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Feasibility of Aptamer-based Sensors for the Real-time Detection of Protein Targets

by Dimitra N. Stratis-Cullum

ARL-TR-3939 **September 2006**

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Dimitra N. Stratis-Cullum
Sensors and Electron Devices Directorate, ARL

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED (From - To)
September 2006	Progress	January to October 2005

4. TITLE AND SUBTITLE
Feasibility of Aptamer-based Sensors for the Real-time Detection of Protein Targets

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Dimitra N. Stratis-Cullum

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
U.S. Army Research Laboratory
ATTN: AMSRD-ARL-SE-EO
2800 Powder Mill Road
Adelphi, MD 20783-1197

8. PERFORMING ORGANIZATION REPORT NUMBER
ARL-TR-3939

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Research Laboratory
2800 Powder Mill Road
Adelphi, MD 20783-1197

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The selective molecular recognition capability and high binding affinity of nucleic acid aptamers is integrated with the signal transduction methodology of molecular beacons for real-time monitoring of a surrogate protein toxin, thrombin. An aptamer recognition element was modified to exist in a stem-loop configuration in the absence of a protein target, and in the presence of the protein target change conformation. Upon binding to the target, a physical separation of the attached fluorophore and quencher molecules is achieved, allowing for an engineered, single-step recognition and signaling system. The aptamer signaling probe was found to exhibit a maximum 12-fold change in signal when hybridized with a complement control, and a 3-fold change in signal with an excess of thrombin. The fluorescence increased with increasing concentration of thrombin, until probe saturation where the fluorescence signal did not increase further, but leveled off in intensity. The signaling probe produced a rapid response, with 70% of the maximum signal achieved within 15 seconds.

15. SUBJECT TERMS
Biosensor, aptamer, FRET, protein

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE	UL	20	Dimitra N. Stratis-Cullum
Unclassified	Unclassified	Unclassified			19b. TELEPHONE NUMBER (*Include area code*) (301) 394-0794

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1. Introduction

The need for rapid biosensing methodologies spans a number of fields ranging from medical diagnostics, drug discovery, and homeland security. For example, the benefits of rapid biological detection can allow for earlier diagnosis and treatment of disease, as well as for an increased response time to a chemical or biological threat attack. Of particular interest is the development of sensing methodologies which are not only rapid, but are capable of specific and sensitive biological identification, and can be integrated into a multiplexed system for analysis of multiple target types on a single detection platform.

A biosensor fundamentally consists of a bioreceptor that specifically recognizes and binds to a target of interest, in combination with some form of transduction mechanism to convert the binding event to a measurable signal (*1-3*). The transduction mechanism can vary widely, depending on the needs of the application. Many use an optical transduction due to the ability to optically label biological recognition elements and perform a sensitive spectroscopic analysis. The bioreceptor can also function in a variety of formats with the most common being nucleic acid (hybridization) and antibody (immunological). In the case of nucleic acid hybridization, specific identification of another nucleic acid target is achieved through complementary base-pair matching, often of the genetic components of an organism (*1*). In the case of antibody-based sensing, specific recognition of an antigen component of an organism or surface epitope of an organism is responsible for the identification (*1*).

Although not commonly employed, nucleic acid aptamers are an emerging chemical and biological recognition approach that offers a number of advantages over traditional bioreceptors. Nucleic acid aptamers are single strands of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), typically 20-100 bases in length that are capable of specific binding to a variety of target types (e.g., chemicals, viruses, toxin, cells, spores) through a combination of shape complementarity and noncovalent chemical interactions (figure 1) (*4-7*). Although the mechanisms for specific binding are similar to antibody:antigen interactions, aptamers are amenable to reproducible mass production and do not require the use of animals. Other distinct advantages over antibody-based sensing include a greater stability for field use, and an ability to be easily chemically modified to allow for a variety of signaling schemes.

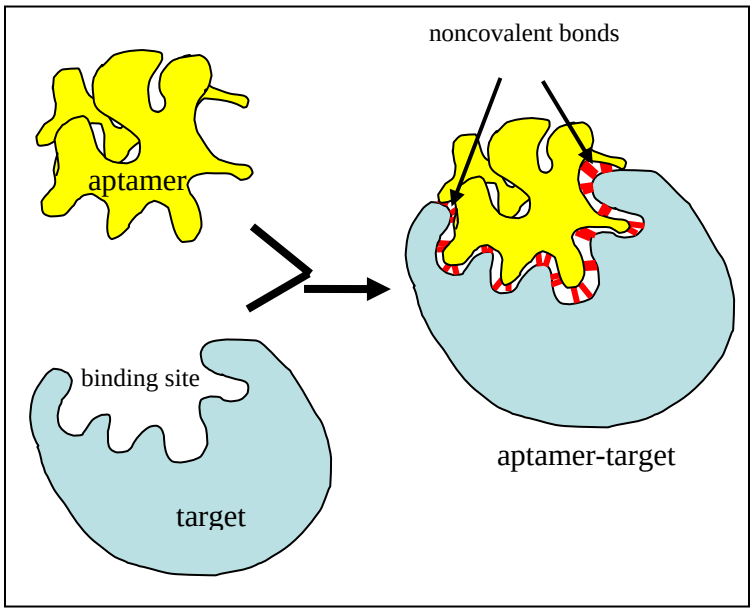

Figure 1. Schematic diagram showing aptamer recognition concept.

In this paper, a new class of rapid signaling probes, referred to as molecular aptamer beacons (MABs) (*8*,*9*), are being explored for the rapid detection of protein targets. The MAB approach combines the selective molecular recognition capability and high binding affinity of synthetic nucleic acid aptamers with the signal transduction method of molecular beacons for real-time monitoring of target species.

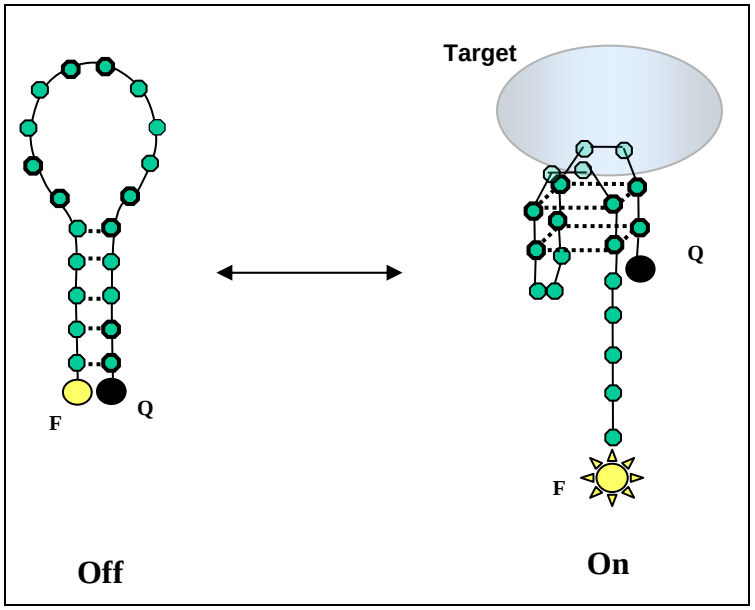

Figure 2. Diagram showing FRET-based signaling scheme.

Similar to a conventional molecular beacon, a conformation change occurs in the MAB upon binding to the target, which spatially separates a fluorophore and a quencher to produce a detectable signal. As shown in figure 2, the signaling probe can exist in different conformational forms and in the absence of the target, the two ends and resulting fluorophore and quencher labels are held in close proximity through careful design of a "stem" portion of the aptamer structure. This close proximity allows for the transfer of energy from the excited fluorophore to the quencher moiety either by a direct energy transfer or a fluorescence resonance energy transfer (FRET) mechanism. For the former, contact between the two moieties is required and the collisions between them distort the energy levels of the excited fluorophore, thereby quenching the fluorescence. In the case of FRET, spectral overlap is required between the fluorophore's emission and the quencher's absorption spectra (*10,11*). The most commonly used quenching moiety is dimethylaminophenylazobenzoic acid (DABCYL), as it is capable of efficiently quenching a large variety of fluorophores through both direct energy transfer and FRET processes (*11*). For both forms of energy transfer, the distance between the fluorophore and quencher dramatically affects the interaction, with typical interactions occurring up to distances as great as 100 Å (*12*). Upon selective binding to the target, the conformational change that occurs physically separates the two ends beyond the förster radius, such that an increase in overall fluorescence intensity is observed.

2. Materials and Methods

2.1 Materials

All oligonucleotides probes used in this work as well as Tris EDTA buffer, pH 7.5, were purchased through Integrated DNA Technologies (www.idtdna.com). Specifically, table 1 shows the custom synthesized sequences (probes and controls) used in this paper with 5' and 3' labels indicated. All sequences were purified using HPLC. The sequences were resuspended in Tris EDTA buffer to a concentration of 100 μMolar and stored in single use aliquots at -20 ºC. Prior to use, each aliquot was heated using a thermocycler to 99 ºC for 3 minutes, and then cooled to room temperature to remove any secondary structure. Thrombin (product number T-1063), 1000 units, was purchased from Sigma and stored as a 10 μM stock in PBS with 20% glycerol pH 7.4 at -20 ºC in single use aliquots until use.

Table 1. OLIGONUCLEOTIDE probes.

Sequence Description	5'-Sequence-3'-*
Thrombin MAB Probe	5'FAM-CCAACGGTTGGTGTGGTTGG-DABCYL3'
Complement	5'-CCAACCACACCAACC-3'
Scambled Dual-labeled Contro	5' FAM-TGTGTGTGTGTTGGTGT-DABCYL3'

* FAM=6-carboxyfluorescein
DABCYL=dimethylaminophenylazobenzioic

2.2 Measurement System

All measurements were performed using a Biotek fluorescence microplate reader equipped with excitation filter 485(20) nm, and emission filter 528(20) nm for excitation of the FAM label.

2.3 Hybridization Studies

For all hybridization experiments, equivalent concentration (5 μM at dilution) of aptamer probe and complement were incubated first for 99 ºC for 3 minutes, followed by 50 ºC for 10 minutes before cooling to room temperature. After cooling, the hybridized mixture was diluted for measurement on the fluorescence microplate reader to a concentration of 40 nM.

2.4 Thrombin Studies

Thrombin binding studies were performed using a fixed concentration of aptamer probe at 40 nM. Various concentrations of thrombin were mixed through 15 second vortex, and incubated at room temperature with the aptamer probe in a 100 μL reaction volume for 10 minutes. After incubation, replicate measurements of the resulting fluorescence signals were obtained by transfer to a 96 well plate and subsequent measurement. Time-response experiments were conducted by preparing thrombin buffer solutions at the desired concentration to produce a final volume after addition of the probe of 250 μL in IDTE buffer. In each case, the aptamer probe concentration was fixed at 40 nM and the volume of the aptamer probe required to achieve this was quite small(1 μL) due to the high concentration of the stock solution.

3. Results and Discussion

In order to investigate the feasibility of direct and rapid protein detection using the aptamer signaling approach, several probes were synthesized with a commonly used fluorophore quencher pair (FAM/DABCYL) that is known in the literature to produce good quenching efficiency of the fluorophore (*13, 14*). The other advantage to this system is that the attachment chemistries and stability of the conjugated probes are well understood. For this work, a stem-loop design was adopted from the literature and is designed to bind to the thrombin protein target. As described in figure 2, in the absence of the target, the probe is designed to exist in a

conformation where the ends are held in close proximity, enabling the quenching of the fluorescence. After introduction of the target, the probe ends physically separate, and the resulting fluorescence is recorded, indicating the presence of the target protein. In addition to the signaling probes, several control probes and experiments were conducted to further investigate the performance of the system.

3.1 Complement-Induced Probe Signaling

To fully separate the fluorophore and quencher ends of the aptamer probe, a traditional hybridization experiment was conducted in which a complementary oligonucleotides sequence to the aptamer portion of the probe was conducted. The resulting change in fluorescence signal of the FAM label is shown in figure 3. It is clear upon comparison of the probe (noted as MB for molecular beacon) plus complement (noted as Comp) produced a much larger fluorescence signal, compared to the probe alone in the buffer solution (MB+buffer). The buffer conditions were selected based on other work conducted in this area and the aptamer probe and all samples were subjected to identical temperature conditions. Each bar represents the average of 4 replicate measurements, and $\pm 1\sigma$ error bars displayed. Specifically, approximately a 12-fold change in fluorescence intensity was observed upon equimolar introduction of the complement control. Also shown in figure 3 for comparison, are the scrambled sequence results with complement. The scrambled sequence consisted of a randomized sequence of bases, with equivalent FAM and DABCYL labels. It is important to note that the fluorescence intensity of this control probe remained constant (data not shown) when comparing the scrambled probe results in the absence of the complement, to the results in the presence of the complement. In other words, the scrambled sequence exhibited no signaling capability, as in the case of the stem-loop aptamer probe, which was designed to exist in an “off” state in the absence of the target and an “on” state in the presence of the target when the two ends of the probe physically separate to produce a measurable signal.

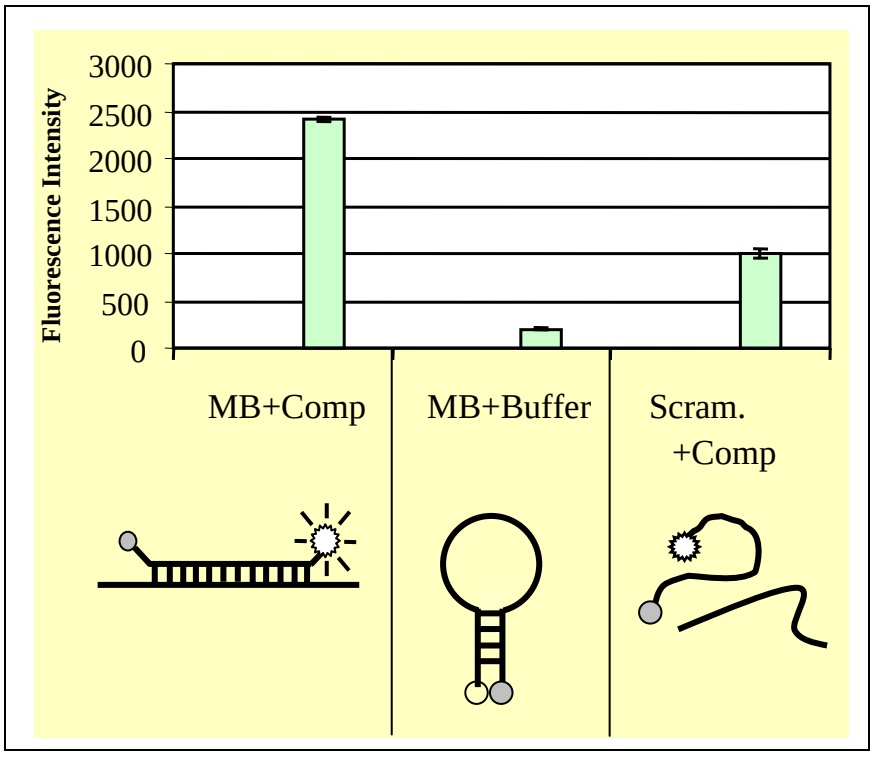

Figure 3. Demonstration of conformational signaling aptamer through complementary hybridization.

After determining the change in fluorescence signal when fully extending the probe using a complementary sequence, the change in fluorescence binding upon introduction of the protein target was investigated. The thrombin aptamer used here has been one of the most widely studied aptamer system and consequently much information is known about the conformational changes to different tertiary structures that occur under different buffer, temperature, ionic strength, and target binding conditions. For example, the unmodified thrombin aptamer (i.e., no stem-loop signaling configuration), has been shown to produce a G-quartet configuration when bound to the thrombin target, as shown in the schematic diagram of figure 2. Other ionic strength, buffer, and temperature conditions have also been found capable of controlling the shift in equilibrium between the G-quartet and other forms. In the stem-loop configuration used here, the G-quartet formation is minimized in the absence of the target. In other words, conditions such as ionic strength changes that would normally shift the equilibrium to the G-quartet form are minimized through the incorporation of the stem (*8,9*). However, the stem potion of the probe necessitates careful design, in order to allow for the improved "off" state in the absence of the target, but it also must be able to open up to shift to the G-quartet form to bind to the thrombin target.

3.2 Protein-Induced Probe Signaling

Figure 4 shows the resulting change in fluorescence signal of the same aptamer signaling probe shown in figure 3, but with introduction to the thrombin protein target. In each case, the resulting fluorescence signal represents the average of 4 replicate measurements. It is clear that

there is an increase in fluorescence signal upon introduction of the thrombin protein, when compared to the probe alone in the buffer solution. However, the maximum change in fluorescence is less (3-fold) than that observed using the complement (12-fold), suggesting that there is not as much separation between the fluorophore and quencher or that the equilibrium shift is not as great. Also shown in figure 4 are results for two different concentrations of thrombin, 20 nM and 100 nM. For both measurements a 40 nM probe concentration was used. Therefore, the 100 nM thrombin is in excess of the probe concentration and the 20 nM thrombin is less than the probe concentration.

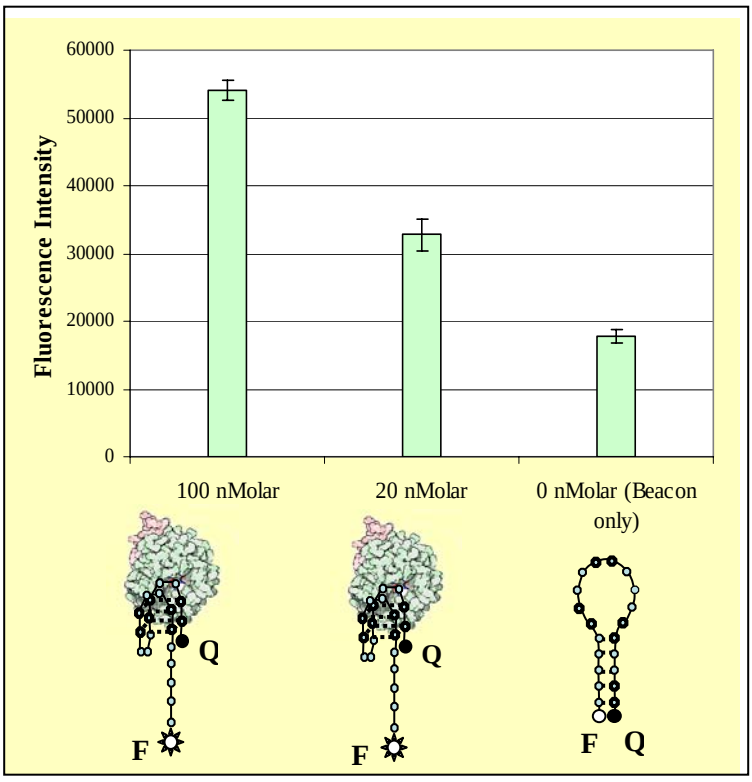

Figure 4. Demonstration of aptamer binding with thrombin protein target.

3.3 Signal Dependence on Protein Concentration

To further investigate the change in fluorescence as a function of thrombin concentration, a series of thrombin dilutions were investigated under identical conditions including a fixed concentration of 40 nM signaling aptamer. As shown in figure 5, there is an increase in fluorescence intensity with increasing thrombin concentration until the 1:1 equivalent thrombin:probe concentration is reached at 40 nM and the intensity levels off since the probes have been saturated with thrombin target. In each case, the average of 3 replicate measurements is shown with $\pm 1\sigma$ error bars. Within this error, the 10 nM concentration of thrombin is distinguishable from the blank measurement that was performed in the absence of thrombin target. Some shifting of the dynamic range is possible by changing the concentration of the

aptamer probe. However, this background level of fluorescence is quite significant and suggests further improvements to the "off" state of the probe are needed in order to allow for a more sensitive analysis as well as a larger dynamic range of performance.

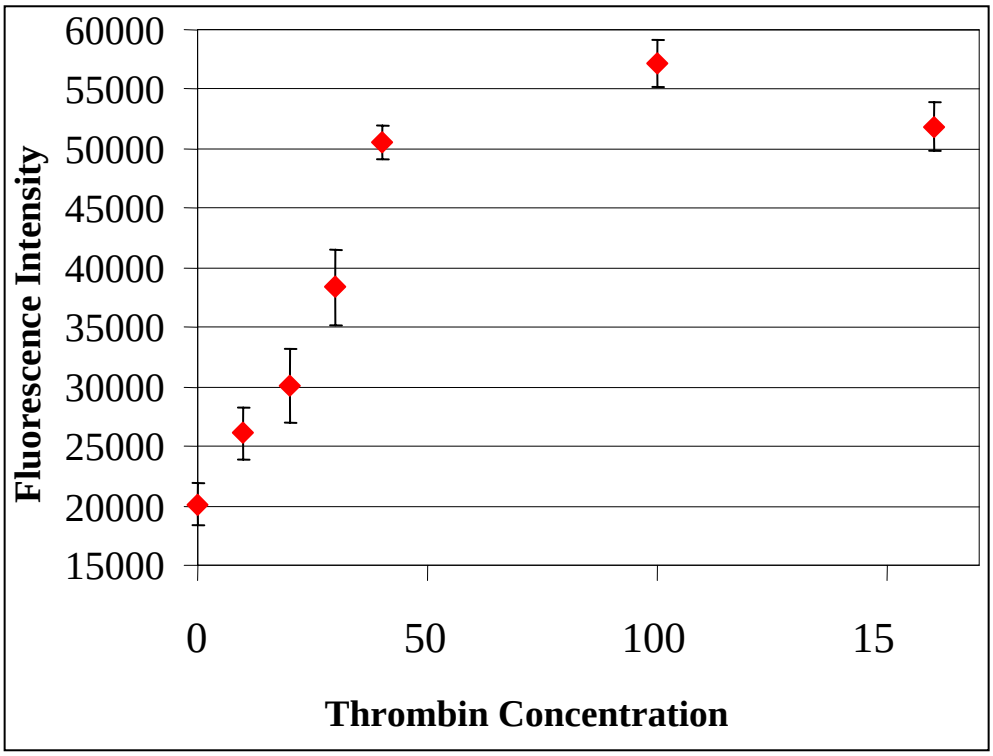

Figure 5. Change in fluorescence intensity using a 40 nM signaling aptamer concentration, with varying thrombin protein target concentration.

3.4 Rapid Signaling Capability

The results of figures 3, 4, and 5 were all achieved after incubation with the target for several minutes before recording the resulting fluorescence signal. Although several minutes is quite rapid more most analyses, the power of this method is in the real-time or near-real-time signaling capability of the single-step bind and signal concept. To illustrate this, an experiment was performed where the thrombin target and buffer were placed in various microplate wells and a small volume of aptamer probe was introduced to the system. These results are shown in figure 6, where the squares represent two replicate wells that contained approximately a 2-fold excess of thrombin target plus probe and the triangles represent two replicate wells that contained the probe only. In each case, the fluorescence is normalized to the maximum observed fluorescence intensity, and the signal is monitored over a period of 20 minutes.

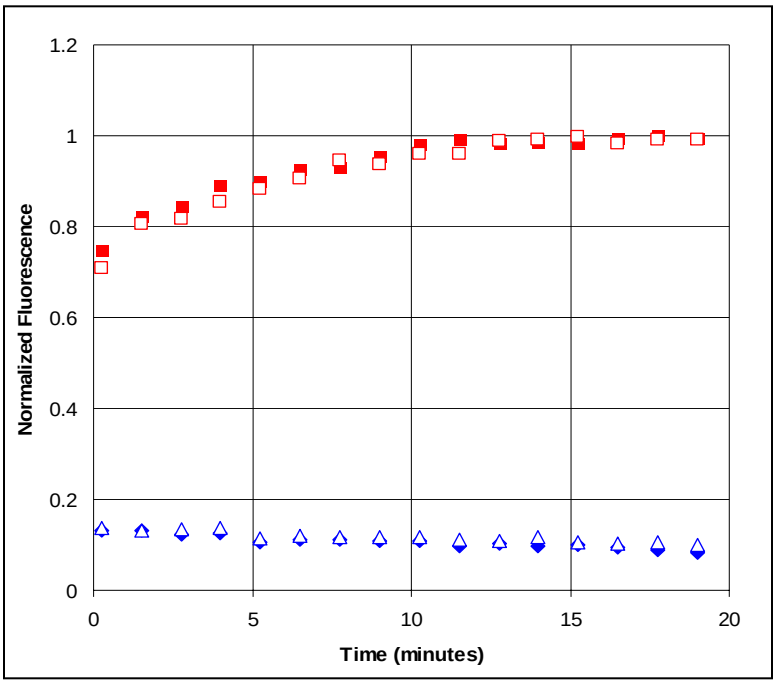

Figure 6. Rapid time response of signaling aptamer with target (squares) compared to signaling aptamer control (triangles).

As can be seen in figure 6, the fluorescence of the wells containing only aptamer probe remain relatively constant, and consistent in behavior. After introduction of the aptamer probe to the thrombin containing wells, there is an immediate increase in fluorescence intensity observed. Specifically, approximately 70% of the maximum fluorescence increase was achieved within the first 15 seconds. This sharp increase is followed by a slower increase in fluorescence intensity over the next 5-10 minutes which is most likely a diffusion limited process. These results clearly show the rapid signaling capability of this type of approach and, if combined with improvements with overall signal-to-background of the probe, performance could be very powerful in a number of fields requiring rapid quantitation of protein targets.

4. Conclusions

These studies support the claim that nucleic acid aptamers combined with a FRET-signaling approach can allow for a rapid analysis of protein samples. A maximum 3-fold change in fluorescence intensity was observed upon introduction of the thrombin target, compared to the 12-fold change that was achieved using the complement control. This suggests there is room for improvement in terms of the signal-to-background ratio achieved. Future work could include investigation of alternate quenching methods for an improved “off” state or lower background as well as improvements to the “on” state through advanced modeling studies.

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