View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by The University of North Carolina at Greensboro

OPTIMIZATION OF A PS2.M-BASED CATALYTIC BEACON IN PREPARATION FOR USE AS A DEVICE TO EXPLORE mRNA SECONDARY STRUCTURES

A thesis presented to the faculty of the Graduate School of Western Carolina University in partial fulfillment of the requirements for the degree of Master of Science in Biology.

By

Donald Franklin Patterson

Director: Dr. Christopher T. Coburn
Assistant Professor of Biology
Biology Department

Committee Members: Dr. Sabine J. Rundle, Biology
Dr. Sean P. O'Connell, Biology

July 2010

ACKNOWLEDGMENTS

I would like to thank Dr. Chris Coburn for all of his help and patience involved in completion of this project. Additionally I would like to thank Drs. Sabine Rundle and Sean O'Connell for helping with the project and for serving on my committee, and Dr. Lori Seischab for serving as a reader of this thesis. Last, I would like to thank my loving wife, my five dogs, and the cat.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

OPTIMIZATION OF A PS2.M CATALYTIC BEACON IN PREPARATION FOR USE AS A DEVICE TO EXPLORE mRNA SECONDARY STRUCTURES

Donald Franklin Patterson, M.S.

Western Carolina University, July 2010

Director: Dr. Christopher T. Coburn

The DNA oligonucleotide PS2.M has been previously reported to have nanomolar affinity for hemin. The PS2.M-hemin complex then exhibits peroxidase activity. It was predicted that this sequence could be used in a catalytic beacon to facilitate the development of a laboratory tool that would be effective at determining potential siRNA target sites. The PS2.M-based beacon used for this study forms a stem-loop structure that opens upon hybridization with a single-stranded target sequence. Once open, the PS2.M part of the beacon can fold, locking hemin within a three level planer arrangement. Once hemin is in place, the structure is catalytically active and can catalyze the oxidization of the chromogenic substrate 2,2,'-azino-bis (3-ethylebenzthiazoline-6-sulphonic acid) (ABTS) in the presence of hydrogen peroxide. Thus, binding of the beacon can be monitored with absorbance readings at 414 nm. Although molecular beacons with a stem-loop structure could potentially be used for this type of test, the cost would be prohibitively expensive for most labs. Molecular beacons cost over $300 a piece, and many of them would be needed to conduct an exploration for a suitable siRNA target site within an mRNA. In contrast, a PS2.M-based beacon would only cost approximately

$12. In general, development and optimization of a PS2.M-based catalytic beacon could provide a more economical and improved means of establishing siRNA target site accessibility within an mRNA. It was concluded that K^+ and Mg^+ are required for folding of the PS2.M beacon. The substrate ABTS offered the highest absorbance values at a concentration of 3.2 mM. Also, H_2O_2 concentrations of 3.6 mM and an extension of the beacon stem by 10 nucleotides will result in reduced non-specific background absorbance activity.

INTRODUCTION

1. RNA Interference

The process of RNA interference (RNAi) is a highly regulated enzyme-mediated pathway that uses small double-stranded RNA molecules to post transcriptionally regulate gene expression in eukaryotic cells. Two small molecules, microRNA (miRNA) and small interfering RNA (siRNA) have both been shown to interfere with the expression of specific genes through base pairing with complementary messenger RNAs (mRNAs) (Xiao et al. 2004). Both molecules have been found in plants, yeast, and some animals, with the response being viewed from an evolutionary standpoint as a defense mechanism against virus attack (Chinnapen and Sen 2004). The mechanism has been appropriated by researchers as an effective tool for the analysis of gene function and is believed to have therapeutic potential for silencing genes associated with human disease. As of May 2010, over 150 startup companies were involved in the development of siRNAs as a therapeutic tool and several siRNAs are currently under review in human clinical trials, including treatments for advanced solid tumors, macular degeneration, and metastatic melanoma (Tafer et al. 2010).

The mechanism of RNAi was initially elucidated in the nematode *Caenorhabditis elegans* (Stormo 2008). As shown in Figure 1, the process of RNA interference begins in the cell with production of double-stranded RNA (dsRNA). The dsRNA may be produced by transcription of a non-coding gene from the cell's own genome or as an intermediate in the replication of a virus within the cell. In either case, the dsRNA is recognized in the cytoplasm and cleaved into short segments approximately 20-22

nucleotides long by the enzyme Dicer. If the dsRNA originates from transcription of a regulatory non-coding gene, the short fragments are referred to as miRNAs. Otherwise, they are referred to as siRNAs.

Once Dicer has produced the short dsRNAs (either an miRNA or siRNA), the machinery of gene silencing is the same for both. The short dsRNA associates with a protein called Argonaute and additional proteins to form an active RNA-induced silencing complex (RISC). As the complex assembles, one strand known as the passenger strand is destroyed. The remaining strand, called the guide strand, conducts RISC to a complementary mRNA. After base pairing with its target sequence, the mRNA is cleaved by Argonaute, resulting in either its rapid degradation or prevention of translation. The released RISC complex can then bind to another complementary mRNA; therefore several mRNAs can be destroyed by one miRNA or siRNA (Li et al. 2009).

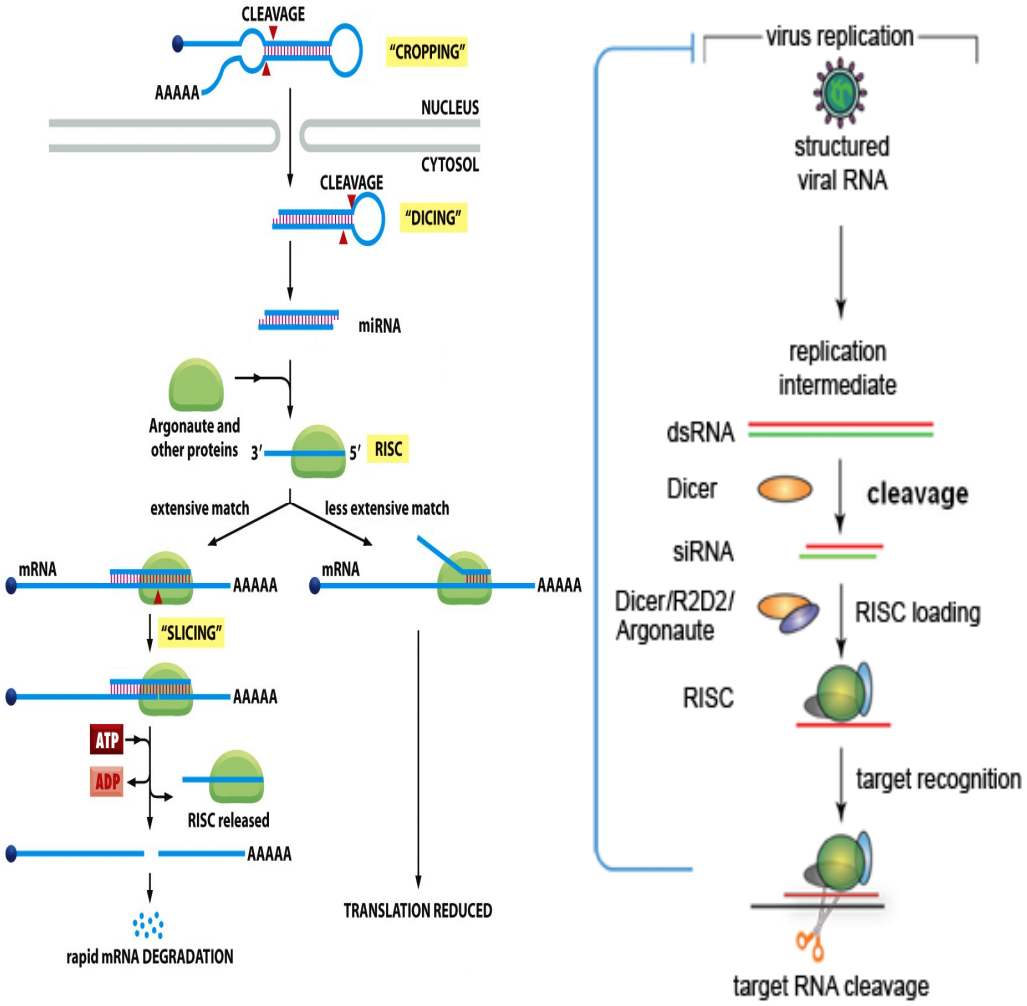

Figure 1. The process of RNA interference. **A.** RNAi is initiated when a natively expressed sequence is cleaved by the enzyme Dicer at the hair-pin section producing an miRNA. The RISC complex associates with these short nucleotide sequences and targets complementary mRNA sequences for silencing. **B.** RNAi can also occur through exogenously or endogenously produced siRNAs, with the pathway being the same as miRNA after entrance into the cytoplasm. Adapted from Alberts et al. 2008.

2. Effect of mRNA Secondary Structure on siRNA Efficiency

A limiting factor in the ability of an siRNA to silence a gene is the accessibility of the target site to the RISC complex. Single-stranded loops within the secondary structure of an mRNA are the best areas to target, as they increase the likelihood that hybridization with siRNA will occur. From an experimental point of view, the task of finding a suitable target site within an mRNA is normally performed in a trial and error fashion by introducing an siRNA into cultured cells and developing an assay to determine if the siRNA was able to decrease or eliminate expression of the protein. The trial and error approach to siRNA development can take several weeks and is labor intensive, making it very costly. Several laboratories have sought to increase their odds of success by using RNA secondary structure prediction algorithms to help identify potentially accessible target sites (Travascio et al. 1998). RNA structure prediction, however, is infamously unreliable and cannot take into account the tertiary structure, which could impede access of the RISC complex.

3. PS2.M Catalytic Beacon

Due to the unreliability of prediction algorithms and lack of an efficient laboratory technique, this thesis describes the development of a catalytic beacon to explore the accessibility of potential target sites within an mRNA. The PS2.M catalytic beacon was borrowed from the previous work of Travascio et al. (1998) and adapted as a potential device for identifying single stranded regions within an mRNA suitable for RNAi. The mechanism of this detection method is outlined in Figure 2.

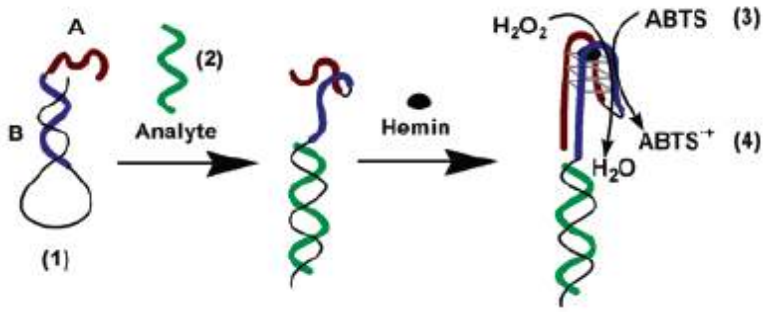

Figure 2. Mechanism of target oligonucleotide detection by the catalytic beacon. PS2.M-based catalytic beacons form a stem-loop structure. When added to a solution containing the single stranded target oligonucleotide, the loop hybridizes with the target sequence, causing the stem to open. This releases the PS2.M sequence (segments A and B), allowing it to bind hemin. The presence of the target oligonucleotide may be determined by assaying for peroxidase activity. In this example, peroxidase activity is measured by monitoring oxidation of the chromogenic substrate ABTS (Xiao et al. 2004).

The beacon (1) is a stem-loop structure constructed from a short DNA oligonucleotide. The loop is complementary to a target single-stranded RNA (2) and is flanked by two self-complementary sequences, forming the stem. The catalytic component of the beacon is composed of a hemin-binding aptamer (HBA) referred to as PS2.M. The PS2.M sequence contains 18 nucleotides and has been reported to bind hemin with nanomolar affinity (Travascio et al. 1998). In the stem-loop configuration, PS2.M is held inactive, as one segment (B) is bound to a complementary strand. However, if the target is present, hybridization will occur with the loop region, forcing the stem to open. This results in release of segment B, allowing the PS2.M sequence to bind hemin and fold into its active conformation. With hemin bound, PS2.M is a functional DNAzyme capable of peroxidase activity. In this reaction, the reduction of H_2O_2 to water is coupled to the oxidation of ABTS to $ABTS^{\bullet+}$. The oxidation of ABTS can be detected by monitoring the change in absorbance at 414 nm with a spectrophotometer.

Incorporating a DNAzyme into a beacon offers advantages over classical molecular beacons. Molecular beacons also use a stem-loop approach, but with a fluorophore at one end and a quencher at the other. Hybridization to a target sequence causes the loop structure to open, separating the quencher from the fluorophore. This separation allows the fluorophore to emit a fluorescent signal that can be detected by fluorescence spectroscopy. The key advantage that justifies development and optimization of a PS2.M based catalytic beacon is cost; a PS2.M based catalytic beacon would cost under $12, compared to over $300 for a similar molecular beacon. Because many sites within an mRNA might have to be probed before a suitable single-stranded

site is discovered, the cost of using molecular beacons for this purpose would be prohibitive for most laboratories. Given the detection provided by the PS2.M beacon and its low cost, it is an ideal structure to develop for this application.

4. Peroxidases

Peroxidases are enzymes found throughout all biological kingdoms. Their usefulness in the molecular laboratory is demonstrated by the widespread use of horseradish peroxidase isolated from the root of the horseradish plant (Travascio et al. 2001). Peroxidases are hemoproteins that function through oxygen redox exchanges to catalyze the reduction of peroxides. The widespread use of these enzymes is due to their ability to couple the reduction of a peroxide to the oxidation of a reporter substrate, producing a change in color. This shift in absorption of light allows the reaction to be monitored in real time using a spectrophotometer.

5. G-Quadruplex Structures

The catalytic beacon PS2.M is based around a guanine-rich DNA sequence that is able to fold into a four sided box structure that can bind a hemin molecule with high affinity and specificity (Figure 3). Each of the structural levels of this G-Quadruplex structure is called a guanine-quartet unit (G-quartent). As the name infers, each corner is composed of guanine bases that are linked together by 4 hydrogen bonds between each corner. Up to eight levels of these square guanine sections are known to be formed by stacking, with three present in the PS2.M beacon (Figure 3). Phosphodiester bonds between the corners of the stacked G-quartents provide a rigid support system (Xio et al.

2004). The stacking is stabilized by charged dipole interactions between the eight oxygen atoms and a cation located between two G-quartent levels. The cation most favored for incorporation within the cavity of the folded structure is potassium.

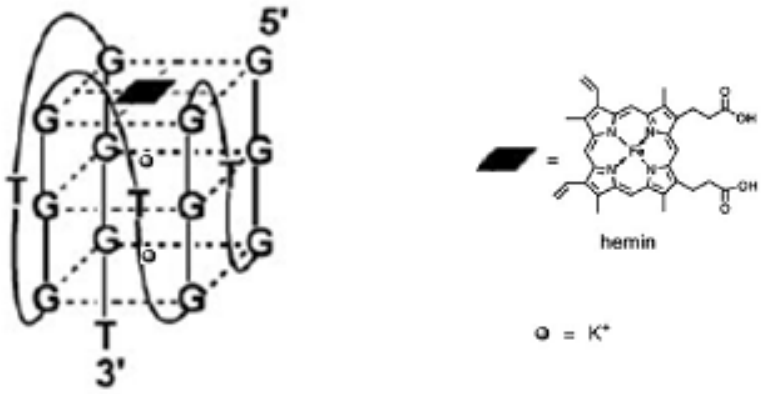

Figure 3. Structure of PS2.M. Hemin is stabilized by guanine in a box-like configuration called a guanine-quartet unit. This planar arrangement enables stacking that is stabilized with metal ions present between the units (Travascio et al. 1998).

SPECIFIC AIMS

The goal of this project was to optimize the peroxidase reaction catalyzed by the PS2.M-based beacon to a point where it could be used to explore mRNA secondary structures. Therefore, the strongest absorbance signal possible was an important goal, along with reducing non-specific activation of the beacon. Any published developments that included improvements were acknowledged during the design phase of the project.

It is our hope that the assay can be used in the future to open the door to financially limited labs so that research into the development and use of siRNAs can be more accessible. Ideally, then labs with limited resources and facilities should be capable of performing the reaction. For example, the developed protocol only requires a -20^{o}C freezer, a pH meter, a spectrophotometer, and calibrated pipettes. The wavelength at which the reaction is monitored matches a filter commonly found in most spectrophotometers. Components not absolutely necessary for the peroxidase reaction have been eliminated.

SIGNIFICANCE

At present no other publications have explored the possibility of using a catalytic beacon as a device for exploring mRNA secondary structure accessibility. Algorithmic approaches at structure prediction have been the standard, but results have been far from ideal. It has been shown that the ability of the RNA-induced silencing complex (RISC) to efficiently reduce translation of an mRNA is directly related to its ability to access the mRNA target site (Williamson 1994). RISC cannot unfold structural RNA, so the siRNA must target the complex to a single-stranded region of the target mRNA (Thornalley et al. 1983). Therefore, experiments are necessary to see if a PS2.M-based beacon could be used to probe an mRNA for accessible single-stranded sites. The initial work presented here offers the hope that a PS2.M-based catalytic beacon could be developed that would accurately and dependably predict accessible RNAi target sites. A system capable of detecting such sites would hold great therapeutic potential.

MATERIALS AND METHODS

1. Preparation and Storage of Stock DNA Oligonucleotides and Beacon

All DNA oligonucleotides (PS2.M, the PS2.M beacons and control and target oligonucleotides) were purchased from Sigma-Aldrich (St Louis, MO), with a purity of over 99.1% guaranteed by several size-purification steps performed at their facilities. All were received dissolved in water at a concentration of 100 μM. Stocks were divided into 30 μL aliquots and stored in a Styrofoam container at -20^{o}C. The PS2.M, PS2.M beacon, target oligonucleotide, and control DNA oligonucleotide sequences are given in Table 1.

Table 1. Nucleotide sequences of PS2.M, the PS2.M beacon, target DNA, target RNA, and control DNA. The PS2.M region of the beacon is shown underlined.

Component	**Nucleotide Sequence**
PS2.M	5'TGGGTAGGGCGGGTTGGG3'
PS2.M Beacon	5'CCCTACCCACGTCGTGGTATTC<u>TGGGTAGGGCGGGTTGGG</u>3'
Target DNA	5'GAATACCACGACG3'
Target RNA	5'GUUTUCCUCGUCG3'
Control DNA	5'CCTACAGGTATTA3'

2. Preparation and Storage of Reaction Reagents

Reagents used in optimized protocol

Hemin stocks (5 mM) were prepared by dissolving the solid in dimethyl sulfoxide (DMSO) and the concentration of the solution was confirmed with absorbance measurements using the Beer-Lambert law and an extinction coefficient of $\varepsilon(407\ nm) = 174\ mM^{-1}cm^{-1}$ (Collier 1979). Hemin stock solutions were aliquoted into 10 μL amounts, stored at -20°C, and covered to block exposure to light. When stored in this manner, the solution is stable for up to 30 days (Yoshida and Shimazono 1965).

An 83 mM stock of ABTS in water was prepared fresh before the start of each series of reactions. A 0.3% H_2O_2 (w/v) stock was prepared from the supplied 30% (w/v) reagent grade H_2O_2 solution by dilution with water before each reaction. Twenty-five mM HEPES buffer, pH 7.4, containing 200 mM NaCl and 20 mM KCl was used for both the reaction mix and the start mix. For each experiment, two different reaction mixes were prepared, one containing the beacon sequence and the other containing the negative control sequence.

Additional reagents tested

Buffers

Initial experiments in which the pH was varied to measure the effect of H^+ concentration on catalytic beacon activity were performed with Tris-HCl buffer. Twenty-five mM Tris-HCl buffers were prepared at pH 6.0, 6.5, 7.0, 7.5, and 8.0.

Chromogenic Substrates

All peroxidase substrates were purchased from Sigma Aldrich (St. Louis, MO). For these experiments, all parameters of the protocol remained the same but the new concentration of the substrate was taken into account and modifications made to the volume were balanced with the addition or subtraction of water. The concentrations of stock solutions and the final concentrations present in the peroxidase reactions are shown in Table 2 for each substrate.

Table 2. Concentrations of additional chromogenic substrates tested.

Substrate	Stock Solution (in water)	Final Concentration in Peroxidase Reaction
5-Aminosalicylic acid	87.5 mM	7 mM
3,3'-Diaminobenzidine (DAB)	13.88 mM	1 mM
Guaiacol	40 mM	3.2 mM
Luminol	50 mM	0.5 mM
o-Phenylenediamine	112.5 mM and 70.8	13.5 mM and 8.5 mM
Pyrogallol	40 mM	3.2 mM

3. The Optimized PS2.M Beacon Reaction Protocol

Procedure

Oligonucleotide stocks were removed from storage at -20^{o}C and allowed to thaw on ice. Aliquots of the beacon, target oligonucleotide, and control oligonucleotide stocks were diluted with an equal volume of 25 mM HEPES buffer (pH 7.4). Twelve microliters of the diluted beacon were transferred to a 0.2 mL PCR tube, which was placed in a PCR machine (GeneAMp PCR System 2400) and heated at 95^{o}C for 10 minutes. Immediately after heating, the PCR tubes were removed and 0.86 μL of the

heat-treated beacon was transferred to microcentrifuge tubes (one for each planned reaction) containing 39 μL of reaction mix (25 mM HEPES, pH 7.4, 20 mM KCl, 200 mM NaCl, 3.2 mM ABTS, 0.43 μM hemin). Ten microliters of either the diluted target or diluted control oligonucleotide were added, and the solutions were incubated for 90 minutes at room temperature. The entire content of each tube was then transferred to wells of a clear, flat bottomed 96-well plate (Nunc F, Nalge Nunc International, Rochester, NY). Using a multi-channel pipetter, 50 μL of start mix (25 mM HEPES, pH 7.4, 20 mM KCl, 200 mM NaCl, 3.2 mM H_2O_2) was transferred into the reaction mix for a total reaction volume of 100 μL. After mixing, absorbance measurements were taken at a wavelength of 414 nm every 15 seconds using the SpectraMax 190 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA).

Table 3. The optimized PS2.M beacon protocol. This protocol was developed to allow six reactions to be run in parallel.

Reaction Mix Stock Solutions	Volume for 1 reaction (40 μL):	Volume for 7 reactions (280 μL):	Final Concentrations
0.1 M HEPES, pH 7.4	12.5 μL	87.5 μL	25 mM
0.5 M KCl	2 μL	14 μL	20 mM
1 M NaCl	10 μL	70 μL	200 mM
0.083 M ABTS	3.9 μL	27 μL	3.2 mM
50 μM hemin in DMSO	0.86 μL	6 μL	0.43 μM
50 μM Beacon (heat treated for 10 minutes at 95^{o}C)	0.86 μL	6 μL	0.43 μM
water	10.7 μL	69.5 μL	

- Prepare 40 μL of reaction mix in a separate tube for each planned reaction as outlined above. Add 10 μL of 50 μM target or control oligonucleotide to each reaction tube. Incubate for 90 minutes at room temperature. Transfer to the wells of a clear, 96-well plate.

Start Mix Stock Solutions	Volume for 1 Reaction (50 μL):	Volume for 10 reactions (500 μL):	Final Concentrations
0.1 M HEPES, pH 7.4	12.5 μL	125 μL	25 mM
0.5 M KCl	2 μL	20 μL	20 mM
1 M NaCl	10 μL	100 μL	200 mM
0.3% (w/v) H_2O_2	3.6 μL	36 μL	3.2 mM
water	21.9 μL	219 μL	

- Start the reaction by adding 50 μL of start mix to each well. Monitor the absorption every 15 seconds at 414 nm for 15 minutes.

PS2.M Assays

For the PS2.M assays, an aliquot of the PS2.M stock was diluted with an equal volume of 100 mM Tris-HCl (pH 8.0) and heat treated at 95^{o}C for 10 minutes. The PS2.M was then added to a reaction mix and allowed to incubate at room temperature for 30 minutes before initiation of the reaction with H_2O_2. The reactions were initially prepared in 100 mM Tris-HCl buffer at pH 8.0, but other 100 mM Tris-HCl and 25 mM HEPES buffers at various pHs were tested. Final concentrations of 200 mM NaCl, 3.2 mM ABTS, 50 μM hemin, and 50 μM PS2.M were maintained for each reaction. The final KCl concentration was varied from 0 mM to 20 mM, and reactions were performed with or without 0.05% (v/v) Triton X-100. All reactions were initiated by addition of H_2O_2 to 3.6 mM. Absorbance measurements were taken at a wavelength of 414 nm every 15 seconds using the SpectraMax 190 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA).

RESULTS

1. DNAzyme Activity Using Published Protocols

Efforts were initially made to reproduce results obtained in a previous publication (Xiao et al. 2003). However, attempts to reproduce these experimental results were unsuccessful despite following the published protocol exactly and even contacting one of the authors for advice. Although some activity was detected (Figure 4) the results were not as reproducible as expected.

As shown in Figure 4, a steady increase in absorbance at 414 nm was detected for both the target DNA as well as the control DNA. With no target or control DNA present, the absorbance remained steady at 0.02. Although reproducible, the results from this protocol showed that it was not an effective detection method since the absorbance at 414 nm never exceeded 0.1. Variations were made in buffer pHs from these published protocols, but all reagents remained the same during the first 6 weeks of experimentation.

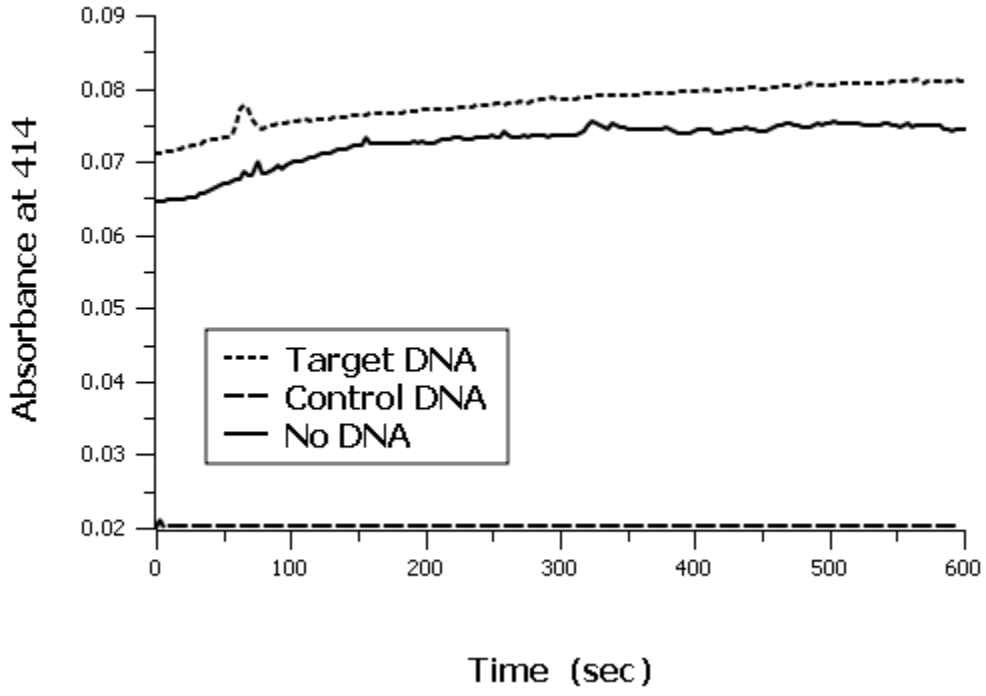

Figure 4. Initial results obtained with the PS2.M-based catalytic beacon. The assays were performed according to Xiao et al. 2003. Each reaction contained 0.43 μM beacon, 0.43 μM hemin, 3.2 mM ABTS, 3.2 mM H_2O_2, and 20 mM $MgCl_2$ in a 100 mM Tris-HCl buffer at pH 8.0. Although beacon activity was detected, the absorbance was weak and there was little discrimination between the target and control oligonucleotides.

2. Combining Methods from Other PS2.M Published Results

Since the results described in Section 1 were unsatisfactory, the protocol was modified with elements incorporated from structural studies of PS2.M alone (Travascio et al. 1998, Nakayama and Sintim 2009). Also, due to the prohibitively high price of RNA oligonucleotides, much of the optimization work was performed using only DNA oligonucleotides. Modifications to the protocol included changing the buffer from Tris-HCl to HEPES. Despite this change, however, increased absorbance values were not obtained until the salt present in both the reaction mix and the start mix was changed from $MgCl_2$ to NaCl and KCl. These ions appear to be necessary for proper folding of the PS2.M sequence.

Figure 5 shows the first results achieved with 200 mM NaCl and 20 mM KCl in HEPES buffer. The effect of buffer pH on the reaction was explored at pH values of 6.5, 7.0, 7.4, and 8.0. However, a significant change in absorbance was not observed, and a pH of 7.4 was chosen since this was the pH reported in the published protocols. A full spectral read (380-710 nm) was performed to ensure the chosen wavelength (414 nm) offered the highest absorbance readings. While a marginal improvement in absorbance measurements was obtained at 420 nm, in order to compare results to related publications in which the PS2.M beacon was used, the wavelength was kept at 414 nm. After repeated experiments during which the reaction was monitored for an hour, it was determined that the rate of the reaction stabilized within 10 minutes following initiation, and consequently the monitoring time was reduced to 15 minutes. The final protocol with the optimized concentrations of reagents is given in Table 3.

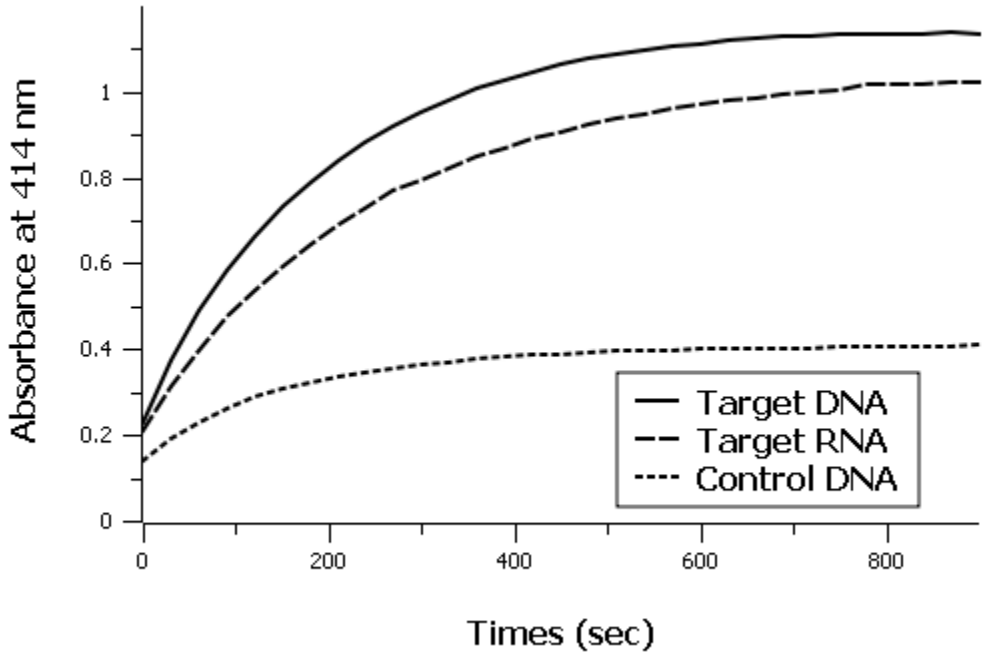

Figure 5. Initial results obtained with the modified protocol. The reactions were run in parallel. Each contained 0.43 μM beacon, 0.43 μM hemin, 3.2 mM ABTS, 3.2 mM H_2O_2, 20 mM KCl, and 200 mM NaCl in a 25 mM HEPES buffer at pH 7.4. The assays were performed in the presence of 5 μM target or control oligonucleotide, as indicated.

After repeating the experiment several times to ensure the results were reproducible, the optimization process continued with assessment of the effect of changes in ABTS concentration. The published value of 3.8 mM was tested along with an increase in concentration of 4.6 mM and a decrease of 3.2 mM. These reactions were performed in triplicate and results were found to be highly reproducible. Figure 6 details the concentration of 3.2 mM reporting the highest spectral values, while also increasing the target sequence specificity. Therefore, an improvement in the overall absorbance activity for the RNA was reported.

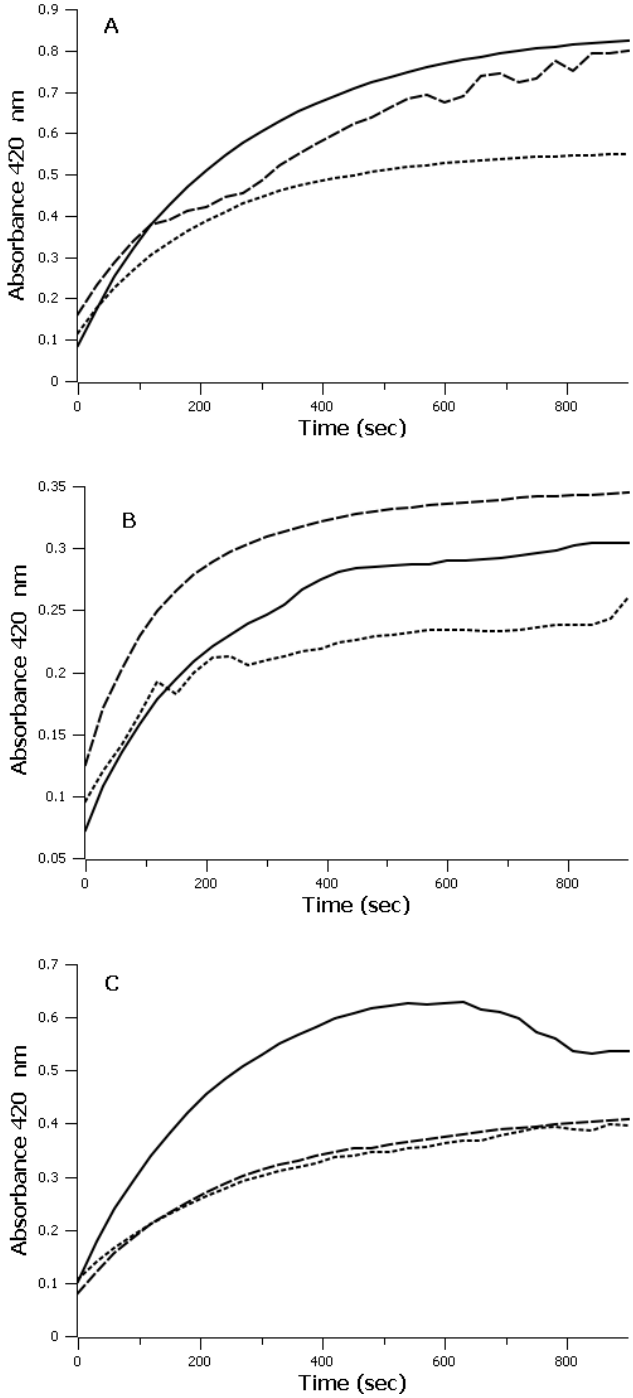

Figure 6. Results obtained varying the concentration of ABTS. All reactions were run in parallel. Each contained 0.43 μM beacon, 0.43 μM hemin, 3.2 mM H_2O_2, 20 mM KCl, and 200 mM NaCl in a 25 mM HEPES buffer at pH 7.4. The ABTS concentration was 3.2 mM (solid line), 3.8 mM (dashed line), or 4.6 mM (dotted line). Assays were performed with 5 μM (A) target DNA oligonucleotide, (B) control DNA oligonucleotide, or (C) target RNA oligonucleotide at each ABTS concentration (Data not to scale)

The optimization process continued with assessment of the effect of changes in the H_2O_2 concentration on the reaction rate. The concentration was modified from the value given by the Travascio protocol by either increasing or decreasing the H_2O_2 concentration by 20% (Figure 7). Reactions with the control sequence, DNA target sequence, and RNA target sequence were compared to assess the effects of these concentration changes. With an established value of 3.2 mM for ABTS, the H_2O_2 concentration that resulted in the highest level of absorbance for the target DNA was 3.6 mM. The concentration of H_2O_2 that gave the greatest response for the target RNA was determined to be 5.1 mM.

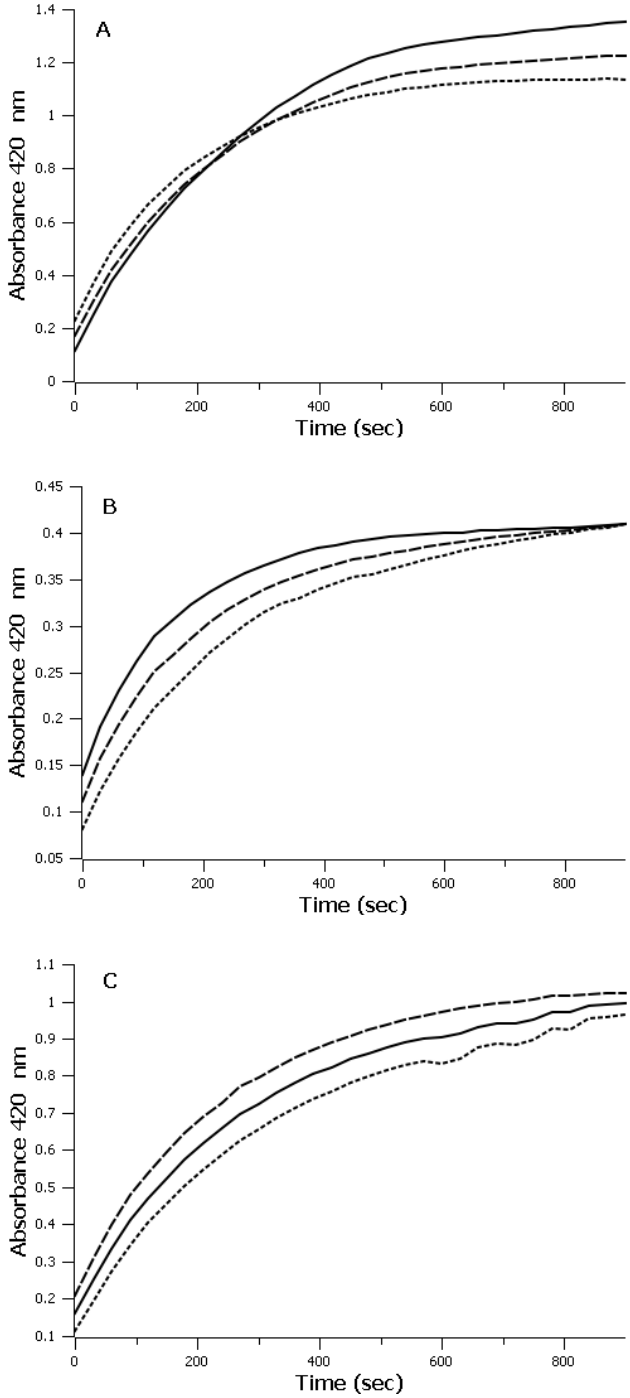

Figure 7. Results obtained varying the concentration of H_2O_2. All reactions were run in parallel. Each contained 0.43 μM beacon, 0.43 μM hemin, 3.2 mM ABTS, 20 mM KCl, and 200 mM NaCl in a 25 mM HEPES buffer at pH 7.4. The H_2O_2 concentration was 3.6 mM (solid line), 4.3 mM (dotted line), or 5.1 mM (dashed line). Assays were performed with 5 μM (A) target DNA oligonucleotide, (B) control DNA oligonucleotide, or (C) target RNA oligonucleotide at each H_2O_2 concentration. (Data not to scale)

At this point, essential reagent concentrations had been established, along with the most effective buffer, so incubation times were explored. As indicated in Table 3, the reaction mix was incubated at room temperature after heating for 10 minutes at 95^{o}C. This allows time for the beacon to hybridize with its target (if present) and fold into its peroxidase-active conformation. Incubation times reported in previous publications did not exceed 1 hour and included 5, 30, and 60 minutes. To assess the effect of incubation period on the reaction, incubation periods of 5, 30, 60, 90 and 120 minutes were tested. The relationship between incubation period and product formation for the target DNA sequence is shown in Figure 8 and for the RNA target sequence in Figure 9.

The protocol was also tested with other common peroxidase reporter substrates. Most showed no peroxidase activity, including DAB, TMB, TMPD, guaiacol, pyrogallol, and luminol (data not shown). Two alternate substrates did show activity. These were o-phenylenediamine and 5-aminosalicylic acid (Figures 10 and 11), but neither worked as well as ABTS.

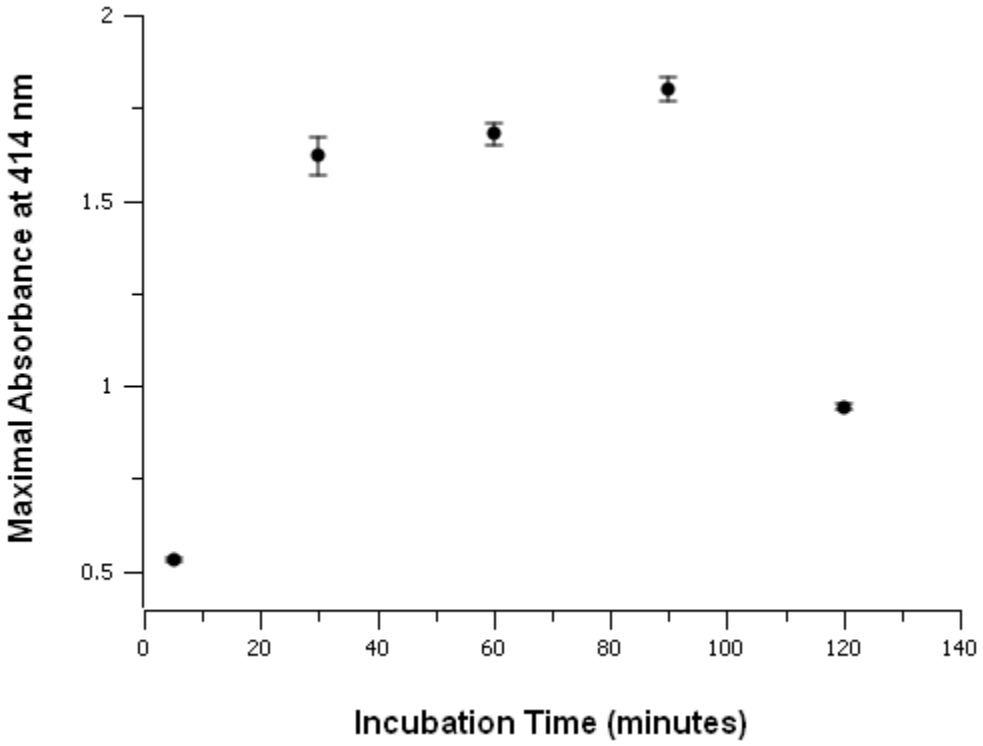

Figure 8. Effect of incubation time on beacon activity in the presence of target DNA oligonucleotide. The reactions were run in parallel. Each contained 0.43 μM beacon, 0.43 μM hemin, 5 μM target DNA oligonucleotide, 3.2 mM ABTS, 3.6 mM H_2O_2, 20 mM KCl, and 200 mM NaCl in a 25 mM HEPES buffer at pH 7.4. The incubation time between heat treatment of the beacon and initiation of the reaction was varied as indicated. The maximal absorbance attained following each incubation time was determined by fitting the raw data to a hyperbola.

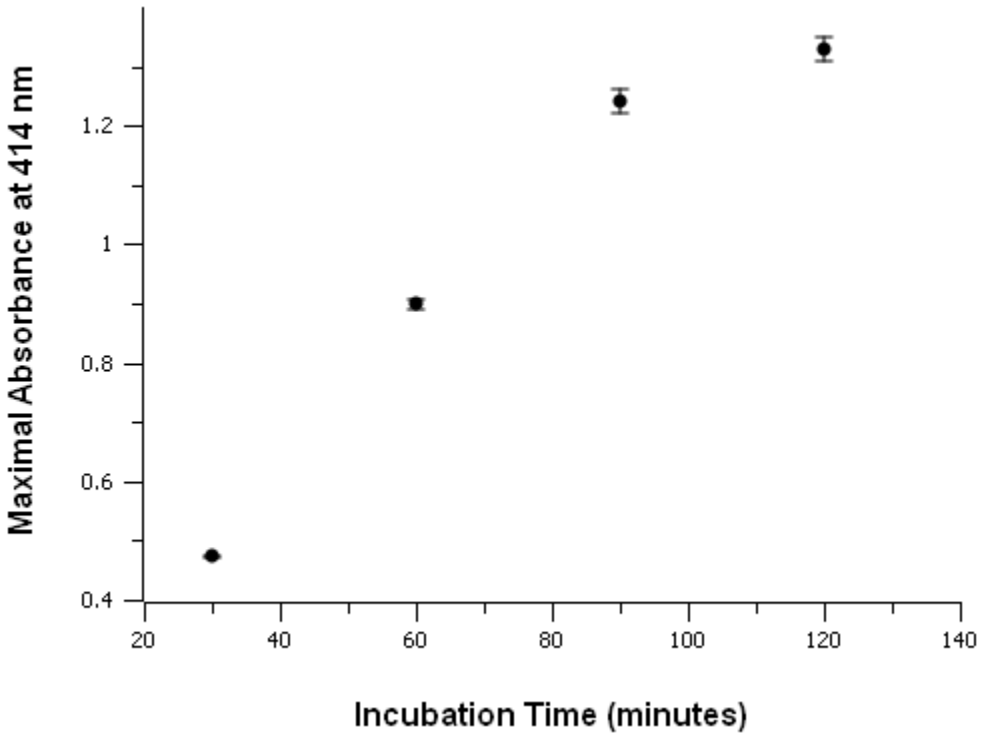

Figure 9. Effect of incubation time on beacon activity in the presence of target RNA oligonucleotide. The reactions were run in parallel. Each contained 0.43 μM beacon, 0.43 μM hemin, 5 μM target RNA oligonucleotide, 3.2 mM ABTS, 3.6 mM H_2O_2, 20 mM KCl, and 200 mM NaCl in a 25 mM HEPES buffer at pH 7.4. The incubation time between heat treatment of the beacon and initiation of the reaction was varied as indicated. The maximal absorbance attained following each incubation time was determined by fitting the raw data to a hyperbola.

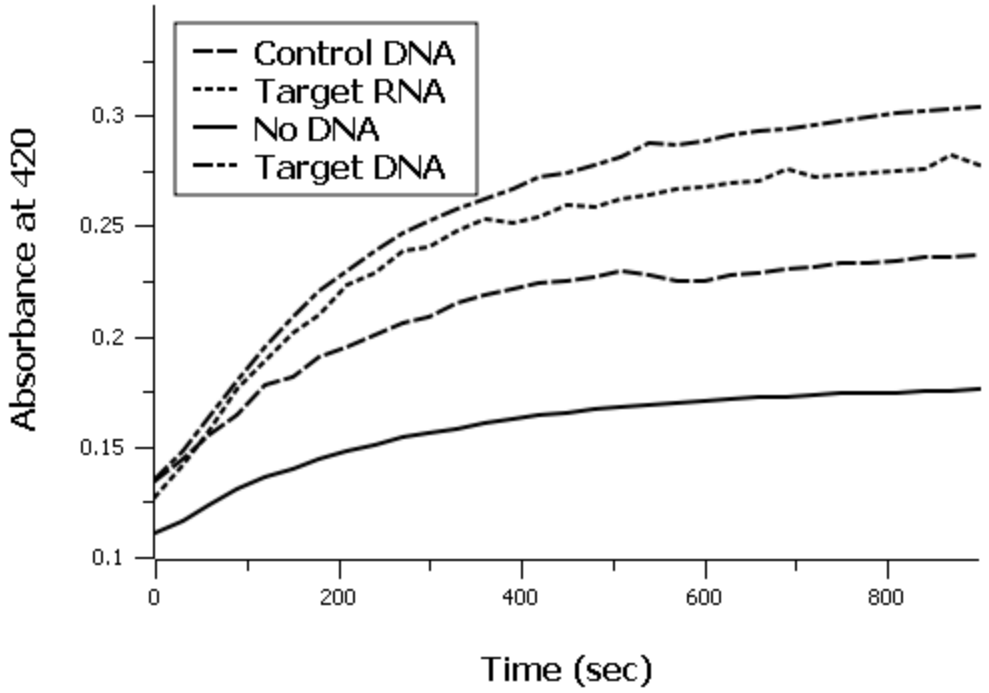

Figure 10. Results obtained with o-phenylenediamine as the chromogenic substrate. All reactions were run in parallel. Each contained 0.43 μM beacon, 0.43 μM hemin, 13.5 mM o-phenylenediamine, 3.6 mM H_2O_2, 20 mM KCl, and 200 mM NaCl in a 25 mM HEPES buffer at pH 7.4. Assays were performed in the presence of 5 μM target or control oligonucleotide, as indicated. The absorbance was monitored at 420 nm.

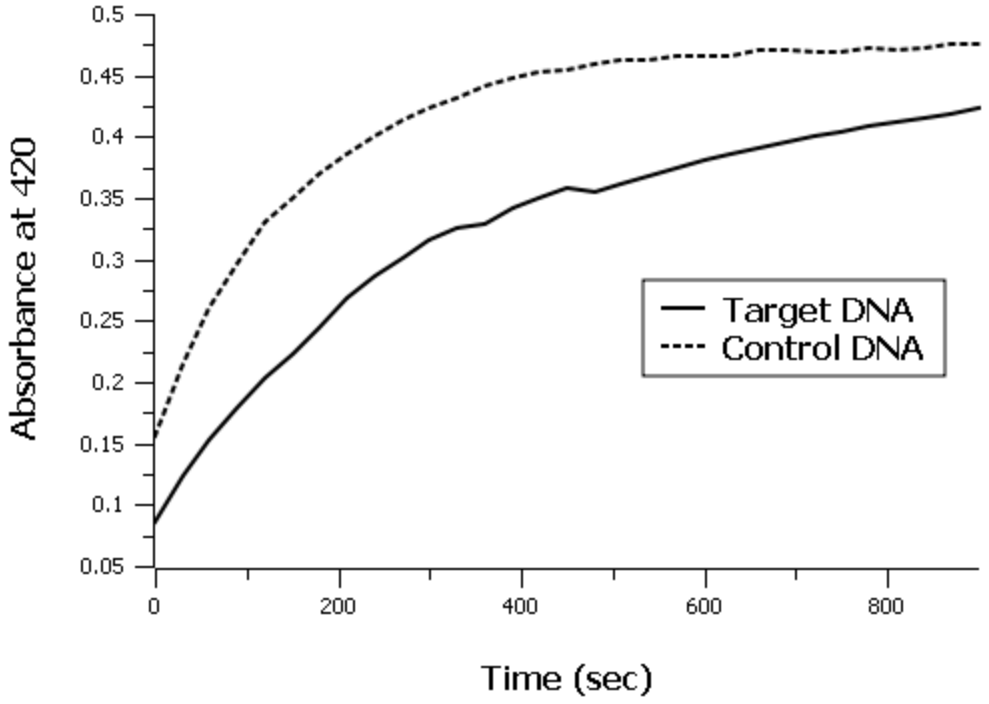

Figure 11. Results obtained with 5-aminosalicyclic acid as the chromogenic substrate. All reactions were run in parallel. Each contained 0.43 μM beacon, 0.43 μM hemin, 7 mM 5-aminosalicyclic acid, 3.6 mM H_2O_2, 20 mM KCl, and 200 mM NaCl in a 25 mM HEPES buffer at pH 7.4. Assays were performed in the presence of 5 μM target or control oligonucleotide, as indicated. The absorbance was monitored at 420 nm.

3. Effect of Stem Length on the Assay

Ideally, the assay would produce a high signal in the presence of target and no signal (i.e. no change in absorbance) in the absence of target. Because we saw some activity in the presence of a non-target control oligonucleotide, we sought to maximize the ratio of target signal to non-target signal at 15 minutes post-initiation. To do this, we varied the stem length of the beacon from 8 and 13 nucleotides. The results are shown in Figure 12.

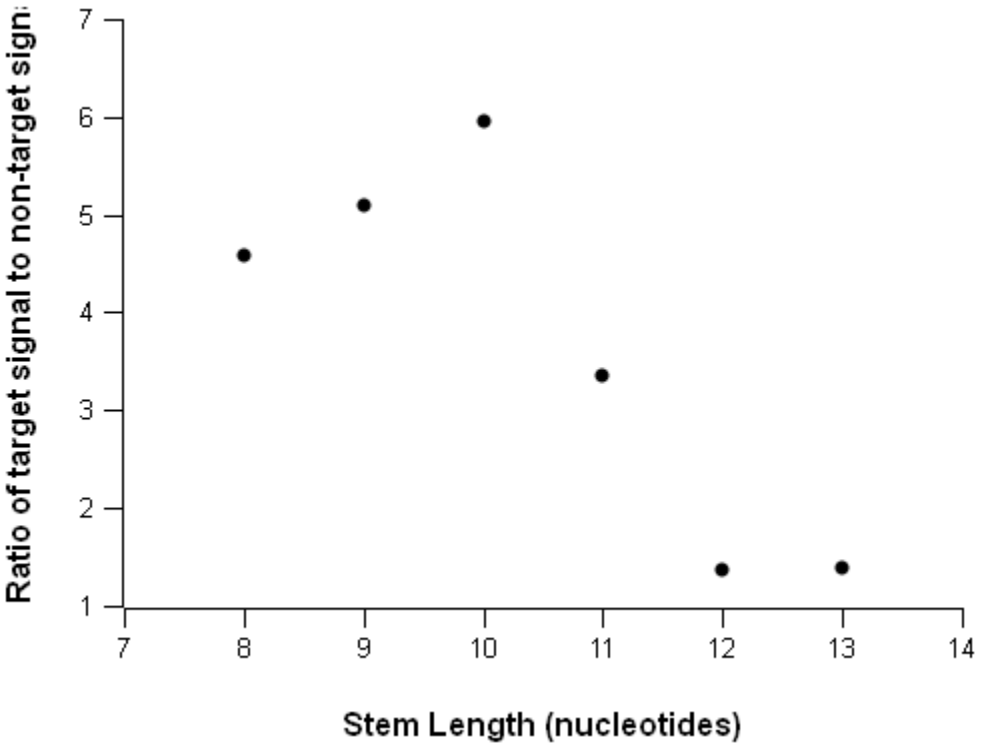

Figure 12. Effect of stem length on beacon activity. The reactions were run in parallel. Each contained 0.43 μM beacon, 0.43 μM hemin, 5 μM target or control DNA oligonucleotide, 3.2 mM ABTS, 3.6 mM H_2O_2, 20 mM KCl, and 200 mM NaCl in a 25 mM HEPES buffer at pH 7.4. The stem-length of the beacon was varied from 8 to 13 base pairs as indicated. Each reaction was performed in triplicate and the ratio of target signal to non-target signal was determined from the average absorbance measured for each at 15 minutes after the start of the reaction.

DISCUSSION

1. Optimizing the PS2.M Catalytic Beacon Assay

Basis for PS2.M Selection for Beacon

This study was conducted to optimize the use of the PS2.M sequence in a catalytic beacon for the detection of a single stranded target nucleic acid sequence. This was the first step before initial trials could be performed to explore natively-folded mRNA secondary structures. In the absence of target, the PS2.M component of the catalytic beacon is held in an inactive conformation by hybridizing within the stem of the stem-loop structure. Hybridization of the loop with the target sequence forces the stem open, releasing the PS2.M sequence. The PS2.M sequence must then fold with hemin into its active conformation. Therefore, the conditions required for folding were explored because they would result in improvements in overall peroxidase activity.

Results Obtained Following Published PS2.M Protocols

Attempts were made to extend the published protocol that originally detailed the PS2.M catalytic beacon, but after several months this approach was abandoned. After having difficulties following the methods as outlined by Xiao *et al* (Xiao et al. 2004), I requested the exact protocol from the author and repeated it exactly with results typical of those shown in Figure 4. The maximal absorbance in the presence of target was much lower than reported, but clearly, some activity from the beacon was occurring due to the fact that the control sequence did elicit somewhat less absorbance at 414 nm.

Effect of Salts on the Peroxidase Reaction

Since we initially had such difficulty obtaining results similar to those previously reported for a PS2.M-based beacon, I decided to focus our attempts on assaying the PS2.M sequence by itself. Other researchers, interested in the structures of PS2.M and related HBAs, have shown that both NaCl and KCl are necessary for the two-step folding process required to achieve the active conformation of the PS2.M DNAzyme. To bind hemin, the sequence must form a guanine-quadruplex structure of stacked G-quartents. The stacked G-quartents form a hollow space that is the exact size and shape to allow chelation of potassium, and it is also possible that the free energies of hydration prefer potassium over sodium ions. Indeed, the folding of each G-quartent requires a specific bond angle at each of the guanine sequences and correct alignment does not occur unless potassium ions are present (Micheli 2009).

Since catalysis will not occur unless potassium is present, PS2.M reactions were performed to determine the best possible KCl concentration. Quantities as little as 3 mM were found to result in DNAzyme activity (data not shown). The lack of potassium cations in the first set of experiments is probably why the beacon gave such poor results.

Stabilization of Hemin in an Aqueous Solution and Buffer pH

One difficulty in stabilizing hemin in aqueous solutions is the poor water solubility of hemin. Therefore, hemin had to be prepared in DMSO to prevent clumping and reduce spikes during spectral readings. As mentioned in other hemin protocols, aggregation of hemin in aqueous solutions may result in its chemical properties being changed (Xiao et al. 2004). Therefore, attempts to dissolve hemin in aqueous solutions

will result in an equilibrium occurring, so that the only a fraction of the dissolved hemin is available as the monomer to complex with PS2.M.

Because the solubility of hemin is somewhat pH dependent, we explored the effect of buffer pH on the reaction, trying values of 6.5, 7.0, 7.4, and 8.0. However, a significant change in the ability to discriminate between target and control nucleotides was not observed. We decided to continue with a pH of 7.4 because we are developing this assay to probe native mRNA structures, which formed within the cell at a slightly alkaline pH.

In addition to testing the effects of salt and pH, we removed Triton X-100 from the protocol. Although it was previously reported that the addition of the detergent would reduce background signal, its removal from the reaction mixture had no affect on absorbance values in our hands (Travascio et al. 1998).

Optimization of ABTS Concentration

Once I had the PS2.M assay working, I returned to experimenting with the PS2.M-based catalytic beacon. My next step was to optimize spectrophotometric detection of the PS2.M catalytic beacon activity by determining the ABTS concentration that would produce the highest absorbance. The peroxidase reaction proceeds according to the following scheme:

HOOH + ABTS Ý HOH + $ABTS^{\bullet+}$ + H_2O.

In the course of the reaction, the reduction of hydrogen peroxide is linked to the oxidation of ABTS. The concentrations of both are critical to the level of reactivity, each

working in synergy with the other. If ABTS is the limiting factor, then I would expect that a higher concentration of ABTS would provide a stronger signal.

To test this, I performed the assay with three concentrations of ABTS: 3.2 mM, 3.8 mM and 4.6 mM. Each assay included 4.3 mM H_2O_2. The results, shown in Figure 6, were somewhat surprising. A greater signal was obtained using a concentration of ABTS 25% lower than the H_2O_2 concentration. At higher concentrations, ABTS appears to inhibit the reaction. It may be that the two negatively charged sulfonic acid groups of ABTS (each with a pKa of 2.08) attracted potassium ions preferentially to sodium ions (Thomas et al. 2003). If so, at the higher concentration, ABTS might interfere with proper folding of the PS2.M. Regardless of the cause, we used 3.2 mM ABTS in all subsequent experiments.

Optimization of Hydrogen Peroxide Concentration

Once the concentration of ABTS was established, work turned toward achieving the same goal with H_2O_2. We performed the assay with three H_2O_2 concentrations: 3.6 mM, 4.3 mM, and 5.1 mM. The 3.6 mM H_2O_2 concentration, which nearly matches the ABTS concentration, showed some improvement in detecting the DNA oligonucleotide target, so this concentration was used in all subsequent experiments.

Effect of Incubation Time

Further improvements were made to the protocol by studying the incubation times necessary for the PS2.M beacon to fold into the correct active configuration. Previously it was reported that 20 minutes is adequate to allow the PS2.M sequence to completely

fold with hemin into the active DNAzyme (Travascio et al. 1998). We tested the following incubation times: 5, 30, 60, 90, and 120 minutes.

The results differed greatly between assays for the DNA and RNA oligonucleotide targets. It is apparent in assays for the DNA target that 5 minutes is not enough time to allow all the available beacon to fold into the active conformation. The peak absorbance following a 5 minute incubation was less than one third that attained following a 90 minute incubation. We also found that, in general, the reaction progress curves are less reproducible when using incubation times under 60 minutes. Although a 60 minute incubation time is adequate for detection of an unpaired DNA sequence, assays with the RNA target showed that a 90 minute incubation time clearly resulted in a superior signal. Since we wish to use the assay to detect unpaired RNA sequences, we decided to use 90 minute incubation in all subsequent assays. Once 90 minutes was decided upon, the experiment was repeated twice more with only a 0.5% variance between reactions (data not shown).

2. Trials with Various Substrates

Out of eight additional chromogenic substrates assayed, only two worked with the beacon protocol. None of them were able to provide detection comparable to that obtained with ABTS. Although these substrates have been shown to provide adequate detection with other peroxidases, clearly for PS2.M, ABTS provides the best detection. Perhaps ABTS can form a more stable interaction with the porphyrin ring of the hemin.

3. Effect of Stem Length

To further optimize use of the PS2.M sequence in a catalytic beacon, attempts were made to maximize the ratio of target-specific activation to nonspecific activation. This was done by varying the length of the stem region. A shorter stem should make it easier for the beacon to open and hybridize with its target sequence. It might also be expected to increase the level of non-specific activation, especially if the folded PS2.M is energetically more stable than the hybridized stem.

Ideally, hydrogen bonding in the stem region should be strong enough to inhibit activation in the absence of target but weak enough to unfold and enable hybridization to occur. The sequences used for these experiments are given in Table 4, with the stem-forming regions underlined. The experimental results (Figure 12) clearly show that a stem length of 10 oligonucleotides is optimal under these conditions. At stem lengths longer than this, the stem is too stable and appears to interfere with hybridization.

Table 4. Tested PS2.M based beacons with varying nucleotide lengths. Stem-forming sequences are underlined.

Oligo Name	**Beacon Sequence**
8stem_bGAPDH.378	CCTACCCAGACGAACATGGGGGCATCATGGGTAGGGCGGGTTGGG
9stem_bGAPDH.378	CCCTACCCAGACGAACATGGGGGCATCATGGGTAGGGCGGGTTGGG
10stem_bGAPDH.378	GCCCTACCCAGACGAACATGGGGGCATCATGGGTAGGGCGGGTTGGG
11stem_bGAPDH.378	CGCCCTACCCAGACGAACATGGGGGCATCATGGGTAGGGCGGGTTGGG
12stem_bGAPDH.378	CCGCCCTACCCAGACGAACATGGGGGCATCATGGGTAGGGCGGGTTGGG
13stem_bGAPDH.378	CCCGCCCTACCCAGACGAACATGGGGGCATCATGGGTAGGGCGGGTTGGG

FUTURE RESEARCH

Optimizing the PS2.M beacon was only the first step in development of it as a detection device. Its ability to detect unpaired sequences within an mRNA has not yet been demonstrated. No clues have been found that indicate the catalytic beacon cannot be used for this purpose, but without future experiments this cannot be confirmed. The next phase of experiments would study the influence that RNA secondary structure has on beacon activation. This could be accomplished by performing reactions that include RNA sequences with known secondary structures. Different RNAs containing the same target sequence but with accessibility to the sequence reduced by a stepwise increase in secondary structure could be used. By running multiple RNA reactions with the same target sequence in parallel, any changes in the peroxidase activity could be attributed exclusively to the ability of the beacon to locate the target and access the RNA secondary structure. It is expected that the stability of the secondary structure (as determined by its ΔG) should be the deciding factor in assessing the accessibility to the catalytic beacon. As with the peroxidase reaction performed in this work, a non-related RNA sequence would also be required to function as a negative control so that the level of non-specific peroxidase activity could be determined.

Next, future experiments must explore the use of the catalytic bacon to look for target sites within an actual mRNA. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA would be an obvious choice. The mRNA from this gene has been targeted at 75 different sites for RNAi, and the silencing efficiency at each site has been measured (Robins et al. 2005). Catalytic beacons could be designed against a number of

these sites and assayed in separate reactions for their ability to detect these sites within the length of the GAPDH mRNA (1310 nucleotides). It is expected that the level of activation of a catalytic beacon would correlate positively with the siRNA silencing efficiency for that site. If this is found to be true then it would suggest that the PS2.M-based catalytic beacon could be used to facilitate identification of effective siRNA target sites within an mRNA.

APPENDICES

APPENDIX A: OPTIMIZED PROTOCOL

Procedure:

Dilute Beacon, Control and Target Stock Nucleotides from 100 µM to 50 µM

1. Remove 5µL for each reaction to be performed from the SIGMA stock nucleotide solution, and transfer to a new PCR thin walled microcentrifuge tube. These tubes should be labeled either T Dilution or C Dilution

2. Add 5 µL of 25 mM HEPES buffer solution for each reaction to be performed and place into the PCR tube with the stock solution.

3. For dilution of the beacon, remove 1 µL of the stock beacon for each reaction and add an equal amount of 100 mM HEPES buffer.

Prepare the master mix

1. Label a 1.5 mL microcentrifuge tube as the master mix.

2. Add 12.5 µL 100 mM HEPES buffer for each reaction.

3. Add 10.7 µL of water for each reaction.

4. Add 2 µL of 0.5 M KCl for each reaction.

5. Add 10 µL of 1 M NaCl for each reaction.

6. Add 1 µL of 50 µM hemin in DMSO for each reaction.

Preparation and heating process (for unfolding beacon)

1. Label a PCR tube for each reaction. For reactions that will include the control DNA, add C to the end of the label.

2. Add 40 µL of the master mix to each PCR tube.

3. If the reaction is to contain beacon, add 1 µL from the diluted beacon stock. Otherwise, add 1 µL water.

4. Place each PCR tube into the PCR machine and run the program for heating at 95^{o}C for 10 minutes.

Incubation (refolding and beacon formation phase)

1. Remove tubes from the PCR machine and add 3.9 µL of 83 mM ABTS to each tube.

2. Remove 10 µL from the control DNA stock dilution and add to each PCR tube labeled with a C.

3. Remove 10 µL from the target DNA stock dilution and add to each PCR tube not labeled with a C.

4. Allow the tubes to sit at room temperature for 90 minutes.

5. Prepare the software for monitoring the reaction. Under template, label each well according to the reaction placed in it. Measure the absorbance at 414 nm. Mix before the first reading only. Read every 15 seconds and set the duration of the reaction to 15 minutes. Highlight each row of wells to be measured.

Prepare the start mix (for a total volume of 500 µL)

1. Five minutes before the end of the incubation period label a fresh 1.5 mL microcentrifuge tube as the start mix.

2. Add 125 µL of 100 mM HEPES.

3. Add 20 µL of 0.5 M KCl.

4. Add 100 µL of 1 M NaCl.

5. Add 219 µL of water.

6. Add 36 µL of 0.3% H_2O_2.

7. Transfer the contents of the start mix tube to a trough container so all tips from the multi-channel pipette can remove start mix.

Start the reaction

1. Transfer 50 µL from each PCR tube and place into a fresh well of the 96-well plate, and place the plate into the tray of the spectrophotometer.

2. Using a multi-channel pipette, withdraw 50 µL of start mix into each tip.

3. Carefully pipette the start mix into the wells.

4. Click read in the Softmax software.

APPENDIX B: STOCK SOLUTIONS

Make 100 mL of a 100 mM HEPES stock solution:

$$\frac{238.3\ g}{mol} \times \frac{0.1\ mol}{L} \times 0.1\ L = 2.383\ g\ HEPES\ (free\ acid)$$

Add water to 90 mL.
Adjust the pH to 7.4 with 1 N NaOH.
Add water to 100 mL.

Make 100 mL of a 0.5 M KCl stock solution:

$$\frac{74.551\ g}{mol} \times \frac{0.5\ mol}{L} \times 0.1\ L = 3.7276\ g\ KCl$$

Add water to 100 mL.

Make 100 mL of a 1 M NaCl stock solution:

$$\frac{58.443\ g}{mol} \times \frac{1\ mol}{L} \times 0.1\ L = 5.8443\ g\ NaCl$$

Add water to 100 mL.

Make 1 mL of a 50 µM hemin stock solution:

First make a 10 mM stock solution:

$$\frac{651.96g}{mol} \times \frac{0.01\ mol}{L} \times 0.001\ L = 0.0065\ g\ hemin\ in\ 1\ mL\ DMSO$$

Dilute this to make the 50 µM stock solution:

Add 5 µL of 10 mM hemin to 995 µL DMSO.

Use the NanoDrop spectrophotometer to check the absorbance at 405 nm. The absorbance should be 0.87 for a 1 mm path length. If it is not, adjust the concentration accordingly with DMSO.

The stock solution is good for 7 days if refrigerated and protected from light.
It is good for 1 month if frozen and protected from light.

Make 1.5 mL of an 83 mM ABTS stock solution:

$$\frac{548.68\ g}{mol} \times \frac{0.083\ mol}{L} \times 0.0015\ L = 0.0683\ g\ \text{ABTS diammonium salt}$$

Add water to 1.5 mL.

Make 0.3 % (w/v) H_2O_2 from a 30% (w/v) H_2O_2 supplied solution:

Add 10 µL 30% H_2O_2 to 990 µL water.

REFERENCES

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, and Walter P. Molecular Biology of the Cell. 5th ed. Florence (KY): Taylor & Francis, Inc.; 2007. 13,824 p.

Bianco S, Musetti C, Waldeck A, Sparapani S, Seitz JD, Krapcho AP, Palumbo M, Sissi C. 2010. Bis-phenanthroline derivatives as suitable scaffolds for effective G-quadruplex recognition. Dalton Transactions. 39(25): 5833-5841.

Chinnapen DJ, Sen D. 2002. Hemin-stimulated docking of cytochrome c to a hemin-DNA aptamer complex. Biochemistry. 41(16): 5202-5212.

Collier GS, Pratt JM, De Wet CR, Tshabalala CF. 1979. Studies on haemin in dimethyl sulphoxide/water mixtures. Biochemical Journal. 179(2): 281-289.

Deng M, Zhang D, Zhou Y, Zhou X. 2008. Highly effective colorimetric and visual detection of nucleic acids using an asymmetrically split peroxidase DNAzyme. Journal of the American Chemical Society. 130(39): 13095-13102.

Li T, Dong S, Wang E. 2009. G-quadruplex aptamers with peroxidase-like DNAzyme functions: which is the best and how does it work? Chemistry – An Asian Journal. 4(6): 918-922.

Li T, Shi L, Wang E, Dong S. 2009. Multifunctional G-quadruplex aptamers and their application to protein detection. Chemistry. 15(4): 1036-1042.

Micheli E, D'Ambrosio D, Franceschin M, Savino M. 2009. Water soluble cationic perylene derivatives as possible telomerase inhibitors: the search for selective G-quadruplex targeting. Mini Reviews in Medicinal Chemistry. 9(14): 1622-1632

Nakayama S, Sintim HO. 2009. Colorimetric split G-quadruplex probes for nucleic acid sensing: improving reconstituted DNAzyme's catalytic efficiency via probe remodeling. Journal of the American Chemical Society. 131(29): 10320-10333.

Reynolds A, Leake D, Boese Q, Scaringe S, Marshall WS, Khvorova A. 2004. Rational siRNA design for RNA interference. Nature Biotechnology. 22(3): 326-330.

Robins H, Li Y, Padgett RW. 2005. Incorporating structure to predict microRNA targets. Proceedings of the National Academy of Sciences U S A. 102(11): 4006-4009.

Stormo GD. 2006. An overview of RNA structure prediction and applications to RNA gene prediction and RNAi design. Current Protocols in Bioinformatics. Chapter 12:Unit 12.1.

Tafer H, Ameres SL, Obernosterer G, Gebeshuber CA, Schroeder R, Martinez J, Hofacker IL. 2008. The impact of target site accessibility on the design of effective siRNAs. Nature Biotechnology. 26(5): 578-583.

Thornalley J, Trottar RJ, Stern A. 1983. Free radical involvement in the oxidative phenomena induced by tert-butyl hydroperoxide in erythrocytes. Biochimica Biophysica Acta. 759: 16–22.

Tian Y, Mao C. 2005. DNAzyme amplification of molecular beacon signal. 67(3): 532-537.

Thomas J, Drake M, Paddock JR, Conklin S, Johnson J, Seliskar CJ, Halsall HB, Heineman WR. 2004. Characterization of ABTS at a Polymer-Modified Electrode. Electroanalysis. 16(7): 547-555.

Travascio P, Li Y, Sen D. 1998. DNA-enhanced peroxidase activity of a DNA-aptamer-hemin complex. Chemistry and Biology. 9: 505-517.

Travascio P, Bennet AJ, Wang DY, Sen D. 1999. A ribozyme and a catalytic DNA with peroxidase activity: active sites versus cofactor-binding sites. Chemistry and Biology. 11: 779-787.

Travascio P, Witting P, Mauk AG, Sen D. 2001. The peroxidase activity of a hemin-DNA oligonucleotide complex: free radical damage to specific guanine bases of the DNA. Journal of the American Chemical Society. 123(7): 1337-1348.

Williamson JR. 1994. G-quartet structures in telomeric DNA. Annual Review of Biophysics and Biomolecular Structure. 23: 703–730.

Xiao Y, Pavlov V, Niazov T, Dishon A, Kotler M, Willner I. 2004. Catalytic beacons for the detection of DNA and telomerase activity. Journal of the American Chemical Society. 126(24): 7430-7431.

Yoshida M, Shimazono N. 1965. Studies on the chromatographic separation of hemin alpha and protohemin from heart muscle. Journal of Biochemistry. 58(1): 39-46.