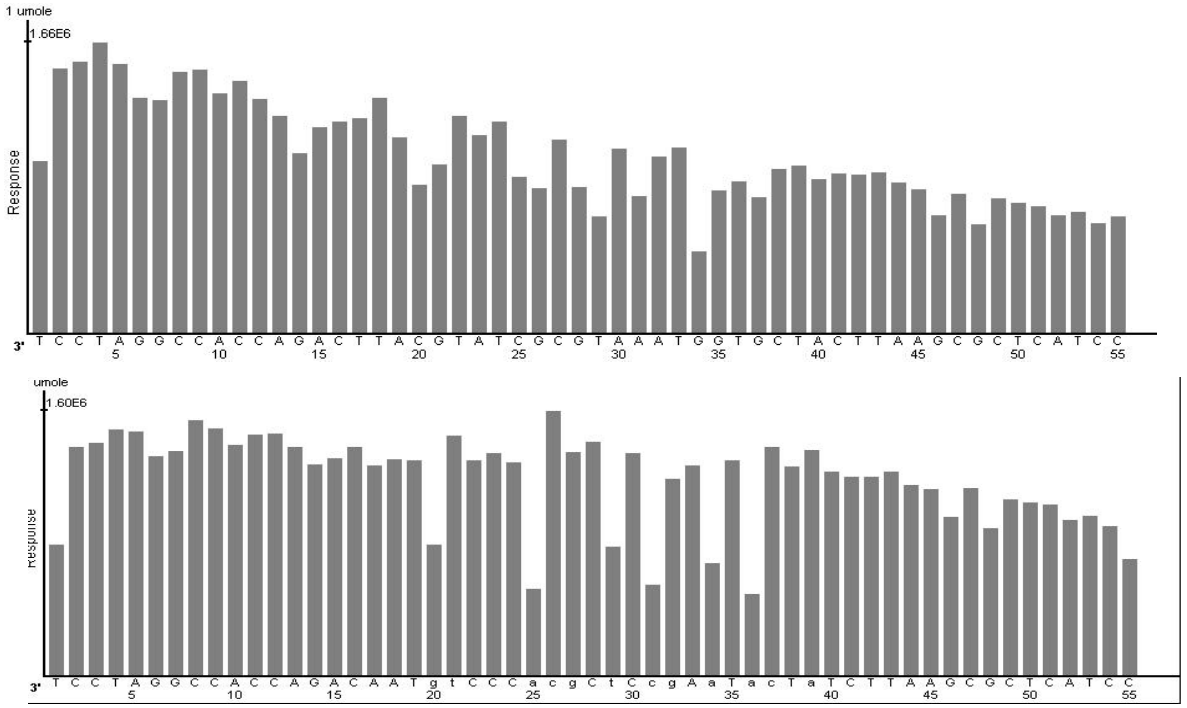
>WNVED3 beads lib column 1 (1bp - 54bp, direct)

5'- CCT ACT CGC GAA TTC TAT CAT AAG CCT CGC ACC CTG TAA CAG ACC ACC GGA TCC -3'

>WNVED3 beads lib column 2 (1bp - 54bp, direct)

5'- CCT ACT CGC GAA TTC ATC GTG AGA AAT GCG CTA TGC ATT CAG ACCACC GGA TCC-3'

Figure 4-4: The coupling yield determined by the dimethoxytrityl cation assay



Above: WNVED3 lib column 1 yield. Bottom: WNVED3 lib column 2

It is worth noting that at each manually operated site, the yield assay is disrupted and shown in Fig 4-4, this is understandable due to column loading and unloading, but this poses one potential problem: in ODN synthesis, a yield of greater than 99% is needed in order to gain a long chain. For example, if each step has a yield of 99.7%, after 55 steps, only 86% of the ODNs have the correct sequence, and this yield decreases exponentially. Even at a yield of 99% for each step, after 55 steps, only 57% yield can be gained. So a manually synthesized library is definitely fine for testing purposes, but a robust reproducible mechanical method should be applied to further thioaptamer selection studies.

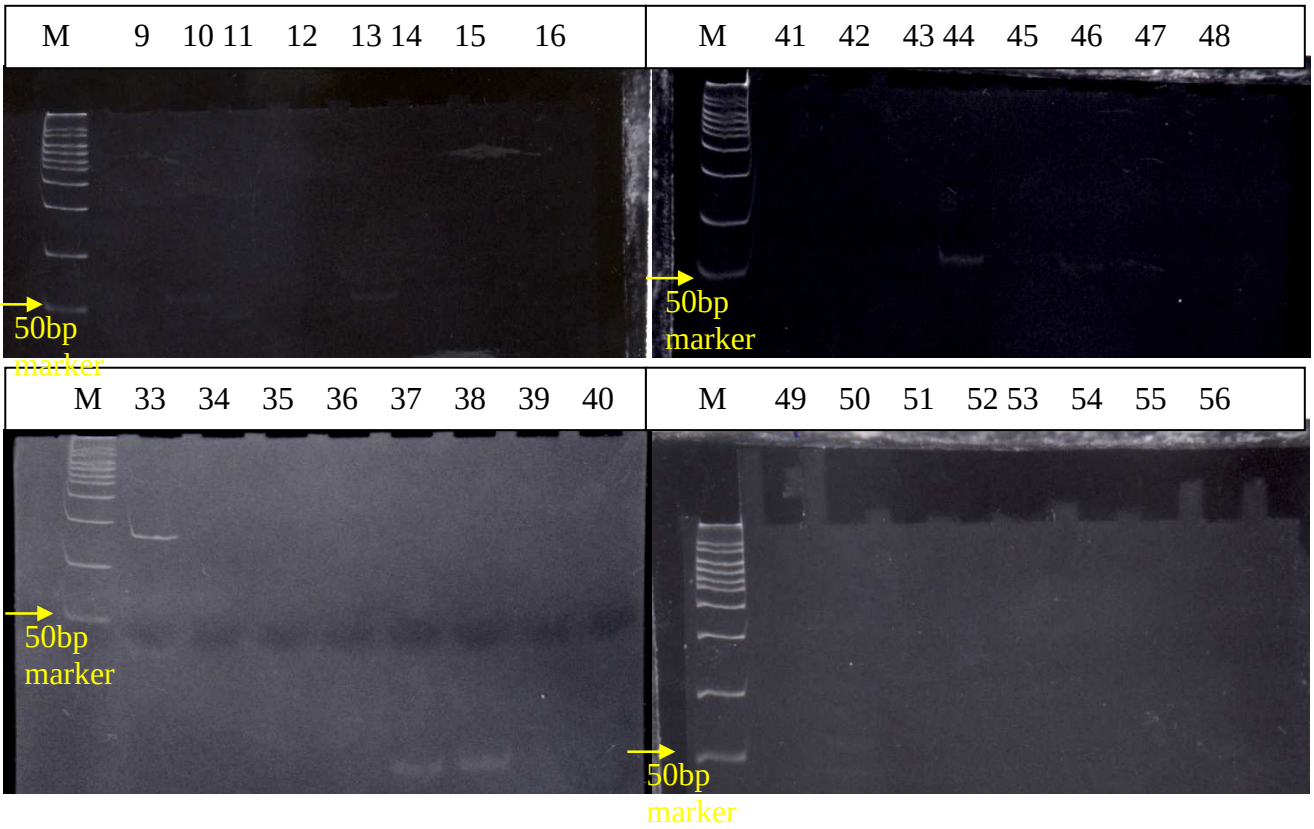
Complementary strand synthesis

Klenow fragment was used to fill the 5' overhang. 1 umol primer (5'-GGA TCC GGT GGT CTG-3') was mixed with 1/2 of the synthesized beads in 1x PCR buffer ($MgCl_2$ is added to 2 mM final concentration). 36 units of Klenow and 80 umol of each dNTP are added and the reaction is incubated at 37 ^oC with mild shaking (100 rpm) for 24 hrs. Beads were then washed by 1x vPBS and centrifuged twice.

One-Bead One PCR

A single bead was selected under microscope and then washed with 8M urea (pH 7.2) to remove any contaminations (possible proteins) and then was amplified by PCR: Each vial contains one selected bead as template in 1x PCR buffer (2 mM $MgCl_2$, 2.5 u Taq polymerase, 0.08 mM each dNTP and 0.4mM of each primer. Primer sequences were 5'-CCT ACT CGC GAA TTC-3' and 5'-GGA TCC GGT GGT CTG-3'. Thermal cycles were 94 ^oC heat shock for 5 min, 35 cycles of 94 ^oC for 2 min, 35 ^oC for 2 min and 72 ^oC for 2 min, and a final extension at 72 ^oC for 7 min. PCR products were analyzed on 15% native PAGE gels and those with correct length were purified by molecular weight filters (Centricon) to remove polymerase and additional primers.

Figure 4-5: Selected One-Bead One-PCR



All markers are from 50bp-200bp, all samples used one single bead as template.

It is as expected that a portion of selected beads showed the correct length (Fig 4-5: sample 10, 14, 44, 45, 46, 47, 50, and 56), while the strength of the gel band. This may reflect that some sequences may form internal secondary structures such as hairpins that may interference with the PCR reaction. Interestingly, sample 33 showed a much longer band about 150 bp long. A cross-linking between PCR product may induce this effect, and since we are dealing with a randomized library, this is in fact one of the possible side-effect that could be expected.

In conclusion, a random library was constructed, and even though the manual operation complicated the aptamer/thioaptamer synthesis process, this library is well suited for pioneering testing of the 2D layout bead based selection.

4.2 EMSA ASSAY ON BEADS

With a well investigated protein-aptamer/thioaptamer model system, NF-kappa B dimers p50/p65, p/50/p50 and p65/p65 which bind specifically to Ig-kappaB aptamer and XBY6 thioaptamer, we were able to study and optimize functional assays on bead to establish the 2D layout method for selecting aptamers and thioaptamers. Furthermore, electrophoretic mobility shift assays have been successfully tested *in vitro*, so the next rational step was to study this assay in a on-bead scenario.

As with the EMSA assay used in section 2.3, SYBR green and SYORO ruby staining dye was utilized.

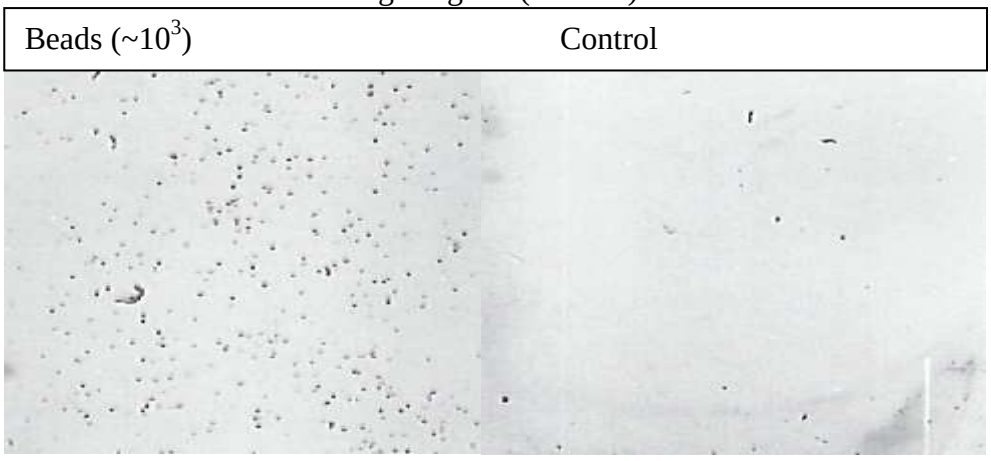
On bead EMSA assay

Selected loading of sample beads (Ig-kappa B dsDNA beads, ssDNA beads with or without primers) was incubated with serial dilutions (1 ul) of purified recombinant NF-

kappB RelA (p65) or p50 in a 20 ul reaction under standard reaction conditions (20 mM Hepes, pH 7.5, 50 mM KCl, 2.5 mM $MgCl_2$ 20 mM DTT, 10% glycerol, since poly I-C can interfere with SYBR Green it is not included in the reaction buffer). After 1 hour, the reaction buffer was dispersed into a native 15% polyacrylamide gel. Beads were mixed i with soluble PAGE mixture before adding the last catalyst. (Either 10% APS or TEMED as the last step). Upon adding the final catalyst, the solution was immediately vortexed to uniformly distribute the beads in solution and the solution was injected into a Bio-Rad mini-gel (8.6 x 6.8 cm x 0.5mm). Following solidification, the gels (15% PAGE) were stained with SYBR Green for 20 minutes and the subsequence protein staining used SYPRO Ruby overnight (the staining process was protected from light). The gels were then imaged on a FluorChem 8800 system with excitation at 304nm or by white light if needed.

SYBR Green Staining of Beads

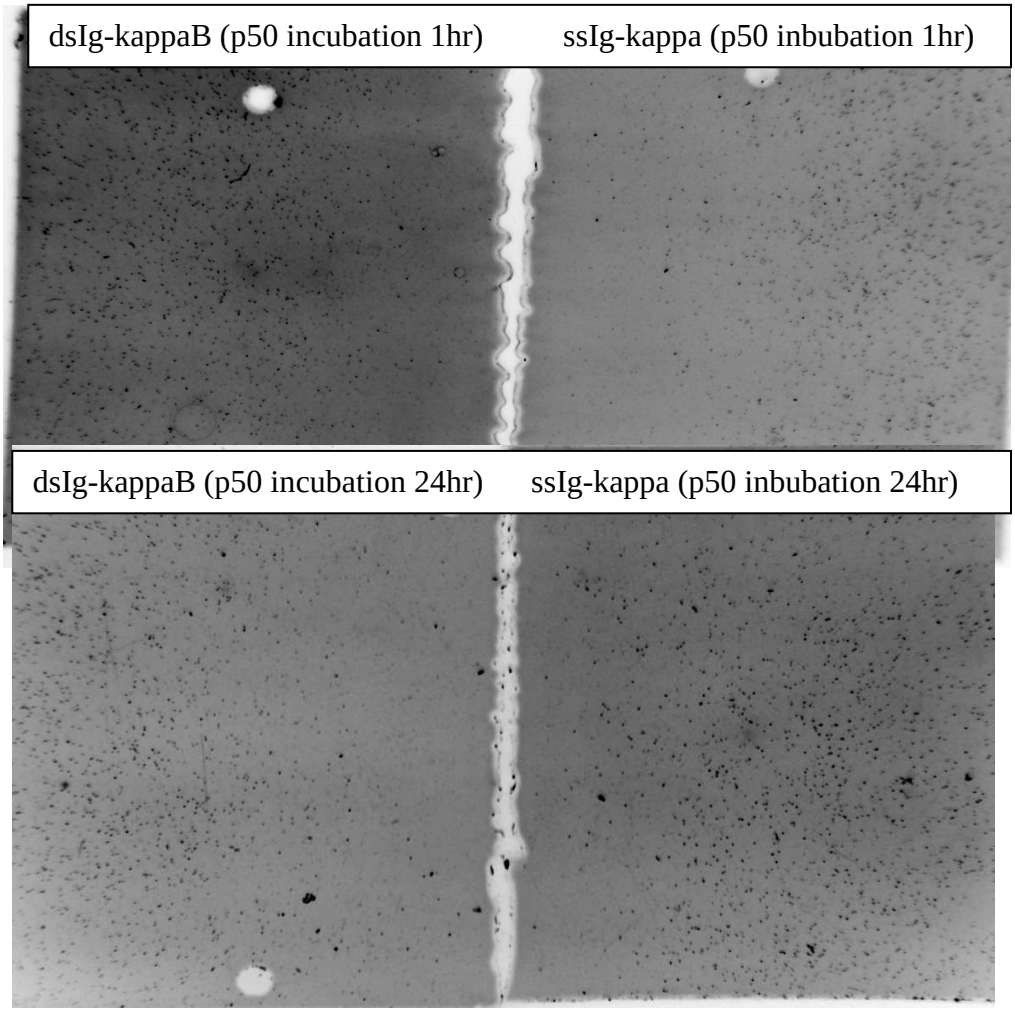
Figure 4-6: SYBR Green staining of Ig-κB (dsDNA) beads



1/1000 of totally synthesized beads (~10^3 beads) incubated with p50 for 3hrs at room temperature. The control gel contains no beads.

Regarding SYBR Green dye, our experiments (Fig 4-6) showed the signal/noise of bead staining is similar to fluorescein attached DNA, so SYBR Green should be possibly a good candidate for the selection process. Since positive beads were detected from staining, not all beads produce the same level of signal (Fluorescein-attached beads are homogenous regarding their signal). The reason why empty beads were not used as a control is that empty beads are hydrophobic and not easily distributed into the gel. Also, we have noticed that at this loading level, beads begin clustering in areas that are hard to hand-pick at this density. This is one important technical aspect that should be carefully decided, and this factor also depends on the downstream operation techniques. As our initial research had to be operated in a manual fashion, we consider this loading as the limit for effective selection.

Figure 4-7: SYBR Green staining of beads with double strands (Klenow synthesized) and single strand Ig-kappa B after incubation with p50 (1:20) dilution



All gels are loaded with 1/100 totally synthesized beads incubated with p50 (1:20 dilution) at room temperature. Above: 1hr incubation; Bottom: 24hrs incubation.

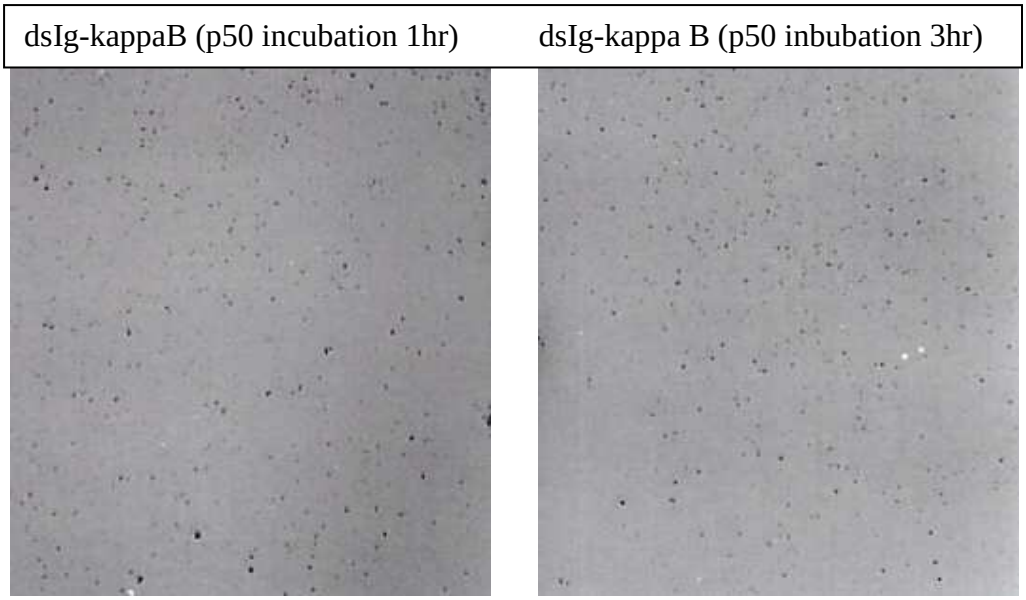
The signal intensity (Fig 4-7) can tell us which beads have dsDNA attached, as indicated here by darker staining in these 2 experiments, which can serve as a way to verify that the Klenow fragment synthesized the second strand on bead. It is easy to verify annealing experiments as mentioned earlier, (see section 4.1.1), but in the scenario

of beads with random sequence, we need to use polymerase to synthesis the second strand. This assay is the first assay that is able to solve this.

Interestingly, after a Klenow reaction of 24 hrs, the beads with dsDNA did not show stronger but a little bit weaker signal than the beads with 1 hr. One possibility is that SYBR Green could possibly interfere with the binding reaction, thus more incubation time with p50 could cause less staining of SYBR Green with beads.

SYPRO Ruby Staining of Beads

Figure 4-8: SYPRO Ruby staining of beads with double strands (Klenow synthesized) after incubation with p50 (1:20 dilution)



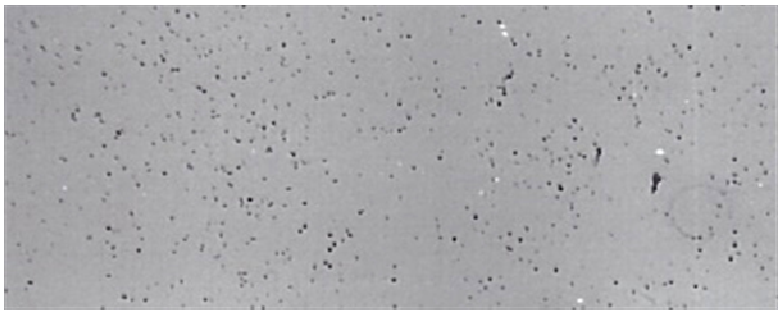
All gels are loaded with 10^3 beads. Beads are incubated with p50.

SYPRO Ruby is not as strong as SYBR Green, so the signal/noise is apparently reduced, and the homogeneity of the bead signals is also decreased. It is still acceptable for future selection process, especially in combination with SYBR Green, since SYBR

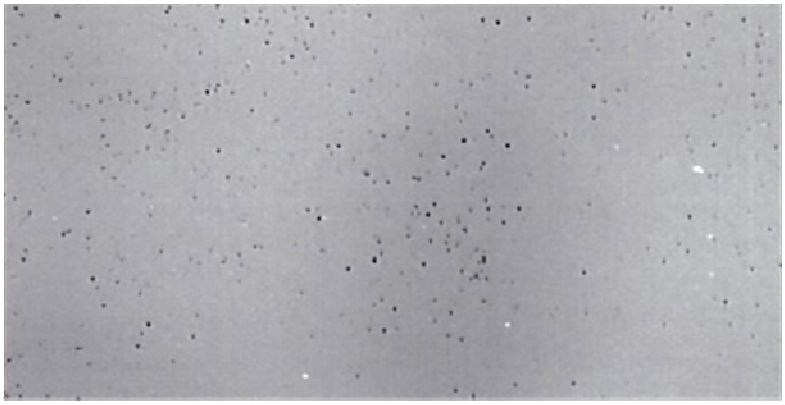
Green can be used to single out those noisy spots on the gel that are in fact not beads. Also, protein is probably not totally washed off from the gel, given the dark background.

Figure 4-9: SYPRO Ruby staining of beads of double strands and single strand Ig-kappa B after incubation with p50 (1:20) dilution

ds Ig-kappa B beads bound to p50 (10^3 beads loaded)



ss Ig-kappa B beads bound to p50 (10^3 beads loaded)



All gels are loaded with 10^3 beads. Above: double strand Ig-kappa B beads, Bottom: single stand Ig-kappa B beads.

Further comparison of double strand beads with single strand beads under the SYPRO Ruby staining technique confirmed previous report that single strand Ig-kappa B binds to p50 homodimer weakly, as shown in Fig 4-9.

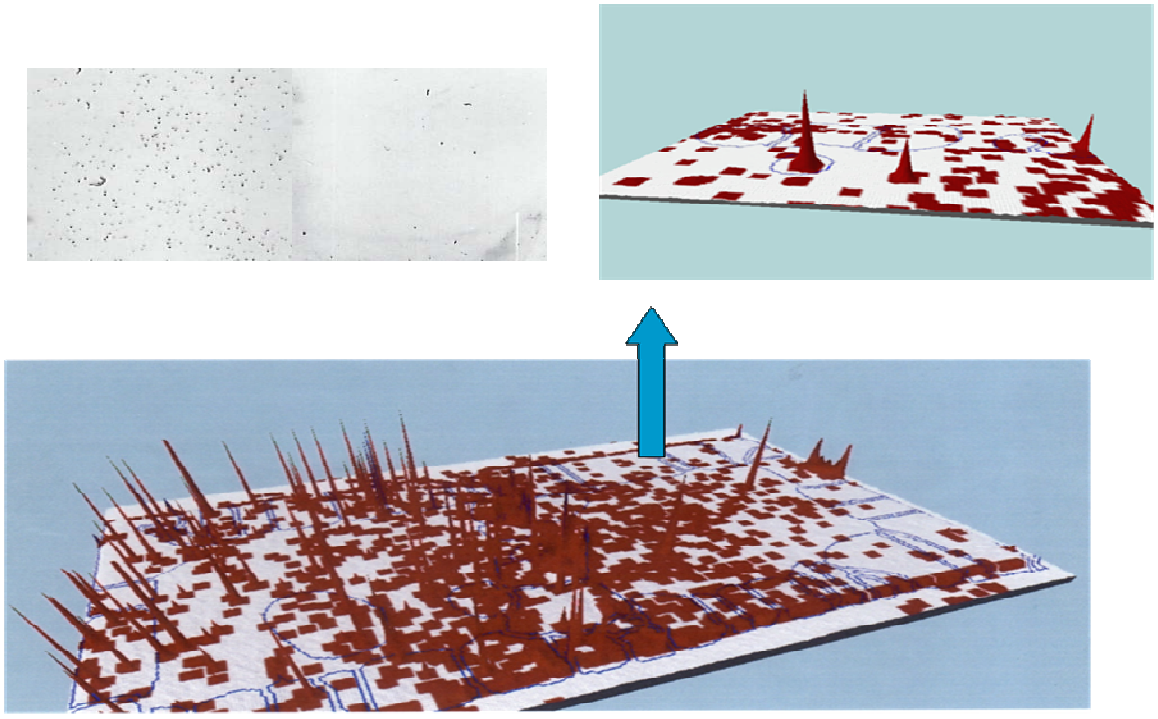
3D visualization for automatic image processing

Admittedly, EMSA-like staining using SYBR Green and SYPRO Ruby generated results with hard-to-interpret visual data, as previous gels results have shown. Also, to further the 2D layout selection of thioaptamers, an automatic solution for identifying positive beads is needed. If an image transformation is applied and we see the gel in three-dimensional fashion, the results are much easier to interpret. (See Fig 4-10)

3D image generating process

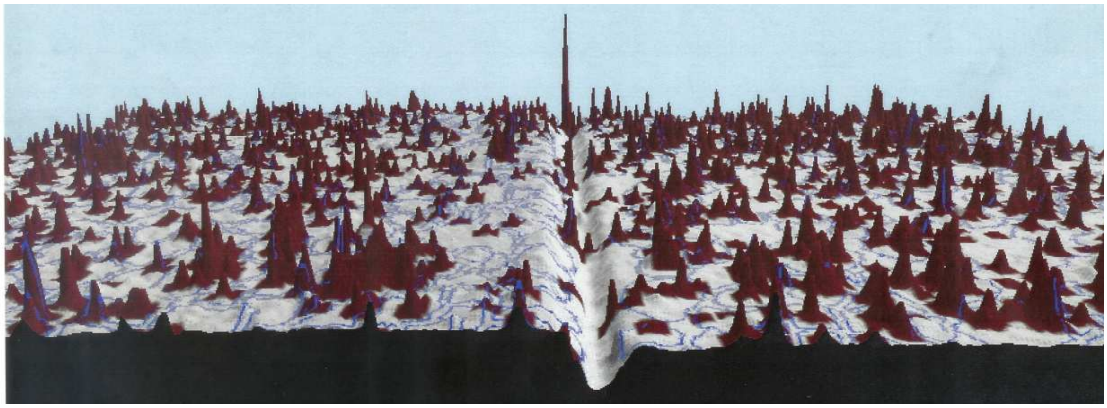
Gel image is taken in a tiff format (16bit grey scale) by.FluorChem 8800 system with excitation at 304nm or by white light if needed. 3D generating engine is Progenesis PG240 V2006 (Software from Nonlinear Dynamics Inc). Peaks are indicated in red (Fig 4-10 and Fig 4-11). Progenesis also gave the clustering of spots from data (indicated in blue lines in Fig 4-10 and Fig 4-11), this is designed for analysis of 2D protein electrophoresis, so basically it gave the regions the software considered as spots of protein. Peaks were frequently identified as “noise” in this setting, but in fact it is the spot we are interested in.

Figure 4-10: 3D visualization of 2D layout of beads (SYBR Green staining)



3D transformation of Fig. 4-6, upright shows an area of interest can be selected as input for a robotic bead picking using automatic gel excision.

Figure 4-11:3D visualization of 2D layout of beads (SYPRO Ruby staining)



3D transformation of Fig. 4-8

Undoubtedly, 3D transformation makes the selection process much easier as the peaks are indications of beads, and in this way, the noise is easier to screen out, since noise usually is presented not as a sharp peak but more like “mountains”. Nevertheless, an algorithm is needed to help facilitate the screening process so a mass bead library (was may as 10^8 different beads with different proteins bound) could be screened and positive beads could be identified in a timely fashion.

In conclusion, the EMSA-like staining strategy is well suited for the screening process, but parameters need to be optimized for better signal/noise ratios. Other alternatives may generate a better signal/noise ratio, but at least this staining strategy could serve as co-screening techniques to facilitate noise reduction and positive bead identification.

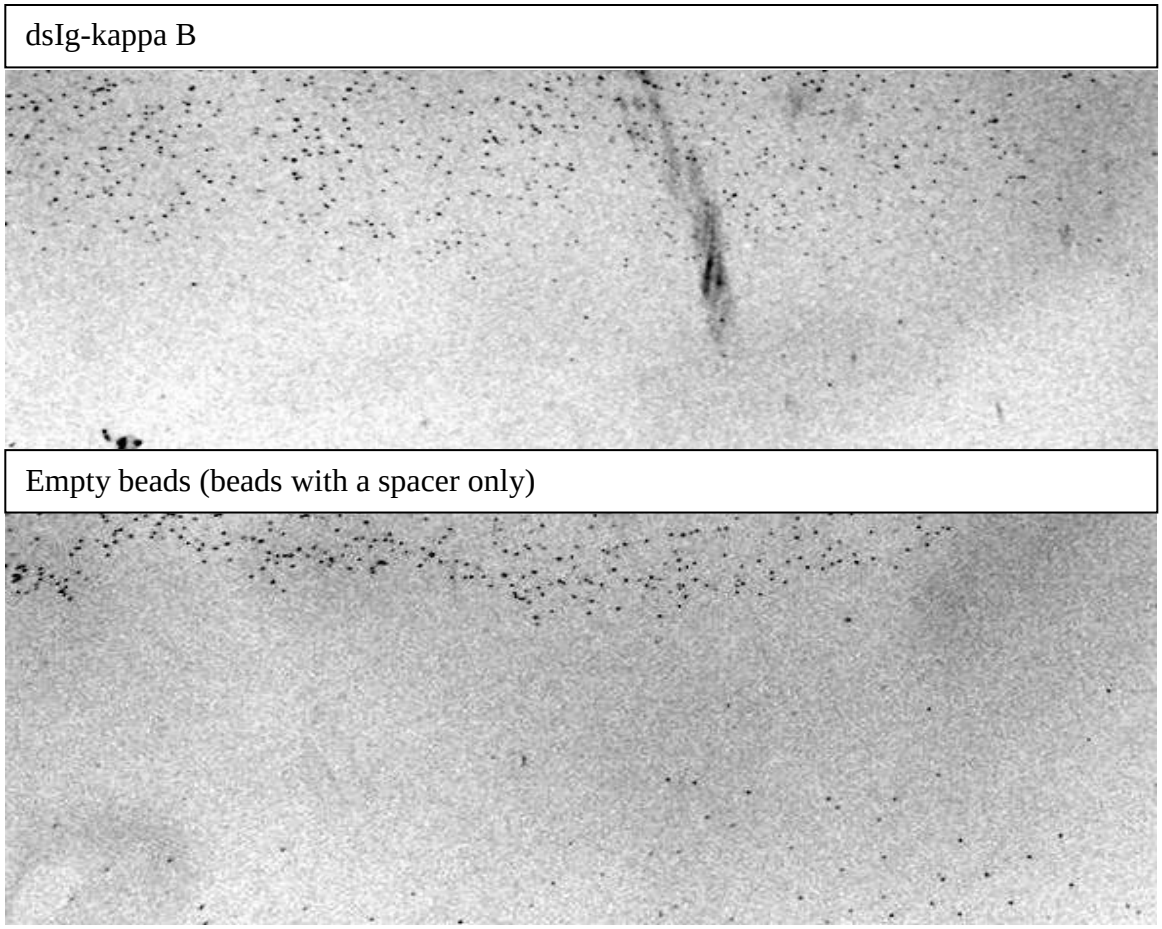
4.3 IMMUNOFLUORESCENCE ASSAY ON BEADS

Besides the EMSA-like staining technique, another promising method could be a various sandwich assay, as discussed earlier (see section 2.4.2). In this experiment, a sandwich immunofluorescence assay is tested.

Bead based immunofluorescence assay

Beads were washed twice with PBS+0.1% Tween-20 to block non-specific binding. Then the beads were incubated with serial concentrations of p50 homodimer, p65 homodimer or p50/p65 heterodimer for hours at room temperature with occasional agitation (<200 rpm). The beads were washed again. Rabbit IgG antibody targeting p65 (sc-372, Santa Cruz. Biotech) or p50 (sc-114, Santa Cruz biotech) was incubated with beads at 1:100 dilution for 2h at room temperature with occasional shaking. The beads were then washed three times. Alexa Fluro 488 labeled Goat anti-Rabbit IgG (500uL-2mg/ml Molecular Probes) at 1:50 dilution was incubated with beads (ds Ig-kappa B beads with only spacer, beads with 10bp random sequence serve as a control) in the vial for 1h at room temperature with occasional shaking. Beads are then washed. The beads were then loaded onto a native 15% polyacrylamide gel. Beads are immobilized into 15% PAGE gel by mixing beads with soluble PAGE mixture before adding the catalyst (either 10% APS or TEMED). Upon adding the final catalyst, the solution was immediately vortexed to homogenize the bead distribution and the solution was injected into a mini-gel 8.6 x 6.8 cm x 0.5mm (Bio-Rad). The gel was then imaged on a FluorChem 8800 system. The image system has no specific 488nm excitation profile, white light is used instead.

Figure 4-12: ds-Ig-kappa B beads incubated with p50, detected by immunofluorescence assay



Immunofluorescence assay on beads: **Above**: dsIg kappa B incubated with p50, p50 is detected by anti-p50 antibody, and the signal is detected by Alex Fluro 488. **Bottom**: Empty beads incubated with antibody p50 and anti-antibody linked with Alex Fluro 488. (The loading of empty beads is 1/80 of totally synthesized beads, and the loading of Ig kappa B beads is 1/128 of totally synthesized beads) Each batch of totally synthesized beads is estimated to contain 10^6 beads.

The result showed beads with ds Ig-kappaB has slightly higher positive rates compared to the control beads. This signal/noise needs to be improved before it is applicable for selection purposes. But since the image system used here do not have a specific excitation wavelength at 488nm, strong white light is used to excite the beads. It is believed that white light excitation can cause interference and diffraction as well as additional excitation on the beads and thus induce the noise signal. A specific excitation at 488nm should be able to decrease the noise.

Chapter 5: Summary

The work reported in this thesis describes development of a 2D layout of one-bead-one thioaptamer/aptamer library.

A model system is first established based on one of the well-investigated binding partner in aptamer/thioaptamer research: NF-kappa B protein and Ig-kappa B sequence and its derivative XBY-6. NF-kappa B protein p50 and p65, which are considered the central mediator of immune response, is cloned, purified and characterized. Its natural binding partner, the Ig-kappa B binding motif, is constructed on the bead via flanking between primers or directly attaching with a spacer. XBY-6, the leading thioaptamer that showed highest binding affinity and also the specificity towards NF-kappa B protein, or RHD domain in essence, is synthesized with a biotin label at the 5' end of its forward strand. Six phosphorodithoate modifications at T position in its sequence is the key element that increases the binding affinity. Both binding partners are verified: For XBY-6, electrophoretic mobility shift assay revealed its high binding affinity (0.18 nM), and specificity. For Ig-kappaB, immobilized consensus sequence on 96 well plate has successfully pulled down p50 and p65 and was detected by antibody system by ELISA assay.

In addition, the purification process of various thioaptamers, especially those with 5' end modification with functional groups, is investigated. Several functional groups' effects on the thioaptamer purification process is characterized. In short, a tandem system using FPLC with strong ion exchange column followed by HPLC with PRP-1 column is found effective to purify these 5'-functionalized thioaptamers.

The successful buildup of these model systems led us to investigate possible functional assays on a 2D layout bead library, which is considered a promising alternative to original aptamer/thioaptamer selection technique. Both simple species bead library (Ig-kappa B beads library) and a small scale randomized bead library (2^{12}=4096 different species as designed) were constructed and verified. Especially, to construct a double strand bead library, annealing on bead and Klenow synthesis on bead are utilized and later confirmed by different experiment. In fact, this is the first evidence that the second strand is able to successfully attach to the single stand bead library.

In terms of the final 2D layout selection platform development, this idea is first justified in our fluorescein attached bead library and several parameters are optimized for increasing signal to noise ratio.

2D layout selection of thioaptamer/aptamer beads library is further developed: Two kinds of assays are tested for on-bead screening: EMSA-like staining technique using SYBR Green and SYPRO Ruby and immunofluorescence assay. The EMSA-like assay first helped us verify the Klenow synthesis on bead and then showed as a promising method for selecting thioaptamers. The immunofluorescence assay is also giving some encouraging results.

The importance of this project is that it paves the way of realization for the one-bead-one-thioaptamer library selection concept, which was derived 5 years ago. Thioaptamers have shown important features that are is suitable for drug candidates and also give us the possibility to arbitrarily target proteins of our interest. The therapeutic and diagnostic prospect of thioaptamers is great, but the development of thioaptamers is currently limited by the need for an effective large scale high-throughput screening of thousands of billions of thioaptamers.

The techniques developed in this project shall serve as an initial point for large scale selection process. Future directions may involve the development of modern algorithms for automatically identifying positive beads in the 2D gels, as well as a robotic automation for excision of small spots that contains our beads of interest. Those are more an aspect of engineering rather than scientific development, so the major progress could be expected in a short time.

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