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Excimer-Monomer Switching Molecular Beacon: The Study on Synthetic Cryptosporidium DNA Detection, Thermodynamics, and Magnesium Effects

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EXCIMER-MONOMER SWITCHING MOLECULAR BEACON: THE STUDY ON SYNTHETIC
CRYPTOSPORIDIUM DNA DETECTION, THERMODYNAMICS, AND MAGNESIUM EFFECTS

By

Michael L. Davis

A thesis submitted in partial fulfillment
of the requirements for the degree
of
MASTER OF SCIENCE
in
Biological Engineering

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2014
ABSTRACT

Excimer-Monomer Switching Molecular Beacon: The Study on Synthetic Cryptosporidium DNA Detection, Thermodynamics, and Magnesium Effects

By

Michael L. Davis, Master of Science
Utah State University, 2014

Major Professor: Dr. Anhong Zhou
Department: Biological Engineering

Cryptosporidium parvum is a deadly waterborne protozoan parasite that invades the gastrointestinal tract of humans and causes severe to life-threatening gastroenteric disease. Due to the ubiquitous nature of Cryptosporidium parvum in the world's water, it is necessary to determine the source of an outbreak. Rapid detection and identification of various genotypes of Cryptosporidium are a valuable goal in determining the source of the pathogen in a human epidemic. Exploitation of gene sequences specific to species is a powerful tool detecting pathogens.

Molecular beacons are one of these tools for high selectivity and specificity detection of DNA and RNA. Molecular beacon is a single strand of DNA that forms a stem and loop structure, where the stem holds the DNA together and the loop detects a target sequence. This molecular beacon detection is determined by the changes of fluorescent emissions of fluorescent dye linked to the ends of the stem. In this thesis work, a new and novel molecular beacon was designed to detect the specific sequences from the heat shock protein gene of Cryptosporidium parvum that infects humans. This probe is synthesized by the conjugation of pyrene molecules to both ends of the stem which leads to a unique feature of pyrene excimer-monomer switching molecular beacon upon the hybridization of the loop sequence with the target DNA sequence.
This thesis systematically investigates the physical binding (e.g., quantum yield) and thermodynamic properties, including enthalpy, entropy, and free energy of this excimer-monomer switching molecular beacon in the presences of complimentary, mismatched, and damaged DNA, respectively, in the three phases: phase one is the molecular beacon in the stem and loop structure, phase two is the molecular beacon hybridized to its target DNA, and phase three is the molecular beacon in a random coil. The effect of magnesium concentration on the binding and thermodynamic properties was also investigated. Finally, as a comparison, a conventional fluorescence resonance energy transfer-based molecular beacon with a fluorophore at the 5’ end and quencher at the 3’ end was used to assess selectivity and sensitivity in detection of DNA-DNA hybridization.

(117 pages)
PUBLIC ABSTRACT

Excimer-Monomer Switching Molecular Beacon: The Study on Synthetic Cryptosporidium DNA Detection, Thermodynamics, and Magnesium Effects

Michael Davis

The purpose of this study is to quantify and determine the binding of a molecular DNA probe or molecular beacon used in the detection of a known waterborne pathogen Cryptosporidium parvum. The objectives of this study are to determine the stability, sensitivity, and selectivity of the molecular beacon in detecting synthesized Cryptosporidium DNA sequences. The designed probe is based on a two-fluorophore system that is different than similar molecular beacons that utilize a fluorophore and quencher detection method. A comparative study between the two probes was also performed to demonstrate enhanced selectivity of the fluorophore based molecular beacon. Magnesium ions are known to affect the stability of DNA, and investigation was also performed to determine the effects on the stability by a thermodynamic analysis of the fluorophore based molecular beacon.

C. parvum is a waterborne pathogen that affects public water supplies and water recreational facilities. To prevent outbreaks or for identification of an unknown pathogen DNA biosensors are being developed. The fluorophore based molecular beacon is a DNA biosensor that can allow for the needed selectivity and sensitivity in DNA detection in the identification and detection of a pathogen in a water supply. This research is aimed at further quantifying the capabilities of a fluorophore based molecular beacon.

The proposed work was funded by the Utah water initiative and the Utah Water Research Laboratory.
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Michael L. Davis
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A.21: A) The effect of Mg$^{2+}$ concentration on EMS-MB emissions at 85 °C, the left arrow indicates general trend of monomer emission and the right arrow indicates general trend of excimer emission due to Mg$^{2+}$ increase, and B) is the effect of Mg$^{2+}$ concentration on monomer and excimer emissions at 85 °C.
LIST OF ABBREVIATIONS

MB = Molecular Beacon
EMS-MB = Excimer-monomer switching molecular beacon
QF-MB = Quencher free molecular beacon
FAM = Fluorescein
TMR = tetramethyl rhodamine
MB2 = FAM and TMR based molecular beacon
T0 = Complimentary target sequence
T1 = One base mismatch target sequence
T5 = Five base mismatch target sequence
oxo-G = Oxidatively damaged target sequence
40ssDNA = 40 base single stranded DNA target sequence
40dsDNA = 40 base pair double stranded DNA target sequence
ctDNA = thymus genomic DNA
dsEMS-MB = Double stranded DNA with same sequence as EMS-MB and T0 duplex
FL= Fluorescence
INTRODUCTION AND LITERATURE REVIEW

Introduction and Background

_Cryptosporidium parvum_ is a deadly waterborne protozoan parasite that invades the gastrointestinal tract of humans and causes severe to life-threatening gastroenteric and diarrheal disease (1,2). Due to the ubiquitous nature of _C. parvum_ in the world’s water, multiple outbreaks occur every year. When an outbreak occurs it is necessary to determine the source in order to prevent further spread and infection of the parasite (3). Unfortunately, there is no effective antimicrobial treatment to eliminate this pathogen. Rapid detection and identification of various genotypes of _Cryptosporidium_ could be a valuable goal in determining the source of the pathogen in a human epidemic. The genomes for various species of _C. parvum_ have been sequenced. In particular, the nucleotide sequence of heat shock protein (_hsp_) 70 genes from various species of _C. parvum_ has been identified. These sequences are found with accession numbers AF221528 to AF221543 in the GenBank database (4). Exploitation of gene sequences specific to species is a powerful tool in detection of this kind of pathogens.

Molecular beacons (MB) provide a powerful tool in detection of DNA and RNA, and are useful in detecting single nucleotide polymorphisms (SNP) in DNA sequences (5). In this study, a new and novel molecular beacon was designed to detect the _hsp_ gene of _C. parvum_ that infects humans. This probe is a light switching probe or excimer monomer switching MB (EMS-MB) by conjugation of two pyrene molecules on the 5’ and 3’ ends of MB. Hybridization of DNA causes the excimer and monomer emission intensities to switch, which makes possibility of improvement of specificity and selectivity in detecting the DNA-DNA hybridization.

Magnesium cations have been shown to affect the stability of DNA and are integral in nucleic acid folding processes (6). These ions have also been shown to have a dominating effect over other ions in DNA stabilization (7). For this reason, it is proposed to study the effects of varying concentrations of magnesium ions on the stability of the EMS-MB. Stabilizing effects of
the magnesium ions on the EMS-MB will be determined by its thermodynamic properties, emission spectra, and melting profiles. The thermodynamic properties of the stem will be calculated from fluorescent denaturation profiles of the molecular beacon for each of the magnesium ion concentrations.

Literature Review

Cryptosporidium and Current Detection Methods

*Cryptosporidium* (crypto) is an infectious enteric waterborne pathogen. Infection by crypto is known as cryptosporidiosis and can last up to 2 weeks or longer in people who are immunocompromised. It is estimated that crypto has a 50% infection rate in humans. Infections are due to ingestion of oocysts and very few oocysts can lead to infection. Infection is caused when the oocyst in the small intestine release four sporozoites. The sporozoites then attach to the epithelium. Merozoites are then developed from the sporozoites and can reproduce via production of oocysts. Thin wall oocysts are generated and continue the infection in the host. Thick walled oocysts are excreted and can lead to outbreaks in public water supplies. Outbreaks can occur due to improper filtration and flocculation of waste treatment water as was the case in an outbreak in Milwaukee in 1993 (8). Crypto oocysts are smaller than other pathogen oocysts such as *Giardia* increasing the difficulty in effectively removing crypto oocysts (9). The oocysts have also been shown to survive for months and to be extremely resilient to various chemical treatments. For most chemical treatments to be successful the concentration and exposure time is increased. This increase in concentration and exposure time often is different than what is suggested by manufactures of disinfectants. An example is chlorine, which is used in water treatment systems, where the higher concentrations of chlorine needed to kill the oocyst isn’t recommended (2).

In the event of an outbreak it is important to quickly detect the culprit pathogen (9). Various crypto detection methods exist. The first method is to obtain samples and screen for oocysts via
staining processes utilizing acid fast stains. These kits though can lead to false positives due to the low specificity in this detection method, due to the potential of multiple species being stained (10). Some other kits utilize direct fluorescent antibody assay and enzyme immunoassay kits (11) . These kits are also only valuable to a clinical diagnosis where those persons infected with cryptosporidiosis excrete billions of oocysts, and not in testing water supplies where smaller populations of oocysts might exist (10).

Various groups have been working on detection methods for determining the amount of viable oocyst in water supplies where concentrations are potentially small. These methods are based on the genomic sequence of various species of Cryptosporidium and involve polymerase chain reactions (PCR). PCR methods though often require the separation of oocyst from water samples, due to contaminates in water supplies that can interfere with the PCR reaction(10). To reduce the interference of contaminates reverse transcription PCR (RT-PCR) is used. RT-PCR is the process where mRNA is collected from viable oocyst, and converted to DNA via a reverse transcriptase, and amplified with PCR. The hsp 70 gene is a popular target of these assays since if the viable oocyst is placed in a temperature pressure environment, then multiple strands of hsp 70 mRNA become available for detection (12-14).

Real time RT-PCR allows for the quantification of detected DNA as the sample is amplified. Multiple methods of real time RT-PCR exists such as the use of DNA binding dyes, hybridization probes, hydrolysis probes, and molecular beacons. DNA binding dyes fluoresce when bound to dsDNA, this allows for quantification of DNA after each cycle, but also eliminates the specificity of probes in DNA detection. Hybridization probes utilize two linear probes where a fluorophore is attached to the 3’ end of one probe and another probe with a fluorophore attached to the 5’ end. When the probes are separate in a solution, the background fluorescence that is emitted is ignored by the monitoring system. But when the probes bind to the amplicon they line up head to toe so that the two fluorophores are in close proximity. The fluorescent energy of one probe is transferred to the second probe via fluorescent energy transfer (FRET), and a new detectable emission is released from the second probe that is detected by the monitoring system. The
hybridization probes allow for specificity but require the synthesis of two probes, which could be more costly. Hydrolysis probe is a linear probe with a fluorophore and quencher attached to the 5’ and 3’ ends respectively, the close proximity quenches the fluorescence. When the strand is extended by the polymerase, the probe is hydrolyzed and the fluorophore is separated from the quencher and fluorescence is returned and measured for DNA quantification. The hydrolysis probes are destroyed in this process and the amount of probes in a solution would need to be determined. A molecular beacon consists of a fluorophore and quencher on the 5’ and 3’ ends of the stem and loop structure of an ssDNA strand. When the probe hybridizes with the amplicon, fluorescence is restored and DNA amounts can be quantified. The molecular beacon allows for specificity and can be reused throughout the PCR process (15).

Molecular beacons (MB) provide a simplistic method of determining target DNA and quantity in real time PCR with high specificity to a target sequence. Melting temperature of DNA duplexes plays an important role in PCR. It is also important to know the melting temperature of the molecular beacon when considering primer design for PCR. In general, the probe should have melting temperature approximately 10 °C greater than the primers used in the reaction, so that they hybridize before the primers (15). A choice of fluorophores, quenchers, and stem sequence are key factors in probe design. While real time RT-PCR provides an amplification of DNA to detect and observe expressed genes, the ultimate goal would to be able to detect pathogenic DNA without amplification. Detection without amplification has led to the development of single molecule based DNA sensing. Molecular beacons are a form of single molecule DNA sensing. Table 1.1 is a quick comparison of the advantages and disadvantages of the various methods available for detection of Cryptosporidium parvum that have been discussed.

**DNA Biosensors and Molecular Beacons**

**DNA Biosensors**

DNA detection has become a high area of interest and research that has greatly increased understanding on human diseases, mutations, drug discovery, evolution, pathogens, and so on.
There is a movement of biosensors designed to specifically detect gene sequences of DNA with high specificity and selectivity. These DNA biosensors consist of sensor surface modifications sensors, electrochemical sensors, microarray, and optical sensors. Surface DNA sensors involve sensors such as surface Plasmon resonance (SPR) and quartz crystal microbalances (QCM), where either DNA or other agent is bound to the surface of sensor allowing for a change either in refractive index (SPR) or mass (QCM) to be detected when an event such as hybridization takes place in real time (16-18). Electrochemical DNA sensors apply different potentials to DNA and the current of the reaction is measured. Redox reactions have been shown to detect DNA hybridization. Electrochemical DNA sensors have also been used to assess DNA detection limits, cystic fibrosis, and electrochemical properties of DNA such interfacial electron transfer between electrolytes, DNA, and surfaces (18-22). Electrochemical DNA sensors have potential in increased DNA detection and there is much hope for this field in the future. Microarrays consist of multiple oligos attached to glass chips at high densities to observe hybridization of DNA, gene expression, disease detection and pathogen detection. A scanner is used to measure the fluorescent intensities from the chip. Bioinformatics is then used to analyze the vast amounts of data obtained from these chips (18). Optical DNA biosensors often involve the detection of a fluorophore that interacts with DNA such as ethidium bromide where fluorescence is different base on where it is bound or unbound to dsDNA. Another important optical biosensor is the use of molecular beacons which provide high specificity and selectivity when detecting DNA (18). The focus of this research will be on this form of optical biosensor.

Conventional and Quencher Free Molecular Beacons

Conventional molecular beacons are single stranded DNA (ssDNA) sequences designed to form a stem and loop structure, also known as a hairpin. Attached to the 5’ and 3’ ends of the stem are the fluorophore and quencher molecules respectively. When the fluorophore and quencher are in close proximity due to the hairpin and pairing structure, fluorescent emissions are minimized due to fluorescent resonant energy transfer (FRET). When the molecular beacon undergoes hybridization with its target DNA, the fluorophore ("F") and quencher ("Q") are
separated and fluorescent emissions can be detected upon use of the proper excitation wavelength, as seen in Fig. 1.1 (23).

Table 1.1: Comparison of various Cryptosporidium detection methods

<table>
<thead>
<tr>
<th>Methods</th>
<th>Water Sample</th>
<th>Clinical Diagnosis</th>
<th>Oocyst Sample Size</th>
<th>Specificity</th>
<th>Selectivity</th>
<th>Species Differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid Fast Stain (10)</td>
<td>X</td>
<td>X</td>
<td>Large</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Fluorescent Antibody Assay (11)</td>
<td>X</td>
<td>X</td>
<td>Small</td>
<td>High</td>
<td>Medium to High</td>
<td>Low</td>
</tr>
<tr>
<td>Enzyme Immunoassay (10,11)</td>
<td>X</td>
<td>X</td>
<td>Small</td>
<td>High</td>
<td>Medium to High</td>
<td>Low</td>
</tr>
<tr>
<td>PCR Methods (10)</td>
<td>X</td>
<td>X</td>
<td>Very Small</td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Molecular Beacons (15)</td>
<td>X</td>
<td>X</td>
<td>Very Small</td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
</tbody>
</table>

Fig. 1.1: Molecular schematic of operation (23)

Molecular beacons have been explored in a variety of applications of DNA and RNA detection. Due to the stability of the stem, the only sequence that can pull the stem apart efficiently is the complementary sequence specific to the loop sequence. Sequences with nucleotide mismatch, results in an unstable probe. This instability can be detected by the
fluorescent output. This property allows molecular beacons to detect SNPs, which are often the case for diseases and differences in species of organisms (24). Molecular beacons when coupled with polymerase chain reaction (PCR) allows for quick detection of a DNA sequence of gene mutations. This process has been used to successfully and with great specificity detect multiple types of pathogens such as West Nile virus, *Salmonella*, Hepatitis B, and *E. coli* (25-28).

However, the conventional fluorophore and quencher based molecular beacons often have issues with residual fluorescence when in the stem and loop structure. In theory the fluorophore should be completely quenched and no fluorescence should be detected. However, residual fluorescence can persist as a result of the fluorophore and quencher pairs, molecular beacon synthesis, and fluorophore attachment (23,29). Multiple variations on the conventional beacon have been overcome this issue. One that has successfully quenches 99.7 % of the fluorescent emissions are MBs that utilize super quenchers where multiple quenchers are attached to the 3’ end of stem this also has been shown to enhance the signal to noise ratio (S/N) (23,30).

Another interesting approach to resolve the issue of residual fluorescence is the use of quencher free molecular beacons (QF-MBs). These probes are oligos that don’t have a quencher molecule, but utilizes one or more fluorophores attached anywhere on the beacon. For instance a fluorophore can be attached directly to the loop or the stem of a MB usually by incorporating a fluorescently modified base. The fluorescence is quenched by a photoelectron transfer (PET) between the fluorophore and neighboring nucleotides. Since the base guanine has the lowest oxidative potential of bases or its ability to donate electrons, fluorescent emission of the fluorophore is quenched when in close proximity to this base (31). When the probe undergoes hybridization the fluorophore is then separated from the base and an increase in fluorescence emission is detectable (23,31,32). The need for a guanine to be close to the fluorescent nucleotide could make MB design harder when trying to detect a certain gene.

Another QF-MB is the two-fluorophore system. In this case two fluorophores are attached to the 5’ and 3’ ends of the stem of the MB. The MB is designed so that as it undergoes the conformational change from open to close the fluorescence of one fluorophore increases while
the other decreases. A spectrum overlap is needed between these fluorophores. This change is caused by FRET between the two fluorophores. An example of this is the paring of the fluorophores fluorescein (FAM) and tetramethyl rhodamine (TMR). When FAM and TMR are in close proximity in the hairpin structure the energy emitted from FAM is transferred to TMR; or in other words FAM is the electron donor and TMR is the electron acceptor. When the MB undergoes hybridization to a complimentary target the FAM and TMR fluorophores are separated so that FRET doesn’t occur between them and different fluorescent emission spectrum is detectable. This allows one to take the ratio of the open to closed fluorescent emissions from the two fluorophores. This ratio of the two fluorophores will be larger than just a single fluorophore ratio of open to close mode, which leads to better detection sensitivity. Other benefits of a two-fluorophore system includes less photobleaching, potentially broader detection range, less influence of optical geometries, less environment effects, and enhances quantification (23,29).

An excimer monomer switching molecular beacon (EMS-MB) is another form of a quencher free two-fluorophore MB. Instead of using two different fluorophores the same fluorophore is attached to the 5’ and 3 ends of the stem by a short linker molecule, usually pyrene is used. EMS-MBs shift fluorescence emission intensities as the beacon hybridizes with its target DNA. Monomer and excimer fluorescence emissions are emitted by the aromatic hydrocarbon pyrene, depending on the spatial arrangements of pyrene moieties upon excitation (33). Monomer emissions come from un-stacked pyrene moieties, and excimer emissions come from π stacked pyrene moieties (34). Fig. 1.2 demonstrates the spatial arrangements of pyrene moieties and the type of emission. EMS-MBs have an advantage over FRET base molecular beacons in that FRET-MBs either have no fluorescence or have a residual fluorescence. With EMS-MB there is the monomer and excimer fluorescence that are detectable, where with two emission peaks a ratio can be determined that will help reduce false signals. Because of the differences in lifetime measurements of pyrene monomer and excimer emissions and new developments in single molecule detection based on lifetime measurements, EMS-MBs have a strong potential in
detecting a gene sequence in a genome without PCR amplification because of their ability to detect small amounts of targets without amplification (33).

![Monomer Emission](image1) ![Excimer Emission](image2)

Fig. 1.2: Pyrene moieties conformation for monomer (left) and excimer (right) emissions

The use of pyrene as a fluorophore in detecting DNA has some distinct advantages such as high quantum yield, increased lifetime, the large difference between monomer and excimer emissions, and the stability of π stacked pyrenes. The π-stacked pyrene moieties also add stability to a stem and loop structure of DNA, by helping keep this conformation (33,35). Pyrene has also been used in multiple other optical biosensors such as protein folding, DNA and RNA detection, protein-protein interactions, biological membrane monitoring, and is non-mutagenic allowing for potential in vivo studies (35-38).

**MBs Thermodynamics**

The thermal stability of molecular beacon-target hybrid in a solution can be viewed in three phases (See Fig. 1.3): phase 1) bound to target, phase 2) free of target in hairpin form, and phase 3) free of target in random coil formation. The transition between phase 2 and 3 will be used to determine the stability of the stem. Using a two-state (phase) model, the disassociation constants between phase 2 and 3 can be determined using Eq. 1.1. Where, β is the characteristic...
fluorescence intensity of the MB in the hairpin form, $\lambda$ is the characteristic fluorescence intensity in the random coil form, and $F$ is the fluorescence intensity at a given temperature (39).

\[ K_{2\rightarrow 3} = \frac{F - \beta}{\lambda - F} \]  
\[ (1.1) \]

Eq. 1.1 is applied to the free energy ($\Delta G$) equation (Eq. 1.2) and then linearized (Eq. 1.3) to determine enthalpy ($\Delta H$) and entropy ($\Delta S$) of the beacon for the transition from phase 2 to 3.

Where, $T$ is temperature and $R$ is the gas constant (39).

\[ \Delta G = -RT \ln K(T) = \Delta H - T\Delta S \]  
\[ (1.2) \]

\[ R \ln \left( \frac{F - \beta}{\lambda - F} \right) = -\Delta H_{2\rightarrow 3} \frac{1}{T} + \Delta S_{2\rightarrow 3} \]  
\[ (1.3) \]

The thermodynamic properties for phase 1 to 2 transition can be determined in a similar way. Since the melting temperature is defined as the temperature at which half of the targets are still attached to the MB the disassociation constant $K_{12}$ is determined by Eq. 1.4 and therefore Eq.
1.2 can be rewritten as shown in Eq. 1.5. Where \( T_0 \) is the molar target concentration and \( MB \) is the molar molecular beacon concentration, and \( Tm \) is the melting temperature (39). At the quantitative information of the thermodynamics will provide insights into the stability of the MB alone and in the presence of targets. A stable MB is important when trying to design new probes to detect SNPs with high specificity.

\[
K_{12} = T_0 - 0.5[MB] \tag{1.4}
\]

\[
R \ln(T_0 - 0.5[MB]) = -\Delta H_{12} \frac{1}{T_m} - \Delta S_{12} \tag{1.5}
\]

To our best of knowledge, there is no literature reported the systematical study of thermodynamics of EMS-MB and how the thermodynamic behaviors are affected by the presence of magnesium ions.

Motivation of Research

Due to the threat that Cryptosporidium parvum poses to the health of millions around the world, development of a rapid and quick detection method are needed. As discussed, multiple diagnostic tests exist for detection of an infection within individuals, but as of yet there is no quick and rapid onsite detection of a pathogen available for outbreak prevention. Fluorescent probes such as MBs have the potential in detecting small amounts of DNA within an entire genome without amplification especially those of the class known as EMS-MBs (33). This capability gives EMS-MBs an edge that merits further and extensive research on their physical and chemical properties, selectivity, and sensitivity in the detection of DNA such as that of the pathogen Cryptosporidium. Quick detection of DNA in a water source allows for the capability of preventing
and quickly identifying the source of an outbreak. Such prevention could eliminate outbreaks such as the outbreak that affected 400,000 people in Milwaukee, Wisconsin (3,9).

The proposed research is to evaluate the physical and chemical properties, selectivity, and sensitivity of an EMS-MB designed to detect 20 bases (in the loop of MB) of the hsp 70 gene of the human pathogen Cryptosporidium parvum. To accomplish this evaluation of the EMS-MB the following objectives were achieved:

- The stem and loop structure of the EMS-MB was verified with thermal denaturation profiles to observe the changes in fluorescence and the effect of concentration on output intensity.
- The effect of magnesium on the stability of the EMS-MB was assessed by determination of the thermodynamic properties of the EMS-MB.
- The selectivity of the EMS-MB was observed by hybridization studies of the EMS-MB in the presence of a complimentary 20 base target (T0), a 1 base mismatch target (T1), a five base mismatch target (T5), an oxidatively damaged complimentary target (oxo-G), a 40 base ssDNA with 20 base complimentary target (40ssDNA), and a 40 base pair complimentary dsDNA strand (40dsDNA).
- The sensitivity of the EMS-MB was determined by a concentration titration of the probe in the presence of T0 while varying various concentrations of genomic calf thymus DNA (average size 13 kb, from Sigma Co.). Further thermodynamic properties are determined for the EMS-MB in the presence of T0 and T1.
- The melting temperature was rapidly determined from fluorescent thermal denaturation profiles and applied to calculate the chemical thermodynamic properties. The effect of magnesium on the stability of the EMS-MB in the presence of T0 and T1 was also determined by measurements of the melting temperature.
The insight brought by these experiments allow for a better understanding of the physio-chemical properties of the EMS-MB that is particularly selected from human genotype of C. parvum. This knowledge is of a benefit to those who are looking for increased, rapid and onsite detection methods of other genotypes of this waterborne pathogen (by simply changing the sequences of loop and stem of EMS-MB that are specific to the genotype of interest). Further research into “lab on a chip” based sensors incorporating EMS-MBs has a strong potential in rapid detection of organisms.
CHAPTER 2
EMS-MB PROPERTIES

Objective

The objective of this Chapter is to gain a basic understanding of our designed EMS-MB. This will verify that our EMS-MB is functioning properly after synthesis. To accomplish this we will investigate the EMS-MB and its changes from Phase 2 to 3. Phase 2 is the EMS-MB in its stem and loop form and phase 3 is the EMS-MB in a random coil phase (Fig. 2.1). As the EMS-MB changes from phase 2 to 3, the excimer emission intensity should decrease, while the monomer intensity increases due to the separation of the pyrene moieties. Verifying this step will demonstrate the EMS-MB in the desired stem and loop phase. Investigation of the thermodynamic properties of EMS-MB before and after hybridization with the target sequence will allow for a look at the energy requirements and properties of the EMS-MB.

The quantum yield of the EMS-MB will also be determined. The quantum yield is the measurement ratio of the amount of photons emitted to the amount of photons absorbed. So a higher quantum yield would indicate a brighter and more desirable probe that would be easier for detection by using fluorescence emission method. The quantum yield is determined by relating the EMS-MBs absorption and fluorescent spectra with a known reference for a fluorophore with similar absorption and emission properties, in this case 9, 10 diphenylanthracene (DPA).

![EMS-MB Phase 2 to Phase 3](image)

Fig. 2.1 EMS-MB Phase 2 to Phase 3
Materials and Methods

**EMS-MB Emission Spectra**

The EMS-MB was obtained from Invitrogen (see Table 4.1). 46 nmoles of lyophilized EMS-MB was reconstituted to a 100 μM solution in 20 mM Tris-HCl pH 8 solutions (EMS-MB stock). A MB buffer of 20 mM Tris-HCl pH 8, 50 mM KCl and 5 mM MgCl2 were prepared for all MB samples used in the following experiments (33). A Jobin Yvon Fluoromax-3 spectrafluorometer with attached thermal control unit was used for these experiments. Samples were excited at a wavelength of 345 nm (λ_exc), and emission intensities was measured from 357 to 650 nm (λ_em) at a 1 nm step (λ_step). Three 200 nM EMS-MB and three 2 μM EMS-MB samples were prepared in MB buffer for a total sample volume of 400 μL from the EMS-MB stock solution. Thermal denaturation profiles for each sample were recorded from 10-90-10°C at 5°C increments. Each sample was allowed to equilibrate for 4.5 minutes at each temperature step. Data were collected by Fluorescence software (Irvine, CA) then analyzed using Microsoft Excel and Origin Pro 8.0 (North Hampton, MA).

**EMS-MB Quantum Yield**

A series of 0, 200, 400, 600, 800, and 1000 nM samples of EMS-MB probe were prepared in aqueous MB buffer, and a 30 45 60 and 75 nM samples of DPA were prepared in cyclohexane (Sigma, USA). Absorption spectra were measured from 300 to 400 nm and fluorescent emission spectra were recorded from 357-650 nm with excitation wavelength of 345 nm for each sample. The quantum yield was determined from Eq. 2.1 where Φ_F is the quantum yield of the unknown fluorophore, Φ_r is the quantum yield of the reference fluorophore (0.95), Grad_F is the gradient for the unknown fluorophore (determined by plotting the absorbance against the integrated area of the emission spectra), Grad_r is the gradient for the reference fluorophore, η is the refractive index of the unknown fluorophores buffer solution (1.333), and η_r is the refractive index of the reference fluorophores buffer solution (1.423) (40).
\[ \phi_F = \phi_I \frac{\text{Grad} \eta^2}{\text{Grad} \, \eta^2} \] (2.1)

Results

200 nM EMS-MB Thermal Denaturation Profiles

Fig. 2.2 is the recorded thermal denaturation profiles for 200 nM EMS-MB in MB buffer for run 1. Thermal denaturation profiles for run 2 and 3 data for the same EMS-MB concentration are found in the Appendix (see Fig. A.1 and Fig. A.2). Note that the excimer peak at 485 nm is decreasing with temperature increase and increasing with temperature decrease. The monomer peak at 396 nm also is decreasing.

Fig. 2.2 200 nM EMS-MB thermal denaturation profile (\(\lambda_{\text{exc}}=345\)nm, \(\lambda_{\text{em}}=357-650\) nm) run 1, arrow indicates direction from heating to cooling from 10-90-10 °C
2 μM EMS-MB Thermal Denaturation Profile

Fig. 2.3 is the recorded thermal denaturation profiles for 2 μM EMS-MB samples for run 1. The thermal denaturation profiles for run 2 and run 3 can be found in the appendix (see Fig. A.3 and Fig. A.4). The trend is similar to the 200 nM samples. The spectra however, are less noisy due to the increase fluorescence counts from the higher concentration of EMS-MB.

Fig. 2.3 2 μM EMS-MB thermal denaturation profile (λ\text{exc}=345nm, λ\text{em}=357-650 nm) run 1, arrow indicates direction from heating to cooling from 10-90-10 °C

200 nM Thermal Denaturation Profiles Excimer and Monomer Peak Intensities

Fig. 2.4 is an example of the monomer and excimer peak intensities for 200 nM EMS-MB samples taken from run 1. Both the heating and cooling trends are plotted over each other to show that there isn't a significant amount of hysteresis occurring during the run. However, when calculating the thermodynamic properties in the next section both heating and cooling will be calculated and averaged for the final comparison.

Fig. 2.5 is an example of the 2 μM excimer and monomer peak intensities taken from run 1. The heating and cooling are overlaid to show that there is not a significant amount of hysteresis for our purposed in determining the thermodynamic properties in the next section.
Fig. 2.4 200 nM EMS-MB monomer and excimer peak intensities heating and cooling overlay, arrows indicate direction of heating and cooling ($\lambda_{\text{exc}}$=345nm, $\lambda_{\text{em}}$=357-650 nm)

Fig. 2.5 2 μM EMS-MB monomer and excimer peak intensities heating and cooling overlay, arrows indicate direction of heating and cooling ($\lambda_{\text{exc}}$=345nm, $\lambda_{\text{em}}$=357-650 nm)

**Thermodynamic Properties**

The enthalpy ($\Delta H$), entropy ($\Delta S$), and free energy ($\Delta G$) for the 200 nM and 2 μM EMS-MB sample were calculated according to the thermodynamic methods discussed in the literature.
review. Excimer peak emissions were used to calculate these values, because of its representation of the phase changes of the EMS-MB.

The disassociation constant was determined for the 200 nM and 2 μM EMS-MB solutions at each temperature step (see Fig. A.5 and Fig. A.6). The disassociation constant (K_d) was then used to in the determination of the enthalpy and entropy for each samples heating and cooling spectra, then averaged.

Fig. 2.6 and Fig. 2.7 are the Van Hoff diagrams for the 200 nM and 2 μM EMS-MB solutions respectively. These figures are a representative for each of the sample runs. The Enthalpy (∆H) is determined from the slope and entropy (∆S) from the intercept. Table 2.1 is the average enthalpy (∆H) and entropy (∆S) determined from all three runs and their heating and cooling profiles. Fig. 2.8 is the free energy (∆G) calculated from the enthalpy (∆H) and entropy (∆S) averages over the temperature range.

Fig. 2.6 Van Hoff diagram to determine enthalpy (∆H) and entropy (∆S) of 200 nM EMS-MB (see Eq. 1.3)
Fig. 2.7 Van Hoff diagram to determine enthalpy ($\Delta H$) and entropy ($\Delta S$) of 2 $\mu$M EMS-MB (see Eq 1.3)

Table 2.1: Comparison of calculated enthalpy ($\Delta H$) and entropy ($\Delta S$) of EMS-MB

<table>
<thead>
<tr>
<th>Conc.</th>
<th>$\Delta H$ Average±SD (kcal/mol)</th>
<th>$\Delta S$ Average±SD (cal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 $\mu$M</td>
<td>16.979 ± 0.899</td>
<td>53.579 ± 2.359</td>
</tr>
<tr>
<td>200 nM</td>
<td>16.259 ± 0.381</td>
<td>52.726 ± 0.823</td>
</tr>
</tbody>
</table>

EMS-MB Quantum Yield

The absorbance spectra for the EMS-MB concentration samples are recorded in Fig. 2.9. The absorbance increase as the concentration increases this is to be expected since more fluorophores are present more photons should be absorbed. The peaks seen in the absorption spectra indicate points with the strongest absorption for the pyrene moieties. The fluorescent emission spectra are seen in Fig. 2.10 where the intensity increases with the increase in sample concentration. The absorbance at 345nm for each EMS-MB concentration was plotted against
the integrated fluorescent emission area and the slope was determined to be \(2.948 \text{E}9\) (See Fig. 2.11). Fig. 2.12 is the absorbance spectra measured for the DPA concentrations where the absorption also increases with the increase in sample concentrations. However, in the fluorescent emission spectra the emission intensity at around 420 nm decreases with an increase in DPA concentration (see Fig. 2.13), this results in a negative slope \((-2.665 \text{E}9\) when plotting the absorption (345nm) against the integrated fluorescent spectrum of Fig. 2.14. The slopes of Fig. 2.11 and Fig. 2.14 equal the \(\text{Grad}_F\) and \(\text{Grad}_r\) variables. The \(\Phi_F\) for the EMS-MB was determined to be 0.92 (see Eq. 2.2)

![Free energy diagram](image)

**Fig. 2.8** Free energy (\(\Delta G\)) diagram for 200 nM and 2 \(\mu\)M EMS-MB (see Eq. 1.2)
Fig. 2.9 UV absorbance spectra of EMS-MB in MB buffer, Arrow indicates concentration increase

Fig. 2.10 fluorescence emission spectra ($\lambda_{\text{exc}}=345\text{nm}$, $\lambda_{\text{em}}=357-650\text{ nm}$) of EMS-MB in MB buffer, where arrow indicates concentration increase
Fig. 2.11: EMS-MB Absorbance (345nm) against integrated fluorescence spectra where

\[ \text{Grad}_F = 2.948 \times 10^9 \]

Fig. 2.12: UV absorbance spectra of DPA at different concentrations where arrow indicates concentration increase
Fig. 2.13: DPA concentration fluorescence emission spectra where arrow indicates concentration increase.

Fig. 2.14: DPA Absorbance (345nm) against integrated fluorescent spectra where $Grad_r = 2.665E9$. 
Discussion

Thermal Denaturation Profiles

The EMS-MB as it changes from the stem and loop structure (phase 2) to a random coil (phase 3) is represented in Fig. 2.2. As the EMS-MB changes from phase 2 to 3 the excimer emission peak intensity (485 nm) decreases as seen in Fig. 2.4 and Fig. 2.5. The decrease is due to the pyrene fluorophores change in physical conformation of their π-stacking in the stem and loop structure (34) (see Fig. 2.1). However, the monomer emission peak intensity also decreased for both concentrations of EMS-MB, which was expected to increase (Fig. 2.4 and Fig. 2.5). This decrease in monomer emission intensity is most likely due to the increased temperature and its effect on the fluorescence intensity of pyrene. This can be corrected by running a thermal denaturation profile on just a pyrene moiety and correcting the collected spectrum (41). In our case pyrene moieties weren’t synthesized to perform this correction to the data. Nevertheless, the monomer and excimer peak intensities switch which indicates the conformation change from phase 2 to 3. This switch is more visible in the 2 μM EMS-MB conformation due to an increase in concentration and emission counts. This increase in concentration also resulted in a smoother spectra reading as seen in comparing Fig. 2.2 with Fig. A.1 through Fig. A.4 of the EMS-MB concentrations.

Thermodynamic Properties and Quantum Yield

A comparison of the 200 nM and 2 μM EMS-MB thermodynamic properties indicates that there isn’t a large difference in their thermodynamic properties. As expected though an increase of the disassociation constant occurred as the temperature increased (See Fig. A.5 and Fig. A.6). These results indicate that the thermodynamic properties of the transition between phase 1 to phase 2 aren’t affected by the concentration of the EMS-MB. The negative free energy for both the concentrations indicates that this is a thermodynamically favorable reaction.
The quantum yield was determined to be 0.92. This is a high quantum yield and indicates that 92% of the absorbed photons are emitted through fluorescence. This high quantum yield is desirable so that the emission spectra can easily be recorded and enhanced sensitivity can be achieved.

Conclusion

The synthesized EMS-MB has the desired properties of an excimer-monomer switching molecular beacon. The excimer emission responds in the desired way, while the monomer still decreases. This decrease is mostly likely due to the intrinsic fluorescent properties of pyrene in relation to temperature. The switching of the monomer and excimer intensities however indicates the probe is switching its conformation from a stem and loop structure to as random coil. The EMS-MB also has a high quantum yield that will enhance the detection of target DNA and allow for enhanced sensitivity while detecting the conformational changes of the EMS-MB.
CHAPTER 3
MAGNESIUM ION EFFECT ON THERMODYNAMIC PROPERTIES OF THE EMS-MB

Objective

The purpose of this chapter is to apply Eq. 1.3 to the fluorescence output of each magnesium ion concentration to determine the effect of magnesium on the stability using the thermodynamic properties. Excimer fluorescence emission intensities will be used in Eq. 1.3 to determine the enthalpy (ΔH) and entropy (ΔS). The stability of the EMS-MB in the magnesium concentrations will be determined by the enthalpy, because this is the amount of energy needed for the reaction to take place. It is assumed that lower enthalpy indicates increased stability of the EMS-MB (39). SAS/STAT ® software (Cary, NC) will be used to analyze the differences between the magnesium concentrations effect on the thermodynamic properties of the EMS-MB.

Material and Methods

46 nmoles of the lyophilized of EMS-MB was reconstituted to a 100 μM stock concentration in a 20 mM Tris HCl at pH 8, and gently shaken to allow for complete reconstitution. From the 100 μM stock solution, 500 μL of a 2 μM stock EMS-MB solution was prepared by combining 10 μL from the 100 μM stock with 490 μL of molecular grade H₂O (Sigma, MO). This solution was placed in a hot water bath of 90 °C for 5 minutes then allowed to cool to room temperature for 15 minutes. Varying buffer solutions with different magnesium concentrations were prepared as shown in Table 3.1

To obtain an EMS-MB concentration of 200 nM 40 μL of the 2 μM EMS-MB stock was combined with 360 μL of magnesium buffer. Three samples of EMS-MB were prepared for each magnesium ion concentration.

The FluoroMax-3 spectrofluorometer was set to excite the sample with a wavelength of 345 nm and scan the emission spectrum from 357 nm to 650 nm measuring at an increment of 1 nm.
Each 300 μL EMS-MB sample went through a thermal denaturation profile from 10-90-10°C at 5°C increments. At each step the sample was allowed to equilibrate to temperature for 4.5 minutes and then the emission spectra was collected. The data was transferred to Excel and analyzed, then plotted in Origin Pro 8.0. It was also further analyzed with SAS/STAT software.

### Table 3.1: Magnesium buffer stock solutions

<table>
<thead>
<tr>
<th>Vial</th>
<th>Mg (mM)</th>
<th>MgCl₂ (μL)</th>
<th>KCl (mL)</th>
<th>Tris (mL)</th>
<th>water (mL)</th>
<th>total (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.25</td>
<td>0.5</td>
<td>23.25</td>
<td>25</td>
</tr>
<tr>
<td>1</td>
<td>0.5</td>
<td>12.5</td>
<td>1.25</td>
<td>0.5</td>
<td>23.24</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>25</td>
<td>1.25</td>
<td>0.5</td>
<td>23.32</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>50</td>
<td>1.25</td>
<td>0.5</td>
<td>23.20</td>
<td>25</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>75</td>
<td>1.25</td>
<td>0.5</td>
<td>23.18</td>
<td>25</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>100</td>
<td>1.25</td>
<td>0.5</td>
<td>23.15</td>
<td>25</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>125</td>
<td>1.25</td>
<td>0.5</td>
<td>23.13</td>
<td>25</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>250</td>
<td>1.25</td>
<td>0.5</td>
<td>23.00</td>
<td>25</td>
</tr>
<tr>
<td>8</td>
<td>20</td>
<td>500</td>
<td>1.25</td>
<td>0.5</td>
<td>22.75</td>
<td>25</td>
</tr>
<tr>
<td>9</td>
<td>50</td>
<td>1250</td>
<td>1.25</td>
<td>0.5</td>
<td>22.00</td>
<td>25</td>
</tr>
<tr>
<td>10</td>
<td>125</td>
<td>3125</td>
<td>1.25</td>
<td>0.5</td>
<td>20.13</td>
<td>25</td>
</tr>
</tbody>
</table>

### Results

*Spectra of Monomer and Excimer Emissions at Different Mg²⁺ Concentrations*

The spectrum collected for each magnesium ion concentration was normalized to 1 at 650 nm. This will allow for intensity correction due to magnesium concentration, and thus provides easier comparison between the collected magnesium EMS-MB spectra. To get a glimpse the data was combined for each temperature step to allow for a better view of the magnesium effect. Fig. 3.1 and Fig. 3.2 are from run 1 as an example of the three runs. Fig. 3.1 and Fig. 3.2 represent the coldest temperature and the hottest temperature for the sample run; see Appendix for further detail in the effect of temperature on the magnesium concentrations (see Fig. A.7 through Fig. A.21). Differences in the three runs occur but the overall trend is visible and similar in
each sample run. Each temperature/magnesium spectrum is followed by the monomer and excimer peak emissions to allow for a more distinct look at the peak trends.

Fig. 3.1: A) The effect of Mg\(^{2+}\) concentration on EMS-MB emissions at 10 °C, the left arrow indicates general trend of monomer emission and the right arrow indicates general trend of excimer emission due to Mg\(^{2+}\) increase, and B) is the effect of Mg\(^{2+}\) concentration on monomer and excimer emissions at 10°C.

Fig. 3.2: A) The effect of Mg\(^{2+}\) concentration on EMS-MB emissions at 90 °C, the left arrow indicates general trend of monomer emission and the right arrow indicates general trend of excimer emission due to Mg\(^{2+}\) increase, and B) is the effect of Mg\(^{2+}\) concentration on monomer and excimer emissions at 90 °C.
**Thermodynamic properties**

Enthalpy (ΔH) and Entropy (ΔS) were determined for each magnesium ion concentration within the thermal denaturation profiles. Fig. 3.3 demonstrates a Van’t Hoff graph for the varying magnesium ion concentrations. Then enthalpy (ΔH) was determined from the slope of a linear fit of the data as was the entropy (ΔS) from the y-intercept. Enthalpy (ΔH) and entropy (ΔS) were then determined for each sample and averaged for each sample heating and cooling run. The average enthalpy (ΔH) and entropy (ΔS) are found in Table 3.2. These values were then used to calculate the free energy (ΔG) for each magnesium ion concentration as seen in Fig. 3.4, where the free energy is plotted verses the temperature.

![Van't Hoff plot with temperature ranges for varying Mg²⁺ concentrations](image)

**SAS/STAT Software Analysis and Results**

A random effects model with one random factor was used to analyze the enthalpies of heating and cooling for each of the magnesium ion effect on the EMS-MB that are summarized in Table 3.2. Eq. 3.1 is the random effects equation where, $Y_{ij}$ is the effect due to the jth run that
received magnesium treatment $i$. $\mu$ is the overall grand mean, $M_i$ is the effect due to the magnesium concentration, and $\varepsilon_{ij}$ is the random error. Where $i=1,2,3,\ldots,11$ and $j=1,2,3,4,5,6$

Table 3.2: Average enthalpy ($\Delta H$) and entropy ($\Delta S$) for temperature ranges for varying Mg$^{2+}$ concentrations

<table>
<thead>
<tr>
<th>Conc.</th>
<th>$\Delta H \pm SD$ (kcal/mol)</th>
<th>$\Delta S \pm SD$ (cal/ K mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0 mM</td>
<td>18.3 ± 0.8</td>
<td>59.7 ± 2.8</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>17.8 ± 1.2</td>
<td>58.0 ± 4.1</td>
</tr>
<tr>
<td>1 mM</td>
<td>17.0 ± 5.5</td>
<td>55.4 ± 2.2</td>
</tr>
<tr>
<td>2 mM</td>
<td>16.0 ± 0.8</td>
<td>52.1 ± 2.3</td>
</tr>
<tr>
<td>3 mM</td>
<td>16.0 ± 0.5</td>
<td>52.0 ± 1.1</td>
</tr>
<tr>
<td>4 mM</td>
<td>15.8 ± 0.6</td>
<td>51.3 ± 1.4</td>
</tr>
<tr>
<td>5 mM</td>
<td>16.1 ± 0.4</td>
<td>52.4 ± 0.8</td>
</tr>
<tr>
<td>10 mM</td>
<td>16.4 ± 0.3</td>
<td>53.0 ± 0.7</td>
</tr>
<tr>
<td>20 mM</td>
<td>16.6 ± 0.3</td>
<td>53.5 ± 1.3</td>
</tr>
<tr>
<td>50 mM</td>
<td>16.9 ± 0.4</td>
<td>54.7 ± 1.6</td>
</tr>
<tr>
<td>125 mM</td>
<td>16.5 ± 0.7</td>
<td>53.5 ± 1.9</td>
</tr>
</tbody>
</table>

Fig. 3.4: Free energy ($\Delta G$) versus temperature for Mg$^{2+}$ concentrations
\[ Y_{ij} = \mu + M_i + \varepsilon_{ij} \quad (3.1) \]

The statistical assumptions of normality and homoscedasticity were observed before moving on with the analysis.

ANOVA analysis was performed to determine if there were significant differences between the magnesium concentrations. Statistically significant differences will be determined by the p-value of the model. P-values less than 0.05 indicate statistically significant differences while p-values greater than 0.05 indicate less statistically significant differences. If the p-value indicates there is significant difference between the variables, a post hoc means analysis using the REGWQ method will be used to determine where the differences occur among the concentrations.

Table 3.3 displays the ANOVA results using the random effects model described above. The p-value is less than 0.0001; therefore a post hoc means analysis was performed using the REGW function of SAS software to identify the differences and reported in Table 3.4: Means comparison of \( \Delta H \) for effect of \( \text{Mg}^{2+} \) on EMS-MB Table 3.4.

<table>
<thead>
<tr>
<th>Source</th>
<th>F</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F</th>
<th>P &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>0</td>
<td>37.31</td>
<td>3.73</td>
<td>9.23</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Error</td>
<td>5</td>
<td>22.24</td>
<td>0.40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected Total</td>
<td>5</td>
<td>59.55</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Discussion

**Thermal Denaturation Profiles**

When analyzing the denaturation profiles of the EMS-MB in the varying concentrations some noticeable effects of the magnesium on the EMS-MB can be seen. At the lower temperatures between 15-30 °C the monomer and excimer emissions are similar in intensity for magnesium concentrations 0-10 mM this indicates that a majority of the EMS-MB is in their stem and loop conformation. While at the higher magnesium ion concentrations greater than 10 mM, the monomer emissions increase while the excimer emissions decrease (see Fig. 3.1, Fig. 3.2, and Fig A.7 through Fig. A.10). This would indicate that the magnesium ions are affecting the bonding of the stem to some degree that the pyrene moieties are being separated from their π-stacking. At higher temperatures of the thermal profiles (35-90°C), the excimer emission constantly decrease until the emissions level out, at which point the EMS-MB is in the random coil phase. The monomer emission, in general for this higher temperature range, decreases rapidly from 0-5 mM Mg^{2+} and then levels out from 10 mM-125 mM Mg^{2+} (Fig. A.11 through Fig. A.21). This indicates the magnesium ions have a stabilizing effect on the monomer emission intensity. Further analysis of the thermodynamic properties and the free energy will give further insights into the EMS-MB and its properties.

**Thermodynamics**

A careful look at the thermodynamic properties of the phase 2 to phase 3 conformation change of the EMS-MB reveals characteristics of the effect of magnesium. The free energy (ΔG) appears very similar for each of the ion concentrations (see Fig. 3.4). This then merits the need to take a closer look at the stability using the enthalpies (ΔH) of the reaction, where a lower enthalpy indicates stability. In order to distinguish any differences between the recorded enthalpies a statistical analysis was performed.
Temperature Range Statistical Analysis

The means comparison analysis for magnesium effect on the EMS-MB indicates that there is no significant difference between 0 mM and 0.5 mM magnesium ion concentrations. This is shown by the link of the letter A of Table 3.4 between these two concentrations. There is also no significant difference among 0.5, 1 and 50 mM magnesium ion concentrations as indicated by the link of letter B in Table 3.4. There is also no significant difference in enthalpy for concentrations 50, 20, 125, 10, 5, 2, 3, and 4 mM magnesium concentrations as indicated by the link between the letters C and D of Table 3.4, with 4 mM with the lowest enthalpy, the lower the enthalpy the more stable the molecular beacon. It is also noted that magnesium ion concentrations greater than 1 mM have a greater stabilizing effect on the molecular beacon (Table 3.4). As seen the 4 mM concentration has the optimum stability for the entire thermal denaturation profile.

Conclusion

Magnesium ions due affect the stability of the EMS-MB as it undergoes conformational changes from phase 2 to phase 3. This can be seen when comparing the thermodynamic properties of the samples with magnesium to the control sample; this is confirmed with the ANOVA analysis for the enthalpy of the samples. Magnesium ion concentrations greater than 0.5 mM resulted in a decrease of enthalpy. This indicates an increase in the stability of the EMS-MB. The lowest enthalpy occurred at 4 mM magnesium concentration; however the enthalpy at 4 mM concentration was not significantly different from other concentrations that are greater than 0.5 mM. Use of a magnesium ion concentration greater than 4 mM Mg$^{2+}$ will provide maximum stability of the EMS-MB.
Table 3.4: Means comparison of ΔH for effect of Mg$^{2+}$ on EMS-MB

<table>
<thead>
<tr>
<th>REGWQ Grouping</th>
<th>Mean (kcal/mol)</th>
<th>N</th>
<th>Mg$^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>18.30</td>
<td>6</td>
<td>0.0mM</td>
</tr>
<tr>
<td>B</td>
<td>17.77</td>
<td>6</td>
<td>0.5mM</td>
</tr>
<tr>
<td>B</td>
<td>17.01</td>
<td>6</td>
<td>1mM</td>
</tr>
<tr>
<td>B</td>
<td>16.86</td>
<td>6</td>
<td>50mM</td>
</tr>
<tr>
<td>C</td>
<td>16.62</td>
<td>6</td>
<td>20mM</td>
</tr>
<tr>
<td>C</td>
<td>16.45</td>
<td>6</td>
<td>125mM</td>
</tr>
<tr>
<td>C</td>
<td>16.38</td>
<td>6</td>
<td>10mM</td>
</tr>
<tr>
<td>C</td>
<td>16.12</td>
<td>6</td>
<td>5mM</td>
</tr>
<tr>
<td>C</td>
<td>16.00</td>
<td>6</td>
<td>2mM</td>
</tr>
<tr>
<td>C</td>
<td>15.97</td>
<td>6</td>
<td>3mM</td>
</tr>
<tr>
<td>D</td>
<td>15.77</td>
<td>6</td>
<td>4mM</td>
</tr>
</tbody>
</table>
CHAPTER 4:
EMS-MB HYBRIDIZATION STUDIES

Objective

The objective of this chapter is to determine the specificity of the EMS-MB in the presence of target DNA. The target DNA in this case is synthetic DNA that is specific to the hsp 70 gene of the human pathogen Cryptosporidium parvum. Four synthetic DNA target sequences will be examined for the specificity and selectivity of the designed EMS-MB. The first is a completely complimentary sequence identical to 20 bp of the hsp 70 gene (T0). The second is a modified one mismatch base of the complimentary target (T1). The third is a five base mismatch (T5) (see Table 4.1). A fourth is a 40 bases single stranded DNA (ssDNA) which contains the 20 bases complimentary to the EMS-MB (40ssDNA). The fifth is a 40 base pair double stranded DNA (dsDNA) that contains the 20 bases complimentary to the EMS-MB (40dsDNA). To determine selectivity of the EMS-MB multiple samples will be prepared with the EMS-MB in the presence of varying target concentrations for each of the designed targets. The specificity will be determined by a target concentration titration of EMS-MB and T0 in the presence of varying concentrations of calf thymus genomic DNA (ctDNA). The results of T0, T1, and T5 will be compared to another molecular beacon (MB2) with two different fluorophores FAM and TMR attached to the 5’ and 3’ ends respectively, instead of the pyrene fluorophores attached to the stem of EMS-MB (see Fig. 4.1). MB2 also allows for a ratiometric comparison of the FAM and TMR emission peaks as does our EMS-MB with its monomer and excimer peak emission intensities. This will allow for a comparison of a fluorophore quencher based MB (MB2) and a two -fluorophore based MB (EMS-MB).
The sixth target sequence will also be assessed, which is based on damaged DNA caused by oxidation. One of the most common damages to DNA is the oxidation of the 8th Carbon in the purine base guanine (42). We will determine if the EMS-MB is capable of detecting this damaged DNA, by detecting the hybridization of the EMS-MB with a new complimentary DNA target sequence with an oxidized guanine base (oxo-G).

Material and Methods

**Synthesis and Stock Prep**

Both EMS-MB and MB2 were synthesized by Invitrogen (Carlsbad, CA) and prepared as a 2 µM stock solution. The EMS-MB was prepared in a 2 µM stock solution as in previous chapters in the prepared MB buffer. The complimentary (T0), 1 base mis-matched (T1), 5 base mis-matched (T5), 40 base ssDNA (40ssDNA), and 40 bp dsDNA (40dsDNA) were synthesized by Syngen (Hayward, CA). The oxo-G sequence was synthesized by the DNA Peptide Core Research Facility at the University of Utah (Salt Lake City, UT). The base pair sequence and description of these targets can be seen in Table 4.1. T0 and T5 base sequences were obtained from Genbank accession numbers AF221535 and AF221539, respectively. The 40ssDNA and 40dsDNA base sequences were also obtained from Genbank accession number AF221535. The
T0, T1, T5, 40ssDNA, and 40dsDNA sequences were received lyophilized and were each reconstituted to a 200 µM target solution in 20 mM Tris-HCl pH 8 (Sigma). A 50 µM oxo-G stock solution was prepared from 23 nmoles of lyophilized DNA in 20 mM Tris-HCl pH 8.0. A 2 µM sample was then prepared as a dilution from the 200 and 50 µM stock solution for each of the target sequences. Stock solutions for the EMS-MB, MB2 and targets were kept frozen in a –20 °C freezer in between 2 µM sample preparation.

**EMS-MB Target Hybridization**

The effects of target concentration were determined by preparing a 200 nM EMS-MB in the presences of the varying target concentrations (0, 40, 80, 120, 160, 200, and 400 nM) for T0, T1, T5, 40ssDNA, 40dsDNA, and oxo-G in MB buffer solution. Each EMS-MB target solution will be excited with 345 nm wavelength and emission spectra measured from 357-650nm. Monomer peaks at 396nm and excimer peaks at 485 nm will be recorded for emission spectrum of each sample. A ratio analysis of the monomer to excimer peak analysis will be used to determine hybridization affects of the EMS-MB and the synthesized targets. This analysis will be done for repeated runs of each EMS-MB to target concentration. The monomer to excimer ratio of each spectrum will be averaged and the standard error will be determined. This ratio analysis will allow for intensity variations between samples to be taken into consideration. The monomer to excimer ratio is more indicative of the EMS-MB phase in the presence of its targets.

**EMS-MB:T0 Hybridization in Presence of Genomic ctDNA**

To determine the selectivity a 200 nM sample of EMS-MB was prepared with varying T0 concentrations (0, 40, 80, 120, 160, 200, and 400 nM) in the presence of genomic calf thymus DNA (ctDNA) at each concentration of 0, 0.25, 0.75.1.25, and 2.5 µg/ml in MB buffer solution. The stock ctDNA was obtained from Sigma (USA), and its concentration was determined by UV-Vis spectroscopy at absorption wavelength of 260 nm and diluted accordingly to achieve the desired concentrations.
**MB2 Target Hybridization**

The effects of hybridization for MB2 were determined by preparing a 200 nM solution in the presence of varying target concentrations (0, 40, 80, 120, 160, 200, and 400 nM) for T0, T1 and T5 in MB buffer. Samples were excited with a 494 nm wavelength and spectrum recorded from 504-700 nm. One scan was run for each sample due to amount of MB2 available. A ratiometric analysis of the emission peaks of FAM (518 nm) and TMR (578 nm) peaks will provide the conformation information of MB2, or in other words, whether the probe is open or closed. When the probe is open the ratio of FAM to TMR will be larger due to increased fluorescence of the FAM fluorophore as it is separated from its quencher TMR. A lower ratio indicates that the two fluorophores are still in close proximity and the beacon is in the stem and loop structure.

**EMS-MB to MB2 comparison**

The intensity ratios of MB2 will be compared to those of EMS-MB to determine which MB structure would provide a better detection performance. To compare the detection response of the probes, a ratio known as the contrast ratio will be used. The contrast ratio is a ratio of the intensities of emission peaks (monomer to excimer for EMS-MB, and FAM to TMR for MB2) of the open beacon to the ratio of the emission peaks of the closed beacon. Eq. 4.1 gives the contrast ratio equation for the EMS-MB and Eq. 4.2 gives the contrast ratio for MB2 (29). The contrast ratio will be obtained for each of the beacons in the presence of targets except oxo-G since MB2 hybridization to oxo-G wasn’t investigated due to limitations of MB2 sample volumes.

**EMB-MB:**

\[
\text{Contrast Ratio} = \frac{l_{395\text{open}}}{l_{395\text{closed}}} / \frac{l_{485\text{open}}}{l_{485\text{closed}}} \tag{4.1}
\]

**FAM-TMR (MB2):**

\[
\text{Contrast Ratio} = \frac{l_{518\text{open}}}{l_{518\text{closed}}} / \frac{l_{578\text{open}}}{l_{578\text{closed}}} \tag{4.2}
\]
Table 4.1: EMS-MB and targets sequences with description, bold type bases indicate mismatch from complimentary sequence.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Sequence (5’ – 3’)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMS-MB</td>
<td>(1-Pyrenyl)(C_6CONHC_3)ACGGCCAATACTTTC TCTCGCCAGGCCG(T(C_3NH_2COC_5)) (1-Pyrenyl)</td>
<td>Loop sequence (20 bases) is complementary to human genotype of C. parvum</td>
</tr>
<tr>
<td>T0</td>
<td>CTGGCGAGAG AAATGTATTG</td>
<td>Corresponds to the base positions between 507 to 526 of hsp 70 gene in human genotype, (AF221535)</td>
</tr>
<tr>
<td>T1</td>
<td>CTGGTGAGAG AAATGTATTG</td>
<td>Control sequence (single base mismatched)</td>
</tr>
<tr>
<td>T5</td>
<td>CTGGTGGAAG AAATGTTCTT</td>
<td>Corresponds to the base positions between 504 to 523 of hsp 70 gene in C. baileyi genotype, (AF221539)</td>
</tr>
<tr>
<td>40ssDNA</td>
<td>AAGAAAGGAA CTGGCGAGAG AAATGTATTG ATCTTCGATT</td>
<td>Corresponds to the base positions between 497-537 of hsp 70 gene in C. parvum, (AF221535)</td>
</tr>
<tr>
<td>40dsDNA</td>
<td>sense- AAGAAAGGAA CTGGCGAGAG AAATGTATTG ATCTTCGATT anti-sense- AATCGAAGAT CAATACATTCTCCTGAGAG TTCTTTTCTT</td>
<td>Corresponds to the base positions between 497-537 of hsp 70 gene in C. parvum, (AF221535) with complimentary anti-sense for dsDNA</td>
</tr>
<tr>
<td>oxo-G</td>
<td>CTGGCGAGAG AAATGTATTG</td>
<td>Damaged DNA</td>
</tr>
</tbody>
</table>

Results

**EMS-MB Target Hybridizations**

**EMS-MB: T0**

Fig. 4.2A is an example spectrum for the EMS-MB: T0 titration from run 1 of the four sample runs that emission spectra were recorded. Fig. 4.2B is an example of the monomer (396 nm) and excimer (485 nm) peaks for the EMS-MB: T0 titration. It’s important to notice the switch in monomer and excimer intensities in the presences of targets. The average ratio of the monomer and excimer intensities are seen in Fig. 4.2C with the error bars representing the standard error.
at each titration dilution. The overall trend is that we see a decrease in excimer emission intensity and an increase in monomer emission intensity.

Fig. 4.2: A) The effect of T0 target concentration on EMS-MB, the left arrow indicates general trend of monomer emission and the right arrow indicates general trend of excimer emission due to target concentration increase, B) monomer and excimer peak emission intensities, and C) average ratio of monomer to excimer intensities (n = 4)

**EMS-MB:T1**

Fig. 4.3A shows example of emission spectra for the EMS-MB:T1 titration from run 1 of the four sample runs. Fig. 4.3B is an example of the monomer and excimer peaks for the EMS-MB:T1 titration. The average ratio of the monomer and excimer intensities are seen in Fig. 4.3C with the error bars representing the standard error at each titration dilution. The trend for the
hybridization of EMS-MB:T1 samples are similar to EMS-MB:T0 samples where the excimer intensity decreases while the monomer intensity increases with increased target concentrations.

Fig. 4.3: A) The effect of T1 target concentration on EMS-MB, the left arrow indicates general trend of monomer emission and the right arrow indicates general trend of excimer emission due to target concentration increase, B) monomer and excimer peak emission intensities, and C) average ratio of monomer to excimer intensities (n = 4)

EMS-MB:T5

Fig. 4.4A is an example spectra for the EMS-MB:T5 titration from run 1 of the four sample runs. Fig. 4.4B is an example of the monomer and excimer peaks for the EMS-MB:T5 titration. The average ratio of the monomer and excimer intensities are seen in Fig. 4.4C with the error bars representing the standard error at each titration dilution over the four experimental runs.
The monomer and excimer peak intensities are very similar throughout the titration until the target concentration is the same or doubled the EMS-MB concentration. At this point the excimer peak slightly decreases as the monomer slightly increases.

![Graph showing the change in peak intensities with target concentration](image)

**Fig. 4.4**: A) The effect of T5 target concentration on EMS-MB, no arrows indicates an unobservable trend due to concentration. B) monomer and excimer peak emission intensities, and C) average ratio of monomer to excimer intensities (n = 4)

**EMS-MB: 40ssDNA**

As seen in Fig. 4.5, the general trend for the 40ssDNA target concentration titration is that the excimer peak decreases and the monomer peak has a slight increase over the 40ssDNA target concentration. A closer look is seen in Fig. 4.5B where the monomer and excimer peaks are plotted against the increase in target concentration. The monomer to excimer ratio is shown
in Fig. 4.5C where the increase in ratio with concentration indicates that more EMS-MBs are hybridizing with the target.

Fig. 4.5: A) The effect of 40ssDNA target concentration on EMS-MB, the left arrow indicates general trend of monomer emission and the right arrow indicates general trend of excimer emission due to target concentration increase, B) monomer and excimer peak emission intensities, and C) average ratio of monomer to excimer intensities (n = 3)

**EMS-MB:40dsDNA**

An example of the emission spectra recorded from the three runs is given in Fig. 4.6A. A closer look at the monomer and excimer peaks reveals an initial decrease in excimer intensity when the EMS-MB is mixed in the presence of 40dsDNA, then the intensity levels off, without ever reaching the initial intensity of the beacon alone (see Fig. 4.6B). The monomer to excimer
ratio in Fig. 4.6C shows an increase in the monomer to excimer ratio after the 40dsDNA is
introduced to the EMS-MB, but no further significant increase is observed with increasing
40dsDNA concentration.

Fig. 4.6: A) The effect of 40dsDNA target concentration on EMS-MB, no left arrow indicates
lack of a general trend of monomer emission and the right arrow indicates general trend of
excimer emission due to target concentration increase, B) monomer and excimer peak emission
intensities, and C) average ratio of monomer to excimer intensities(n = 3)

EMS-MB:oxo-G

Fig. 4.7A presents example spectra for the EMS-MB:oxo-G titration from run 1 of the three
sample runs. Fig. 4.7B is an example of the monomer and excimer peaks for the EMS-MB:oxo-G
titration. The average ratios of the monomer and excimer intensities are seen in Fig. 4.7C with
the error bars, representing the standard error at each titration dilution over the experimental
runs. The monomer and excimer peak emissions are fairly similar throughout the concentration titration with no significant decrease or increase in excimer and monomer emissions.

Fig. 4.7: A) The effect of oxo-G target concentration on EMS-MB, no arrows indicates an unobservable trend due to concentration B) monomer and excimer peak emission intensities, and C) average ratio of monomer to excimer intensities (n = 3)

*EMS-MB Target Hybridization Comparison*

The monomer to excimer ratio for each EMS-MB target duplex is plotted together in Fig. 4.8. The increase in the ratio for EMS-MB:T0 and EMS-MB:T1 indicates that hybridization between the probe and these target sequences is somehow occurring. However, EMS-MB:T5 and EMS-MB:oxo-G have minimal hybridization between the probe and these targets. The overall
preference for target hybridization with the same 20 base target is in this order: T0 > T1 > T5 > oxo-G.

The EMS-MB showed strong hybridization to the 40ssDNA target, while not showing a large amount of hybridization to the 40dsDNA target, as shown in Fig. 4.9. It also shows that 40ssDNA acts similarly to the case of T0 target until the higher concentration (400 nM target) is reached, there appears to be an increase in binding or an increase in the separation of the pyrene moieties attached to the EMS-MB as indicated by the increase monomer to excimer ratio.

![Fig. 4.8: EMS-MB monomer to excimer ratio for each of the target concentration titrations in MB buffer. [EMS-MB] = 200 nM](image-url)
Fig. 4.9: EMS-MB monomer to excimer ratio comparison for concentration titrations of
40ssDNA, 40dsDNA and 20 base ssDNA T0 in MB buffer. [EMS-MB] = 200 nM

EMS-MB : T0 Titration in Presence of Genomic ctDNA

Fig. 4.10 is the average of the monomer to excimer ratios of three runs for each EMS-MB:
T0 concentration at four different ctDNA concentrations. Overall, it shows an increase trend
before 1:1 ratio and then levels off over the increase of genomic ctDNA concentrations,
regardless the standard error becomes larger when the hybridization takes place in the presence
of larger strands of genomic ctDNA.

MB2 target Hybridizations

MB2:T0

Fig.4.11A shows the spectra for the MB2:T0 concentration titration. Where the samples were
excited with a wavelength of 494 nm (λ_{exc}=494 nm), and emission intensities were recorded from
504 to 700 nm (λ_{em}=504-700 nm). Fig. 4.11B is an example of the FAM (518 nm) and TMR (578
nm) emission peaks from the MB2:T0 concentration titration. Notice the increase in the FAM
peak as the target concentration increases. The ratio of the FAM and TMR intensities are
presented in Fig. 4.11C. The FAM emission intensity increases with the increased target concentration, indicating that fluorescence is being restored as it is separated from the quencher.

Fig. 4.10: EMS-MB monomer to excimer ratio for T0 concentration titration in the presence of varying genomic ctDNA concentrations (0, 0.25, 0.75, 1.25, and 2.5 μg/ml) in MB buffer. [EMS-MB] = 200 nM

MB2:T1

Fig. 4.12A gives the spectra for the MB2:T1 concentration titration. Fig. 4.12B is an example of the FAM and TMR peak intensities from the MB2:T1 concentration titration. It is found that the FAM peak increases as the target T1 concentration increases till 100 nM. In contrast, TMR peak stays relatively stable over the T1 concentrations. The ratio of the FAM and TMR intensities are seen in Fig. 4.12C. Here the trend is similar to MB2:T0 where the ratio levels off when T1 concentration is above 100 nM.
Fig. 4.11: A) The effect of T0 target concentration on MB2, the left arrow indicates general trend of FAM emission (518 nm) and the right arrow indicates general trend of TMR emission (578 nm) due to target concentration increase, B) FAM and TMR peak emission intensities, and C) average ratio of FAM to TMR emission intensities

**MB2:T5**

The emission spectra for the MB2:T5 titration is seen in Fig. 4.13A. Fig. 4.13B is the FAM and TMR peak intensities from the MB2:T5 concentration titration. The FAM peak increases with an increase in target concentration. The ratio of the FAM and TMR intensities is seen in Fig. 4.13C, which shows a small increase in the ratio. There appears to be a slight increase in FAM fluorescence as the T5 target concentration is increased, but isn’t as significant as the MB2:T0 and MB2:T1 emission changes as can be seen Fig. 4.14, where the FAM/TMR ratio is plotted for each target concentration. The MB2:T0 and MB2:T1 have a larger increase in the ratio indicating a much stronger hybridization of the probe to target.
**Fig. 4.12:** A) The effect of T1 target concentration on MB2, the left arrow indicates general trend of FAM emission (518 nm) and the right arrow indicates general trend of TMR emission (578 nm) due to target concentration increase, B) FAM and TMR peak emission intensities, and C) average ratio of FAM to TMR emission intensities

**Comparison of EMS-MB with MB2**

Fig. 4.15 is a comparison plot of the ratiometric analysis of EMS-MB and MB2 in the presences of target DNA sequences (T0, T1, and T5). The emission ratio is representative of the monomer to excimer ratio of the EMS-MB and the FAM to TMR ratio for MB2. Table 4.2 is the contrast ratio determined for both EMS-MB and MB2 with their target concentrations. The contrast ratio for the EMS-MB and MB2 samples are fairly similar for this set of experiments.
Fig. 4.13: A) The effect of T5 target concentration on MB2, the left arrow indicates general trend of FAM emission (518 nm) and the lack of a right arrow indicates no general trend of TMR emission (578 nm) due to target concentration increase, B) FAM and TMR peak emission intensities, and C) average ratio of FAM to TMR emission intensities.

Fig. 4.14: Comparison of FAM (518 nm) to TMR (578 nm) emission peak ratio of MB2 hybridization with targets T0, T1, T5 in MB buffer. [MB2] = 200 nM.
Discussion

EMS-MB Target Hybridization

The EMS-MB in the presence of its complimentary target demonstrated the desired characteristics of our pyrene based MB. The excimer emission decreased with the increasing presence of T0, which indicates that more and more EMS-MB molecules were hybridizing with the target, as seen in Fig. 4.2A. A better look at this switch between monomer and excimer peak intensities can be seen in Fig. 4.2B, where once the EMS-MB is in the presence of T0 the excimer intensity increases and the monomer intensity decreases, causing the excimer and monomer intensity switch. The detection range is linear from a 1:0.2 to 1:1 ratio while the detection levels off near the 1:2 ratio of EMS-MB to targets. This can be seen in Fig. 4.2C where since the monomer increases and the excimer decreases with concentration, the ratio will increase as more EMS-MBs are bound to targets.

![Fig. 4.15: Ratiometric comparison for EMS-MB and MB2 in the presence of targets T0 (black), T1 (Red), and T5 (blue) in MB buffer. Both molecular beacons are at 200 nM.](image-url)
Table 4.2: Emission intensity ratio of EMS-MB and MB2 in the presence of targets T0, T1, T5. [EMS-MB, MB2] = 200 nM, [T0, T1, T5] = 400 nM

<table>
<thead>
<tr>
<th>Target</th>
<th>EMS-MB</th>
<th>MB2</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>1.937</td>
<td>2.119</td>
</tr>
<tr>
<td>T1</td>
<td>1.701</td>
<td>2.136</td>
</tr>
<tr>
<td>T5</td>
<td>1.136</td>
<td>1.359</td>
</tr>
</tbody>
</table>

Similar trends of hybridization of the probe and its targets can be seen in the presences of the one base mismatch target T1. Fig. 4.3A and Fig. 4.3B look similar to Fig. 4.2A, and Fig. 4.2B respectively. The difference between the T0 and T1 targets is better seen in the monomer excimer ratio of Fig. 4.3C where the values are not as high, indicating less of a response in the hybridization.

The EMS-MB in the presences of a 5 base mismatch appears to have minimal binding (See Fig. 4.4A and Fig. 4.4B), as the monomer and excimer peaks don’t appear to vary much with increasing concentration. Some hybridization appears to take place when examining Fig. 4.4C where an increase in monomer and decrease in excimer can be detected by the ratio analysis. This increase although is easily distinguishable when plotted alone, but when plotted in comparison with the EMS-MB in the presence of T0 and T1 a distinction between each of the targets binding affinity with the EMS-MB is apparent (see Fig. 4.8). This difference would allow for distinction of pathogens that infect humans versus a variation in species that could infect other animals. This is seen when comparing the binding of the EMS-MB to T0 and T5 (T5 corresponds to the genotype of C. baileyi). The response is dramatically different in T0 and allows for specificity in species detection. An EMS-MB probe can be developed in the detection for other pathogens or desired DNA target sequences.

Often the desired gene sequence to detect isn’t exactly 20 bases, but actually much larger and is in the format of double stranded sequences. To observe the EMS-MBs ability to
detect the 20 base sequence of a longer DNA strand, the 40ssDNA and 40dsDNA targets were synthesized. The EMS-MB successfully hybridized to the 40ssDNA target as seen in Fig.4.5.

Some hybridization took place between the EMS-MB and 40dsDNA target, but hybridization doesn’t increase with an increase in target concentration, as it does for the 40ssDNA target (see Fig. 4.6). This indicates that the 40dsDNA strand is more stable than the 20 base pair hybridization that takes place with the EMS-MB and its targets. This would indicate that if dsDNA was present in a sample the strands would have to be separated in order to accurately detect the desired sequence using the EMS-MB. The 40ssDNA target also appears to have a stronger binding effect at the highest concentration when compared to T0 (see Fig. 4.9). This however might not be true it could be that the 10 bases on either side of the target sequence are just preventing the pyrene moieties of the EMS-MB from coming closer and thus enhancing the monomer to excimer ratio.

There appears to be no hybridization taking place between the EMS-MB and the oxo-G target. The overall spectra demonstrate that there isn’t a lot of change in the emission intensity of the monomer and excimer peaks (see Fig. 4.7A and B). Even a look at the ratio of the monomer and excimer peaks doesn’t reveal any significant hybridization event (see Fig. 4.7C). Since the oxo-G sequence is complimentary to the detection sequence of the EMS-MB, it is important to take a closer look at the effect of oxidized carbon of the guanine base and how it affects the bonds and base conformation in a GC bond.

Fig. 4.16 is the molecular structure of a normal Watson-Crick GC base pair bond without oxidative damage. In this conformation the normal 3 hydrogen bonds can form between the GC base pairs. However, if the guanine is oxidized an anti and syn conformation can arise as seen in Fig. 4.17 and Fig. 4.18, respectively. The anti conformation is less stable due to the interaction of the oxygen interacting with the phosphate backbone. This interaction is the most likely cause to have the 8-oxoG base change into the syn conformation. The oxo-G (syn) conformation allows for only two hydrogen bonds to form in the GC pairing. However, it is also shown that the oxoG (syn) can form a paring with the base adenosine. This pairing of bases is known as a Hoogsteen base
pairing. This base pairing occurs when a polymerase misreads the G base and incorporates an A in place of C into the DNA sequence, which gives rise to mutations in the DNA (42). The fact that our EMS-MB isn’t hybridizing with our oxo-G sequence indicates that the G is in the syn conformation. This implies that the GC bond at the oxidative G doesn’t want to form, and as a result no hybridization is taking place. Further molecular modeling could be performed to determine if the 8-oxoG base prefers to bind to an A or C, but seen in our case the target sequence isn’t binding with the base C of the EMS-MB.

Fig. 4.16: GC base pair in normal Watson-Crick base pairing

Fig. 4.17: 8-oxo GC base pair anti conformation, oxidative damage indicated by arrow.
Fig. 4.18: 8-oxo GC base pair syn conformation, oxidative damage indicated by arrow.

The EMS-MB binds more intensely to the complimentary target and allows for a distinction of one base mismatch. Where a single base differs in a codon of gene sequence is known as a single nucleotide polymorphism (SNP). SNPs have been linked to various diseases and are important to detect in the screening of diseases, where conventional MBs can also be used (24). The detection capability of pyrene-based MB is comparable to other MB (33).

EMS-MB: T0 Titration in Presence of ctDNA

The selectivity of the EMS-MB was determined by observing the hybridization of the probe with T0 in the presence of varying amounts of genomic ctDNA with an average size of 13kb. The concentration titrations of EMS-MB and T0 in the varying ctDNA concentrations demonstrated that the EMS-MB has high selectivity for a 20 base DNA sequence as represented by T0. The genomic ctDNA didn’t impede the EMS-MBs ability to detect the target sequence as seen when looking at the monomer and excimer ratios in Fig. 4.10. This demonstrates that the EMS-MB can detect small amounts of DNA amidst larger groups of DNA, which is a possible scenario in the detection of Cryptosporidium parvum DNA from a water sample. This would be necessary for the potential use of the EMS-MB without DNA amplification in the detection of Cryptosporidium (33).
**MB2 Target Hybridization**

MB2 is an example of a conventional MB with a fluorophore (FAM) at the 5’ end and quencher (TMR) at the 3’ end of the MB. However both FAM and TMR are fluorophores and the fluorescent emission of FAM is quenched by TMR which also emits fluorescent profile, and allows for a ratiometric analysis the probes hybridization capabilities by taking the ratio of the FAM emission peak where fluorescence increases as it is separated from the quencher TMR emission peak which remains fairly constant. This ratiometric detection method is seen when MB2 is in the presence of T0 and T1 (Fig. 4.11 and Fig. 4.12). The FAM peak at 518 nm increases fluorescence as the FAM fluorophore is separated from the TMR quencher, which peak remains relatively constant at 578 nm as the amount hybridization increases. A closer look at this change is seen by investigating at the emission of the FAM and TMR peaks alone as seen in Fig. 4.11B and Fig. 4.12B for MB2 in the presence of T0 and T1 respectively. The ratio of FAM to TMR peaks relates both peaks to a quick method of determining the detection range and specificity of the MB2 in the presence of T0 and T1 are seen in Fig.4.11C and Fig. 4.12C. However, there isn’t much change in the peaks when MB2 is in the presence of T5 as see in Fig. 4.13. Once again there appears to be an increase in the ratio of FAM to TMR as T5 concentrations increases (see Fig. 4.13C), but when compared to the ratios of MB2: T0 and MB2:T1 there is a significant difference (see Fig. 4.14). It is also interesting to note that the differences in the ratios of MB2:T0 and MB2:T1 are very similar over the entire target concentration range. This closeness indicates that it might be hard to distinguish a 1 bp difference in the target concentration for MB2.

**Comparison of EMS-MB and MB2 Detection**

To compare the two molecular beacons, the contrast ratio (Eq. 4.1) was determined from the ratio of the open to closed beacons, which was taken from the monomer to excimer peak ratio for the EMS-MB and from the FAM to TMR ratio for MB2. Fig. 4.15 is a compilation of the ratios collected for the MBs in the presences of targets where the larger ratio number is representative of the MBs in the open conformation, and the smaller ratio number is representative of the MBs in
the closed conformation. Thus, values for open beacons were taken from the ratios at the highest target concentration and values for closed beacons were taken from MBs without the presence of targets. Generally a higher contrast ratio is desired to indicate that the beacons are opening and hybridizing to the target concentrations. As seen in Table 4.2, the values are slightly higher for MB2, but they are both very similar in values. It was also difficult to distinguish 1bp difference for MB2 in our experiments (see Fig. 4.15). With its enhanced ability to distinguish 1 bp difference, the EMS-MB has an advantage (see Fig. 4.15) over conventional MB with two fluorophores.

Conclusion

The EMS-MB is able to distinguish between complimentary targets, one base mismatch targets, and a five base mismatch hsp 70 gene sequences of human genotypes of Cryptosporidium. This allows for a probe with high specificity for its target sequence. The EMS-MB was better able to distinguish a 1 base mismatch better than a conventional MB as demonstrated with MB2 with two fluorophores. They both however perform well in the hybridization to targets as demonstrated by the contrast ratio of the probes.

The oxidative damaged DNA was unable to hybridize to the EMS-MB. This is most likely due to the conformation that occurs with the damaged GC base pair. Magnesium also didn’t affect the stability of this interaction. The interaction with other metals in the hybridization could be looked at for future research. Since the damaged DNA reacted similar to the hybridization with T5 would be difficult to distinguish the two strands.

The EMS-MB provides a quick detection of synthetic Cryptosporidium parvum DNA. It has high selectivity and specificity in detection of target DNA. This probe design could be used in other DNA detection applications such as other pathogens.
CHAPTER 5
EMS-MB AND TARGET THERMODYNAMICS AND MELTING STUDIES

Objective

A key parameter in DNA detection is the melting temperature (T<sub>m</sub>) of DNA duplex. Melting temperatures are used for thermodynamic analysis, and operating procedures in real time PCR DNA detection. A novel feature of our EMS-MB is that the melting temperature is rapidly determined during a thermal denaturation profile of the EMS-MB in the presence of its target DNA sequence. This new rapid detection of the melting temperature allows for saved time, reduced experimental cost, and extended datable concentration range of target sequences. Melting temperature of DNA also provides a quick method in determining the stability of a DNA duplex, in general the higher the melting temperature, the more stable the duplex. The determination of the melting temperature from the fluorescence spectrometry (FL) method was verified using the standard UV-Vis spectrophotometry methods. With the T<sub>m</sub> the thermodynamic properties will be determined.

Material and Methods

EMS-MB Melting Temperature for FL and UV-Vis Measurements

The melting temperature for the EMS-MB (200 nM) in the presence of the target sequences (400 nM) in MB buffer is determined using fluorescent thermal denaturation profiles and verified with UV-Vis denaturation profiles. The fluorescent thermal denaturation profiles samples will be heated from 10 °C to 90 °C and allowed to equilibrate for 4 minutes. Spectra will be recorded at 1°C increments during the cooling process and allowed to equilibrate for four minutes at each temperature step.

A Shimadzu UV-1800 spectrophotometer with attached temperature control unit is used for UV-Vis denaturation profiles. The EMS-MB will be prepared at a 1:2 (1µM:2µM) dilution to target
DNA sequences. The oxo-G sample was not included in this measurement since no hybridization was detected in neither concentration titrations nor melting profiles. Samples will be heated from 15-105 °C at a rate of 0.5 °C/ min and absorption will be measured at 260 nm. T_m analysis software will be used to measure the T_m for each EMS-MB target sample using an average method provided in the software. We will also assess the effects of magnesium on the melting temperature from both fluorescence and UV melting temperature methods. Magnesium concentrations from 0-125 mM will be examined and an average of three runs for each sample will be used.

In order to determine if the fluorophores (pyrene) of the EMS-MB have an effect on melting temperatures, a sample of double stranded DNA (designated as dsEMS-MB) with the exact sequence of the EMS-MB:T0 duplex was synthesized by Syngen (Hayward, CA.), refer to Table 4.1 for sequence of EMS-MB:T0. T_m will be measured using UV-Vis spectrophotometry and determined for the varying magnesium concentrations (0-125 mM).

**Thermodynamics of EMS-MB Phase 1 to 2**

As discussed in Tech Chapter II the MBs exist in three phases: phase 1 where the MB is bound to DNA, phase 2 where the MB is in the stem and loop structure, and phase 3 where the MB is in a random coil phase. Phase 2 to 3 thermodynamics was discussed in Tech Chapter II; in this chapter the phase 1 to 2 thermodynamics will be investigated and discussed. In order to relate the phase 2 to 3 thermodynamic properties to the recorded data a thermal denaturation profile for the EMS-MB was recorded with 1°C steps between recorded spectra and the thermodynamic parameters were calculated in Tech Chapter II. Thermodynamic parameters in transition Phase 1 to 2 were calculated for EMS-MB: T0 and EMS-MB: T1. The thermodynamic parameters are based on the disassociation constant at the melting temperature of the probe and target duplex. Since the melting temperature is defined as the temperature where half of the probes and targets are separated the dissociation constant (k_{f,t}) is determined by Eq. 5.1 where Ta is the concentration of targets and MB is the concentration of probes. The dissociation
constant can then be combined with the free energy equation (Eq. 5.2) and linearized to determine the enthalpy and entropy at the melting temperature (Eq. 5.3). Where \( R \) is the universal gas constant, \( T_m \) is melting temperature (41). The \( T_m \) will be determined using measurements from the spectrofluorometry for concentrations at [EMS-MB] = 200 nM, [T0] and [T1] =0.4 \( \mu \)M. The \( T_m \) will be used in Eq. 5.3 to determine the enthalpy (\( \Delta H \)) and entropy (\( \Delta S \)) of the reaction. The enthalpy and entropy will then be used to determine the free energy (\( \Delta G \)) of the reaction from Eq.5.2.

\[
k_{12} = [Ta] - 0.5[MB] \quad (5.1)
\]

\[
\Delta G = -RT \ln k = \Delta H - T \Delta S \quad (5.2)
\]

\[
\ln([Ta] - 0.5[MB]) = -\frac{1}{T_m} \Delta H_{12} + \Delta S_{12} \quad (5.3)
\]

Results

EMS-MB Fluorescence Melting Profiles

EMS-MB: T0 Thermal Melting Profiles

Fig. 5.1 gives the spectra recorded for the thermal denaturation profile of a 200 nM EMS-MB in the presence of 400 nM T0 target DNA. A change in the excimer emission can be seen as the EMS-MB reaches a higher temperature. To get a better look, the excimer and monomer peaks were plotted against temperature in Fig. 5.2. The excimer emission initially increases then decreases again for a few degrees before increasing. The excimer intensity is representative of the physical conformation of the pyrene moieties.
Fig. 5.1: EMS-MB: T0 thermal denaturation profile, where the arrow indicates that data were recorded while sample was cooled.

Fig. 5.2: EMS-MB: T0 monomer and excimer peaks from thermal denaturation profile, where the arrow indicates that data were recorded as sample was cooled.
EMS-MB: T1 Thermal Melting Profiles

Fig. 5.3 shows the spectra recorded for the thermal denaturation profile of a 200 nM EMS-MB in the presence of 400 nM T1 target DNA. The excimer and monomer peaks were plotted against temperature in Fig. 5.4 and follow a trend as seen in the EMS-MB:T0 thermal melts. The interesting features are the peak that occurs at a higher temperature of the excimer emissions. This peak is thought to represent the melting temperature of the probe target duplex. This is verified later with UV-Vis spectrophotometry.

Fig. 5.3: EMS-MB: T1 thermal denaturation profile, where the arrow indicates that data were recorded while sample was cooled

EMS-MB:T5 Thermal Melting Profiles

Fig. 5.5 illustrates the spectra recorded for the thermal denaturation profile of a 200 nM EMS-MB in the presence of 400 nM T5 target DNA. The melt profile most resembles the EMS-MB only where the monomer and excimer emissions steadily decrease as the temperature is
increased (see Fig. 5.6). This indicates that there is no reformation of the stem and loop structure after any hybridization with the target.

**EMS-MB:oxo-G Thermal Melting Profile**

The melting profile for EMS-MB (200 nM) in the presence of oxo-G (400 nM) is reported in Fig. 5.7. The monomer and excimer emission intensities continually decrease as the temperature of the solution is increased (see Fig. 5.8). This decrease in excimer indicates that the pyrene moieties are increasingly separating from each other as the DNA strands melt. There is no increase in the excimer emission at a higher temperature as there is when the EMS-MB is in the presence of T1 and T5 (see Fig. 5.1 and Fig. 5.3). This continual decrease indicates that no hybridization is taking place. This indicates that the decrease in excimer emission is more of a result of the temperature increase rather than any hybridization between the probe and target.
Fig. 5.5: EMS-MB: T5 thermal denaturation profile, where the arrow indicates that data were recorded while sample was cooled.

Fig. 5.6: EMS-MB: T5 monomer and excimer peaks from thermal denaturation profile, where the arrow indicates that data were recorded as sample was cooled.
**EMS-MB: Targets Comparison**

Since the excimer emission peak changes was more pronounced in the EMS-MB to target hybridizations a comparison plot of the EMS-MB to the targets was plotted, as seen in Fig. 5.9. The excimer peak occurs for the EMS-MB in the presence of targets, and a hint of one can be seen in the presence of T5. While the EMS-MB by itself shows no such increase in the excimer peak as the temperature increases. The EMS-MB:oxo-G solution follows similar to the EMS-MB only melts. The lack of change in the oxo-G profile indicates minimal hybridization if any. The general trend of stability of the melting profiles is T0 > T1 > T5 > oxo-G due to the location of the excimer peak that occurs at higher temperatures as the EMS-MB hybridizes with its target (see Fig. 5.9).
Fig. 5.8: EMS-MB: oxo-G monomer and excimer peaks from thermal denaturation profile, where the arrow indicates that data were recorded as sample was cooled.

Fig. 5.9: Comparison of EMS-MB: targets thermal denaturation in MB buffer. The arrow indicates the direction the data were recorded as the sample was cooled. [target] = 200 nM, [EMS-MB] = 400 nM.
**Magnesium Effect on EMS-MB: Target Hybrids Melting Profiles**

The excimer peaks were recorded from thermal denaturation profiles of the EMS-MB hybrids (200 nM) with T0 and T1 (400 nM) in presence of magnesium concentrations ranging from 0-125 mM (in the form of MgCl$_2$). Profiles were normalized so that the relative intensity equals 1 at 90 °C. Fig. 5.10 is the excimer peak melting profile for run 1 of the two runs for the EMS-MB: T0 hybrid under Mg titrations. Fig. 5.11 is the excimer peak thermal melting profile for run 1 of the two runs of the EMS-MB: T1 under Mg titrations. Another repeat data not shown but will be used in comparing $T_m$ to those obtained from UV-Vis spectrophotometry method.

![Graph showing excimer melting profiles](image)

**Fig. 5.10**: EMS-MB: T0 (200 nM:400 nM) Excimer Melting profile for magnesium titrations.

Mg$^{2+}$ is increased from 0 to 125 mM.

**UV-Vis Spectrophotometry**

Thermal melts were determined for the EMS-MB in the presence of T0, T1, and T5 using UV-Vis thermal melts where absorbance was measured at 260 nm (see Fig. 5.12). The $T_m$ analysis software was used to determine the $T_m$, however $T_m$ was only determinable for EMS-MB:T0 and EMS-MB:T1.
Fig. 5.11: EMS-MB: T1 (200 nM: 400 nM) Excimer Melting profile for magnesium titrations. Mg$^{2+}$ is increased from 0 to 125 mM.

Fig. 5.13A, B, and C represent the magnesium concentration titrations for EMS-MB:T0, EMS-MB:T1 and dsEMS-MB, respectively. In general, the T$_m$ increases for each sample as the magnesium ion concentration increases. These figures are a sample of one of the three runs that were used for each DNA sample. The T$_m$ values determined are reported in the following section where a comparison of FL and UV-Vis measured T$_m$ values is performed.

Fig. 5.12: UV-Vis thermal melting profile of EMS-MB in the presence of targets
Fig. 5.13: The effect of Mg$^{2+}$ ion concentration on UV-Vis thermal melting of A)EMS-MB: T0, B)EMS-MB: T1, and C) dsEMS-MB, where [Mg$^{2+}$] = 0, 0.5, 1, 2, 3, 4, 5, 10, 20, 50, 125 mM, [EMS-MB] = 1 μM, [T0] = 2 μM, [T1]=2 μM, and [dsEMS-MB]= 2 μM in MB buffer solution.

Comparison of Fluorescence and UV-Vis Measured $T_m$

A comparison of melting temperatures obtained from fluorescence thermal denaturation profiles and UV-Vis thermal melting profiles was observed for the EMS-MB in the presence of targets and varying Mg$^{2+}$ concentrations. Table 5.1 is a comparison of the melting temperatures ($T_m$) determined from the fluorescence (FL) and UV-Vis melting methods. Further analyses were accomplished with comparing the $T_m$ values determined from Mg$^{2+}$ titrations. Mg$^{2+}$ was chosen because of its known affects on DNA stability (7). The $T_m$ determined from the UV-VIS and FL method for EMS-MB: T0 at varying Mg$^{2+}$ concentrations is shown in Fig. 5.14A, while EMS-MB:T1 is presented in Fig. 5.14B, where the standard error shown was determined from the average of the runs.
The $T_m$ determined from the FL method was also compared to a double stranded DNA (dsEMS-MB) with the same DNA sequence as the EMS-MB:T0 duplex, but without the attached fluorophores.

Fig. 5.15 is a comparison of the measured $T_m$ values from the FL method using the EMS-MB: T0 sample and the $T_m$ for the dsESM-MB determined using the UV-Vis method. To further compare the $T_m$, they were averaged and the standard error was determined from the values obtained from the sample runs, the maximum standard error for the comparison of averages was only 1% (see Fig. 5.16).

<table>
<thead>
<tr>
<th>Sample</th>
<th>UV-Vis (°C)</th>
<th>Fluorescence (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMS-MB</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>EMS-MB:T0</td>
<td>61.39 ± 0.71</td>
<td>62.5 ± 0.65</td>
</tr>
<tr>
<td>EMS-MB:T1</td>
<td>51.921 ± 3.9</td>
<td>50 ± 0.0</td>
</tr>
<tr>
<td>EMS-MB:T5</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Note: $T_m$ value from UV melt is referred to the temperature at the midpoint of UV absorbance. The $T_m$ value from the Fluorescence curve refers to the excimer peak emission.

**Thermodynamics of EMS-MB**

The thermodynamics for the EMS-MB only was determined from the Van’t Hoff plot in Fig. 5.17 where measurements were taken at 1 °C increments. Using Eq. 5.3 the thermodynamics were determined for the EMS-MB: T0 and EMS-MB: T1 hybridizations as seen in Fig. 5.18. The enthalpy ($\Delta H$) and entropy ($\Delta S$) of the reactions are listed in Table 5.2. These parameters were used in Eq. 5.2 to determine the free energy ($\Delta G$) over the temperature range. The free energy
of these phase transitions is seen in Fig. 5.19, where the random coil phase or phase 3 is represented by $\Delta G = 0$ kcal/mol because no reaction is taking place.

Fig. 5.14: $T_m$ measured with FL peak and UV-Vis for A) EMS-MB: T0, and B) EMS-MB: T1 in the presence of Mg$^{2+}$ Concentrations (0 to 125 mM)

Fig. 5.15: $T_m$ comparison for EMS-MB:T0 duplex and dsEMS-MB in the presence of varying Mg$^{2+}$ concentrations (0 to 125 mM)
Fig. 5.16: T_m profiles obtained from the averaging the datasets of EMS-MB:T0 by FL method and for dsEMS-MB by UV-Vis method

Fig. 5.17: EMS-MB only Van’t Hoff Diagram. The slope is used to determine ΔH; while the intercept is used to determine ΔS
Table 5.2: Enthalpy ($\Delta H$) and Entropy ($\Delta S$) for EMS-MB in the presence of targets

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\Delta H$ (kcal/mol)</th>
<th>$\Delta S$ (cal/mol K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMS-MB only</td>
<td>22.1</td>
<td>69.6</td>
</tr>
<tr>
<td>EMS-MB:T1</td>
<td>121.9</td>
<td>352.6</td>
</tr>
<tr>
<td>EMS-MB:T0</td>
<td>146.4</td>
<td>406.1</td>
</tr>
</tbody>
</table>

Discussion

**EMS-MB target and Mg$^{2+}$ Melting Curves**

The EMS-MB in the presence of targets when put through a thermal denaturation profile revealed interesting features of the excimer peak as the temperature increased. The excimer peak is initially high at lower temperatures but as the temperature increases the EMS-MB opens and hybridization can occur when targets are present. A trough appears for melts of EMS-MB:T0 and EMS-MB:T1 as seen in Fig. 5.1 and Fig. 5.3. The low point of this trough represents when...
the EMS-MB is the most stable in its hybridized form to the target DNA. A look at just the excimer peaks in Fig. 5.2 and Fig. 5.4 shows the trough of the spectra clearly. It’s also interesting to note that EMS-MB: T0 is more stable at a higher temperature than EMS-MB:T1 as indicated by the shift of the emission spectra towards higher temperatures.

![Graph showing free energy vs. temperature for EMS-MB only (phase 2), EMS-MB: T0 (phase 1), and EMS-MB:T1 (phase 1). Where a ΔG = 0 represents the random coil phase (phase 3)](image)

A novel feature of the EMS-MB appears in Fig. 5.2 and Fig. 5.4 where after the low point of the trough the excimer fluorescent emission increases in intensity. An increase in excimer emission indicates that a portion of the EMS-MBs are reforming into the stem and loop structure. This increase continues for a few degrees until the energy from the increase temperature causes the EMS-MB to pull apart and enter the random coil phase. The peak after the trough is thought to be representative of the melting temperature of the EMS-MB and the target sequence. This peak also appears at a higher temperature for EMS-MB: T0 hybrid than from EMS-MB:T1 hybrids. This again demonstrates the increased stability of the EMS-MB:T0 duplex. However, this trough and peak is not distinguishable for EMS-MB:T5 thermal melts, neither in EMS-MB
oxo-G thermal melts (see Fig. 5.5 and Fig. 5.7). A slight trough and peak occurs early on (<20 °C) in the EMS-MB:T5 hybrid as seen in Fig. 5.6, but due to the lack of stability in this bond any hybridization is quickly lost as the temperature increases. This confirms with what was seen in the EMS-MB: T5 and EMS-MB: oxo-G target titrations where minimal hybridization occurred.

To confirm that this peak at higher temperature isn't a result of other factors, the melting profile for EMS-MB only was also measured. When comparing the profiles in Fig. 5.9 the excimer intensities of the EMS-MB: T0 and EMS-MB: T1 solutions line up with the EMS-MB only melt after the peak that represents the T\textsubscript{m}. Since no trough or peak occurs in the EMS-MB only run it is determined that once the probe begins melting it doesn’t reform to the stem and loop structure as the temperature increases. However, for the EMS-MB: T0 and EMS-MB: T1 samples to match the EMS-MB only profile, indicates that there still exists a fraction of the EMS-MBs in the stem and loop structure, and that the EMS-MB:T0 and EMS-MB:T1 solutions reform a fraction of the EMS-MBs into the stem and loop structure. Since the peak doesn’t keep increasing after this point, this indicates the point where the beacon is unraveling and entering the random coil phase.

Magnesium often plays an important role in DNA stability, the melting temperatures (T\textsubscript{m}) were determined for EMS-MB:T0 and EMS-MB:T1 in the presence of varying magnesium ion concentrations (0-125 mM). Fig.5.14A and Fig. 5.14B represent the excimer peak intensities of the thermo melts for the magnesium ion concentrations titrations for EMS-MB:T0 and EMS-MB:T1 respectively. They both demonstrate the trough and peak trend as before and reveal that as the magnesium ion concentration increases the Tm increases, especially a remarkable increase of T\textsubscript{m} is observed in the Mg\textsuperscript{2+} ion concentration lower than 20 mM, and subsequent level off is observed when Mg\textsuperscript{2+} ion concentration at above 20 mM . The positive charge of the magnesium ion possibly interacts with the negatively charged phosphate backbone of the DNA molecular structure by adding extra stability to the hydrogen bonds that form in a DNA duplex. The magnesium is creating a stabilizing effect and is allowing for increased T\textsubscript{m} for the probe target duplex of EMS-MB:T0 and EMS-MB:T1.
**UV-Vis verification for EMS-MB $T_m$**

In order to verify that the $T_m$ determined from the fluorescent thermal denaturation profiles is accurate, UV-Vis spectrophotometry thermal melts were used to determine the Tm of the EMS-MB in the presence of targets and in varying magnesium ion concentrations (see Fig. 5.13A and B). We also verified the $T_m$ with a synthesized DNA duplex (designated as “dsEMS-MB”) consisting of the EMS-MB DNA sequence and T0 sequence in varying concentrations of Mg$^{2+}$ (see Fig. 5.13C).

Table 5.1 summarizes the $T_m$ values of EMS-MB in the presence of each targets (T0, T1, and T5). These values are obtained from FL and UV-Vis methods. It is found that both methods give extremely similar $T_m$ results. Further comparison was accomplished through magnesium ion titrations. Fig. 5.14A and B represent the $T_m$ measured for both methods. The $T_m$ quickly increase as the magnesium ion concentration increases (till 20 mM) then it reaches a saturation point when Mg ion concentration is above 20 mM.

The duplex DNA of the dsEMS-MB (which has no conjugated fluorophore dyes) was used to prove that the fluorophores attached to the EMS-MB have a minimal effect on the melting temperature of the probe target duplex.

Fig.5.15 is a comparison of the $T_m$ value determined by FL and UV-vis methods for dsEMS-MB at varying Mg$^{2+}$ concentrations. It can be seen that for both measurement methods, that the trend is that the Mg$^{2+}$ ions quickly increases the $T_m$ at lower concentrations (< 20 mM). Where at the higher Mg$^{2+}$ concentrations the Tm increase is slower as the DNA becomes saturated with ions(>20 mM). Fig. 5.16 is the average of the FL measured $T_m$ values for EMS-MB:T0 hybrids and for the $T_m$ values determined with UV-Vis for dsEMS-MB . By averaging these $T_m$ and analyzing the standard error, concentrations where the $T_m$ values are different. When compared the standard error reaches a maximum of 1%. This difference is small enough that it can be said that the pyrene fluorophores attached to the 5’ and 3’ ends of the EMS-MB don’t significantly affect the melting temperature of the duplex. This information further justifies the use the excimer peak as the melting temperature of the EMS-MB probe.
**Thermodynamics of EMS-MB**

Since the $T_m$ determined from fluorescent thermal denaturation profiles was verified by using UV-Vis spectrophotometry melting profiles, the $T_m$ values can be used in determining the thermodynamic properties of the phase 1 to phase 2 transitions for the EMS-MB. The enthalpy ($\Delta H$) and entropy ($\Delta S$) for the EMS-MB only were determined as before in Chapter 2, but the temperature step size was changed to a 1 °C increment. Fig. 5.17 is the Van’t Hoff plot for the EMS-MB only where the enthalpy ($\Delta H$) (22.1 kcal/mol) was determined from the slope of the line, and the entropy ($\Delta S$) (69.6 cal/mol k) from the intercept, according to Eq. 5.3. Using to plot the Van’t Hoff plots of Fig. 5.18 the enthalpy (146.9 and 121.9 kcal/mol) and entropy (406.1 and 352.6 cal/mol k) were determined for EMS-MB:T0 and EMS-MB:T1 from the slope and intercept, respectively. These values are expected, since enthalpy is dependent on the number of bonds formed between base pairs, and entropy is dependent on the number of conformations possible in a given phase (41).

The values of enthalpy ($\Delta H$) and entropy ($\Delta S$) listed in Table 5.2 were used in Eq. 5.2 to plot the free energy as shown in Fig. 5.19. In general, the phase that dominates at a given temperature is the one with the lowest free energy (41). This indicates that the EMS-MB:T0 phase is preferred over varying temperatures than if a 1 base mismatch was present or any other target.

**Conclusion**

The novel feature for quickly determining the $T_m$ from an EMS-MB fluorescence thermal profile allows for the saving of time and equipment. Often $T_m$ is determined in real time PCR machines but these machines are often limited in the ranges of excitation for fluorophores. Often it is required to use a certain set of fluorophores. This limits the range and capabilities of MBs that can be employed in these experiments. The EMS-MB allows for a quick fluorescence based
T<sub>m</sub> detection method along with the ability to determine the hybridization to the desired target in real time.

This T<sub>m</sub> is a valuable component in determining the thermodynamic properties of MBs. The EMS-MB:T0 was shown to be the most stable format of the EMS-MB: target samples. This indicates that the most bonds were formed for the EMS-MB: T0 duplex and is stable over a wider range of temperature than the other EMS-MB:target duplexes.

The bivalent cation magnesium has a quantifiable effect on the hybridization of the EMS-MB to its target DNA sequences. In general, the stability of the probe target duplex increased as the magnesium ion concentration increased until the concentration level reached a saturation point (usually after a 20 mM under our experimental conditions). After this 20 mM point, the effect on Tm wasn’t as great.

Our EMS-MB provides rapid quantification of specificity, selectivity, and stability in the detection of synthetic DNA sequences. As shown in the detection of targets, and the melting studies comparison of UV-Vis to fluorescence.
CHAPTER 6
GENERAL CONCLUSION

This research demonstrates the physical and thermodynamic properties of the EMS-MB. The EMS-MB was successfully synthesized so that the beacon exists in the three phases of molecular beacons. The EMS-MB forms the desired stem and loop structure. This means that when the pyrene fluorophores attached to the stem form are in close proximity an excimer emission peak is more pronounced. In contrast when the stems are pulled separated, whether by heat or hybridization, the excimer emission peak intensity decreases, while the monomer peak intensity increases. The EMS-MB also has a high quantum yield, which is allows for easier detection of the EMS-MBs conformation.

I have also shown that magnesium ion has a stabilizing effect on the EMS-MB, with minimal concentrations. This was demonstrated by the determination of the thermodynamic properties of enthalpy. An addition of greater than 0.5 mM magnesium ion induced a stabilization effect on the EMS-MB. This had a maximum stabilizing effect at 4 mM magnesium ion, however ANOVA analysis demonstrated that the 4 mM solution didn’t have a significant difference from other magnesium ion concentrations.

The EMS-MB successfully demonstrated detection of the hsp 70 of Cryptosporidium parvum. The EMS-MB demonstrates high specificity with its ability to detect a difference with one base mismatch in the target DNA. The EMS-MB also demonstrated selectivity, by detecting the complimentary target DNA amidst large amounts of calf thymus DNA.

The EMS-MB was also shown to be comparable to conventional MBs. This was determined by observing the hybridization of both beacons to DNA targets, and demonstrated by determining a contrast ration. The advantage of the EMS-MB was that of a greater ability to distinguish between 1 base mismatch of the target DNA. This selectivity is important when searching for mutations and when distinguishing between species.

The detection of oxidative damaged DNA provided some interesting results. The oxidative damaged complimentary DNA showed minimal hybridization to the EMS-MB. The initial
results indicate that the oxidized G base results in a base conformation change due to steric hindrance. This conformation change of the G base potentially makes a conformation change of the target sequence, resulting in minimal detection.

The Tm of the EMS-MB can be rapidly determined from thermal denaturation melts using fluorescent spectroscopy, without the aid of extra calculations. This allows for the elimination of the use of a UV-VIS spectroscopy, an industry standard, in determining DNA melting temperatures.

Finally the thermodynamic properties of the EMS-MB were observed for the hybridization to target sequences. A complimentary base demonstrated the most stability between the EMS-MB:Target duplex. This was also demonstrated by the rapid determination of Tm by using only fluorescent spectroscopy.

Our EMS-MB provides rapid quantification of specificity, selectivity, and stability in the detection of synthetic DNA sequences. As shown in the detection of targets, and the melting studies comparison of UV-Vis to fluorescence. The EMS-MB shows great potential for rapid pathogen detection from minimal amounts of DNA.
CHAPTER 7
ENGINEERING PRINCIPLES and FUTURE RESEARCH

The main engineering principles gained from this thesis work, is in the set up of design controls. The first step in this process is to determine what type of results and functionality do we need to achieve for the EMS-MB. This was achieved by the design of the EMS-MB to specifically target a pathogen that is known to cause problems in water sources around the world. The functionality requires the EMS-MB to detect a gene that has been well studied in the species of *C. parvum*.

The second step in this process is to determine the feasibility of the EMS-MB to meet the requirements necessary for the detection of the pathogen DNA. Experiments were designed to address the specificity, selectivity, and the stability required for DNA detection. This is an iterative process where multiple experiments and test configurations were performed to determine the specificity, selectivity, stability of the EMS-MB.

The final steps would be for the EMS-MB to be applied in a real world scenario to determine if the feasibility experiments match. This was not addressed in this thesis, and future research and work will need to be performed. Some of the areas of future research that would be a benefit to this project are as follows:

- Temperature dsDNA research: Expand ability of EMS-MB to detect pathogen DNA when it is in its double stranded form. This would provide the potential of detecting the pathogen DNA, without separating the DNA into single strands
- Genomic DNA studies: Gaining a better understanding of the different target areas, could enhance the detection and selectivity of molecular beacons.
- Thermal profiling with PCR: Determine EMS-MB ability in detecting DNA during a PCR chain reaction. This could help with rapid detection and when
coupled with lab-on-a-chip technologies could provide effective detection methods in the field.

- Determine Oxo-G hybridization probe: Modeling of hybridization with an EMS-MB with damaged DNA could provide insights into detection of mutations and mechanisms for what’s causing the inability for hybridization to occur as seen in this thesis.
REFERENCES


Fig. A.1: 200 nM EMS-MB thermal denaturation profile ($\lambda_{\text{exc}}=345\text{nm}, \lambda_{\text{em}}=357-650\ \text{nm}$)
run 2, arrow indicates direction from heating to cooling from 10-90-10 °C

Fig. A.2: 200 nM EMS-MB thermal denaturation profile ($\lambda_{\text{exc}}=345\text{nm}, \lambda_{\text{em}}=357-650\ \text{nm}$)
run 3, arrow indicates direction from heating to cooling from 10-90-10 °C
Fig. A.3: 2 μM EMS-MB thermal denaturation profile ($\lambda_{\text{exc}}=345\text{nm}$, $\lambda_{\text{em}}=357-650\text{ nm}$) run 2, arrow indicates direction from heating to cooling from 10-90-10 °C

Fig. A.4: 2 μM EMS-MB thermal denaturation profile ($\lambda_{\text{exc}}=345\text{nm}$, $\lambda_{\text{em}}=357-650\text{ nm}$) run 3, arrow indicates direction from heating to cooling from 10-90-10 °C
Fig. A.5: $K_{23}$ for 200 nM EMS-MB

Fig. A.6: $K_{23}$ for 2 μM EMS-MB
Fig. A.7: A) The effect of Mg$^{2+}$ concentration on EMS-MB emissions at 15 °C, the left arrow indicates general trend of monomer emission and the right arrow indicates general trend of excimer emission due to Mg$^{2+}$ increase, and B) is the effect of Mg$^{2+}$ concentration on monomer and excimer emissions at 15 °C.

Fig. A.8: A) The effect of Mg$^{2+}$ concentration on EMS-MB emissions at 20 °C, the left arrow indicates general trend of monomer emission and the right arrow indicates general trend of excimer emission due to Mg$^{2+}$ increase, and B) is the effect of Mg$^{2+}$ concentration on monomer and excimer emissions at 20 °C.
Fig. A.9: A) The effect of Mg$^{2+}$ concentration on EMS-MB emissions at 25 °C, the left arrow indicates general trend of monomer emission and the right arrow indicates general trend of excimer emission due to Mg$^{2+}$ increase, and B) is the effect of Mg$^{2+}$ concentration on monomer and excimer emissions at 25 °C

Fig. A.10: A) The effect of Mg$^{2+}$ concentration on EMS-MB emission at 30 °C, the left arrow indicates general trend of monomer emission and the right arrow indicates general trend of excimer emission due to Mg$^{2+}$ increase, and B) is the effect of Mg$^{2+}$ concentration on monomer and excimer emissions at 30 °C
Fig. A.11: A) The effect of Mg\(^{2+}\) concentration on EMS-MB emissions at 35 °C, the left arrow indicates general trend of monomer emission and the right arrow indicates general trend of excimer emission due to Mg\(^{2+}\) increase, and B) is the effect of Mg\(^{2+}\) concentration on monomer and excimer emissions at 35 °C.

Fig. A.12: A) The effect of Mg\(^{2+}\) concentration on EMS-MB emissions at 40 °C, the left arrow indicates general trend of monomer emission and the right arrow indicates general trend of excimer emission due to Mg\(^{2+}\) increase, and B) is the effect of Mg\(^{2+}\) concentration on monomer and excimer emissions at 40 °C.
Fig. A.13: A) The effect of Mg²⁺ concentration on EMS-MB emissions at 45 °C, the left arrow indicates general trend of monomer emission and the right arrow indicates general trend of excimer emission due to Mg²⁺ increase, and B) is the effect of Mg²⁺ concentration on monomer and excimer emissions at 45 °C.

Fig. A.14: A) The effect of Mg²⁺ concentration on EMS-MB emissions at 50 °C, the left arrow indicates general trend of monomer emission and the right arrow indicates general trend of excimer emission due to Mg²⁺ increase, and B) is the effect of Mg²⁺ concentration on monomer and excimer emissions at 50 °C.
Fig. A.15: A) The effect of Mg\(^{2+}\) concentration on EMS-MB emissions at 55 °C, the left arrow indicates general trend of monomer emission and the right arrow indicates general trend of excimer emission due to Mg\(^{2+}\) increase, and B) is the effect of Mg\(^{2+}\) concentration on monomer and excimer emissions at 55 °C.

Fig. A.16: A) The effect of Mg\(^{2+}\) concentration on EMS-MB emissions at 60 °C, the left arrow indicates general trend of monomer emission and the right arrow indicates general trend of excimer emission due to Mg\(^{2+}\) increase, and B) is the effect of Mg\(^{2+}\) concentration on monomer and excimer emissions at 60 °C.
Fig. A.17: A) The effect of Mg$^{2+}$ concentration on EMS-MB emissions at 65 °C, the left arrow indicates general trend of monomer emission and the right arrow indicates general trend of excimer emission due to Mg$^{2+}$ increase, and B) is the effect of Mg$^{2+}$ concentration on monomer and excimer emissions at 65 °C.

Fig. A.18: A) The effect of Mg$^{2+}$ concentration on EMS-MB emissions at 70 °C, the left arrow indicates general trend of monomer emission and the right arrow indicates general trend of excimer emission due to Mg$^{2+}$ increase, and B) is the effect of Mg$^{2+}$ concentration on monomer and excimer emissions at 70 °C.
Fig. A.19: A) The effect of Mg\(^{2+}\) concentration on EMS-MB emissions at 75 °C, the left arrow indicates general trend of monomer emission and the right arrow indicates general trend of excimer emission due to Mg\(^{2+}\) increase, and B) is the effect of Mg\(^{2+}\) concentration on monomer and excimer emissions at 75 °C.

Fig. A.20: A) The effect of Mg\(^{2+}\) concentration on EMS-MB emissions at 80 °C, the left arrow indicates general trend of monomer emission and the right arrow indicates general trend of excimer emission due to Mg\(^{2+}\) increase, and B) is the effect of Mg\(^{2+}\) concentration on monomer and excimer emissions at 80 °C.
Fig. A.21: A) The effect of Mg$^{2+}$ concentration on EMS-MB emissions at 85 °C, the left arrow indicates general trend of monomer emission and the right arrow indicates general trend of excimer emission due to Mg$^{2+}$ increase, and B) is the effect of Mg$^{2+}$ concentration on monomer and excimer emissions at 85 °C.