Split Aptameric Turn-On Fluorescence Sensor for Detection of Sequence Specific Nucleic Acid at Ambient Temperature

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SPLIT APTAMERIC TURN-ON FLUORESCENCE SENSOR FOR DETECTION OF SEQUENCE SPECIFIC NUCLEIC ACID

by

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ABSTRACT

Nucleic acid amplification tests (NAATs) enable sensitive detection of low density infections that microscopy and rapid diagnostic test are not capable of detecting. They enable quantitative and qualitative nucleic acid detection, genotype analysis, and single nucleotide polymorphism (SNP) detection. Current state of the art molecular probes used with NAATs include molecular beacon (MB), Taqman and its variations. This work presents novel molecular probe designed from Spinach and Dapoxyl aptamers. The aptamers are split into two parts (split aptamer), allowing greater sensitivity and selectivity towards fully complimentary nucleic acid analyte. The major advantage of split aptamer probe over state-of-the art fluorescent probes is its high selectivity: in the presence of a single base mismatched analyte, it produces only background fluorescence, even at room temperature. SSA is a promising tool for label-free analysis of nucleic acids at ambient temperatures.

Split spinach aptamer (SSA) probes and split dapoxyl aptamer (SDA) for fluorescent analysis of nucleic acids were designed and tested. In both split aptamer design, two RNA or RNA/DNA or DNA strands hybridized to a specific nucleic acid analyte and formed a binding site for fluorescent dye, which was accompanied by up to 270-fold and 69-fold increase in fluorescence. SSA consisted entirely of ribonucleotides which potentially be expressed in live cells and used for imaging of specific mRNAs. For in vitro RNA/DNA analysis, SDA consisting of entirely DNA are preferable due to greater chemical stability, lower synthetic cost and reduced ability to form intramolecular structures. Additionally, we designed two DNA strands that function as an adapter.
for SSA and demonstrated how a single universal spinach aptamer (USSA) probe can be used to
detect multiple (potentially any) nucleic acid sequences. USSA can be used for cost-efficient and
highly selective analysis of even folded DNA and RNA analytes, as well as for the readout of
outputs of DNA logic circuits.
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CHAPTER 1 : INTRODUCTION

Single nucleotide polymorphisms (SNPs)

A difference in single nucleotide is referred to as single nucleotide polymorphisms (SNPs).\(^1\) In humans, SNPs occur once in every three hundred nucleotides and most SNPs have no effect on health or development as long as the SNPs exist in the non-active transcription site.\(^1\) If the SNPs occur in the active transcription site, they can result in genetic disorder.\(^1\) In addition to humans, SNPs also occur in other living organisms. One such example is Tuberculosis (TB), a disease caused by a group of opportunistic pathogens in the Mycobacterium tuberculosis complex (MTBC), which includes M. tuberculosis (M.tb), M. canetti, M. microti, M. bovis, M. caprae, M. pinnipedi, and M. africanum.\(^2-3\) Of those, M.tb is a pathogenic bacterial species in the Mycobacteriaceae known as one of top ten causes of death worldwide. In 2016, the World Health Organization (WHO) reported 1.7 million deaths due to M.tb. Over 95% of M.tb deaths occur in developing countries wherein patients are financially unable to afford the diagnostics and anti-TB drug treatment, which takes six to nine months to fully cure.\(^4\) TB can spread by air when a patient has infection around their airway, leading to further spread of the disease. The delay in treatment and spread of disease led to evolution of multidrug-resistant tuberculosis (MDR-TB).\(^3\) MDR-TB is developed when a bactericidal drug is not able to completely cure the TB as a result of acquisition of SNPs in the genome responsible for bacterial drug resistance.\(^3\) Even though M.tb has become a curable disease, its ability spread easily by air and to mutate into multidrug-resistant tuberculosis
has caused proliferation of the bacteria. One way to stop the spread of such bacteria is to reduce the diagnostic time and to make the medical examination affordable.

Importance of identifying of viruses

Zika virus is a mosquito-borne flavivirus that was first identified in Uganda in 1947 in monkeys through a network that monitored yellow fever. It was later identified in humans in 1952 in Uganda and the United Republic of Tanzania. Outbreaks of Zika virus disease have been recorded in Africa, the Americas, Asia and the Pacific. Zika virus are spread through infected *Aedes* mosquito bites and sexual intercourse from infected individuals. The symptoms of Zika virus infection are mild and will last for 2-7 days. Currently, there is no vaccine for the disease and is cured by the body’s own defense system. Although Zika infection is not lethal to many individual, Zika infection to the fetus causes abnormality to the brain of infants. World Health Organization (WHO) has concluded that Zika virus infection during pregnancy is a cause of congenital brain abnormalities, including microcephaly; and that Zika virus is a trigger of Guillain-Barré syndrome. Therefore, identification of the Zika virus and others without vaccine is necessary to stop the spread of the disease.

Current instantaneous hybridization probes

Recombinant deoxyribonucleic acid (DNA) technology and the availability of equipment such as Next Generation Sequencing has led to development of various nucleic acid hybridization tools. Molecular beacon (MB) and Taqman probes are one of such nucleic acid hybridization
probes, which hybridize to a specific target analyte sequence by the presence of complementary nucleic acid sequences. Once they hybridize to the target, molecular marker tagged onto the hybridization probe will emit a signal that can be read by fluorimeter, colorimeter, electrometer etc. 9-14 They are used to identify the presence of specific nucleic acid sequence and are often used as markers for nucleic acid amplification.11-12, 15-17 WTO has recommended the use of Xpert MTB/RIF (Cepheid, Sunnyvale, CA, United States), a molecular beacon based assay for identifying multidrug-resistant tuberculosis (MDR-TB), for initial diagnostic on individuals suspected of having multidrug-resistant tuberculosis or HIV associated tuberculosis.18

Aptameric hybridization sensor

Aptamers are short single stranded DNA or RNA that are engineered by screening from a large oligonucleotide pool, a technique known as systematic evolution of ligands by exponential enrichment (SELEX), to bind towards specific target molecule by intermolecular affinity.19-22 Size of target molecule can range from a single molecule to a size of a single protein and whole cells.23-27 The intermolecular forces associated with aptamer and its target are attraction and repulsion forces that act between neighboring particles that includes electrostatic interactions, hydrogen bonds, aromatic stacking interactions, and Van der Waals forces.22, 24-25, 27 Aptamers were first developed in 1990 by two independent labs of Gold and Szotak.20-21 Due to the ease and quick selection process compared to the traditional antibodies, aptamers have been developed to replace antibodies by binding to the antigen.24 Aptamers are also used as hybridization probes; one such example is the Spinach Aptamer,28-29 an RNA aptamer design to mimic the activity of green
fluorescent protein. They were developed to fluorescently track RNA in vivo to investigate the complex cellular functions of mRNA, rRNA, and other various RNAs.28

Split aptameric sensor for selective nucleic acid analysis

To have aptamers functioning as a turn-on sensor, the aptamer must be turned-off in absence of the target. This is accomplished by splitting the aptamer complex in half.30 By doing so, split aptamer complexes are unable to form the target binding site unless additional external force is applied. External force could be additional intermolecular interaction from Watson Crick base pairs or additional aptameric target complex.25 Target binding site of aptamers often forms into internal bulge with a G-quadruplex structure, which forms guanine tetrads by Hoogsteen hydrogen bonds assisting in binding of the target molecule by creating channels between the pairs of tetrads.29,31-33 By separating the internal bulge, the aptameric complex loses the intermolecular force associated with the tetrads inhibiting formation of target binding pockets until the addition of the target complex occurs.30 Nucleic acid strands, which hybridize to complementary nucleic acids, herein called binding arms, are attached to the aptameric sequence responsible for forming the internal bulge.

Unlike the MB probes9-13, aptamers are not labeled with molecular marker;34-37 as such, they do not need to go through time consuming and expensive HPLC purification. This quality makes aptameric sensors attractive as a molecular diagnostic tool to replace more expensive MB and Taqman probes. By splitting the aptameric complex in two parts, the sensitivity and selectivity of
aptamer towards DNA or RNA analytes are improved, adding to the other advantages including low-cost, chemical stability, and simple modular design.

The following chapters describe the design of split aptamers of Spinach and Dapoxyl with high sensitivity and selectivity towards various DNA and RNA analytes. We have also designed universal split aptamer where addition of DNA strands act as adapters for the main split aptamer and the various target sequences, eliminating the need for re-design of the split aptamer complex for each new analyte.

List of References


CHAPTER 2 : SPLIT SPINACH APTAMER FOR HIGHLY SELECTIVE RECOGNITION OF DNA AND RNA AT AMBIENT TEMPERATURES

Preface

This first complete draft of this chapter was written by N.K. Comments from coauthors and reviewers were incorporated into the final version presented here. In this chapter, supplemental data is merged into the relevant sections of the publication.

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Abstract

Split spinach aptamer (SSA) probes for fluorescent analysis of nucleic acids were designed and tested. In SSA design, two RNA or RNA/DNA strands hybridized to a specific nucleic acid analyte and formed a binding site for DFHBI dye, which was accompanied by up to 270-fold increase in fluorescence. The major advantage of the SSA probe over state-of-the-art fluorescent probes is high selectivity: it produces only the background fluorescence in the presence of single base mismatched analyte even at room temperature. SSA is a promising tool for label-free analysis of nucleic acids at ambient temperatures.

Introduction

Hybridization probes that fluoresce upon binding to specific nucleic acid sequences (instantaneous probes) have attracted significant attention due to the possibility of immediate detection of specific nucleic acids in mix-and-read format i.e. without the need for time-consuming and effort-intensive downstream analysis (e.g. by electrophoresis).[1] Practically significant representatives of such probes include adjacent hybridization probes,[2] molecular beacon (MB) probes,[3] and their variations.[3c,4] Special emphasis is given to the development of the probes that enable RNA detection in live cells.[5] Such probes should operate under physiological conditions (pH, salt concentration, temperature) and should be selective enough to fluoresce only in the presence of specific RNA. Here, we report on new fluorescent probes that operate in the mix-and-read format. The major advantages of the probes are (i) label-free design and (ii) high selectivity of DNA and RNA recognition under physiological conditions.
The probes were designed based on the recently isolated spinach aptamer,\textsuperscript{[6]} an RNA molecule with the affinity to a low-fluorescent dye 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI, Fig. 2-6). Binding of DFHBI to the aptamer increases its fluorescence.\textsuperscript{[6]} To design the split spinach aptamer (SSA) probes, we divided the structure of spinach aptamer into two parts (SSA\textsubscript{f} and SSA\textsubscript{m}) and linked each part with an analyte-binding arm, a fragment complementary to DNA or RNA analyte (dashed lines in Fig. 2-6A). Splitting of the dye-binding core of the aptamer prevented DFHBI from binding, and the fluorescence of the dye remained low, when no nucleic acid analyte was present. Hybridization of strands SSA\textsubscript{f} and SSA\textsubscript{m} to the adjacent fragments of the analyte stabilized the DFHBI-binding site, thus resulting in tighter binding of the dye to the aptameric core, which was accompanied by the increase in fluoresce (Fig. 2-6A bottom).

Two types of probes were designed: SSA\textsuperscript{r} and SSA\textsuperscript{d}. SSA\textsuperscript{r} consisted entirely of ribonucleotides. To enable greater conformational flexibility, diuridylate (UU) linker was used to attach the analyte-binding arm of strand m to the aptamer half-core (Fig. 2-6A). The linker was crucial to prevent the interference between the hybridization of the strand to the analyte and the correct formation of the aptamer’s DFHBI binding pocket (see examples of less successful SSA designs in supporting materials, Fig. 2-1,2,3). SSA\textsuperscript{r} can potentially be expressed in live cells and used for imaging of specific mRNAs.\textsuperscript{[5]} On the other hand, for \textit{in vitro} RNA/DNA analysis, DNA probes are preferable due to greater chemical stability, lower synthetic cost and reduced ability to form intramolecular structures. Therefore, in our SSA\textsuperscript{d} design the analyte-binding arms were made of deoxyribonucleotides, and connected to the aptameric portion of SSA\textsuperscript{d\_m} via triethylene glycol linker. In this proof-of-concept study, we targeted a fragment of \textit{inhA} gene from \textit{Mycobacterium}
tuberculosis (M.tb), which contains point mutation associated with M.tb resistance to one of the key drug of tuberculosis treatment – isoniazid. In this work, a C->T mutation was targeted using the following DNA and RNA analytes: matched DNA analyte $A_d^m$ (5'-GCG GCA TGG GTA TGG GCC ACT GAC A C A ACA CAA GGA C) and a single-base mismatched $A_d^{mm}$ (5'-GCG GCA TGG GTA TGG GCC ACT GAC A T A ACA CAA GGA C), as well as their RNA counterparts $A_r^m$ and $A_r^{mm}$.

Split (binary) hybridization probes have attracted significant attention due to their high selectivity in recognition of nucleic acids at ambient temperatures,\[2,7,8\] which otherwise is difficult to achieve. In split probe design, one of the analyte-binding arms can be short (7-9 nucleotides in this study, see SI for more details) to form stable hybrid only with a perfectly matched sequence, while the entire recognition site remains long (e.g. 28 nucleotides in this study). Earlier, we introduced the strategy of split aptameric probes for nucleic acid recognition by designing split malachite green (MGA) aptamer probe.\[8a\] The probe was proven to be a versatile tool of RNA nanotechnology and synthetic biology.\[9\] The disadvantages of split MGA probe was low fluorescence intensity and strong photobleaching, which limited its practical applications. Spinach aptamer isolated by Paige et al.\[6\] has attracted significant attention both as a tool for fluorescent monitoring of endogenous RNA in live cells\[10\] and as a sensor platform for detection of biological molecules and metal ions in vitro.\[11\] Self-reconstituting split spinach aptamer constructs were designed recently for fluorescent monitoring of RNA assembly, functional imaging of viral genome trafficking and for monitoring of ribozyme activity.\[12\] However, these constructs did not operate as mismatch-selective sensors for nucleic acid analysis. Additionally, structural switching spinach
aptamer-based monolith sensors were developed for the detection of RNA and DNA. However, long RNA probes have a disadvantage of misfolding in non-functional structure, which can be as much as 80% in case of spinach aptamer, and lead to low (ca. 5) turn-on ratio. They may also respond slowly due to the time required for structural switch between the two energetically close RNA conformations. Even though spinach molecular beacon has been shown to differentiate several specific single nucleotide substitutions, in general, structural switching constructs are shown to poorly differentiate single base mismatches. Moreover, long RNA structure-switching sensors require tedious optimization and are expensive commercial products. Finally, structural switching spinach construct was able to detect only about 25 nM analytes even when sophisticated amplification strategies are implemented.

Material and Methods

All oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). DNAse/RNase free water was purchased from Fisher Scientific and used for all assays including buffers, and for dissolution of oligonucleotides. Concentrations of oligonucleotide were determined based on UV light absorption at 260 nm. DFHBI was purchased from Lucerna, Inc. (New York, NY), KCl and MgCl₂ were purchased from Fisher Scientific. Trizma Hydrochloride (Tris-HCl), pH 7.40 was purchased from Sigma Aldrich. Two 2× Spinach buffer were prepared: Spinach-20 mM 2×buffer contained 20 mM Tris-HCl, pH 7.4, 200 mM KCl, 10 mM MgCl₂; 100 mM Mg Spinach 2×buffer contained 20 mM Tris-Cl, pH7.4, 200 mM KCl, 100 mM MgCl₂. All fluorescent spectra were taken using Fluorescence Spectrometer LS55 (PerkinElmer). Otherwise noted, excitation wavelength was set to 450 nm and emission was taken at 500 nm.
Table 1 Oligonucleotides used in the study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aₘᵣ</td>
<td>5’-GCG GCA UGG GUA UGG GCC ACU GAC A_CA ACA CAA GGA C</td>
<td>SD</td>
</tr>
<tr>
<td>Aₘᵣₘₘ</td>
<td>5’-GCG GCA UGG GUA UGG GCC ACU GAC AUA ACA CAA GGA C</td>
<td>SD</td>
</tr>
<tr>
<td>Aₖᵣ</td>
<td>5’-gcg gca tgg gta tgg gcc act gac aca aca caa gga c</td>
<td>SD</td>
</tr>
<tr>
<td>Aₖᵣₘₘ</td>
<td>5’-gcg gca tgg gta tgg gcc act aₜₐ aca caa gga c</td>
<td>SD</td>
</tr>
<tr>
<td>SSAₖᵣₘ</td>
<td>5’-gtg tgt tgt /TEG/ UGG UGA AGG ACG GGU CCA GU</td>
<td>SD</td>
</tr>
<tr>
<td>SSAₖᵣₖ</td>
<td>5’-ACU GUU GAG UAG AGU GUG AGC UCC Gca gtg gcc cat acc catgc</td>
<td>SD</td>
</tr>
<tr>
<td>SSAₖᵣₖ₁</td>
<td>5’-gtg tgt tgt UGG UGA AGG ACG GGU CCA GU</td>
<td>SD</td>
</tr>
<tr>
<td>SSAₖᵣₖ₂</td>
<td>5’-GUU A_UU UGG UGA AGG ACG GGU CCA GU</td>
<td>SD</td>
</tr>
<tr>
<td>SSAₖᵣₖ₃</td>
<td>5’-GUG U_UG UUU GGU GAA GGA CGG GUC CAG U</td>
<td>SD</td>
</tr>
<tr>
<td>SSAₖᵣₖ₄</td>
<td>5’-GUU GUG UUU UGG UGA AGG ACG GGU CCA GU</td>
<td>SD</td>
</tr>
<tr>
<td>SSAₖᵣₖ₅</td>
<td>5’-ACU GUU GAG UAG AGU GUG AGC UCC GUU CAG UGG CCC AUA CC</td>
<td>SD</td>
</tr>
<tr>
<td>SSAₖᵣₖ₆</td>
<td>5’-ACU GUU GAG UAG AGU GUG AGC UCC GCA GUG GCC CAU ACC</td>
<td>SD</td>
</tr>
</tbody>
</table>

TEG-triethylene glycol linkers; SD, standard desalting; SNS sites are underlined; DNA sequences are in low case; linkers between analyte binding arms and the aptameric portion of SSA strands are shown in cyan.
1. Detailed Experimental Procedure

Fluorescent assay. For SSA\textsubscript{d}, DFHBI (2 μM) SSA\textsubscript{d,m} (3.6 μM) and SSA\textsubscript{d,f} (2 μM) strands and matched or mismatched analyte (5.5 μM) were added to 30 μL of 20 mM Mg\textsubscript{2+}×spinach buffer (20 mM Tris-Cl, pH 7.4, 200 mM KCl, 10 mM MgCl\textsubscript{2}). Total volume was adjusted to 60 μL by water. Control samples contained only DFHBI (2 μM) or DFHBI (2 μM) and SSA\textsubscript{d,m} (3.6 μM) and SSA\textsubscript{d,f} (2 μM) strands. For SSA\textsuperscript{r}, DFHBI (1 μM) SSA\textsuperscript{r,m} (3.6 μM) and SSA\textsuperscript{r,f} (2.6 μM) strands and matched or mismatched analyte (1.38 μM) were added to 30 μL of 20 mM Mg\textsubscript{2+}×spinach buffer (20 mM Tris-HCl, pH 7.4, 200 mM KCl, 10 mM MgCl\textsubscript{2}). Total volume was adjusted to 60 μL by water. Control samples contained only DFHBI (1 μM) or DFHBI (1 μM) SSA\textsuperscript{r,m} (3.6 μM) and SSA\textsuperscript{r,f} (2.6 μM) strands. All samples were incubated at room (22.5°C) temperature. Fluorescent spectra were recorded at 430-600 nm, with excitation at 450 nm after indicated incubation times. Data of three independent experiments were processed using Microsoft Excel.

Time dependence of SSA fluorescent response. DFHBI (2 μM for SSA\textsubscript{d}, 1 μM for SSA\textsuperscript{r}), SSA\textsubscript{d,m} (3.6 μM) and SSA\textsubscript{d,f} (2 μM) strands, or SSA\textsuperscript{r,m} (3.6 μM) and SSA\textsuperscript{r,f} (2.6 μM) strands and matched or mismatched analytes (275 nM each) were added to 30 μL of 2×spinach buffer and adjusted to 60 μL by addition of H\textsubscript{2}O. Fluorescence measurements were taken after 0, 1, 2, 5, 10, 25, 40, 55, 65 and 80 min. Control samples contained only DFHBI (2 μM for SSA\textsubscript{d}, 1 μM for SSA\textsuperscript{r}) or DFHBI (2 μM for SSA\textsubscript{d}, 1 μM for SSA\textsuperscript{r} and SSA\textsubscript{d,m} (3.6 μM) and SSA\textsubscript{d,f} (2 μM) strands, or SSA\textsuperscript{r,m} (3.6 μM) and SSA\textsuperscript{r,f} (2.6 μM) strands. Data of three independent experiments were processed using Microsoft Excel.

Determining Limit of Detection. DFHBI (2 μM for SSA\textsubscript{d}, 1 μM for SSA\textsuperscript{r}), SSA\textsubscript{d,m} (3.6 μM) and SSA\textsubscript{d,f} (2 μM) strands, or SSA\textsuperscript{r,m} (3.6 μM) and SSA\textsuperscript{r,f} (2.6 μM) strands and matched analyte (1.4, 5.5, 14, 28, 138, 275 nM or 5.5 μM) were added to 30 μL of 2×spinach buffer and water was added to the final volume of 60 μL. Control samples contained only DFHBI (2 μM for SSA\textsubscript{d}, 1 μM for SSA\textsuperscript{r}) or DFHBI (2 μM for SSA\textsubscript{d}, 1 μM for SSA\textsuperscript{r} and SSA\textsubscript{d,m} (3.6 μM) and SSA\textsubscript{d,f} (2 μM) strands, or SSA\textsuperscript{r,m} (3.6 μM) and SSA\textsuperscript{r,f} (2.6 μM) strands. Fluorescent spectra were measured for SSA\textsubscript{d} after 60 and for SSA\textsuperscript{r} after 30 min, respectively. Data of three independent experiments were processed using Microsoft Excel.

Selectivity Assay. DFHBI (2 μM for SSA\textsubscript{d}, 1 μM for SSA\textsuperscript{r}), SSA\textsubscript{d,m} (3.6 μM) and SSA\textsubscript{d,f} (2 μM) strands, or SSA\textsuperscript{r,m} (3.6 μM) and SSA\textsuperscript{r,f} (2.6 μM) strands and matched analyte or
mismatched analyte (275 nM each) were added to 30 μL of 2×spinach buffer and water was added to final volume of 60 μL. Control samples contained only DFHBI (2 μM for SSA$d$, 1 μM for SSA$r$) or DFHBI (2 μM for SSA$d$, 1 μM for SSA$r$) and SSA$d_m$ (3.6 μM) and SSA$d_f$ (2 μM) strands, or SSA$r_m$ (3.6 μM) and SSA$r_f$ (2.6 μM) strands. Fluorescent spectra were measured after 60 and 30 min for SSA$d$ and SSA$r$, respectively. Data of three independent experiments were processed using Microsoft Excel.

**Selectivity at 37°C.** Reaction mixtures were prepared as described for room temperature above. Fluorescence was recorded after 30 min incubation at 37°C. Data of three independent experiments were processed using Microsoft Excel.

### 2. Figure 2-1. Design of SSA

![Figure 2-1 Design of SSA](image)

A) Primary and secondary structure of spinach aptamer as it was published by Huang et al[1] and the structure of DFHBI. B) Primary and predicted secondary structure of SSA$d$ in complex with DNA analyte. Dotted line is the triethylene glycol linker that connects analyte binding arm of SSA$d_m$ with the aptameric part of the strand. Ribunucleotides are shown in upper case; deoxyribonucleotide are in low case. SNP position is indicated in red.
3. **Figure 2-2 and 2-3. Suboptimal designs of SSA.**

We tested the effect of the nature of the linkers between analyte binding arms and the aptameric part of SSA on the performance of SSA\(^d\). Fluorescent outputs of 4 different combinations of SSA strands are shown in Fig. S2. Only one combination (panel B) was able to differentiate \(A_{dm}^d\) and \(A_{mm}^d\) with modest differentiation factor. We conclude that presence of a linker in strand m is essential for providing sufficient flexibility for the formation of DFHBI-binding site in the SSA-analyte complex. At the same time, UU linker in strand f abolished fluorescence signal, which indicates that the additional flexibility in connection analyte binding arm of strand f with the aptamer reduces stability of the DFHBI-binding site.
Figure 2-2 Fluorescence complexes of SSA with the DNA analyte and responses of the four different combinations of SSA strands m and f to the presence of fully matched analyte (A^d_m) and mismatch analyte (A^d_mm). Fluorescence data shows dye only (blue), SSA_m and SSA_f (red), SSA with A^d_m (green), SSA with A^d_mm (purple). Data for each combination is shown on the right side of each panel. Each sample contained DFHBI (1 μM), SSA^d_m (3.6 μM) and SSA^d_f (2 μM) strands, or SSA^f_m (3.6 μM) and SSA^f_f (2.6 μM) strands in the absence (red bars) or presence of A^d_m (275 nM, green bars) or A^d_mm (275 nM, purple bars) in 60 μL of spinach buffer. Control sample contained DFHBI (1 μM) (blue bars). SNP sites are indicated in red. Fluorescent spectra were measured after 30 min of incubation at 22.5°C. Data of three independent experiments were processed using Microsoft Excel.

Several all-RNA constructs were tested in recognition of fully matched and single base mismatched analytes as shown in Fig S3. When m-strand was contained a 9-nucleotide analyte binding arm, a good selectivity towards DNA analyte (middle bar graph) was achieved. However,
such construct displayed only modest selectivity in recognition of RNA analytes (right bar graph). Therefore, analyte binding arm of m strand was shortened to 7 nucleotides (Fig. 2-3 B and C). This improved the selectivity towards RNA analytes as illustrated by the fluorescent response shown in Fig. 2-3 B and C, right panels. This change in the design illustrates how SSA selectivity can be adjusted to the particular experimental conditions.

Figure 2-3 Fluorescence complexes of SSAs with the RNA analyte and responses of the 3 SSA stands m, m1,m2 and SSA_f to the presence of fully matched analyte (A^m) and mismatch analyte (A^mm). Fluorescence data shows dye only (blue), SSA_m and SSA_f (red), SSA with A^m (green), SSA with A^mm (purple). DFHBI (1 μM), SSA_m (3.6 μM) and SSA_f (2.6 μM) strands and matched analyte (275 nM) or mismatched analyte (275 nM) were added to 30 μL of 2x spinach buffer and water was added to final volume of 60 μL. Control samples contained only DFHBI (1μM) or DFHBI (1 μM) SSA_m (3.6 μM) and SSA_f (2.6 μM) strands. SNP sites are indicated in red. Fluorescent spectra were measured after 30 min of incubation at 22.5°C. Data of three independent experiments were processed using Microsoft Excel.
4. **Figure 2-4. Response of SSA′ to the presence of DNA analyte**

![Figure 2-4](image)

Figure 2-4 Recognition of the DNA analyte by SSA′

A) Time dependence. B) Limit of detection. C) Selectivity. Samples contained **DFHBI** (1 μM), **SSA′_m** (3.6 μM) and **SSA′_f** (2.6 μM) strands and 275 nM **A_d_m** (green bars) or 275 nM **A_d_mm** (purple bars) in 60 μL of 1×spinach buffer. Control samples contained only **DFHBI** (1 μM) (blue bars) or **DFHBI** (1 μM), **SSA′_m1** (3.6 μM) and **SSA′_f** (2.6 μM) (red bars) strands. Fluorescent spectra were measured after 30 min of incubation at 22.5°C. Data of three independent experiments were processed using Microsoft Excel.

5. **Figure 2-5: Selectivity of SSA′ at 37°C**

![Figure 2-5](image)

Figure 2-5 Selectivity of SSAr RNA analyte at 37°C

Samples contained **DFHBI** (1 μM), **SSA′_m** (3.6 μM) and **SSA′_f** (2.6 μM) strands and 275 nM **A_d_m** (green bars) or 275 nM **A_d_mm** (purple bars) in 60 μL of 1×spinach buffer. Control samples contained only **DFHBI** (1 μM) (blue bars) or **DFHBI** (1 μM) **SSA′_m** (3.6 μM) and **SSA′_f** (2.6 μM) (red bars) strands. Fluorescent spectra were measured after 30 min of incubation at 22.5°C. Data of three independent experiments were processed using Microsoft Excel.
μM) (red bars) strands. Fluorescence measured after 30 min incubation at 37°C. The overall fluorescent intensities were lower that at 22°C due to the instability of DFHBI-binding site of spinach aptamer at 37°C.[2]

1. References


Results

Emission spectra of SSA probes recorded in the absence or presence of the fully matched analytes demonstrated turn-on ratio (Fon/Foff) up to 270 and 76 for SSA' and SSA'd probes, respectively (Fig. 1B,C), which exceeds that of the instantaneous probes currently used for nucleic acid analysis.[2,3c,7] Importantly, the increase in fluorescence was observed immediately after addition of analytes (Fig. 2-7A). Both SSA'd and SSA' demonstrated limits of detection (LODs) in the low nanomolar range: 1.8 nM for SSA'/A'm, 5.3 nM for SSA'd/A'm or 1.5 nM SSA'd/A'm (Figs. 2-7B and 2-4). These LODs fall in the range of that reported for a typical MB probe.[3c] Further we demonstrated that SSA probes are highly selective and can differentiate single base substituted analytes even at room temperature. Indeed, no fluorescence above the background was observed when fully matched analyte was replaced with a single based mismatched one (Fig. 2-8 A and B). The high selectivity of the analyte recognition can be visually monitored upon light irradiation of the samples (Fig. 2-8C). This high selectivity of
SSA was preserved at 37°C (Fig. 2-5), which might be important for future application of the probe for RNA monitoring in live cells. Overall, the performance of SSA probes is comparable with currently used state-of-the-art practically useful MB probes in terms of LOD, but SSA probes have better turn-on ratios and selectivity at ambient temperatures.[3c]

Figure 2-6 General design and fluorescent response of the split spinach aptamer (SSA) probe and the fluorescent reposed of SSA and SSA probes

A) Two strands, SSA_m and SSA_f hybridize to a specific DNA or RNA analyte and re-form a binding site for DFHBI organic dye. Binding of the dye by the aptamer results in fluorescent increase. Dashed lines represent analyte-binding arms, which were DNA in SSA or RNA in SSA. Dotted line is either diuridylate (UU) linker for SSA or triethylene glycol for SSA (see Fig. S1 for detailed design). B) Fluorescent response of SSA_m (2.6 µM), SSA_f (3.6 µM) and DFHBI dye (1 µM) in the absence or presence of fully matched analyte A (1.38 µM). Emission spectrum (λ_ex = 450 nm) were recorded after 90 min of incubation. C) Fluorescent response of SSA_m (2 µM), SSA_f (3.6 µM) and DFHBI dye (2 µM) in the absence or presence of fully matched DNA analyte A_m (5.5 µM). Emission spectrum (λ_ex = 450 nm) were recorded after 30 min of incubation.
Figure 2-7 Kinetics and limit of detection (LOD) of the SSA probes

A) Time dependence of fluorescent response of SSA$^r$ and RNA analytes (left) and SSA$^d$ and DNA analytes (right). The apparent difference in signal-to-noise ratio in comparison with Figure 1 B and C is due to the different analyte concentrations. Reaction mixtures contained: 1 μM DFHBI, 2.6 μM SSA$^r_m$ and 3.6 μM SSA$^r_f$, 275 nM RNA analytes; or 2 μM DFHBI, 2 μM SSA$^d_m$ and 3.6 μM SSA$^d_f$, 275 nM DNA analytes. B) Limits of detection (LOD) for SSA$^r$ and SSA$^d$ after 30 min of incubation. Averaged data from three independent experiments with standard deviations are presented.
Figure 2-8 Selectivity of the SSA probes

A) Fluorescence response of SSA in the presence of 100 nM of either matched (A_m) or single base mismatched (A(mm)) RNA analytes. B) Fluorescence response of SSA in the presence of 100 nM of either matched (A_m) or single base mismatched (A(mm)) DNA analytes. The data are average values of 3 independent experiments with standard deviations. C) Photograph of the SSA samples from panel B upon excitation with transilluminator. The controls samples were as follows: DFHBI, DFHBI dye only; SAA, DFHBI dye, SSA_m, and SSA_f (no analyte). The concentrations were as specified in Figure 2 legend.

Discussion

In this work, we took advantage of the recently published X-ray structure of spinach aptamer,[16] which reviled actual folding of spinach aptamer and localized the G- quadruplex -based binding site for DHFBI. Our prior attempts to design SSA based on predicted secondary structure[6] were
unsuccessful. Practically important features of SSA probes are the following. (i) High selectivity at ambient temperatures. (ii) Mix-and-read reporting format with up to 270-fold turn-on ratios, which is better than that of other mix-and-read probes including adjacent\textsuperscript{[2]} and MB probes.\textsuperscript{[3]} (iii) LOD in low nanomolar range, which is about one order of magnitude better than that for structure switching spinach sensors.\textsuperscript{[13b]} (iv) Label-free design: there is no need for conjugation of oligonucleotides with a fluorophore or quencher dye, which eliminates the need for purification of SSA strands prior to usage (note that all SSA\textsubscript{f} and SSA\textsubscript{m} stands used in this study were only desalted after solid-phase synthesis, see Table S1). (v) It is easy to tailor SSA probes for recognition of each new analyte by simple change of the analyte-binding arms. (vi) Finally, being short, RNA or RNA/DNA strands, SSA components can be conveniently obtained from industrial suppliers of custom-made nucleic acids.

In conclusion, we have designed two label-free fluorescent probes for nucleic acid analysis. The probes demonstrate superior performance in comparison with relevant state-of-the-art probes. The probes can be custom-designed and purchased as synthetic products, which makes them affordable by any laboratory. The applicability of SSA for \textit{in vivo} detection of single nucleotide differences in RNA or in PCR products are the subjects of the follow up studies.

Acknowledgements

We are grateful to Dr. Gerasimova for discussion and careful reading of the manuscript. Funding from NSF CCF (1423219) and NIH NIAID R15AI10388001A1 is greatly appreciated.
List of References


CHAPTER 3 : A UNIVERSAL SPLIT SPINACH APTAMER (USSA) FOR NUCLEIC ACID ANALYSIS AND DNA COMPUTATION

Preface

This first complete draft of this chapter was written by N.K. Comments from coauthors and reviewers were incorporated into the final version presented here. In this chapter, supplemental data is merged into the relevant sections of the publication.

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Abstract

We demonstrate how a single universal spinach aptamer (USSA) probe can be used to detect multiple (potentially any) nucleic acid sequences. USSA can be used for cost-efficient and highly selective analysis of even folded DNA and RNA analytes, as well as for the readout of outputs of DNA logic circuits.

Introduction

Instantaneous hybridization probes that fluoresce upon binding to specific nucleic acid sequences have become invaluable tools in molecular diagnostics, fundamental biological studies, as well as to analyse specific structures in DNA nanotechnology and molecular computation. All currently used representatives of such probes include strand displacement probes, adjacent hybridization and molecular beacon (MB) probes. In all these probes, fluorescently labelled oligonucleotides bind directly to the analysed DNA or RNA sequences. Such traditional approach requires design and synthesis of new labelled probe for each new target analyte. Moreover, the fluorophore-labelled probes must be HPLC purified after automated DNA synthesis, which is essential to maintain low background fluorescence. The high cost of synthesis and purification led to the development of the concept of universal hybridization probes, in which the expensive labelled oligonucleotides hybridize to nucleic acid targets via adaptor strands: unlabelled DNA strands that do not required HPLC purification. It is believed that the universal platform promises to contribute to affordable point-of-care diagnostics of infectious diseases. An additional approach for the affordable diagnostics is to use label-free probes to avoid expensive DNA-dye conjugation and
HPLC purification. This concept has already produced a number of intriguing designs.\textsuperscript{7b,9} Our recent development - split spinach aptamer (SSA) probe\textsuperscript{10} – is a highly selective label-free probe or nucleic acid detection. Two RNA strands of the SSA probe hybridize to a target DNA or RNA sequence and form a binding site for 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI).\textsuperscript{11} The fluorescence of DFHBI increases up to 270 times upon binding to the aptamer.\textsuperscript{10} Along with the label-free design, the SSA probe offers high selectivity towards single nucleotide substitutions (SNS) at ambient temperatures. However, the probe directly binds to the analysed nucleic acid, so to detect another analyte, two new RNA strands need to be optimized. Synthetic RNA strands is a relatively expensive commercial product, which may limit applicability of SSA in \textit{in vitro} assays. Here we demonstrate how SSA can be converted into a universal SSA (USSA) by binding to the analysed DNA and RNA sequences via inexpensive DNA adaptor strands. Furthermore, we constructed USSA-based logic gates for molecular computation. The proposed design is one of the most affordable and versatile instantaneous probe developed so far.

USSA takes advantage of $\text{SSA}_m$ and $\text{SSA}_f$ strands, which indirectly bind to an arbitrary nucleic acid analyte via two adaptor strands, $\text{Adp}_m$ and $\text{Adp}_f$ (Fig. 3-5A). In this design, $\text{SSA}_m$ and $\text{SSA}_f$ strands do not contact analyte and can be used universally as a reporting module given that the adaptor stands are tailored for specific recognition of each new analyzed sequence. First, we used two microRNA (miRNA) sequences - \textit{miRNA99a} (5’-AAC CCG UAG AUC CGA UCU UGU G) and \textit{miRNA100} (5’-AAC CCG UAG AUC CGA ACU UGU G) – as model analytes (Table 2). These two sequences belong to the same family of miRNAs and differ in a single nucleotide (underlined above). It was shown earlier that the level of these miRNAs changes in
case of several types of human cancer.\textsuperscript{12} We also used the DNA of these miRNA sequences, \textit{miDNA99a} and \textit{miDNA100} (Table 2) to ensure that the same USSA probe is suitable for the analysis of both RNA and DNA targets.

The design of the adaptor strands was optimized to enable efficient complex formation only in the presence of a specific miRNA analyte. For example, it was found that Adp\textsubscript{m} with 6 nucleotides complementary to SSA\textsubscript{f} produced the greatest analyte dependent fluorescent response (see Figures 3-2,3,4 for less successful designs). A triethyleneglycol (TEG) linker connected the SSA-binding arm and analyte-binding arm in Adp\textsubscript{m} was found to be essential for high analyte-dependent fluorescent response. The TEG linkers supposedly provided conformational flexibility to the multi-stranded associate to ensure proper orientation of the adaptor strands for accurate folding of the DFHBI-binding site in response to the analyte presence.

Further, we explored versatility of the USSA probe by designing nucleic acid logic gates with more sophisticated response than the signal-on USSA sensors described above. Inspired by undoubted success of modern electronic computers, we follow the others\textsuperscript{15} who believe that molecular computation can help to build smaller computers that consume less power. DNA is considered an advantageous material for building molecular computers, since a DNA computer can be easily adopted for the analysis of complex mixtures of DNA sequences. During the last decade, instantaneous hybridization probes have been used in DNA and RNA nanotechnology and molecular computation for detection of specific nucleic acid complexes. For example, MB probes was shown to be a convenient tool to report the readout of digital output of DNA logic circuits.\textsuperscript{4} Here we demonstrate how USSA can be used in DNA computation. For the proof of concept, we
chose to design a NOR gate, which in computation is considered a monotonic functionally complete operation: any logic operation can be created but connecting several NOR gates in computational circuits. According to the Boolean logic,\textsuperscript{16} NOR gate produces high output (digital 1) in the absence of inputs while addition of at least one or both of the two inputs (I\textsubscript{1} or I\textsubscript{2}) results in a lost of a signal (digital 0). The design of USSA NOR gate is shown in Figure 4A. Four DNA strands form a florescent complex in the absence of input strands. The complex is stabilized by hybridization of \texttt{Adp\_f\_NOR1} and \texttt{Adp\_m\_NOR1} strands. I\textsubscript{1} or I\textsubscript{2} would bind \texttt{Adp\_f\_NOR1} and/or \texttt{Adp\_m\_NOR1} and destabilize the complex. Indeed, fluorescent response of this construct corresponds to the expected digital behavior of NOR gate with high signal produced only in the absence of inputs (Fig. 3-8B). This example demonstrates of USSA can be easily tailored for the desired application including molecular computation.

Material and Methods

1. Materials and instruments. All oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). DNAse/RNase free water was purchased from Fisher Scientific and used for all assays including buffers, and for dissolution of oligonucleotides. Concentrations of oligonucleotide were determined based on UV light absorption at 260 nm. DFHBI was purchased from Lucerna, Inc. (New York, NY), KCl and MgCl\textsubscript{2} were purchased from Fisher Scientific. Trizma Hydrochloride (Tris-HCl), pH 7.40 was purchased from Sigma Aldrich. Two 2× Spinach buffer were prepared: Spinach-20 mM 2×buffer contained 20 mM Tris-HCl, pH 7.4, 200 mM KCl, 10 mM MgCl\textsubscript{2}; 100 mM Mg Spinach 2×buffer contained 20 mM Tris-HCl, pH 7.4, 200 mM KCl, 100 mM MgCl\textsubscript{2}. All fluorescent spectra were taken using Fluorescence Spectrometer LS55 (PerkinElmer). Otherwise noted, excitation wavelength was set to 450 nm and emission was taken at 500 nm.
Table 2 Oligonucleotides used in this study

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3. **Detailed Experimental Procedure**

**General Fluorescent assay for miDNA and miRNA.** For DNA analyte, DFHBI (1 μM) SSA\_m (3.6 μM) and SSA\_f (2 μM) strands, Adp\_m (0.8 μM) and Adp\_f (4.0 μM) and matched or mismatched analyte were added to 30 μL of 20 mM Mg\textsubscript{2+} spinach buffer (20 mM Tris-Cl, pH 7.4, 200 mM KCl, 10 mM MgCl\textsubscript{2}). Total volume was adjusted to 60 μL by water. Control samples contained only DFHBI (1 μM) or DFHBI (1 μM) and SSA\_m (3.6 μM) and SSA\_f (2 μM) strands. For RNA analyte, DFHBI (0.5 μM) SSA\_m (3.6 μM) and SSA\_f (2.0 μM) strands and matched or mismatched analyte (1 μM) were added to 30 μL of 100 mM Mg\textsubscript{2+} spinach buffer (20 mM Tris-Cl, pH 7.4, 200 mM KCl, 100 mM MgCl\textsubscript{2}). Total volume was adjusted to 60 μL by water. Control samples contained only DFHBI (0.5 μM) or DFHBI (0.5 μM) SSA\_m (3.6 μM) and SSA\_f (2.0 μM) Adp\_m (0.8 μM) and Adp\_f (4.0 μM) strands. All samples were incubated at room (22.5°C) temperature. Fluorescent spectra were recorded at 430-600 nm, with excitation at 450 nm after indicated incubation times. Data of three independent experiments were processed using Microsoft Excel.

**General Fluorescent assay for Mtb.F11 and Mtb.KZN.** DFHBI (2 μM) SSA\_m (3.6 μM) and SSA\_f (2 μM) strands, Adp\_m (0.8 μM) and Adp\_f (4.0 μM) and matched or mismatched analyte were added to 30 μL of 100 mM Mg\textsubscript{2+} spinach buffer (20 mM Tris-Cl, pH 7.4, 200 mM KCl, 100 mM MgCl\textsubscript{2}). Total volume was adjusted to 60 μL by water. Control samples contained only DFHBI (2 μM) or DFHBI (2 μM) and SSA\_m (3.6 μM) and SSA\_f (2 μM) strands. All samples
were incubated at room (22.5°C) temperature. Fluorescent spectra were recorded at 430-600 nm, with excitation at 450 nm after indicated incubation times. Data of three independent experiments were processed using Microsoft Excel.

**Time dependence of USSA fluorescent response.** DFHBI, SSA_m and SSA_f strands, Adp_m and Adp_f and matched or mismatched analytes (400 nM for DNA analyte, 100 nM for RNA analyte) were added to 30 μL of 2× spinach buffer and adjusted to 60 μL by addition of H2O. Fluorescence measurements were taken after 0, 1, 2, 5, 10, 25 and 40 min. Control samples were ran parallel with the samples. Data of three independent experiments were processed using Microsoft Excel.

**Determining Limit of Detection.** DFHBI, SSA_m and SSA_f strands, Adp_m and Adp_f and matched DNA analyte (1.4, 5.5, 14, 28, 138, 275 nM or 5.5 μM) or matched RNA analyte (0.5, 2, 5, 10, 50, 100, 250 nM, 1 μL) were added to 30 μL of 2× spinach buffer and adjusted to 60 μL by addition of H2O. Fluorescent spectra were measured after 30 min. Control samples were ran parallel with the samples. Data of three independent experiments were processed using Microsoft Excel.

**Selectivity Assay.** For miDNA and miRNA, DFHBI, SSA_m and SSA_f strands, Adp_m and Adp_f, matched or mismatched analyte (100 nM) were added to 30 μL of 2× spinach buffer and adjusted to 60 μL by addition of H2O. For Mtb.F11 and Mtb.KZN, DFHBI, SSA_m and SSA_f strands, Adp_m and Adp_f, matched or mismatched analyte (200 nM) were added to 30 μL of 2× spinach buffer and adjusted to 60 μL by addition of H2O. Control samples were ran parallel with the samples. Fluorescent spectra were measured after 30 min. Data of three independent experiments were processed using Microsoft Excel.

**NOT and NOR logic gates.** DFHBI (0.5 μM), SSA_m and SSA_f strands (2.0 μM), Adp_f_NOR1 (1.0 μM) and Adp_m_NOR1 (0.3 μM), Input I1 and I2 (1.0 μM) were added to 30 μL of 100mM Mg 2× spinach buffer and adjusted to 60 μL by addition of H2O. Control samples were ran parallel with the samples. Fluorescent spectra were measured after 30 min. Data of three independent experiments were processed using Microsoft Excel.
4. Figure 3-1. Design of USSA

Design of USSA for miRNA analyte indicated as follows; SSA-black, adp_m-blue, adp_m-orange. Dotted black line is the triethylene glycol linkers. Ribunucleotides are shown in upper case; deoxyribonucleotide are in low case. SNP location on miRNA underlined.

We initially designed the adapter strands as indicated in Figure 3-2A but showed no selectivity towards fully matched analyte. Therefore strand f (Adp_m in Figure 3-1 and the main paper) crossed over and was made complementary to SSA_f by 4 nucleotides which reduced the fluorescence signal of single mismatched analyte (Figure 3-2B). We also inserted a triethyleneglycol (TEG) linker connecting the SSA binding arm and the analyte binding arm which allowed great increase in the selectivity of the USSA (Figure 3-3C). Magnesium concentration in Spinach buffer was increased from 5 mM to 50 mM which allowed greater stability for hybridization which inducing higher signal for fully matched analyte (Figure 3-4). For any TEG containing strand combination, lower concentration of adapter-f was used which allowed lowering single mismatch analyte signal (Figure 3-4). We concluded that USSA in which strand_f is
complementary to SSA_f by 6 nucleotides produced the greatest analyte dependent fluorescent increase (Figure 3-4).

5. Figure 3-2: Suboptimal designs of USSA analyzing Mtb. F11/KZN analyte

![Figure 3-2](image)

Figure 3-2 Fluorescence complexes of USSA with *inhA* DNA analyte and responses of the two different combinations of stands m and f.

Data for each combination is shown on the right side of each panel. Samples contained 3.6 µM SSA_f; 2.0 µM SSA_m; 4.0 µM Adp_f; 4.0µM Adp_m; 6.0 µM analyte; 20 µM DFHBI; in Spinach Buffer. Emission spectrum (excitation 450 nm) were collected after 30 min at 22.5°C. Fluorescence data shows dye only (blue), dye and USSA (red), dye and USSA with Am (green), dye and USSA with Amm (purple). SNP sites are indicated in red.

Figure 3-3: Suboptimal designs of USSA analyzing Mtb. F11/KZN analyte

![Figure 3-3](image)

Figure 3-3 Fluorescence complexes of USSA with *inhA* DNA analyte and responses of the three different combinations of stands m and f.

Data for each combination is shown on the right side of each panel. Samples contained 3.6 µM SSA_f; 2.0 µM SSA_m; 4.0 µM Adp_f; 4.0µM Adp_m; 6.0 µM analyte; 10 µM DFHBI; in
Spinach Buffer. Emission spectrum (excitation 450 nm) were collected after 30 min at 22.5°C. Fluorescence data shows dye only (blue), dye and USSA (red), dye and USSA with A_m (green), dye and USSA with A_{mm} (purple). SNP sites are indicated in red.

**Figure 3-4: Suboptimal designs of USSA analyzing Mtb. F11/KZN analyte**

![Figure 3-4](image)

Figure 3-4 Fluorescence complexes of USSA with *inhA* DNA analyte and responses of the two different combinations of stands m and f.

Data for each combination is shown on the right side of each panel. Samples contained 3.6 µM SSA_f; 2.0 µM SSA_m; 0.4 µM Adp_f; 4.0µM Adp_m; 6.0 µM analyte; 5 µM DFHBI; in 50 mM Mg-Spinach Buffer. Emission spectrum (excitation 450 nm) were collected after 30 min at 22.5°C. Fluorescence data shows dye only (blue), dye and USSA (red), dye and USSA with A_m (green), dye and USSA with A_{mm} (purple). SNP sites are indicated in red.

**Results**

USSA produced fluorescent output immediately (in ~10 sec) after mixing (Fig. 3-5B). Typically, hybridization for instantaneous probes is completed in 15-25 min, while for the USSA the plateau was not reached even after 3 hrs of incubation (Fig. 3-5B). Longer equilibration time for USSA is the result of complex multi-stranded design. Even though traditionally probes that equilibrate faster are considered advantageous,^{12} recently we have reported that slow hybridization kinetics favor differentiation of SNS at ambient temperatures^{13} and, therefore, can be advantageous for room-temperature diagnostic assays.
USSA produced high fluorescence in the presence of fully matched DNA and RNA analytes (Figure 3-6A). Importantly, a single base mismatched miDNA100 and miRNA100 produced signal only slightly above the background resulting in a differentiation factor (DF) of 152 and 84, respectively (Fig. 3-6A). To demonstrate that SSA_m and SSA_f strands can be used for the analysis of other DNA and RNA sequences, we chose another practically significant, but otherwise arbitrary DNA sequence of *Mycobacterium tuberculosis* (*Mtb*) *rpoB* gene. Point mutation in this gene are known to confer the bacteria to be resistant to antibiotic rifampin. We made the analyte-binding arms of USSA to recognize the fragment of wild type *rpoB* gene (F11 analyte). As a single-base mismatched analyte (KZN) we chose a sequence with C>T mutation in 526 codon of the gene. Only one change was made to adopt USSA to the new analytes: the analyte-binding arms of the new adaptor strands Adp_m2 and Adp_f2 were made to be complementary to F11 (Fig. 3-7A). The new construct was found to retain high selectivity of the analyte recognition with DF = 61 (Figure 3-7B).
Figure 3-5 Design of universal split spinach aptamer (USSA) and its fluorescent response

A) SSA_m and SSA_f hybridize to the analyte via adaptor strands Adp_m and Adp_f and re-form a binding site for DFHBI, which is accompanied by increase in fluorescence. Single nucleotide substitution (SNS) is shown as a red bar (see Fig. S1 for more details). B) Fluorescence response of the probe to the presence of 0.8 µM miRNA or miDNA 99a analyte within over 180 min. Samples contained 0.5 µM DFHBI, 3.6 µM SSA_f, 2.0 µM SSA_m, 4.0 µM Adp_f, 0.8 µM Adp_m in the absence (no analyte) or presence of either 0.8 µM miDNA99a or 0.8 µM miRNA99a analytes. Emission was registered at 500 nm (λ_ex = 450 nm).
Figure 3-6 Selectivity (A) and limits of detection (B) for USSA with miRNA and miDNA analytes

A) Selectivity and signal to background ratio of USSA. Composition of the hybridization samples were from left to right; dye only, dye and USSA, dye and USSA with A_m, dye and USSA with A_mm. Emission at \( \lambda = 500 \text{ nM} \) (\( \lambda_{\text{ex}} = 450 \text{ nm} \)) were collected after 60 min of incubation at room temperature.
Figure 3-7 USSA for highly selective recognition of alternative analytes

A) Design of USSA for hybridization to F11 and KZM. Deoxyribonucleotides are shown in low case. Non-canonical base pair are shown by dotes are reported earlier.\textsuperscript{11b} Dashed lines represents triethylene glycol linkers.  B) Selectivity of USSA with Mtb. F11/KZN analyte. The samples contained 1.0 µM SSA\_f; 1.0 µM SSA\_m; 0.05 µM Adp\_f; 1.0 µM Adp\_m; 6 µM DFHBI in the absence (bar 2) or presence of 0.2 µM. F11 or KZN analytes. Bar 1 (control) is demonstrates fluorescence of 6 µM DFHBI. Fluorescent response at 500 nm (\(\lambda_{\text{ex}} = 450\) nm) was collected after 30 min of incubation at room temperature (22\(^{\circ}\)C).
Figure 3-8 Application of USSA for instantaneous detection of output NOR DNA logic gate

A) Design: in the absence of DNA inputs $I_1$ and $I_2$ the association of the 4 strands SSA_m, SSA_f, Adp_F_NOR1, Adp_m_NOR1 forms stable DFHBI binding-site, which is destabilized in the presence of either $I_1$ or $I_2$ or both inputs. Deoxyribonucleotides are shown in low case. Dashed lines represent triethylene glycol linkers. B) Fluorescent response of NOR gate. The samples contained 2.0 µM SSA_m; 2.0 µM SSA_f; 1 µM Adp_F_NOR1; 0.3 µM Adp_m_NOR1; 1 µM Input $I_1$ and $I_2$; 0.5 µM DFHBI. Fluorescent response at 500 nm ($\lambda_{ex} = 450$ nm) was collected after 30 min of incubation at room temperature.

Discussion

In this study we introduced a universal probe that can detect potentially any nucleic acid sequence with high signal-to-background response and high selectivity even at ambient temperature. The only adjustment required for the analysis of each new analyte is the sequence of two label-free adaptor strands. The construct is versatile, which was demonstrated by converting it into a NOR DNA logic gates for molecular computation in straightforward and low-cost format. We hope that
USSA described here will become an affordable multipurpose alternative to molecular beacon probes.

Acknowledgements

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List of References


12 (a) C. Li, Y. Gao, K. Zhang, J. Chen, S. Han, B. Feng, R. Wang and L. Chen, Cell. Physiol. Biochem., 2015, 6, 2143–2159; (b) C. Qin, R. Y. Huang and Z. X. Wang, Tumor Biol., 2015, 3,


CHAPTER 4: SPLIT BINARY DAPOXYL APTAMER FOR DETECTION OF SEQUENCE SPECIFIC NUCLEIC ACIDS

Abstract

There has been numerous fluorescent light up RNA aptamers often used to visualize cellular activity in vivo. DNA light up aptamer, having greater chemical stability compared to RNA aptamers, are useful when analyzing biological fluid and extracellular molecular imaging. Herein, we developed a split DNA aptamer (SDA) designed from modified DAP-10. Two DNA strands of SDA hybridize to a specific nucleic acid and reforms the Dapoxyl dye binding site. Dapoxyl than emits fluorescence towards target nucleic acid sequence with single nucleotide resolution as seen by turn-on ratio up to 69. SDA is a cheap, quick, and stable tool that will function as an alternative to the current hybridization probes.

Introduction

Sequence specific fluorescence sensors such as Molecular Beacon (MB) and Taqman probes have been widely used in real time PCR for sequence specific detection of nucleic acids. However, these probes are expensive due to conjugation of the fluorophore to the probe followed by HPLC purification. Also these probes are known to have high background fluorescence due to the incomplete quenching. Label-free aptameric probes have been
developed to eliminate the need for separation, purification, and unexpected cleavage or conformational changes often seen in labeled oligonucleotide.\textsuperscript{28, 51}

Earlier we have developed the concept of split aptameric probes for nucleic acid analysis (Scheme 1A).\textsuperscript{52} It takes advantage of the aptamers that can bind a non-fluorescent dyes and increase their fluorescence.\textsuperscript{28-29, 52} Binary aptameric probe consists of two unlabeled nucleic acid strands that bind a complementary DNA or RNA analyte sequence and forms a dye-binding site. Following dye binding increases sample fluorescence. Previously published constructs used either RNA – based aptamers\textsuperscript{31, 53} including malachite green\textsuperscript{9, 33, 54-57} and spinach.\textsuperscript{28, 51} However, RNA are high cost and has less chemical stability compared to DNA

Recently a DNA aptamer, DAP-10, was selected to bind daphoxyl dyes (Scheme 1C).\textsuperscript{47, 58} Daphoxyl dye is a water-soluble fluorescent solvatochromic dye with long emission wavelength, large extinction coefficient, high fluorescence quantum yield, large Stokes shift and excellent photostability.\textsuperscript{58} Daphoxyl dyes are highly environment sensitive being weakly fluorescent in water but are strongly fluorescent upon constraints such as certain organic solvents (e.g. acetone).\textsuperscript{58} Kato, \textit{et al.} develop a DNA aptamer which restricts the rotational conformational changes of Daphoxyl dye in water, allowing greater than 722-fold fluorescence enhancement and a substantially low $K_d$ value of 7.6 ± 1.2 nM.\textsuperscript{47} Furthermore, DAP-10 was designed as to function as an turn-on label-free sensor by fusing analyte binding aptamer to the DAP-10 via short stem and obtained fluorescence enhancement turn-on signal ($F/F_0$) of 12.4.\textsuperscript{47}

In this study, the hybridization probes were designed based on DAP-10. DAP-10 was divided into two strands (SDA\textsubscript{f} and SDA\textsubscript{m}) and analyte binding arm was attached to each component
(Scheme 1A, B). Splitting dye-binding loop of the aptamer prevented Dapoxyl dye from binding to two aptamer strands, and maintained minimum background signal. The analyte binding arm of SDA_f and SDA_m hybridizes to its complimentary nucleic acid sequence on the analyte, thus reforming the dapoxyl binding site leading to fluorescence. One analyte binding arm is composed of short DNA sequences to enables differentiation of fully matched oligonucleotide toward single nucleic acid polymorphism (SNP) at room temperature thus selectivity is achieved. Also, the analyte binding arms were attached with a linker, spacer made of polyetheneglycal (PEG) or dinucleotide uninvolved in Watson Click pairing towards the neighboring nucleotides, to give flexibility when hybridizing to an analyte. Sequence specific fluorescence sensors such as Molecular Beacon (MB) and Taqman Probe have been widely used for real time detection of sequence specific nucleic acid, small molecules and proteins. However, preparation of these probes has been expensive and time consuming due to conjugation of the fluorophore to the probe resulting in separation and purification. Also MB commonly has high background fluorescence and incomplete quenching. Label-free aptameric probes have been developed to eliminate the need for separation, purification, and unexpected cleavage or conformational changes often seen in labeled oligonucleotide. Furthermore, ‘turn-on’ label-free probes have low background signal, fluorophore alone is non-fluorescent, no quenching necessary and oligonucleotide: dye ratio can easily be optimized. There has been various turn-on label-free RNA aptamer selected like malachite green and spinach. However, RNA are high cost and has less chemical stability compared to DNA.
Scheme 1 Design of SDA and structure of Dapoxyl fluorophores

A) General Scheme of SDA. SDA_m (blue) contains triethylene glycal linker indicated in dashed line. SDA_f (purple) contains dinucleotide linker indicated in solid buldge. B) Detailed sequence of SDA with fully matched analyte (A_m). SNP site is indicated in red. C) Structure of Dapoxyl-F (top) Dapoxyl-SEDA (bottom)

Among the different designs and length of linker tested, using dithymine linker on one SDA and triethylene glycol linker on the other SDA gave the best fluorescence turn-on signal (Figure 1). We have synthesized dapoxyl sulfonyl fluorine (dapoxyl-F), the intermediate product of dapoxyl sulfonyl ethylenediamine synthesis procedure, and Dapoxyl sulfonyl ethylenediamine (dapoxyl-SEDA) as part of label free split DNA aptamer probe (SDA). These dyes contain electron-donating dimethylamino group at position 4 of the 5-phenyl ring and electron-withdrawing
sulfonyl group at position 4 of the 2-phenyl ring creating an electron transfer system from the 5-phenyl to the 2-phenyl ring.\textsuperscript{47} In this proof-of-concept study, we targeted a fragment of \textit{inhA} gene from \textit{Mycobacterium tuberculosis} (\textit{Mtb}), which contains point mutation associated with \textit{Mtb} resistance to one of the key drug of tuberculosis treatment – isoniazid. In this work, a C->T mutation was targeted using the following DNA analytes: matched DNA analyte $A_m$ (5’-GCG GCA TGG GTA TGG GCC ACT A C A ACA CAA GGA C) and a single-base mismatched $A_{mm}$ (5’-GCG GCA TGG GTA TGG GCC ACT GAC A \textsuperscript{T} A ACA CAA GGA C) The locations of the SNP are underlined.

Material and Methods

All oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). DNAse free water was purchased from Fisher Scientific and used for all assays including buffers, and for dissolution of oligonucleotides. Concentrations of oligonucleotide were determined based on UV light absorpt ion at 260 nm. 2-(-4’-Fluorosulfonylbenzoylamino)-4’-dimethylaminoacetophenone (\textit{dapoxyl-SEDA}) was synthesized in the laboratory. KCl and MgCl\textsubscript{2} were purchased from Fisher Scientific. Trizma Hydrochloride (Tris-HCl), pH 7.40 was purchased from Sigma Aldrich. Spinach buffer contained 20 mM Tris-HCl, pH 7.4, 200 mM KCl, 10 mM MgCl\textsubscript{2}. All fluorescent spectra were taken using Fluorescence Spectrometer LS55 (PerkinElmer). Otherwise noted, excitation wavelength was set to 390 nm and emission was taken at 505 nm.

Table 3 Oligonucleotides used in this study

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iSp9, triethylene glycol linkers; iSp18, triethylene glycol linkers; SD, standard desalting; SNS sites are underlined; RNA sequences are in upper case; linkers between analyte binding arms and the aptameric portion of SSA strands are shown in cyan.

**Detailed Experimental Procedure**

**General Fluorescent assay for inhA Analyte.** Dapoxyl-SEDA (0.32 μM) SDA_m (1 μM) and SDA_f (1 μM) strands, and matched or mismatched analyte were added to 30 μL of Spinach buffer (20 mM Tris-Cl, pH 7.4, 200 mM KCl, 10 mM MgCl₂). Total volume was adjusted to 60 μL by water. Control samples contained only dapoxyl-SEDA (0.32 μM) or dapoxyl-SEDA (0.32 μM) and SDA_m (1 μM) and SDA_f (1 μM) strands. All samples were incubated at room (22.5°C) temperature. Fluorescent spectra were recorded at 450-600 nm, with excitation at 390 nm after indicated incubation times. Data of three independent experiments were processed using Microsoft Excel.

**General Fluorescent assay for DAP-10.** Dapoxyl-F (0.5 μM) and DAP-10 (0.58 μM) were added to 30 μL of Spinach buffer (20 mM Tris-Cl, pH 7.4, 200 mM KCl, 10 mM MgCl₂). Total volume was adjusted to 60 μL by water. Control samples contained only dapoxyl-F (0.5 μM). All samples
were incubated at room (22.5°C) temperature. Fluorescent spectra were recorded indicated incubation times. Data of three independent experiments were processed using Microsoft Excel.

**General Fluorescent assay for Amel X and Amel Y.** Dapoxyl-F (0.5 μM) amel_m (1 μM) and amel_f (1 μM) strands, and Amel X or Amel Y were added to 30 μL of Spinach buffer (20 mM Tris-Cl, pH 7.4, 200 mM KCl, 10 mM MgCl₂). Total volume was adjusted to 60 μL by water. Control samples contained only dapoxyl-F (0.5 μM) or dapoxyl-F (0.5 μM) and amel_m (1 μM) and amel_f (1 μM) strands. All samples were incubated at room (22.5°C) temperature. Fluorescent spectra were recorded after indicated incubation times. Data of three independent experiments were processed using Microsoft Excel.

**General Fluorescent assay for LAMP_Amel Y 152.** Dapoxyl-F (0.5 μM), LAMP_m (1 μM) and LAMP_f (1 μM) strands, and pcr amplified LAMP_Amel Y152 analyte were added to 30 μL of Spinach buffer (20 mM Tris-Cl, pH 7.4, 200 mM KCl, 10 mM MgCl₂). Total volume was adjusted to 60 μL by water. Control samples contained only dapoxyl-F (0.5 μM) or dapoxyl-F (0.5 μM) and LAMP_m (1 μM) and LAMP_f (1 μM) strands. All samples were incubated at room (22.5°C) temperature. Fluorescent spectra were recorded after indicated incubation times. Data of three independent experiments were processed using Microsoft Excel.

**Time dependence of SDA fluorescent response.** Dapoxyl-SEDA (0.32 μM) SDA_m (1 μM) and SDA_f (1 μM) strands, and matched or mismatched analyte (0.25 μM) were added to 30 μL of Spinach buffer (20 mM Tris-Cl, pH 7.4, 200 mM KCl, 10 mM MgCl₂). Total volume was adjusted to 60 μL by water. Control samples contained only dapoxyl-SEDA (0.32 μM) or dapoxyl-SEDA (0.32 μM) and SDA_m (1 μM) and SDA_f (1 μM) strands. Fluorescence measurements were taken
every 10 sec for 80 min or after 10, 30, 50, 70, 90, 110, 130, 150, 170, 200, 230, and 260 min. All samples were incubated at room (22.5°C) temperature.

**Determining Limit of Detection.** Dapoxyl-SEDA (0.32 μM) SDA_m (1 μM) and SDA_f (1 μM) strands, and matched (0.5, 2, 5, 10, 50, 100, and 250 nM) or mismatched analyte (0.5, 2, 5, 10, 50, 100, and 250 nM) were added to 30 μL of Spinach buffer (20 mM Tris-Cl, pH 7.4, 200 mM KCl, 10 mM MgCl₂). Total volume was adjusted to 60 μL by water. Control samples contained only dapoxyl-SEDA (0.32 μM) or dapoxyl-SEDA (0.32 μM) and SDA_m (1 μM) and SDA_f (1 μM) strands. Fluorescent spectra were measured after 60 min. Control samples were ran parallel with the samples. Data of three independent experiments were processed using Microsoft Excel.

**Selectivity Assay.** Dapoxyl-SEDA (0.32 μM) SDA_m (1 μM) and SDA_f (1 μM) strands, and matched or mismatched analyte (0.25 μM) were added to 30 μL of Spinach buffer (20 mM Tris-Cl, pH 7.4, 200 mM KCl, 10 mM MgCl₂). Total volume was adjusted to 60 μL by water. Control samples contained only dapoxyl-SEDA (0.32 μM) or dapoxyl-SEDA (0.32 μM) SDA_m (1 μM) and SDA_f (1 μM) strands. All samples were incubated at room (22.5°C) temperature. Fluorescent spectra were recorded after 60 min incubation. Data of three independent experiments were processed using Microsoft Excel.
**PCR amplification for LAMP_Amel Y 152.** LAMP_Amel Y 152 (1 nM) was PCR amplified for 21 cycles [15 sec, 94°C: 30 sec, 47.7°C: 60 sec, 68°C]. PAGE gel was ran to confirm the PCR product. Asymmetric PCR was run with 20:1 ratio of Primers.

Table 4 Oligonucleotides used in PCR

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| LAMP Primer 1   | cca gtt taa gct ctg atg gtt | SD |
| LAMP Primer 2   | ttt tgc cet ttc atg gaa c | SD |
### LAMP protocol

Table 5 Oligonucleotides used in LAMP

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### NASBA protocol

PAGE gel (Figure SI 10)

NABA reaction prepared by master mix for the purpose of two controls and 5 different concentrations for each target of 10 kb RNA of Zika Virus (MR-766) for the purpose of antisense amplification of 84 bases target and 141 bases target. 1pg ,0.1 pg, 0.01 pg, 0.001pg and 0.0001 pg concentrations of 10 Kb RNA of zika virus (MR-766) were prepared in DNase, RNase and Protease free water (Fisher; Catalog No. BP2484-100), in ice path. Master mix of 46.9 µL of 3X
NASBA reaction buffer for NEC-1-24 (Lifescience; SKU: NEBC-24), 23.1 µL of 6X nucleotide mix for NEC-1-24 (Lifescience; SKU: NECN-24), 7 µL of 5 µM forward primer1 (5’-AAT TCT AAT ACG ACT CAC TAT AGG GAG AAG GGC ATA GCG GGA TGA TTG GAT A-3’), and 7 µL of 5 µM reverse primer84 (5’-GCT CTT GGT GAA TTA GGC GTA-3’) in case of targeting antisense amplification of 84 bases; reverse primer 141 (5’-CCT GTC CTT GGT TCA CAG TC-3’) in case of targeting antisense amplification of 141 bases, were mixed using mini-centrifuge then partitioned on 7 microtubes as 12 µL of the mixture in each tube then 3 µL of DNase, RNase and Protease free water was added to each of the two controls tubes, while 1.6 µL of 0.0001 pg, 0.001 pg, 0.01 pg, 0.1 pg and 1 pg Zika Virus RNA was added and 0.4 µL DNase, RNase and Protease free water were added to the five reaction tubes in ascending order, consequently. All the tubes were incubated for 2 minutes at 65 °C for annealing purpose then cooled down in 41°C water path for 10 minutes, followed by adding 5 µL of NASBA enzyme cocktail of wet kit for NEC-1-24 (Lifescience; SKU: NEC-1-24) for each tube and incubate in 41 °C for 90 minutes. Contents of analyte tubes were mixed after incubation in one tube. Concentration of the NASBA reacted analyte mixture was measured using Promega Quantus™ Fluorometer (Reference: E6150). Gel electrophoresis was performed after the 90 minutes incubation, using 2% agarose gel where agarose of molecular biology grade (Fisher; Catalog No. BP160-100) was dissolved by microwaving for 1 minute in 1xTBE buffer (89mM Tris, 89 mM Boric acid, and 2 mM EDTA) that was previously prepared in DNase and Protease free water (Fisher Catalog No. BP2470-1) and casted in owl gel system and left to cool. Once the gel solidified, samples were prepared for gel loading. The marker prepared by mixing 2 µL (1 µg) 50-1000 bases ssRNA Ladder (500 µg/mL) 3 µL (DNase, RNase, and Protease free water), and 5 µL 2X RNA ladder loading buffer
(BioLabs, Catalog No. N0364S) then heated at 65 °C for 5 minutes followed by chill in ice for 2 minutes then loaded in the gel. Negative Controls and NASBA products were prepared for loading by mixing 5 µL of each with 5 µL of 2X RNA ladder loading buffer (BioLabs, Catalog No. N0364S) then heated at 65 °C for 5 minutes followed by chill in ice for 2 minutes then loaded in the gel. Gel electrophoresis was performed in 1X TBE buffer and 75 volts. Gel was imaged by Bio Rad molecular imager (Model No. Universal Hood II).

**Experimental Procedure for NASBA Purification**

NASBA product was mixed with Chloform/Phenol as 1:1 volume ratio, followed by vortex for 30 sec and centrifuged for 10 minutes. The supernatant was incubated with 1.4 mL of 2% lithium perchlorate in acetone for 1 hour at -20 °C followed by centrifuging for 10 minutes and drying the precipitate and re-dissolve it in DNase, RNase, and Protease free water.
Figure SI 10. Gel electrophoresis of NASBA amplified Zika Virus' (MR-766) RNA in 2% Agarose stained with GelRed. The Marker of the gel is 50-1000 SSRNA. A1, and A2: Negative controls of NASBA reaction that targeted antisense amplification of 84 bases; these two controls contain Forward primer 1 (0.25 µM), reverse primer 84 (0.25 µM), 6.7 µL 3X NASBA reaction buffer for NEC-1-24 (TrisHCl, pH 8.5@ 25ºC; MgCl₂; KCl; DTT; Dimethyl sulfoxide), 3.3 µL 6X nucleotide mix for NEC-1-24, and 5 µL NASBA enzyme cocktail of wet kit for NEC-1-24 (Avian Myeloblastosis Virus Reverse Transcriptase (AMV RT), RNase H and T7 RNA Ploymerase (T7RNAP) in high molecular weight sugar matrix), 3 µL DNase, RNase and protease free water. B1, and B2: Negative controls of NASBA reaction that targeted antisense amplification of 141 bases; these two controls contain Forward primer 1 (0.25 µM), reverse primer 141 (0.25 µM), 6.7 µL 3X NASBA reaction buffer for NEC-1-24 (TrisHCl, pH 8.5@ 25ºC; MgCl₂; KCl; DTT;
Dimethyl sulfoxide), 3.3 µL 6X nucleotide mix for NEC-1-24, and 5 µL NASBA enzyme cocktail of wet kit for NEC-1-24 (Avian Myeloblastosis Virus Reverse Transcriptase (AMV RT), RNase H and T7 RNA Ploymerase (T7RNAP) in high molecular weight sugar matrix), 3 µL DNase, RNase and protease free water. A: NASBA reaction that targeted antisense amplification of 84 bases; it contains Forward primer 1 (0.25 µM), reverse primer 84 (0.25 µM), 6.7 µL 3X NASBA reaction buffer for NEC-1-24 (TrisHCl, pH 8.5@ 25ºC; MgCl²; KCl; DTT; Dimethyl sulfoxide), 3.3 µL 6X nucleotide mix for NEC-1-24, 0.02 pg/µL of 10 kb Zika Virus’ RNA (MR-766), and 5 µL NASBA enzyme cocktail of wet kit for NEC-1-24 (Avian Myeloblastosis Virus Reverse Transcriptase (AMV RT), RNase H and T7 RNA Ploymerase (T7RNAP) in high molecular weight sugar matrix), 0.4 µL DNase, RNase and protease free water. B: NASBA reaction that targeted antisense amplification of 84 bases; it contains Forward primer 1 (0.25 µM), reverse primer 84 (0.25 µM), 6.7 µL 3X NASBA reaction buffer for NEC-1-24 (TrisHCl, pH 8.5@ 25ºC; MgCl²; KCl; DTT; Dimethyl sulfoxide), 3.3 µL 6X nucleotide mix for NEC-1-24, 0.02 pg/µL of 10 kb Zika Virus’ RNA (MR-766), and 5 µL NASBA enzyme cocktail of wet kit for NEC-1-24 (Avian Myeloblastosis Virus Reverse Transcriptase (AMV RT), RNase H and T7 RNA Ploymerase (T7RNAP) in high molecular weight sugar matrix), 0.4 µL DNase, RNase and protease free water.

**Synthesis of Dapoxyl sulfonyl fluoride.** Dapoxyl sulfonyl fluoride was synthesized step by step according to Diwu.¹ H-NMR (Figure SI 8)
4. Figure 4-1. Detailed Design of SDA and Comparison with Full Dap-10

Figure 4-1 Detailed design of SDA
A) Design of SDA. Deleted nucleotide is indicated in red; Dotted lines indicate binding arms; Bent line indicate linkers; B) Fluorescence of synthetized dapoxyl-F with Full Dap-10. The samples contained 0.5 µM of dapoxyl-F, 1µM DAP-10 in Spinach Buffer. All readings were taken after 10 min of incubation.
Figure 4-2. Limit of detection of SDA with dapoxyl-F

A) Time dependence for SDA. The samples contained 0.5 µM of dapoxyl-F, 1µM SDA. Measurements were taken in the presence of 1 µM of Am; B) Limit of detection for SDA with dapoxyl-F. Data consists of the average of 3 independent experiments with the concentrations of 0.5 µM dapoxyl-F, and 1 µM SDA. All readings were taken after 60 min of incubation. C) Fluorescence turn-on ratio and Dr. 0.5 µM dapoxyl-F, 1 µM SDA_f, 0.2 µM SDA_m and 0.1 µM of Am or Amm.
Figure 4-3. SDA Fluorescence data for Amel Y

The samples contained 0.5 µM of *dapoxyl*-F, 1µM SDA. Measurements were taken in the presence of 1 µM of Am or Amm.
Figure 4-4 Fluorescence data for Zika

A) Fluorescence turn-on ratio of SDA with Zika. 0.5 µM dapoxyl-F, 0.5 µM SDA_f, 4 µM SDA_m and 0.5 µM of Zika 50. B) LOD of SDA with Zika. Data consists of the average of 3 independent experiments with the concentrations of 0.5 µM dapoxyl-F, and 0.5 µM SDA_f, 4 µM SDA_m. All readings were taken after 60 min of incubation.
Figure 4-5. Suboptimal designs of SDA

A-D) Four different combinations of linkers for each SDA probe against DNA analyte was tested to see how it will affect the fluorescence of **dapoxyl-F**. The fluorescence data for each combination is shown on the right next to each design. Linker is indicated in gray dot, triethylene glycol; bold dot, trithymine.
ZIKA sequences*

Figure 4-6 Design of SDA towards Zika sample

Dapoxyl NMR Data
Figure 4-7 Dapoxyl H-NMR

*Material and Methods References*

Results

Figure 4-8 Fluorescence intensity of SDA with six different variations of probe m and f

Optimization of the linker between aptamer sequence and analyte binding arm in SDA_f strand: p, triethylene glycol linkers; tt, dithymine linker. Samples contained 1μM SDA_f, 1μM SDA_m, and 0.5 μM dapoxyl-F in Spinach Buffer (20 mM Tris-Cl, pH 7.4, 200 mM KCl, 10 mM MgCl_2) in the absence (light grey bars) or presence (dark grey bars) of 1 μM A_m analyte. Fluorescence taken after 40 min of incubation. Refer to material and methods for sequence of strands.

Upon synthesis of dapoxyl-SEDA, intermediate compound dapoxyl-F (Figure 4-8A) was tested for its ability to function as a turn-on fluorophore against Dap-10 aptamer.

Dapoxyl-F fluorescence was compared with Full Dap-10 aptamer (Figure 4-1). Maximum fluorescence plateau was obtained within 150 min at emission peak 505 nm and excitation peak 385 nm (Figure 4-2A). Limit of detection (LOD) was 9.70 nM (Figure 4-2B).

Emission spectra of SDA probes recorded in the absence or presence of fully matched analyte demonstrated turn-on ratio (F/F_0) up to 63.5 with a differentiation factor (D_f) of 17 (Figure 4-2C), which was calculated according to the formula:
Maximum fluorescence was obtained by **dapoxyl-SEDA** within 10 min at emission peak 503 nm and excitation peak 390 nm (Figure 4-10A). Dapoxyl fluorescence was compared with SDA, full Dap-10 aptamer. LOD was 1.2 nM (Figure 4-10B) which is comparable to that of the current hybridization probes.

![Figure 4-9 Characterization of SDA](image)

**Figure 4-9 Characterization of SDA**

A) Response of the SDA sensor dependent on time. The samples contained 0.32 µM of **dapoxyl-SEDA**, 1µM SDA_f, and 0.4 µM SDA_m in the absence (dashed line) or presence of 0.25 µM of matched A_m (solid line) or single base mismatch A_mmm; B) Analyte Dependence and Limit of detection for SDA with **dapoxyl-SEDA**. Data consists of the average of 3 independent experiments with the concentrations of 0.32 µM **dapoxyl-SEDA**, and 1 µM SDA_f, 0.4 µM SDA_m. All readings were taken after 10 min of incubation.

Emission spectra of SDA probes recorded in the absence or presence of fully matched analyte demonstrated turn-on ratio (F/F_0) up to 69, with a differentiation factor (D_f) of 19 at analyte concentration of 250 nM (Figure 4-11).
Figure 4-10 SDA Fluorescence performance.

Data consists of the average of 3 independent experiments with the concentrations of 0.32 μM of \textit{dapoxyl-SEDA}, 1μM SDA\_f, and 0.4 μM SDA\_m. Measurements were taken in the presence of 0.25 μM of A\_m or A\_mm. All readings were taken after 10 min of incubation.

To show that SDA approach is capable of detecting other analytes, two additional analyte Amel Y and Zika were tested using \textit{dapoxyl-F} and \textit{dapoxyl-SEDA} respectively. Amel Y (5’- CCC AGT TTA A GC TCT GAT GGT TGG CCT CAA GCC TGT GTT G CTC CAG CAC CCT CCT GCC TGA CCA TTC GGA TTG ACT CTT TCC TCC TAA ATA TGG CTG TAA GTT TAT TCA T TC ATG AAC CAC TGC TCA GGA AGG TTC CAT GAA AGG GCA AA A A) is expressed in enamel gene on human chromosome Y. Zika 50 (5’- AGC GGG ATG ATT GGA TAT GAA ACT GAC GAA GAT AG AGC GA A AGT CGA GGT) is a sequence corresponding to fragment of zika virus strain. New strands f and m were designed to be complementary to the corresponded analyte sequences. SDA showed F/F\_0 up to 183 for Amel Y (Figure SI 3) and F/F\_0 up to 53 for Zika. (Figure SI 4). Even though the maximum fluorescence intensity is ~130 for both Amel Y and
Zika, difference in background dye fluorescence intensity accounts for the variation of $F/F_0$. Analysis of Zika was conducted using nucleic acid sequence based amplification (NASBA) Reaction Buffer (Life Sciences Advanced Technologies Inc.) which compared to Spinach Buffer, NASBA Reaction Buffer increased the intensity of background dye fluorescence possibly due to presence of dimethyl sulfoxide. To show that SDA is capable of analyzing RNA, sequence corresponding to miRNA (5’- AACC GUAGA UCCGA UCUUG UG) were tested.

Discussion

In this study we took the design of recently published light-up fluorophore-DNA aptamer pair. We divided the DAP-10-42 into two separate parts by the mutational analysis on a loop of the possible hairpin structure of DAP-10-42 which revealed information about the secondary and tertiary structure of DAP-10-42 conducted by Kato et al. and attached a binding arm to act as hybridization probes.

Comparing the two dyes $F/F_0$ and $D_f$ was in the same range, however Dapoxyl-SEDA has superior detection limit. Dapoxyl-F performed $F/F_0$ up to 63.5 with a $D_f$ of 17 and LOD 9.7 nM; Dapoxyl-SEDA performed $F/F_0$ up to 69 with a $D_f$ of 19 and LOD 1.2 nM.

Most hybridization probes require amplification of sample before analysis. SDA was tested to function as probe for real time amplification of nucleic acid. Dapoxyl dye was able to maintain function up to 40°C, therefore we have tested the product of asymmetric PCR, LAMP amplification, and NASBA with SDA as an initial step for usage as real-time analysis probe. Initially, Amel Y was amplified by asymmetric PCR producing dsDNA and fragments of ssDNA.
aiming to detect ssDNA by SDA. However, ssDNA asymmetric PCR products exist with dsDNA that hybridization of SDA is constantly competing against fully complement dsDNA strands. Along with the poor LOD, SDA was not able to detect target sequence. Next, Amel Y was amplified by Loop mediated isothermal amplification (LAMP) to produce long dsDNA encoding the target nucleic acid sequence. Typically, dsDNA is analyzed by hybridization probes by melting the hydrogen bonds between the dsDNA and controlling the melting temperature of the probes. Since dsDNA has greater stability due to fully complement base pairs, SDA was not able to detect dsDNA at ambient temperature. Finally, Zika 138 (5’-GCAGUCAAGCAAGCUGGAAGUGUAUCUCGGGAGCUAUCUUUAAGAUGGAAAACAUCAUGUGAGAUCAGUAGAAGGGGAGCUCAACGCAAUCCUGGAA GAGAAUGGAGUUCAACUGACCGGUUGUG) was amplified by NASBA producing fragments of target RNA sequence of various lengths.

Two different fluorophores were used with SDA. Although the $F/F_0$ and $D_l$ was comparable to each other, dapoxyl-SEDA has superior LOD. Dapoxyl-F had performance of $F/F_0$ up to 63.5 with a $D_l$ of 17 and LOD 9.7 nM; Dapoxyl-SEDA had performance of $F/F_0$ up to 69 with a $D_l$ of 19 and LOD 1.2 nM.

Limitation of SDA is the following: Although having high turn-on ratio, SDA has poor LOD. As for any aptameric sensor, the target molecule is bound by intermolecular forces, it goes through constant association and dissociation with the aptamer. Typical dissociation constants of aptameric complex is picomolar to mid nanomolar range. Therefore, LOD of SDA is influenced by poor dissociation constant due to split aptameric complex. Split aptameric probes can easily dissociate
from the aptamer target complex more so than the fully bound aptameric complex, leading to poor fluorescence associated with the poor LOD.

Label free SDA offers high selectivity and specificity towards full complimentary analyte offering straightforward and fast response. Single deoxyribonucleic acid is 6-8 times less expensive than RNA from commercial vendors. Therefore, DNA based sensors will lower the cost of designing and mass producing sensors for diagnostic tests.

**List of References**


CHAPTER 5 : CONCLUSION

Design of SSA

In this work, we took advantage of the recently published X-ray structure of spinach aptamer, which revealed actual folding of spinach aptamer and localized the G-quadruplex-based binding site for DFHBI. Our initial attempts to design SSA based on predicted secondary structure were unsuccessful. By localizing the G-quadruplex, we were able to successfully separate the binding site for DFHBI. In addition to the separation, RNA and DNA binding arms complementary to target nucleic acids were added. The binding arm and the core aptameric DFHBI binding sequences of SSA were initially attached without any spacer. However, the experimental data revealed that formation of core DFHBI binding pocket was limited due to flexibility hindrance of nucleic acids (Figure 2-2A). Triethylene glycol or a dinucleotide spacer was added to increase flexible rotation between the core DFHBI binding sequences and the binding arms. We have tested 4 different combinations of spacer on SSA_m and SSA_f (Figure 2-2). Highest turn-on signal was obtained with spacer on strand-f, and no spacer on strand-m. Interestingly, specificity was greater for design with spacer on strand-f rather than on strand-m (Figure 2-2C). This can be explained by the greater conformational flexibility of the spacer that led to less structural hindrance from secondary and tertiary structure of the spinach structure. The spacer was crucial to prevent the interference between the hybridization of the strand to the analyte and induce correct formation of the aptamer’s DFHBI binding pocket. Therefore, when designing binary split aptamer, customization of the presence and length of spacer is necessary for each individual aptamer.
Using wobble base pairs as single nucleotide mismatch analyte

We limited our mismatch analyte base pair to guanine uracil. Our theory behind using a single mismatch pair to conclude that SSA can discriminate all other mismatch combination is that guanine uracil forms a wobble base pair.\textsuperscript{59} Even though wobble base pairs do not follow the Watson-Crick base pair rules, the pairs of two RNA forms thermodynamically stable base pairs comparable to that of Watson-Crick base pairs.\textsuperscript{59} Therefore, if SSA is capable of discriminating fully matched analyte from thermodynamically stable single mismatch wobble base pairs, the other mismatch pair that does not form wobble base pairing will be easily discriminated. This was proved by experimental data when SSA\textsuperscript{r} was tested against RNA analyte. Discrimination factor of SSA\textsuperscript{r} against fully matched and single mismatch RNA analyte was poor compared to discrimination against SSA\textsuperscript{r} with adenine cytosine mismatch analyte (Figure 2-3B, C). When the mismatch base pair was changed from guanine uracil to adenine cytosine, SSA\textsuperscript{r} complex formation in the presence of mismatch analyte was reduced to noise level (Figure 2-3C). Additional investigations on designing RNA binding arm is necessary to fully differentiate those that had partial complex formation with the mismatch analyte.

Analyte binding arm

High selectivity of split aptamer is achieved by the design of analyte binding arms. In split aptamer design, one of the analyte-binding arms is made short (7-9 nucleotides in this study) to form a stable hybrid with only a perfectly matched sequence, while the entire recognition site remains long (e.g. 19 nucleotides on Figure 2-1). Short strand forms hydrogen bonds with the analyte only.
if all the nucleotides are matching to the analyte sequence. If one or more mismatch nucleotide exists on the analyte sequence, the binding arm and the target has less force to form a stable bond, leading to dissociation. The dissociation and association of the bond are sensitive to temperature. In our work, we designed our sensors to function at ambient temperature, therefore 7-9 nucleotide was used. By increasing the nucleotide number on the short binding arm, the split aptamers could potentially be used at higher temperature so long as the fluorophore could withstand the temperature without losing its activity. On the other hand, long strand is responsible for opening the highly folded analyte sequence and expose the sequence complementary to the short strand. When analyzing long target analyte, often times the concentration of the long strand is as much as the target analyte being analysed so that majority of analyte strands are bound to the long strand, thereby increasing the efficiency of short strand selectivity. Split aptamers were able to detect analytes of various length (Figure 4-2,3,4). Compared to the short target analyte, detection of longer length target analyte required longer incubation time for the split aptamer to obtain its maximum fluorescence. This indicates that upon incubation with folded target analyte, binding arms on the split aptamers act as strand displacement probes competing between the secondary structures of the target analyte.

Design of SDA

In our next study, we employed the design of a recently published light-up fluorophore-DNA aptamer pair. We divided DAP-10-42 into two separate parts by the mutational analysis on a loop of the possible hairpin structure of DAP-10-42 that revealed information about the secondary and tertiary structure of DAP-10-42, and attached a binding arm in the same way as SSA. The
initial design with spacer on strand_f differentiated A_m from A_mm but with limited differentiation D_r~3 (Figure 4-5). We than tested different combinations of spacer on strand_f and strand_m. The highest differentiation factor was obtained from spacer on both strand_f and strand_m. For cost efficiency, we decided to use triethyleneglycal on strand_f and dithymine on strand_m. The initial DAP-10-42 was modified by adding 6 base pair stem in addition to the original stem base which stabilized the two split pieces.

Upon synthesis of Dapoxyl-SEDA, intermediate compound Dapoxyl-F was also used as the fluorescent marker for SDA. Comparing the two dyes, F/F_0 and D_r were in the same range but Dapoxyl-SEDA has superior detection limit. Dapoxyl-F performed F/F_0 up to 63.5 with a D_r of 17 and LOD 9.7 nM; Dapoxyl-SEDA performed F/F_0 up to 69 with a D_r of 19 and LOD 1.2 nM.

SDA was tested to function as probe for real time amplification of nucleic acid. Dapoxyl dye was able to maintain function up to 40°C, thus we tested the product of asymmetric PCR, LAMP amplification, and NASBA with SDA as an initial step for usage as real-time analysis probe. We continue to work at designing SDA that are compatible for dsDNA and 84 nts RNA at ambient temperature.

In conclusion, we have designed label-free fluorescent probes for nucleic acid analysis. SSA consists entirely of RNA or RNA/DNA and SDA entirely from DNA. The probes demonstrate superior performance in comparison to state-of-the-art probes. Important practical features of split aptameric probes include: (i) High selectivity at ambient temperatures. (ii) Mix-and-read reporting format with up to 270-fold and 69-fold turn-on ratios respectively. (iii) LOD in low nanomolar range, which is about one order of magnitude better than that for structure switching spinach
sensors.\textsuperscript{53} (iv) Label-free design: there is no need for conjugation of oligonucleotides with a fluorophore or quencher dye, which eliminates the need for purification of split aptameric strands prior to usage (note that all strand\_f and strand\_m stands used in this study were only desalted after solid-phase synthesis). (v) Facile tailoring of SSA probes for recognition of each new analyte by simple change of the analyte-binding arms. (vi) Finally, split aptameric components can be conveniently obtained from industrial suppliers of custom-made nucleic acids.

Design of USSA

In this work, we introduced two label-free adaptor DNA strands that can be used with the recently designed SSA to function as universal probe capable of detecting potentially any nucleic acid sequence with high signal to background response and high selectivity even at ambient temperature. The design of the adaptor strands was optimized to enable efficient complex formation only in the presence of a specific analyte. Our initial attempt on designing Adp\_m and Adp\_f failed when the USSA formed a 5 way junction with SSA, adapter strands, and the target analyte (Figure 3-2A). We than shifted the adapter binding location which separated the 3 way junction formed by SSA and the 4 way junction formed by SSA analyte binding arm, adapter strands, and new target analyte (Figure 3-2B). In order for SSA and adapter strands to communicate to each other, 3 way junction and 4 way junction had to be in close proximity so as to maintain the function of the split aptamer. For example, it was found that Adp\_m with 6 nucleotides complementary to SSA\_f produced the greatest analyte dependent fluorescent response (see Figures 3-2,3,4 for less successful designs). A triethylene glycol (TEG) linker
connected the SSA-binding arm and analyte-binding arm in Adp_m was found to be essential for high analyte-dependent fluorescent response. The TEG linkers supposedly provided conformational flexibility to the multi-stranded associate to ensure proper orientation of the adaptor strands for accurate folding of the DFHBI–binding site in response to the analyte presence.

The construct is versatile, which was demonstrated by converting it into a NOR DNA logic gate for molecular computation in straightforward and low-cost manner (Figure 3-8).

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