USING FLUORESCENCE ANISOTROPY FOR SENSITIVE PLATELET-DERIVED GROWTH FACTOR DETECTION BASED ON MOLECULAR APTAMERS

By

ZEHUI CAO

A THESIS PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2002
ACKNOWLEDGMENTS

I would like to thank my parents for what they have gone through to bring me up to where I am now, and my wife, Qian, for her support and patience all the time. I would also like to thank my advisor, Dr. Weihong Tan, for his confidence in me and the encouragement he gave me to continue my study, Dr. Xiaohong Fang for her guidance in the early PDGF work, Dr. James Winefordner for his kind help and understanding during my difficult times, the members of my advisory committee for their helpful guidance and suggestions, and the Tan research group.
TABLE OF CONTENTS

ACKNOWLEDGMENTS ........................................................................................................ iii

ABSTRACT ......................................................................................................................... vi

CHAPTER

1 INTRODUCTION ........................................................................................................ 1
   Introduction to PDGF Protein ......................................................................................... 2
   Aptamer as a Probe for Protein ....................................................................................... 3
   Fluorescence Anisotropy ................................................................................................ 5

2 MOLECULAR APTAMER FOR REAL-TIME PDGF DETECTION USING
   FLUORESCENCE ANISOTROPY ................................................................................... 8
   Experimental Section ...................................................................................................... 8
      Materials ..................................................................................................................... 8
      Instrumentation ........................................................................................................... 9
      Anisotropy Measurements .......................................................................................... 9
   Results and Discussions ................................................................................................ 10
      Design of an Fluorescent Aptamer Probe ................................................................. 10
      Binding of the Aptamer to PDGF ............................................................................. 11
      Real-Time Binding Detection Using Fluorescence Anisotropy ............................... 12
      Effects of Mg$^{2+}$ on the Binding Assay .................................................................. 14
      Effect of Temperature on the Binding Assay ........................................................... 16
      Detection of PDGF-BB in Homogeneous Solution .................................................. 17
      Selectivity of the Aptamer Probe .............................................................................. 21
   Conclusions................................................................................................................... 23

3 DEVELOPMENT OF FLUORESCENCE ANISOTROPY IMAGING SYSTEM
   FOR PROTEIN ARRAYS............................................................................................... 25
   Protein Arrays ............................................................................................................... 25
   Fluorescence Anisotropy Imaging ................................................................................ 28
   Experimental Section .................................................................................................... 30
      Materials ................................................................................................................... 30
      Anisotropy Imaging Setup ........................................................................................ 31
      Acquisitions of Anisotropy Images ......................................................................... 32
   Results and Discussions .............................................................................................. 35
Proteins play very important roles in almost all functions of life. Detection of proteins has drawn great attention from scientists for many years. However, analytical methods for real-time protein detection in homogeneous solutions are scarce. The recent development of molecular aptamers, combined with fluorescence techniques, may provide an easy and efficient approach to sensitive protein analysis. Aptamers are small oligonucleotides which have high affinity and high selectivity to their target molecules. They are isolated by the systematic evolution of ligands by exponential enrichment (SELEX) process. We have designed a fluorescein-labeled aptamer for the detection of platelet-derived growth factor (PDGF) protein which has the potential as a cancer indicator. Fluorescence anisotropy was used as the detection method. Fluorescent molecules, when excited by plane-polarized light, will give an emission that has been depolarized to a certain extent. This can be described in terms of anisotropy. Factors that can change the rotational diffusion of the fluorescent molecules will affect their
anisotropy, including their molecular weights and the properties of the surrounding environment. Fluorescence anisotropy technique is ideal for aptamer/protein binding assays where there is a large molecular weight change. By combining aptamers and fluorescence anisotropy, we are able to achieve highly sensitive PDGF detection with high selectivity. The detection limit was about 0.22 nM. We then extended this technique into a two dimensional format by building a fluorescence anisotropy imaging system. This format enables the simultaneous anisotropy measurements of multiple samples. We demonstrated the ability of this system to detect multiple proteins in one sample. With some modifications and improvements, the anisotropy imaging technique may have the potential to be a very effective and easily implemented approach to high throughput and multiplex protein analysis in an array format.
CHAPTER 1
INTRODUCTION

Proteins are macromolecules that consist of one or more unbranched chains of amino acids. Although typical proteins contain 200-300 amino acids, they can be much smaller or much larger. Proteins are the building blocks of life. Almost every function in living cells depends on proteins. Those include catalysis of all biochemical reactions, construction of cells, motion of cells and organisms, transportation of materials in body fluid and many more. Since proteins realize their functions through interactions with other molecules, it is highly important to understand those interactions in order to find out how proteins work in living cells. Many techniques have been developed to detect and analyze proteins. Some of them, such as electrophoresis and affinity chromatography, have provided a good way to separate and detect proteins. However, they lack the ability of monitoring protein interactions in real time and in homogeneous solutions. Fluorescence techniques, on the other hand, have shown great capability in detecting and studying protein functions in their native environments. This ensures a more direct and precise understanding of protein interactions. Many protein probes have been used for protein detection using fluorescence techniques. Some of which are extracted from animals that have the inherent ability of binding to certain proteins, such as antibodies. They have great affinity and selectivity towards proteins, but they have more restricted requirements for their surrounding environments in order to function properly. Other probes have been synthesized or selected by scientists, such as aptamers. Those synthetic protein probe molecules can also bind to proteins with high affinity and high selectivity,
but they are more robust and much easier to obtain. In our work, we were trying to develop a fluorescence detection technique, particularly a fluorescence anisotropy technique, for proteins using aptamers as the probe. We later extended and modified this technique to an array format as we tried to examine the possibility of using fluorescence anisotropy for simple and accurate high throughput protein screening and multiplex protein detection. We specifically developed our technique for platelet-derived growth factor (PDGF) detection because of the great interest in this protein for cancer studies.

**Introduction to PDGF Protein**

Understanding disease-related proteins could be the very first and most important step in disease studies and drug discovery. As increasing attention has been focused on cancer diagnosis, it is of great interest to find out more about those proteins that may be related to cancers. One of which is platelet-derived growth factor (PDGF) found in many human cell types. Its biological function is to stimulate the division and proliferation of the cells through binding of its receptors on cell membranes. It is also believed to play a role in intercellular signaling [1]. PDGF has several isoforms, among which are PDGF-AA, PDGF-BB and PDGF-AB. Those isoforms consist of the two subunits, PDGF-A chain and PDGF-B chain.

The three isoforms act differently in specific situations. PDGF-BB has been shown to be actively involved in cell transformation process and in tumor growth and progression [2-6]. PDGF-BB, when bound to its receptor on the cell membrane, activates phosphatidylinositol 3-kinase (PI3-K) inside the cell, which eventually leads to cell growth. PDGF-BB is expressed at undetectable low levels in normal cell [7] while it is often found over-expressed or mutated in a malignant tumor [8]. Because of the potential
as a cancer indicator, its detection has been attempted using the traditional antibody based radioisotopic methods and ELISA techniques [3-6].

**Aptamer as a Probe for Protein**

For the last several decades, antibodies have been the most important probes for a variety of molecular recognition applications. Many diagnostic tests based on antibodies are routinely conducted in laboratories and clinics. The great success in these applications is the result of their very high sensitivity and high selectivity to the target molecules. However, antibody technology also has some major disadvantages. It relies on the animal host to produce antibodies, which means high cost, low efficiency and the very limited number of target molecules that can be detected. Antibodies are very sensitive to their surrounding environments and often easily undergo irreversible denaturation. Regeneration of antibodies is usually not easy, which also contributes to the high cost. In some cases, ligand density is very limited by the antibodies are large molecules. It is also relatively more difficult to directionally immobilize antibodies onto a surface, which may be critical in some applications.

Recent development of the systematic evolution of ligands by exponential enrichment (SELEX) process, however, may provide solutions to some of the problems associated with antibodies. This process has the ability to isolate the oligonucleotide sequences that recognize virtually any class of target molecules with high sensitivity and high selectivity [9]. The resulting oligonucleotide ligands were given the name “aptamers,” which comes from the Latin word “aptus,” meaning “to fit.” The SELEX process begins with a library of synthesized oligonucleotides usually containing $10^{14}$ to $10^{15}$ random sequences. This library is then incubated with the target molecule of interest under certain conditions. The sequences that interact with and bind to the target
molecules are isolated for next round of incubation. This process is repeated until a sequence that binds to the target with highest selectivity and affinity is determined. SELEX enables the discovery of ligands to virtually any target molecules of interest. Some aptamers have been well studied, such as the aptamer that specifically interacts with human $\alpha$-thrombin. New aptamers are also being discovered for a variety of different target molecules [10]. Although most aptamers are exploited to study proteins, there are some applications that use aptamers for detecting smaller molecules such as cocaine [11].

Compared to antibodies, aptamers have similar high affinity and selectivity for proteins [12,13]. What makes aptamers so useful is that they have several important advantages over antibodies. First, their production is easier, cheaper and not limited by the animal hosts. Because oligonucleotides have more stable structures than proteins, aptamers can withstand harsher experimental conditions than antibodies and can be stored and reused without causing much degradation. Aptamers can be easily labeled or modified in different ways for different molecular recognition applications. They can also be easily immobilized onto solid surfaces without much change in the binding affinities to proteins. In one of the examples, the aptamer for human $\alpha$-thrombin was labeled with a fluorophore and then immobilized onto glass surface for high sensitivity thrombin detection [14]. In another example, the $\alpha$-thrombin aptamer was labeled with a fluorophore and a fluorescence quencher at its two ends to form an aptamer beacon [15]. The change in the aptamer’s conformation upon binding to $\alpha$-thrombin brought the fluorophore and quencher far away from each other, thus causing the restoration of fluorescence. Highly sensitive thrombin detection was also achieved based on this
scheme. Unlike antibodies, aptamers can also be used to inhibit target protein’s normal functions by occupying the active binding sites of the protein. This gives aptamers the potential to be drug candidates for numerous diseases. Traditional protein inhibitors either are too toxic or lack good specificity. Aptamers, on the other hand, are just small DNAs or RNAs with high affinity and high selectivity for proteins. Thus, they should be very safe and effective. Some work has been done to investigate possibility of aptamers inhibiting the activity of HIV virus [10,16].

High-affinity aptamers for PDGF-B chain have been reported using SELEX [17]. Several single-stranded DNA sequences were found to bind to PDGF-AB and PDGF-BB with high affinity ($K_d \approx 10^{-10}$ M) while to PDGF-AA with lower affinity ($K_d > 10^{-8}$ M). Most of the ligands were found to have a structure of a three-way helix junction with a three-nucleotide loop at the branch point.

**Fluorescence Anisotropy**

Fluorescence anisotropy has been effectively used previously to study interactions between macromolecules. Upon excitation with polarized light, some samples will have polarized emission. The extent of polarization of the emission is described in terms of anisotropy ($r$) [18]. The theory behind the polarized emission can be explained as following: when excited by a polarized light, the sample molecules that have absorption transition moments oriented along the electric vector of the incident light are preferentially excited. Those excited molecules may rotate to other directions before returning from the excited state to the ground state and emitting light, thus causing a depolarized emission. This depolarization is dependent on the extent of the rotational diffusion of the excited molecules. Two factors are believed to affect the diffusive motion of a molecule. One is the viscosity of the solvent surrounding the molecule and the other
is the size of the molecule itself. A small fluorophore molecule in a solvent with low viscosity will be almost fully depolarized on the excited state and exhibit a non-polarized emission. The anisotropy in this case is close to zero.

To measure the anisotropy of a sample, the sample is excited with a vertically polarized light. The intensity of the vertically polarized component \( I_{VV} \) and horizontally polarized component \( I_{VH} \) of the emission are measured with a polarizer on the emission side. The anisotropy \( r \) is then calculated using the following equation:

\[
r = \frac{I_{VV} - I_{VH}}{I_{VV} + 2I_{VH}}
\]

where the subscripts V and H refer to the orientation (vertical or horizontal) of the polarizers for the intensity measurements, with the first subscript indicating the position of the excitation polarizer and the second for the emission polarizer.

Note that even though from this equation, the range of anisotropy value is from 0 to 1, in a real-world sample, with the molecules evenly distributed in all directions, the maximal anisotropy one can observe with one-photon excitation is 0.4.

Fluorescence anisotropy is a simple signaling method for binding assays. In a typical binding assay, a small dye-labeled probe molecule, such as an aptamer, binds to a large target molecule, such as a protein. The increased molecular size will slow down the rotational movement of the dye molecule linked to the probe, thus causing a more polarized emission. This polarization, or the binding event, can be reflected by an increase in the measured anisotropy value. Traditional binding assays like ELISA require multiple steps and complex procedures. With fluorescence anisotropy, the mixing of the binding probe and the target is the only step needed. Anisotropy measurement can then be performed directly on the sample mixture and the result will clearly show whether
there is binding between the probe and the target. Compared to techniques such as surface plasmon resonance (SPR) and total internal reflection fluorescence (TIRF), fluorescence anisotropy is insensitive to changes of the refractive index of the sample solution. Compared to other fluorescence based techniques for biomolecular interaction study, fluorescence anisotropy requires only one fluorescent dye molecule on the probe. Because all that is needed to give a signal change in anisotropy is simply the change in the size of the molecule linked to the dye, there is no need to worry about whether the conformational change of the probe after the binding can give a signal, such as in molecular beacon techniques. Furthermore, because fluorescence anisotropy is a rationing technique, some problems associated with fluorescence intensity techniques, such as photobleaching, nonuniform illumination and unstable light source, are not of major concern.

Applications of fluorescence anisotropy technique are not limited to protein detection. Fluorescence anisotropy has also been used to study biomolecular interactions in order to understand some biological processes [19]. Although in a lot of cases, fluorescence anisotropy is used to study protein-DNA interactions, it is also suitable for protein-protein [20], DNA-DNA and other type of interactions. Theoretically, as long as there is a molecular weight change after a binding process, there should be an anisotropy change for the fluorescent molecule. This is not limited to small fluorescent molecules binding to large ones. Some work was done using fluorescent protein molecules to study protein-DNA interaction [21].
CHAPTER 2
MOLECULAR APTAMER FOR REAL-TIME PDGF DETECTION USING FLUORESCENCE ANISOTROPY

Despite the presence of current techniques for protein detection, there is still few nonisotropic and sensitive methods for real time protein analysis in homogeneous solutions. In this part of our work, we have developed an easy and effective way to specifically analyze PDGF due to its potential significance in cancer research.

Experimental Section

Materials

The fluorescein-labeled PDGF aptamer was customer-designed and then synthesized by Trilink Biotechnologies (San Diego, CA). The sequence of the aptamer is 5’-fluorescein-CAGGC TACGG CACGT AGAGC ATCAC CATGA TCCTG. Recombinant human PDGF-BB, PDGF-AB, and PDGF-AA were purchased from R&D Systems (Minneapolis, MN). They were dissolved in 4 mM HCl and then diluted in a Tris buffer before use. Other recombinant human growth factors, epidermal growth factor (EGF), and insulin-like growth factor 1 (IGF-1), were bought from Roche (Indianapolis, IN). Human bovine serum albumin (BSA), human hemoglobin (HEM), porcine lactic dehydrogenase (LDH), horse myoglobin (MYO), chicken lysozyme (LYS), and human γ-thrombin (THR) and other chemicals were from Sigma (St. Louis, MO). The buffer we used consisted of 20 mM Tris-HCl (pH 7.1), 140 mM NaCl, 5 mM KCl, 1 mM CaCl$_2$, and 1 mM MgCl$_2$ to simulate the ionic strength under physiological conditions. Superpurified water was used to prepare all of the solutions.
Instrumentation

Fluorescence measurements were performed on a Fluorolog-Tau-3 spectrofluorometer (Jobin Yvon, Inc., Edison, NJ) equipped with a thermostat accurate to 0.1 °C. All experiments were carried out at 37 °C unless otherwise specified. The sample cell was a 100-µL cuvette. The fluorescence intensity of the aptamer was monitored by exciting the sample (fluorescein label) at 470 nm and measuring the emission at 520 nm. Slits for both the excitation and emission were set to 10 nm. Corrections were also made for potential effects on sample concentrations caused by dilutions in the titration experiments. To achieve a better analytical sensitivity of PDGF by the anisotropy measurement, the emission monochromator box in the spectrofluorometer was removed. A 515-nm long-pass filter (Oriel, Stratford, CT) and a 525-nm bandpass filter (Chroma, Brattleboro, VT) were put in front of the PMT to select the desired fluorescence signal from the polarizer. This increased the optical signal collection efficiency, as compared with the spectrometer’s original optical path.

Anisotropy Measurements

Fluorescence anisotropy was measured using the L-format configuration (Figure 2-1). The anisotropy \( r \) is calculated according to the following equation:

\[
r = \frac{(I_{VV} - G I_{VH})}{(I_{VV} + 2G I_{VH})}
\]

where the subscripts V and H refer to the orientation (vertical or horizontal) of the polarizers for the intensity measurements, with the first subscript indicating the position of the excitation polarizer and the second for the emission polarizer. G is the G-factor of the spectrofluorometer, which is calculated as \( G = I_{HV}/I_{HH} \). G-factor represents the ratio of the sensitivities of the detection system for vertically and horizontally polarized light.
G-factor is dependent on the emission wavelength and can be determined using a standard sample with known anisotropy.

![Diagram of L-format measurements of fluorescence anisotropy](image)

**Figure 2-1 Schematic diagram for L-format measurements of fluorescence anisotropy**

In all of our experiments, each anisotropy data point was the average of six measurements with an integration time of 1 second. The relative standard deviation was <2% for all measurements.

**Results and Discussions**

**Design of an Fluorescent Aptamer Probe**

High-affinity aptamers for PDGF-B chain have been developed to inhibit binding of PDGF-BB to its receptor [17]. The consensus secondary structure motif of the PDGF aptamers is a three-way helix junction with a conserved single-stranded loop at the branch point.

The helix junction domain of the aptamer represents the core of the structural motif required for high-affinity binding. The binding of the aptamers has been studied by radiolabeling an aptamer using a nitrocellulose filter-binding method. To construct a
fluorescent probe for PDGF-BB based on the aptamers, we adopted a 35-base single-
stranded DNA sequence similar to the reported 36-base aptamer [17]. The 5’ end of the
sequence was covalently linked to a fluorescein molecule as a fluorescence reporter. The
structure of the aptamer is shown in Figure 2-2.

![Figure 2-2 Structure of the fluorescein-labeled aptamer probe.](image)

The fluorescein label is far away from the helix junction, which is the binding
center for PDGF-B [17]. So it will not affect the binding affinity of the aptamer. Our gel
electrophoresis result clearly showed the effective binding of the dye-labeled aptamer to
PDGF-BB.

**Binding of the Aptamer to PDGF**

The binding between the aptamer and the PDGF-BB molecule was confirmed by
gel electrophoresis (Figure 2-3). The experiment was done on a 4-20% precast gradient
polyacrylamide gel (Bio-Rad, Hercules, CA). A running buffer of 0.5× TB (Bio-Rad) was
used. The gel was prerun at 60 V for 10 min, and then samples were introduced and run
at 150 V for 70 min. Fluorescence images were taken with a Kodak DC290 digital camera (Eastman Kodak, Rochester, NY) and a UV illuminator. On the gel image, the right lane contained the fluorescein-labeled aptamer and resulted in only one bright band. The left lane, which had dye-labeled aptamer and unlabeled PDGF-BB, showed two bands. The weaker band appeared at the same position as the bright band on the right lane, indicating the unbound aptamer. The brighter band on the left lane moved much slower than the aptamer band, which reveals the binding complex of the aptamer to the protein. With the ratio of the aptamer:PDGF being 1:10, only a very small fraction of the aptamer was unbound.

Figure 2-3 Gel electrophoresis of the aptamer and its binding complex with PDGF. The sample injected to the right lane is 0.1 µM aptamer, and that in the left lane was the mixture of 0.1 µM aptamer and 1.0 µM PDGF.

**Real-Time Binding Detection Using Fluorescence Anisotropy**

The fluorescein-labeled aptamer is a relatively small molecule compared to PDGF-BB molecule (M.W.=25000). When the aptamer binds to the protein, the increase in overall size and molecular weight will greatly slow down the rotation of the fluorescein
molecule. The slower rotational diffusion will result in a lower ability of the fluorescein molecule to depolarize the incident polarized excitation. Consequently, the anisotropy of the emission will also increase to reflect the slower motion of the dye itself. Based on this, we believe the fluorescence anisotropy should be a simple and reliable method for studying the interaction between aptamer and PDGF molecules, and for detecting the protein as well. Our anisotropy measurement showed a significant increase in anisotropy with the addition of PDGF-BB to the aptamer solution (Figure 2-4).

Figure 2-4 Anisotropy change upon the binding of PDGF to the aptamer. The concentration of the aptamer is 0.1 µM. A 1.0 µM PDGF solution was added at time 0 s. The time resolution for the data collecting was 3.3 s.

As shown in this figure, with the addition of PDGF-BB, the anisotropy increased more than 2-fold. In a time period of about 3 seconds, anisotropy increase was observed and the anisotropy value remained stable after that, meaning that the binding between the aptamer and the PDGF was fast and stable. Control experiments were conducted with just fluorescein dye solutions and PDGF under the same conditions as above. No anisotropy change was observed, confirming that the anisotropy increase in the aptamer experiment
was really due to the interaction between the aptamer and the protein. All measurements were done in real-time; no pre-separation was needed, and no need to label the target protein. It should be mentioned that after the binding, the overall fluorescence intensity decreased about 30%. Since there was no intensity decrease in the fluorescein dye and PDGF control experiment, we concluded that this intensity decrease was due to the formation of the binding complex, which brought the dye molecule and protein close to each other. The oligo bases may also have an effect if they were closer to the dye after the binding.

**Effects of Mg\textsuperscript{2+} on the Binding Assay**

Metal ions in the solution often have a significant impact on the binding of a single-stranded DNA (ssDNA) to a protein. A series of experiments designed to study the effects of Mg\textsuperscript{2+} ions on the binding between the aptamer and PDGF molecules were carried out. The concentration of Mg\textsuperscript{2+} was varied in a solution with only the fluorescein-labeled aptamer and also in a solution with same amount of aptamer and excess PDGF-BB molecules. The anisotropies of the solutions were measured at each Mg\textsuperscript{2+} concentration and results are shown in Figure 2-5.

The results showed that the anisotropy of the fluorescein-labeled aptamer increased significantly as Mg\textsuperscript{2+} concentration increased. This is because the divalent metal ions stabilize the three-way helix structure of the free aptamer [17], thus hindering the rotational rate of the labeled fluorophore linked to the aptamer.

In the case of aptamer/PDGF binding complex, the Mg\textsuperscript{2+} had a very small effect on the anisotropy until its concentration reached 2 mM. With higher Mg\textsuperscript{2+} concentrations, the anisotropy of the solution began to decrease, which might be the result of the effects of high ionic strength on the conformation of the protein.
Figure 2-5 Effects of Mg$^{2+}$ concentration on sample’s anisotropy. (A) 0.1 µM aptamer in buffer; (B) 0.1 µM aptamer and 1.0 µM PDGF-BB in buffer.

The combination of the two effects of Mg$^{2+}$ is shown in Figure 2-6. It shows that at different Mg$^{2+}$ concentration, the anisotropy change between the aptamer and the aptamer/PDGF binding complex is different. Higher Mg$^{2+}$ concentration results in a smaller anisotropy change. A larger anisotropy change is desirable when detecting protein. However, in order to study the ability of our method to detect PDGF-BB under physiological conditions, we still chose to use a buffer with 1 mM Mg$^{2+}$. 
Figure 2-6 Effect of MgCl$_2$ on the aptamer/PDGF binding. The concentrations of the aptamer and PDGF were $0.1 \mu$M and $1.0 \mu$M respectively.

**Effect of Temperature on the Binding Assay**

Temperature also has its effect on a fluorescent molecule’s anisotropy. As temperature increases, the rotation ability of a molecule will increase and the viscosity of the surrounding solvent will decrease. Both factors contribute to a lower fluorescence anisotropy. Since temperature will have an effect on the anisotropies of both aptamer and the aptamer/PDGF binding complex, we did experiments to find out what the overall effect would be on this binding process. Experiments were carried out in a similar manner as that in the study of Mg$^{2+}$ effect. Anisotropies of both an aptamer solution and an aptamer/PDGF binding complex solution were measured at different temperatures, as shown in Figure 2-7. Even though higher temperature decreased the anisotropies of both aptamer and the aptamer/PDGF binding complex (data not shown), a gradual increase in the anisotropy difference between the two species was observed. However, at 40 °C,
there was a significant decrease in the anisotropy of the binding complex (data not shown), which contributed to the sharp decrease at 40 °C in Figure 2-7.

![Figure 2-7](image)

**Figure 2-7** Effect of temperature on the anisotropy change during the aptamer/PDGF-BB binding process. Solutions contained 0.1 µM aptamer and 1.0 µM PDGF-BB in buffer.

This may indicate that at this temperature, the three-way helix junction structure of the aptamer, which is essential to the aptamer/ PDGF binding, is much less stable. As a result, more aptamer molecules became unbound, and the anisotropy decreased. In fact, 40 °C is close to the reported melting temperature of the aptamer (about 44 °C), at which the aptamer changes from the folded structure to an unfolded one [17]. This result also shows that fluorescence anisotropy is not just a detection technique. It can also be a useful tool for studying and understanding the conformational changes of molecules in some biological processes.

**Detection of PDGF-BB in Homogeneous Solution**

To test if the fluorescence anisotropy method is a practical way to quantitatively detect PDGF-BB in homogeneous solution, we carried out a series of titration experiments to construct a calibration curve. The experiments were performed by adding
aliquots of PDGF-BB stock solution to an aptamer solution. Potential dilution effects on sample concentrations during the titration experiments were corrected. A control experiment was also conducted using exactly the same procedures and conditions as in the PDGF titration experiments, with the only difference being that we used the buffer alone to do the titration instead of the PDGF-BB stock solution. The results are shown in Figure 2-8.

![Figure 2-8 Titration of the aptamer with PDGF solutions in the concentration range of 0-1.0 µM; blank buffer. The aptamer concentration is 0.1 µM.](image)

The control experiment showed a stable anisotropy value and no increase, meaning that volume increase or aptamer concentration decrease does not affect the anisotropy of the aptamer. With the addition of PDGF-BB, the anisotropy of the aptamer solution greatly increased, indicating the binding between the aptamer and the PDGF-BB.

This increase, however, is not linear to the PDGF concentration. This is because the measured anisotropy of a sample at a certain wavelength is actually the average anisotropy of all the components in this sample that have emission at this wavelength.
For example, for a solution containing a dye-labeled aptamer and its target protein, if the binding is a 1:1 ratio reaction, there will be only two species that may have fluorescence anisotropy: the aptamer itself and the aptamer/protein binding complex. And the anisotropy of this solution \( (r) \) can be calculated using the following equation:

\[
r = (1-\chi) \cdot r_{\text{aptamer}} + \chi \cdot r_{\text{complex}} \quad \text{(Equation 1)}
\]

where \( r_{\text{aptamer}} \) and \( r_{\text{complex}} \) are the anisotropies of pure aptamer and pure binding complex respectively, and \( \chi \) is the fraction of the total aptamer that is bound to the protein to form the binding complex. It is clear that the anisotropy of such a system is related to the aptamer/protein concentration ratio and the constant of the binding reaction. In a more complex system, more components need to be considered in the above equation. For PDGF-BB and its aptamer, the titration curve we obtained (Figure 2-8) indicated that the interaction between aptamer and PDGF-BB might not be monophasic, but rather biphasic, as there seems to be two plateaus in the curve. This is in agreement with the previous report that studied the aptamer/PDGF binding using radioisotropic technique [17]. The reason for this biphasic binding is proposed to be the coexistence of two noninterconverting components of the aptamer that bind to the PDGF with different affinities.

Despite the biphasic curve in a range of PDGF-BB concentration up to 1 µM, at lower PDGF-BB concentration (0-100 nM) there is a good linear relationship between the anisotropy and the amount of PDGF-BB. Clinical studies showed that the concentration of PDGF is about 0.4-0.7 nM in human serum and 0.008-0.04 nM in human plasma [4-6]. However, the PDGF concentration in the local tumor area should be higher than that in the blood as the PDGF has not diffused into blood. To demonstrate that our method is
capable of detecting PDGF in that concentration range, we made some modifications to our experiments. Before modification, our detection limit was ~2 nM of PDGF-BB. We first removed the monochromator box on the emission side of the spectrofluorometer and put a band-pass filter directly between the sample and the PMT detector. This resulted in a broader emission wavelength range, a shorter optical path, and a more efficient optical signal collection. The second thing we changed was using an initial aptamer concentration of 2 nM, instead of 0.1 µM we used before. According to Equation 1 and the principles behind it, the anisotropy change caused by the addition of PDGF is not related solely to the PDGF concentration. It also depends on the initial aptamer concentration. The lower aptamer concentration, the less PDGF is needed to achieve a similar level of anisotropy change. Because of this reason, as long as our detection system is sensitive enough to give sufficient signal for 2 nM aptamer, we should be able to detect a PDGF-BB concentration much lower than 2 nM. We carried out a series of experiments with the modifications mentioned above, and obtained a linear curve in a lower PDGF-BB concentration range (Figure 2-9). The sensitivity of our method was greatly improved as indicated in the figure. The detection limit was calculated to be ~0.22 nM of PDGF-BB when 2 nM fluorescein-labeled aptamer was used in the binding assay. We believe this detection limit will be feasible for PDGF detection in blood serum samples and in local tumor fluid samples. But in order to be useful for PDGF detection in clinical blood plasma sample, our method still needs to be improved. Possible approaches could be optimization of system design and optical detection, using a dye with higher fluorescence intensity and using a better light source, such as a laser.
Figure 2-9 Titration of the aptamer with PDGF solutions in the concentration range of 0-1.25 nM. The aptamer concentration is 0.2 nM.

**Selectivity of the Aptamer Probe**

It is essential for a protein probe to have the ability to distinguish between its target protein and other proteins in order to be of practical use. While the aptamer sequence has been reported to be highly selective for PDGF by other techniques, it is still important for us to test the selectivity of the probe using fluorescence anisotropy. We need to show that the fluorescent label and the anisotropy method do not affect in any way the selectivity of the aptamer probe for PDGF. We carried out a series of experiments where excess of several common extracellular proteins, such as albumin, hemoglobin, myoglobin and lysozyme, etc., were added to aptamer solutions and the anisotropy changes were recorded. These changes were then compared to the anisotropy change caused by PDGF. The result is shown in Figure 2-10. It is clear that compared to PDGF, at similar concentrations, other proteins showed no or little anisotropy increase due to the aptamer’s specificity for PDGF.
Figure 2-10 Binding selectivity of the aptamer. Different extracellular proteins were compared with PDGF-B chain (PDGF) in their capability to change the aptamer’s anisotropy. The 5 fold proteins (moles) are added into 0.02 μM aptamer solution at 25°C.

Other experiments were done to compare the affinities of the aptamer to PDGF-BB and to other growth factors which may coexist with PDGF-BB in clinical samples. As shown in Figure 2-11, the aptamer did not bind to epidermal growth factor (EGF), or insulin-like growth factor-I (IGF1), indicating that the aptamer is highly selective for PDGF-BB. The other two isoforms of PDGF, PDGF-AA and PDGF-AB, however, showed some anisotropy increase. But their affinities are much lower than that of PDGF-BB. The PDGF A chain and B chain have 60% similarity in their amino acid sequence [22]. This may explain the anisotropy change caused by PDGF-AA and PAGF-AB. The fact that the A chain is more acidic than B chain, however, may be the reason for its lower affinity to the aptamer.
Conclusions

In this part of our work, we have developed a molecular aptamer for real-time detection of the oncoprotein PDGF in homogeneous solution using a fluorescence anisotropy method. A fluorescein-labeled PDGF aptamer was designed and used as a probe to observe its anisotropy increase upon binding to its target protein. The significant increase in anisotropy was attributed to the large difference in molecular size between the free fluorescein-labeled aptamer and its PDGF binding complex. This difference is significant enough to allow the molecular binding to be quantified for protein detection in real-time. The assay is highly selective and can detect PDGF down to 0.22 nM. The assay is quick and can detect PDGF without separation. Anisotropy measurements are ideally suited for measuring the binding of small aptamer probes with protein macromolecules. This work demonstrates the potential applications of dye-labeled aptamers for oncoprotein and disease-related protein detection in clinical studies. This assay can be used in a noncompetitive homogeneous assay format. The same assay concept can also be
used for biosensors by immobilization of the aptamer onto a solid surface for in vivo or in vitro protein monitoring [23]. Recently, the in vivo instability of aptamers in a biological fluid containing nucleases has been circumvented by chemical modification of the bases, particularly by substitutions at the 2’ position of the sugar [24], allowing aptamers to function adequately in biological fluids. This will ensure the application of aptamer-based analytical methods in real biological samples.
CHAPTER 3
DEVELOPMENT OF FLUORESCENCE ANISOTROPY IMAGING SYSTEM FOR PROTEIN ARRAYS

As databases for many sequenced genomes have been built, people begin to realize it is time to move to the next level: to understand more about proteins and their functions in life. Whereas genes contain the information for life, the encoded proteins and RNAs fulfill nearly all the functions, from replication to regulation. It is well recognized that the complexity of the human proteome far exceeds that of the genome. The number of different molecular protein species in the human body is likely to be at least 500,000. To be able to deal with such a large amount of proteins, it is very important to have new techniques that can realize protein analysis with high throughput. Not long ago, the term “proteomics” was proposed to define the large-scale study of the proteins expressed by a genome. Although microarray technology has enabled rapid development of genomics, it is not as easy, if not much more difficult, to apply similar techniques to proteomics. In this part of our work, we try to develop a novel technique based on anisotropy measurements using imaging technique, which has the potential to realize high throughput and multiplex protein analysis.

Protein Arrays

Protein analysis has been done using a variety of techniques in small scale. They include techniques based on probe-protein binding, such as enzyme-linked immunosorbent assay (ELISA), and techniques based on protein separations, such as gel electrophoresis. Those techniques have showed good sensitivity and selectivity in protein
analysis, but in order to do large-scale protein studies, they still need to be modified or improved. A recent approach is the combination of 2-dimentional gel electrophoresis and mass spectrometry. This enables the analysis of multiple proteins in a short period of time. However, it also has some major drawbacks. First, it is a destructive technique, meaning you will lose some of your protein samples. Second, it may not be able to collect and analyze all the protein species if they are expressed at low abundance. It can not provide reliable quantitative results. Lastly, while it is effective in separating and isolating individual proteins, it does not yield much information about the interactions between proteins and other biomolecules in real-time.

Protein arrays, on the other hand, have been rapidly developed to address some of the problems faced by other techniques. Protein arrays are solid-phase ligand binding assay systems using immobilized proteins on surfaces which include glass, membranes, microtiter wells, mass spectrometer plates, and beads or other particles. The assays are highly parallel and often miniaturized. Their advantages include being rapid and automatable, capable of high sensitivity, economical on reagents, and yielding a lot of data for a single experiment. Protein array is expected to be a very important technology in proteomics thanks to its ability to make possible high throughput protein detections as well as parallel multiplex screening of interactions between multiple proteins and other biomolecules. Some work has demonstrated extraordinary power of protein chips to analyze thousands of proteins at the same time [25]. The capture ligands used in protein arrays are often antibodies, but may also be proteins, enzyme-substrates, receptor-ligands and aptamers [26]. Compared to antibodies, aptamers have the advantages of ease of
production by automated oligonucleotide synthesis, robust nature of the nucleic acids, and easy modification for expanded applications.

Even though it has a great potential and some successful applications, the protein array technique is still facing some challenges. Traditional protein arrays, or protein chips, when compared to DNA arrays, have some major drawbacks due to protein’s inherent properties. While DNA molecules are stable even in some harsh experimental conditions, proteins are much more fragile and very sensitive to their surrounding environment and will be easily damaged if not treated carefully. DNA molecules can be readily immobilized onto several kinds of surfaces without much loss of biological functions; proteins are much easier to be denatured when close to a solid surface, which will result in the loss of their abilities to react with their ligands. In traditional protein arrays where the detection system is based on fluorescent intensity or other intensity measurements, the immobilization of the capture ligand is inevitable. People usually need to immobilize the ligands on a chip, and then incubate the chip with the analytes. After the target protein in the sample binds to the ligands on the chip surface, all the unbound molecules need to be washed away. The protein on the chip will then be stained with a stain reagent. Finally the stain generates intensity signals on the detection system, which indicates the presence of the target protein. All these procedures are the result of the detection methods adopted by the array system. In our work, a new detection scheme has been developed for protein arrays based on fluorescence anisotropy which does not require the ligand immobilization and is very simple to operate. This will also allow us to circumvent the problems associated with denaturation of proteins, either the ligand or the target, when immobilized to the solid surface.
Fluorescence Anisotropy Imaging

As described in Chapter 1, fluorescence anisotropy is a unique technique that has many distinctive features. Unlike intensity based fluorescence techniques, the excitation in fluorescence anisotropy is a plane-polarized light. Depending on the size of the fluorescent molecules in the sample or the viscosity of the sample solution, the emission will have different intensities at two polarization planes, one parallel to the excitation and the other perpendicular to it. The difference is measured and calculated using following equation:

\[ r = \frac{I_{VV} - I_{VH}}{I_{VV} + 2I_{VH}} \]

where the subscripts V and H refer to the orientation (vertical or horizontal) of the polarizers for the intensity measurements, with the first subscript indicating the position of the excitation polarizer and the second for the emission polarizer. It is important to know that in a real-world measurement, the detection system may have different sensitivity to polarized light at different directions. So the actual equation used for anisotropy calculation is:

\[ r = \frac{I_{VV} - G I_{VH}}{I_{VV} + 2G I_{VH}} \]

where G is the G-factor of the detection system, which is calculated as \( G = I_{HV}/I_{HH} \). G-factor represents the ratio of the sensitivities of the detection system for vertically and horizontally polarized light. G-factor is dependent on the emission wavelength and can be determined using a standard sample with known anisotropy.

For a fluorophore-labeled probe, after it binds to its target protein, the increase of the overall weight of the binding complex will greatly decrease the fluorophore’s ability to rotate and result in a higher fluorescence anisotropy value. Intensity-based techniques can not tell the difference after the binding because only the total emission of the
fluorophore is measured. However, an anisotropy system can easily tell if there is a binding process even if the total emissions of the bound and unbound fluorophores are about the same. Most of the current protein arrays require a long process of immobilization, incubation, washing and staining, in order to see the binding between the ligand and protein. By using fluorescence anisotropy, on the other hand, we can detect the binding event as soon as the ligand and the protein are mixed together. This method also has the potential to detect not only the DNA-protein interaction but also protein-protein and RNA-protein interactions.

Fluorescence anisotropy measurements are mostly done in fluorometers, where the type of the sample container is fixed and simultaneous multiple sample measurements cannot be easily implemented. The idea presented here is that if we could use a plate with wells on it that have different samples in them and then construct an anisotropy image of the plate, we should be able to look at samples with different anisotropies within one image. It has been reported that an anisotropy imaging method has been developed to study single molecules [27]. However, it was done with a very limited scale of samples, and should not be suitable for applications like protein arrays. Other work has been done to study protein interactions and detect proteins with high throughput using fluorescence polarization (FP) [28]. It was based on a similar method to fluorescence anisotropy and utilized a plate reader to measure samples one by one, instead of using one-time imaging system to measure all samples at the same time. In this part of our work, we have developed a system that can take anisotropy images of fluorescent samples and make it a potentially better detection method for protein arrays.
In order to obtain anisotropy images, two polarizers need to be coupled to an imaging system. One is the excitation polarizer that provides a polarized light source. Another is a polarizer on the emission side. While the excitation polarizer should be fixed in position, the emission polarizer need to placed in a way so that we can change its position to get two polarization planes, one parallel to the polarization plane of excitation light and the other perpendicular to it. By changing the position of the emission polarizer, two images can be obtained, on which each pixel does not show the real emission of the sample at that position, but rather $I_{VV}$ or $I_{VH}$ of the total emission. An image processing software will be used to calculate the two images at pixel level to get an anisotropy image. The value of each pixel on this anisotropy image represents the anisotropy of the sample at that spot.

**Experimental Section**

**Materials**

The carboxytetramethylrhodamine (TAMRA)-labeled PDGF aptamer was customer-designed and then synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The sequence of the aptamer is 5’- CAGGC TACGG CACGT AGAGC ATCAC CATGA TCCTG -3’- TAMRA. A TMARA-labeled human α-thrombin aptamer, with a sequence of 5’- TTTGG TTGGT GT GGT TGGT -3’- TAMRA, and another TAMRA-labeled oligonucleotide with a random sequence of 5’- CGGTA GTACC AAGTC CAGGT -3’- TAMRA as a control DNA were also synthesized by Integrated DNA Technologies, Inc. Recombinant human PDGF-BB, PDGF-AB, and PDGF-AA were purchased from R&D Systems (Minneapolis, MN) and they were dissolved in 4 mM HCl and then diluted in a Tris buffer before use. Human bovine serum albumin (BSA) and epidermal growth factor (EGF) were bought from Roche (Indianapolis, IN).
Other chemicals were from Sigma (St. Louis, MO). Buffer used in all experiments consisted of 10 mM Tris-HCl (pH 7.5), 75 mM NaCl, and 2.5 mM KCl. Superpurified water was used to prepare all of the solutions. All experiments were done at room temperature.

**Anisotropy Imaging Setup**

A schematic diagram of the anisotropy imaging system is shown in Figure 3-1. An intensified charge-coupled device (ICCD) (Roper Scientific, EEV 512 × 1024 FT) was used to capture all images. A home-made cylindrical polarizer holder was placed under the ICCD. There was a 90° slit on the outer surface of the holder around the axis of the cylinder through which we could rotate a polarizer fixed inside the holder. Rotation of the polarizer from on end of the slit to the other could change between two emission polarization planes perpendicular to each other. A TV zoom lens from Edmund Industrial Optics (Barrington, NJ) was also placed beneath the ICCD to help look at a relatively large area. A mercury lamp by Olympus America Inc. (Melville, NY) was used as the light source. The light coming out of the lamp was guided by a fiber bundle to the sample plate. A polarizer was placed just before the outlet of the fiber bundle as the excitation polarizer. All polarizers were from Edmund Industrial Optics. A 520-550 nm bandpass filter from Olympus America Inc. was used for excitation. A 590 nm longpass filter (from Olympus America Inc.) and a 600 nm shortpass filter from Oriel Instruments (Stratford, CT) were used for emission.

The sample plate was made of black plastic (DELRIN acetal resin) with dimensions of 20 mm×20 mm×2 mm. A 6×6 array of small wells was mechanically made on the plate. Each well is 1.5 mm in diameter and 0.75 mm deep, and it holds about 1.5 µL of liquid sample.
A Fluorolog-Tau-3 spectrofluorometer (Jobin Yvon, Inc., Edison, NJ) was also used to perform some fluorescence anisotropy measurements. In those cases, a 50 µL quartz cuvette was used to hold the sample solution. Excitation and emission wavelength were set to be 545 nm and 595 nm respectively for TAMRA. Bandpasses for both the excitation and emission were set to 10 nm.

![Figure 3-1 Schematic diagram of the anisotropy imaging system. An ICCD with a zoom lens is the detector. A fiber bundle coupled to a mercury lamp provides the excitation light.](image)

**Acquisitions of Anisotropy Images**

Alignment of polarizers is done as following. A sample of very high anisotropy, such as a TAMRA dye solution with 80% (V/V) glycerol is used. First, the emission polarizer is fixed at one end of the 90° slit on the polarizer holder. Then the position of the excitation polarizer is adjusted until the lowest fluorescent signal is observed. The current position of the emission polarization should be perpendicular to that of the
excitation polarization, which will only allow the $I_{VH}$ signal of the sample to reach the detector. With rotation of the emission polarizer for 90°, the $I_{VV}$ component of the emission will then be collected by the detector. Same approach was also reported by other group [29]. By using ICCD as the detector, two images can be obtained, with one containing the $I_{VV}$ part of the emission and the other containing the $I_{VH}$ part. We may call them Image $I_{VV}$ and Image $I_{VH}$, or $I_{IVV}$ and $I_{IVH}$ respectively.

1.5 µL of sample solution is added to the well on the sample plate. With the excitation on, we obtain two images of the sample, $I_{IVV}$ and $I_{IVH}$, by rotating the emission polarizer. The exposure time is the same for the two polarization images of the same sample, but may vary from 0.05 sec to 3 sec from sample to sample depending on the sample intensity. The image capturing software is Winview (Roper Scientific).

Data processing is done using a Java-based computer program called ImageJ (http://rsb.info.nih.gov/ij/index.html). It was developed by the Research Services Branch at National Institute of Mental Health and National Institute of Neurological Disorders and Stroke. It has many image processing functions and allows you to develop plugins for your own image processing needs. We developed two plugins for the anisotropy imaging experiments. One plugin is used to clear the background area of the image $I_{IVV}$ and $I_{IVH}$ to zero. This is needed because the background noise on the two images is low and highly fluctuating. If it were not set to zero, on the final anisotropy image, the background would show very random anisotropies, from very low to very high. The real anisotropy signal from the wells would be buried in this background. Another plugin we developed is used to directly calculate an anisotropy image $I_r$ with image $I_{IVV}$ and $I_{IVH}$ and a user-specified G-factor using the following equation:
\[ I_r = \frac{(I_{IVV} - G \cdot I_{IVH})}{(I_{IVV} + 2G \cdot I_{IVH})} \]

In the anisotropy image, the value of each pixel represents the fluorescence anisotropy of the sample at that position. To get quantitative results from the anisotropy image, the value of the pixels within the area of one sample is averaged and the average value is used to represent the actual anisotropy of the sample. The final anisotropy image might be rescaled or cut off of some trivial areas that are not related to the real signal for the sake of better presentation of data.

In order to get reliable anisotropy values from our anisotropy imaging system, it is important to know the G-factor of the system. However, it is not easy to measure the G-factor directly using the anisotropy imaging system. We solved this problem by correlating the anisotropy values measured by the imaging system to the anisotropies we get from the same sample using a spectrofluorometer that has the anisotropy measurement capability. This spectrofluorometer is able to determine the G-factor by measuring \( I_{HV} \) and \( I_{HH} \), and thus can get relatively accurate anisotropy values for a fluorescent sample. We set the excitation and emission wavelengths on the spectrofluorometer to be similar to those on the anisotropy imaging system so that the two systems should yield similar anisotropy values for the same sample. We then used the spectrofluorometer to determine the fluorescence anisotropies of the free protein-binding ligand solutions. These ligand solutions with known anisotropies were used as references in all experiments conducted on the anisotropy imaging system. G-factors of the system were chosen to make sure the calculated anisotropies for the reference solutions were the same as what we obtained from the spectrofluorometer. In this way,
the results obtained from the anisotropy imaging system should be parallel to those from the spectrofluorometer.

**Results and Discussions**

**Experimental Considerations**

The carboxytetramethylrhodamine (TAMRA) dye was chosen to be the fluorescent label for all the ligands for different proteins. TAMRA is much more stable than some of the other dyes such as fluorescein, which means we will have less severe photobleaching problem. This enables us to use relatively longer exposure time to obtain higher signals. Another advantage of TAMRA is that it can be readily excited by the intense 546 nm spectral line from mercury-arc microscope lamps [30] and often shows brighter intensity than fluorescein.

We use two TAMRA-labeled aptamers as the ligands for two proteins. One of them is the PDGF-aptamer that binds selectively to PDGF-BB protein. It has a 35 base sequence and has a three-way helix junction structure as shown in Figure 2-2. Another aptamer is the aptamer for human $\alpha$-thrombin. It has a 15 base sequence and is believed to fold into a chair-form quadruplex with the 5’ and 3’ ends in the corners of the quadruplex and two stacked G-quartets linked by TT and TGT loops [31,32]. The aptamer was found to bind selectively to human $\alpha$-thrombin with high affinity [33]. Aptamers are ideal for protein binding assay because of its high selectivity and affinity to their target protein. Compared to antibodies, aptamers have the advantages of low cost, easy handling and stable structure. We here use aptamers as the binding ligands for proteins and use fluorescence anisotropy imaging as the detection method since the binding of the aptamer to target protein results in a larger molecular weight and greater anisotropy.
**Anisotropy Imaging of TAMRA-Glycerol Solutions**

To test the ability of our system to detect anisotropies of multiple samples simultaneously, we used TAMRA dye solutions with different glycerol concentrations (V/V). Viscosity of glycerol is much higher than that of pure water. With glycerol concentration increases, the viscosity of the solutions also increases. As the rotational diffusion of the TAMRA dye molecules is restricted by the higher viscosity of the solution, the measured anisotropies of the dye solutions should show an increase. Figure 3-2 shows an anisotropy image of eight TAMRA solutions in eight wells on the sample plate with glycerol concentration ranging from 10% to 80%.

We can see clearly that the difference in anisotropy between two solutions can be seen directly from the image. By comparing the colors of two samples on the same anisotropy image, we can tell the anisotropy difference and the information associated with the difference, such as differences in molecular size and solution viscosity. In the case of TAMRA-glycerol solutions, the anisotropy difference is caused by the difference in the viscosity of the solutions.

On the anisotropy image, pixels within one sample showed a distribution of anisotropy values. It is most likely due to signal fluctuation of each small photosensor on the ICCD during the data acquisition process. However, this distribution is not totally random but rather centered around a certain anisotropy value. To get the quantitative results from the anisotropy image, we averaged the anisotropy values of each pixel within one sample to get the overall anisotropy of the sample. The results are shown in Figure 3-3.
Figure 3-2 Anisotropy image of 200 nM TAMRA solutions with different glycerol concentrations. The table shows concentrations of glycerol (V/V) and the relative positions of the eight solutions. Exposure time was 0.05 sec.

<table>
<thead>
<tr>
<th>Concentration (V/V)</th>
<th>Relative Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>80%</td>
<td>10%</td>
</tr>
<tr>
<td>70%</td>
<td>20%</td>
</tr>
<tr>
<td>60%</td>
<td>30%</td>
</tr>
<tr>
<td>50%</td>
<td>40%</td>
</tr>
</tbody>
</table>

Figure 3-3 Anisotropy vs. glycerol concentration (V/V) in 200 nM TAMRA solutions. Data was averaged from four anisotropy images.

The anisotropy values obtained from the anisotropy imaging system showed quite small error bars, partially due to the high fluorescence of the TAMRA solutions. The anisotropy increases as the glycerol concentration increases. Similar curves were also obtained as a confirmation from the spectrofluorometer, which was supposed to give rather accurate anisotropy measurements. It is worth noting that from the quantitative
curve and the anisotropy image, an anisotropy difference of as little as 0.015 can be seen with ease from the anisotropy image by just comparing colors. The imaging system provides a qualitatively very straightforward and quantitatively rather accurate way to examine multiple samples simultaneously.

**Anisotropy Imaging for Aptamer-Protein Binding Assay**

In our previously work, we used the spectrofluorometer to do all the anisotropy measurements for PDGF and aptamer binding study. Sample solutions of at least 50 µL were used in order to fit in a cuvette or to get enough signals. With the anisotropy imaging system’s ability to conduct multiple anisotropy measurements with very small amount of samples, we should be able to place more than one solution with different aptamer/PDGF ratios in the plate wells and look at the extents of the binding reaction reflected by the anisotropy values in all wells at the same time. This will be quite useful and convenient when it comes to studying protein binding process under different conditions. We carried out some experiments where the anisotropies of a series of solutions with the same aptamer concentration but different PDGF concentrations were determined at one time using anisotropy imaging. An anisotropy image is shown in Figure 3-4. A binding curve was also constructed based on the image, as shown in Figure 3-5.
Figure 3-4 Anisotropy image of eight 50 nM PDGF-aptamer solutions with different PDGF-BB concentrations. The table on the right shows the concentration of the sample in nM at the corresponding position on the anisotropy image.

Figure 3-5 Anisotropy vs. PDGF-BB concentration in 50 nM TAMRA-labeled PDGF-aptamer solutions. Data was averaged from 5 anisotropy images. Exposure time was 3 sec.

Compare the shape of the curve in Figure 3-5 to the curve we obtained previously using the spectrofluorometer (Figure 2-8), we can see that in the PDGF-BB concentration
range of from 0 to $6 \times C_{\text{PDGF-aptamer}}$ (0 to 0.6 µM in Figure 2-8 and 0 to 300 nM in Figure 3-5), these two curves are very much alike. We did not go to higher PDGF concentration here since we just wanted to demonstrate the capability of the anisotropy imaging system in protein binding assay. This curve was also repeatable on the spectrofluorometer.

Compared to the anisotropy image we got from the TAMRA-glycerol solutions, the anisotropy image for aptamer/PDGF binding solutions showed less distinctive colors between samples. This is probably because at a much lower fluorescence intensity level and with a much longer exposure time, the signal fluctuation of the pixels on the ICCD became more severe. Photobleaching of the TAMRA dye at low concentration might also have played a role in this problem. The result of this problem is a much larger anisotropy distribution within one sample on the anisotropy image. This is very much like broadened peaks in chromatography and there is color overlapping between two adjacent samples on the anisotropy image. This is why we see similar colors between samples. However, the color difference is still discernable and the averaged anisotropy values of the samples still showed significant change as PDGF-BB concentration increased as shown in Figure 3-5. Similar reasons can be used to explain the larger error bars in Figure 3-5 than in Figure 3-3.

In a system where we use multiple sample wells and compare signals from different wells for analyte detection, the well-to-well signal variation should be the indicator of the noise of this assay system [28]. The well-to-well anisotropy variation of our system was tested to be <0.002 by measuring the anisotropy of the same sample in multiple wells. To qualify for a real signal, the anisotropy should be 3 times of this deviation, which accounts for an anisotropy difference of 0.006. Applying this to the
curve in Figure 3-5, we determined the detection limit of our anisotropy imaging system for PDGF-BB to be around 13 nM. Since we are detecting a sample of only 1.5 µL in each well, the mole detection limit should be $13 \text{ nM} \times 1.5 \text{ µL} \approx 2.0 \times 10^{-14} \text{ mole} = 20 \text{ fmole}$ of PDGF-BB. This sensitivity can still be improved by optimization of the optical path, changing to a near IR dye with higher fluorescence and less photobleaching, and using high power laser as the light source.

**Selectivity of Aptamer/PDGF Binding on the Anisotropy Imaging System**

We carried out a series of experiments to demonstrate that the selectivity of the aptamer/PDGF-BB binding can be preserved on the anisotropy system. First, 50 nM PDGF-aptamer solutions with 300 nM of different proteins were added to six wells. The resulting anisotropy image is shown in Figure 3-6.

![Anisotropy image of 50 nM PDGF-aptamer solutions with 300 nM different proteins.](image)

The relative anisotropy changes over pure aptamer solution caused by other proteins compared to PDGF-BB are shown in Figure 3-7. We can see that on the
anisotropy imaging system, the PDGF-aptamer still showed very good selectivity for PDGF. At similar concentrations as PDGF-BB, EGF and BSA showed very little or no anisotropy increase while PDGF-AA and PDGF-AB showed some increase which is agreeable with previous result in Figure 2-11.

Figure 3-7 Comparing the binding capability of the PDGF-aptamer to PDGF-BB and to other proteins such as PDGF-AA, PDGF-AB, epidermal growth factor (EGF), and bovine serum albumin (BSA). The aptamer and protein concentrations are 50 nM and 300 nM respectively.

Another experiment was designed to show that only PDGF-aptamer binds to PDGF-BB while other dye-labeled oligonucleotides have little affinity to PDGF-BB. An anisotropy image is shown in Figure 3-8. We used a TAMRA-labeled control DNA with a random sequence and the TAMRA-labeled human α-thrombin aptamer as ligands to bind to the same concentration of PDGF-BB. As shown clearly in Figure 3-8, no anisotropy increase was observed for control DNA/PDGF-BB or thrombin-aptamer/PDGF-BB. Only PDGF-aptamer showed significant anisotropy increase with the addition of PDGF-BB. This shows that aptamers are ideal binding ligands for protein
binding assay. By combining aptamers with fluorescence anisotropy techniques, we should be able to provide an easy and effective way for proteomics studies.

Figure 3-8 Anisotropy image for comparison of binding ability of PDGF-BB to different TAMRA-labeled oligonucleotides [a control DNA sequence (ctrDNA), the human α-thrombin aptamer (tAPT) and PDGF-aptamer (pAPT)]. The first column is 50 nM pure oligonucleotide solutions. The second column is 50 nM oligonucleotides plus 50 nM PDGF-BB. The table on the right shows the samples’ relative locations on the anisotropy image and their anisotropy values.

**Detection of Protein Mixture**

In protein array or protein chip techniques, it is important to be able to not only detect proteins with high throughput but also detect multiple proteins in one sample simultaneously. In order to demonstrate that the anisotropy imaging system can easily realize multiplex protein detection, we conducted a simplified example of the detection of a protein mixture. The anisotropy image of this experiment is shown in Figure 3-9.
Figure 3-9 Anisotropy image of 50 nM different TAMRA-labeled oligonucleotides [a control DNA sequence (ctrDNA), the human $\alpha$-thrombin aptamer (tAPT) and PDGF-aptamer (pAPT)] with different proteins or protein mixture (50 nM human $\alpha$-thrombin, 100 nM PDGF-BB and the mixture of these two). The table shows all samples’ relative locations on the anisotropy image and their anisotropy values.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Anisotropy Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ctrDNA</td>
<td>0.096</td>
</tr>
<tr>
<td>ctrDNA + thrombin</td>
<td>0.090</td>
</tr>
<tr>
<td>ctrDNA + PDGF-BB</td>
<td>0.092</td>
</tr>
<tr>
<td>ctrDNA + mixture</td>
<td>0.096</td>
</tr>
<tr>
<td>pAPT</td>
<td>0.109</td>
</tr>
<tr>
<td>pAPT + thrombin</td>
<td>0.107</td>
</tr>
<tr>
<td>pAPT + PDGF-BB</td>
<td>0.142</td>
</tr>
<tr>
<td>pAPT + mixture</td>
<td>0.129</td>
</tr>
<tr>
<td>tAPT</td>
<td>0.057</td>
</tr>
<tr>
<td>tAPT + thrombin</td>
<td>0.088</td>
</tr>
<tr>
<td>tAPT + PDGF-BB</td>
<td>0.052</td>
</tr>
<tr>
<td>tAPT + mixture</td>
<td>0.081</td>
</tr>
</tbody>
</table>

In this experiment, human $\alpha$-thrombin, PDGF-BB and their mixture were added separately to three TAMRA-labeled oligonucleotide sequences (a control DNA sequence, the thrombin-aptamer and the PDGF-aptamer) and resulting anisotropy image was recorded. It is clear from Figure 3-9 that the control DNA showed little anisotropy
increase when mixed with any of the proteins, indicating there was minimal interaction between the control DNA and the proteins. The thrombin-aptamer showed anisotropy increase with addition of thrombin or thrombin/PDGF-BB mixture, but no increase with addition of PDGF alone, meaning that it was $\alpha$-thrombin that caused the anisotropy increase. Similar results were observed for PDGF-aptamer, which showed anisotropy increase only with PDGF-BB and thrombin/PDGF-BB mixture. This experiment demonstrated that such an array of aptamers can be used to accurately detect proteins in a mixture sample when the concentrations of the proteins are within comparable ranges. A sample that causes the thrombin-aptamer an anisotropy increase, but not other DNA sequences, would probably contain $\alpha$-thrombin but not other proteins. Similar things can be stated for PDGF-aptamer and PDGF-BB. On the other hand, a sample that increases the anisotropies of both thrombin-aptamer and PDGF-aptamer, would probably contain a mixture of both proteins. This is the simplest example of detecting protein mixtures. By using more aptamers or antibodies targeted for more proteins of interest and making a larger array, we can use anisotropy imaging technique to easily detect more proteins in one sample.

**Conclusions**

We have developed a novel technique that has the potential to be used to build a very simple but effective protein array. It uses an imaging system to measure fluorescence anisotropy in a 2-dimensional format, which enables anisotropy measurements of multiple samples in an array format. Due to fluorescence anisotropy technique’s unique ability of effectively detecting protein interactions with other molecules, anisotropy imaging technique has the possibility of making a protein chip much simpler than conventional protein chips. It does not require tedious procedures that
are necessary in most of the current protein chips, such as immobilization, washing and protein staining. At the same time, it has the same ability to realize high throughput and multiplex protein detection which is essential in a wide range of applications in immunodiagnostics, protein function and interaction screening, and drug discovery.

Although we used aptamers as the ligands for proteins, the application of anisotropy imaging is not limited by aptamers. Basically, anything that exhibits a significant molecular weight increase after binding to proteins can be used as a protein ligand in anisotropy imaging, which includes antibody, antigen, DNA, RNA, and protein. This ensures the versatility of anisotropy imaging in a variety of applications.
CHAPTER 4
SUMMARY AND FUTURE WORK

Summary

We have demonstrated the possibility of detecting platelet-derived growth factor (PDGF) using aptamer based fluorescence anisotropy technique. This method has been proved to be highly sensitive, highly selective and very simple. We then extended this method to a 2-dimentional array format for possible high throughput and multiplex protein analysis by developing a fluorescence anisotropy imaging system. This system allowed us to examine multiple aptamer/protein binding samples simultaneously. Very small amount of samples were needed and all measurements could be done in seconds. We also used this system to demonstrate detection of protein mixtures. We believe this anisotropy imaging technique has the potential to help build very simple yet highly efficient protein arrays for various applications.

Future Work

Improving and Refining the Techniques

The fluorescence anisotropy techniques we used in our work can be improved in many aspects. First, we could use a better fluorescent dye such as the Cy5 dye for all our experiments. It has much longer excitation and emission wavelengths than TAMRA. Longer excitation wavelength will cause less photobleaching and sample autofluorescence problem. It also dramatically reduces light scattering in biological samples as light scattering is inversely proportional to the fourth power of wavelength. Since light scattering is the major source of error, this will greatly improve the accuracy
and sensitivity of our anisotropy measurements. We can also use a high power ion laser as the light source for those longer wavelength dyes. Since the power of ion lasers is much higher and more stable than that of the lamp we currently use, a much better sensitivity is expected.

For the anisotropy imaging system, we may need to optimize it for better sensitivity and easier handling. Currently in our system, the fluorescence signals from the sample plate have to travel a quite long distance before reaching the ICCD detector. This contributes to the relatively low optical efficiency and sensitivity of the system. We should be able to improve the sensitivity by using a way to image large area with a much shorter optical path. We may also improve our image processing software to combine background subtraction and image calculation into one simple step, instead of two steps we currently use. In order to be used in real world high throughput protein analysis, the automated sample handling capability and a more miniaturized sample plate should be included in our system.

**Applications of the Anisotropy Imaging System in Cancer Diagnosis**

A cancer cell is a cell that grows out of control. Since growth factors are believed to play a role in intercellular communication and regulating cell growth and division, people are interested in studying the relationship between cancer and growth factors. However, study showed that cancer is often related to the level of not just one growth factor, but rather levels of several growth factors and other related proteins in human body [34]. The ability of our anisotropy imaging system to detect multiple proteins in a mixture may make it useful as a simple and quick cancer diagnostic method. Body fluid samples of patients with no cancer, early stage cancer and late stage cancer can be added to different probe solutions targeted to different cancer related proteins. Levels of those
proteins can then be determined using the anisotropy imaging system. A database can be built in a short period of time for study of the specific cancer.

**Anisotropy Imaging Technique for Real-Time Cell Imaging**

It will be of great interest if one can obtain anisotropy images of fluorescent samples in real-time. Some optical devices have been developed that are able to divide the emission from the sample into two images of different polarization state using a polarization beam splitter. They can cast the two images side by side on a CCD detector. By using new software that allows real-time image calculation within one image, we will be able to construct anisotropy images in real time. This technique may be very useful in monitoring binding processes inside or outside living cells. For example, real-time monitoring of PDGF secretion from a cancer cell may be very difficult for other techniques. Using real-time anisotropy imaging technique, we may have dye-labeled PDGF aptamer in the cell culture medium and monitor any anisotropy change that indicates the release of PDGF from the cell. Further more, we might also be able to monitor PDGF transportation between cells. This might help improve our understanding of intercellular signaling. We believe that the real significant applications of the fluorescence anisotropy imaging technique may be in the field of real-time monitoring of biological processes at cell level. This is a field that is worth more attention and investigation. The experience we gained from our anisotropy imaging experiments might help us a lot in building a system for real-time cell imaging.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Zehui Cao is from Zhuzhou, Hunan Province, China. He spent 18 years in that same province before he traveled almost 1000 miles to Nanjing, a city in eastern China, for college study. He got his B.S. degree in chemistry from Nanjing University in 1998. He spent one more year at the graduate school of Nanjing University studying organic chemistry before he finally realized he was more attracted to control panels than to flasks and beakers. So he traveled again over 10000 miles to University of Florida to study analytical chemistry. He likes what he is doing and would like to pursue a Ph.D. degree after graduation.