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Kevin Harrell 16 April 2012

Conditional Deoxyribozyme Gene Knockdown:

Investigating DNA Displacement Reactions on Gold Nanoparticles

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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Here we present the development of a conditional RNA hydrolysis catalyst with the potential for cellular gene knockdown through the use of DNA displacement reactions on gold nanoparticles. The activity of catalytic oligonucleotides, or deoxyribozymes, that hydrolyze the phosphodiester backbone of specific RNA strands, is triggered by using a secondary input signal. DNAzymes are widely investigated for their potential in gene regulation. One of the major challenges pertinent to DNAzymes in particular, and RNA interference techniques in general, is the ability to act on specific cell types without affecting others. To overcome the issue of selectivity while also addressing the need for cellular delivery, we use DNA functionalized gold nanoparticles, along with DNA displacement reactions to achieve a conditional response which is triggered by the presence of a specific switch strand. The DNAzyme was shown to be inactive when hybridized to a capture strand functionalized on a gold nanoparticle, and active in solution once displaced from the capture particle by the specific switch strand. The initial rate constant of DNAzyme activity was shown to increase up to 14-fold upon addition of appropriate concentrations of switch strand. Thus, the catalyst is triggered by the presence of a switch strand. We have also shown that the system is highly selective and is unlikely to be activated by other strands. This system has broad potential for clinical applications in that it can be selectively activated based on a cell's genetic and molecular profile. Gene knockdown will only occur in the presence of specific mRNA markers, allowing the system to differentiate between cells. Therapeutic techniques could be developed from this system to treat or kill cancerous cells while leaving healthy cells unaltered.

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Introduction:

Formulated by Francis Crick in 1958, the central dogma of molecular biology revolutionized the modern conception of cellular function and is at the root of much of the biochemical research of the past several decades. While his idea was initially met with skepticism, in 1970 Crick defended his work with an article in *Nature,* solidifying his theory as a bedrock principle for understanding much of cellular process and function [1]. In his papers, Crick elucidated the general concept of information transfer in cells, where DNA is transcribed into RNA, which is then translated into specific proteins. Scientists have been trying to discover and understand the specific mechanisms of this process ever since.

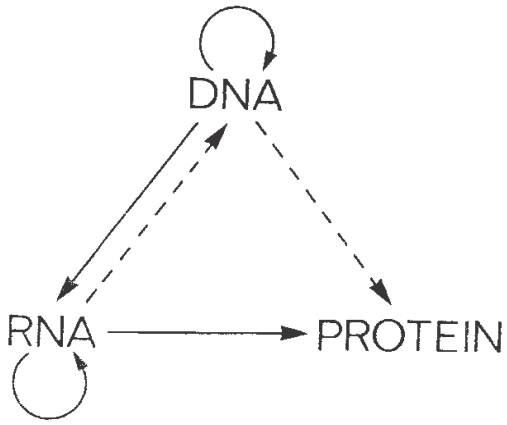


Figure 1: The central dogma of molecular biology. Reprinted with permission from [1]. Copyright © 1970 Nature Publishing Group.

Much research has been done to understand the specific translation and transcription processes themselves, while others have investigated cellular systems that influence the central dogma.

There is great research utility in understanding the central dogma. Scientists employ many methods of mimicking, enhancing, or interfering with the processes of transcription and translation to gain control of cellular functions. One large area of research that is applicable in clinical and laboratory settings is gene knockdown. This involves the interruption of the second step of the central dogma, hydrolyzing mRNA and thus

preventing proteins from being expressed. This thesis explores the synthesis and characterization of a model system which may be used for gene knockdown by combining several well-studied types of chemistry. This system is highly selective and conditional, and will be capable of preventing specific proteins from being expressed in cells only in the presence of a second, signaling mRNA.

Gene Knockdown

Gene knockdown itself is a well-studied and widely applicable field. Scientists use many different methods to prevent specific protein expression, with the end goal of controlling cellular functions. The ability to prevent gene expression has been shown useful in controlling the fate of cells and their responses.

These gene regulation techniques have vast clinical applications in that many cancers are caused by mis-regulation of various receptors, such as the epidermal growth factor receptor (EGFR), and consequently, these techniques may allow for prevention or even cure [2]. In his review on this subject, David Corey gives the example of the antisense oligonucleotide fomivirsen which is FDA approved for the treatment of cytomegalovirus retinitis [3]. Figure 2 shows some of the general methods for gene silencing described by Corey.

One method for gene regulation involves the use of antisense oligonucleotides which selectively sequester the desired mRNA strands, sterically preventing them from being translated into protein. These antisense strands can also form an RNA-DNA duplex, which is selectively recognized by RNase H and then cleaved. Advanced versions of this technique use “gapamers” which is a mixed strand with DNA in the center and modified RNA at the termini. This renders the antisense nucleotide less

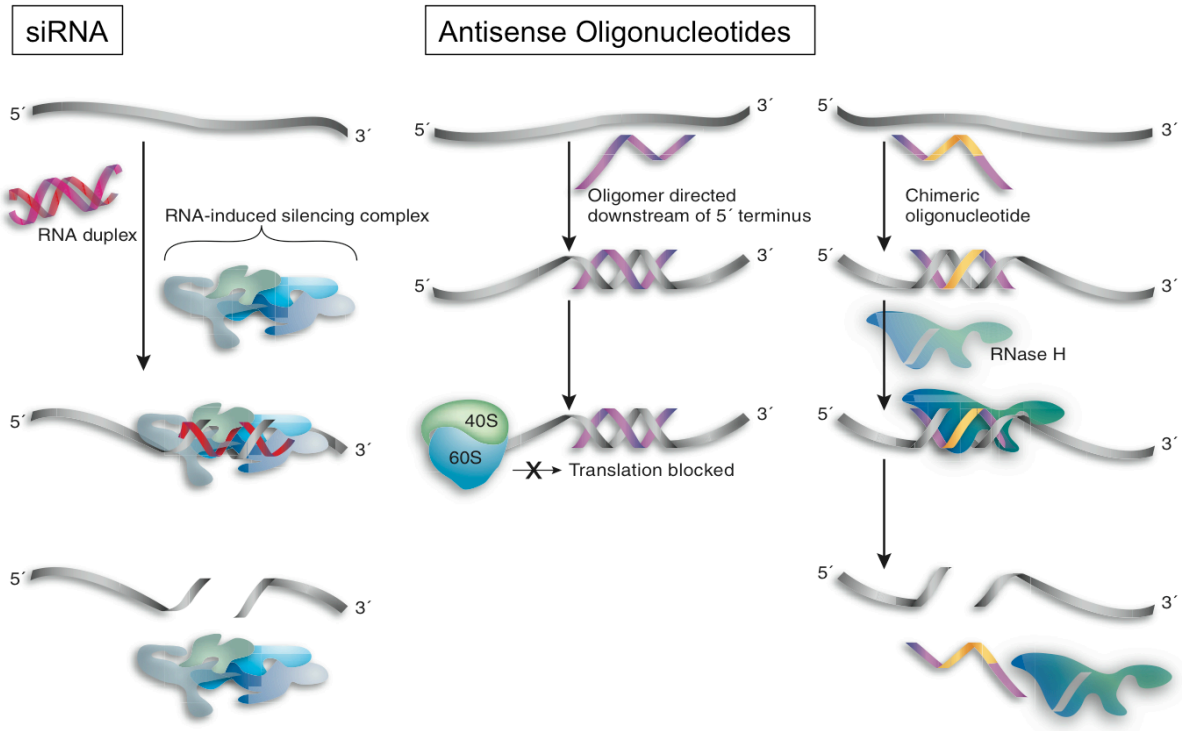


Figure 2: Schematic showing some of the most commonly used methods for gene knockdown. Reprinted with permission from [3]. Copyright © 2007 Nature Publishing Group.

susceptible to nucleases, while still allowing the DNA portion to recruit the RNase H for cleavage. These antisense strands can also be chemically modified in various ways to increase affinity for the silencing target or increase resistance to degradation. The most widely used approach for gene regulation takes advantage of endogenous cellular mechanisms in which double stranded small interfering RNAs (siRNAs) recruit a RNA-induced silencing complex (RISC). The RISC then uses the RNA to hybridize to a specific mRNA and cleaves it. While this technique has great potential, challenges of stability *in vivo,* and delivery of the siRNA need to be addressed before the clinical application can be realized.

Deoxyribozymes

An alternative method for gene regulation involves the use of catalytically active strands of DNA which can also be used to cleave the phosphodiester bonds of mRNA [4]. Catalytically active nucleic acid sequences are used in biology for the efficient processing of RNA strands. “Ribozymes,” RNA-cleaving RNA strands, were shown in the early 1980s to be naturally occurring and active in cells [5]. In 1989, Sidney Altman and Thomas Cech were awarded the Nobel Prize in Chemistry “for their discovery of catalytic properties of RNA [6]. Fairly recently, researchers have furthered this concept and used *in vitro* screening to select for catalytically active sequences of DNA, called deoxyribozymes (DNAzymes), that are also capable of RNA phosphoesterase catalytic activity [4]. While most of the work with DNAzymes has dealt with their cleavage properties, DNAzymes have also been described that perform RNA ligation and other chemistry [7].

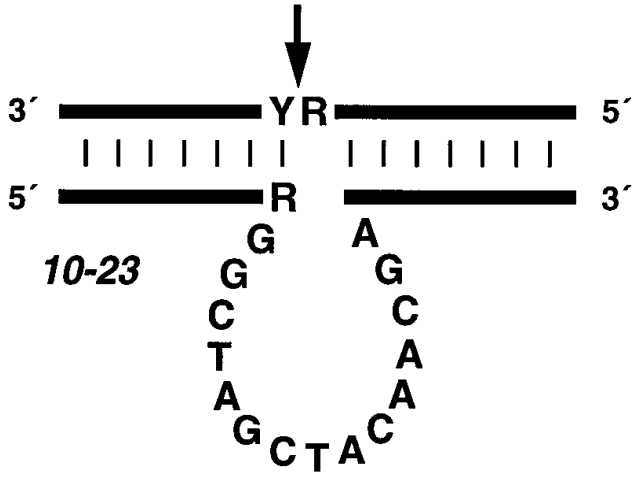


Figure 3: Schematic showing the structure of the 10-23 DNAzyme. The arrow indicates the site of hydrolysis. R and Y represent purine and pyrimidine bases, respectively. Reprinted with permission from [8]. Copyright © 1997 National Academy of Sciences.

One of the most well studied sequences is the “10-23” DNAzyme. First discovered by Santoro and Joyce in 1997, 10-23 was screened from a library of 10^{14} nucleic acids, and shown to have potent enzymatic capabilities for substrate RNA cleavage [8].

The 10-23 DNAzyme was shown to be dependent on

coordination with a divalent Mg^{2+} ion, and though the exact conformation of the catalytic sequence is unknown, it is often represented in the literature as the loop structure shown in Figure 3 [9]. The 10-23 is a 15 base pair sequence known as the “catalytic core” which is essential to catalytic activity. The flanking sequences, called binding or recognition arms, do not have a required sequence and can be adapted to hybridize with the desired RNA, thus making the 10-23 able to specifically cleave almost any desired RNA sequence. The one requirement of the RNA strand is a purine-pyrimidine dinucleotide at the catalytic site, as shown in Figure 3.

While the mechanism of cleavage has yet to be fully elucidated, studies have shown that the catalytic core sequence is the essential portion for catalysis. In 2002, Kurreck *et al.* described a series of mutagenesis experiments in which they separately substituted each base in the catalytic core of the 10-23 DNAzyme [10]. They found that the sequence substitution of any of the bases shown in Figure 3 led to a loss of activity, though some residues were discovered to be more essential than others. Beginning from the 5’ end of the DNAzyme, if the first 6 bases (GGCTAG) were substituted, an almost complete loss of activity was observed. All of the guanines were found to be essential, while many of the 7-12 bases (CTACAA) showed moderate activity when substituted. In particular, the thymine at the 8 position showed 75-90% activity when substituted with any other nucleotide base, compared to the fully functional DNAzyme. These results show that the catalytic core is essential to catalysis of the target RNA strands and help elucidate the mechanism of activity of this important DNAzyme.

The applications and utility of these DNAzymes are fairly broad. DNAzymes are relatively stable in the cellular environment, and can be tailored to selectively cleave

RNA targets. Though useful, DNAzymes also have their limitations. The DNAzyme has a low rate of turnover and performs its catalysis slowly compared to enzymes [11]. As a nucleic acid, it is also susceptible to degradation in the cellular environment by nucleases. A significant challenge is in delivery of the DNAzyme [11]. Transporting the DNAzyme into a cell so that it can cleave the desired mRNA target can be difficult.

DNA Functionalized Gold Nanoparticles

In the past several decades, much research has been done on the utility of gold nanoparticles functionalized with oligonucleotide strands [12]. These functionalized gold nanoparticles are used in a variety of applications, but when used as a cellular delivery system they provide many distinct advantages.

First, oligonucleotide-gold nanoparticle conjugates have been shown to readily cross the cell membrane and enter mammalian cells [13]. While the exact mechanism is unknown, Chithrani and Chan in 2007 suggested that the particles become coated with proteins and are then endocytosed through a clathrin-mediated process [14].

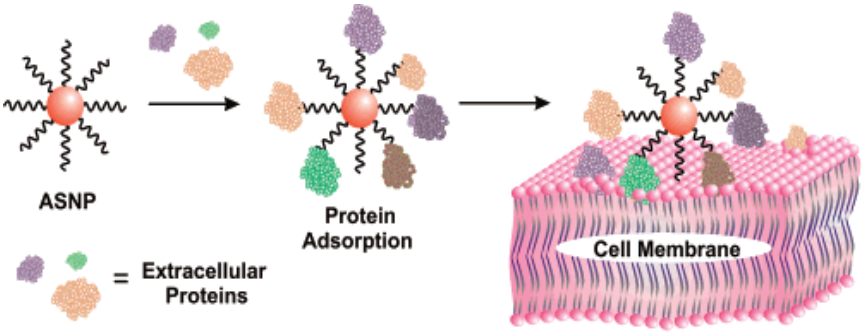


Figure 4: Proposed mechanism of cellular uptake of oligonucleotide functionalized gold nanoparticles. Reprinted with permission from [15]. Copyright © 2007 American Chemical Society.

The authors of this work observed this endocytosis behavior with transferrin coated nanoparticles and hypothesized a similar mechanism of uptake where oligonucleotide-functionalized particles bind various extracellular proteins and are

endocytosed into the cell. Other groups have shown that uptake is dependent on the size of the nanoparticles used and the density of oligonucleotide functionalization [13,15]. Furthermore, some groups have used functionalized particles to deliver reagents into the nucleus of the cell [16].

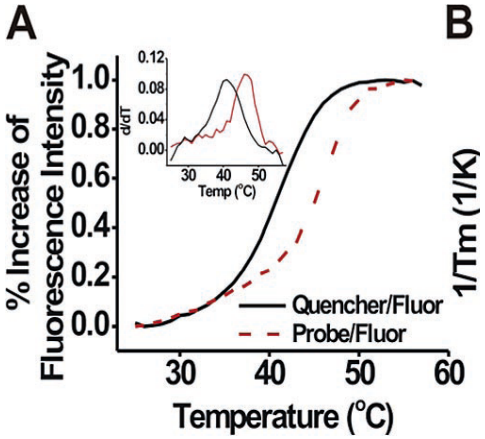


Figure 5: Melting curves comparing AuNP functionalized and free oligonucleotide duplexes. Reprinted with permission from [17]. Copyright © 2005 American Chemical Society.

Another advantage of DNA-gold nanoparticle conjugates is that oligonucleotide duplexes functionalized onto a nanoparticle have been shown to be more stable than duplexes that are free in solution [17]. Figure 5 shows melting curves for both nanoparticle functionalized and solution strands. The AuNP functionalized oligonucleotides were observed to have a higher melting temperature and were considered more stable than the soluble strands. The nanoparticle functionalized functionalized strands were also shown to have a lower (more negative) value for ΔG^{o}, thus indicating greater duplex

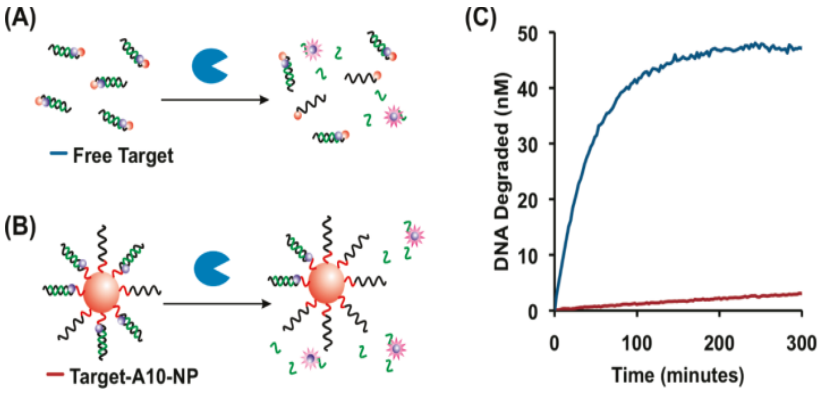


Figure 6: (A) and (B) are schematics showing the activity of nucleases on soluble duplex strands and AuNP-modified duplex strands, respectively. (C) Representative plot showing the kinetics of DNA hydrolysis for (A) and (B). Reprinted with permission from [19]. Copyright © 2011 American Chemical Society.

stability and therefore enhanced binding affinity. The Mirkin group has also investigated the melting properties of these DNA-gold nanoparticle complexes[18]. They observed that nanoparticle functionalized duplex DNA exhibited extraordinarily sharp melting curves when compared to analogous duplexes in solution.

Oligonucleotides functionalized onto gold nanoparticles have also been demonstrated by Mirkin *et al.* to be selectively resistant toward nucleases [19]. Once again comparing functionalized and free DNA strands, Figure 6 shows that nuclease activity is drastically reduced when DNA is functionalized onto the particle.

Lastly, oligonucleotide functionalized nanoparticles have been used with different systems for gene regulation [20,21]. Using an antisense DNA strand to sequester the desired mRNA strands, Mirkin *et al.* were able to demonstrate up to a 20% knockdown of enhanced green fluorescent protein (EGFP) in a cellular system. In these same experiments, they note that they do not observe significant toxicity to the cells when the DNA-functionalized nanoparticles are internalized. All of these advantages make DNA functionalized gold nanoparticles an ideal system for the delivery of DNAzymes into cells.

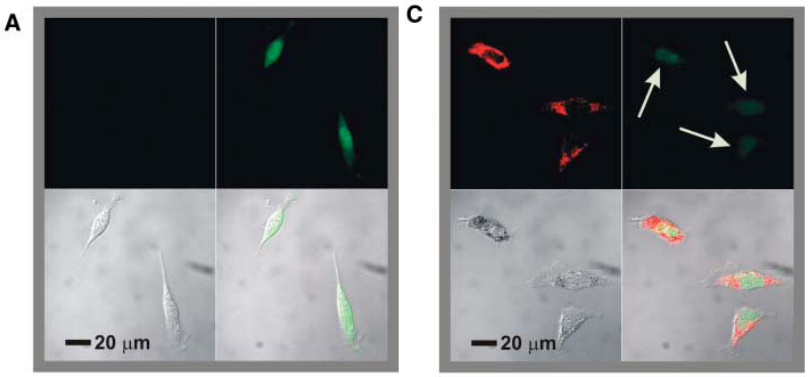


Figure 7: A: Untreated cells expressing EGFP. C: Cells treated with nanoparticle functionalized antisense DNA showing 20% EGFP knockdown. Reprinted with permission from [20]. Copyright © 2006 AAAS.

Though gene knockdown is a useful tool, it is often applied as a blanket treatment to all cells encountered. It is desirable to develop a gene knockdown system

which could distinguish between different cells based on their genetic profile. In other words, the system would only be active when triggered by specific gene products that are expressed within a cell.

Conditional Response Signaling

This thesis describes a conditional system, or one which produces signal output only under specific conditions or in the presence of certain signaling molecules. These types of systems are highly desirable since conditional signaling allows for specific cell recognition and screening, thus certain functions are performed on particular cells but not others. This has important application in clinical settings, and especially in cancer research and treatment. Several previously developed conditional systems have served as the inspiration for the work presented in this thesis.

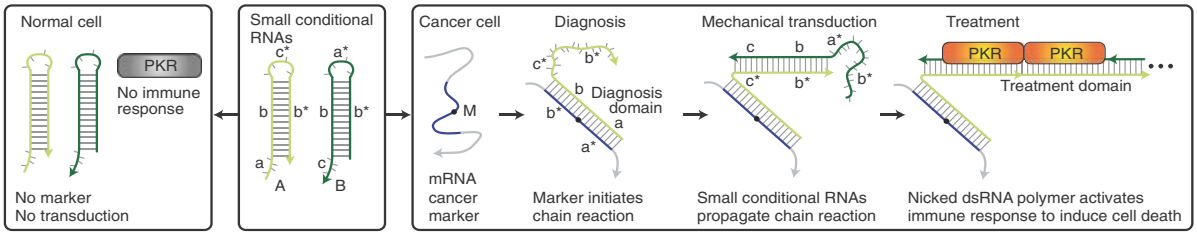


Figure 8: Schematic representation of a conditional system for cell death. A cancer mRNA marker causes the polymerization of small RNAs which then induce apoptosis by activating an immune response through PKR. Reprinted with permission from [22]. Copyright © 2010 National Academy of Sciences.

Venkataraman *et al.* described a system using small conditional RNA strands that are activated by a separate mRNA cancer marker [22]. In the presence of the cancer marker, the small RNA strands hybridize and trigger the polymerization of monomer RNA into a long chain polymer of RNA. This RNA chain has been shown to activate Protein Kinase R (PKR) which in turn activates an immune response that induces apoptosis.

This system is useful because it can selectively target cancer cells and kill them. The system shows no activity or cell death in the absence of the specific cancer marker, and they report a 20 to 100-fold population reduction in cells with the marker present.

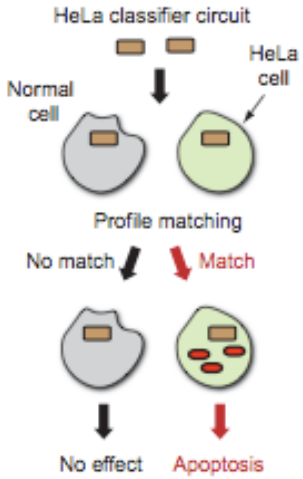


Figure 9: Schematic showing a conditional system for cell death using a synthetic classifier circuit. Used with permission from [23]. Copyright © 2011 AAAS.

A conditional system was also described by Xie *et al.* in 2011 [23]. In this system, they program an RNAi-based logic circuit to detect the levels of several RNA markers endogenous to HeLa cells (cervical cancer cells). If certain levels are detected, then apoptosis is induced. They have shown that their system can selectively kill HeLa cells with the select markers with no effect on the healthy cells. This has enormous implications for future cancer research and clinical treatments and will allow scientists to develop similar systems that can differentiate between healthy and cancerous cells in the body.

Innovation

Here we demonstrate the synthesis of a novel conditional system with the potential for gene knockdown using DNAzyme-gold nanoparticle conjugates. We have combined the well-studied chemistries described above to create a new system that can perform cleavage of a target only in the presence of a specific switch strand. Figure 10 shows the general scheme of this system.

An oligonucleotide, called the capture strand, is immobilized onto the gold nanoparticle using a 3’ thiol modification. The DNAzyme, whose recognition arms and

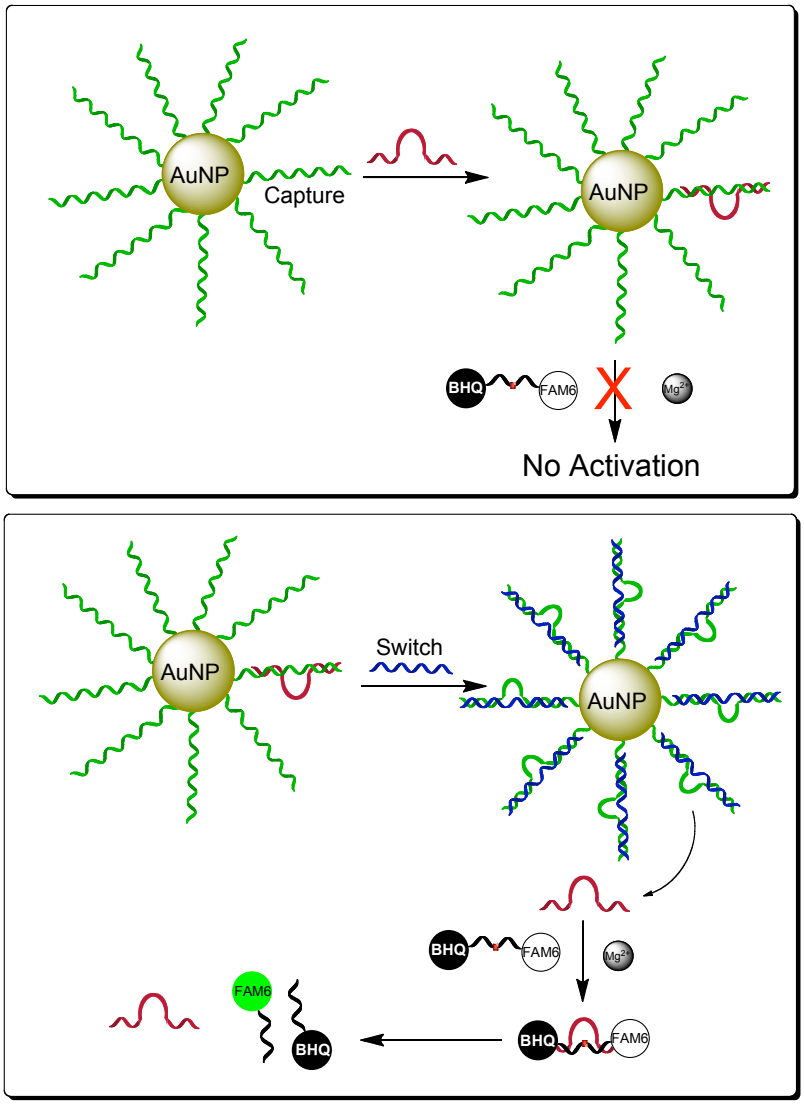
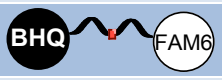
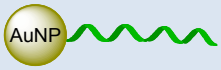


Figure 10: Schematic of the conditional system with the potential for gene knockdown using DNA displacement.

two bases of the catalytic core are complementary to part of the capture strand, is then hybridized to the capture strand. This leads to a deactivation of the DNAzyme, and it is not able to cleave the target strand (mRNA in cells or model substrate for *in vitro* studies). A switch strand (signaling mRNA in cells or oligonucleotide in solution) is complementary to other portions of the capture strand and can displace the DNAzyme from the nanoparticle complex. Only in the presence of a switch strand is the DNAzyme displaced and able to cleave the target. This is a conditional system that can selectively cleave a target RNA only in the presence of another RNA marker. The DNA sequences used in these experiments are listed in Table 1.

Table 1: DNA sequences used in experiments. All strands were custom synthesized by Integrated DNA Technologies.

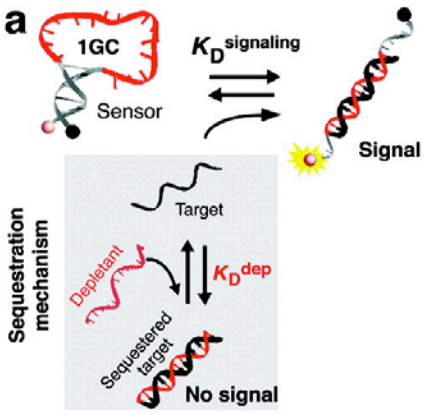
Name	Representation	Sequence
DNAzyme		5'- GCA CCC AGG CTA GCT ACA ACG ACT CTC TC -3'
Capture		5'- ATC GAC GTT GAG AGA GTC ACC TGG GTC GAT ACC GCA ATG CTT GC-3'
Switch		5'- GCA AGC ATT GCG GTA TAC TCT CTC AAC GTC GAT -3'
Target		5'- FAM - GAG AGA GrArU GGG TGC - BHQ -3'
Thiolated Capture		5'- ATC GAC GTT GAG AGA GTC ACC TGG GTC GAT ACC GCA ATG CTT GCT TTT TTT TTT - Thiol -3'
Switch: 21bp		5'- NNN NNN ATT GCG GTA TAC TCT CTC AAC NNN NNN -3'
Switch: 15bp		5'- NNN NNN NNN GCG GTA TAC TCT CTC NNN NNN NNN -3'
Switch: 9bp		5'- NNN NNN NNN NNN GTA TAC TCT NNN NNN NNN NNN -3'
Switch: 6bp		5'- NNN NNN NNN NNN NNN TAC NNN NNN NNN NNN NNN -3'
Switch: 0bp		5'- NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN -3'

The conditional response of the DNAzyme derived from maximizing the number of Watson-Crick base pairings. Two equilibria form the basis of this system, and can be used to trigger catalytic activity, based on conditions. The first equilibrium is the binding between the capture strand and the DNAzyme. This is a 16 base pair interaction with a predicted ΔG value of -20.38 kcal/mol. The second equilibrium is the 32 base pair hybridization of the switch and capture strands. This was predicted to have a ΔG value of -44.81 kcal/mol. The ΔG values were calculated using an online calculator of oligonucleotide properties using nearest neighbor approximation with 300 mM NaCl and 50 mM $MgCl_2$ [24].

It is important to note that this is the minimum (most favorable) value possible for these hybridizations. These calculations assume a perfect duplex formation, and our system does not allow for this. Both equilibria have loops that destabilize the duplex and make binding less favorable. Though these ΔG values represent an over estimation, the base pairing is known to be an extremely favorable thermodynamic state and can be achieved with high affinity and specificity.

These two competing hybridizations drive the conditional system described above. The capture strand serves at first as a sequestering mechanism, and the equilibrium is pushed to bind the DNAzyme, preventing it from cleaving the target. Upon the addition of the switch strand however, the DNAzyme is displaced because the capture-switch duplex is more stable. This balance creates the conditional component of the system, using hybridization to activate and deactivate the desired catalytic phosphoesterase activity.

While the thermodynamic calculations presented above give a scope of the system, the concept of strand displacement is also essential. In describing the equilibrium between the capture and the switch, it must be considered that some of the capture strands will already be hybridized to DNAzyme. Thus enters strand displacement, in that it is more favorable for the switch to be bound and so the DNAzyme will be displaced from the capture strand. This is the difference between the active and inactive forms of the system. The Mirkin group has shown that strand displacement is also kinetically favorable on the AuNP surface [25]. They show that the capture-switch hybridization rate is actually increased with the presence of pre-hybridized DNAzyme.



A similar system using two equilibria was described by Kevin Plaxco [26]. Figure 11 shows the two equilibrium system analogous to the one presented above. This system is being used as a molecular beacon in describing extended dynamic ranges, but the basic principles of thermodynamics are the same.

Figure 11: Schematic showing the competing equilibria between a molecular beacon, target, and a depletant strand. Used with permission from [26]. Copyright © 2012 American Chemical Society

Experimental:

DNA Sequences

All DNA sequences presented in Table 1 were purchased from Integrated DNA Technologies. The target sequence contains the FAM (6-carboxy fluorescein) fluorophore, which has an excitation wavelength at 495nm and an emission wavelength of 520nm. It also contains two RNA bases (rA and rU) which serve as the cleavage site in the model substrate. Lastly, the target contains a Black Hole Quencher, which is designed to quench FAM. The DNAzyme was adapted from the reported catalytic 10-23 DNAzyme [8]. The capture and switch strands for the *in vitro* studies were based on DNA sequences used to study strand displacement, to allow for efficient release of the DNAzyme [25].

Gold Nanoparticle synthesis

Gold nanoparticles were synthesized according to procedures described by Mirkin *et al.* [27]. A two-neck round bottom flask was cleaned with aqua regia, rinsed and dried in the oven. This was placed on a hot plate and connected to a reflux condenser using Teflon tape. 500mL of 1mM hydrogen tetrachloroaurate (III) trihydrate was prepared and then poured into the flask. Water was flowed through the condenser, and the solution was brought to a rigorous boil while stirring. A 50mL solution of 38.8 mM sodium citrate tribasic dihydrate was prepared. Once the gold solution was refluxing vigorously, the citrate solution was added quickly and the mixture was allowed to reflux for 15 minutes, the solution was observed to change from yellow to clear, to black, to purple, and finally to deep red. After the 15 minutes, the heat was removed and the reaction was allowed to cool to room temperature. The solution was filtered through a 0.45μm acetate filter and placed into a clean amber storage bottle. These particles were characterized by UV-vis absorbance at 520nm as well as TEM as shown in Figure 12. The particles were observed to have a mean diameter of 15nm and to be of mostly uniform shape and size.

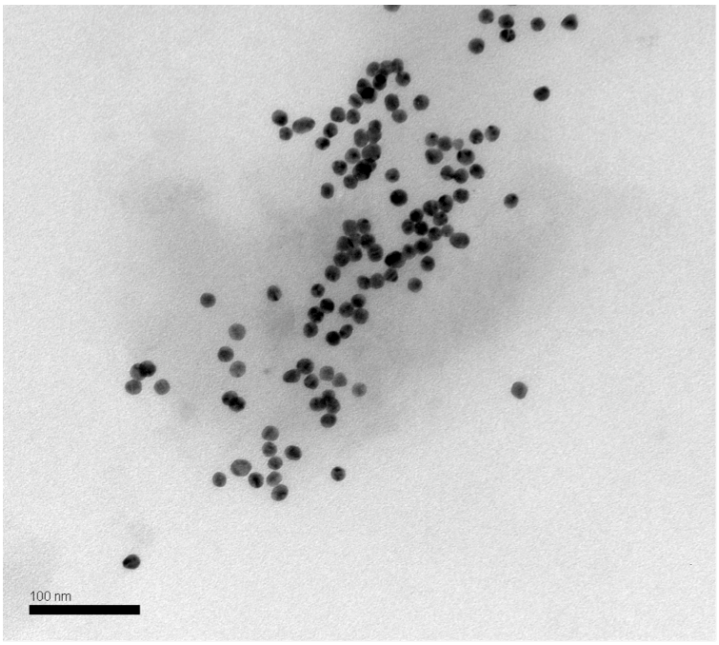


Figure 12: TEM image of 15 nm gold nanoparticles.

Gold Nanoparticle DNA Functionalization

The procedure used to functionalized oligonucleotides onto the gold nanoparticle surface was adapted from the Hill and Mirkin protocol [27]. Some experimental procedures from a subsequent paper from the Mirkin lab detailing how to achieve maximum functionalization density on the particle were used to optimize this protocol [28].

5 nanomoles of thiolated DNA were dried down using a speedvac. A 170 mM (pH 8.0) solution of disulfide cleavage buffer, 10 mM (pH 7.0) salting buffer, 100 mM (pH 7.0) phosphate adjustment buffer, and a 10% (wt/vol) SDS surfactant solution were prepared. A 0.1M solution of dithiothreitol (DTT) was prepared in the disulfide cleavage buffer. 100 μL of DTT solution was added to the dried DNA, wrapped in foil, and let sit at room temperature for 2 to 3 hours with occasional vortexing. During this period, a small Nap-5 column was flushed with NANOpure water, filling the column at least 3 times. The 100 μL of DNA was added to the column, and allowed to flow into the column. 400 μL of NANOpure water was added to the column and allowed to flow through uncollected. 950 μL of water was added to the column and fractions of 3 to 4 drops were collected in individual microtubes. Each of these fractions were measured with the UV-vis Nanodrop spectrophotometer to detect the presence of the DNA as signaled by absorbance at 260 nm. The concentration was measured using Beer's Law, $A=\varepsilon CL$. From the fractions, 40 nmol were extracted and placed into a clean and dry 20 mL vial. 1 mL of the previously synthesized gold nanoparticles were added to the DNA, wrapped in foil, and left overnight on a gently shaking orbital shaker.

Phosphate adjustment buffer was added to obtain a final phosphate concentration of 9 mM. Surfacant solution was added to obtain a final SDS concentration of ~0.1% (wt/vol). The solution was rewrapped in foil and left on the orbital shaker for an additional 30 minutes. The amount of salting buffer needed to obtain a salt final concentration of 0.8 M was calculated. This total amount was divided into six aliquots, added over 2 hours at 20 minute intervals. After the last salt addition, the particles were allowed to equilibrate overnight at room temperature. Functionalized nanoparticles should have the deep red color of the unfunctionalized particles, and should not crash out of solution.

The particles were then divided into fractions in microtubes and spun down in a centrifuge for 40 minutes at 13500 rpm. The nanoparticles were observed to form a dense pellet at the bottom of the tubes. The supernatant was removed to remove any excess thiolated DNA free in solution, and the functionalized nanoparticles were resuspended in NANOpure water. This process was repeated 4 more times to remove a maximum amount of free DNA. On the last round, the nanoparticles were resuspended in less water to concentrate the nanoparticle solution enough for use in the forthcoming catalytic studies.

Density Assay

Once the nanoparticles were functionalized, the functionalization density was then calculated. A commercial assay kit was purchased from Invitrogen that uses a fluorescent reagent that selectively binds to single-stranded DNA. First, a concentration calibration curve was created using thiolated DNA strands. Figure 13 shows this

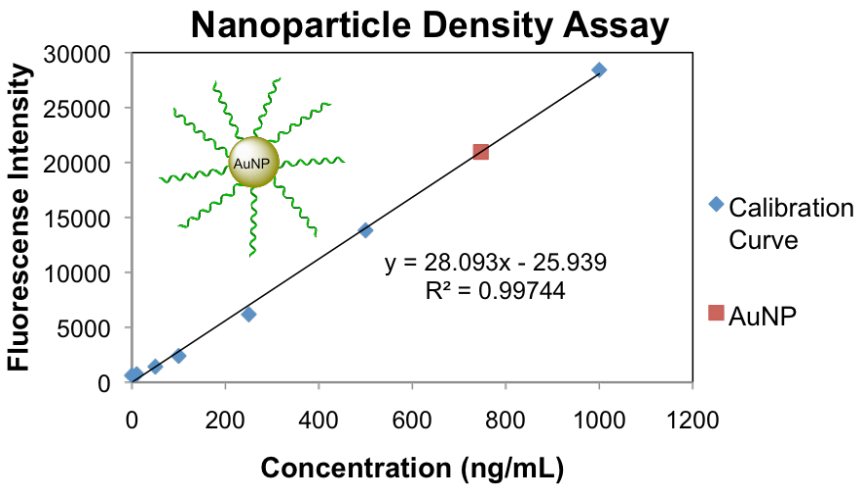


Figure 13: DNA concentration calibration curve and nanoparticle functionalization density measurements.

calibration curve. To measure the DNA functionalization density, the gold in a solution of known concentration was dissolved using high molar concentration of potassium cyanide (KCN). The DNA previously on the particle was then free in solution and the concentration was measured using the fluorescent reagent. This result is compared to the calibration curve to obtain a concentration of DNA. This concentration is compared to the original concentration of the nanoparticles to obtain the number of strands per particle. Figure 13 shows the measurements taken from the dissolved nanoparticle solution, and these values give an average of 97 strands per particle.

Catalytic Experiments

To test our system, catalytic experiments were run using a fluorescence 96 well-plate reader, testing different combinations of DNA strands as controls for the system.

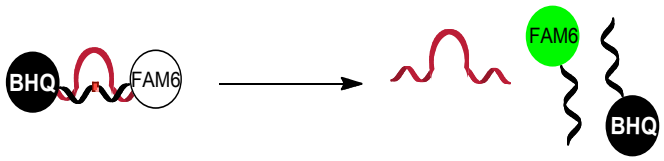


Figure 14: Model substrate used to simulate RNA cleavage and detect DNAzyme activity.

To detect catalysis a model substrate was used as shown in Figure 14. This

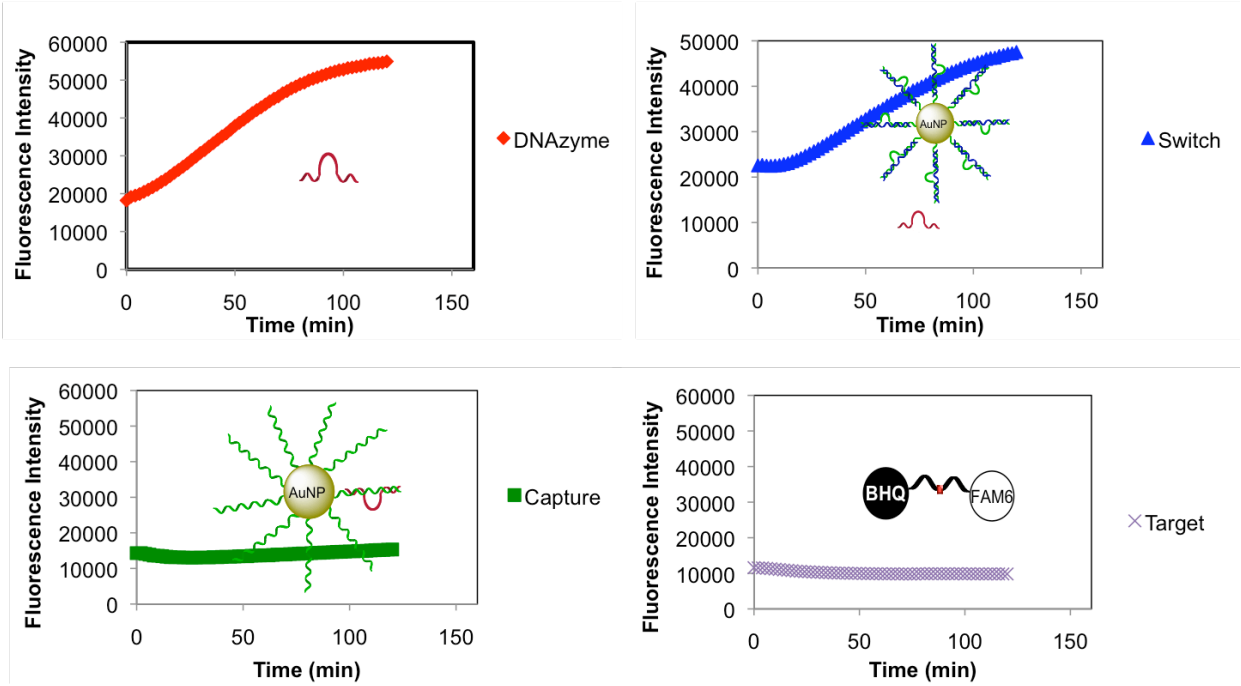


Figure 15: Plots showing the catalytic experiments performed along with representative results.

target strand consists of a specific DNA sequence, and two RNA bases which act as the cleavage site. The strand is modified with a FAM fluorophore at the 5' end and a Black Hole Quencher at the 3' end. If the target is intact, low fluorescence is observed, but once cleaved by the DNAzyme, the fluorophore is free to diffuse away from the quencher and increase the fluorescent signal. In the catalytic experiment, four different runs were tested as represented in Figure 15. The first run, represented by red diamonds and termed "DNAzyme," contained just free DNAzyme, target, and salts. This served as a positive control and was expected to show activity. Run 2, represented by green squares and termed "capture," contained DNAzyme, capture strand, target, and salts. This run should not show much activity above the background reaction, as the DNAzyme remains sequestered by the capture strand. Run 3, represented by blue triangles and termed "switch," is the full system. It contained DNAzyme, switch, capture, target, and salts and was expected to show activity since the switch strand should

displace the DNAzyme off of the capture and allow for target cleavage. The last run, represented by purple Xs and termed “Target,” contained target and salts. This served as a negative control to account for photobleaching and target self-cleavage.

Results:

Solution Studies

To prove the concept of conditional response gene knockdown, we first performed studies with DNA free in solution, using the model substrate described above to detect target cleavage and DNAzyme activity. The procedure for all solution experiments involved adding Tris buffer (pH 7.4), NaCl, water, DNAzyme, and capture strand first. This mixture was thoroughly mixed, heated to 75°C for 15 minutes, and then allowed to slowly cool to room temperature. Switch and target strands were then added to the appropriate experiments. Magnesium was added last, to activate the DNAzyme. All experiments were run with 300 mM NaCl, 20 mM Tris buffer, and 50 mM $MgCl_2$.

Figure 16 demonstrates the effects of heating on the proposed conditional response system. Experiment A was run at 25ºC and did not show a conditional response. Experiment B was run at 37ºC and did exhibit a conditional response. In experiment A, the capture run is inactive and the free DNAzyme run is active, but the switch run does not show activity. The switch strand is known to be partially complementary to the target strand (by 7 base pairs). When hybridized, the quencher and fluorophore are a greater distance apart than the free target, leading to the observed increased fluorescence. As the run progresses, slight heating from the plate reader being set to 25°C causes melting of this hybridization, accounting for the

observed decrease in fluorescence over time. This hybridization effect is competing with the observation of catalytic activity of the DNAzyme.

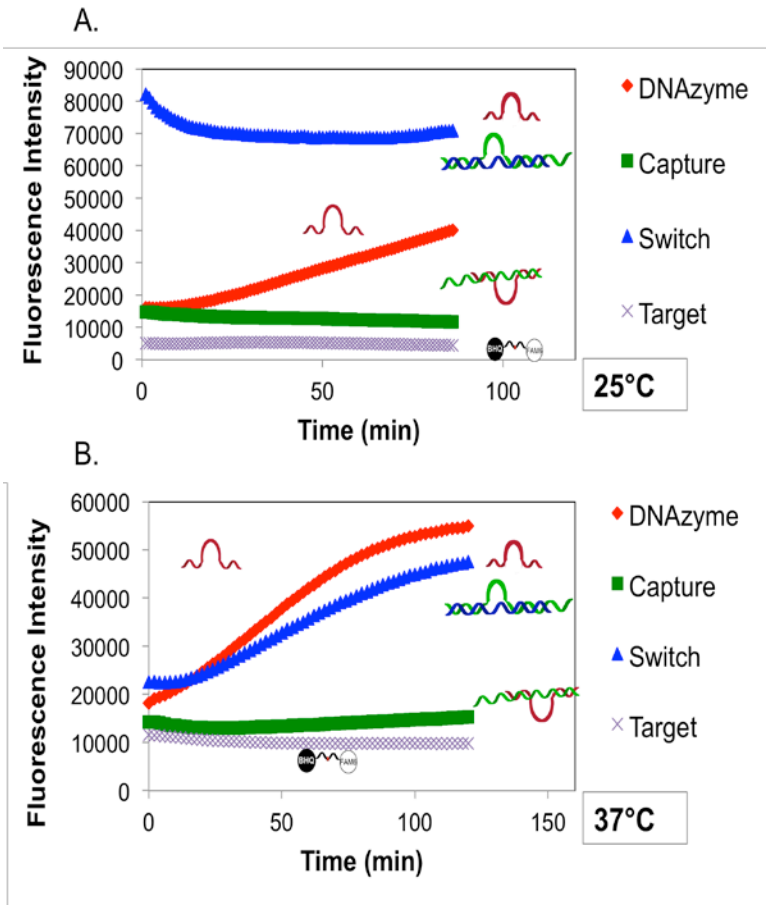


Figure 16: The effects of heating on the solution conditional response system. Run with 100 nM DNAzyme, 10 μM capture, 10 μM switch, and 1 μM target.

We altered the procedure described above for experiment B. Before activating the DNAzyme by adding magnesium and beginning the catalytic activity, the plate was placed into the reader and allowed to warm to 37°C for 10 minutes. The plate was then removed, the magnesium quickly added, and the plate reinserted into the reader to begin measurements. Experiment B demonstrated a conditional response. The DNAzyme run showed activity, while the capture run showed no activity, and the switch run showed activity. This result is significant because it shows that the conditional system is possible, where there is only activity in the presence of the switch strand and no activity in its absence.

The next step was to decrease the concentrations of the DNA strands to a nanomolar range that can be used in a nanoparticle based system. Gold nanoparticles

can only be concentrated into the nanomolar range before they begin to crash out of solution, thus the DNA must by be at lower concentrations than in the previous experiments. Figure 17 shows the experiment run with lower concentrations.

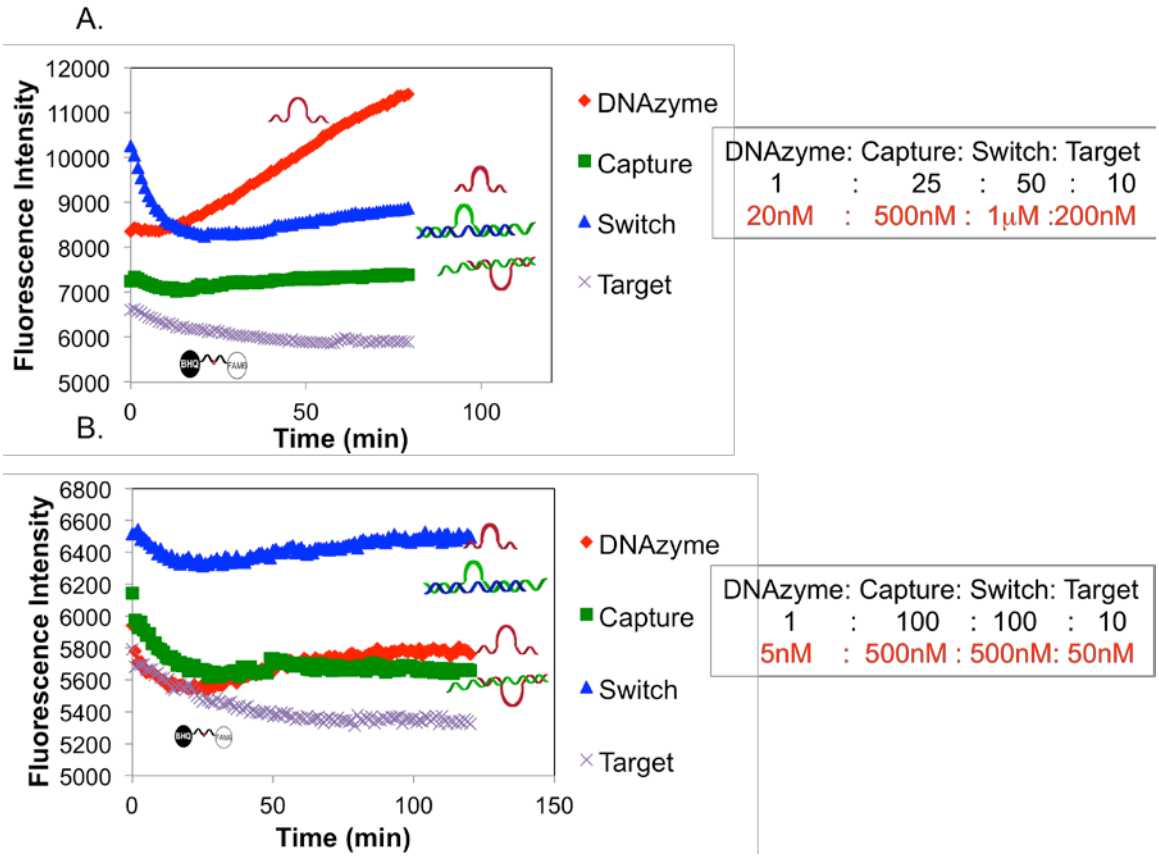


Figure 17: Effect of adjusting the molar ratios of DNA components on the solution conditional system. Experiments run at 37°C.

In Figure 17, experiment A does not exhibit a conditional response with the displaced DNAzyme not showing activity, and the captured DNAzyme showing activity. By adjusting the ratios of the DNA strands, we allow the equilibriums to give the desired conditional response. In examining the slopes of the DNAzyme, capture, and switch runs for experiment B, it did exhibit a conditional response, but with low signal. To achieve greater signal output, the target concentrations were increased.

Figure 18 shows a plot of catalytic activity when with the target strand concentration is increased to 500 nM. In this case, we did observe a conditional response under these conditions. The DNAzyme showed activity, the capture no activity, and the switch activity. This experiment, in particular, shows an initial decrease in activity that stabilizes after 50 minutes. This effect may be due to the heating of the plate and the fact that heating decreases fluorescence. Even though the plate is allowed to heat before catalysis, it must be removed to add the magnesium. During this time the plate cools and must reheat once the experiment begins, causing the observed initiation phase.

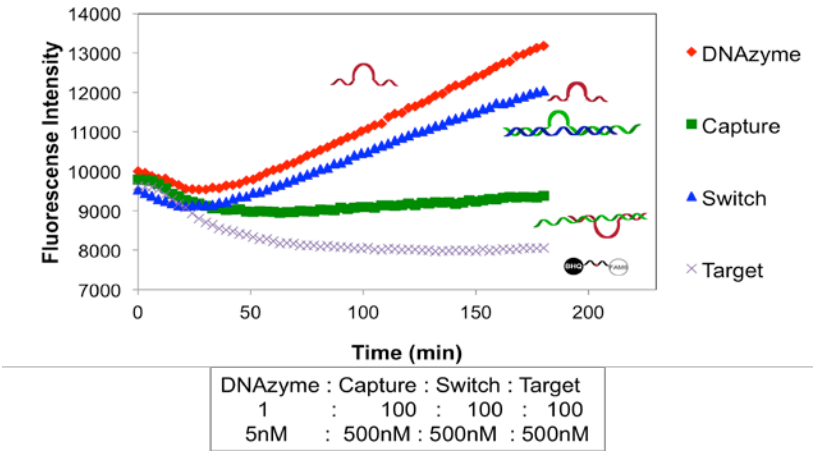


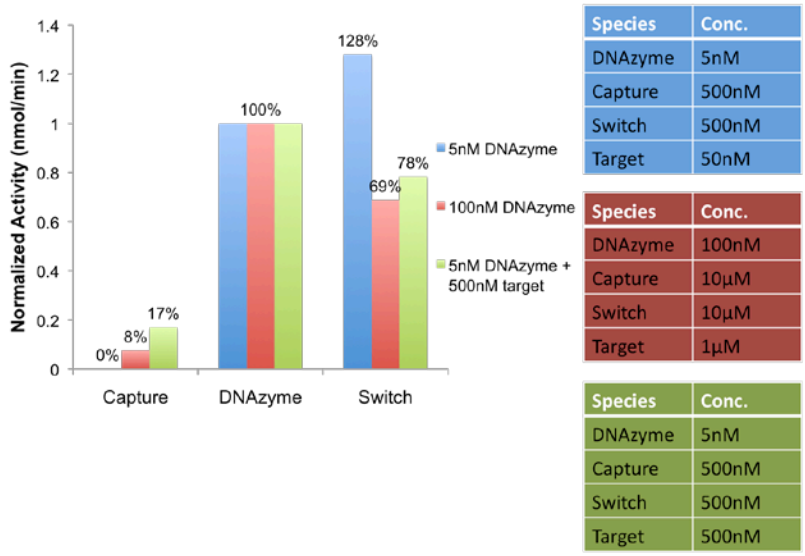
Figure 18: Observed conditional response with target strand increased to 500 nM.

With careful observation, the capture run (green) is seen to have a slight upward slope, indicating a small amount of activity. This is explained by the fact that the system is flooded with target and the equilibrium will be pushed in the direction of having some DNAzyme displaced from the capture strand, and thus cleaving a small amount of target.

To compare the different experiments, the slopes of the runs were measured after the initiation phase and normalized to the activity of the free DNAzyme. Figure 19

shows a comparison of the normalized activities of the system under different conditions.

The graph shows the normalized activities of the capture and switch runs as compared to the free DNAzyme in solution which was set at 100% activity. All of these experiments show a conditional response signal. As noted above, the high target experiment has a larger background reaction than then compared with other runs. The dilute experiment shown in blue has both the highest and lowest reactivity also as noted above, this experiment showed very low signal output and was thus much noisier than the other experiments.



Species	Conc.
DNAzyme	5nM
Capture	500nM
Switch	500nM
Target	50nM

Species	Conc.
DNAzyme	100nM
Capture	10µM
Switch	10µM
Target	1µM

Species	Conc.
DNAzyme	5nM
Capture	500nM
Switch	500nM
Target	500nM

Figure 19: A comparison of the conditions of solution experiments that demonstrated conditional response signaling.

Before moving to a nanoparticle system, an experiment was run to test how the system was affected by varying concentrations of the switch strand. Figure 20 shows the results of this experiment. A catalytic run of free DNAzyme was used as a positive control, while an inactive DNAzyme with no switch strand added was run as a negative control.

Some DNAzyme activity is observed in the negative control, as observed in previous experiments. These results indicate that as switch strand concentration

increases, DNAzyme activity increases. Little difference is observed between the 500 nM and the 1 μM runs, with only a 2% activity difference in slope. This could be explained by the fact that there is only a finite amount of DNAzyme in the system. Once there is sufficient switch strand to displace the maximum amount of DNAzyme, a plateau effect will be observed where additional switch strand will not increase activity. Even the run with 1 μM switch does not regain the full activity of free DNAzyme. While full activity is not expected due to equilibrium with the capture strand, this effect is partially masked by the target-switch hybridization described above.

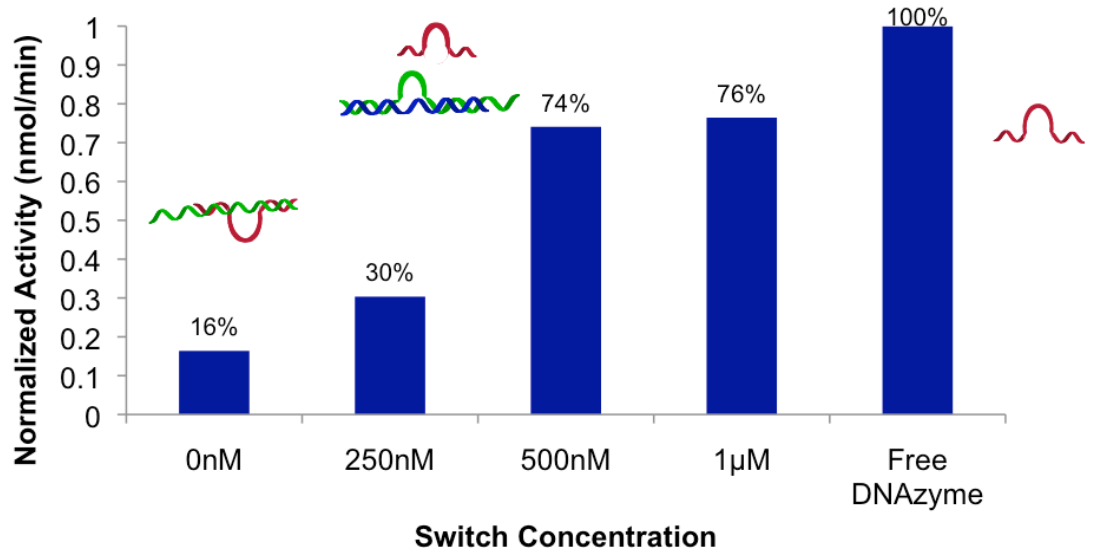
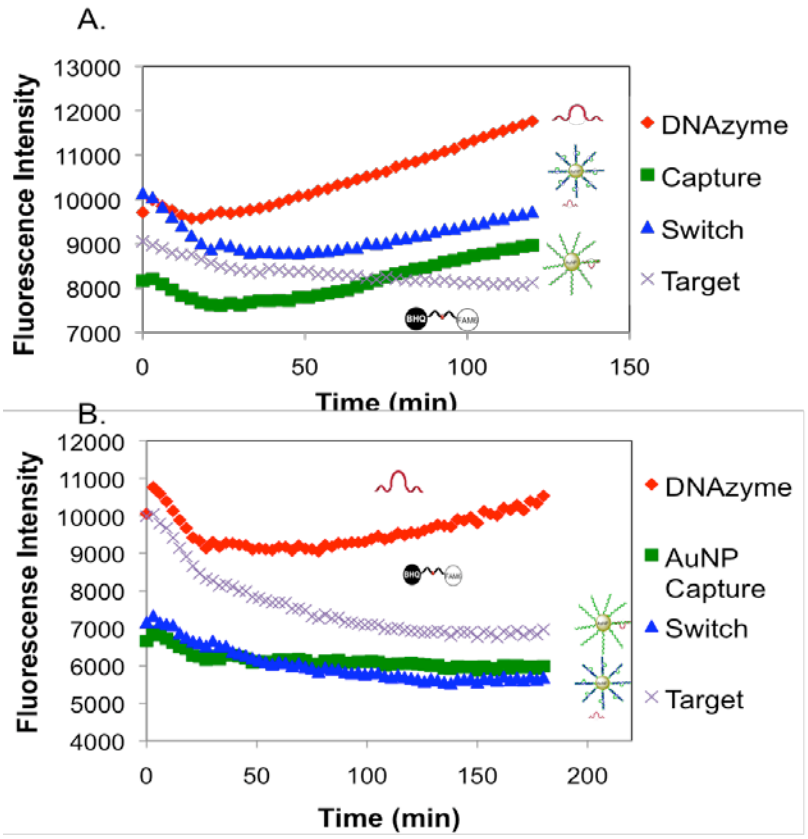


Figure 20: Effect of different concentrations of switch strand. All experiments run with 5 nM DNAzyme, 500 nM capture, and 500 nM target.

Several of the experiments presented above indicate a successful conditional response system, which can cleave an RNA model substrate. The next step was then to functionalize the capture strand onto a nanoparticle.

Nanoparticle Studies

The thiolated capture strand was successfully functionalized onto gold nanoparticles, using the procedure described in the experimental section, and the functionalization density was found to be approximately 100 strands per particle. Once the particles were functionalized and characterized, experiments were run with the conditions from Figure 18 but with the capture strand now immobilized onto the nanoparticle surface. Figure 21 shows the results of these experiments.



Species	Conc.
DNAzyme	5nM
AuNP	5nM
Switch	500nM
Target	500nM

Species	Conc.
DNAzyme	5nM
AuNP	10nM
Switch	500nM
Target	500nM

Figure 21: Kinetic plots showing the effect of increasing the concentration of capture strand functionalized gold nanoparticles on the conditional response system.

These experiments were not heated to 75°C and cooled as had been done in the solution studies, since the literature indicates that heating can cause the strands to dissociate from the particle [29]. Experiment A did not exhibit a conditional response in

that the DNAzyme and capture showed activity, but the negative control with just nanoparticle functionalized strands also showed significant catalytic activity.

Functionalized gold nanoparticles are often depicted with all of the strands standing perpendicular to the particle surface, though this is often not the case. Studies have shown that as much as 60 to 70% of strands on a particle are not available for binding [30]. This may partially explain the increased background reaction, since there are now 60% fewer capture strands available. Experiment B addresses this problem by increasing the concentration of nanoparticles to 10 nM and thus increasing the capture strand concentration.

Figure 21 B also did not show a conditional response because the switch strand was unable to displace the DNAzyme. The switch strand concentration was then increased to 1 μM to drive the equilibrium of the system towards the DNAzyme being displaced from the nanoparticle. Figure 22 shows this result with the increased switch concentration.

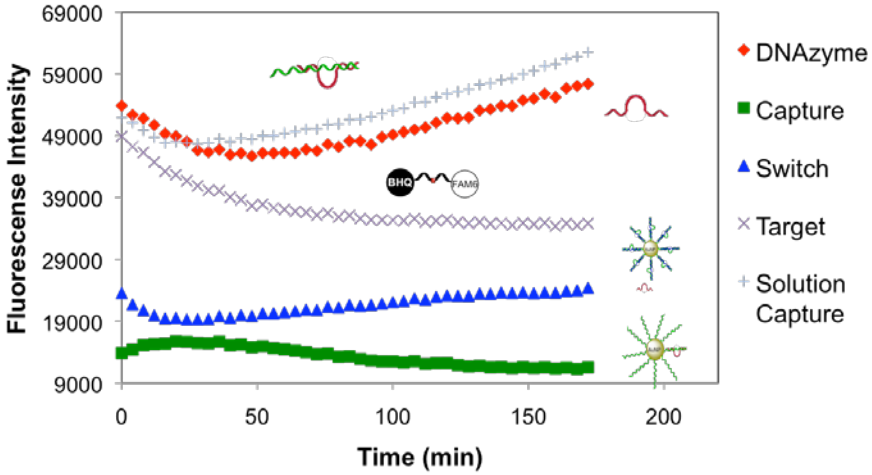


Figure 22: Observed conditional response signal. Run with 5 nM DNAzyme, 10 nM AuNP, 1 μM switch, and 500 nM target.

Under these conditions, the system exhibits a conditional response signal. The free DNAzyme showed activity, the captured DNAzyme showed no activity, and the displaced DNAzyme

activity. An additional control run was included with this experiment (represented by blue crosses) that contained DNAzyme and free capture strands. This control showed catalytic activity, though the captured DNAzyme was expected to be inactive. A subsequent solution experiment under these conditions also showed increased activity in the capture run, leading to the conclusion that the heating to 75°C and cooling to allow for capture-DNAzyme annealing is an essential step. To keep a minimal amount of DNA from coming off of the nanoparticle, the heating time was decreased from 15 minutes to 5 minutes.

Now that a conditional response system was demonstrated on a nanoparticle, the system needed to be optimized to achieve a maximum-fold increase of the active displaced DNAzyme over the inactive captured DNAzyme. This was accomplished by adjusting the molar ratios of the different DNA strands involved in the system. To achieve a final concentration of capture strand of 500nM, a 12.5 nM gold nanoparticle solution was used based on the measured 100 capture strands per nanoparticle, and an estimation of about 40% of strands being available for hybridization. Figure 23 shows the results from the subsequent experiment run with the adjusted ratios.

These results represent the slope of a kinetic curve measured over 180 minutes and have been background subtracted from the activity of target self-cleavage to account for photobleaching.

Figure 23 A shows a clear conditional response with activation only in the presence of the switch strand, with a 14-fold greater cleavage rate in the presence of the switch strand. To further optimize the rate enhancement, the amount of switch strand was increased to 1.25 μM so that it was at a ratio of 1:1 with the capture strands

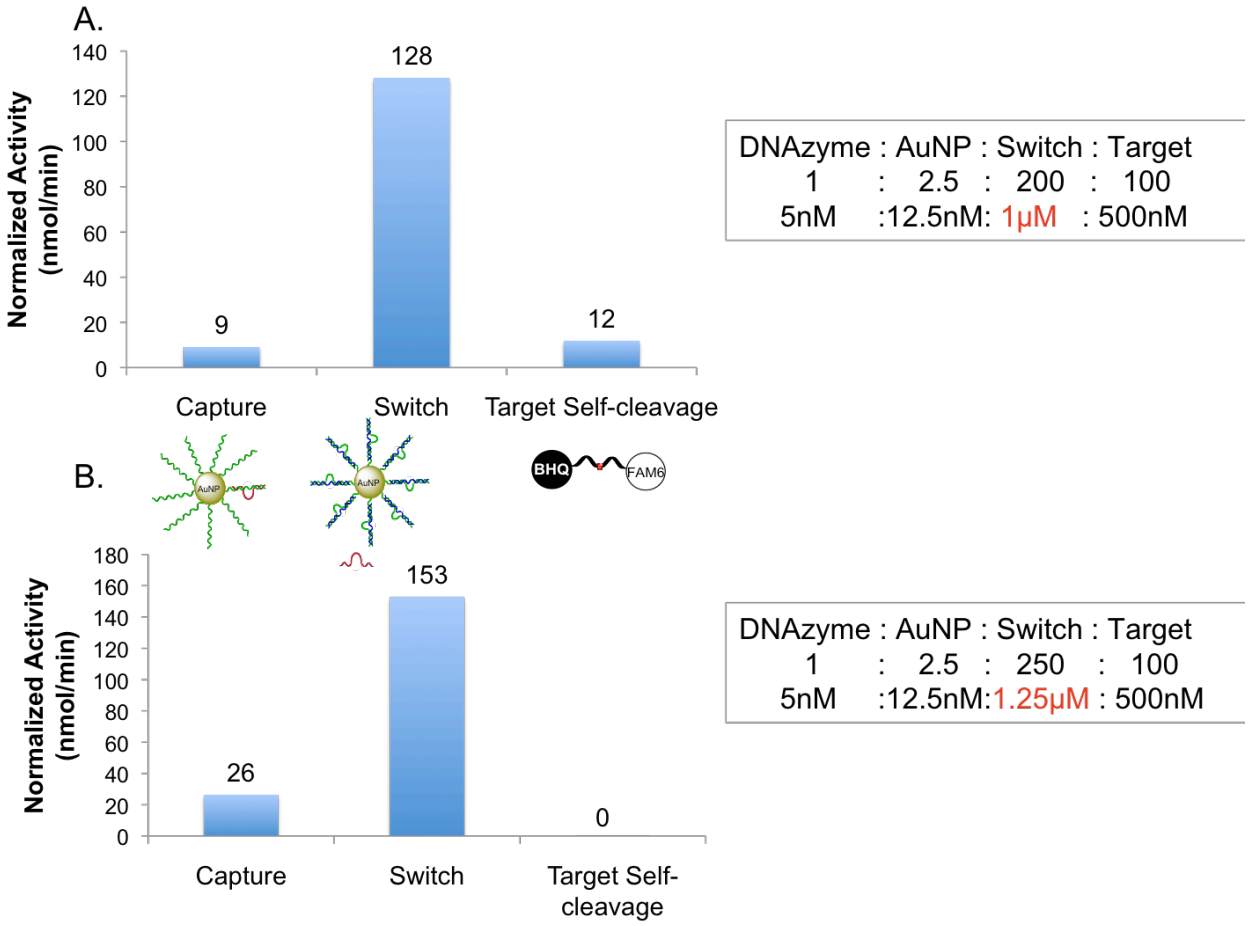


Figure 23: Effect of increasing switch concentration on conditional response in nanoparticle system.

(Figure 23 B). This allowed the maximum amount of DNAzyme to be displaced and thus the maximum rate of cleavage. Figure 23B also shows a conditional response, but with only a 6-fold observed enhancement. Figure 24 shows the subsequent experiments in which the concentration of switch strand was further increased to 1.5 μM.

These results once again demonstrate a conditional response system with an average of 14-fold rate enhancement of displaced over captured DNAzyme. This is the maximum rate observed with the conditional *in vitro* nanoparticle system.

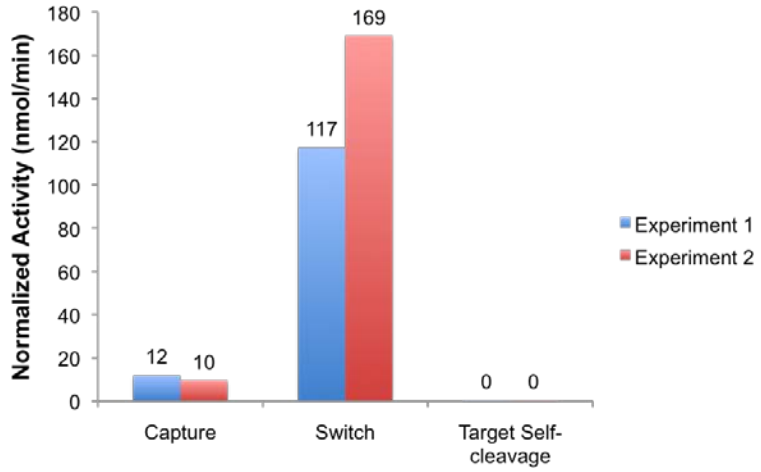


Figure 24: Observed conditional response signaling in nanoparticle system. Run with 5 nM DNAzyme, 12.5 nM AuNP, 500 nM switch, and 1.5 μM target.

It is important to note that the complete system does not regain the full activity of free DNAzyme in solution. This is due to the fact that gold nanoparticles decrease fluorescence through a filtering effect. The FAM fluorophore absorbs at 495nm and emits at 520nm and the gold particles absorb at 520nm. Some of the light emitted by the fluorophore will be absorbed by the nanoparticles, thus decreasing the overall fluorescence observed. To test the activity of DNAzyme in the presence of gold particles, the procedure of the experiment was altered. Instead of adding the DNAzyme and allowing it to anneal to the capture, the switch strand was added first and allowed to anneal to the capture strands on the

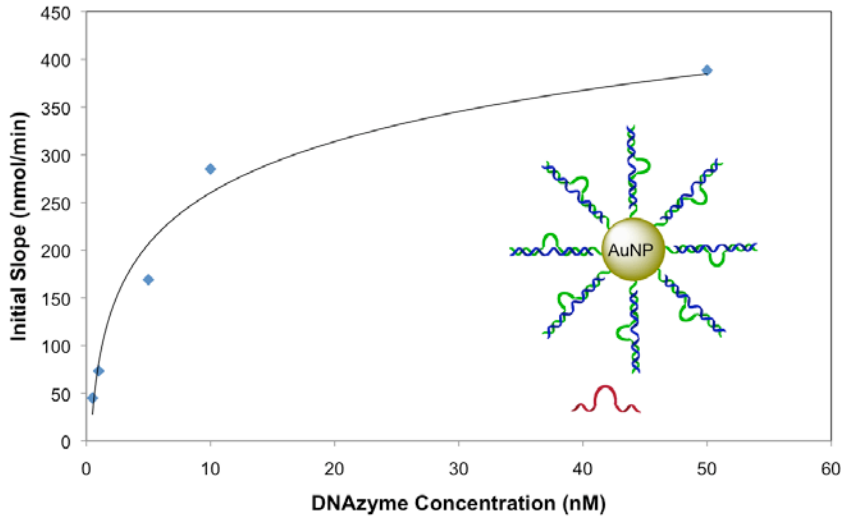


Figure 25: Calibration of activity based on DNAzyme concentration. Run with 12.5 nM AuNP, 500 nM switch, and 1.5 μM target.

nanoparticle. Afterwards, different concentrations of DNAzyme were added into the solution to obtain a calibration curve that compares activity slope to concentration of DNAzyme in the presence of the functionalized nanoparticles. These results are shown in Figure 25.

All of the runs shown above use a concentration of 5nM DNAzyme, and based on this curve, the switch run should have a slope of about 170. An experiment run at the same time (shown as the red bars in Figure 24) as this calibration showed a switch run with a background subtracted slope of 168 which is in agreement with the value for 5 nM of DNAzyme showing that the switch experiments are effectively reactivating the DNAzyme. Michaelis-Menton kinetics predict that this calibration should be linear since we are increasing the concentration of our "enzyme." This is not observed because as the DNAzyme concentration increases, the equilibrium will be pushed to have some DNAzyme hybridized to the capture strand even in the presence of excess switch strand. This sequestering of "enzyme" accounts for the non-linear behavior observed.

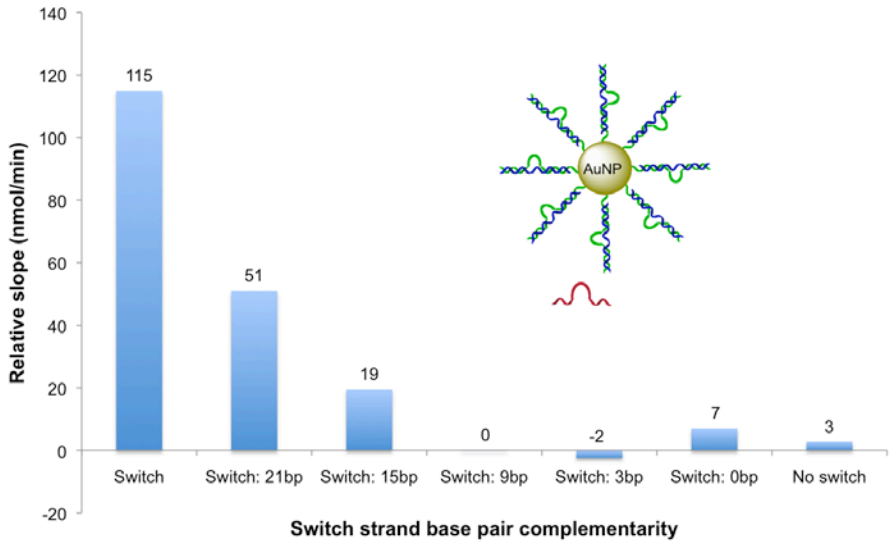


Figure 26: Switch strand selectivity through variable complementary sequences. Run with 5 nM DNAzyme, 12.5 nM AuNP, and 500 nM switch, and 1.5 μM target.

In the future biological applications

of this system, the switch strand will be designed as a specific mRNA marker. Other mRNAs with similar sequences could displace some of the DNAzyme and cause unwanted cleavage of the target mRNA as a false positive. To test this possibility, the system was run with switch strands of different complementarities. Figure 26 shows the results of this experiment. The switch strand fully complementary to the capture strand shows the highest activity and the activity decreases with less complementarity, as expected. Once the number of complementary base pairs drops below 15, the binding of the DNAzyme to the capture is the more favorable state, and no activity is observed. While the system can be varied in the number of complementary base pairs as needed, with the current setup only an mRNA with a specific 15 base pair sequence could give a significant false positive reaction over the small observed background cleavage. It is unlikely that the system would be active in the presence of anything other than the desired mRNA marker.

Conclusions:

These results indicate the successful synthesis, characterization, and optimization of an *in vitro* conditional system with the potential for *in vivo* gene knockdown using gold nanoparticle strand displacement reactions. The system has been shown to only cleave the target sequence in the presence of the switch strand with minimal background reaction observed. To achieve the inactive state, heating the system for 5 minutes and allowing for slow annealing is an essential step for DNAzyme-capture hybridization. The ratios of the different components of the system, and by extension the equilibria of affinity base pairing is what drives the system. The

concentrations of the different DNA strands can be used to push the equilibria into the active or inactive states of the system. The maximum rate achieved presented above in the *in vitro* nanoparticle system is about 14-fold faster than the inactive system and about 75-fold faster than the rate of target self-cleavage. We have also shown that only the specific switch strand will cause significant DNAzyme displacement.

Future Direction:

With a characterized *in vivo* system, the next step is to move the system into cellular *in vivo* studies. While we have achieved as much as 14-fold increase with the nanoparticle studies, to be successful in a cell, the effect needs to be increased further. Increasing the amount of DNAzyme could increase the cleavage rate, but it would also increase the background reaction observed, lessening the conditional effect. As a model cellular system, the target mRNA will be tailored to knockdown green fluorescent protein (GFP). This provides an easily detectable method to observe if the gene knockdown has been successful. The switch strands could be the mRNA for any sort of protein that is commonly over expressed and signals cancerous cell activity such as the epidermal growth factor receptor (EGFR).

There are many future clinical and research applications of this system. It has the ability to selectively target and identify cells expressing certain genes. This allows it to act on particular kinds of cells (such as cancerous ones) and not on others. Once active, the system can be tailored to knockdown specific gene targets. The target protein could be an essential transcription factor, preventing cell proliferation, or something that prevents cell death, and thus induce apoptosis. This ability to

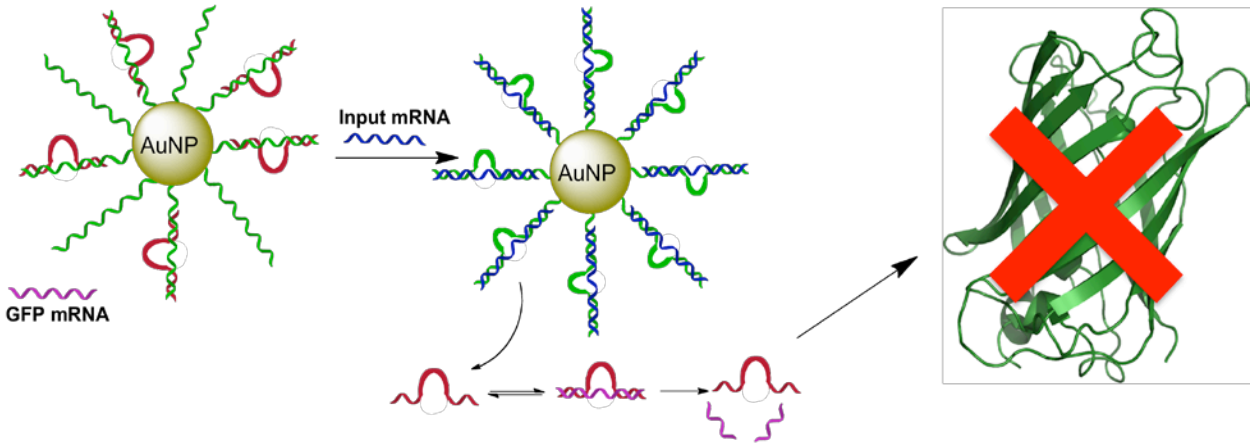


Figure 27: Proposed scheme for GFP knockdown in a cellular system.

knockdown specific proteins rather than just kill the cell could be very useful in regaining cell function, or even preventing cancer in at-risk cells. As a specific example, the over expression of EGFR has been shown to cause constitutive cell division which leads to many forms of cancer. If this mRNA is detected, then the system will be switched to the active state, and the DNAzyme is free to act on other receptors to prevent the cell from dividing uncontrollably. This system could also be adapted to work on similar targets described by Xie *et al.* as discussed above [23]. Specific cancer makers in HeLa cells could activate the system, which could then knockdown specific proteins that prevent apoptosis. This system could also be used to deliver multiple DNAzymes, designed to knockdown a variety of genes when activated in the presence of a switch strand.

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