CHARACTERIZATION OF A GTP-BINDING DNA APTAMER
CHARACTERIZATION OF A GTP-BINDING DNA APTAMER

By Lawrence Lin
B.Sc., McMaster University, 2009

A thesis submitted to the School of Graduate Studies in partial fulfillment of the degree Master of Science

McMaster University
© Copyright by Lawrence Lin 2011
Master of Science (2011)  McMaster University
(Biochemistry and Biomedical Sciences)  Hamilton, Ontario

TITLE: Characterization of a GTP-binding DNA aptamer
AUTHOR: Lawrence Lin, B.Sc. (McMaster University)
SUPERVISOR: Professor Yingfu Li
NUMBER OF PAGES: ix, 67
ABSTRACT

Aptamers are single-stranded nucleic acids that can bind a wide variety of targets. The target-binding events involve structural changes of an aptamers and have been exploited to create fluorescent reporters. Aptamer-based fluorescent sensors are highly sensitive and selective and can be adapted as signaling components in detection assays. Previously, an aptamer that binds GTP was isolated by in vitro selection from a random-sequence DNA library. Guanosine-5’-triphosphates (GTP) is an important biological cofactor that is widely utilized by many biological receptors and enzymes. Assays that monitor these receptors and enzymes, such as radioactive binding assays using GTP analog $[^{35}\text{S}]$ GTPγS, are not homogeneous and required extensive sample treatments. In contrast, fluorescent reporters made using functional nucleic acids, such as aptamers, are convenient to use and provide real-time detection for a plethora of targets. If the latter detection method could be applied for the detection of GTP, this would provide a more simple and convenient alternative to current methods.

In this thesis, we explore the possibility of designing a novel assay that unifies aptamer characterization with structure-switching. We used this assay to analyze the sequence requirement and recognition specificity of this GTP-binding DNA aptamer. And, we found that binding activity can be retained with up to 40% of nucleotides removed and the sequence identity can be simplified to only guanine and thymine residues. The sequence information about this aptamer can facilitate the development of an efficient fluorescence-signaling aptamer for real-time detection of GTP.
ACKNOWLEDGEMENT

I would like to express my gratitude to my supervisor, Dr. Yingfu Li, for his guidance, insights, and support throughout my graduate study. I would also like to thank my committee members, Drs. John Brennan and Paul Berti, for their support and valuable discussions about my research.

I want to extend my appreciations to all past and present Li lab members, especially Dr. Monsur Ali, Sergio Aguirre, Dr. William Chiuman, Pui Sai Lau, Kha Tram, and Jeffrey Lam, for their stimulating suggestions, encouragements, and assistance during the course of my research.

Lastly, I want to thank Lisa Kush for all her help and organizing all of my meetings.
TABLE OF CONTENT

ABSTRACT iii
ACKNOWLEDGEMENT iv
LIST OF FIGURES vii
LIST OF ABBREVIATIONS ix

CHAPTER 1: INTRODUCTION

1.1 Guanosine-5’-triphosphate (GTP) and G-protein-coupled receptor (GPCR) 1
1.1.1 GPCR drug screening assays 3
1.2 Functional Nucleic acids (FNAs) and In vitro selection 7
1.3 Fluorescent Aptamer-based Sensors
   1.3.1 Aptamers and sensor anatomy 10
   1.3.2 Label-free approach 11
   1.3.3 Single-labeled signaling aptamers 14
   1.3.4 Double-labeled signaling aptamers 15
   1.3.5 Structure-switching signaling aptamers 18
1.4 Applications using Aptamers 20
1.5 Research Objective 25

CHAPTER 2: APTAMER CHARACTERIZATION BY STRUCTURE-SWITCHING

2.1 Introduction 26
2.2 Experimental Section 28
2.3 Results and Discussion
2.3.1 Sequence and properties of the original GTP-binding aptamer 31
2.3.2 Design of duplex assemblies 33
2.3.3 Denaturation profile 33
2.3.4 High temperature enhanced structure-switching 35
2.3.5 Aptamer specificity is not affected by 5’ duplex 38
2.3.6 The miniaturized mutant – G1.5 40
2.3.7 The guanine-thymine-exclusive aptamer 48
2.3.8 GTP titration and pH dependency 53
2.3.9 Conclusion 56

CHAPTER 3: CONCLUDING REMARKS 60

REFERENCES 62
LIST OF FIGURES

CHAPTER 1

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Generic in vitro selection schematic</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>Detection using organic dye</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>Detection using cationic polymer</td>
<td>13</td>
</tr>
<tr>
<td>4</td>
<td>Single-labeled signaling aptamer</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>Aptamer beacon design</td>
<td>17</td>
</tr>
<tr>
<td>6</td>
<td>Self-assemble aptamer design</td>
<td>18</td>
</tr>
<tr>
<td>7</td>
<td>Structure-switching signaling aptamer</td>
<td>19</td>
</tr>
<tr>
<td>8</td>
<td>Aptamer displacement assay</td>
<td>22</td>
</tr>
</tbody>
</table>

CHAPTER 2

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>Sequence of original GTP-binding aptamer and design of duplex assemblies</td>
<td>31</td>
</tr>
<tr>
<td>10</td>
<td>Denaturation profile of duplex assemblies</td>
<td>35</td>
</tr>
<tr>
<td>11</td>
<td>Temperature optimization</td>
<td>37</td>
</tr>
<tr>
<td>12</td>
<td>Specificity analysis of 5’ duplex assembly</td>
<td>39</td>
</tr>
<tr>
<td>13</td>
<td>Sequence for truncation analysis</td>
<td>40</td>
</tr>
<tr>
<td>14</td>
<td>Setup of temperature-switching assay on 96-well plate</td>
<td>42</td>
</tr>
<tr>
<td>15</td>
<td>Plate image of truncation analysis</td>
<td>44</td>
</tr>
<tr>
<td>16</td>
<td>Sequence for substitution analysis</td>
<td>45</td>
</tr>
<tr>
<td>17</td>
<td>Substitution analysis on fluorometer</td>
<td>47</td>
</tr>
<tr>
<td>18</td>
<td>Sequence for nonessential mutation analysis</td>
<td>48</td>
</tr>
<tr>
<td>Figure 19</td>
<td>Nonessential mutation analysis</td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>--------------------------------</td>
<td></td>
</tr>
<tr>
<td>Figure 20</td>
<td>Guanine-thymine exclusive aptamer</td>
<td></td>
</tr>
<tr>
<td>Figure 21</td>
<td>Fluorescence background of active mutants</td>
<td></td>
</tr>
<tr>
<td>Figure 22</td>
<td>Real-time detection of GTP</td>
<td></td>
</tr>
<tr>
<td>Figure 23</td>
<td>Effect of pH on binding activity</td>
<td></td>
</tr>
<tr>
<td>Figure 24</td>
<td>Sequence comparison</td>
<td></td>
</tr>
</tbody>
</table>
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARF</td>
<td>ADP ribosylation factor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5’-triphosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine-5’-monophosphate</td>
</tr>
<tr>
<td>CTP</td>
<td>Cytidine-5’-triphosphate</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine-5’-diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine Exchange Factor</td>
</tr>
<tr>
<td>GMP</td>
<td>Guanosine-5’-monophosphate</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein-coupled receptor</td>
</tr>
<tr>
<td>GRK</td>
<td>G-protein-coupled receptor kinase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine-5’-triphosphate</td>
</tr>
<tr>
<td>HIV-1 RT</td>
<td>Reverse transcriptase of human immunodeficiency virus type 1</td>
</tr>
<tr>
<td>ITP</td>
<td>Inosine-5’-triphosphate</td>
</tr>
<tr>
<td>NTP</td>
<td>Nucleoside-5’-triphosphate</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>SELEX</td>
<td>Systematic Evolution of Ligands by Exponential Enrichment</td>
</tr>
<tr>
<td>UTP</td>
<td>Uridine-5’-triphosphate</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION

1.1 Guanosine-5’-triphosphate (GTP) and G-protein-coupled receptor (GPCR)

Cofactors and metabolites are abundant in biological systems and these small molecules can influence an arsenal of cellular activities including, cell signaling, energy production, macromolecule synthesis, and gene regulations. The proper maintenance in these molecular events contributes to normal cell functions and abnormal regulations can promote the onset of cancer, inflammation, and immune disorders. The molecular regulators in these processes are often carried out by receptors and enzymes, such as G-protein-coupled receptors (GPCR) and protein kinases, and have been regarded as drug targets by pharmaceutical companies. It has been reported that nearly half of current drugs are aimed at GPCRs and this group of receptors represent one of the largest superfamilies in the human genome (Leifert et al 2005). Dysregulation of GPCRs has been linked with diseases such as Alzheimer’s disease, cardiovascular, and cancer (Banks and Harvey 2002). A hallmark of GPCRs is their 7 transmembrane domains and these receptors respond to various excellular stimuli including light, odorants, neurotransmitters, and hormones (Ritter and Hall 2009). GPCRs are associated with G-protein subunits (Gα, Gβ, and Gγ) as a complex and responsible for regulating their activity by catalyzing the exchange of GTP for GDP on its neighbor Gα subunit (Bridges and Lindsley 2008).

The canonical view of GPCR signaling pathway begins with the binding of an external stimulus to the extracellular region or the transmembrane domain of the receptor (Ritter
and Hall 2009). This ligand-binding event induces a conformational change of GPCR that is coupled to its catalytic activity, which triggers the exchange of GDP for GTP on the receptor-bound $G\alpha$ subunit. The activated $G\alpha$ subunit (GTP-bound) then promotes the dissociation of other subunits ($G\beta$, and $G\gamma$) from the receptor and as a result, the liberated G-protein subunits further advances the signaling cascade by activating downstream effectors, such as adenylyl cyclases, phospholipases, and ion channels (Leifert et al. 2005). In essence, GPCRs are involved with the relay of extracellular stimuli to intracellular signaling event, which in turn, govern a wide array of downstream biological events. A more complete coverage of the GPCR signaling pathway has been provided elsewhere (Ritter and Hall 2009; Tesmer 2010).

Structural changes of these receptors are an interesting phenomenon. Biased conformational changes of GPCRs can be induced as a response to the binding of different ligands and invoke the corresponding downstream signaling pathways (Tesmer 2010). Depending on the conformational state of the receptor, GPCRs can respond to other targets such as the GPCR kinase (GRK) (Ritter and Hall 2009; Tesmer 2010). These kinases are responsible for receptor degradation by phosphorylating GPCRs. Upon receptor phosphorylation by GRKs, adaptor proteins and clathrin are recruited, which packages GPCRs into endosomes for internalization. Ultimately, receptor endocytosis leads to the termination of the GPCR signaling pathway (Ritter and Hall 2009). Unfortunately, the phenomenon of how the ligand-binding event influences biased conformational change and the subsequent synchronization with its catalytic activity has
not yet been fully elucidated (Marshall and Congreve 2010). To a large extent, this is due to scarce structural information about GPCRs and as a result, drug development against these receptors remained a challenge (Marshall and Congreve 2010).

1.1.1 GPCR drug screening assays

Despite the limited availability of structural information on GPCRs, numerous GPCR drug screening assays have been developed. The mode of detection of these assays can be broadly classified into four different levels of the GPCR signaling pathway: ligand binding (level I), conformational change of receptor (level II), concentration of GTP bound to Gα subunit (level III), and the dissociation of G-protein subunits from GPCR (level IV) (Leifert et al. 2005). These assays are designed to monitor GPCR activation during the early stages of signaling. However, homogeneous and cell-free assay formats are the most desirable because they are amenable to high-throughput screening, which provide an efficient route to analyze a large chemical library (Leifert et al. 2005). Furthermore, drug discovery tools are transitioning towards automation, miniaturization, improved sensitivity and robustness, and inexpensive and nonradioactive assays and assay reagents (Leifert et al. 2005). The advantages and disadvantages for each level of assays are discussed below.

Level I assays were developed to probe interactions between the receptor and its ligand (Leifert et al. 2005). A variety of techniques can be used to monitor this site of interest such as fluorescence polarization (Banks and Harvey 2002) and flow cytometry (Waller
et al. 2003). Fluorescence polarization assays have the advantages of being less prone to environmental changes, such as pH, and offer high signal-to-noise ratio than standard fluorescence intensity assays (Leifert et al. 2005). In addition, they are homogeneous in nature, which eliminate intermediate steps that required separation of free and bound ligands. Homogeneous assay format are also amenable to high-throughput screening (HTS). These assays relied on ligands modified with chemical moiety (ie. fluorophore) as the source for signal output (Leifert et al. 2005). However, not all ligands are suitable for such modifications and as a result, these methods cannot be adopted for all members of the GPCR family. Waller and colleagues reported a particle-based approach that functionalized dextran beads with a dihydroalprenolol ligand that binds a recombinant protein containing both a β2-adrenergic receptor and a green fluorescent protein (GFP) (Waller et al. 2003). Flow cytometry was then used to determine the binding between ligand and receptor by measuring the fluorescence on the bead surface from GFP.

Unlike level I assays, fluorogenic moieties can be directly attach to the receptor and therefore, level II assays detect conformational changes of GPCRs induced by ligand binding (Leifert et al. 2005). There are several drawbacks of this method; for example, site-specific labeling of fluorophores on the receptor is restricted to conformational changes that occur within the vicinity of the fluorophores. Furthermore, the attachment of fluorophores on the receptor is dependent on the availability of structural information. Very few reported assays from the literature belong to level II.
Alternatively, GPCR activation can also be assessed by monitoring the early stages of the signal transduction cascade, such as the exchange of GDP for GTP. Level III assays are the most common and monitor the activation of Gα subunit, which is activated by GPCR (Leifert et al. 2005). These methods utilize nonhydrolyzable [35S] GTPγS to examine the concentration of GTP that is bound to the Gα subunit. However, when large chemical libraries screening is needed, it may encounter health concerns, such as the safe handling and waste disposal of high volume radioactive materials (Leifert et al. 2005). Under this setting, fluorescent nucleotide analogs may be a better substitute for [35S] GTPγS, which converts the assay into a nonradioactive format (Remmers et al. 1994; Remmers and Neubig 1996). For example, methylisatoic acid can be conjugated at the ribose sugar of GTP to yield N-methylanthaniloyl GTP (MANT-GTP) (Leifert et al. 2005). The guanine nucleotide exchange event can then be monitored based on fluorescence resonance energy transfer (FRET) where tryptophan residues of Gα protein act as the donor and MANT-GTP is the acceptor (Remmers et al. 1994). The detection limit of these fluorescence-based assays is usually in the nM range (Remmers et al. 1994; Remmers and Neubig 1996). In a recent report, a nonradioactive and homogeneous method was developed that utilizes Eu-GTP and detection was based on quenching resonance energy transfer (QRET) (Rozwandowicz-jansen et al. 2010). Limitations of level III assays include varying affinities and compatibility between different Gα proteins when fluorogenic GTP analogs are used and often separation steps are associated with radioactive assay format to partition free GTP and Gα-bound GTP (Leifert et al. 2005).
Level IV assays monitored the assembly and disassembly of the heterotrimeric complex (G-protein:GPCR) as a response of binding between ligand and receptor (Leifert et al. 2005). These assay systems required immobilization of a complex component to a solid support and then the remaining components are added until the full heterotrimeric complex is formed. One example showed the tethering of his-tagged $\beta_1\gamma_2$ subunits to $\mathrm{Ni}^{2+}$-NTA modified dextran beads and as a result, these bead surfaces are capable of binding the $\mathrm{G}\alpha$ subunit, which in turn, bring along a recombinant protein of $\beta_2$-adrenergic receptor and green fluorescent protein (GFP) (Simons et al. 2003). The assembled complex on beads can then be sorted using flow cytometry based on fluorescence that reports the effect of ligand-receptor binding on the assembly and disassembly of these heterotrimeric complexes. Information gathered at this level reflects the downstream effects of ligand-receptor binding rather than simple ligand-receptor interactions (Leifert et al. 2005). The results can potentially enhance our understanding about the structural folding of GPCR and also elucidate novel target sites for drug development. However, these systems may be difficult to set up because care must be taken during the modifications of the N or C termini of the immobilized protein to avoid improper orientation or structural folding. In addition, recombinant receptors may be difficult to express and the size increase can also create steric hindrance with other complex components during assembly.

GPCRs are membrane proteins and its associated production, purification, and manipulations can be expensive and challenging (Leifert et al. 2005). The popularity of
level III assays is attributed to its mode of detection at the Gα subunit level and therefore, assay reagents are more common and convenient to gather. For example, radioactive GTP analogs are commercially available and purified or modified Gα subunits required less sophisticated protocols to obtain in comparison to GPCR (Leifert et al. 2005). Results from level III and IV assays are also more informative because details about downstream signaling events are provided. This has implication when the identity of the ligand needs to define as agonist or antagonist where level I assays cannot differentiate (Leifert et al. 2005).

1.2 Functional Nucleic Acids (FNAs) and In vitro selection

Nucleic acids are long known to play pivotal roles in vast cellular processes necessary to sustain life. It is well understood that DNA safeguards the genetic material in a rigid double-helical structure inside cells. But the genetic alphabets can also orchestrate extraordinary functions in single-stranded nucleic acids such as molecular recognition and catalysis. Nucleic acids with these capabilities are termed functional nucleic acids (FNAs) (Navani and Li 2006; Joyce 2007; Liu et al. 2009). Some examples include DNA or RNA aptamers, DNAzymes, ribozymes, and more recently, riboswitches (Navani and Li 2006; Joyce 2007; Liu et al. 2009; Breaker 2004). In 1982, the Nobel Prize was awarded to Thomas Czech and Sidney Altman for the discovery of the first ribozyme (Kruger et al. 1982; Guerrier-Takada et al. 1983). This revolutionary finding introduced nucleic acids to the realm of biological catalysis. In 1990, the molecular recognition potential of nucleic acids was unveiled by a technique known as in vitro selection or SELEX (Systematic
Evolution of Ligands by Exponential Enrichment) (Ellington and Szostak 1990; Tuerk and Gold 1990).

In vitro selection or SELEX is a selective amplification method that allows the isolation of FNAs through repeated rounds of enrichment (figure 1) (Ellington and Szostak 1990; Tuerk and Gold 1990; Joyce 2007). Typically, a large pool of random DNA or RNA molecules is placed under selection pressure to partition inactive sequences from active sequences and only the isolated sequences are enriched by amplification. Depending on the selection context, various molecular tools are suitable, such as polymerase chain reaction (PCR) and affinity chromatography, to perform amplification, mutation, and selection on nucleic acids (Joyce 2007; Wilson and Szostak 1999). The progress of selection is usually tracked with isotopes and the genotype that dictates the desired function or the phenotype is ultimately revealed upon cloning and sequencing. Details of this technique have been extensively reviewed elsewhere (Joyce 2007; Wilson and Szostak 1999). The following sections (1.3.1 – 1.3.5) review some of the common rational sensor designs using aptamers. It is important to note that some of the presented materials (sections 1.3.1 – 1.3.5) originated from a book chapter titled, “Functional nucleic acids for fluorescence-based biosensing applications”.
Figure 1.
1.3 Fluorescent Aptamer-based Sensors

1.3.1 Aptamers and Sensors Anatomy

The term ‘aptamer’ derived from a Latin word ‘Aptus’ meaning ‘to fit’ (Ellington and Szostak 1990). They are single-stranded DNA or RNA molecules that recognize a vast array of targets, ranging from metal ions to whole cells, with high specificity and affinity. Aptamers can undergo adaptive binding, which is a transition from flexible single-stranded nucleic acids to distinct high-order structures in complex with their target (Hermann and Patel 2000). This exquisite ability has been explored for engineering molecular probes on various sensing platforms (Song et al. 2008; Cho et al. 2009; Mok and Li 2010).

Aptamers are an attractive candidate in this area because the nucleic acid nature allowed their chemical synthesis and modifications to be simple and cost-effective (Liu et al. 2009; Nutiu and Li 2005). For example, chemical moieties can be introduced at specific sites for conjugation, immobilization, and detection purposes. Nucleic acids that are chemically synthesized or modified often exhibit minimal batch-to-batch variations (Liu et al. 2009; Cho et al. 2009). Over the years, a plethora of signaling schemes have been reported based on optical, electrochemical, and amplification detection methods (Liu et al. 2009; Navani and Li 2006). However, detection by fluorescence remained a popular choice because fluorophores and quenchers can be directly labeled onto an aptamer without affecting its binding activity and a wide range of fluorophores and quenchers can be purchased commercially. Many rational design strategies have also been developed and published that allows simple conversion of aptamers into fluorescence probes. The
rational designs of fluorescence-based aptamer sensors and their utility are presented here.

A sensor has two essential components that work in concert – a molecular recognition element (MRE) that binds a specific target and this ligand-binding event is tethered to a measurable signal, which is generated by a sensor element. Desirable characteristics of an ideal sensor are high sensitivity and specificity, large dynamic range, cost-effective, fast response time, stable and most importantly, simple operation (Liu et al. 2009). Sensors crafted from aptamers can adequately fulfill these requirements.

1.3.2 Label-free Approach

One simple, label-free fluorescent detection method utilizes DNA intercalating dyes or cationic conjugated polymer to probe changes in DNA conformations. Certain organic dyes, such as TOTO, YOYO, and ethidium bromide, are able to intercalate into double-stranded DNA and the intercalated dyes often exhibit significantly different fluorescence properties. Thus, organic dyes can be used to monitor the formation or dissociation of double-helical elements of an aptamer as a result of ligand binding.

Fang and co-workers used the TOTO dye to monitor an oncprotein, platelet-derived growth factor BB (PDGF-BB) (Zhou et al. 2006) (figure 2). A fluorescence signal is present in the absence of PDGF-BB because the dye is able to intercalate with the double-stranded region in the PDGF-binding aptamer. When PDGF-BB is added to the sample,
the binding between PDGF-BB and the aptamer promotes a conformation change that displaces bound intercalating dyes causing a decrease in fluorescence signal. This assay demonstrated a simple and convenient method to monitor protein and nucleic acid interactions without the need of covalent labeling of either the analyte or the aptamer. Furthermore, the assay exhibits excellent sensitivity with a reported detection limit of 0.1 nM.

Figure 2.

*Detection using organic dye.* In the absence of target, the binding between the aptamer (black line) and the DNA binding dye (round disks) produces a fluorescence signal (left). The presence of the target causes the dissociation of the dye and as a result, the fluorescence signal is decreased (right).

Leclere and colleagues used polythiophene, a cationic conjugated polymer, to probe interactions between an anti-thrombin aptamer and the thrombin protein (Ho et al. 2004) (figure 3). The mechanism for detection is based on electrostatic interactions between the positively charged polymer with the negatively charge phosphate backbone found in DNA or RNA. Polythiophene is water-soluble and fluoresces as a free polymer. When it interacts with single-stranded DNA to form a duplex, the electrostatic interactions promote aggregation and the fluorescence signal becomes quenched. When thrombin is introduced, the aptamer folds into a guanine quadruplex structure to engage binding with
the protein and therefore, the resulting complex is only weakly associated with the polymer allowing the fluorescence signal to be restored.

Figure 3.
Detection using cationic polymer. In the absence of target, the binding between the aptamer (black line) and the cationic conjugated polymer (rod shape) quenches the fluorescence signal (left). The binding between target (star) and aptamer displaces the cationic conjugated polymer and the fluorescence signal is restored (right).

The label-free methods illustrate the idea that detection assays and sensors do not always require sophisticated reagents or fancy equipments. These studies utilized an external reagent (organic dye or cationic conjugated polymer) in conjunction with an aptamer to function as a sensor. The label-free approach offers a sensitive way for target detection without complicated rational designs although these methods do have significant drawbacks.

Label-free detection assays are susceptible to interference with other components in a sample and certain organic dyes, such as ethidium bromide, are mutagenic and therefore, toxic for in vivo applications (Liu et al. 2009). Furthermore, label-free methods cannot precisely probe a local conformation change in an aptamer and consequently, the fluorescence enhancement becomes difficult to optimize under different in vitro and in vivo conditions (Liu et al. 2009). This suggests that rational designs of sensor that can
accurately pinpoint local conformation changes in an aptamer are needed for optimum biosensing under different experimental conditions.

1.3.3 Single-labeled Signaling Aptamers

Fluorescence enhancement is governed by changes in the fluorophore’s spectroscopic properties and therefore, susceptible to an aptamer’s conformational change which can cause significant perturbations in a fluorophore’s local electronic environment (Nutiu and Li 2004).

Ellington’s group illustrated this concept of single-labeled signaling aptamers with modified constructs of ATP-binding aptamer that are labeled with a single fluorophore (Jhaveri et al. 2000) (figure 4). The rational design was based on the three dimensional structure of this aptamer (Hermann and Patel 2000). However, the signaling probes developed in this study only showed moderate fluorescence enhancement upon ATP binding. Ellington and co-workers then went on to perform an in vitro selection experiment to isolate ATP-binding aptamers containing fluorophore modified nucleotide analogues (Jhaveri et al. 2000). The isolated signaling aptamers exhibit a 200-fold increase in binding affinity and a 2-fold fluorescence enhancement at saturating ATP concentration.

Early attempts to convert aptamers into signaling probes required optimization steps based on the structure of the aptamer and significant fluorescence enhancement is not
always observed in the presence of target (Nutiu and Li 2004). In addition, the site of attachment for a fluorophore on an aptamer is critical for both target recognition and fluorescence enhancement (Liu et al. 2009). If a fluorophore is labeled at a location where no conformational change takes place during ligand binding, it will not produce detectable fluorescence enhancement when the cognate target is added. On the other hand, if a fluorophore is introduced at a conserved nucleotide, it may abolish the aptamer’s binding affinity. The major limitations of the single-labeled approach include high background signal and sensor design is dependent on structural information for a specific aptamer. Therefore, this approach can be difficult to generalize for different aptamers.

Figure 4.
*Single-labeled signaling aptamer.* Detection using an aptamer (blue line) modified with a fluorophore (F). The interactions between target (star) and aptamer alter the fluorophore’s electronic environment, which leads to increase in fluorescence signal (right).

1.3.4 Double-labeled Signaling Aptamers

This approach involves labeling an aptamer with either a fluorophore and quencher pair for fluorescence quenching or a pair of fluorophores for fluorescence resonance energy transfer (FRET). This strategy often exploits conformational changes of aptamers to perturb spatial arrangements between the fluorophore/quencher or fluorophore/fluorophore pair; consequently, this allows the synchronization of the ligand-
binding event with signal transduction. The aptamer beacon and self-assemble strategies are reviewed here.

The aptamer beacon approach was based on the molecular beacon design that was originally developed for the detection of nucleic acid sequence through hybridizations (Tyagi and Kramer 1996; Wang et al. 2009). An aptamer beacon consists of a target recognition region flanked by complementary ends that are labeled with a fluorophore and a quencher (figure 5). In the absence of target, the aptamer beacon folds into a hairpin structure so that the fluorophore and quencher are forced into close proximity for fluorescence quenching. In the presence of target, the binding between an aptamer and its target promotes a conformational change that disrupts the hairpin structure allowing the fluorescence signal to be restored. Hamaguchi et al. adapted the aptamer beacon design to construct a double-labeled aptamer that binds thrombin (Hamaguchi et al. 2001). Complementary sequences were added at terminal ends, which facilitate the formation of a hairpin structure in the absence of thrombin. Under this conformation, the fluorophore and quencher located on the terminal ends are closely positioned to achieve efficient fluorescence quenching. When thrombin is introduced, this aptamer undergoes a transition from the hairpin structure to the guanine quadruplex structure so that the fluorophore and quencher are separated and this spatial rearrangement restores the fluorescence signal. This fluorescent reporter shows a 2.5-fold increase in fluorescence when thrombin is present.
Figure 5.
Aptamer beacon design. Aptamer (black line) is doubly labeled with a fluorophore (F) and a quencher (Q) appended on the terminal ends (gray lines). In the absence of target (star), fluorescence is low because the fluorophore and the quencher are oriented in close proximity (left). In presence of target, the binding between the aptamer and target disrupts the hairpin structure and a fluorescence signal results (right).

The self-assemble design required an aptamer to be split into 2 oligonucleotides and each oligonucleotide is either labeled with a fluorophore or a quencher (figure 6). Signal transduction is coupled to the ligand-dependent formation of a stable target-bound complex. Stojanovic and colleagues made sensors that detect cocaine and ATP using the self-assemble design (Stojanovic et al. 2006). In the absence of cognate target, the fluorophore-labeled and quencher-labeled oligonucleotides do not assemble and therefore, a fluorescence signal is produced. In the presence of cognate target, these oligonucleotides undergo ligand-dependent self-assembly causing the fluorophore and quencher to be in close proximity and consequently, the fluorescence signal is reduced. Yamamoto and coworkers constructed an aptamer beacon for the Tat protein of HIV that undergoes ligand-dependent self-assembly (Yamamoto et al. 2000). The Tat-binding aptamer was separated into 2 oligonucleotides, one strand was double-labeled with a fluorophore and a quencher and the other was unmodified. In the absence of Tat protein, the labeled strand forms a hairpin structure (like an aptamer beacon) and low fluorescence
signal is produced. In the presence of Tat, the unmodified strand forms a complex with the labeled strand producing significant fluorescence enhancement (up to 14-fold).

![Figure 6](image)

**Figure 6.**
Self-assemble aptamer design. An aptamer is split into two single-strand oligonucleotides, one labeled a fluorophore (F) and the other with a quencher (Q). High fluorescence background results in the absence of target (left). These oligonucleotides assemble into a complex with the target (star) and the fluorescence background is decreased (right).

The above examples have illustrated that aptamers labeled with a fluorophore-quencher pair can effectively reduce the background level of fluorescence. However, the compatibility between the binding structure of an aptamer and the structural restrictions imposed by a given design can greatly affect the performance of the aptamer. For example, aptamers that cannot be split are not compatible with the self-assemble approach.

### 1.3.5 Structure-switching Signaling Aptamers

Significant fluorescence enhancement from the aforementioned design strategies can be difficult to achieve because specific structural information of an aptamer is required to produce signaling aptamers with optimal settings. Nutiu and Li came up with a more generalizable rational design strategy for making “structure-switching” fluorescent aptamer reporters (Nutiu and Li 2003) (figure 7). This method utilizes fluorescence-
labeled DNA aptamers and quencher-labeled complementary DNA strands. The production of a fluorescence signal is dependent on structural differences between the bound and unbound states of an aptamer. In the absence of the ligand for the aptamer, a weak DNA duplex is formed between the fluorophore-labeled aptamer and the quencher-labeled oligonucleotide, and this conformation allows the fluorophore and quencher to be in close proximity and therefore, the fluorescence from the fluorophore is quenched. In the presence of the ligand, the complex is formed between the aptamer and its ligand causing the dissociation of the quencher-labeled oligonucleotide from the fluorophore-labeled aptamer with a concomitant increase in the fluorescence signal.

**Figure 7.**
*Structure-switching signaling aptamer.* Target (star) promotes structure-switching of an aptamer from a DNA-DNA duplex (left) to a DNA-target complex (right). The dissociation of quencher-labeled DNA strand (QDNA) from the aptamer is accompanied with an increase in fluorescence signal. F and Q denote fluorophore and quencher, respectively.

To demonstrate the structure-switching concept, aptamers that bind ATP (Huizenga and Szostak 1995) and thrombin (Macaya et al. 1993) were converted into structure-switching reporters (Nutiu and Li 2003). These fluorescent probes were able to provide real-time detection and delivered over 10-fold fluorescence enhancement when the cognate target was introduced. This suggests that the structure-switching rational design does not impose structure limitations that an aptamer needs to adapt in order to be signaling competent.
Similar to other rational design methods, structure-switching also have an inherent caveat, that is, the native binding affinity of an aptamer can be affected when the aptamer is confined in a duplex structure. Strategies to overcome this limitation have been reported in greater detail elsewhere (Nutiu and Li 2005). In general, the number of base pairs occupied by the complementary DNA can be optimized to suit the temperature at which the sensor will operate or mismatch base pairs can be employed to form flawed duplex to facilitate the structure-switching process. Alternatively, the structure-switching concept can be implemented with in vitro selection to isolate aptamers with both molecular recognition and structure-switching abilities (Nutiu and Li 2005; Oh et al. 2010). Tan and co-workers showed an approach using fluorophore-labeled aptamers that are covalently attached with their quencher-label complementary DNA through a PEG linker (Tang et al. 2008). Their study showed that the number of base pairs between the complementary DNA and the aptamer can be reduced and potentially make the structure-switching process easier. Aptamers that bind ATP and thrombin were chosen to demonstrate this working principle. The ATP and the thrombin fluorescent probes showed a 30-fold and 16-fold fluorescence enhancement in the presence of cognate target, respectively.

### 1.4 Applications using Aptamers

So far, aptamers are isolated by in vitro selection and can be readily converted into fluorescent sensors based on the aforementioned rational designs. These modified oligonucleotides can also serve as signaling components for bioanalytical applications. This integration brings the benefits of aptamer-based sensors to an assay; in essence, the
aptamer-based assays can potentially provide high sensitivity and selectivity, fast response time, multiplex and real-time detection, and most importantly, versatile for any target of choice in a homogeneous format. For proof-of-principle purposes, structure-switching signaling aptamers have been explored in applications such as sol gel entrapment (Rupcich et al. 2005), nanoengines for precise delivery (Nutiu and Li 2005), monitoring enzymatic reaction (Nutiu et al. 2004), and high-throughput screening for small molecule inhibitors (Elowe et al. 2006). In addition to detection by fluorescence, structure-switching aptamers can also be used in other sensing formats such as electrochemical or colorimetric detections (Navani and Li 2006; Liu et al. 2009).

A formidable challenge for delivering aptamers inside cells is crossing the cell membrane (Famulok et al. 2007; Famulok 2009; Keefe et al. 2010). Nucleic acids are large in size and highly negative charged relative to small molecules. Unfortunately, aptamers are also futile under in vivo conditions because they are often sought out and destroyed by nucleases. To cope with these limitations, Famulok and colleagues have developed an aptamer displacement assay that allows the inhibitory function of aptamers that target proteins to be indirectly ‘transferred’ to small molecules by screening methods (Hafner et al. 2006). The assay was employed to identify the first antagonist for cytohesins, SecinH3 (Hafner et al. 2006). Cytohesins are small guanine exchange factors (GEFs) that stimulate ADP ribosylation factors (ARFs), which in turn, regulate a dazzling array of cellular events. The signaling component in this screen was a cytohesin-binding aptamer labeled with a fluorescent tag and detection was based on fluorescence polarization (figure 8).
Figure 8.  
Aptamer displacement assay. The addition of a small molecule (orange rings) displaces an aptamer (green line) that is bound to a protein (blue oval) and registers a fluorescence signal.

The aptamer displacement assay can also equip with an allosteric ribozyme to detect binding (Yamazaki et al. 2007). For example, an allosteric ribozyme consists of an aptamer that recognizes the HIV-1 RT protein and a ribozyme was employed as the signaling component. In this case, the catalytic activity of the ribozyme is modulated by an aptamer and therefore, the formation of a protein-aptamer complex renders the ribozyme inactive. However, if a small molecule displaces the aptamer from the protein, it would activate the ribozyme and trigger the cleavage of a fluorophore-and quencher-labeled oligonucleotide substrate and register a fluorescent signal.

The incorporation of aptamer-based sensors into high-throughput screening has identified novel inhibitors for these clinical relevant proteins. This combination precipitated an elegant approach to overcome current obstacles in aptamer technology; however, its effectiveness is dependent on unique aptamers for their inhibitory and signaling profiles against the target of interest. In general, enzyme inhibition can be detected indirectly by
monitoring the different steps of a biochemical reaction, such as changes in the cofactor, substrate, or product concentrations. If these detection parameters are adapted using aptamers that bind small molecules, the screening may become universal for enzymes that utilize common metabolites or cofactors. For example, aptamers that bind small molecules, such as GTP, may fulfill this purpose (Davis and Szostak 2002; Nutiu and Li 2005). Nonetheless, the aptamer displacement assay represents an innovative solution to package the inhibition effect of an aptamer into a smaller footprint by exploring chemical space.

Here, a general paradigm is presented about how aptamer-based sensors are made and the potential impact of their integration in sensing applications as the signaling component. Aptamers and their siblings in the FNA family have also expanded into other disciplines including sensing (Navani and Li 2006; Liu et al. 2009), therapeutics (Keefe et al. 2010; Famulok et al. 2007), nanotechnology (Nutiu and Li 2005; Lu and Liu 2006; Aldaye et al. 2008; Weizmann et al. 2008; Aldaye et al. 2009), and drug discovery (Famulok et al. 2007; Hafner et al. 2006; Yamazaki 2007). To date, an arsenal of aptamers has been discovered and it is expected that more and better aptamers will continue to be developed. The aptamer displacement assay developed by Famulok and colleagues has unraveled an exciting niche for aptamer-based applications – the convergence of signaling and inhibition capabilities of aptamers to penetrate restrictions imposed by biological systems for therapeutics and drug delivery. This work was made possible chiefly due to the availability of aptamers modified with the signaling moieties to serve as the sensing
component in the assay. In addition, this method exemplified that the multidisciplinary nature of FNAs can be united to workaround common limitations.

An immense reservoir of aptamers has been isolated using in vitro selection for almost any target of choice and these single-stranded nucleic acids can be coupled with a diverse detection schemes to transduce ligand-binding with signal output. The growth of the aptamer sensing field may suggest that its current state is on the cusp of transition from proof-of-principle examples to real-world applications. Therefore, the appearance of aptamers as molecular probes in highly demanded applications might be on the horizon.
1.5 Research Objective

The research endeavours embodied in this thesis focused on the development of a homogeneous single-step assay that bridges aptamer characterization with structure-switching. In this work, a previously isolated GTP-binding aptamer was employed to deduce a minimal functional construct, binding specificity, and its working conditions. These attributes are reported using a structure-switching design, which also reflects the aptamer’s performance as a sensor. Details about the experimental design and the results about this method are discussed in chapter 2. The implications of this work broaden our understanding about the molecular properties of aptamers and this information may open up potential utilities of this GTP-binding aptamer such as a sensor that generates optical output in bioanalytical assays.
CHAPTER 2: APTAMER CHARACTERIZATION BY STRUCTURE-SWITCHING

2.1 Introduction

As described in chapter 1, the functional nucleic acids (FNAs) family comprised of DNA or RNA aptamers, DNAzymes, ribozymes, and more recently, riboswitches (Wilson and Szostak 1999; Breaker 2004). In general, these oligonucleotides can be found in nature or created artificially. The latter employed a technique termed in vitro selection or SELEX and it is a selective amplification method that allows the isolation of FNAs through repeated rounds of enrichment (Ellington and Szostak 1990; Tuerk and Gold 1990; Klug and Famulok 1994). The general paradigm of this process begins with a random DNA or RNA library where unwanted sequences are separated from rare sequences under a predefined selection context. Isolated sequences are usually enriched by means of amplification. The selection context can vary greatly and compatible with many biochemical tools, such as polymerase chain reaction (PCR) and affinity chromatography, to harvest unique sequences with the desire function(s) (Wilson and Szostak 1999; Joyce 2007). Cloning and sequencing are then used to decipher the molecular identity of these sequences.

Aptamers are single-stranded DNA or RNA molecules that can bind a wide variety of targets with high specificity and affinity (Ellington and Szostak 1990; Tuerk and Gold 1990; Klug and Famulok 1994). These nucleic acid receptors can be equipped with signaling moieties, such as fluorophores and quenchers, and these modifications allow the
aptamer to tether its molecular recognition ability to a detectable signal (Klussmann 2006; Liu et al. 2009). Detection by fluorescence offers a convenient solution to monitor this process and many rational design strategies are available to engineer aptamers into fluorescent probes (see section 1.3). The DNA aptamer that can recognize ATP has been developed into various fluorescent probes and these signaling molecules can be integrated with high-throughput screening assays to aid drug discovery. The pharmaceutical company, Archemix, has adopted an RNA variant of this ATP-binding aptamer in an assay that screens for kinase inhibitors (Srinivasan et al. 2004). Analogously, GTP is an important small molecule that participates in various biological activities, such as RNA and protein syntheses. However, it is chiefly utilized as a secondary messenger for signal transduction in sophisticated cellular events (Ritter and Hall 2009). DNA and RNA aptamers that can recognize GTP have been isolated by in vitro selection (Davis and Szostak 2002; Nutiu and Li 2005). The synthetic production of DNA aptamers is simple, cost-effective, and offers superior chemical stability over its RNA counterpart (Liu et al. 2009). In spite of this, the development of sensors that detect GTP based on these aptamers has not yet been fully explored. The limited availability of sequence and structural information may have hindered the transition of aptamers into sensors. Here, we performed a characterization analysis for the GTP-binding DNA aptamer to examine its sequence requirement and recognition specificity. Our method minimizes the need for enzymatic reactions, gel purifications, and follow-up steps found in conventional protocols. This characterization approach leverages upon the structure-switching concept, which was used to convert aptamers into fluorescent reporters.
2.2 Experimental Section

Oligonucleotide and reagents

Standard and modified (fluorophore-and quencher-labeled) DNA oligonucleotides (including aptamer mutants) were purchased from Integrated DNA Technologies (IDT) and purified on a 10% denaturing (8M urea) polyacrylamide gel electrophoresis (PAGE). The concentrations of all purified oligonucleotides were determined spectroscopically. Nucleoside triphosphates (ATP, UTP, CTP, ITP, and GTP) were purchased from Fermentas, and guanine derivatives (GDP, GMP, cGMP, and guanosine) were purchased through Sigma Aldrich.

Fluorescence measurements

All DNA terminal duplex assemblies were made with the following concentrations of oligonucleotides: 40 nM of FDNA, 80 nM of DNA aptamer, and 120 nM of QDNA. This duplex assembly ratio was implemented in accordance with a previous protocol (Nutiu and Li 2003) such that most of the FDNA molecules would hybridize and in effect, reduce the fluorescence background. These oligonucleotides were mixed in 1× selection buffer (300 mM NaCl, 50 mM KCl, 10 mM MgCl₂, 50 mM Tris-HCl, pH 8.3) and denatured at 90 °C for 10 minutes and then slowly cooled to room temperature to allow proper DNA hybridization. The assay volume was 100 µL in all cases unless otherwise specified. The excitation and emission wavelengths were set at 495 nm and at 520 nm, respectively. The fluorescence intensities were measured using a Cary Eclipse.
Fluorescence Spectrophotometer (Varian). All fluorescence assays were performed in triplicate to yield an average reading.

The denaturation profile for the 5’ and 3’ terminal duplex assemblies were obtained by heating each sample at a rate of 2 °C/min from 20 °C to 70 °C. The fluorescence intensity from each sample was measured for every 1 °C increase in temperature.

The temperature-switching assay is a 3-step process: 1) background fluorescence measurement, 2) target addition and temperature elevation, and 3) reduce temperature to resolve aptamer-target complex. First, an initial background fluorescence reading for each DNA sample was recorded for 5 minutes at 22 °C. Next, the temperature was elevated to either 37, 42, or 55 °C followed by the addition of 0.5 mM GTP (or other NTPs) and the fluorescence intensity of each sample was recorded for 10 minutes. Finally, the temperature was lowered to 22 °C and the fluorescence intensity in each sample were recorded for 15 minutes. The fluorescence intensity in each step was recorded every minute until the end of the entire experiment. For the specificity test, all final NTP concentration in each fluorescence sample was 0.5 mM.

*Temperature-switching assay on 96-well plate*

The experimental setups of temperature-switching assay were conducted as described above. Exceptions include reduction of sample volume from 100 µL to 50 µL per well and the duration of the cooling step (22 °C) was increased from 15 minutes to 1 hour.
(prior to plate imaging), which provided a better fluorescence separation between samples containing GTP or selection buffer. Plates were scanned on a Molecular Dynamics Typhoon 9200 imager with the laser set to 532 nm. Plate images were quantified with Molecular Dynamics Image Quant version 5.2 software.

Real-time fluorescence assays

Components of DNA duplex were assembled as 40 nM of FDNA, 80 nM of DNA aptamer, and 120 nM of QDNA. Each sample was incubated for 5 minutes to obtain a background fluorescence reading prior to the addition of 0.5 mM GTP. Samples in selection buffer (SB) were used as a control and contained no GTP. For pH dependency analysis, duplex assemblies were prepared in selection buffer with pH adjusted to 6.5, 7.5, or 8.3. For GTP titration, the addition of GTP varied from 0.1 to 2 mM. Sample volumes were 100 µL for all real-time fluorescence assays.
2.3 Results and Discussion

2.3.1 Sequence and properties of the original GTP-binding aptamer

The GTP-binding DNA aptamer used in this study was previously isolated by in vitro selection (Nutiu and Li 2005). The aptamer is 49 nucleotides in length (excluding primer-binding sites) that can be divided primarily into 2 domains: a fixed domain (red; 15 nucleotides) and 2 variable domains (green; 10 and 20 nucleotides) as shown in figure 9A. This aptamer was demonstrated to function as a real-time fluorescent reporter for GTP when hybridized with fluorophore-and quencher-labeled oligonucleotides and delivers up to 2-fold fluorescence enhancement in the presence of 1 mM GTP. Here, we sought to elucidate a minimal construct and also identify conserved nucleotides that are required for molecular recognition. Consequently, these sequence information may enable the development of a more signaling efficient fluorescent reporter for GTP.
B.

![Diagram of aptamer structure]

C.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ – 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDNA1</td>
<td>[F] GCG GAG CGT GGC AGG</td>
</tr>
<tr>
<td>QDNA1</td>
<td>ACC CCT CAG TG [Q]</td>
</tr>
<tr>
<td>E1</td>
<td>CCT GCC ACG CTC CGC ACT GAG GGG TGG TTT CCG CAG CGA TTC TTG ATC GCG GAA GTC GGT GGG GAG GGT</td>
</tr>
<tr>
<td>FDNA2</td>
<td>GAT GCG GGT GCC AAG CTT A [F]</td>
</tr>
<tr>
<td>QDNA2</td>
<td>[Q] GTG ACT ACC C</td>
</tr>
<tr>
<td>E2</td>
<td>GGG GTG GTT TCC GCA GCG ATT CTT GAT CGC GGA AGT CGG TGG GGA GGG TAG TCA CTA AGC TTG GCA CCC GCA TC</td>
</tr>
</tbody>
</table>

Figure 9
A. Sequence of original GTP-binding DNA aptamer. The aptamer consists of 3 domains: 2 variable domains (green nucleotides) and a fixed domain (red nucleotides). The blue nucleotides were originally implemented as a linker region. B. Design of duplex assemblies. Each terminal duplex assembly consists of 3 components: FDNA, QDNA, and the GTP-binding aptamer. C. Sequence of oligonucleotides used for duplex assembly. E1 and E2 are aptamers with extra nucleotides added to the 5’ and 3’ termini, respectively. F and Q denotes fluorophore and quencher, respectively.
2.3.2 Design of duplex assemblies

Structure-switching is an elegant solution to convert aptamers into signaling probes and this idea exploits the exquisite ability of aptamers to adopt 2 unique conformations: helical duplex with an antisense sequence and a distinct tertiary structure that is bound with its target (Nutiu and Li 2003). Fluorescence is often used to monitor the transition between these states of an aptamer. To elucidate a minimal construct and also critical residues required for binding activity, mutation analyses, such as truncations, single-base deletions, and substitutions, need to be conducted on the aptamer. For this purpose, extra nucleotides (blue) were added at either the 5’ or the 3’ ends of the original GTP-binding aptamer (figure 9b). This arrangement would facilitate the formation of terminal DNA duplexes and each duplex assembly consists of a fluorophore modified DNA (FDNA), a quencher modified DNA (QDNA), and the original GTP-binding aptamer (green). These duplex assemblies would enable us to manipulate every nucleotide in the sequence and determine its effect on molecular recognition by fluorescence. For example, the 5’ duplex assembly can be used to study the effect of various mutations on the 3’ end and vice versa. The sequence for individual oligonucleotides that were used to form the duplex assemblies can be found in figure 9c.

2.3.3 Denaturation profile

To determine whether the modified oligonucleotides (FDNAs and QDNAs) could accurately anneal at the predicted regions on the GTP-binding aptamer, we conducted a DNA denaturation test that examined the correlation between temperature and
fluorescence intensity (figure 10). Each component of the duplex assembly, FDNA, aptamer, and QDNA, were prepared in selection buffer with a ratio of 1:2:3 and this proportion was previously used to minimize the background fluorescence (Nutiu and Li 2003; Lau et al. 2010). The duplex assemblies (filled triangles and squares) were assessed independently and samples that lack the QDNA were included as controls (open triangles and squares). Based on the denaturation profile, we observed a positive correlation between temperature increase and fluorescence intensity for both duplex assemblies. This trend reflects a shift in equilibrium from assembled DNA duplexes to denatured oligonucleotides as a response to temperature elevation and more importantly, fluorescence can be used to monitor the dissociation of short oligonucleotides from the aptamer. Similar to previous structure-switching studies (Nutiu and Li 2003), the fluorescence from the control samples (open triangles and squares) also decreased over the course of the experiment and ultimately, exhibits a similar fluorescence as the samples containing the full duplex assembly (filled triangles and squares). Here, our results suggest that the modified oligonucleotides can precisely hybridize with the aptamer and the proper assembly of our duplex designs is crucial for subsequent characterization analyses.
Figure 10

Denaturation profile of duplex assemblies. The formation of the desired DNA duplex was assessed using a thermal test monitored by fluorescence. 5’ assembly (filled square) and 3’ assembly (filled triangle) contained FDNA, QDNA, and the GTP-binding aptamer. Control samples were designated as 5’ assembly (open square) and 3’ assembly (open triangle) and contained no QDNA.

2.3.4 High temperature enhanced structure-switching

To investigate the structure-switching ability of our duplex designs, we tested these duplex assemblies using the temperature-switching assay (figure 11). The procedure of this assay has been previously described (Nutiu and Li 2003). Briefly, the assembled oligonucleotides are subjected to a temperature cycling process such that the DNA duplex becomes unstable at an elevated temperature and this helps the aptamer to structure switch (i.e. engage binding with GTP). The temperature is then lowered to resolve the state of the aptamer (i.e. bound with QDNA or GTP). The most crucial parameter to consider for this assay is temperature because an identical set of temperatures for both 5’ and 3’ duplex assemblies needs to be established and the quenching and dequenching of fluorescence should correlate with the DNA duplex and the target complex, respectively.
In the first step of our temperature-switching assay, the background fluorescence for each duplex assembly was recorded at 22 °C (figure 1). This temperature was selected because the original aptamer was isolated at room temperature (22 °C) by in vitro selection (Nutiu and Li 2005). Next, we increased the temperature to 37 (triangles) or 55 (circles) °C to determine the optimum temperature that will facilitate structure-switching. These temperatures were selected based on the denaturation profile (figure 10) and the theoretical melting temperature \(T_m\) of the DNA duplex between the QDNAs and the aptamer. Finally, the temperature is lowered back to 22 °C, dequenching of fluorescence corresponds to the complex state, which suggests the aptamer is able to structure switch at the selected temperature. Should the aptamer reforms the duplex structure, the fluorescence from the fluorophore will be quenched. Therefore, fluorescence is employed to differentiate the state of an aptamer at the end of the assay. Consequently, this set of temperatures will be applied for mutation analyses to identify active mutants.

We tested these temperatures for both the 5’ and 3’ end duplex assemblies in either buffer (open) or 0.5 mM GTP (filled) (figure 11a and 11b). Our 5’ duplex assembly was found to be capable of structure-switching at 37, and 55 °C (figure 11a); however, the most noticeable difference in fluorescence intensity between samples with GTP and selection buffer was observed at 55 °C. The gradual increase in fluorescence at 37 °C appeared to be a real-time signaling response to GTP and will be addressed later in the paper. In contrast, the 3’ duplex assembly switched best at 55 °C, whereas structure-switching at 37 °C was negligible (figure 11b). The data showed that the addition of nucleotides to either
the 5’ or 3’ ends do not abolish binding activity. Although both our duplex designs can form a target complex with GTP, the 5’ duplex assembly appeared to exhibit higher fluorescence enhancement than the 3’ duplex assembly under the same temperature and target concentration (0.5 mM GTP). Therefore, 22/55/22 °C is the optimum temperature set to resolve the duplex (selection buffer) or complex (GTP) states of the aptamer using the temperature-switching assay. It is important to note that the purpose of temperature optimization was to attain the greatest separation in fluorescence intensity between samples with and without GTP for both duplex assemblies. This would avoid ambiguous scenarios when determining whether a particular mutant is active or inactive during later studies. Herein, we adopted 22/55/22 °C for all of our subsequent mutants analyses with the temperature-switching assays.

Figure 11

Temperature optimization. A. The 5’ duplex assembly was examined at 37 °C (GTP – filled triangle and SB – open triangle) and 55 °C (GTP – filled circle and SB – open circle). B. The 3’ duplex assembly was tested at 37 °C (GTP – filled triangle and SB – open triangle) and 55 °C (GTP – filled circle and SB – open circle). SB is selection buffer. Arrow indicates the addition of 0.5 mM GTP at the fifth minute. 55 °C provided
the greatest separation between samples containing GTP and selection buffer for both 5’ and 3’ duplex assemblies.

2.3.5 Aptamer specificity is not affected by 5’ duplex

Next, we assessed whether the addition of extra nucleotides on the 5’ end would affect the specificity of the original aptamer when assembled into the 5’ duplex. We tested the aptamer against ATP, CTP, GTP, ITP, and UTP under the same conditions using the temperature-switching assay. Figure 12a showed the sample with GTP (black filled squares) exhibits higher fluorescence than the samples containing either selection buffer (SB) or the other NTPs. This indicates that when the original aptamer is assembled into the 5’ duplex, it responded exclusively to GTP (ie. in a target complex). All the samples in selection buffer (SB) or NTPs reformed into the 5’ duplex upon cooling to 22 °C. Therefore, our results demonstrate that the 5’ duplex assembly does not affect the specificity of the original GTP-binding aptamer.
Figure 12
A. *Specificity analysis of 5’ duplex assembly.* The 5’ duplex assembly was tested against GTP (black square), CTP (orange triangle), ATP (blue circle), UTP (red diamond), ITP (green cross) and SB (grey circle). The 5’ duplex assembly with GTP (black square) is the only sample that is able to form a complex with the aptamer. B. *Specificity analysis of 5’ duplex assembly against guanine analogs.* Guanine derivatives were used to further assess the specificity of the aptamer using the 5’ duplex assembly. The aptamer was tested against guanosine (purple square), GTP (black square), GDP (blue square), GMP (red square), cGMP (orange square), and SB (grey circle). SB is selection buffer. All target concentrations were 0.5 mM.

The specificity of the aptamer for similar guanine derivatives has not been tested previously and we would like to examine if the aptamer can discriminate other functional groups such as the phosphate groups on GTP. The same specificity test was adapted to analyze the aptamer’s specificity towards GDP, GMP, cGMP, and guanosine (figure 12b). We found that all samples containing the guanine derivatives were able to form a complex with the aptamer indicated by the increased in fluorescence. The ability of the aptamer to engage binding with all these guanine analogs may suggests that molecular recognition takes place between the aptamer and the nitrogenous base portion of these small molecules.
2.3.6 The miniaturized mutant – G1.5

Here, truncation studies were performed in an effort to identify a minimal functional construct. Truncated mutants were designed with minor deletions from either the 5’ or 3’ end and then assembled into the opposite duplex to study binding activity (see design of duplex assemblies). However, an intriguing question was whether the embedded fixed domain (red) was involved with the binding of GTP. Predefined nucleotides in the fixed domain may inadvertently participate in target binding or the length of these residues enables precise spatial orientation of essential nucleotides from the variable domains that bind GTP. To examine the role of the fixed domain, mutants with truncations in this region were also constructed. Some of our representative truncated mutants (G1.1 – G.15) are shown in figure 13.

![Figure 13](image)

**Figure 13**
Sequence for truncation analysis. Sequence of representative mutants, G1.1 – G1.5, used in the truncation studies. The WT 5’ is the original GTP-binding aptamer (49 nucleotides) and provided for comparison. For simplicity, FDNA binding-site and blue nucleotides are omitted.
In an attempt to increase the scale of the assay, the format of the temperature-switching assay was transferred from fluorometer-based to 96-well plates and this allowed multiple mutants to be screened simultaneously. As a result, we can be more efficient with the gathering of sequence information about the aptamer. Figure 14 provides a schematic that illustrate the plate layout in reference to our standard temperature-switching assay. Experimental conditions were identical to the previous assays (see materials and methods) except changes were made to the sample volume and the duration at the 22 °C cooling step. The sample volume was lowered from the original 100 µL to 50 µL and this reduction can be economical when testing a large quantity of mutants in triplicates, especially when common FDNAs and QDNAs are employed for the entire screening process. Based on our previous fluorescence data (figure 11), we observed that 15 minutes of the 22 °C cooling step may be insufficient for the duplex assemblies (in selection buffer) to completely refold into the duplex. Inadequate fluorescence separation between samples with and without GTP may cause ambiguity in discriminating DNA duplex from target complex. Therefore, the duration of the 22 °C cooling step was increased from 15 minutes to 1 hour in order for the signal to equilibrate and produce a better fluorescence separation.
A. Setup of temperature-switching assay on 96-well plate. Green wells are controls that assess the background fluorescence and the wells contained the duplex assemblies prior to any treatments. Yellow and red wells correspond to duplex assemblies that have been heated in the absence or presence of GTP, respectively.

B. Plate layout for the temperature-switching assay. Each mutant was tested according to the diagram shown in A.

Figure 14
Our truncation analysis showed that mutants became inactive (G1.1 – G1.4) when 2 or 4 nucleotides are deleted from either 5’ or 3’ termini (figure 13 and 15). The deletions from these sequences may suggest important residues located at these termini. Interestingly, we isolated an active mutant 5’ (G1.5) that can tolerate the removal of the entire fixed domain, which spanned from T7 to G29 (figure 17a and b). The plate image showed that, in the presence of GTP, G1.5 exhibits similar fluorescence enhancement as the 5’ WT. The positive control, 5’WT, was the original aptamer (assembled into the 5’ duplex) and was also used to maintain relative fluorescence between different plates. The G1.5 mutant showed that nucleotides from 49 to 27 are disposable but more importantly, both variable domains can coexist as a single continuous domain to carry out molecular recognition.
Figure 15
A. Plate image of truncation analysis. Image was put together from different plates that were screened independently and separated by the grey line. The positive control, 5’WT, is the original GTP-binding aptamer. The same colour code in figure 14 was used to designate the treated samples. See main text for more details. B. Quantification of plate images. The wells containing individual mutants were quantified using the ImageQuant software (version 5.2) and normalized using the 5’ WT.
Next, we created a series of mutants that contained a single point mutation (figure 16) to deduce the essential residues of G1.5. The point mutation replaces each base with a thymine for every nucleotide in this 27-nucleotide mutant. We chose thymine because it exhibits weaker base pairing and it is known for its flexibility and the lack of secondary structures. Structural studies (Hermann and Patel 2000) suggest that essential residues provide molecular interactions for either structural support or target binding in aptamers, such as stacking, hydrogen-bonding, and dipole-dipole, and therefore these interactions will most likely be perturbed in mutants with critical residues replaced by thymines.

Mutants were assembled into duplex assemblies and screened for active sequences using the temperature-switching assay on 96-well plates. Mutants with point mutations on the 5’ end were analyzed using the 3’ duplex assembly (T1, T2, T3, and T4) and the remaining residues were examined with the 5’ duplex assembly (T6 – T8, T9-10, T11–T13, T15 – T17, T19 – T26).

![Figure 16](image)

**Figure 16**
*Sequence for substitution analysis.* Sequence of mutants with single point mutation. G1.5 is provided as a reference. See main text for detail.
We conducted the substitution analysis using the temperature-switching assay on fluorometer (figure 17). Here, the relative fluorescence was calculated based on a single time point at the 30th minute and expressed as fold over 5’ WT. In this case, the ratio for relative fluorescence, \( RF = \frac{(F_{\text{mutant, GTP}} - F_{\text{mutant, SB}})}{(F_{5'\text{WT}, GTP} - F_{5'\text{WT}, SB})} \) where \( F_{\text{mutant, GTP}} \), \( F_{\text{mutant, SB}} \), \( F_{5'\text{WT}, GTP} \), and \( F_{5'\text{WT}, SB} \) are the recorded fluorescence intensities for the mutant of interest and 5’WT. For inactive mutants, the DNA duplex is reformed at the end of the temperature-switching assay and therefore, the fluorescence separation between samples containing GTP and SB will be minimal. This negligible difference is divided by the fluorescence difference from samples with GTP and SB of 5’WT, which generates an insignificant value (~ 0). The mutants employed here were tested in triplicate and the average was used for calculations with the error bar provided (figure 17). We identified 5 clusters of essential guanine residues (T1 – T4, T6 – T7, T16 – T17, T19 – T22, and T24 – T26) in the aptamer and 2 of these clusters (T1 – T4 and T24 – T26) resided near the 5’ and 3’ termini. This observation augments our previous truncation analysis in which small deletions on either terminus immediately abolish binding activity. The conservation of guanines at the 5’ and 3’ ends may reflect the previous in vitro selection context in which the fixed domain fragmented the variable domains. Consequently, the binding of GTP may instruct cooperative molecular recognition between the variable domains and therefore, sequences that contained the proper residues in these termini were selected. We also found 7 nonessential nucleotides that occupied the middle region of the aptamer. To a lesser extent, this cluster of residues further supports the view of cooperative molecular recognition via 5’ and 3’ termini nucleotides. More
importantly, mutations at these nonessential positions (T11 – T13, and T15) enabled these mutants to display higher fluorescence enhancement than the original aptamer (5’ WT). Improved fluorescence enhancement may correlate with better GTP binding. In addition, we also identified an active mutant with a single base deletion at position 27, ΔT27.

Collectively, our substitution results highlighted 5 clusters of guanine residues that are important for binding GTP and also 9 nonessential nucleotides (T11 – T13, T15, T23 and T27) that can be independently mutated without impairing binding. The length of G1.5 can be either 27 or 26 nucleotides but the effect of removing thymine at position 27 needs to be further investigated.

**Figure 17**

*Substitution analysis on fluorometer.* Graph was compiled based on results collected from mutants with single-point mutation using the fluorometer. Each mutant was tested in triplicate with the relative fluorescence was expressed as fold over 5’ WT.
2.3.7 The guanine-thymine exclusive aptamer

Previously, we showed that 9 out of the 27 nucleotides in the aptamer are nonessential and speculated that the aptamer may engage target binding with conserved nucleotides located at the 5’ and 3’ termini. To investigate the aptamer’s sequence tolerance, we generated mutants that contained multiple nonessential nucleotides. These constructs contained 2 thymine residues (T9 – T10), 3 thymine residues with T27 deletion (T9 – T10ΔT27), and 4 thymine residues with T27 deletion (T8T9 – T10T15ΔT27). Figure 18 shows the sequence of these mutants.

![Sequence of mutants](Figure 18)

*Figure 18*

*Sequence for nonessential mutation analysis.* The sequence of mutants with different nonessential mutations is shown.

The mutants were assembled into 5’ duplex and tested for binding. In the absence of GTP, we observed that mutants with higher incorporation of thymine residues (T9 – T10 vs. T8T9 – T10T15ΔT27) resist moderately when refolding back to DNA duplex (figure 19). Nonetheless, all constructs were found to be active, which suggest that some of these nonessential nucleotides could be accumulated without abolishing binding activity.
Figure 19
Nonessential mutation analysis. Mutants with different nonessential mutations were tested using the temperature-switching assay performed on the fluorometer. Samples were tested in either selection buffer (open circle) or GTP (closed circle).

The substitution analysis and active mutants showed that only guanine residues are essential for binding and this may suggest the aptamer adopt a guanine quadruplex for target binding (Davis 2007). The guanine quadruplex is an unique structure found in nucleic acids and folds using predominately guanine and thymine residues. To gain further insights into the sequence requirement, we replaced all nonessential nucleotides with thymines and constructed a mutant consists of only guanine and thymine residues (figure 20). In the presence of GTP, we found this mutant to exhibit the lowest fluorescence enhancement among all active sequences. In the absence of GTP, the excessive thymine residues rendered the aptamer almost incapable of refolding into the DNA duplex, which is characterized by a negligible decrease in fluorescence. This behavior is analogous to the previous active mutants containing multiple nonessential residues.
Here, we would like to propose 2 models that may help to explain the observed attributes of this guanine-thymine exclusive aptamer. Excessive sequence manipulations can abolish or alter the binding properties of the aptamer and this has a negative impact on the aptamer’s ability to structure switch. Alternatively, the simplification of sequence to guanine and thymine residues could favour stable conformations or secondary structures that compete with the formation of DNA duplex. The former could be influenced by the flexibility of the aptamer, which is increased when more thymine residues are incorporated. Enhanced flexibility may exert destabilization effects on the complex that is formed between the aptamer and GTP. As such, disruption of target complex translates to a reduction in binding affinity, which can impair the aptamer’s ability to structure switch.

The latter could associate with duplex instability due to the formation of alternative DNA structures. For example, guanine-rich sequences have been reported to undergo oligomerization (Boese and Breaker 2007) and also adopt guanine quadruplex (Davis 2004) structures that are more stable than DNA duplex. In the absence of target, a competition between the intermolecular DNA duplex and alternative folding structures may exist and this conflict occurs at the expense of DNA duplex stability. One possibility may involve a competition for common nucleotides that are required for both the proper folding of DNA duplex and the alternative conformations. As a result, an equilibrium is created between such structures because the DNA duplex and alternative conformations cannot coexist within the aptamer due to the sharing of common nucleotides.
Results from the temperature-switching assay showed distinctive features that appeared to be supportive of the latter conjecture. First, the guanine-thymine exclusive aptamer showed approximately 3-fold higher background fluorescence than most of the other active sequences (figure 21). Second, this aptamer exhibits a staircase trend in which the fluorescence was increased at each step of the temperature-switching assay when GTP was present (figure 20). It is important to note that this trend was not observed from the original GTP-binding aptamer. Previous studies showed that when the ATP-binding DNA aptamer was converted into a structure-switching signaling aptamer, it also displayed a similar trend when examined using the temperature-switching assay (Nutiu and Li 2003). Lastly, the inability of the aptamer to reform the DNA duplex may suggest the formation of more stable alternative structures. Taken together, the elevated background fluorescence, inability to reform DNA duplex, and the staircase trend are more likely to indicate stable alternative structures that compete with the DNA duplex rather than the inability of the aptamer to structure switch. Therefore, this set of results appeared to conflict the former conjecture.

Although we addressed these molecular events independently, their effects on the aptamer may not be mutually exclusive. Criteria that fulfill either model do not necessarily indicate a better GTP-binding aptamer. From our substitution analysis, we did observe some mutations (T11, T12, T13, T15, and T23) that gave rise to higher fluorescence enhancement than the 5’ WT. However, the effect from the accumulation of these mutations on binding activity and fluorescence enhancement remain unresolved.
Nevertheless, we were successful in the creation of a GTP-binding aptamer using only guanine and thymine residues.

![GTP-binding aptamer](image)

**Figure 20**

*Guanine-thymine exclusive aptamer.* Inset (top) showed the sequence of the mutant containing only guanine and thymine residues, nucleotides outlined in green are required for binding GTP. The aptamer was tested using the temperature-switching assay. Samples containing GTP and SB are represented by black and grey circles, respectively. SB is selection buffer. GTP concentration was 0.5 mM.
Figure 21
Fluorescence background of active mutants. The fluorescence background taken during the first step of the temperature-switching assay (figure 22) for different active mutants. Bars are shown as an average of triplicate with the error bar indicated. 5’ WT is the original GTP-binding aptamer.

2.3.8 GTP titration and pH dependency

Our temperature optimization results showed that the 5’ duplex of the original aptamer was capable of responding to GTP in real-time at 37 °C (figure 11). To explore the possibility of real-time detection with this assembly design, we examined real-time detection using GTP concentrations that varied from 0.1 mM to 2 mM (figure 22). Our 5’ duplex exhibited a 1.8-fold fluorescence enhancement at 0.1 mM and up to 3-fold when the GTP concentration reached 2 mM. The fluorescence from the control, SB, remained unchanged over the course of the experiment. Previously, this GTP-binding aptamer was reported to deliver 2-fold fluorescence enhancement at 1 mM GTP (Nutiu and Li 2005). Here, the same aptamer with the duplex relocated to the 5’ terminal displayed similar
fluorescence enhancement at one-tenth the GTP concentration. The improvement was attributed from better fluorescence quenching by positioning the fluorophore and quencher in close proximity. However, the 5’ duplex using the original aptamer appeared to have an attenuated response and narrow dynamic range.

![Graph showing fluorescence enhancement over time](image)

**Figure 22**

*Real-time detection of GTP.* 5’ duplex of the original GTP-binding aptamer was tested at varying GTP concentration in real-time. In all cases, GTP was added at the fifth minute (arrow).

To elucidate the working conditions of this aptamer, we examined the binding activity at different pH. The aforementioned 5’ duplex was tested at pH 6.5, 7.5, and 8.3 (figure 23). At pH 8.3, the original aptamer generated a 3-fold fluorescence enhancement at 0.5 mM
GTP. As we lower the pH to 7.5, we noticed an approximate 14% dropped in fluorescence signal. At pH 6.5, the fluorescence enhancement further decreased to 43%. The responsiveness of the aptamer to 0.5 mM GTP appeared to be optimum at pH 8.3. The results suggest the increased acidic of the solution negatively impacts the fluorescence enhancement of the aptamer. This trend is not surprising because the binding activity of aptamer is usually governed by conditions that mirrored the in vitro selection environment.

Figure 23
*Effect of pH on binding activity.* 5’ duplex of the original GTP-binding aptamer was tested under different pH. In all cases, GTP was added at the fifth minute (arrow).
2.3.9 Conclusion

Aptamer characterization by structure-switching is a rapid and efficient approach to identify the minimal sequence and conserved nucleotides in an aptamer. The implementation of structure-switching for aptamer characterization leverages upon 2 important aspects of nucleic acid aptamers: 1) the dual capability of aptamers to form DNA duplex and target complex 2) precise positioning of fluorophore-and quencher-labeled oligonucleotides by base pairing with an aptamer to conveys its the molecular recognition properties into an optical signal. We also made our system cost-effective by utilizing common modified oligonucleotides (FDNAs and QDNAs) to detect binding activity of various mutants with fluorescence. A major challenge for structure-switching designs is the customization of the antisense (QDNA) length for individual mutants and this process can be expensive, time-consuming, and laborious. To simplify the assay design, our system adopted the temperature-switching assay to avoid unnecessary optimizations and therefore, the same antisense, QDNA, was used to test all the mutants in our study. We chose a relatively short QDNA to fulfill 2 purposes: 1) low background fluorescence is achieved through a relatively stable DNA duplex that is formed between the aptamer and QDNA and more importantly, this antisense is also susceptible to be displaced when the target is introduced, and 2) reduced QDNA length exposes more residues of the aptamer for mutational analyses.

Finally, we showed that the combination of terminal duplex assemblies and the temperature-changing assay could be an effective strategy to elucidate active mutants and
the efficiency of the assay can be further increase by utilizing 96-well plates to accommodate high volume screening of mutants. Conventional aptamer characterization methods include binding affinity assays and post-selection experiments (Hall et al. 2009; Allali-Hasani et al. 2007; Kanadadai 2009). In general, these multi-step methods are low-throughput and involved various preparation steps. In contrast, aptamer characterization by structure-switching offers a single-step method and avoids enzymatic reactions for labeling, repetitive intermediate purification steps, and sample analysis (such as scintillation counting for measuring for binding activity).

To date, numerous DNA and RNA aptamers have been isolated by in vitro selection for almost any target of choice. However, only a handful of these nucleic acid receptors have successfully transitioned into useful molecular tools. This limitation, in part, results from insufficient structural studies and has delayed the adoption of aptamers in applications, such as sensing. Aptamer characterization by structure-switching may bridge this gap by providing the necessary sequence information of existing aptamers in a simple and convenient format, which can aid the development of efficient fluorescent probes. For example, unrefined sequences may contain extra nucleotides that interfere with the proper folding of active structure and impairs the sensitivity and response time of the sensor. Although we have only demonstrated this method using the GTP-binding DNA aptamer, structure-switching is also amenable for RNA aptamer and therefore, it may be possible to adapt this strategy for RNA aptamer characterization (Lau et al. 2010).
The original GTP-binding DNA aptamer was 49 nucleotides in length (excluding primer-binding sites) and consists of a 15-nucleotide fixed domain that is embedded between 2 variable domains (figure 24). Truncation studies revealed a 27-nucleotide functional variant (G1.5) that binds GTP, which suggests that the fixed domain is dispensable. More importantly, this minimal construct demonstrated that both variable domains could function as one continuous domain to carry out molecular recognition. Substitution analysis further identified 5 clusters of guanine residues in this region that are required for function. Finally, the construction of a guanine-thymine exclusive aptamer showed that reducing sequence diversity to only thymine and guanine residues is sufficient to bind GTP. This nucleotide-depleted aptamer contained an 8-thymine region that is flanked by two highly conserved guanine rich regions. This arrangement closely mirrored the fixed domain that separates the variable domains in the original aptamer. Sequence information from both of these aptamers may suggest that the fixed domain of the original aptamer may actually be 30 nucleotides in length that spanned from T7 to C37. Taken together, it might be possible that conserved residues localized near the termini are involved with target binding and nucleotides that resided in the middle of the aptamer are simply placeholders. The GTP-binding aptamer offers a high degree of sequence tolerance, in which the genetic alphabet can be simplified to contain only guanine and thymine and the sequence length can be reduced from 49 to 26 nucleotides. In addition, the aptamer is highly specific for the guanosine nitrogen base and this precise molecular recognition can be achieved with 5 uniquely arranged clusters of guanine residues. Future research endeavour should be directed to examine whether this guanine-thymine exclusive aptamer
is capable of adopting a guanine quadruplex motif to engage target binding. This motif is commonly found in other functional nucleic acids such as the aptamer that binds thrombin (Macaya et al. 1993) and DNAzymes can catalyze self-phosphorylation (McManus and Li 2008).

Figure 24
Sequence comparison. The original GTP-binding aptamer (top), G1.5 mutant (middle), and the guanine thymine exclusive aptamer (bottom).
CHAPTER 3: CONCLUDING REMARKS

It has been over 2 decades since the first in vitro selection experiment was conducted using modern biochemical techniques (such as PCR). In these years, we have witness the exciting discoveries of the remarkable capabilities of nucleic acids, clever strategies that harness these functions as molecular detection tools, and most currently, the emergence of aptamer-based assays. Functional nucleic acids and aptamers have clearly established themselves to be more capable than simple biopolymers that store biological information.

In chapter 1, we discussed about the biological relevance of GTP such as the GPCR signaling pathway and briefly described the methods that are used to monitor GPCR activity. We then introduced functional nucleic acids and aptamers and the common methods researchers used to convert them into detection tools. Aptamers can be modified extensively to convey its molecular recognition properties to a measurable signal. We used the aptamer displacement assay as an example to illustrate how aptamers can function as the signaling component in assays. Aptamers hold great promise for assay development because their synthesis is simple and inexpensive and therefore, these factors would minimize the cost associated with the production of the resulting assay. And, as demonstrated aptamer sensors are suitable for homogeneous and nonradiative assay format.

In chapter 2, we discussed about the development of a simple characterization assay that can examine the sequence properties of an aptamer. In general, aptamers selected using in
vitro selection undergo a characterization process followed by a sensor design that converts the aptamer into a signaling probe. Each individual step is time-consuming and laborious. Common characterization methods only probe how mutational analysis influence binding and therefore, these assays do not reflect the effect of sequence manipulation on the performance of the aptamer as a sensor. In addition, intermediate purification steps are inevitable because the methods required enzymatic reactions to process different mutants in order to assess binding (Hall et al. 2009; Allali-Hasani et al. 2007; Kanadadai 2009; Yang et al. 2011). In contrast, our work showed that both steps could be replaced with a simple procedure when characterization is conducted using a sensor design. In this assay, we were able to demonstrate simultaneous screening for various truncated mutants in a nonradioactive and homogeneous format. We were successful in identifying a minimal functional construct along with the conserved nucleotides of a GTP-binding DNA aptamer. Understanding the molecular properties of this aptamer may enable the development of an efficient GTP sensor. Future work should focus on using the identified sequence properties for this GTP-binding aptamer to design a signaling efficient sensor for GTP.
REFERENCES


65


