Sensing Ribonuclease H Activity with DNA Nanoswitches

Ruju Trivedi
University at Albany, State University of New York, rtrivedi@albany.edu

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Sensing Ribonuclease H Activity with DNA Nanoswitches

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Ruju Trivedi

Research Mentor: Arun Richard Chandrasekaran, Ph.D.
Research Advisor: Ken Halvorsen, Ph.D.
Second Reader: Elise Gervais, Ph.D.

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Abstract

Ribonuclease H (RNase H) is a damage-repair protein and ribonuclease that specifically catalyzes the hydrolysis of RNA in an RNA/DNA duplex and breaks down RNA/DNA junctions. It plays an important role in a variety of biological processes including DNA replication, DNA repair, and transcription. It is also pivotal in anti-HIV drug development and the analysis of cellular processes and has been shown to be a potential therapeutic target for various neoplastic diseases. This thesis discusses a unique assay based on DNA nanoswitches to detect RNase H levels and activity. The assay is based on conformational changes of DNA nanoswitches in the presence of RNase H. Using gel electrophoresis, we study the kinetics of cleavage, sensitivity and specificity of detection of RNase H. We also show the utility of the assay in screening potential RNase H inhibitors. This novel biosensing platform is low-cost, label-free, and will be a useful tool to detect RNase levels and identify RNase contamination in biological samples.
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Chapter 1: Introduction

In the 1950s the structure of DNA was solved by James Watson and Francis Crick, along with the help of the research done by Rosalind Franklin and her team (1). The canonical DNA double helix we all know is a linear chain, but the strands of DNA can interact in a very programmable way to provide a wide of variety structures. Scientists have designed synthetic DNA strands to self-assemble into complex arrangements such as various shapes and structures (2). A reason for using DNA in the biotechnology industry is the ability to manipulate DNA using enzymes such as ligases or restrictions enzymes (3). Furthermore, DNA can be functionalized with other units such as proteins, small molecules or inorganic materials (2). Some proteins have been successfully used for design and self-assembly, but the process can become very complex using proteins due to the wide variety of the 20 amino acids which are used as building blocks for proteins themselves (4). DNA, on the other hand, only has four different nucleotides based on the Watson and Crick model which in turn makes it more efficient and easier to use (4).

DNA is widely known as the genetic material but is also used as a viable material for bottom up construction of nanoscale shapes and structures (5). With its nanoscale size, persistence length, and self-assembly by its specific base pairing, DNA forms the basis of constructing a wide variety of configurations with defined shapes and sizes. The DNA duplex itself has a diameter of ~2 nm, a helical pitch of ~3.4 to 3.6 nm, and a persistence length of ~50 nm (6). DNA also has better stability than other naturally occurring nucleic acids such as RNA, making it more suitable as a building block. DNA also provides other forms of assembly such as non-Watson–Crick base pairing (7), protein binding (8), and enzymatic activity (9). DNA is a highly viable molecule for assembly not only because of its specificity or programmability but also because of its ability to
function under a wide range of temperatures and various other conditions. Using DNA nanotechnology, scientists have created a variety of nanostructures including DNA objects (such as truncated octahedron (10), tetrahedron (11), cube (12) and icosahedron (13), two-dimensional (14, 15) and three-dimensional crystal lattices (16-20), and nanotubes (21). In recent years, one of the most important breakthroughs in area of structural DNA nanotechnology was the use of a long viral genome-derived “scaffold” DNA strand for assembly of numerous structures (22). Using this concept of “DNA Origami”, a long strand of DNA is folded with the help of hundreds of short complementary strands to create two- and three-dimensional shapes and structures (23-26).
Chapter 2: Biosensing Applications of DNA Nanotechnology

Over the past several years, numerous new applications for DNA nanotechnology have been identified. Many of these applications reside in the field of medicine and diagnostics where DNA nanostructures are used for biosensing. Nanotechnology has opened up many doors towards highly sensitive detection of biological molecules and various toxic agents (27). This type of technology can also be used for therapeutic targeting and screening. The simplest practice of DNA-based biosensing involves using single stranded DNA to hybridize different complementary sequences. Within the area of biosensing, the hybridization event is converted into an optical, electrical, or coupled response by labeling oligonucleotides with fluorescent dyes (27). DNA-based biosensors rely on specific recognition sites between a substrate and the target (eg: nucleic acid or protein detection) or programmable conformational changes (eg: pH sensing). The majority of DNA or nucleic acid-based biosensors involve hybridization of a DNA or RNA strand to a complementary region on the nucleic acid (28).

Biosensing strategies involve (i) fluorescence, (ii) FRET, (iii) nanoparticle-based, (iv) electrochemical, (v) gel electrophoresis, (vi) atomic force microscope (AFM), (vii) surface-enhanced Raman spectroscopy (SERS) based and many more (29). Fluorescence based biosensing has been used to detect conformational changes in a DNA nanostructure by tagging the component DNA strands with a fluorophore-quencher pair. These fluorescence-based biosensors have been used to detect antibodies and protein targets including the HIV biomarker anti-p17 antibody (30). Such fluorescence-based biosensors have also been used for pH detection (31). Other biosensing methods include Fluorescence Resonance Energy Transfer (FRET) in which fluorescence signals
are created for recognition of molecular association and separation in the 1–10 nm range (32). One such FRET based example is a DNA nanomachine that senses pH inside living cells (33).

Researchers have also used nanoparticle-based biosensing which relies on metallic nanoparticles (NPs) that exhibit color changes between individual nanoparticles and accumulated clusters (34). This feature has also been used in colorimetric assays based on DNA-functionalized gold nanoparticles (AuNPs) that provide an optical readout or a visual color change (35). Some AuNPs assays have also been used to detect Pb (II) ions (36) and other NP based visual color change assays have been used as biosensors for identifying various nucleic acid sequences (35). There are also DNA-based electrochemical sensors that use nanoscale interactions between a target and a recognition layer. For example, DNA tetrahedron biosensors are used in combination with surface-based assays for electrochemical detection of nucleic acids (37). This biosensing strategy detection has potential use in immunological sensing, cancer cells, and HIV DNA. There has also been development of electrochemical aptamer-based sensors that can be used for the detection of proteins, small molecules, and inorganic ions (38).

Atomic force microscopy (AFM) has been used to analyze two-dimensional structures made from DNA, and in some biosensing examples. Results of AFM are more evident with DNA origami structures that change conformation or where a visual marker is present on target interaction (39). Surface-enhanced Raman scattering (SERS) is another technique that provides enhanced Raman signals through electromagnetic interactions between the analyte molecules and metal surface (40). Previous SERS methods have been used to detect ATP molecules and single-stranded DNA (41). Another strategy that is less-often used is based on gel electrophoresis. For example, gel-
electrophoresis has been used to detect specific nucleic acid sequences in which a conformational change in a DNA nanoswitch can be detected on an agarose gel (42).

The detection of various biomarkers is crucial to the identification and diagnosis of various diseases. This thesis describes the use of a DNA nanoswitch to detect the enzyme ribonuclease H (RNase H) which is an endoribonuclease that can hydrolyze RNA fragments in DNA/RNA hybrid duplexes through an endonucleolytic mechanism.
Chapter 3: DNA Nanoswitch-based Ribonuclease Assay

RNase H can be found in almost all organisms including bacteria, archaea, and eukaryotes. It is an endogenous enzyme that is found in the nucleus and cytoplasm of many cells (43). RNase H play an important role in the biochemical processes that are associated with DNA replication, gene expression, and DNA repair where DNA/RNA hybrids can occur in the human body. Additionally, RNase H degrades DNA/RNA hybrids generated during viral replication (44). The importance of this enzyme is also shown by its existence in organisms from Escherichia coli to humans (45). Unlike in prokaryotes and in various other single-cell eukaryotes, in higher eukaryotes RNase H is essential for development as well. Since RNaseH also has the specific function of cleaving the RNA strand of a DNA/RNA hybrid it will yield a 3’-hydroxyl and a 5’-phosphate at the hydrolysis site (45). Although the physiological role of RNase H has not been entirely elucidated, recent studies suggest that the enzyme’s function is associated with DNA replication. The importance of this enzyme is also shown by its existence in organisms from Escherichia coli to humans (45). The RNase H enzyme has also been shown to be related to some diseases and is a potential therapeutic target. For example, with the involvement of the RNase H, the retroviral RNA in the HIV virus could possibly be reversed into double-stranded DNA which could be inserted into the host cell chromosome and eventually result in the disease (46). The possible involvement of RNase H in HIV replication makes it pivotal in anti-HIV drug development and analysis. Various methods including gel electrophoresis (47), high-performance liquid chromatography (HPLC) (48), colorimetry (49), and fluorometry (50) have been used to determine RNase H activity in relevant biological fluids. In our lab we have used DNA nanoswitches and the approach of gel-electrophoresis as a biosensor to detect the presence of RNase H.
In recent years, our lab has developed a simple DNA nanoswitch that has been used in the detection of microRNAs (51, 52), viral RNA detection (53), and for the analysis of biomolecular interactions (54). On binding a target molecule, the DNA nanoswitches undergo a conformational change and this ensuing change can be analyzed through gel electrophoresis (Figure 1). The nanoswitch is a duplex comprised of two strands, one of which is the single stranded DNA scaffold (derived from M13 viral genome) and the other strand is made up of short complementary oligonucleotides (“backbone oligos”). This is the “off” state of the DNA nanoswitch. The DNA nanoswitch is programmable for specific nucleic acid detection by incorporating two detector strands. The sequences of these detectors are designed to be complementary to the target DNA or RNA sequence. Target recognition and binding reconfigures the linear nanoswitch to form a loop, thus changing it to the “on” state. The “on” and “off” states of the DNA nanoswitches migrate differently on an agarose gel providing an easy readout.

![Figure 1. The DNA nanoswitch. This figure shows the basic mechanisms of the DNA nanoswitch. The “off” state of the DNA nanoswitch is a linear stand DNA. The nanoswitch is programmable for specific nucleic acid detection by incorporating two detector strands. Upon target recognition and binding a conformational change occurs and the linear nanoswitch forms a loop, thus changing it to the “on” state. In an agarose gel the "on" and “off” states of the DNA nanoswitches migrate differently providing an efficient way of analysis. Image reproduced from (55).](image-url)
In this project we developed a DNA nanoswitch based assay for detecting RNase H levels and activity in relevant biological fluids. Using gel electrophoresis, we aim to study the kinetics of cleavage, sensitivity and specificity of detection of RNase H, and screen RNase H inhibitors. This assay has potential use in studying ribonuclease activity, screening drugs for disease biomarkers with RNase H activity, and as a lab tool for identifying the presence of RNases in biological samples.
Chapter 4: Materials and Methods

A. Linearizing M13 Scaffold DNA

We mixed 5 µL of single stranded (ss) M13 DNA (M13mp18ssDNA) (New England Biolabs), 2.5 µL the 10x cut smart buffer (New England Biolabs), 1 µL of the cutsite oligo (complementary to BtsCI restriction site at 100 µM) and 16.5 µL of deionized water and annealed 95 °C to 50 °C in a thermocycler (Bio-Rad, USA). When the sample reached 50 °C, BtsCI enzyme was added and incubated at 50 °C for 15 minutes and cooled down to 4 °C. The linearized M13 was then filtered before using it to make the nanoswitch.

B. DNA Nanoswitch Assembly

We mixed 5 µL of linear M13 and 1.2 µL of the total oligo mixtures (containing backbone oligonucleotides and detectors) and annealed from 90 °C to 4 °C for 1 hour in a thermal cycler.

C. RNase H Assay

We mixed the RNA lock strand (2.5 nM final) with DNA nanoswitch to form the looped switches. We then mixed locked nanoswitches with RNase buffer at 1× final concentration. For sensitivity experiments, the enzyme dilutions were made as a serial dilution from the stock RNase H at 5 units/µL. The samples were incubated for 1 hour at 37 °C. Experiments were done in triplicates.

D. RNase H Activity in Biological Fluids

To test RNase H activity in biological fluids, we mixed locked nanoswitches with fetal bovine serum (FBS) at a final of 10% FBS. For cell lysates derived from C2C12 (mouse) and HeLa
(human) cells, we mixed 1 µL of cell lysate to 9 µL of locked nanoswitches. We then added 1 µL of RNase H (at 5 U/µL) and incubated the samples at 37 °C for 15 mins.

**E. Specificity – Testing Other RNases**

We tested the specificity of the assay across various other enzymes and their respective buffers. Along with RNase H, we tested RNase A, RNase T (exonuclease T), and RNase If. The locked nanoswitch was mixed with the respective buffers of each enzyme (to 1× final) and then 1 µL of the enzymes was added into the mixture (1 U for RNase H, T and If and 1 mg/ml for RNase A). The samples were incubated for 15 minutes at 37 °C.

**F. Inhibitor Screening**

We mixed the locked nanoswitches with RNase H buffer and added different concentrations of kanamycin to the samples. We added different enzyme amounts (0.5 and 1 U/µl) to these inhibitor concentration series and incubated the samples for 20 minutes at 37 °C. Then to each mixture, 2 µl of loading dye and 1 µl of Gel Red (1× final) were added and loaded on an agarose gel. The gel was run for 45 mins at room temperature at 75V.

**G. Gel Electrophoresis**

Gels were made from molecular biology grade agarose (Fisher BioReagents) dissolved in 0.5× Tris-borate EDTA (TBE) (Ultra-pure grade, Amresco). We used 0.8% agarose gels run at 75V (constant voltage) for 45 min, typically at room temperature. Gels were imaged using Gel-Doc imaging station. Samples were pre-stained by mixing 1 µL of 1:1000× GelRed stain (Biotium) and 2 µl of loading dye, for every 10 µL sample. Samples were then loaded on agarose gels.
Chapter 5: Results and Discussion

For detecting RNase H, we started by "locking" a DNA nanoswitch with an RNA strand, making it looped (Figure 2a). Once the RNase H has been added to the solution it will cleave the RNA “lock”, causing a conformational change and reconfiguring the nanoswitch from the looped (locked) state to the linear (open) state (Figure 2b). The absence of the locked band on an agarose gel indicates the presence of RNase H (Figure 2c).

![Diagram of DNA nanoswitch and RNase H activity](image)

Figure 2. DNA Based assay for RNase H activity. (a) The basic working principles of the DNA nanoswitch and reconfiguration by RNase H. (b) Once the RNase H is added to the solution, the RNA lock will be cleaved by our enzyme, thus opening the nanoswitch. (c) As seen in the gel, once the enzyme has been added to the solution, the “on” or looped band will no longer be visible.

Using this method, we tested the locking and unlocking of our nanoswitch with our RNA lock and the RNase H. The RNase H specifically cleaves RNA in a DNA/RNA duplex. So, when there is a DNA lock versus an RNA lock, the RNase will only cleave the RNA lock in the DNA/RNA duplex (Figure 3). When the enzyme is added to a nanoswitch with a DNA lock the RNase H has no impact on the DNA lock. In this case the “on” band is still visible in the agarose gel.
Figure 3. Testing unlocking of nanoswitches by RNase H. A DNA nanoswitch locked with a DNA strand is not affected by RNase H. RNase H cleaves the DNA/RNA duplex in a DNA nanoswitch locked by an RNA strand, eliminating the signal of the “on” band.

Now that we tested that our RNase H was specific only to RNA locks and not DNA locks, we used different concentrations of our RNA lock (0.5 to 2.5 nM final) for the best looped percentage (Figure 4). We tested 1-hour incubation of the nanoswitches with the different RNA lock concentrations at 20 °C and found that the “on” band with 2.5 nM RNA lock is much brighter than those for the 0.5 nM or 1 nM concentrations (Figure 4a). However, when we incubated the samples overnight, all three concentrations yielded the same looped intensities (Figure 4b).

Figure 4. Testing Concentrations of the RNA Lock. At 20 °C we tested the various concentrations of RNA lock incubated for (A) 1 hour and (B) overnight. As the gel indicates the “on” band at 2.5 nM is much brighter than the “on” bands shown at 0.5 or 1.0 nM in the 1-hour incubation, however all concentrations provide the same looped intensity when incubated overnight.
We then tested the sensitivity of the assay by incubating the nanoswitches with varying concentrations of RNase H (Figure 5). By testing the sensitivity, we were then able to see that the lowest amount of RNase that is needed for the enzyme to cleave the loop is 0.32 units. Any amount added lower than 0.32 units of RNase the looped band still appears in the gel.

**Figure 5. Sensitivity.** (a) Gel showing looping of the DNA nanoswitch with different amounts of RNase H. (b) Sensitivity plot for the amount of RNase H added and the corresponding signal of the “locked” band seen in the gel in (a).

We next tested the specificity of our assay. We tested the specificity using other enzymes such as RNase A, RNase I, and RNase T (exonuclease T). In our experiment we started with nanoswitches locked by an RNA strand (2.5 nM). Once our enzymes were added, we incubated our samples for 15 minutes at 37 °C and then ran the samples on an agarose gel for 45 minutes at a constant voltage of 75 volts at room temperature. Results shown varied levels of unlooping of the DNA nanoswitch by these RNases (Figure 6). Our results clearly show the specificity of our RNase H enzyme as it is able to cleave the loop in our nanoswitch while RNase T was not able to cleave the loop at all.
Figure 6. Specificity. We tested the specificity of our RNase H against various other enzymes such as RNase A, RNase I$_f$, and RNase Exo T. In comparison to RNase H, RNase A shows similar level of unlooping, RNase I$_f$ shows partial unlooping, and RNase T shows no activity on the nanoswitch.

Since RNase A also cleaved our RNA locked nanoswitch in the specificity test, we tested the addition of NaCl in the reaction. NaCl has been shown to inhibit RNase A from cleaving RNA in a DNA/RNA hybrid and make it more efficient in cleaving only single stranded RNA (43). We ran the experiment once again, but this time added 0.5 nM NaCl with our RNase A solution and analyzed the results (Figure 7). However, this concentration did not inhibit the effect of RNase A. Next, we ran a similar experiment with various concentrations of NaCl (Figure 8a). Results here show that higher concentrations of NaCl inhibit cleavage of the RNA lock strand by RNase A. However, our control experiment showed that NaCl does not only inhibit the RNase A from cleaving our DNA/RNA duplex but also inhibits RNase H (Figure 8b).
Figure 7. Specificity using NaCl to inhibit RNase A activity. Specificity test with different RNases with an additional control for RNase A that contain 0.5 nM NaCl.

Figure 8. Inhibition of RNase activity using NaCl. Higher concentrations of NaCl inhibit not only RNase A (a) but also RNase H (b) in cleaving the RNA in the DNA/RNA duplex.

We also conducted a time series in triplicates in which we used the same concentration of the RNase H to see how effective it would be from 0 to 16 minutes (Figure 9a). Results showed that 2.5 units of RNase H is most effective in the cleaving of the RNA locked nanoswitch rather than 0.5 unit or 1 unit of RNase H. The trend in RNase H cleavage of the nanoswitch is shown in Figure 9b.
Figure 9. Time series of RNase H activity. (A) Gels showing varying concentrations of RNase H incubated for various time periods with the DNA nanoswitch. (B) Plot of time series showing that 2.5 unit RNase H completely cleaves the RNA lock in under 2 minutes.

We then tested a known RNase H inhibitor kanamycin (46). Presence of inhibitors prevent the cleaving of the RNA lock in the nanoswitch (Figure 10). We performed a concentration series using varying concentrations of kanamycin with 0.5-unit RNase H. Results show that kanamycin inhibits the RNA cleavage activity of the enzyme with an IC50 value of 30.6 nM. The inhibition efficiency of kanamycin reported in literature has varied in levels from weak to strong inhibition of RNase H (46, 56), and the inhibition level reported here is within the extremes of the reported numbers.
Next, we tested the nanoswitch assay to detect ribonuclease activity in biological samples. RNase H was added and tested in fetal bovine serum (FBS), confirming that RNase H detection was preserved in 10% FBS and cell lysates from human (HeLa) and murine (C2C12) cell lines (Figure 11a-b). We also performed this assay under various temperatures ranging from 4°C to 37 °C (Figure 11c) showing utility of the assay in a wide variety of temperatures.

Figure 10. Screening RNase H Inhibitors. Inhibition efficiency of kanamycin on RNase H activity.

Figure 11. Testing in biological samples and at various temperatures. (a) Detection of RNase H in 10% FBS and (b) human (HeLa) and murine (C2C12) cell lysates. (c) Represents the results from the assay performed under various different temperatures.
Chapter 6: Conclusion

DNA is a very versatile molecule and can be used for many purposes. In this work, we created a DNA nanoswitch based adaptable biomolecular platform to detect RNase activity. The use of this assay to detect RNase H demonstrates another layer of how the DNA nanoswitches can be manipulated and used. For RNase H detection, the assay provides a simple and effective strategy in which we are able to simply mix in the nanoswitches to a sample and analyze using a gel-based read-out. The approach does not require labeling or amplification, thus making it easy to adapt in any situation without the need for expensive equipment. The use of inhibitors also shows the potential of this assay in drug screening. Further, the assay can be used to detect the presence of RNase contamination in biological samples.
References


