A Highly Active RNA-Cleaving DNAzyme with Accelerated Activity by Ethanol and by Freezing

by

Tianmeng Yu

A thesis

presented to the University of Waterloo

in fulfillment of the

thesis requirement for the degree of

Master of Science

in

Chemistry (Nanotechnology)

Waterloo, Ontario, Canada, 2018

Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

DNAzymes are functional DNA molecules with catalytic activities. The EtNa is an example of a RNA-cleaving DNAzyme that catalyzes the cleavage reaction of a phosphodiester bond in the presence of metal ions, such as Na^+ or Ca^{2+}. It was initially selected during the isopropanol precipitation step of an *in vitro* selection, where the selection library was incubated at low temperature with a high concentration of Na^+ and isopropanol. Although inactive with the intended cofactor, the RNA cleavage activity of the EtNa with Na^+ was determined to be significantly increased in the presence of ethanol. The enhanced activity was due to the lower dielectric constant of ethanol, in turn increasing the electrostatic interaction of Na^+ and the EtNa. The first chapter of this thesis is devoted to the introduction of nucleic acids and DNAzymes. In particular, the previously published work related to the EtNa DNAzyme.

In chapter two, the effect of ethanol on the Ca^{2+}-dependent activity of the EtNa DNAzyme was examined. The cleavage results showed that its activity with low concentrations of Ca^{2+} was enhanced by the addition of ethanol. However, with a concentration higher than 2 mM Ca^{2+}, inhibition of the DNAzyme activity was observed. When testing the effect of ethanol on other RNA-cleaving DNAzymes, no significant increase in activity was shown while 50% ethanol slowed down their RNA cleavage reaction. Therefore, the boosted activity was unique to the EtNa DNAzyme. Besides, the performance of the EtNa based biosensor was improved with the addition of organic solvents. With 30% ethanol, the sensor achieved a detection limit of 1.4 μM Ca^{2+}, which was a 16-fold better than that in water.

In chapter three, the effect of freezing on the activity of the EtNa DNAzyme was characterized. Acceleration in the cleavage reaction was achieved by freezing the EtNa in water

with Na^+. The apparent dissociation constant reached 6.2 mM Na^+ under the frozen condition, which is over 20-fold tighter than that at room temperature. With 10 mM Na^+, the EtNa has a cleavage rate of 0.12 h^{-1} after freezing at -20 °C. This effect is also unique to the EtNa, while all the other tested DNAzymes were inhibited by freezing except for the Na^+-specific NaA43 DNAzyme. Freezing also inhibited the EtNa activity if Ca^{2+} was used, which demonstrated the importance of the metal cofactor to be monovalent. The enhancement in activity can be attributed to the freeze-induced concentration effect of EtNa and monovalent Na^+ in the micropockets formed between ice crystals, whereas divalent metal ions such as Ca^{2+} may misfold DNA under similar conditions.

Taken together, using ethanol, divalent Ca^{2+}, and freezing all achieved the same purpose of enhancing electrostatic interactions between metal species and the DNAzyme. This is summarized in chapter four, where some future directions are outlined.

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Acknowledgments

I would like to express my sincere gratitude and appreciation to my supervisor, Dr. Juewen Liu, for his continued guidance and support throughout this research project. I would also like to express great appreciation to my committee members: Dr. Shirley Tang and Dr. Vivek Maheshwari, for their helpful comments and useful feedback on my research project.

In addition, I am very grateful to have had the opportunity to work with all other members in the Liu research group: Biwu Liu, Po-Jung Jimmy Huang, Yibo Liu, Zijie Zhang, Runjhun Saran, Lingzi Ma, Zhicheng Huang, Yuqing Li, Anand Lopez and Wenhu Zhou. They all have helped me in some way or another to get me where I am today.

Finally, I would like to thank both my parents and Cathy for their unconditional love and support over the past two years. This wouldn't have been possible without them.

Table of Contents

List of Figures

List of Abbreviations

A	adenine
APS	ammonium persulfate
BAPTA	1,2-bis(o-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid
C	cytosine
CaGF	Calcium Green FlAsH
DNA	deoxyribonucleic acid
DNAzyme	deoxyribozyme
dPAGE	denaturing polyacrylamide gel electrophoresis
EDTA	ethylenediaminetetraacetic acid
FAM	carboxyfluorescein
FRET	Förster resonance energy transfer
G	guanine
GFPs	green fluorescence proteins
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
K-EDTA	ethylenediaminetetraacetic acid dipotassium salt
K_d	apparent dissociation constant
LOD	limit of detection
MES	2-(N-morpholino) ethanesulfonic acid
MOPS	3-(*N*-morpholino) propanesulfonic acid
PEG	polyethylene glycol
rA	ribo-adenosine

RNA	ribonucleic acid
RNase P	ribonuclease P
T	thymine
TBE	Tris/boric acid/EDTA
TEMED	Tetramethylethylenediamine
TS	transition state
U	uracil
UV	ultraviolet
λ_{em}	emission wavelength
λ_{ex}	excitation wavelength

Chapter 1. Introduction

1.1. Introduction to nucleic acids

Nucleic acids are a collection of macromolecules that regulate the growth, functioning and reproduction of an organism throughout its lifespan.[1] There are two major types of nucleic acids, DNA (deoxyribonucleic acid) and RNA (ribonucleic acid). A DNA or RNA molecule is made up of monomeric units called nucleotides (Figure 1.1). Each nucleotide is composed of a five-carbon sugar ring attached to a nitrogen-containing nucleobase and a phosphate group. The sugar molecule in a nucleotide can be either a D-ribose (in RNA) or a D-deoxyribose (in DNA). There are two purines in DNA nucleotides, adenine (A) and guanine (G), and two pyrimidines, cytosine (C) and thymine (T). RNA uses adenine, guanine, cytosine and uracil (U) instead of thymine.[2] The phosphate group that is already attached by a phosphodiester bond to the 5′ carbon of one nucleotide is linked by a second phosphodiester bond to the 3′ carbon of the next nucleotide (3′,5′ phosphodiester bond). The polynucleotide formed by this process has an intrinsic directionality, with 5′ phosphate group at one end and 3′ hydroxyl group at the other end.[3]

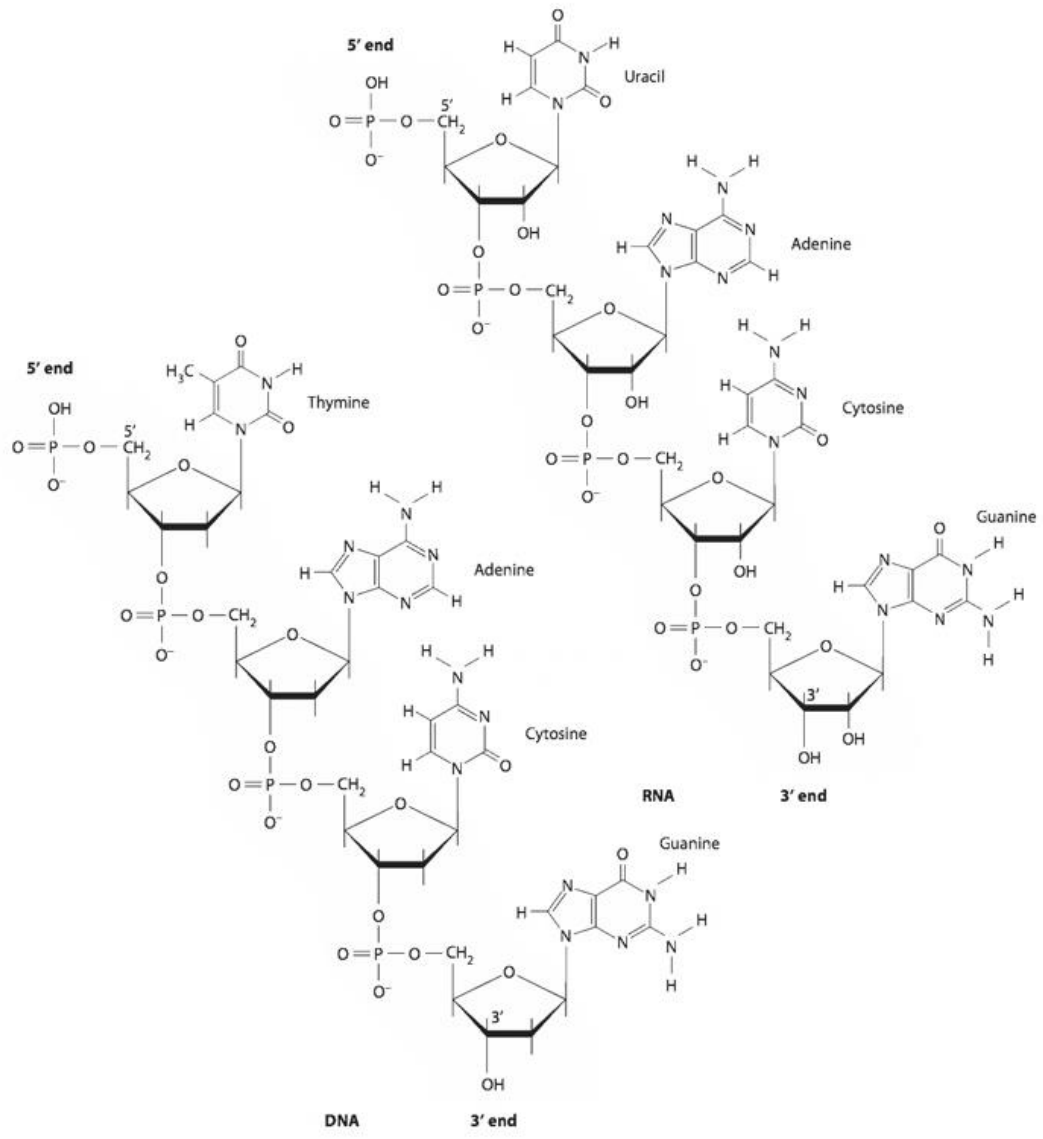

Figure 1.1 The structures of nucleic acids and their nucleotide subunits. 4 DNA nucleotides variants showed on the left and 4 RNA nucleotides variants on the right. (Figure adapted with permission from Ref.[3])

While DNA is mostly studied for their biological roles, in the field of nanotechnology, however, their unique physical and chemical properties are often utilized.[4] Thanks to the specific base pairing mechanism between complementary bases, complicated three-dimensional structures can be formed with high precision when a single-stranded DNA molecule anneals to its counterpart.

The base pairing mechanism depends on the intermolecular interactions between the purine and pyrimidine bases (Figure 1.2). These bases contain carbonyl groups and nitrogen atoms that are capable of hydrogen bonding, where A can form two hydrogen bonds with T and G can form three hydrogen bonds with C. After base pairing, the two complementary chains of DNA would twist together with one of the strands in the direction of 5′ to 3′ and the other from 3′ to 5′.[5]

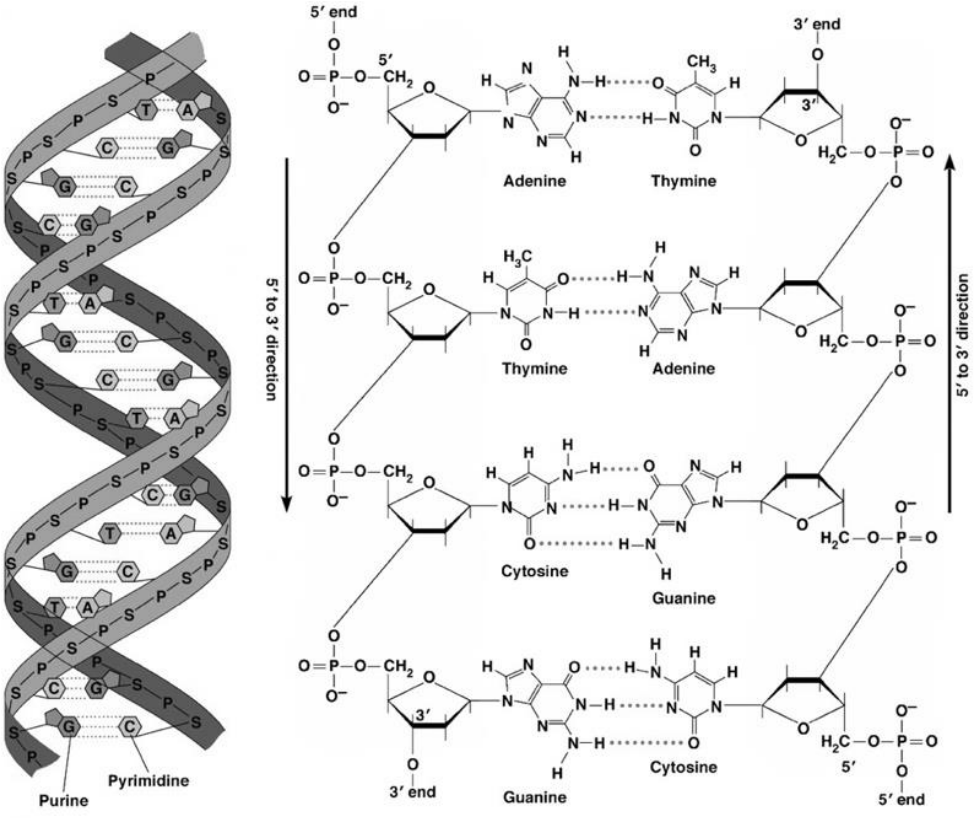

Figure 1.2 The structure of a double-stranded DNA and its base pairing mechanism. Hydrogen bonds can be formed between the bases from one single stranded DNA molecule and its counterpart. (Figure adapted with permission from Ref.[3])

1.2. Introduction RNA-cleaving DNAzymes

1.2.1. Ribozymes and *in vitro* selection

Due to their abilities to form three-dimensional structures, researchers believed that nucleic acids could participate in other biological processes apart from storing genetic information. In 1982, this hypothesis was validated by Cech and his team when they successfully discovered the first case of a naturally-occurring RNA molecule with catalytic activity.[6] Soon after in 1984, Altman and co-workers identified another RNA molecule that could cleave a phosphodiester bond in one of the subunits of ribonuclease P (RNase P) with high accuracy.[7] The term ribozyme was then used to define RNA molecules that are capable of catalyzing specific chemical reactions.[6] Thus far, ribozymes have been found to catalyze only a few reactions in nature, including RNA cleavage,[8] splicing,[9] and peptide bond formation in ribosomes.[10]

After the discovery of ribozymes, many efforts were devoted to isolating new catalytic DNA and RNA molecules from *in vivo* but with very little success. In 1990, three research laboratories developed a revolutionary technique that can select functional nucleic acids artificially, called *in vitro* selection.[11] [12] [13] The process of *in vitro* selection starts with a large population of nucleic acids with partly randomized sequence, followed by a functional screen. The molecules passed the functional screen will then be amplified and screened again. This process will be repeated many rounds until the molecule with specific property dominates the pool. [13] [14] [15] *In vitro* selection enables researchers to identify and obtain functional nucleic acids out of pools that are as large as 10^{16}, making it extremely effective and useful.[16]

1.2.2. Discovery of DNAzymes

By utilizing *in vitro* selection, a new class of functional nucleic acids was identified, called DNAzymes, or deoxyribozymes.[16]. They are DNA molecules that are capable of catalyzing chemical reactions, such as RNA cleavage,[17] DNA cleavage,[18] DNA ligation,[19] DNA

phosphorylation,[20] DNA deglycosylation,[21] and porphyrin metalation.[22] Unlike its counterpart ribozyme, all DNAzymes reported to date are selected artificially, and they have yet to be found in biological systems. The first DNAzyme was discovered by Breaker and Joyce in 1994, named GR-5. [23] [24] Figure 1.3 shows the secondary structure of GR-5. The top strand in green is the substrate strand. It is made almost entirely with DNA nucleotides with just one ribo-adenosine (rA labeled in red) residing in the middle, serving as the RNA cleavage site. The bottom strand labeled in blue is the enzyme strand. It has a catalytic core containing 15 single-stranded nucleotides flanked by two base-pairing regions.[17] GR-5 catalyzes the RNA cleavage reaction in the presence of Pb^{2+} with a catalytic rate as high as 1 min^{-1}, which is 10^5-fold faster than the uncatalyzed reaction.[17]

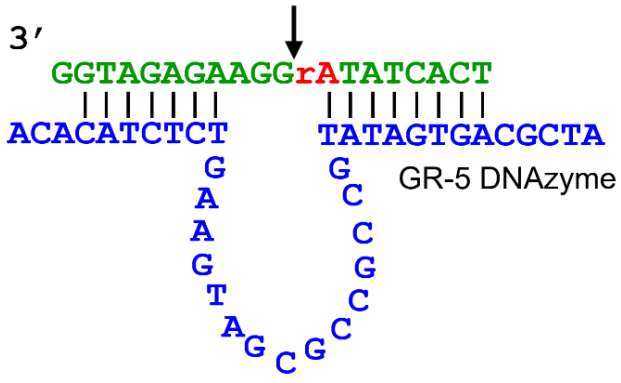

Figure 1.3 Secondary structure of the GR-5 RNA-cleaving DNAzyme. The substrate strand and the enzyme strand are shown in blue and green, respectively. The cleavage site is highlighted in red and the arrow indicates the cleavage position.

1.2.3. Examples of RNA-cleaving DNAzymes

Over the years, many more RNA-cleaving DNAzymes were successfully isolated. The 17E DNAzyme (8-17) is a divalent metal-dependent DNAzyme. Compared to others, the 17E has a small catalytic loop with only 5 DNA nucleotides and a 3-nucleotide long hairpin structure (Figure

1.4). It can catalyze the RNA-cleavage reaction by using either a DNA/RNA chimeric substrate or a full RNA substrate. However, the 17E DNAzyme has poor metal selectivity and can be activated by many divalent metal ions, including Zn^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+} and Pb^{2+}. As a result, its structure was independently reported by more than 10 different research groups.[25 26]

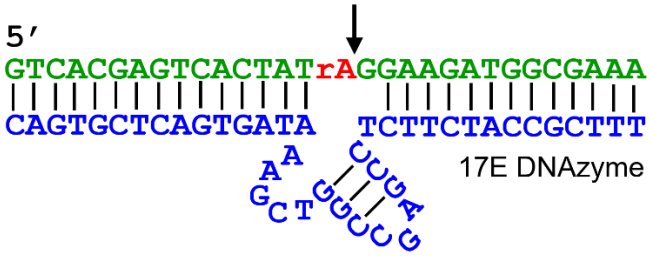

Figure 1.4 Secondary structure of the 17E RNA-cleaving DNAzyme. The substrate strand and the enzyme strand are shown in blue and green, respectively. The cleavage site is highlighted in red and the arrow indicates the cleavage position.

The Tm7 DNAzyme is a trivalent metal-dependent RNA-cleaving DNAzyme. The *in vitro* selection was performed by using three heavy lanthanide ions (Ho^{3+}, Er^{3+} and Tm^{3+}) as the target metal cofactor. The sequencing result suggested a new family of DNAzymes with a single-stranded DNA as the catalytic loop and no hairpin structure (Figure 1.5). It has a very high selectivity towards lanthanides that even Pb^{2+} showed no activity. The Tm7 DNAzyme has a cleavage rate of 1.6 min^{-1} at pH 7.8 with 10 μM Er^{3+}. These features have promoted its utility for constructing a DNAzyme-based biosensor with a limit of detection (LOD) of 14 nM Dy^{3+}.[27]

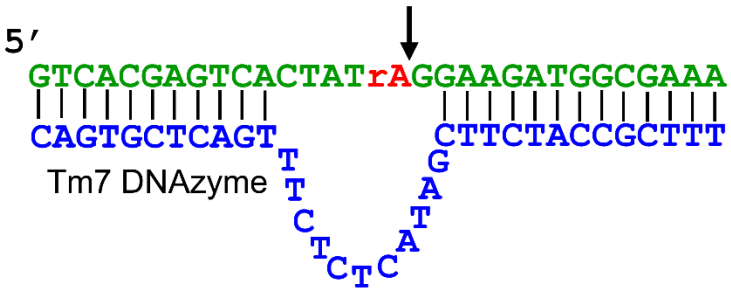

Figure 1.5 Secondary structure of the Tm7 RNA-cleaving DNAzyme. The substrate strand and the enzyme strand are shown in blue and green, respectively. The cleavage site is highlighted in red and the arrow indicates the cleavage position.

In addition to polyvalent metal ions, monovalent ion-responsive DNAzymes were also reported. However, the presence of higher concentrations of these monovalent metal ions is usually required for function.[28] The NaA43 is an example of monovalent metal-dependent DNAzyme (Figure 1.6). Discovered by Dr. Lu and coworkers, the NaA43 is the first reported Na^+-dependent RNA-cleaving DNAzyme. A biosensor designed based on its structure displays exceptionally high selectivity (>10,000-fold) for Na^+ over other competing metal ions with a LOD of 135 μM.[29]

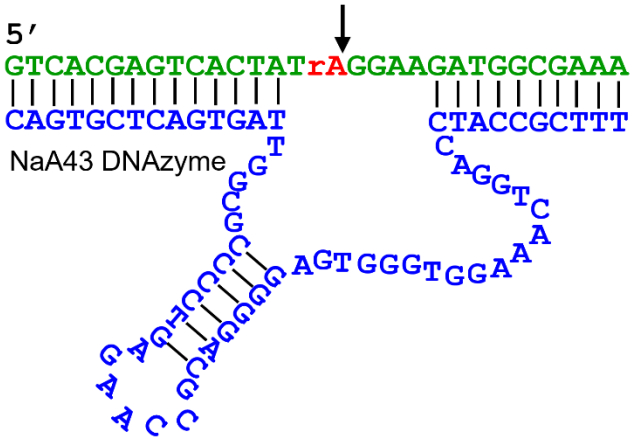

Figure 1.6 Secondary structure of the NaA43 RNA-cleaving DNAzyme. The substrate strand and the enzyme strand are shown in blue and green, respectively. The cleavage site is highlighted in red and the arrow indicates the cleavage position.

1.3. Roles of metal ions in RNA-cleaving DNAzymes

RNA-cleaving DNAzymes catalyze the cleavage reaction of a phosphodiester linkage of the RNA nucleotide in the substrate strand. The cleavage reaction was reported to follow the same mechanism as small self-cleaving ribozymes.[30] The reaction is initiated by the 2′-hydroxyl group acting as an internal nucleophile to attack the adjacent scissile phosphate, producing a negatively charged penta-coordinated intermediate (Figure 1.7, substrate to TS1). The cleavage reaction is completed by the release of the 5′-oxygen followed by the formation of a 2′, 3′-cyclic phosphate and a 5′ -OH terminus (Figure 1.7, TS2 to product). Of two transitions states, TS2 is experimentally proven to be the rate-limiting state.[31] As a result, the presence of metal ions can effectively push the RNA cleavage reaction forward by stabilizing the negatively charged TS2.[32]

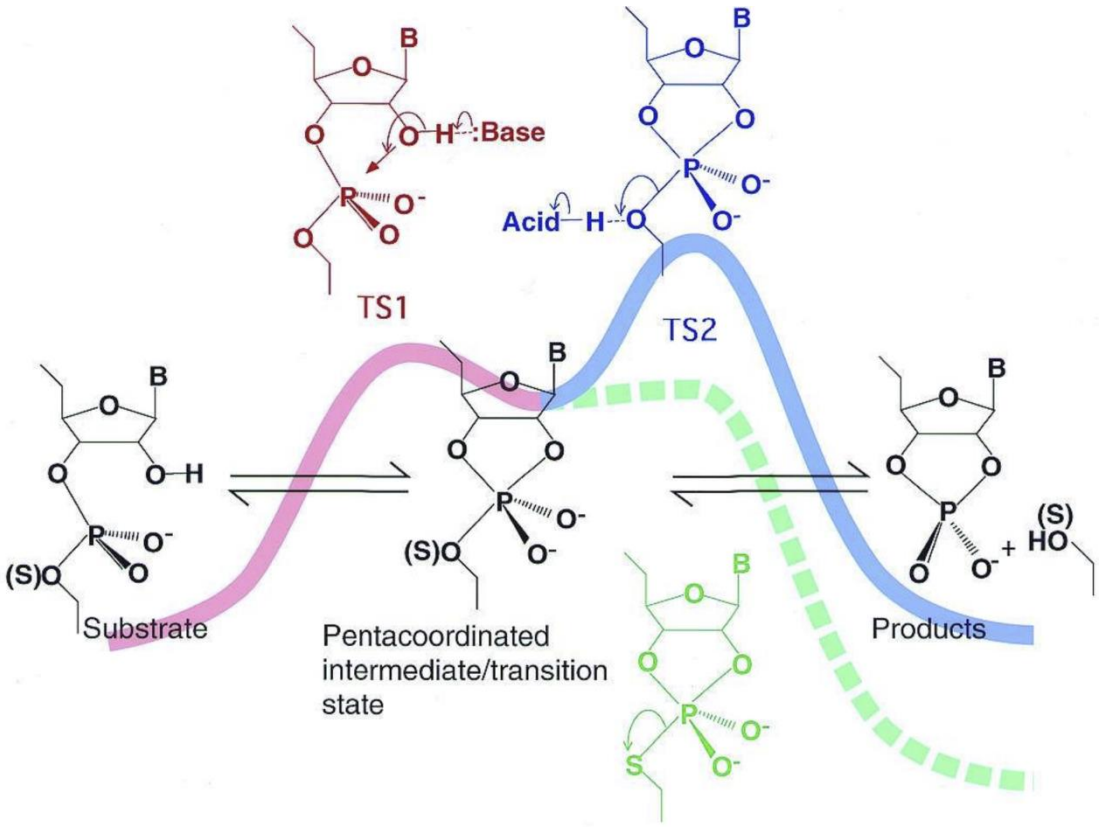

Figure 1.7 Proposed mechanism of the cleavage reaction of a phosphodiester bond in RNA. Firstly, the 2′-hydroxyl group attacks the adjacent scissile phosphate as an nucleophile to form a penta-coordinated transition state (TS1). Then, the 2′, 3′-cyclic phosphate and 5′-hydroxyl RNA termini is formed following the release of the 5′-oxygen (TS2). TS: transition state. (Figure adapted with permission from Ref.[32])

While it is possible for some RNA-cleaving DNAzymes to operate without any metal cofactor,[28] most of them would still require the presence of either monovalent, divalent or trivalent metal ions for proper catalytic function. However, the detailed interaction between DNAzymes and metal ions during catalysis is very complicated and still poorly-understood. Figure 1.8 summarizes some of the possible roles of a metal ion (with Mg^{2+} as an example) that directly participates in the catalysis of the RNA cleavage reaction: (a) the proton from Mg^{2+}-bound water

molecule can neutralize the negative charge of the 5′-oxygen leaving group as a general acid; (b) a metal-coordinated hydroxide can stabilize the proton from 2′-OH; (c) Mg^{2+} can directly coordinate with the 5′-oxygen leaving group as a Lewis acid; (d) a metal ion can act as a Lewis acid to accelerate the deprotonation of 2′-OH by coordinating directly with the oxygen atom; and (e) it can serve as an electrophilic catalyst that increases the electrophilicity of the phosphorous atom by interacting with the two non-bridging oxygen atoms .[32]

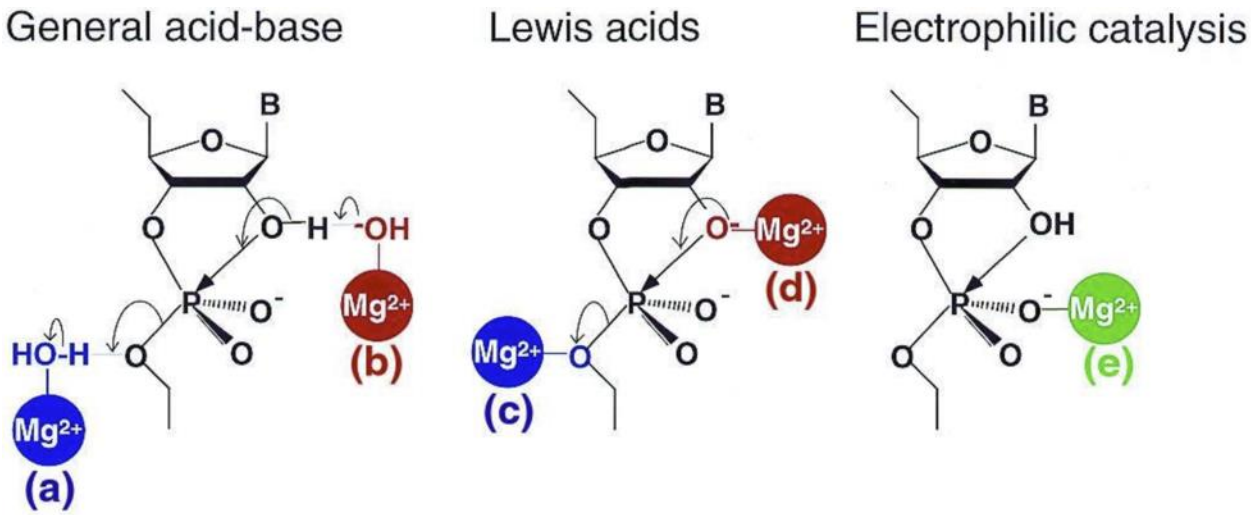

Figure 1.8 Possible catalytic functions of metal ions in the cleavage reaction of a phosphodiester bond. (Figures adapted with permission from Ref.[32])

1.4. The EtNa DNAzyme

1.4.1. Discovery of the EtNa DNAzyme

The EtNa DNAzyme is another example of a RNA-cleaving DNAzyme. Its secondary structure is shown in Figure 1.9. The EtNa was reported by Dr. Liu's group.[33] The original *in vitro* selection was carried out with hemin, a porphyrin metal complex with a ferric ion and a chloride

ligand. After 4 rounds of selection, around 40% of cleavage was identified, with a steady increase to 80% after round 6. However, when incubating the active sequence isolated from round 7 with hemin, no cleavage was observed, suggesting that the cleavage observed during selection was caused by other factors. After the first attempt, a second *in vitro* selection was performed. To minimize the effect of undesired environmental conditions, this time a negative round of selection was carried out before the positive selection. Unfortunately, at the end of round 18, hemin-dependent activity was still failed to achieve. In pursuance of investigating where the cleavage came from, the active sequence from round 18 was purified and sent for sequencing. Then, the EtNa DNAzyme was identified.[33] A series of experiments were conducted on the EtNa DNAzyme to identify the source of cleavage. When incubating the DNAzyme complex with the buffers used during the selection protocol, no cleavage was observed. However, over 50% cleavage was identified during the isopropanol precipitation process, where the sample was incubated with a high concentration of isopropanol (around 70%) and Na^+ (200 mM) in a low-temperature environment (-20 °C).

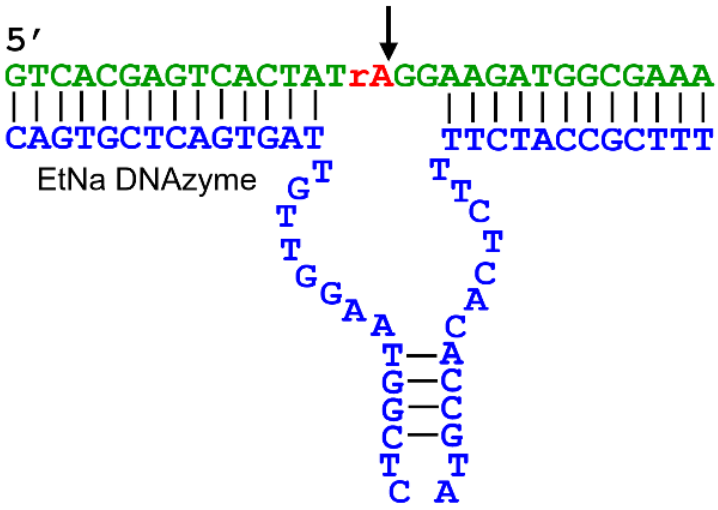

Figure 1.9 The secondary structure of the EtNa DNAzyme. The substrate strand and the enzyme strand are shown in blue and green, respectively. The cleavage site is highlighted in red and the arrow indicates the cleavage position.

1.4.2. Cleavage mechanism of the EtNa with Ca^{2+} and Na^{+}

The EtNa DNAzyme has some unique features compared to others. Firstly, most RNA-cleaving DNAzymes can only utilize either monovalent, divalent or trivalent metal ions for catalysis. Interestingly, the EtNa DNAzyme uses both monovalent and divalent metal ions as cofactors, including Na^{+}, Ca^{2+} and Mg^{2+}. [33-34] In addition, the cleavage rate of the EtNa with 2 mM Ca^{2+} was 90 times faster than that with 2 mM Mg^{2+} (Figure 1.10A).[34] The high selectivity towards Ca^{2+} over Mg^{2+} is rare among DNAzymes because the two ions share very similar chemical properties, both of which are hard Lewis acid with two positive charges.[35] The cleavage results were further analyzed by plotting its logarithm against the logarithm of metal ion concentration (Figure 1.10B). At below 2 mM, the data with Ca^{2+} can be fitted to a straight line with a slope of 1.8 whereas Mg^{2+} has a slope of 0.7. This suggested that the EtNa can cooperatively bind to two

Ca^{2+} ions for catalysis but only one Mg^{2+}. The difference in the number of bonded ions directly contributes to its selectivity towards Ca^{2+}.[34]

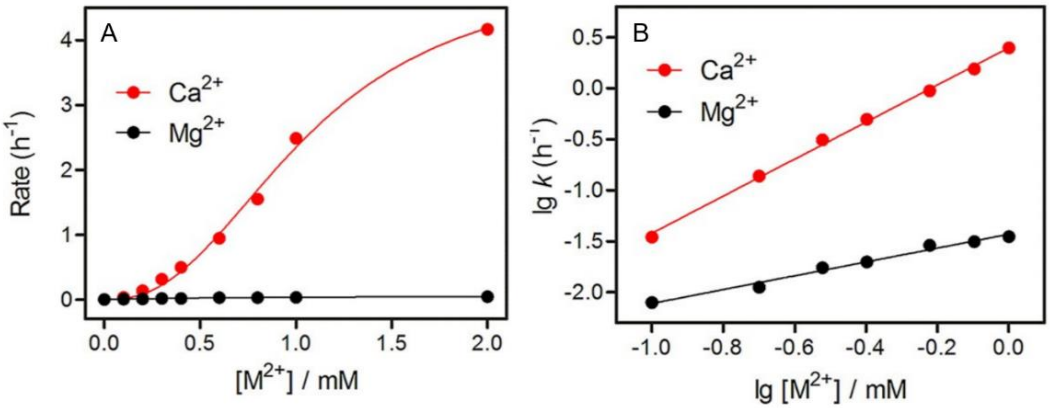

Figure 1.10 (A) Quantification of the cleavage rate of the EtNa DNAzyme with various concentrations of Ca^{2+} and Mg^{2+}. (B) Double-log plot of the cleavage rate of the EtNa against Ca^{2+} and Mg^{2+} concentration. (Figure adapted with permission from Ref.[34])

To further understand the interaction and the cleavage mechanism of the EtNa with Ca^{2+}, a phosphorothioate (PS) modification was introduced at the scissile phosphate of the cleavage site, where one of the two non-bridging oxygen atoms was replaced by a sulfur atom. As a result, two isomers were created, namely R_p and S_p (Figure 1.11A). A strong thio effect was observed with both isomers, yet the EtNa DNAzyme was still active with both modified substrate strands in the presence of 2 mM Ca^{2+} (Figure 1.11B). This result demonstrated that both R_p and S_p oxygen contribute almost equally to the interaction with Ca^{2+} during catalysis. This is also a further indication that the EtNa can bind to two Ca^{2+} ions simultaneously.[34] Further experimentation showed that the Na^+-dependent activity of the EtNa DNAzyme also follows the same mechanism where two Na^+ bind to both of the non-bridging oxygen atoms of the substrate strand during catalysis as electrophilic catalysts.[36] Compared to other metal binding mechanisms, electrophilic

catalysis can be much easier increased by boosted electrostatic interaction between the metal ion and the DNAzyme.

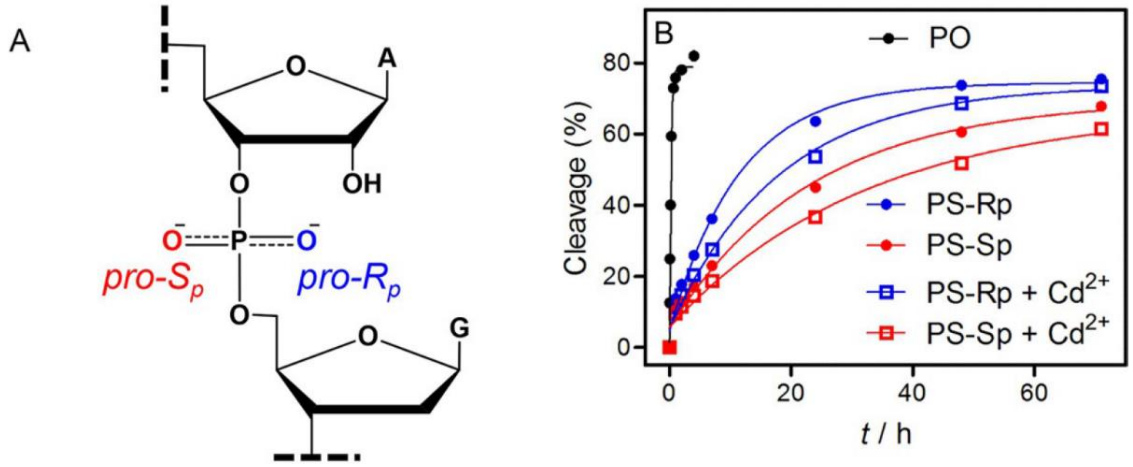

Figure 1.11 (A) Structure of the scissile phosphate linkage and the two modified oxygen positions (R_p and S_p). (B) Cleavage kinetic of the EtNa DNAzyme with the unmodified phosphodiester (PO), phosphorothioate R_p and S_p modified substrate in the presence of 2 mM Ca^{2+} or 50 μM Cd^{2+}. (Figure adapted with permission from Ref.[34])

1.4.3. The improved activity of the EtNa with Na^+ in organic solvents

The catalytic activity of the EtNa DNAzyme with Na^+ has proven to be accelerated significantly in the presence of organic solvents, such as methanol, ethanol or isopropanol (Figure 1.12A). With 4 mM Na^+, the cleavage rate in 54% ethanol is more than 1000-fold faster than that in water. The EtNa DNAzyme can also retain its activity in high concentrations of organic solvents (Figure 1.12B).[33] The mechanism behind such behavior could be that the local Na^+ concentration around the DNAzyme in ethanol is higher due to the lower dielectric constant of organic solvent compared to water, thus promoting the enzymatic activity of the EtNa by providing stronger electrostatic interaction with Na^+.[33]

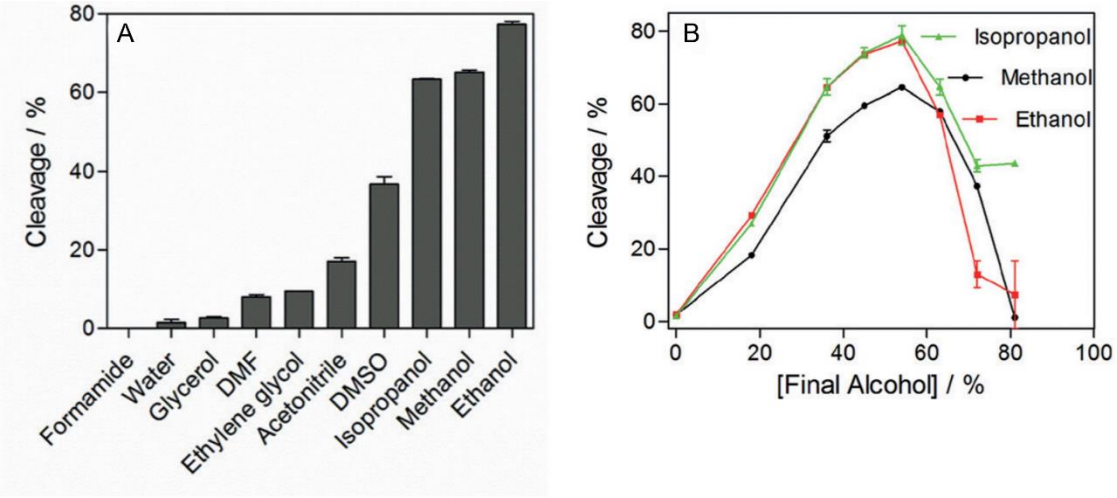

Figure 1.12 (A) The amount of cleavage product after incubating the EtNa DNAzyme for 1 h with various organic solvents. (B) The cleavage product of the EtNa DNAzyme as a function of solvent concentration in ethanol, methanol and isopropanol after 1 h incubation. The reaction buffer contained 50 mM MES (pH 6.0) and 120 mM NaCl for all reactions. (Figure adapted with permission from Ref.[33])

1.5. Research focus of the thesis

Previous studies showed that the EtNa is a RNA-cleaving DNAzyme that works optimally in concentrated organic solvents containing Na^+.[33] In aqueous phase, the EtNa can also use Ca^{2+} as the metal cofactor with high specificity, while its activity with Na^+ was much lower. These two metal ions also share similar roles during the RNA cleavage reaction, which can both be classified as electrophilic catalysts.[34 36] The accelerated activity of the EtNa with Na^+ in organic solvents is most likely due to its binding mechanism. Lower dielectric constant of the organic solvents could strengthen the electrostatic interaction between the Na^+ and the EtNa DNAzyme, thus explaining the higher catalytic activity.

Combination of previous studies suggests a possibility that organic solvents such as ethanol could also enhance the activity of the EtNa with Ca^{2+}. The first research focus of this thesis is to characterize the Ca^{2+}-dependent activity of the EtNa DNAzyme in the presence of ethanol. Based on the results, the performance of the EtNa based biosensor for Ca^{2+} can be improved with the addition of organic solvents. Also, the generality of the effect of ethanol on RNA-cleaving DNAzymes can also be evaluated.

Freezing is a fast and convenient way of persevering organic matters by slowing down chemical reactions. However, many research efforts suggest that freezing can also accelerate certain reactions given the proper condition. Freeze-concentration effect usually contributes to such effect, where low temperature results in a eutectic system of crystalline ice and the interstitial liquid containing concentrated solvents. Most of the previous studies on the impact of freezing on nucleic acids focused on ribozymes with ligation or polymerase activities, while little work was done on RNA-cleaving DNAzymes. In the second study, I aim to have an initial understanding of freezing on the activity of the EtNa DNAzyme. Similar to the previous project, the change in the activity of different RNA-cleaving DNAzymes will be measured to determine the generality of the freezing effect. Meanwhile, the importance of the metal ion cofactor during catalysis in freezing conditions will also be tested.

Chapter 2. The EtNa DNAzyme-based biosensor for Ca^{2+} detection with boosted activity by ethanol

The results presented in this chapter have been published partly as:

Yu, T.; Zhou, W.; Liu, J., Ultrasensitive DNAzyme-Based Ca^{2+} Detection Boosted by Ethanol and a Solvent-Compatible Scaffold for Aptazyme Design. *ChemBioChem* **2018,** *19* (1), 31-36.

2.1. Background information

2.1.1. Common methods for calcium ion detection

Calcium is a particularly important metal in the human body. The development stage of bone tissues requires a large quantity of calcium phosphate crystals ($Ca_{10}(OH)_2(PO_4)_6$) to reinforce its lattice structure.[37] In fact, 99% of total calcium content in the human body is cooperated into bone tissues. This 2.5 pounds of calcium is relatively stable in mass, while it is the body's small fraction of ionic calcium that is responsible for performing physiological functions. Ca^{2+} is one of the most abundant and commonly used second messengers in signal transduction pathways. The net movement of Ca^{2+} from one part of the cell to another would create a concentration gradient, allowing the body to respond to various stimuli accordingly.[38]

Currently, Ca^{2+} measurement and detection rely heavily on instrumental methods, such as atomic absorption spectroscopy, coupled plasma mass spectrometry and colorimetric detection.[39] [40] [41] While these methods can exhibit high sensitivity, selectivity and accuracy, they often require dedicated equipment as well as experienced technicians to operate. Moreover, sophisticated sample pretreatments can also result in long turn-over time, limiting their ability for on-site Ca^{2+} monitoring.

As a result, great strides have been made in developing various biosensors for Ca^{2+} detection. Unlike instrumental methods, biosensors can have short analysis time and high sensitivity at the same time. One of the most well-known biosensors for Ca^{2+} detection is Calcium Green, which is a hybrid molecule between BAPTA and a fluorophore. BAPTA or, 2-bis(o-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid, is an organic molecule that can bind to Ca^{2+} with strong affinity.[42] This is a versatile biosensor for *in vivo* studies because the fluorophore on Calcium Green can be further functionalized for specific applications. For instance, Tsien and coworkers designed a Ca^{2+} indicator molecule based on Calcium Green, called Calcium Green FlAsH (CaGF).[43] The fluorophore was modified with two additional arsenic substituents that can bind to a tetracysteine motif to detect the Ca^{2+} surrounding it.(Figure 2.1A). *In vitro* studies showed that CaGF could bind to Ca^{2+} with an apparent dissociation constant (K_d) of 100 μM.[43] However, one of the major drawbacks of this methodology is that the fluorophore used is very susceptible to heavy metal ions. For instance, the presence of 10 μM Cd^{2+} would cause the fluorescence signal to increase by 50%.[44]

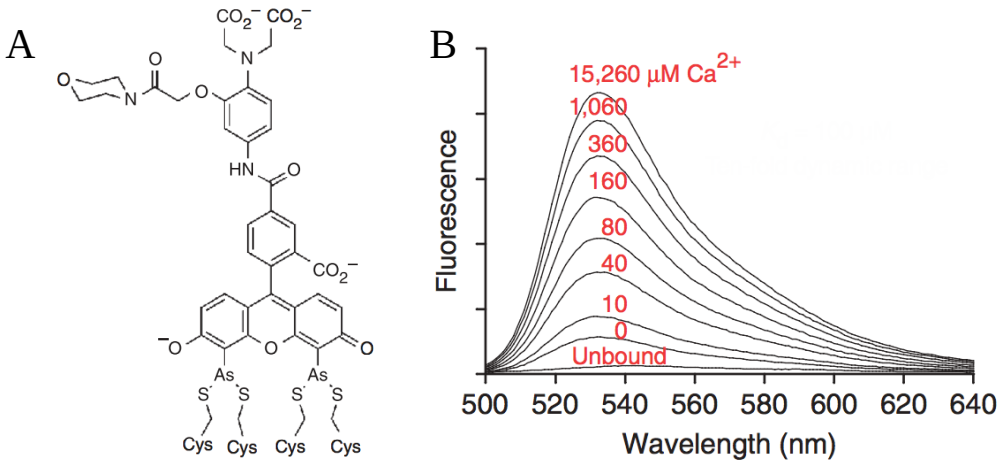

Figure 2.1 (A) The structure of Calcium Green FlAsH (CaGF) molecule. (B) The *in vitro* calibration against calcium ion. In B, fluorescence signal enhancements of the CaGF can be observed with various concentrations of Ca^{2+} labelled in red, with increasing signal intensity from 0 μM to 15,260 μM of Ca^{2+}. (Figure adapted with permission from Ref.[43])

Other than biosensors based on organic molecules, protein-based biosensors like cameleons are also very promising for Ca^{2+} detection. Developed by Miyawaki, cameleons is consisted of a molecule called calmodulin, a calmodulin binding peptide M13 and two green fluorescence proteins (GFPs). When Ca^{2+} is present, calmodulin would bind to it and wrap itself around the M13 binding domain. This would make the two GFPs at both ends moving closer to each other, thus increasing the Förster Resonance Energy Transfer (FRET) between them. Then, the local Ca^{2+} concentration can be determined by fluorescence imaging.[45] Despite having high selectivity, protein-based biosensors like cameleons have prolonged response time comparing to other biosensors due to its intrinsic mechanism, making them undesirable to monitor the real-time Ca^{2+} net movement.[46]

2.1.2. DNAzyme-based biosensors

In the last three decades, the function of DNA has significantly expanded. In particular, DNAzyme has emerged as a powerful platform for biosensor development.[47] They are less expensive and can be readily synthesized compared to proteins, making them versatile and convenient to use. Moreover, the high programmability that DNA molecule offers makes DNAzyme-based biosensor very easy to adapt to optical fibers and chip technology if desired.[48] Comparing to the previously reported methods, an EtNa DNAzyme-based biosensor could have an edge in Ca^{2+} detection owing to all the advantages that nucleic acids have, including excellent selectivity, high stability and versatility in signal generation.[49]

2.1.3. Nucleic acids in organic solvents

DNA molecules are soluble and can maintain their base pairing capabilities in many organic solvents up to a certain concentration. The properties of DNA in ethanol have been extensively studied. With increasing concentrations of ethanol, the DNA duplex is initially destabilized due to a decrease in melting temperature.[50] However, when the amount of ethanol present in the system is higher than the critical ethanol concentration, DNA molecules would start to aggregate. The critical ethanol concentration is related to the type and concentration of cations in the system. Previous studies also showed that the B-form duplex of the DNA molecule could only be maintained in up to around 70% ethanol. With higher ethanol concentrations, DNA will be dehydrated and converts to A-form.[51]

So far, most of the DNAzyme related work was conducted in aqueous phase with only a few reports available in organic solvents. Behera and coworkers performed an *in vitro* selection in 10% methanol and a RNA-ligating DNAzyme was obtained. [52] In addition, a peroxidase-mimicking G-quadruplex DNAzyme was modified with polyethylene glycol (PEG) and it retained

its activity in methanol. [53] Sen and Canale tested the activity of a similar DNAzyme in a series of water-miscible solvents and relatively high sensitivity was also observed. [54]

By introducing organic solvents into the reaction matrix, more types of analytes might be suitable for the DNAzyme-based biosensors. Besides, previous studies illustrated that organic solvent can increase the catalytic activity of the EtNa DNAzyme by increasing the electrostatic interaction between the DNAzyme and the metal ion.[33] Likewise, the sensitivity of the EtNa-based biosensor might be even higher in organic solvents than that in water due to the improved activity.

2.2. Results and discussion

2.2.1. The effect of ethanol on the EtNa DNAzyme with Ca^{2+}

As mentioned in Chapter 1, the EtNa DNAzyme is active with both Ca^{2+} and Na^{+} in water. Also, its activity with Na^{+} accelerated over 1000-fold in the presence of ethanol. To test the effect of ethanol on cleavage activity of the EtNa with Ca^{2+}, the substrate strand was labeled with a carboxyfluorescein (FAM) fluorophore and the cleavage results were analyzed by 15% denatured polyacrylamide gel electrophoresis (dPAGE). A gel micrograph of EtNa with increasing concentrations of Ca^{2+} in 40% ethanol is shown in Figure 2.1A. The bands on the top represent the uncleaved substrate strand, whereas the bottom bands indicate the cleavage product. The intensity of the bands is proportional to the number of nucleic acid molecules. After 1 h of incubation, an increase in the cleavage product was observed with only 5 μM Ca^{2+}. The experiments were repeated three times and the ratio of the cleavage products were quantified in Figure 2.2B (red trace). Initially, the cleavage activity increased significantly with higher concentrations of Ca^{2+} and peaked at around 500 μM. Further addition of Ca^{2+} slowed down the cleavage reaction and full inhibition was observed with 2 mM Ca^{2+}.

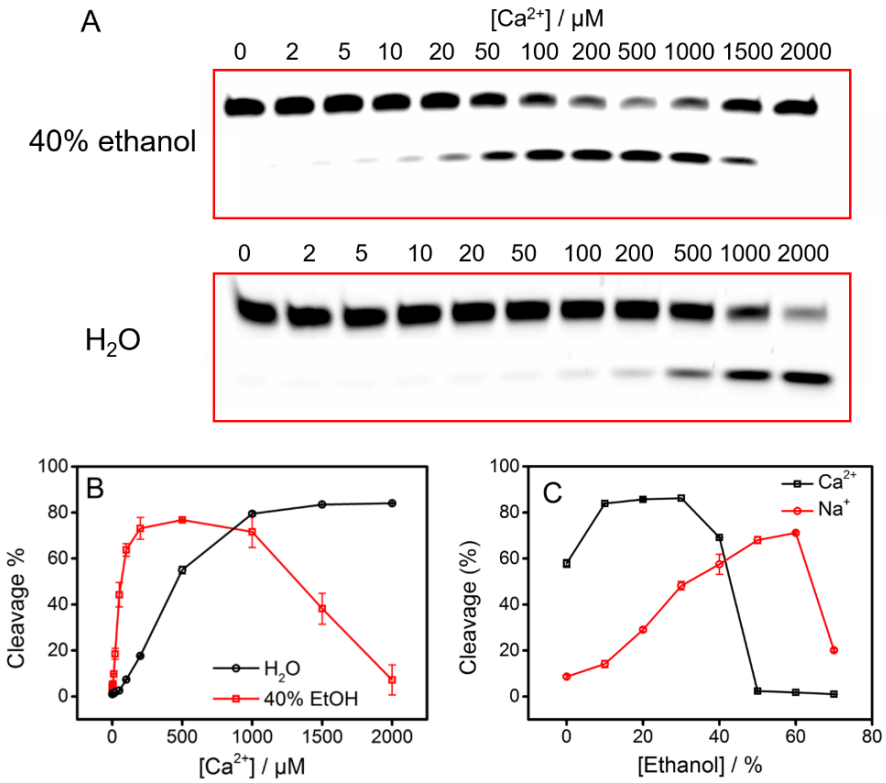

Figure 2.2 (A) Gel micrographs showing the cleavage products of the EtNa DNAzyme with increasing concentrations of Ca^{2+} in both 40% ethanol (top) and in water (bottom). (B) Quantification of the cleavage percentage in (A). (C) Cleavage yield of the EtNa DNAzyme in different concentrations of ethanol with Ca^{2+} (500 μM) or Na^{+} (50 mM).

The same experiment was then repeated in water. The quantification of the gel micrograph (Figure 2.2A, lower panel) is shown in Figure 2.2B (black trace). Comparing to 40% ethanol, the cleavage rate of the EtNa DNAzyme was much lower in water under the same Ca^{2+} concentration, and saturated activity was observed with 2mM Ca^{2+}. Therefore, ethanol has made EtNa much more sensitive to low concentrations of Ca^{2+}, which can be useful for improving Ca^{2+} detection. It can be argued that the interaction between Ca^{2+} and the DNAzyme is stronger in ethanol due to the

lower dielectric constant of organic solvents, which amplifies the electrostatic attraction between Ca^{2+} and negatively charged DNA.

The above experiments were carried out with 40% ethanol. Next, the EtNa DNAzyme activity was characterized in different concentrations of ethanol with 500 μM Ca^{2+} (Figure 2.2C, black trace). After 1 h of incubation, the cleavage yield increased gradually with ethanol concentration until 30%. However, the activity diminished quickly beyond 40% ethanol with full inhibition observed with 50% ethanol. Based on previous reports, the activity of EtNa with Na^{+} peaked at 60% ethanol (Figure 2.2C, red trace).[33] This difference is possibly caused by the higher charge density of Ca^{2+}, which allows more efficient binding to DNA. However, if the concentration of either ethanol or Ca^{2+} is too high, nonspecific condensation might occur, which would induce misfolds of the EtNa DNAzyme into inactive conformations. Such misfolding would require much higher concentrations of both ethanol and Na^{+} to take place.

2.2.2. The effect of ethanol on other RNA-cleaving DNAzymes

Because the EtNa DNAzyme was initially selected in the presence of isopropanol, it might have an inherent influence on the behavior of the DNAzyme, making it more active with ethanol. To test the generality of such enhanced activity, the activity of two other RNA-cleaving DNAzymes were also studied.

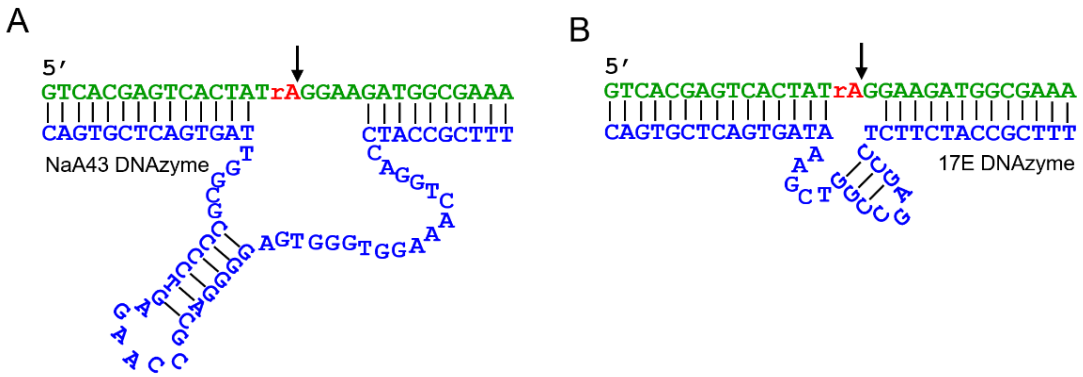

Figure 2.3 The secondary structure of (A) the NaA43 DNAzyme and (B) the 17E DNAzyme

The NaA43 DNAzyme (Figure 2.3A) is another example of a RNA-cleaving DNAzyme that uses Na^+ as the cofactor.[29] When incubated with 100 mM of Na^+, its activity increased slightly with 20% ethanol (Figure 2.4A, red trace) compared to that with water (Figure 2.4A, black trace), but 50% ethanol inhibited the activity by about three-fold (Figure 2.4A, blue trace). The enhancement observed was significantly lower compared to the EtNa. Then, the well studied 17E DNAzyme was tested because it can also be activated with Ca^{2+}. After 2 h of incubation, the enzymatic activity remains approximately the same in water and 20% ethanol. However, 50% ethanol fully inhibited the activity of the 17E DNAzyme (Figure 2.4B).

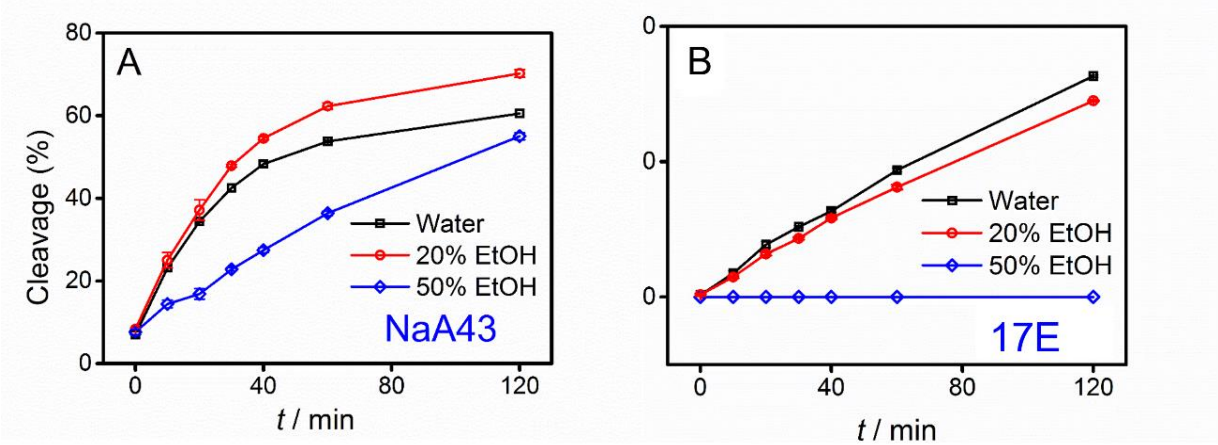

Figure 2.4 Cleavage activity of (A)the NaA43 and (B) the 17E DNAzymes in water, 20% ethanol and 50% ethanol. NaA43 was assayed in 50 mM MOPS pH 7.0 and 100 mM NaCl. The 17E DNAzyme was assayed in 50 mM MES pH 6.0 with 1 mM Ca^{2+}.

2.2.3. Characterization of the EtNa based biosensor for Ca^{2+} detection

Next, the behavior of the EtNa based biosensor for Ca^{2+} detection was characterized in the presence of organic solvents. Since the structure of the EtNa is quite similar to other reported RNA-cleaving DNAzymes, the catalytic beacon strategy was employed (Figure 2.5).[49 55] The 3′-end of the substrate strand was labeled with a carboxyfluorescein (FAM) tag. The 5′-end of the enzyme strand was shortened by 4 nucleotides and labeled with a dark quencher molecule. When the two strands were annealed together, the proximity of the dark quencher to the FAM tag quenched the fluorescence. In the presence of Ca^{2+}, the substrate strand was cleaved at the RNA site and the FAM labeled substrate fragment was dissociated from the complex. Over longer distances, the quenching effect was eliminated and fluorescence signal enhancement was observed.[56] This method has been routinely used for biosensor development in aqueous phase, but this is the first time that it has been used in organic phase.[57]

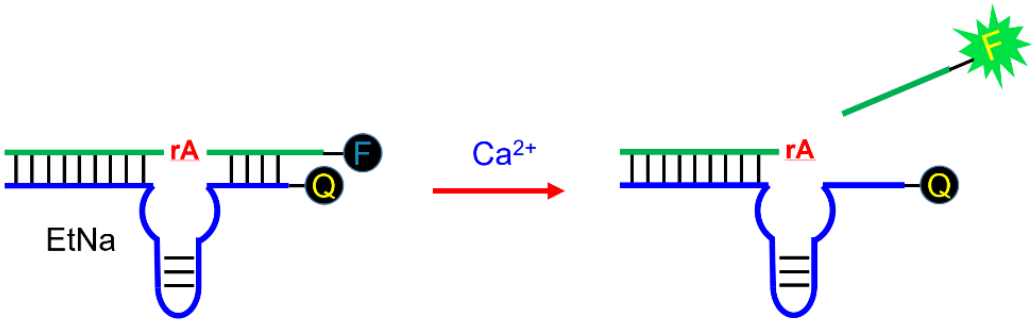

Figure 2.5 A scheme of the EtNa DNAzyme based biosensor. 3′-end of the substrate stand is labelled with a carboxyfluorescein molecule, along with the quencher labelled EtNa enzyme strand at its 5′-end will be used. When the enzyme strand annealing to the substrate strand, proximity of the two labels will quench the fluorescence. In the presence of Ca^{2+} as the cofactor, the substrate strand will be cleaved, and the FAM label will be dissociated from the complex, resulting in the enhanced fluorescence signal.

The detailed structure of the biosensor is shown in Figure 2.6 A. With the addition of 2 μM Ca^{2+}, an increase of the fluorescence signal was observed. The rate of the fluorescence enhancement was progressively faster with higher concentrations of Ca^{2+} and signal saturation was achieved in 20 min (Figure 2.6B). The initial rate of the fluorescence signal was plotted against the Ca^{2+} concentration (Figure 2.6C, black trace). The detection limit was calculated to be 1.4 μM Ca^{2+} based on the signal being greater than three times that of the background variation. Comparing to the behavior of the biosensor with Ca^{2+} in water (Figure 2.6C, red trace), the slope of the initial stage was 18-fold higher in ethanol, which indicates that the biosensor sensitivity in 30% ethanol is 18-fold higher than that it water. In the meantime, the selectivity of the biosensor was also tested. Apart from Ca^{2+}, only Pb^{2+} produced a high fluorescence signal. However, as shown in previous

works, the effect of Pb^{2+} can be easily masked by thiol ligands that does not bind Ca^{2+}.[34] Moreover, no signal was observed with Mg^{2+}, suggesting its excellent metal selectivity was retained in ethanol.

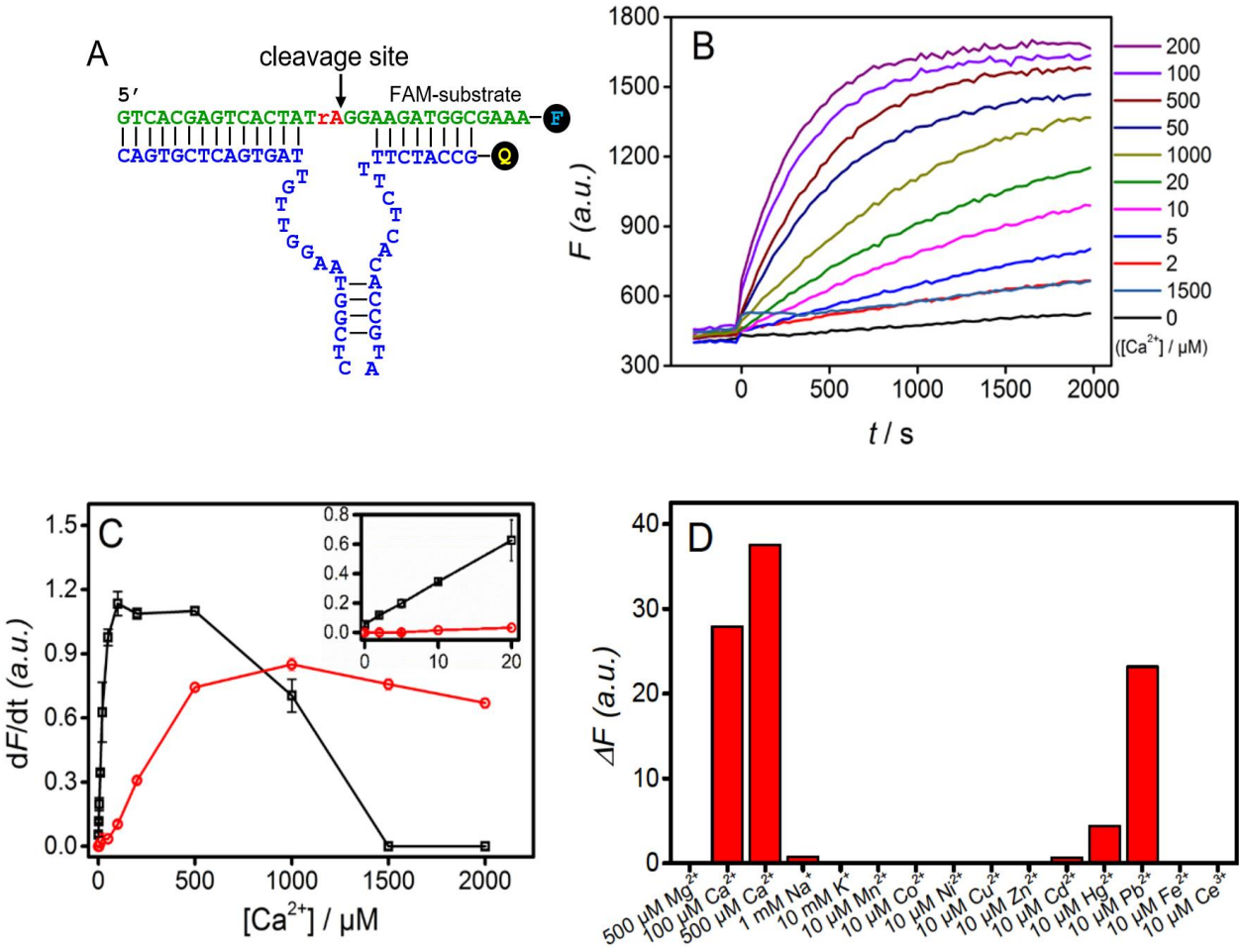

Figure 2.6 (A) The secondary structure of the EtNa DNAzyme-based biosensor with fluorophore and quencher labels. (B) Kinetic trace of the biosensor fluorescence signal with different concentrations of Ca^{2+} in the presence of 30% ethanol. (C) Quantification of the response of the initial rate of the fluorescence enhancement in water and 30% ethanol (from 1 to 6 min after adding Ca^{2+}) Red: in water. Black: in 30% ethanol Inset: the initial fluorescence response with 0 to 20 μM of Ca^{2+}. (D) The sensor response to other metal cations in 30% ethanol. Concentrations of metal ions used: 500 μM Mg^{2+}, 100 and 500 μM Ca^{2+}, 1 mM Na^{+}, 10 mM K^{+}, or 10 μM for the rest of the metal ions.

2.3. Materials and methods

2.3.1. Chemicals

In this work, the DNA samples were all purchased from Integrated DNA Technologies (Coralville, IA). Reagent-grade ethanol was obtained from Fisher Scientific. Magnesium chloride hexahydrate, calcium chloride dihydrate, sodium chloride, potassium chloride, manganese chloride tetrahydrate, cobalt chloride hexahydrate, nickel chloride hexahydrate, copper chloride, zinc chloride, cadmium chloride, mercury perchlorate, lead acetate, iron chloride hexahydrate and cerium trichoride were purchased from Sigma–Aldrich. Their solutions were made by directly dissolving their salts in water. 2-(*N*-morpholino)ethanesulfonic acid (MES), 3-(*N*-morpholino)propanesulfonic acid (MOPS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and ethylenediaminetetraacetic acid (EDTA) disodium salt dehydrate were obtained from Mandel Scientific (Guelph, Ontario, Canada). Urea, 40% acrylamide/bis-acrylamide solution (29:1), 10×TBE (Tris/Borate/EDTA) buffer, tetramethylethylenediamine (TEMED) and ammonia persulfate (APS) were acquired from Bio Basic Inc. (Markham, Ontario, Canada). The pH of the buffers was measured with Denver Instrument UltraBasic pH meter. Lithium hydroxide used for pH adjustment of the buffers were purchased from Alfa Aesar. All solutions, buffers, and gel stocks were prepared with Milli-Q water.

2.3.2. Gel-based activity assays

For the NaA43 DNAzyme, the DNAzyme complex was formed by annealing the FAM-labeled substrate strand (10 μM) and enzyme strand (20 μM) in 50 mM MOPS buffer (pH 7.0) with 90 mM LiCl. For EtNa, 17E and Ce13d, annealing was performed in 50 mM MES buffer (pH 6.0) with 25 mM LiCl. Annealing was performed by warming the mixture at 75°C for 4 min

followed by slow cooling to room temperature in the dark. After that, the annealed complex was stored at 4 °C for 20 min before use. For a typical gel-based activity assay, metal ions (1 μL) were added to an annealed DNAzyme complex (9 μL). After a designated incubation time, the mixture (3 μL) was transferred to urea (11 μL, 8 M) with bromophenol blue to quench the reaction, and the cleavage products were separated by 15% dPAGE. The gel images were documented with a Bio-Rad ChemiDoc MP imaging system.

2.3.3. Biosensor assays

The kinetic studies were carried out in a 96 well plates using a Molecular Device SpectraMax M3 microplate reader. The EtNa DNAzyme-based biosensor were annealed with FAM-labelled substrate (2.5 μM) and quencher-labelled enzyme strand (5 μM) in buffer A (50 mM MES buffer, pH 6, 25 mM LiCl) to form the biosensor complex. For each reaction, the biosensor (2 μL) was diluted in the sensing buffer (98 μL, 10 mM HEPES, pH 7.6, 20 mM LiCl). After 5 min background fluorescence reading (excitation wavelength, λ_{ex}=485 nm; emission wavelength, λ_{em}=525 nm), metal ions with various concentration (1 μL) were added, followed by monitoring the signaling kinetics.

Chapter 3. The EtNa DNAzyme with boosted activity by freezing

The results presented in this chapter have been published as:

Yu, T.; Zhou, W.; Liu, J., A RNA-cleaving Catalytic DNA Accelerated by Freezing. *ChemBioChem* **2018.** 10.1002/cbic.201800049

3.1. Background information

3.1.1. Acceleration of chemical reactions by freezing

Freezing is generally regarded as one of the oldest and most widely used methods of food preservation. It retains the quality of agricultural products over long storage periods, providing a significantly extended shelf life. Under lower temperatures, the migration of molecules is heavily suppressed, thus reducing the overall chemical reactions. However, some of the chemical reactions are known to be accelerated in partially frozen aqueous solutions. One of the earliest reports devoted to the subject was concerned with the preservation of food during frozen storage.[58] In the 1930s, it was reported that protease and esterase could remain active in artificial systems at low temperatures, even in the frozen state.[59] In 1961, it was found that facile imidazole catalyzing the cleavage of β-lactam in penicillin occurred only at temperatures between -5 °C and -30°C in frozen systems. Meanwhile, no cleavage product was observed in supercooled systems, suggesting the formation of ice crystals is essential for the reaction.[60] In the same year, Dr. Berends and his team reported that the photo-induced thymine dimerization reaction could be significantly promoted in a rapidly frozen system. However, the dimers would dissociate upon thawing and aqueous thymine solution is almost entirely immune to the same ultraviolet (UV) irradiation.[61] In the meantime,

some organic reactions are also proved to be accelerated in the unfrozen solution of ice, such as N-nitrosation of dimethylamine and nitrite.[62]

In more recent years, evidence showed that the gluconeogenic carbon bond forming reaction can take place in ice, which greatly contributes to how early metabolism produced the sugar-phosphate central of life.[63] In the field of environmental chemistry, scientists have found certain reactions that occurred in nature can be accelerated in frozen aqueous solutions. These reactions could potentially influence the chemical processing of a wide variety of inorganic components in polar regions.[64] In addition to chemical reactions, the concept of freezing has also been utilized in nanoscience and nanomaterials.[65] For example, directional freezing was proven to be a general and cost-effective method to functionalize gold nanoparticles of different sizes with various DNA molecules. Compare to previously reported methods, freezing does not require any additional reagents while a higher DNA density with high colloidal stability was achieved.[66]

3.1.2. Proposed mechanism of accelerated reaction by freezing

Based on previous findings, different acceleration mechanisms were purposed throughout the years. In 1969, Pincock concluded that freeze-concentration was the only mechanism that boosted the activity of the chemical reactions.[58] Freeze-concentration referred to when the reaction occurred in an unfrozen solution of ice where both solid and liquid coexist above the eutectic point of the water. The micropockets of liquid are spread evenly throughout the entire solid structures as micrometer-sized inclusions. With the formation of ice crystals, the solute can be concentrated in these micropockets. As the temperature is lowered, the micropockets can shrink in size, in turn further concentrating the solutes.[67] For electrochemical reactions, freezing can also introduce freezing potential, which is caused by an unbalanced amount of cation and anions incorporated into the ice lattice, generating an electric potential between the ice crystals and the micropockets.[68]

Meanwhile, in some freezing systems, the freezing potential is neutralized by highly mobile OH^- and H_3O^+, which would result in hydrolysis. Most of the reactions accelerated in the freezing process that has been reported so far can be explained by freezing concentration and hydrolysis.[69] However, based on the suggested mechanism, it is difficult to understand why acceleration of reactions in micropockets for most reactions is not observed.

3.1.3. Functional Nucleic acid and the effect of freezing

Compared to other chemical reactions, functional nucleic acid and their subsequent reactions were less explored in frozen systems until the 20th century. In 2002, Feig and his team reported the first direct evidence for cold denaturation of nucleic acid. However, no freezing occurred in the system since the reaction mixture had 40% methanol.[70] A few years later, Johnston and his team showed that freezing stimulates the self-ligation reaction of the hairpin ribozyme containing 2′,3′-cyclic phosphate and 5′-OH termini. Unlike other reported RNA ligation reactions[71], the hairpin ribozyme does not require any divalent metal ions as the cofactor. In contrast, no ligation occurs either at above 0 °C or in solutions that remain unfrozen when supercooled to subzero temperatures.[72] Holliger and coworkers established a series of papers on isolating ribozymes with RNA polymerase activity directly in ice. The experiment yielded RNA polymerase ribozymes that are specifically adapted to sub-zero temperatures and able to catalyze RNA polymerization reaction in ice at temperatures as low as -19 °C.[73] [74] [75]

3.2. Results and discussion

3.2.1. The effect of freezing on the EtNa DNAzyme with Na^+

To have an initial understanding of freezing on the catalytic activity of DNAzymes, the EtNa was chosen to be the first subject of study for multiple reasons. As mentioned in chapter 1,

the EtNa DNAzyme is highly specific for Na^+ among monovalent metals but it has almost no activity in water when the Na^+ concentration is below 100 mM.[33] In addition, chapter 2 showed that the activity of the EtNa could be significantly enhanced by adding an organic solvent, such as ethanol, attributable to stronger electrostatic interactions between the metal ions and DNA in solutions with a lower dielectric constant.[33] The EtNa also works with a divalent metal, Ca^{2+}, allowing systematic comparing the effect of metal ions.[34] Previous studies suggested that the role of Na^+ or Ca^{2+} was to interact with the scissile phosphate in EtNa to neutralize its negative charges during the transition state.[34 36]

The secondary structure of the EtNa DNAzyme is shown in Figure 3.1A. Aside from EtNa, three other DNAzymes was also included for comparison. The NaA43 DNAzyme (Figure 3.1B) is also specific for Na^+ and it can work with Na^+ alone as well,[76] but the role of Na^+ is to bind to the catalytic core in the enzyme strand.[29] With the same metal requirement but different mechanisms, NaA43 forms an excellent comparison with EtNa. The 17E DNAzyme (Figure 3.1C) works with most divalent metals,[25] while the Tm7 (Figure 3.1D) requires a trivalent lanthanide such as Tm^{3+} for activity.[27] With these DNAzymes, the effect of metal valency upon freezing can be systematically determined.

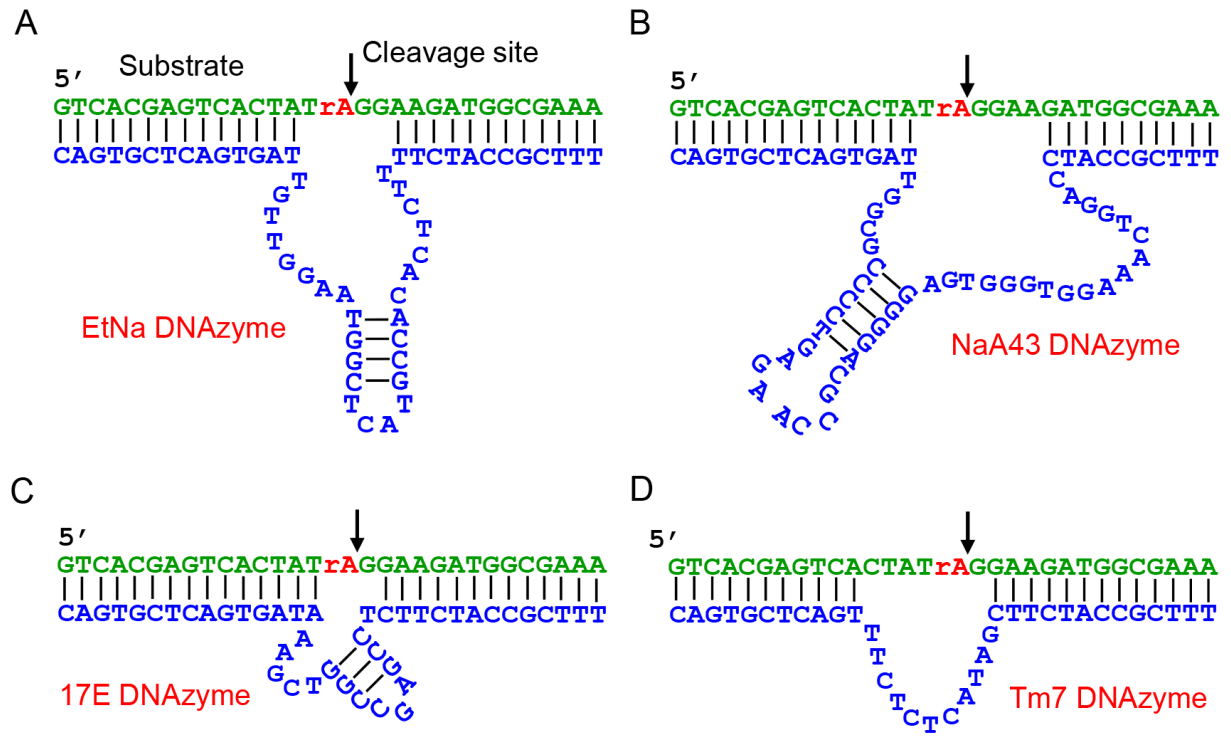

Figure 3.1 The secondary structures of the (A) EtNa, (B) NaA43, (C) 17E, and (D) Tm7 DNAzymes. They all share the same substrate sequence. The EtNa and NaA43 are active with Na^+ alone, while EtNa also works with Ca^{2+}. Tm7 cleaves with a lanthanide like Tm^{3+}, while the 17E DNAzyme requires a divalent metal ion such as Ca^{2+} or Pb^{2+} for activity.

Like chapter 2, the 3′ end of the substrate strand was labeled with a carboxyfluorescein tag (FAM) so that the cleavage product can be quantified by gel electrophoresis. To limit the background cleavage of the EtNa DNAzyme with Na^+ during freezing, the experiments were performed in MES buffer with ionic strength adjusted with LiCl. K- EDTA (ethylenediaminetetraacetic acid dipotassium salt) was also present to eliminate any divalent metal ions. Indeed, very low activity was observed with overnight incubation at -20 °C without any Na^+ (Figure 3.2 A, first lane). By introducing Na^+ into the system, more cleavage product was observed with higher Na^+ concentration, from as low as 1 mM (Figure 3.2 A, second lane). After quantifying

the cleavage yield, saturation can be seen with around 20 mM Na^+ and the K_d was calculated to be about 6.2 mM Na^+ (Figure 3.2B, blue trace).

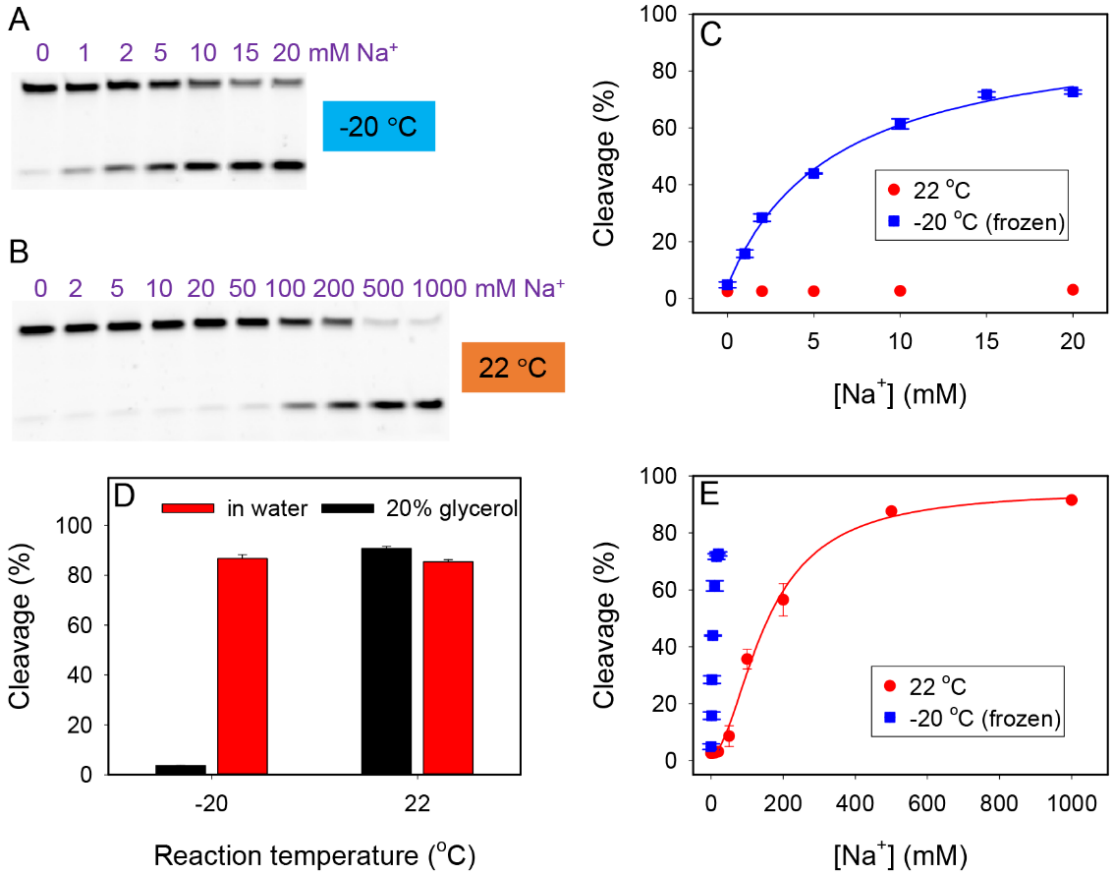

Figure 3.2 Gel micrograph showing the cleavage product of the EtNa DNAzyme in the presence of different Na^+ concentration under overnight incubation in (A) -20 °C and (B) 22°C or room temperature. Quantification of the cleavage percentage from gel micrographs with (C) a small range and (E) a wide range of Na^+ concentration. (D) Comparison of the activity of the EtNa DNAzyme at room temperature with 500 mM Na^+ in water or in 20% glycerol, or at -20 °C with 20 mM Na^+ in water or in 20% glycerol.

Similar experiments were also performed at room temperature for comparison. No cleavage activity was observed even with 50 mM Na^+ after overnight incubation (Figure 3.2B).

The quantification of the cleavage product at room temperature can be found in Figure 3.2E (red trace), and the dissociation constant was estimated to be 153 mM Na^+, which is 24-fold lower than that at -20 °C freezing conditions. This finding strongly suggested that freezing can boost the activity of the EtNa DNAzyme significantly by increasing the binding affinity between the DNAzyme complex and Na^+ ion. Based on the previous findings, it can be hypothesized that the freeze-concentration effect is responsible for the increase in catalytic activity of the EtNa DNAzyme. The local Na^+ concentration in micropockets was significantly increased where the negatively charged DNAzyme was present, resulting in the improved K_d.

During the freezing process, the DNAzyme complex experiences two important physical changes: decrease in the reaction temperature and the formation of ice crystals. To exam the two changes separately, 20% glycerol was introduced to the reaction. It acts as an anti-freezing agent so that the same reaction temperature can be maintained while preventing the formation of any ice crystals.[77] With 20 mM Na^+, the DNAzyme sample did not freeze and no cleavage was observed after overnight incubation at -20 °C (Figure 3.2D). However, with 500 mM Na^+ and at room temperature, the cleavage yield was similar with 20% glycerol or in water. Therefore, the glycerol does not affect the RNA cleavage reaction of the EtNa DNAzyme. The acceleration of the cleavage reaction by freezing was not driven by the low temperature, and the formation of ice crystals is required.

3.2.2. Reaction kinetic studies

In the interest of further exploring the mechanism behind, a series of kinetic experiments were conducted to find out when the cleavage reaction occurred during the overnight incubation. The samples were individually prepared before the addition of 20 mM Na^+, followed by incubating at -20 °C. At the designated time point, the sample was thawed immediately, followed by

quenching the reaction with concentrated urea. In the first hour of incubation, the samples started to freeze. Results from gel electrophoresis revealed that minimal amount of cleavage was produced during this time (Figure 3.3A, second and third lane). After the samples were frozen, the yield of the cleavage product increases with time (Figure 3.2 A, rest of the lanes). To confirm this, another set of samples were prepared. Rather than cooling down from room temperature to -20 °C, the samples were placed into liquid nitrogen initially to speed up the formation of ice crystals. After two minutes of incubation, all samples appeared to be frozen. Then, the samples were incubated at -20 °C like the previous experiments. In this case, cleavage also took place in a time-dependent manner (Figure 3.3B). Therefore, the RNA cleavage reaction of the EtNa DNAzyme did not happen during the freezing process, but rather after the ice crystals were already formed.

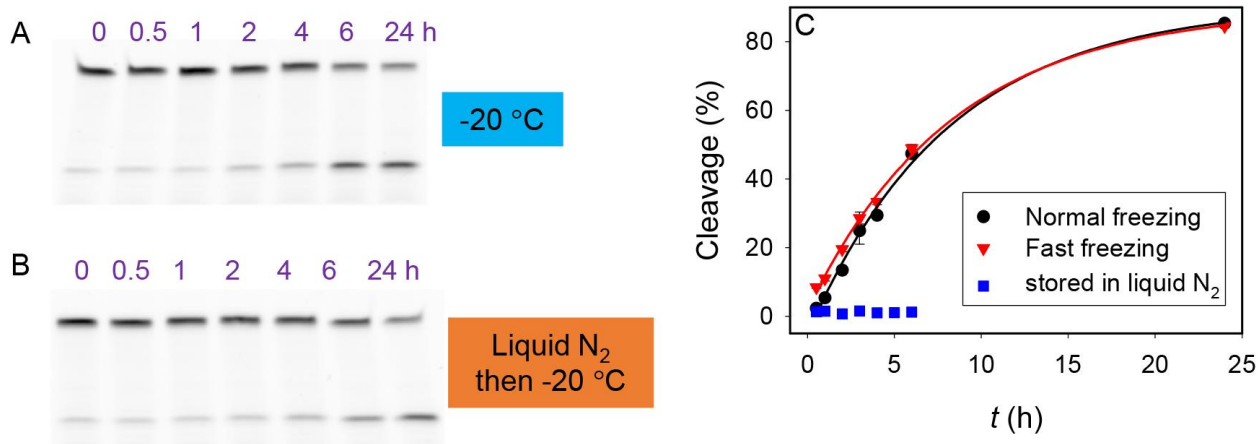

Figure 3.3 (A) Gel micrographs showing the cleavage product of the EtNa DNAzyme with 20 mM Na^+ after certain incubation time where (A) the sample was cooled down from room temperature to -20 °C and (B) the sample was frozen in liquid nitrogen first, then transferred to -20 °C. (C) Quantification of the time-dependent cleavage of the EtNa DNAzyme under three different freezing conditions with 20 mM Na^+.

The quantification of the gel micrographs can be found in Figure 3.3C. The cleavage results agreed with the previous findings with around 50% cleavage in 6 h and over 80% after 24 h incubation at -20 °C. By fitting the cleavage percentage to a first-order kinetic equation, the cleavage rate was calculated to be 0.12 h^{-1}. In addition, similar results were obtained from freezing the samples rapidly with liquid nitrogen, suggesting that liquid nitrogen did not influence the activity of the EtNa DNAzyme by fast freezing the samples. However, keeping the samples in liquid nitrogen for more extended period of times fully inhibit the catalytic activity of the EtNa, since no cleavage was observed after 6 h of incubation (Figure 3.3C, black trace). This is most likely due to the loss of eutectic phase when exposing to extremely low temperature, forfeiting the freeze-concentration effect.

3.2.3. Systematic comparison of the effect of metal ions

In aqueous phase, the EtNa DNAzyme has exceptionally high specificity towards Na^+ over other monovalent metal ions. Upon testing under freezing conditions, the EtNa still showed the most cleavage activity towards Na^+ (Figure 3.4A). For divalent metal ions, EtNa has high selectivity for Ca^{2+} over Mg^{2+} at room temperature. Although the specificity was primarily retained under -20 °C environment, the activity of the EtNa with Ca^{2+} decreased to only about 30% cleavage after overnight incubation (Figure 3.4B). The result implied that the valency of the metal ion might play an essential role during the DNAzyme catalysis in the freezing system. As a result, the effect of Ca^{2+} was systematically studied.

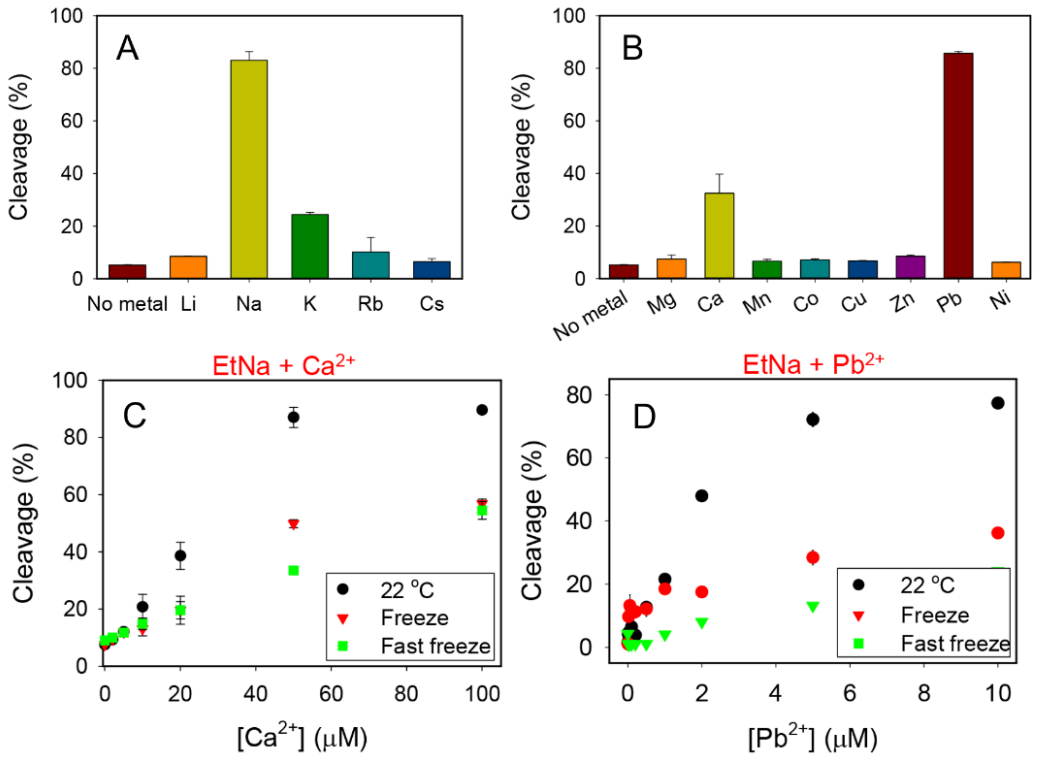

Figure 3.4 The cleavage percentage of the EtNa with various (A) monovalent (10 mM) or (B) divalent (50 μM for Mg^{2+} and Ca^{2+}, 10 μM for the rest) metal ions after overnight -20 °C incubation. Comparison between the effect of freezing on the activity of the EtNa with various concentration of (C) Ca^{2+} and (D) Pb^{2+}.

In the metal selectivity study, 50 μM $Ca^{2+}$was used and it appeared to inhibit the activity of the EtNa after freezing. From the result of Chapter 2, it can be learned that a high concentration of Ca^{2+} can have an adverse effect on the activity of the EtNa in ethanol. It is possible that similar effect could take place under freezing conditions. Therefore, the cleavage yield of the EtNa with 0 to 100 μM Ca^{2+} was measured under three different conditions (Figure 3.4C). In all the concentrations, however, cleavage was faster at room temperature than the frozen samples (comparing the black and red dots). Liquid nitrogen was also used to avoid any cleavage during

the slow freezing process, where the cleavage of the EtNa was even lower (Figure 3.4C, green dots). Therefore, Ca^{2+}-dependent cleavage of the EtNa was not accelerated by freezing. Instead, freezing inhibited the reaction. It might be that divalent Ca^{2+} already interacts with the DNAzyme at an optimal strength at room temperature, and further freezing has made the reaction less optimal.

Moreover, among other divalent metal ions used for the selectivity study, Pb^{2+} displayed the highest activity with over 80% cleavage. With a low pK_a value of Pb^{2+} bound water, Pb^{2+} is active with most RNA-cleaving DNAzymes. In previously conducted activity assays of the EtNa DNAzyme, Pb^{2+} was also appeared to be active.[33] When testing the effect of freezing on Pb^{2+}, similar results were obtained, where the activity of the EtNa was inhibited for all Pb^{2+} concentrations (Figure 3.4D). In addition, the activity of Pb^{2+} can be easily masked by thiol ligands such as mercaptoethanol (Figure 3.5).

Overall, freezing only promoted the activity of the EtNa DNAzyme with Na^+. This might be related to its selection condition. [33] Combined with the observations from Chapter 2, it is plausible that both ethanol and freezing can help concentrating Na^+ ions near the EtNa, thus increasing the sensitivity of the DNAzyme.

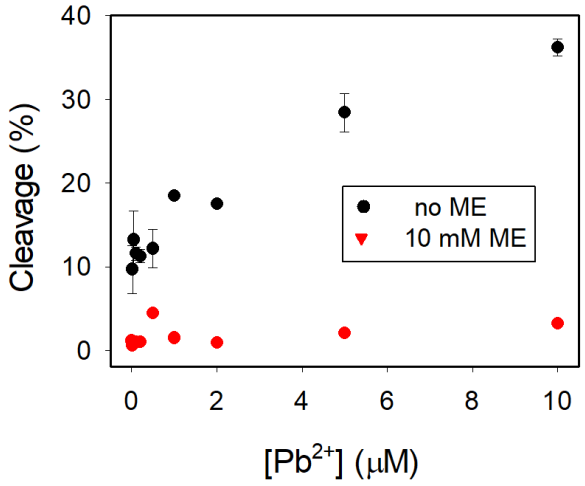

Figure 3.5 Cleavage yield of the EtNa DNAzyme in the presence of Pb^{2+} without or with 10 mM mercaptoethanol after freezing at -20 °C for 6 h.

3.2.4. The effect of freezing on other RNA-cleaving DNAzymes

To evaluate whether the effect of freezing is unique to the EtNa DNAzyme, a few other RNA-cleaving DNAzyme were also tested. The NaA43 is a Na^+-specific DNAzyme with high selectivity and it can cleave the substrate with Na^+ alone.[29] In this regard, it is very similar to EtNa. The NaA43 DNAzyme is a much more efficient DNAzyme in water in the presence of Na^+. Therefore, lower concentrations of Na^+ were used for this experiment (up to 1 mM). Although the frozen samples cleaved more than those at room temperature at each Na^+ concentration (Figure 3.6A), the promotion effect of freezing was not as large as that for the EtNa (for example, Figure 3.2C). The Na^+ specificity of the NaA43 DNAzyme is from the interaction between Na^+ and the sequence in the catalytic core,[29, 36] while Na^+ interacts with the scissile phosphate in the EtNa.[36] It is likely that the electrostatic nature of Na^+ binding to the NaA43 catalytic loop is less critical and thus freezing has a less effect on it.

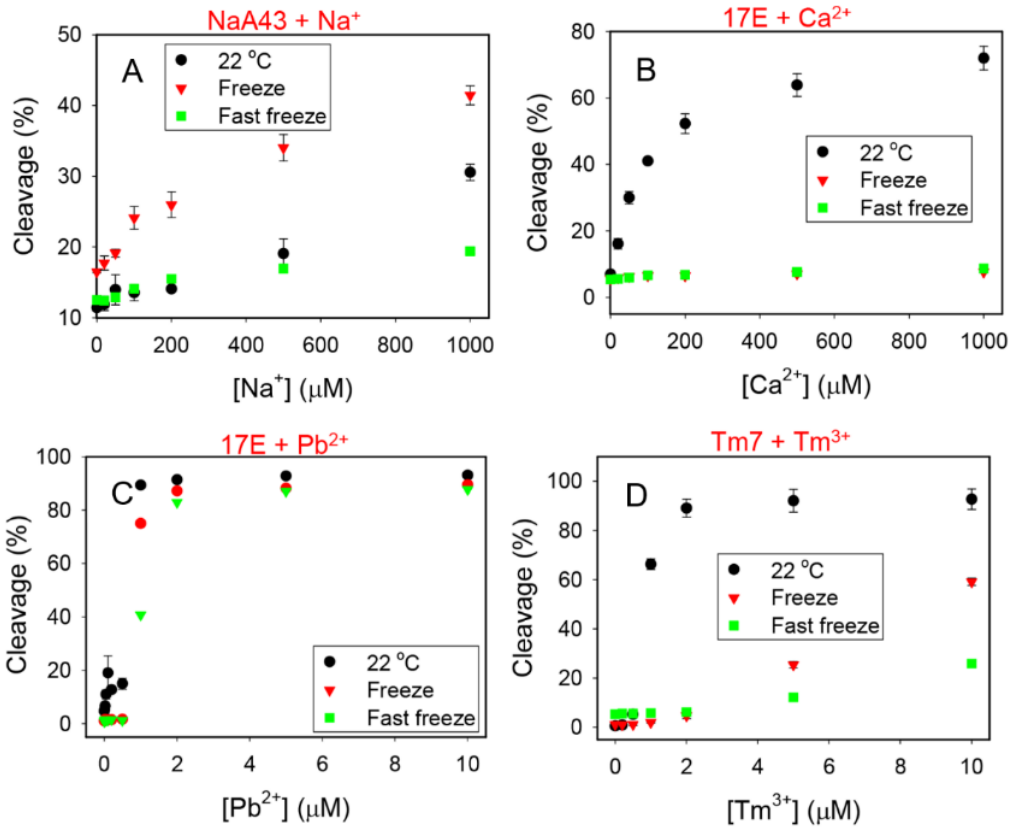

Figure 3.6 The effect of freezing on different DNAzymes and metal ions: (A) NaA43 with Na^+; (B) 17E with Ca^{2+}; (C) 17E with Pb^{2+}; and (D) Tm7 with Tm^{3+}. Each of the experiments were performed with 10 μM of the DNAzyme complex in 20 mM MES pH 6.0. For NaA43 and Tm7, the reactions were allowed for 24 h whereas for 17E, the reactions were quenched after 6 h.

After studying the two Na^+-dependent DNAzymes, the effect of freezing was also characterized for polyvalent metal-dependent DNAzymes. The 17E DNAzyme is active with many divalent metal ions, such as Ca^{2+} and Pb^{2+}.[78] [26] [79] Based on previous biochemical assays, the interaction of Ca^{2+} with the scissile phosphate group is likely to be significant.[80] In this case, however, the activity was fully inhibited by freezing at all tested Ca^{2+} concentrations (Figure 3.6B). On the other hand, the 17E DNAzyme was quite active in the presence of Pb^{2+} after freezing, although the activity at room temperature was still faster (Figure 3.6C).

Finally, the Tm7 DNAzyme was tested, which uses a trivalent lanthanide for activity.[27] In this case, freezing also inhibited the activity (Figure 3.6D). Based on the above results, the acceleration effect of freezing was only observed with monovalent Na^+. Metal ions with higher valency all inhibited the DNAzymes.

3.3. Materials and methods

3.3.1. Chemicals

In this work, the DNA samples were all purchased from Integrated DNA Technologies (Coralville, IA). Magnesium chloride hexahydrate, calcium chloride dihydrate, sodium chloride, potassium chloride, manganese chloride tetrahydrate, cobalt chloride hexahydrate, nickel chloride hexahydrate, copper chloride, zinc chloride, lead acetate, rubidium chloride, cesium chloride and mercaptoethanol were purchased from Sigma–Aldrich. Reagent grade glycerol was purchased from Fisher Scientific. 2-(*N*-morpholino)ethanesulfonic acid (MES), 3-(*N*-morpholino)propanesulfonic acid (MOPS) and ethylenediaminetetraacetic acid dispotassium salt (K-EDTA) dihydrate were obtained from Mandel Scientific (Guelph, Ontario, Canada). Urea, 40% acrylamide/bis-acrylamide solution (29:1), 10×TBE (Tris/Borate/EDTA) buffer, tetramethylethylenediamine (TEMED) and ammonia persulfate (APS) were acquired from Bio Basic Inc. (Markham, Ontario, Canada). The pH of the buffers was measured with Denver Instrument UltraBasic pH meter. Lithium hydroxide used for pH adjustment of the buffers were purchased from Alfa Aesar. All solutions, buffers, and gel stocks were prepared with Milli-Q water.

3.3.2. Gel-based activity assays and freezing assays

For the NaA43 DNAzyme, the DNAzyme complex was formed by annealing the FAM (carboxyfluorescein)-labeled substrate strand (10 μM) and the non-labeled enzyme strand (20 μM)

in 50 mM MOPS buffer (pH 7.0) with 25 mM LiCl. For EtNa, 17E and Tm7 DNAzyme, annealing was performed in 50 mM MES buffer (pH 6.0) with 25 mM LiCl. Annealing was performed by warming the mixture to 75 °C for 4 min followed by slow cooling to room temperature in the dark. Then, the samples were stored in 4 °C environment for 20 min. For a typical gel-based activity assay, 10 μL of the reaction mixture was prepared in the presence of 1 μM of the annealed DNAzyme complex, 20 mM MES buffer (pH 6.0) and metal ion of desired concentration. 1 mM of potassium salt of EDTA was also added to the mixture if monovalent metals ions were used. For a typical freezing experiment, the samples were placed in a -20 °C freezer and the samples appeared to be frozen after 1 h of incubation. For fast freezing experiments, the samples were placed in liquid nitrogen for two min and then immediately transferred to the -20 °C freezer. After the designated incubation time, the samples were thawed right after. 3 μL of the mixture was transferred to 11 μL urea (8 M) with bromophenol blue to quench the reaction, and the cleavage products were separated by a 15% denaturing polyacrylamide gel electrophoresis (dPAGE) experiment. The gel images were documented and quantified with a Bio-Rad ChemiDoc MP imaging system.

Chapter 4. Conclusion and future works

In summary, the activity of the EtNa DNAzyme in ethanol as a function of Ca^{2+} was measured. In addition, its catalytic activity with Ca^{2+} in ethanol were also compared to that in water and Na^{+} in ethanol. In general, the activity was significantly increased at low metal concentrations in ethanol for both Ca^{2+} and Na^{+}. At higher metal concentrations, Ca^{2+} could fully inhibit the cleavage activity of the EtNa with just 2 mM Ca^{2+}. However, the boosted activity in ethanol was a unique property of the EtNa DNAzyme, as no drastically increase in cleavage activity with ethanol was observed of all other RNA-cleaving DNAzymes tested. This work has also demonstrated the unique role of ethanol in improving the sensitivity of the EtNa DNAzyme-based biosensor for Ca^{2+} detection. Compared to in water, the biosensor in 30% ethanol was 18-fold more sensitive to Ca^{2+}.

In the meantime, the catalytic activity of RNA-cleaving DNAzymes in the frozen state was studied. The EtNa DNAzyme was significantly accelerated by freezing at -20 °C in the presence of low concentrations of Na^{+}, and the apparent Na^{+} binding affinity was over 20-fold tighter than that at room temperature. The same DNAzyme was however inhibited by freezing in the presence of Ca^{2+}, suggesting the importance of the valency of metal ions. Most other tested DNAzymes were inhibited by freezing, especially with polyvalent metals. The uniqueness of the EtNa might be attributed to its selection condition, where the selection library was incubated at -20 °C with a high concentration of Na^{+} and isopropanol, providing stronger electrostatic interaction between the metal ion and the DNAzyme. However, the effect of freezing was only examined under three temperatures: room temperature (22 °C), -20 °C and in liquid nitrogen (-196°C). It could be plausible that further decrease in temperature will also reduce the size of the micropockets between

ice crystals, therefore further concentrating the solutes. By maintaining the same Ca^{2+} concentration, higher activity of the EtNa DNAzyme might be observed between 0 °C and -20°C or between -20 °C and -196°C after overnight incubation.

Taken together, the addition of ethanol, using divalent Ca^{2+} instead of Na^{+}, and freezing overnight all achieved higher binding affinities between the metal ion and the DNAzyme by increasing the electrostatic interaction between the DNAzyme and the metal cofactor, in turn promoting the RNA cleavage activity of the EtNa DNAzyme. As a result, it might be possible to intentionally perform *in vitro* selections for new DNAzymes with higher activity sensitivity specifically with organic solvents or freezing conditions as part of the protocol to increase the electrostatic interaction between the DNAzyme and the meta ion.

Chapter 5. Lab safety

General laboratory safety guidelines should always be followed when conducting experiments, including the use of gloves, goggles and lab coats when necessary. Material safety data sheets should be in place and ready to be referred to routinely. Care will be taken to prevent exposure of corrosive materials to skin and eyes, including concentrated acids and bases. All flammable material will be handled in well ventilated areas in the absence of ignition sources. All hazardous chemicals will be handled with great care. All chemicals will be disposed of via their appropriate waste streams and first neutralized if appropriate. The UV light sources used to detect fluorescence can cause damage to both the skin and eyes. Direct exposure will be limited or avoided, and protective eye wear will be used when appropriate. When working with electrophoresis equipment, precautionary measures should be made as they pose potential electrical, chemical and physical safety hazards. Main power supply should be switched off before connecting or disconnecting electrical leads with dry gloved hands

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