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Tuning Aptamer-Switching for Biosensing Malarial Proteins

Abstract

Malaria is a life-threatening disease caused by a protozoan transmitted through mosquito bites. Early diagnosis is essential to start treatment and prevent further transmission. However, current diagnostic methods are expensive, time consuming, and lack the portability required for efficient testing. Emerging methods that are faster and more portable include specialized biosensors called aptasensors. This study compared a range of complementary probes against malaria-binding aptamers to develop an aptamer-switch based platform. One candidate showed a promising increase in fluorescence upon incubating with a malaria target protein. This work has the potential to be incorporated into an aptasensor for rapid detection of malaria infections.

Introduction

Malaria is a potentially lethal parasitic infection present throughout the world. It is mainly concentrated in Africa but is also present in South-East Asia, the Mediterranean, and the Americas to varying degrees[1]. Despite advances in therapeutics and diagnostic methods[2], over 200 million cases and 400,000 deaths were reported globally in 2019[1]. Malaria is caused by five species of the protozoan *Plasmodium*, including *P. falciparum, P. vivax, P. malariae, P. ovale,* and *P. knowlesi*. The *P. falciparum* species is most prevalent and lethal, while *P. vivax* is the widest spread[3]. These protozoans are transmitted between humans through female *Anopheles* mosquito bites[4]. Early during infection, fever, chills, vomiting, and dizziness occur, which, if left untreated, may progress to severe anemia, acute renal failure, pulmonary edema, and even death[5,6].

1.1 Current diagnostic tools for malaria detection

Early diagnosis of malaria and treatment is essential. The gold standard of diagnosis is conventional microscopy of blood smears, which can identify malaria species, parasite stages, and quantify red blood cell counts[7]. However, this process takes an hour at minimum, requires trained staff, and is expensive[8,9]. Rapid diagnostic tests are a common alternative that rely on an antibody-based detection. Although these tests require less training and are faster, developed tests currently only identify two species and are prone to false negatives[7,8]. Polymerase Chain Reaction (PCR)-based tests form a third major diagnostic test that can be used elsewhere for high sensitivity detection to identify species and monitor responses to treatment[9]. However, PCR-based tests are not used in malaria-endemic regions due to high cost, complexity, and personnel requirements[10]. Evidently, there is a need for faster, cheaper, and more portable diagnostic methods.

Recent research has focused on developing new biosensors. Biosensors are devices that detect chemical or biological substances of interest by coupling a biological recognition element with a transducer that converts any recognition into an electrical signal[11]. Often, antibodies are chosen as the biological recognition element, although alternatives such as enzymes and DNA probes may also be used[11]. Biosensors can test for parasites in blood and are being tested for reliability in both saliva and urine as they are non-invasive[12,13]. Malaria biosensors already exist and have higher sensitivity than conventional methods[9,14,15]. However, low antibody stability and short half-life is problematic in the field, in which the uncontrolled environment may cause antibodies to denature irreversibly[16].

1.2 Aptamer-based biosensors

Aptasensors, biosensors that rely on aptamers instead of antibodies[17], provide a promising alternative to antibody-based biosensors. Aptamers are nucleic acid molecules that bind molecular targets with high selectivity, high affinity, and most importantly, improved stability than antibodies; if denatured, they can simply refold once conditions return to conditions in which they were optimized[18]. This stability makes them better candidates for testing in the field than antibodies[16]. Aptamers can be developed into biosensors by incorporating a variety of transducer systems including gold nanoparticles, fluorophores, electrochemical probes, and more[19,20,21].

One of the many ways that fluorescence aptasensors can indicate the presence of protein is by sending a fluorescence signal to the electronics of a smartphone, for instance[22]. For this type of aptasensor to function, the aptamer must change conformation upon target binding to obtain a measurable change in fluorescence[23]. Typical aptamers often must be re-designed, potentially making use of complementary quencher probes, to achieve a significant conformational "switch" (Figure 1).

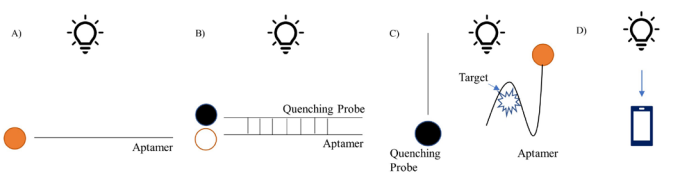


Figure 1. An example of an aptamer-switch system implemented in the development of an aptasensor. In the switch system, the aptamer tagged with a fluorophore emits light (A) and is quenched when a complementary sequence (probe with a fluorescence quencher) is present (B). In the presence of the target protein, the aptamer preferentially binds to the target, releasing the quenching probe. As such, the tagged aptamer emits light again (C). Electronics can convert the fluorescent light signal into a recognizable signal measurement for a smartphone (D).

1.3 Aptamers developed for malaria detection

Several aptamers that have potential for detecting malaria exist or are currently being developed. The majority of aptamers target plasmodium lactate dehydrogenase (PfLDH) because this was the first important diagnostic target identified, although there is now substantial research on glutamate dehydrogenase[24]. Two aptamers towards PfLDH have been developed into aptasensors that can distinguish between *P. falciparum* and *P. vivax*[25,26], providing an excellent tool to identify the plasmodium species for treatment purposes. However, since the PfLDH aptamer cannot detect *P. knowlesi* infection, a strain that requires immediate and aggressive treatment[27], these aptasensors must be used in a multi-panel array with other tests before a negative result can be confirmed[16]. More recently, the glutamate dehydrogenase aptamer has been developed into an aptasensor

that detects *P. falciparum*[28]. Although the system may detect *P. vivax*[29], it remains limited to this strain until research in other species is undertaken. Looking to the future, it would be useful to develop a wide array of malaria-detecting aptamers with varying strain specificity that can be made into aptasensors. For example, an aptamer that broadly detects all malaria strains would enable a rapid preliminary diagnostic method. However, there are no currently reported aptamers with this capability[16,24,30]. Furthermore, additional aptamers specific to each species are needed following an initial diagnosis to detect each type of malaria strain so that doctors can adjust treatment accordingly to each specific strain. Substantial aptamer discovery work has identified additional aptamers that can bind and interact with malaria, but require further development into aptasensors[30].

One potential target for aptamer development is Heat shock protein 90 (Hsp90), an ATP-dependent molecular chaperone that is located on the cell surface and used by *P. falciparum* to regulate its development[31,32]. Although Hsp90 is conserved across many species, PfHsp90 is proposed to be distinct enough from human Hsp90 to allow for selective targeting[31]. Thus, this paper tests PfHsp90 as the target for the aptamer-switch system. Researchers can develop potential aptamers to this target using cell-SELEX (Systematic evolution of ligands by exponential enrichment) or protein-SELEX, an in vitro evolution method[33]. Developing an aptamer-switch system using Hsp90 is our first step towards developing an aptasensor for malaria detection. This paper will explore quenching probes to achieve "aptamer switching" which can be then incorporated into an aptasensor connected to a smartphone. If successful, these aptasensors will provide a cheap, portable method of malaria detection.

Methods

2.1 Materials

Buffers were prepared starting with 1X Phosphate Buffered Saline (PBS) at pH 7.4 from Gibco (Amarillo, TX). The Triton-X100/RPMI Buffer used Triton-X100 from BioBasic (Markham, ON). The Recombinant Hsp90 (*P. falciparum*) partial Protein SPR-122A was ordered from StressMarq Biosciences (Victoria, BC). Two aptamer sequences, Aptamer.1 and Aptamer.2, were selected by collaborators by employing the cell-SELEX method with malaria-infected cells as previously described[30]. All oligonucleotides, including the aptamers, were purchased from Integrated DNA Technologies (Coralville, IA) and are available from the McKeague lab upon request. Sequences are in Table 1. 'FAM' is the fluorophore and 'IABkFQ' is the Iowa Black® fluorescence quencher.

Table 1. Oligonucleotide Names and Sequences

Oligos	Sequence
Quencher.1C8	TTCTCCACCC- 3'Q
Quencher.1C10	CCCTCCACCC- 3'Q
Quencher.1C12	ACCCCTCCACCC- 3'Q
Quencher.2C10	CGTAATAGAC- 3'Q
Quencher.2C12	CCCGTAATAGAC- 3'Q
Quencher.2C14	CCCCCGTAATAGAC- 3'Q
Quencher.1C25mis	AGGCGATTTTTTTTTTTTCCACCC - 3'Q
5'Q Quencher.1C8	5'Q - CAAACTTCTA
5'Q Quencher.1C10	5'Q - CAAACTTCCT
5'Q Quencher.1C12	5'Q - CAAACTTCCTAA
5'Q Quencher.2C10	5'Q - CAGATAATGC
5'Q Quencher.2C12	5'Q - CAGATAATGCCC
5'Q Quencher.2C14	5'Q - CAGATAATGCCCCC

[1]The 3' Quencher (3'Q) is 3IABkFQ; The 5'Quencher (5'Q) is 5IABkFQ

2.2 Preparing Regeants

Binding Buffer was prepared by adding 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 1 mM KCl to PBS pH 7.4. The Binding Buffer for Fluorescence Anisotropy experiments included 0.05% Triton-X100.

The oligonucleotides were diluted with MilliQ water to form 100 µmol/L stock solutions. For the fluorescence-quenching switching experiments, aptamers were made to 600 nM or 1200 nM using Binding Buffer. Quencher probes were made to 1200 nM using Binding Buffer. For Fluorescence Anisotropy experiments, aptamers were made to 10 nM using Binding Buffer with Triton-X.

The target, Heat shock protein 90 (Hsp90), was diluted using the Binding Buffer to 2000 nM or 5000 nM as needed. Hsp90 concentrations were prepared using two-fold serial dilutions and ranged from 5000 nM to 1.56 nM when starting with 5000nM stock solution, or from 2000 nM to 0.1 nM, when starting with 2000 nM stock solution.

2.3 Testing aptamer switching with fluorescence-quenching measurements

Aptamers, buffer, and target Hsp90 protein were added to 96-well plates and incubated for 30 minutes at room temperature to allow Hsp90 binding. Controls included removing one of each of these components and replacing with buffer. After 5 minutes of incubation at room temperature, the fluorescence intensity was measured with the BioTek Cytation 5 Cell Imaging Multi-Mode Reader at excitation and emission wavelengths of 495 nm and 520 nm +/-10 nm, respectively, after 10 seconds of plate shaking. Conditions were prepared in triplicate whenever possible, as indicated. In some cases, duplicates were necessary to reduce material costs and to meet space constraints of a 96 well plate.

2.4 Fluorescence Anisotropy

Binding of the aptamer to the heat shock protein was tested using fluorescence anisotropy. Each aptamer was labelled with a 5'-FAM. Triton-X100 detergent was added to the binding buffer to 0.05% as previously described[34]. Aptamers had a constant concentration of 10 nM. Hsp90 concentrations were prepared using two-fold serial dilutions and varied from 5000 nM to 1.56 nM. The negative control was a 10% glycerol solution in binding buffer that was prepared similarly to Hsp90, using two-fold dilutions to generate solutions ranging from 5000 nM to 1.56 nM. After shaking the plate for 15 minutes at room temperature, the BioTek Cytation 5 Cell Imaging Multi-Mode Reader was used to take polarization measurements using the same procedure as in fluorescence-quenching measurements, but with an extra step to shake the plate for one minute once inside the plate reader. GraphPad Prism 6 was used to plot the difference between polarization values in the presence and absence of Hsp90 against the total concentration of Hsp90 and to generate a binding isotherm using a one-site binding (hyperbola) fit. The dissociation constant, K_D, was reported as the mean across duplicates. This value indicates the strength of interaction between two molecules, with a low K_D indicating strong affinity between target molecules.

Results

3.1 Confirming binding between the aptamers and Hsp90

We first wanted to confirm that Hsp90 bound sufficiently to the aptamers by measuring the dissociation constant, K_D. Several methods are possible to measure interactions between aptamers and their targets[35]; however, since we are interested in developing fluorescence-based aptasensors, measuring aptamer binding with the fluorophore tag is useful to ensure it does not interfere with target binding. As such, we made use of a fluorescence anisotropy assay to measure the aptamer affinity in solution.

The K_D values between Aptamer.1 and Hsp90 and between Aptamer.2 and Hsp90 were 154 nM and 116 nM, respectively (Figure 2). These values represent a reasonably good affinity between Aptamer/Hsp90 binding. Notably, the 10% glycerol control showed no change in polarization. This indicates that binding was between the aptamer and Hsp90 instead of the aptamer and glycerol it came stored in.

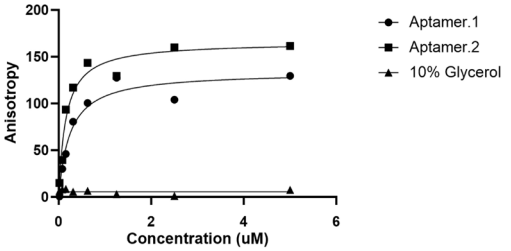


Figure 2. K_D Results of HSP90 Binding to Aptamer.1 or Aptamer.2. The K_D of Aptamer.1 and HSP90 is 116 nM and the K_D of Aptamer.2 and HSP90 is 154 nM. The glycerol control condition showed no change in polarization, suggesting that binding is indeed due to the aptamer/HSP90 interaction.

3.2 Screening for aptamer probe displacement as an "aptamer switch"

We first tested both aptamers with a panel of complementary quencher probes ranging in sizes from 10 to 14 nucleotides (Figure 3A). These quencher probes contained a quencher and were complementary to the 5' end of each aptamer such that the fluorescein present on the 5' end would be quenched. If an aptamer "switch" was to occur, the quencher probe would be released from the aptamer once the target was present and cause an increase in fluorescence signal, as shown in Figure 1. Aptamers (600 nM), quencher probe (1200 nM), and Hsp90 (2000 nM) were all added at the same time, incubated for 20 minutes at room temperature, and fluorescence was measured.

Results yielded excellent quenching but little switching upon addition of Hsp90 (Figure 3B and 3C). Both aptamers showed significant quenching upon addition of the probes. However, there were no significant changes in fluorescence between the Aptamer/Quencher Probe conditions and the Aptamer/Quencher Probe/Hsp90 conditions. This lack of switching means that Hsp90 was not detected.

3.2 Testing quencher probes that bind to the 3' end of the aptamer

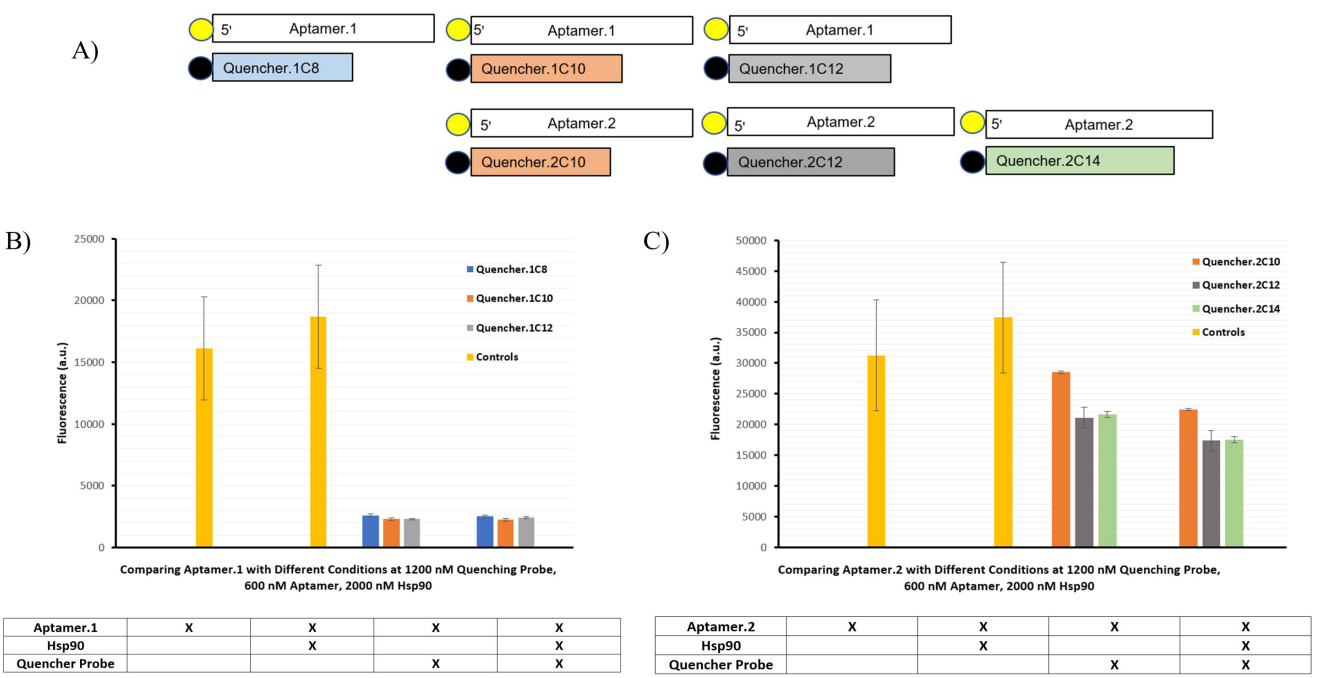
Given that there is no information about the structure of the aptamers and the positions where binding is expected, we hypothesized that the binding site was far away from the 5' end of the aptamer. As such, we designed new quencher probes that interact with the 3' end of the aptamer. Accordingly, we flipped the aptamer design so that the fluorophore was also at the 3' end. We tested 3 designs for each aptamer that ranged from 10 to 12 nucleotides in length (Figure 4A). We specifically tested Aptamer.1 and not Aptamer.2 because we wanted to focus on improving one aptamer at a time in more detail.

Unfortunately, there was still significant quenching but no significant switching (Figure 4B). This suggests that the binding site of the target is not in this region.

3.3 New Quencher Probe Design to Bind the Center of Aptamer.1

Next, we tested our hypothesis that Hsp90 binds to the center of the aptamer sequence by designing a new quencher probe that bound the center of the aptamer. As seen in Figure 5A, the Quencher.1C25mis quencher probe (a 25 nucleotide long strand with a mismatched, non-complementary, region) has two fully complementary sequences to the aptamer, at the 5' end of the aptamer and the center of the aptamer, connected by a non-complementary thymine repeat segment that decreases the otherwise high affinity that would result from a long complementary strand.

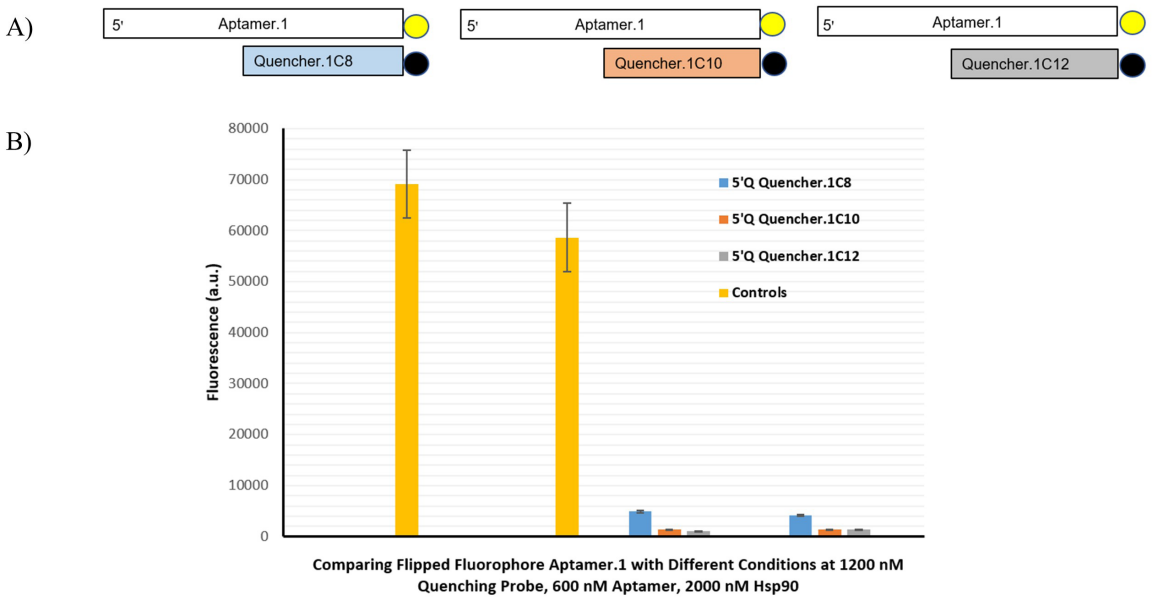
Results from this design showed significant quenching and switching as defined by an increase in fluorescence larger than error bars (Figure 5B). Moreover, a 5-fold increase in switching was seen when Hsp90 binding was favored through an increase in Hsp90 concentration and decrease in quencher probe concentration (Figure 5C), as compared to conditions in Figure 5B. More specifically, we increased the concentration of Hsp90 from 2000 nM to 5000 nM and quencher probe decreased from 1200 nM to 600 nM. This provides evidence that the Hsp90 is indeed resulting in the switch behavior. Overall, these results suggest that Hsp90 does indeed bind to the center of Aptamer.1; when the quencher probe binds to the center of the aptamer, switching occurs because the Hsp90 binds to the same area to displace the quencher probe.



Aptamer.1	X	X	X	X
Hsp90		X		X
Quencher Probe			X	X

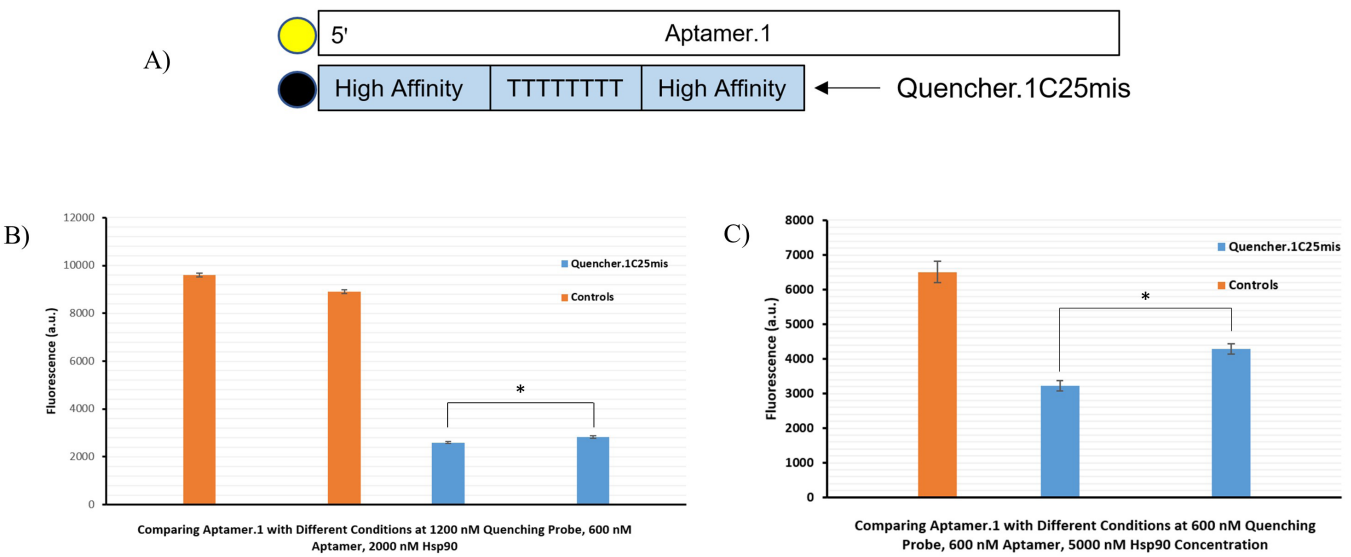
Aptamer.2	X	X	X	X
Hsp90		X		X
Quencher Probe			X	X

Figure 3. Fluorescent changes representing aptamer switching with probes that interact with the 5' end of the aptamers. A) Aptamer.1 and Aptamer.2 were each tested with 3 complementary quenching probes of different lengths. The yellow circle is the fluorescein, the black circle is the quencher. B) The Aptamer.1 aptamer-switch system showed strong quenching upon addition of all quenching probes but no significant change in fluorescence upon Hsp90 introduction. C) The Aptamer.2 aptamer-switch system showed some fluorescence quenching upon addition of the quenching probes but no change in fluorescence upon addition of the target. Error bars represent standard deviation across duplicates.



Flipped Aptamer.1	X	X	X	X
Hsp90		X		X
Quencher Probe			X	X

Figure 4. Fluorescent changes representing aptamer switching with probes that interact with the 3' end of the aptamers. A) Aptamer.1 was tested with 3 complementary probes of different lengths. B) The Aptamer.1 aptamer-switch system showed strong quenching upon addition of all probes but no significant change in fluorescence upon Hsp90 introduction. Error bars represent standard deviation across duplicates.



Aptamer.1	X	X	X	X
Hsp90		X		X
Quencher Probe			X	X

Aptamer.1	X	X	X
Hsp90			X
Quencher Probe		X	X

Figure 5. Fluorescent changes representing aptamer switching with probe that binds the center of Aptamer.1. A) Design of the new probe. There are two complementary sequences, one at the 5' end of the aptamer and one at the center. These are joined by a low-affinity segment. B) Results of the aptamer-switch system shows significant quenching and switching ($p=0.02$) in conditions of 2000 nM Hsp90, 1200 nM Aptamer, and 600 nM Quenching Probe. C) Results of aptamer-switch system in a new condition of raised Hsp90 to 5000 nM and decreased quenching probe to 600 nM also shows significant quenching and switching ($p=0.02$). Error bars represent standard deviation across triplicates. P-values were calculated using a t-test. * indicates $P < .05$.

Discussion

In this study we tested two aptamers for their potential to be incorporated into an aptasensor. Both aptamers displayed high affinity binding to the target Hsp90. With this information in hand, we sought to design complementary quencher probes to the aptamers that would generate a significant conformational "switch" in the presence of Hsp90. Such quencher probes must bind the same location on the aptamer as the target, at a lower affinity than the target, to ensure that the target can successfully displace the quencher probe. This displacement separates the fluorophore on the aptamer from the quencher on the quencher probe, generating an increase in fluorescence indicative of target binding.

Since the chosen aptamers have no structural or binding information, we had to test three novel sets of quencher probe designs to achieve this switching. Our initial designs included quencher probes that interacted with the 5' end of the aptamer only. These showed no change in fluorescence signal in the presence of the target, suggesting that the binding region of the aptamer was not in the 5' end region. We next tested quencher probes that interacted with the 3' end of the aptamer and again observed no binding. This suggested that the majority of the target interaction occurred in the center of the aptamer. Indeed, designing quencher probes that interact with the center of an aptamer but that function as fluorescence-quencher switches is challenging.

By making use of a polyT linker we designed a final quencher probe that bound the center of the aptamer while preserving aptamer "switching". This quencher probe was fully complementary to the aptamer in two short segments, one at the 5' end of the aptamer to maintain proximity between the fluorescein and quencher, and the other at the center of the aptamer, where the target likely binds. The polyT linker reduced the high binding affinity that would have resulted from a long complementary sequence to ensure the target could still displace the long quencher probe.

The significant switching demonstrated with Aptamer.1 and the poly-thymine containing quencher probe provides an exciting design. Before this can be translated into an aptasensor, however, it can be improved. First, the 25-nucleotide quencher probe should be shortened to reduce costs. A structural analysis approach could be used to determine the exact sequence of Hsp90 binding. More specifically, the K_D of a series of alterations to the aptamer could be made and compared to the K_D values found in this experiment. Segments that increase the K_D will be involved in Hsp90 binding, while those that do not change the K_D can be removed. Following structural analysis, the quencher location must be determined. If the quencher must be directly adjacent to the fluorophore for functionality, the fluorophore can be switched from the 5' to the 3' end of the aptamer depending on which location shortens the linker sequence connecting the quencher probe to the quencher. Alternatively, a quencher could be added directly to the short segment and tested to see if it can still act on the fluorophore despite being further away. This structural analysis approach will also provide more overall knowledge about the sequence of these aptamers that can be used towards other applications. In future avenues of research, Aptamer.2 switch systems could be tuned in more detail and compared to the current Aptamer.1 switch-system.

Furthermore, this aptamer-switch system needs to be tested in whichever bodily fluid it will be used in. This is of particular importance in the blood, as aptamers are known to denature easily in blood[18]. It must also be examined for specificity by testing in cell culture or with similar proteins such as other chaperones. Moreover, this aptamer switch system should be tested with all components (Aptamer, Quencher Probe, and Hsp90) added at the same time, instead of with aptamer and HSP90 incubated first for 30 minutes. If the system still works, this will accelerate testing. Last, the system should be tested with live malaria. If all goes well, it can be sent to a biotechnology company to be made into an aptasensor. This aptasensor can be tested in a variety of live malaria strains and compared to other diagnostic tests to assess its use and value.

Conclusion

A novel aptamer-switch system that detects Hsp90, an important protein in malaria, was tuned. Testing has optimized the quencher for the aptamer-switch system and indicated that Hsp90 likely binds to the center of the aptamer sequence. Although further studies are needed to optimize the system and test for stability and specificity, this system shows potential for development into an aptasensor. Once developed, a smartphone-compatible aptasensor could be compared to other aptasensors to determine how the novel target compares to existing targets. If superior, this aptasensor will be used in the field to detect malaria in a cheap, portable method to help combat the spread of malaria.

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