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PHOTO-CONTROL OF DNA OLIGONUCLEOTIDES WITH CAGE COMPOUNDS

A Thesis

Submitted to the Graduate Faculty of the
Louisiana state University and
Agricultural and Mechanical College
In partial fulfillment of the
Requirements for the degree of
Master of Science in Biological and Agricultural Engineering

In

The Department of Biological and Agricultural Engineering

by
Bilal Ghosn
B.S., Louisiana State University, 2002
December 2004

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List of Abbreviations

Abbreviation	Definition
ATP	adenosine triphosphate
DMNPE	1-(4,5-dimethoxy-2-nitrophrnyl) diazoethane
DMF	N,N-dimethyformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediamine-tetraacetic acid
ICAM-1	Intracellular adhesion molecule-1
IPA	isopropanol (isopropyl alcohol)
MB1	Molecular Beacon 1
MeCN	acetonitrile
MeOH	methanol
mRNA	messenger RNA
ODN	oligodeoxynucleotide
PD-ODN	phosphodiester oligonucleotide
PS-ODN	phosphorothioate oligonucleotide
RNase H	Ribonuclease H
UV	ultra violet

Abstract

Many biochemical processes in which DNA and other nucleic acids participate are central to functions in both living cells and in molecular biology assays. While many compounds have been used to regulate the activity of DNA, these strategies are limited to the aqueous-based diffusion of the activator to the target DNA molecule. An improvement to the induction of DNA bioactivity is to move to a light-based modulation. This research demonstrates a light-based technique using a photo-cleavable cage compound to transiently inactivate DNA hybridization. Function can be restored with exposure to near-UV light, allowing for temporal control of DNA oligonucleotide (ODN) activity. This method has demonstrated the control of hybridization in molecular biology assays, and provides the framework for *in vivo* experimentation. A similar light-activated strategy has been shown useful in controlling expression of plasmid transgenes (Monroe 1999). By adapting this method to DNA oligonucleotides (ODNs), we have partially blocked hybridization with the cage compound (1-(4,5-dimethoxy-2-nitrophenyl)ethyl ester (DMNPE) for both phosphodiester and phosphorothioate DNA ODNs. The production and purification of DMNPE-caged DNA ODNs yields products with similar spectrophotometric properties to caged plasmids. In hybridization studies, 20-mer (20 base long) caged DNA ODNs were hybridized with complementary 30-mer molecular beacon probes, and fluorescence measurements were used to assess hybridization of native (non-caged), caged, and caged-light-exposed ODNs. Developments of the molecular beacon assays were studied to improve sensitivity of the assay to caged and caged-flashed ODN hybridization control. Results demonstrated that hybridization can be blocked and subsequently restored by light through the

attachment of the DMNPE cage compound, and were further characterized with gel electrophoresis assays. ODN hybridization was restricted to as little as 2% when compared to native (non-caged) ODNs and restored to up to nearly 80% of the native (non-caged) ODN hybridization activity levels. Additional studies on adduction, purification, and characterization of the DMNPE-caged ODNs were performed to optimize their production and efficacy in controlling hybridization. These results suggest that this light-based technology can be used as a tool for the spatial and temporal regulation of hybridization-based DNA bioactivity, including applications with antisense ODNs as a form of controlled gene therapy.

Chapter 1

Introduction

The use of nucleic acids as tools in molecular engineering and gene therapy has rapidly increased over recent years. Nucleic acids have been developed into powerful tools to achieve many goals never before thought possible. Recently, much of the focus on nucleic acids has been for their use as forms of gene therapy. Recent studies have focused on the use of nucleic acids as a method for treatment for numerous forms of disease (Gewirtz 2001; Opalinska 2002). When expression of genetic therapies is targeted to desired tissues, the treatment is safer and more efficient.

The overall purpose of this research is to develop a method to reversibly control the bioactivity of DNA oligonucleotides to spatially and temporally control gene expression. Our hypothesis is that DNA oligonucleotide hybridization with a complimentary target can be controlled through the use of photo-cleavable cage compounds, such as 1-(4,5-dimethoxy-2-nitrophenyl)ethyl ester (DMNPE) until the cage compound is photo-cleaved from the oligonucleotides by exposure to near-UV light Our study focuses on the development of this control method *in vitro* for DNA oligonucleotides with normal phosphodiester backbones as well as those with sulfur-modified (phosphorothioate) backbones. We aim to demonstrate that reversible caging of the oligonucleotides is possible and efficient through the of use several qualitative and quantitative *in vitro* analyses. We also propose to utilize temperature-varying assays to help provide more sensitive techniques for analysis of the ability to control hybridization of DNA oligonucleotides through caging. This study is intended to provide

a basis for further *in vivo* studies of spatial and temporal control of DNA oligonucleotide hybridization activity through the use of cage compounds.

Cage Compounds

One specific technique being employed to achieve control of nucleic acid hybridization, and the main focus of this research, is the use of photo-cleavable cage compounds. These molecules are compounds that bind to their target with a covalent, yet photo-cleavable bond. Once attached to its target, the cage compound prevents the effector molecule from being reactive, until a time at which it is photo-cleaved and the effector molecule is once again reactive (McCray 1989). It is important to note that these compounds do not literally encompass or form a cage around the effector molecule. In reality, these molecules hinder the reactive abilities of their target by reducing the structural reformation of the effector molecule (McCray 1989; Pelliccioli 2002). Initial studies of cage compounds within biological systems focused on the ability to render ATP biologically inert, and then restore it to a bioactive form (Kaplan 1978). This task was achieved by attaching the cage compound 1-(2-nitrophenyl) ethyl (NPE) to the γ-phosphate of ATP. They detected the efficacy of this strategy by sodium efflux measurements. The ATP was rendered inactive until exposed to 340 nm light, at which point consumption of the ATP was restored. A similar basic mechanism for caged ATP can be seen in Figure 1.1 where the cage compound used is 1-(4,5-dimethoxy-2-nitrophenyl)diazoethane (DMNPE). It should be noted that DMNPE contains two methoxy groups attached to the 4' and 5' carbon atoms on the benzyl ring, and is photo-cleaved by 365 nm light (MolecularProbes 1996).

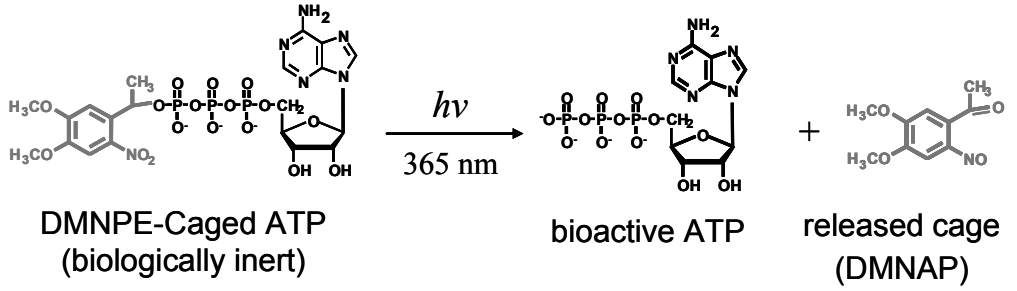

Figure 1.1. Photolysis of ATP caged with a caged compound, DMNPE.

Since the initial experiments with ATP, numerous studies have focused on the use of cage compounds to control bioactivity across several platforms. Time course studies of rapid responses within cells have been greatly helped by the use of cage compounds (McCray 1980; Gee 1998; Choi 2003). Biologically inactive ATP can be introduced into cells in a biological inert form. Upon exposure to light at rapid pulses, restoration of bioactivity can be achieved and the rate at which the ATP is consumed can be controlled and monitored. In this and many other applications, the advantages of using photo-protected or caged species over conventional methods for substrate release are that the caged species can be dispersed throughout the biological target without eliciting the species' normal bioactivity, the concentration and spatial distribution can be controlled, and finally, the temporal release can be varied from seconds to nanoseconds (Givens 1998). There have been many studies that have shown control of other biological processes using cage compounds within cellular studies. These have included transport of proteins, and effects on biological membranes, as well as relaxation of muscle fibers (Patton 1991; Pirrung 1994; Ishihara 1997; Lee 1997). The development of cage compounds for use within biological systems is greatly on the rise.

The use of caged compounds is also being further developed for aiding in control of gene expression and its effects on organisms. Areas of focus for the control of gene expression can be seen with the production of proteins (Monroe 1999; Ando 2001) and bioactivity of hormones (Dorai 1997; Lim 2002; Garcia-Fernandez 2003). Several possible techniques are being studied to block DNA transcription and those that prevent translation of mRNA through degradation (Monroe; Ando 2001; Harborth 2003).

As the utilization of cage compounds has expanded, the structures of caging compounds have been developed based on their specific intended uses. Many are benzyl rings or derivatives with strong leaving groups, commonly bromine, chlorine, or diazonium, each with specific functions and preferred replacements. Some examples of caging compounds are single benzyl rings, such as 1-(2-nitrophenyl)-diazoethane (NPE) and DMNPE, while others are multi-ring compounds such as 6-bromo-7-hydroxycoumarin-4-ylmethly (BHC) (Furuta 1999; reviewed by Pelliccioli 2002). In the case of DMNPE, this cage compound can be photolyzed by photons of wavelength 365 nm (Monroe 1999) similar to that of BHC (Furuta 1999), which differs from those of NPE at 340 nm (Walker 1988). The longer wavelength of light is beneficial for photoactivation in cells and tissues due to less photodamage.

There are few descriptions of applying caging chemistry to nucleic acids to temporally and spatially control function. Cage compounds have been used in the caging of nucleotide analogs (Walker 1988), in the synthesis of bio-chip oligonucleotides (McGall 1996), in studies of the kinetics of DNA repair (Meldrum 1990; Meldrum 1998; Ordoukhanian 2000), and as protecting groups during DNA synthesis (Alvarez 1999). Cage groups have also been shown to control an oligonucleotide

hairpin configuration and indirectly hybridization (Ordoukhanian 1995). The use of cage compounds was also demonstrated in the repair of DNA that has a single strand break at a specific site (Zhang 2001). These studies demonstrate the site-specific caging of a larger molecule by using caged building blocks in standard peptide synthesizers. Cage groups have also been used to temporally control ribozyme and spliceosome activities by including caged adenosine within synthesized RNA oligonucleotides (Chaulk 1998; Chaulk 2001).

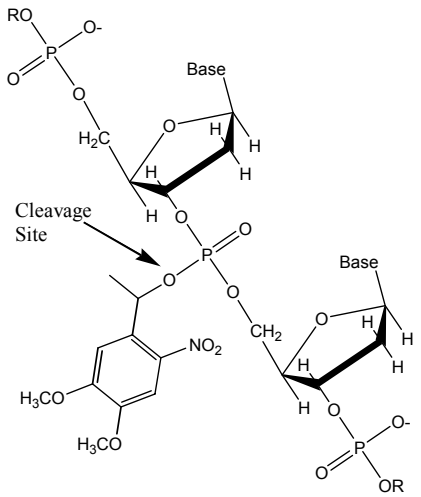

Figure 1. 2 Proposed site of DMNPE adduction to DNA ODN.

Through the attachment of cage compounds, generation of translation and transcription products such as proteins and enzymes can be brought to minimal or no yield by preventing the nucleic acid from forming certain structural conformations. Based on the previous studies of ATP, it is theorized that the attachment of the DMNPE cage compound occurs along the phosphate backbone of the nucleic acid as seen in Figure 1.2 to a protonated non-bridging oxygen of the phosphate backbone. Attachment at this position is consistent with DMNPE's attachment to other moieties such as carboxylic acids and phosphates, or other weak oxy acids (Walker 1988).

In addition to controlling the kinetics of a particular molecular target, caging affords the ability to restrict re-activation to a localized tissue of interest, as demonstrated by the targeting of transgene expression by caging hormone inducers (Cruz 2000; Lin 2002). Control of bioactivity through direct caging of nucleic acids has

been demonstrated with plasmid DNA both *in vitro* and *in vivo* by controlling transgene expression with light exposure (Monroe 1999). More recently, BHC caged mRNA was microinjected into zebrafish embryos to induce expression of certain genes and study the effect of their expression on developmental patterns (Ando 2001). This work has shown that the use of BHC reacted with mRNA coding for green fluorescent protein demonstrated a reduction in translational activity. Once illuminated by photons, translational activity was restored (Ando 2003). This shows a trend to develop increasingly efficient and effective caging compounds, which can be specifically designed to work with complementing sequences of mRNA and DNA. We now present a method for controlling the hybridization activity of short DNA ODNs through the adduction and photo-cleavage cage compounds, thus allowing for spatial and temporal control of ODN bioactivity.

Gene Therapy: Antisense

The use of cage compounds can be noted for its effectiveness in preventing expression of DNA plasmids (Monroe 1999) and mRNA (Ando 2001), and current studies are moving in the direction of performing further tests on other nucleic acids. Both of the aforementioned studies focus on the use of cage compounds with antisense therapy technologies. These compounds may be used with DNA to hybridize and alter transcription (Monroe 1999) and mRNA to prevent translation (Dash 1987; Walder 1988; Liebhaber 1992; Vickers 2001; Sazani 2003), thus preventing or modifying protein production. While traditional drugs and therapies focus on the breakdown or control of proteins already produced, the antisense therapies focus on controlling the production of the proteins on a genetic level (Christoffersen 1995; Koller 2000; Sun 2000; Steele

2003). Antisense oligonucleotides have been studied for several years as treatments for many diseases and genetic disorders (Berg 2002; Alvarez-Salas 2003; Hugle 2003).

Antisense therapy is based on the principles of genetic expression. Strands of mRNA are transcribed from DNA, and are a copy of the "coding" or "sense" strand of the gene. This main form of the therapy uses the complementary or "antisense" strand of the target to hybridize the "sense" strand and prevents production of the protein by blocking or altering translation. Another form of antisense therapy is the use of modified ribozymes that are catalytic RNA derivatives which are sequence specific and are involved in processes such as self-cleavage, ligation, or splicing of mRNA (Kruger 1982; Buzayan 1986; Hutchins 1986; Symons 1987; Sharmeen 1988; Davies 1990).

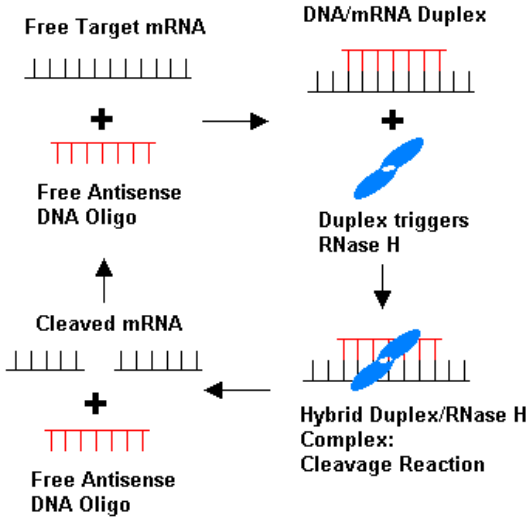

Figure 1.3. Antisense oligonucleotide mechanism through RNase H cleavage of mRNA.

RNAi is another antisense technique, which functions on the premise of the production within cells of short interfering RNAs (siRNAs) that are effectors for targeted gene silencing (Fire 1998; Caplen 2001; Elbashir 2001; Elbashir 2001; Bertrand 2002; Miyagishi 2003).

One specific type of these oligonucleotides is known as antisense oligodeoxynucleotides or antisense ODNs, and they are designed to hybridize with a specific mRNA complement. These antisense ODNS work by hybridizing to their

complementary or target mRNA through basic complimentary Watson-Crick base-pairing (Paterson 1977). Upon hybridization with the target mRNA, translation can be blocked through several mechanisms. These include prevention of the mRNA from being processed through the ribosome (Liebhaber, Cash et al. 1992), modifying the splicing of the mRNA (Sazani and Kole 2003), or an impairment of the polyadenylation of the mRNA (Vickers 2001). The most common mechanism of preventing translation through antisense ODNs is the activation of RNase-H enzyme, which will degrade the mRNA when complexed with the antisense ODNs (Dash 1987; Walder 1988). Here, antisense ODNs are very effective in that the RNase H does not break down the ODN, and so they can continue to hybridize with more target mRNA. An example of this mechanism can be seen in Figure 1.3. Modifications of the ODNs have become a necessity in order to use them for *in vivo* treatments. Cellular defense mechanisms have influenced the efficiency of these tools through degradation of the ODNs within the cell by nucleases (Wickstrom 1986; Akhtar 1991). Modifications of the ODNs to improve efficacy have mainly focused on backbone modifications (Chavany 1995; Agrawal 1997; Summerton 1997; Summerton 1997; Schmajuk 1999) and base modification (Kuwasaki 1996; Herdewijn 2000; Kimber 2003; Sazani and Kole 2003). The most widely used modified DNA in antisense therapies is phosphorothioate ODNs, which replaces a non-binding oxygen on the phosphate back-bone with a sulfur, as shown in Figure 1.4. These modified ODNs have a much greater resistance to digestion by nucleases, and so have advanced the use of antisense therapies immensely. This modified oligonucleotide has been used with many gene therapy

studies and treatments (Chavany 1995; Monia 1996; Butler 1997; de Smet 1999; Yu 2003).

These modified antisense ODNs are being used to help fight disease and several are currently undergoing FDA approval. The first antisense treatment to get FDA approval to date has been Formivirsen (Vitravene), which is a treatment for cytomegalovirus (CMV) retinitis in people with AIDS and was developed by ISIS Pharmaceuticals (Roehr 1998; de Smet 1999). Several other antisense therapies are undergoing FDA approval that focus on combating diseases such as HIV, malignant melanoma, B-cell lymphoma, colon cancer, and lung (Wong-Staal 1998; Amado 1999; Coudert 2001; Cripps 2002; Morris 2002; Adjei 2003). Also, much of the research has recently focused on control and treatment of cancer. One pathway that these antisense drugs are operating through is anti-tumor treatments targeting specific enzymes, by increasing sensitivity of cancer cells to apoptosis inducing agents, and other methods of destroying cells (Monia 1996; Ohta 1996; Dorai 1997). Another method that antisense drugs are approaching treatment is by repairing or correcting improperly transcribed or mutated genes (Dominski 1993; Friedman 1999; van Deutekom 2001).

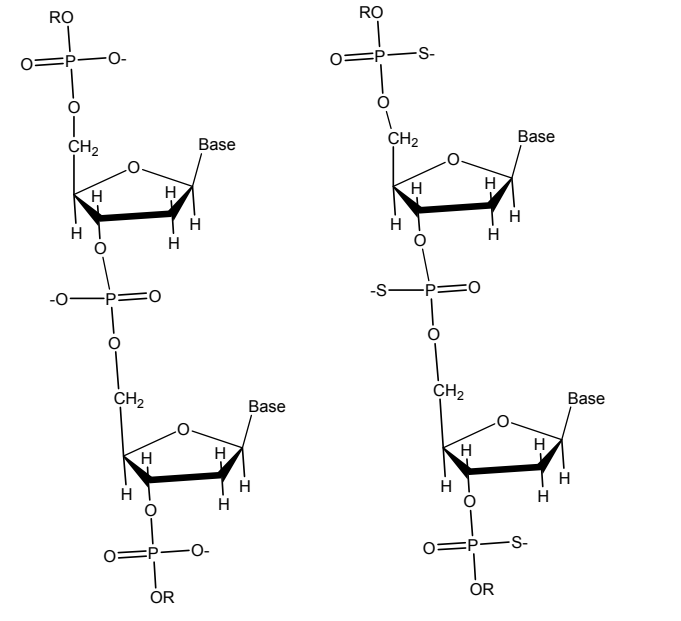

Figure 1.4. Phosphodiester ODN (left) and phosphorothioate modified ODN (right).

One such antisense ODN, which has been very well studied and useful to caging research is Alicaforsen (ISIS 2302), which is being developed by the ISIS Pharmaceuticals. This ODN is currently being researched for its possible medicinal uses against Crohn's Disease and Ulcerative Colitis (Gewirtz 2001). Its main target is the Intracellular Adhesion Molecule-1 (ICAM-1) gene (Bennett 1994). The drug inhibits the expression of this gene, which is a key factor in many autoimmune and inflammatory conditions. ICAM-1 can be found in almost all cells of the human body, and is part of a group of molecules known as Cellular Adhesion Molecules. By measuring the affects of ISIS 2302 and its ability to be caged, possible control of the inhibition of the ICAM-1 gene's expression in living cells can become a reality.

Antisense oligonucleotides although very effective, can repeatedly and continuously prohibit expression of the target gene, and if not controlled can lead to possible complications if the proteins being blocked are essential to other processes within a system. In an effort to develop a control system for this, we have studied the use of photo-cleavable cage compounds to allow for a method to determine spatial and temporal aspects of drug functioning. By attaching these cage compounds to the antisense oligonucleotide, hybridization to its target is blocked. However, once exposed to near UV-light, the cage compound can be removed and thus fully restoring the antisense oligonucleotide into a fully functional form. By this method, we allow for control of the antisense mechanism, while avoiding any loss of potency of the drug. The importance of targeting through the use of cage compounds is the need to avoid blockage of gene expression throughout the entire body. By using photo-cleavable cage

compounds, focused treatments of the antisense can be applied to specific regions of the body.

Molecular Beacons

The detection of hybridization has been an important tool for understanding the properties and potential of nucleic acids. One common technique is the use of UV-absorbance to detect the melt profiles or denaturing of nucleic acids. This method functions on the hyperchromicity characteristic of nucleic acids. Nucleic acids demonstrate high absorbance of light at approximately 260 nm. Hyperchromicity is the principle that when double-stranded DNA or RNA denatures, the absorbance at 260 nm increases, and can be used to detect melting temperatures and hybridization activity. This technique has been utilized in studies on characterization and thermodynamic analysis of duplex DNA (Wu 2000), characteristics of modified nucleic acids such as phosphorothioate ODNs (Stein 1988) and, and DNA hairpin melt studies (Vallone 1999). This technique has also been employed to study the thermal effects on complexes formed with heteromorphic duplex DNA (Riccelli 2003). However, this technique is limited to in-cuvette studies and does not allow real-time study of spatial patterns of hybridization in cells or tissues.

Molecular beacons are extremely powerful tools that are being used for demonstrating the photo-control of hybridization activity of caged ODNs. They allow for differentiation of hybridization activity between native (non-caged), caged, and light-exposed caged ODNs through the use of fluorescence. Fluorescence emissions from the molecular beacon assays can confirm hybridization activity, and thus can provide evidence of control of ODN activity through the attachment of photo-cleavable cage

compounds. Molecular beacons also overcome the limitations of hyperchromicity absorbance methods by permitting *in situ* detection of hybridization activity. Spatial control within cells can be demonstrated once studies are performed to determine the effects of the caged ODNs *in vivo*. The beacons may also be helpful in detecting temporal effects of the caged ODNs by determining the presence of the target mRNAs and its hybridization activity within the cells.

These probes are a revolutionary tool initially developed in 1996 by Tyagi to help identify nucleic acid sequences (Tyagi 1996). Molecular beacons are short single-stranded nucleic acids with self-complementing 5' and 3' ends. The stems generally are four to seven bases in length. In between these self-complementing ends is the sequence complementary to the target of interest. When not in the presence of the target, the molecular beacon takes a stem and loop form. A fluorophore and quencher are attached to the 5' and 3' ends of the oligonucleotide, respectively. When in close proximity to the quencher (stem and loop form), the fluorescence emitted is absorbed,

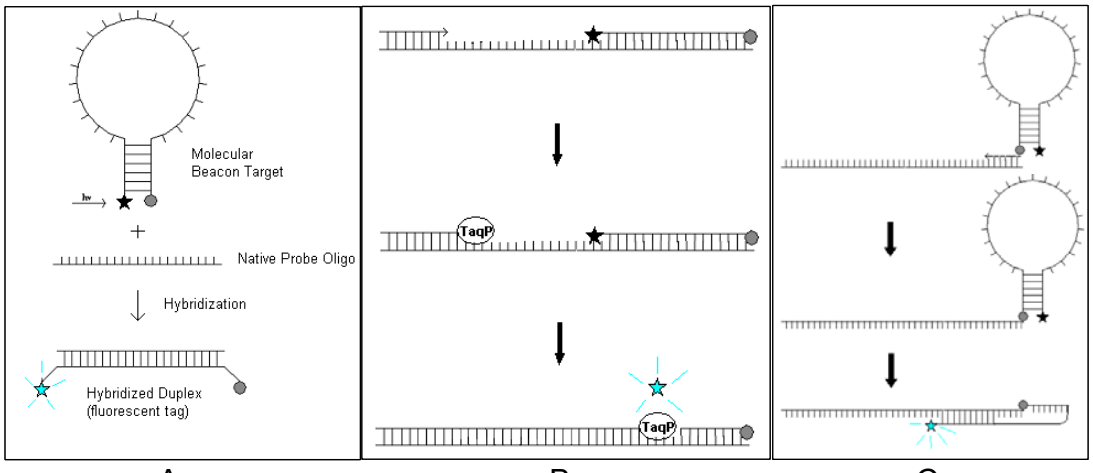

Figure 1.5. Several forms of hybridization probes. These are a basic molecular beacon (A), a TaqMan™ probe (B), and a scorpion beacon (C)

and little to none of it is detected. Hybridized with its target, the fluorophore is

separated from the quencher, and its fluorescence can be detected as can be seen in Figure 1.5. The efficacy of the beacons can be altered based on stem and loop sequence and by the fluorophore and quencher chosen (Tyagi 1998). This includes numerous applications as discussed below including in PCR, cancer research, clinical and protein assays.

PCR-based Applications

One of the most common applications for molecular beacons is the monitoring of PCR reactions in real time. By utilizing these probes in PCR, researchers can monitor the amplification of nucleic acids during the process (Tyagi 1996; Tyagi 1998). When using these probes, they are placed into the reaction mixture, and allowed to remain throughout the amplification process. When at higher temperatures in the cycle, molecular beacons will dissociate from their hairpin loop form, however will not bind to the targets, and thus not interfere with amplification process. Once temperatures have lowered and the amplicon extension has begun, small amounts of the beacon will attach to the synthesized targets and fluorescence can be detected. Any beacon that is not attached to the target will remain dark in its closed-stem form at these lower temperatures. This allows for a sensitive assay to determine the rate of amplification and quantify rare messages in PCR.

There are several advantages of this molecular beacon assay versus other techniques. The use of dyes or stains such as SYBR Green and SYBR Gold (Molecular Probes, Eugene, OR) are limited in that they are non-specific dyes that bind to dsDNA. They produce signals that may not only represent the target sequence, but also incorrectly produced segments as well as dimers of the primers. This leads to an

inability to accurately determine the amount of desired target amplicons in the process. Several other probes have been designed to achieve a more accurate and efficient monitoring of the PCR process. Several of these techniques can be seen in Figure 1.5. TaqMan™ probes are simply a probe sequence labeled with a fluorophore on each end. The first is a donor fluorophore and the other an acceptor fluorophore. Similar to beacons, the fluorophores are quenched when unhybridized to the target, and utilize fluorescence resonance energy transfer (FRET) to achieve this. This technique works by cleavage of the TaqMan™ probe when extension begins, and thus breaking up the probe and releasing the two fluorophores and allowing for fluorescence. The drawback of this technique is that the probe can only be used once, and then is rendered useless. Molecular beacons carry on the characteristic of multiple uses versus the single use of a TaqMan™ probe. Another modified version of this probe is the Scorpion primer. Since the primers are directly linked to the molecular beacons in this type of probe, it allows for very specific to the amplicon produced since the loop section of the beacon is complementary to part of the desired amplicon that will be produced (Thelwell 2000; Taveau 2002).

One example of recent studies of the usage of molecular beacons with RT-PCR is the use of molecular beacons to detect the Y chromosome in single human blastomeres in an effort to determine the sex of an embryo (Pierce 2000). This has also been used as a technique for clone verification (van Schie 2000). Finally there is the ability of the molecular beacons as tools to identify single nucleotide polymorphisms or SNPs (Mhlanga 2001), which is currently an intensifying area of research in the clinical setting due to its high specificity, accessibility, and simplicity. This is also an expanding

area of study in the identification of mutated genes found within small numbers of cells in a population, leading to a great range of uses for this technology (Vogelstein 1999).

One of the greatest advantages that accompanies the use of molecular beacons is multiplexing. The idea of using a multiplex of molecular beacons is quite simple and quite an advance in comparison to previously used assays. Since molecular beacons have an extremely high specificity with the ability to distinguish between single nucleotide mismatches in a target sequence, multiple beacons can be used to accurately identify several variants of target sequences with as little as a single base difference. These probes also have great advantage over other fluorescence stains such as SYBR Green (Molecular Probes, Eugene, OR) in that they can report specific complementary targets, while SYBR Green and other dyes are non-specific DNA binding dyes. An initial study by Marras (1999) probed the accuracy of detection of four molecular beacons, each with different fluorophores, and each differing by as little as one single nucleotide. This study demonstrated the ability to actively differentiate the presence of a specific variant of an amplicon present quickly and efficiently (Marras 1999). Many other studies demonstrated the ability of using multiplexed molecular beacons to quickly and accurately identify specific strains of influenza (Templeton 2003), several types of bacteria (Belanger 2002; Templeton 2003; Varma-Basil 2004), and numerous viruses (Vet 1999; Klerks 2001; Szemes 2002). The use of these techniques with quantitative PCR (Q-PCR) can allow researches to quantitatively determine the levels of specific targets within samples, and thus utilize this in many numerous ways.

In the following sections, adaptations of molecular beacons towards immobilized and other clinical uses will be presented to further underscore this development in the technology.

Thermodynamic and Kinetic Properties

As probes and diagnostic tools, molecular beacons are well known for their specificity. The ability for molecular beacons to distinguish between nucleic acids with a difference of as little as one base pair have been studied since these tools were initially published by Tyagi and Kramer in 1996. In comparison to linear probes, the hairpin stem of the molecular beacons allows for much more selectivity when identifying nucleic acids by reducing the background fluorescence produced by an non-hybridized probe (Tyagi 1998). Since the hybridization of the molecular beacon to its target can be manipulated, this allows for the improvement of the ability of the beacons to identify their targets even in the presence of nucleic acids that may differ by as little as a single or double base mismatch.

This specificity is affected by several conditions including pH, salt concentration, and temperature. It has been shown that the negative effect of a single mismatch on the free energy when two strands of nucleic acids hybridize decreases as the length of the strands increases. A single base mismatch will have more effect on preventing hybridization the shorter the strands of nucleic acids (Aboul-ela 1985). This principle can be seen in molecular beacons, as they are generally short oligonucleotides of about 30 – 40 nucleotides in length. By adjusting the hybridization conditions, one can easily improve the ability of these probes to identify their targets with a high level of selectivity. Of the earlier stated conditions, temperature is the most easily and dynamically

controlled condition to manipulate hybridization reactions. Several studies have provided insight into the thermodynamics and kinetics of molecular beacons (Bonnet 1999; Tsourkas 2002).

There are three forms in which molecular beacons can exist. These three forms are the hybridized duplex, self-hybridized in the hairpin structure, and un-hybridized yet randomly coiled (Bonnet 1999). The likelihood of each state is based on the presence or absence of the target and the reaction conditions, most notably temperature. By altering the temperature, the probes will change their form between the three forms previously mentioned. At temperatures above the melt temperatures of the hybridized duplex, the probes exist in the un-hybridized, randomly coiled form. As the temperature cools in the presence of the target, hybridized duplexes of the probe and target will become more likely along with self-hybridized hairpin forms of the molecular beacons. Because of the free energy “tax” of a single base mismatch in short oligonucleotides, such as the 25-mer molecular beacons shown in the Bonnet (1999) studies, these melt temperatures are much lower. In their study, the single base mismatch was placed in nine different positions within the target sequence to determine the point at which the lowest “tax” on free energy would be present (Bonnet 1999). The mismatch with the lowest strain on the hybridization was the mismatch farthest from the center mismatch, and had a melt temperature near 31 °C. This differed greatly than that of the complementary target, which had a melt temperature of approximately 42 °C. As can be seen in Figure 1.6, once the melt temperature was exceeded, fluorescence rapidly reduces, thus making the detection of the complementary target even more specific.

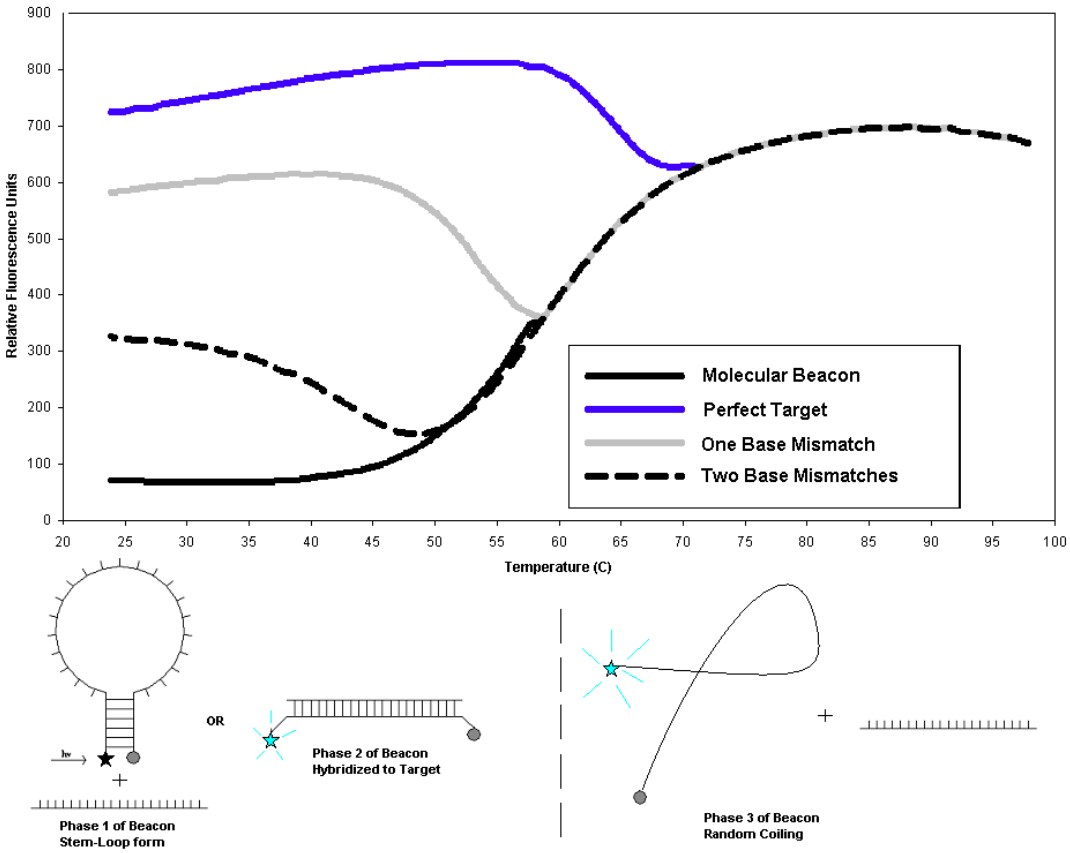

Figure 1.6. Temperature profiles of molecular beacon hybridizations with perfect target, one base mismatch, two base mismatches, and molecular beacon alone (MB1 alone) along with the beacon phases for temperatures.

Other studies have focused on the effects of thermodynamic and kinetic conditions on molecular beacon hybridization to their targets (Tsourkas 2002; Tsourkas 2003). These studies display that not only does the length of the sequence being targeted determine the specificity of the probe, but also the length of the stems, as well as their composition have great effect on the selectiveness of the probes to identify their exact targets. Studies showed that beacons dependent on location, number of mismatches, and lengths of stem altered the specificity of the probe to identify its complementary target. Probes with a stem length of four nucleotides showed much less specificity than those of five or six nucleotides. The probes with stems of six

nucleotides in length also showed an ability to show greater specificity at lower temperatures. Not only did stem lengths affect the amount of fluorescence detected when these probes were hybridized with their targets, they also varied in the amount of time required for the probes to reach their maximum fluorescence. The shorter the stem, the higher the overall fluorescence and the shorter the amount of time required to reach that fluorescence level. This trend alone can be important in designing the probes, based on the response time sought by the user. One final trend noted in these studies shows that as stem length increased, melting temperature of the hybridization duplex reduced. The differences, however, begin to narrow as the probe length increases (Tsourkas 2003).

In 2002, Tsourkas . published a study showing the kinetics of a shared stem molecular beacon versus a conventional molecular beacon(Tsourkas 2002). The initiative was to determine whether a more stabilized fluorophore would help to improve the use of two molecular beacon systems. The shared stem molecular beacons have one stem (in this case the stem attached to the fluorophore) that participates in both the formation of the hairpin as well as the probe target hybridization. This forces the fluorescent dye into a much more constrained position and thus allows for more control, as opposed to random coiling that is seen with traditional probes. The results of this study demonstrated that the melting temperature of the shared stem beacon was higher for both the complementary target and the single mismatch target, thus leading to a stronger hybridization between target and probe in the case of the shared stem probe (Tsourkas 2002). These kinetic and thermodynamic characteristics of molecular

beacons provide insight into the potential of these probes in the field of function-specific probe design.

Clinical Applications

As we have discussed, there are many possibilities for the use of molecular beacons as diagnostic tools in many facets of the scientific community. A promising application for molecular beacons is to detect disease or mutated DNA within an organism. The use of such technology would allow for more accurate and much more rapid results when attempting to diagnose a patient. Many studies have been focused on the use of molecular beacons in the detection and identification of viruses (Lewin 1999; Szuhai 2001; Kostrikis 2002; Jebbink 2003). Several studies have also focused on the use of these molecular tools for diagnosing and helping with the treatment of cancer as well (Arnold 1999; Span 2003; Culha 2004).

Many studies have utilized molecular beacons to detect pathogens (Chen 2000; Fortin 2001; Lanciotti 2001; Belanger 2002). These studies utilize the principles of amplification of target DNA through PCR and then utilize beacons to identify the presence or absence of targets. These probes have also been utilized in research for quantifying viral loads and the replications of viruses through similar studies (Lewin, Vesanen 1999; Yates 2001; Yang 2002). The high specificity of molecular beacons to discriminate between bases with as little as single base differences in their sequences provides researches with a versatile tool. One particular study has been to quantify viral loads of the Human Immunodeficiency (HIV), Hepatitis C (HCV), and Hepatitis B (HBV) viruses with the ability to detect viral loads of as low as 50 copies/mL in the case of HIV and HBV, and 20 IU/mL of the HCV virus (Abravaya 2003). The two most noted

characteristics that make the molecular beacon a great diagnostic tool are the reduction in contamination from post-PCR manual handling and short time required to run the tests.

Use of molecular beacons along with RT-PCR has been used as a tool to diagnose and discriminate between many bacteria, such as *Bordetella pertussis*, *Bordetella parapertussis*, and *Bordetella Holmessi* (Templeton 2003). Earlier studies of the ability of molecular beacons as tools to diagnose infectious diseases demonstrated this ability with the respiratory infectious agent *Mycobacterium tuberculosis* (El-Hajj 2001) and sexually transmitted infectious agents *Chlamydia Trachomatus* (Zhang 2002). This was also demonstrated in a study for the detection of the presence of several species of *Salmonella* through the use of RT-PCR and molecular beacons (Chen 2000). The trend to develop assays as diagnosis for infectious diseases has since greatly increased as the use of such a tool in clinical settings poses such great advancement in health care treatment.

The capability of molecular beacons to be used as genetic assays for other diseases such as Tay-Sachs has also been demonstrated (Rice 2002). This study utilized the probes to detect the specific alleles for single copy genes found within a single cell. By lysing these cells and amplifying the nucleic acids properly through PCR, one molecular beacon was used to detect the presence of either a normal allele, while the other was used to detect the 4 base-pair insertion mutated allele, which is one of the more prominent mutations known to account for a high percentage of Tay Sachs carriers. This study showed a very accurate detection rate of over 99.1% with a run

time of only three hours, thus showing that the use of molecular beacons greatly increased the efficiency of detection of the gene mutation (Rice 2002).

Recent studies have also been focused on the use of molecular beacons as a diagnostic tool for cancer. As a rapid, accurate, and specific diagnostic tool, molecular beacons serve as a great tool for early detection of mutations in genes that can lead to cancer. One example of this is the study by Culha . from 2003 in which they have shown the use of molecular beacons on a miniature biochip for detection of the *BRCA1* gene. As one of the genes known for leading to increased susceptibility to breast cancer if mutated, early detection and identification is an important tool in prevention (Culha 2004). The use of a molecular beacon based RT-PCR assay has also been demonstrated as a valid tool with a predictive value when studying breast cancer (Span 2003). This use of molecular beacons with RT-PCR has also been significant in studying the proteins associated with breast cancer and the ability for metastasis with a tumor by allowing for detection of the presence of the proteins and their amounts within the breast cancer. This leads to the conclusion that higher amounts of these proteins within cancer cells can increase likelihood of metastasis (Arnold 1999).

Applications for molecular beacons as tools with regards to cancer however, have not been limited solely to breast cancer research. Several studies have focused on lung cancer as well as a variety of other variations. One example here is the use of molecular beacons to detect the presence of K-*ras* point mutations in an effort to determine predisposition for the illness. Using molecular beacons to both detect the sense and anti-sense strands of the K-*ras* Exon 1 compared with the commercially available elucigene K-*RAS*7 method showed that this type of assay provides an

accurate and rapid way to screen large numbers of samples in an effort to get early detection (Clayton 2000). Another technique has shown that by simply using human hair shafts and single nucleotide polymorphism PCR, researchers can perform analysis of genotype by using molecular beacons to identify genomic predisposition to diseases such as cancer (Chang 2002). This technology provides an opportunity for quick and non-invasive screening of individuals for genetic mutations that may lead to a greater likelihood of developing a disease, and thus can be treated to attempt to prevent the onset of those illnesses.

Project Aims

The overall purpose of this project is to apply the use of photo-cleavable cage compounds to reversibly control the hybridization activity of DNA oligonucleotides. This method would provide the basis for spatial and temporal control of the activity of such specialized ODNs as antisense ODNs, providing a path for overcoming a drawback of the antisense strategy by allowing for targeting of the ODNs to specific tissues within a system. The use of cage compounds to afford a light induced control of the ODNs hybridization activity may provide a simple method for improved drug delivery and more focused and powerful treatments. Another aim of this project is the development of *in vitro* assays in order to help provide evidence of the reversible caging effect. This is specifically focused on the development of molecular beacon assays based on this tool's high level of sensitivity. Molecular beacons provide a window for potent screening of the temporal control achieved by caging of ODNs with photo-cleavable cage compounds such as DMNPE and its plausible use with targeted antisense therapies.

Chapter 2

Control of DNA Hybridization with Photo-cleavable Adducts

Introduction

The hybridization of nucleic acids with their complementary strands is a rate-limiting step in many biological processes and bioassays including fluorescence *in situ* hybridization (FISH), microarrays, polymerase chain reaction (PCR), DNA-based biosensors, molecular computing, RNAi and nanomachines. Strategies to enhance, limit, or trigger these biological processes often target the hybridization event. It is well known that the extent of DNA hybridization is significantly dependent on temperature and ionic environment, and changes in these have therefore been used to control hybridization. Examples of temperature control are hot-start methods commonly used in PCR. Ionic environmental control of hybridization has been demonstrated through environmental modulation of salts or metal ions (Barnes 2002). Recent work accomplished spatial and temporal control through inductive coupling of oligonucleotides to metal nanocrystals that provide local heating when exposed to an external radio frequency field in order to have a broader ability to manipulate biological functions (Hamad-Schifferli 2002). Here we describe another strategy to control hybridization of nucleic acids with other forms of energy that enable precise regulation at the onset. Light was used to activate oligodeoxynucleotides (ODNs) that were previously inactivated with 1-(4,5-dimethoxy-2-nitrophenyl)diazoethane (DMNPE), a photocleavable “cage” compound that has been shown to control bioactivity of DNA plasmids (Monroe 1999).

Caged compounds have a covalently attached group that can be photocleaved when exposed to specific wavelengths of light. The “caged” term describes the blockade of biological activity rather than a chemical structure(McCray 1989; Pelliccioli 2002). Some studies that have shown control with caged compounds are seen within cellular studies, which include transport of proteins, effects on biological membranes, as well as muscle fibers (Patton 1991; Ramesh 1993; Ishihara 1997; Lee 1997). Caged compounds have been used to study the time course of fast cellular processes induced by a millisecond step increase in the intracellular concentration of a bioactive compound of interest achieved by a pulse of light exposure. In many of these applications, the advantages of using photoprotection over conventional methods for substrate release are that the caged effectors can be dispersed throughout the biological target without eliciting the species normal bioactivity, the concentration and spatial distribution can be controlled, and finally, the temporal release can be varied from seconds to nanoseconds (Givens 1998).

Photoactivatable compounds have been used modulate many aspects of DNA chemistry and biology. O-nitrobenzyl cage compounds were originally used as a protecting group in organic synthesis (Pillai 1980). Caged compounds have now been adapted and used in the caging of nucleotide analogs (Walker 1988), in the synthesis of bio-chip oligonucleotides (McGall 1996), in studies of the kinetics of DNA repair (Meldrum 1990; Meldrum 1998; Ordoukhanian 2000), and as protecting groups during DNA synthesis (Alvarez 1999). Cage groups have also been shown to control an oligonucleotide hairpin configuration and indirectly hybridization (Ordoukhanian 1995). The use of cage compounds has also been seen in the repair of DNA that has a single

strand break at a specific site (Zhang 2001). Inclusion of caged adenosine within synthesized RNA oligonucleotides enables temporal control of ribozyme and spliceosome activities (Chaulk 1998; Chaulk 2001).

We have also shown that direct caging of plasmid DNA with DMNPE can block transcription, allowing for the targeted expression of transgenes *in vitro* and *in vivo* through direct light exposure (Monroe 1999). In addition to controlling the kinetics of a particular molecular target, caging affords the ability to restrict re-activation to a localized tissue of interest, as demonstrated by the targeting of transgene expression by caging hormone inducers or nuclear receptor agonists (Cruz 2000; Lin 2002; Link 2004). More recently, caged mRNA was microinjected in zebrafish embryos to induce expression of certain genes and study the effect of their expression on developmental patterns (Ando 2001; Ando 2003). In this report, we explore the application of photoactivatable cage compounds to reversibly block hybridization of DNA oligonucleotides.

Materials and Methods

ODN Caging with DMNPE

Unless otherwise specified, reagents were purchased from Sigma-Aldrich (St. Louis, MO). 5 mg of 1-(4,5-dimethoxy)-2-nitroacetophenone hydrazone (Molecular Probes, Eugene, OR) and 50 mg of manganese (IV) oxide were gently agitated in 1 mL of N,N-dimethylformamide (DMF) at 25 °C for 20 min. Manganese oxide was removed from the 1-(4,5-dimethoxy-2-nitrophenyl) diazoethane (DMNPE) by filtering the solution through 100 mg of Celite^{TM} supported by glass wool in a 1 cc tuberculin syringe barrel. 100 μl of the filtrate was agitated with 100 μg of the ODN (2μg/μL) in 200 μl of 10mM

Bis-Tris (pH 5.5) for 1 hour at 4 °C. Another 60 μl of the active 1-(4,5-dimethoxy-2-nitrophenyl)-diazoethane (filtrate) was then added and the solution was agitated for 24 hours at 4 °C.

A second set of caging reactions was run in triplicate using phosphorothioate ODNs (PS-ODNs). This reaction followed the same procedure as stated above, however only the initial 100 μl of active DMNPE cage compound was added prior to overnight agitation. Samples were filtered and then characterized in the same format as the initial set.

To remove excess unattached cage compound, caged ODNs were purified using Microcon YM-3 (3000 MW cut-off) centrifugal filters (Millipore, Bilerica, MA). Caged ODNs were spun at $12x10^3$ G for 100 minutes, and then resuspended in 33% DMF, stored at 4 °C, and protected from light by wrapping samples in aluminum foil.

Spectral Scanning Protocol and DMNPE Extinction Coefficient Determination

Absorption spectrophotometry of caged species was used to estimate the degree of caging. Native (non-caged) 20-mer ODN (GCCCAAGCTGGCATCCGTCA, purchased from Integrated DNA Technologies, Coralville, IA) and DMNPE-caged ODN were dissolved in 33% DMF in separate cuvettes (DMNPE-caged ODN: 50 μg/ml; native ODN: 50 μg/ml) and scanned for absorbance from 230 to 500 nm (Thermo Spectronic Genesys 6, Waltham, MA). Spectral scans of caged-flashed ODNs (caged ODNS exposed to light) were also made similarly to the above stated methods. To determine the spectral changes following photoactivation, some of the caged-flashed ODN products were filtered through the Microcon YM-3 filters following light exposure to

remove the released 1-(4,5-dimethoxy-2-nitrosophenyl)-ethanone, and then scanned as described. These samples are referred to as caged-flashed-filtered ODNs.

To approximate the extinction coefficient of DMNPE adducted ODNs, absorbance peaks at 260 and 355 nm of known concentrations of dilutions of DMNPE-caged ATP in 33% DMF were scanned as above. Moles of DMNPE present were calculated and the amount of 260 nm absorbance attributed to the DMNPE cage molecules in the spectra of caged ODNs was determined. These values were then used with Beer's Law to calculate ODN concentration and caging efficiency using the following:

Equations 1 & 2:

- $A_{260\ nm} = [(\varepsilon_{260\ nm,\ DNA})(C_{DNA})(L)] + [(\varepsilon_{260\ nm,\ DMNPE})(C_{DMNPE})(L)]$ (1)

- $A_{355\ nm} = [(\varepsilon_{355\ nm,\ DMNPE})(C_{DMNPE})(L)]$ (2)
  - C = Concentration (M)
  - ε = Extinction coefficient ($M^{-1} \cdot cm^{-1}$)
  - L = Path length (cm)

Equation 3:

- Phosphate Caging Efficiency ($Cage_{eff}$ = percent of phosphates caged)

  - $Cage_{eff} = [(C_{DMNPE} \div C_{DNA}) \div (\#\ of\ Bases\ in\ ODN)] * 100$ (3)

Photoactivation and Gel Electrophoresis of Caged ODNs

ODN (250 ng) was run in a 15% polyacrylamide non-denaturing gel in tris-borate (TBE) buffer (100 mM tris-borate, 2 mM EDTA, pH 8.5) at 70 V for 80 minutes. Caged-flashed samples were prepared by taking aliquots from the 50μg/ml caged-ODN samples and exposing 365 nm light (dose equivalent to 5.6 mJ/cm^2) for 20 minutes prior to electrophoresis. The light source has a peak output at 365 nm and a fluence rate of 4.68 mW/cm^2 at 10 cm (UVP Blak Ray, San Gabriel, CA; Model B 100 AP).

Spectrographic characterization of this lamp confirmed that the emission spectrum is 365 nm ± 8 nm (USB2000 Fiber Optic Spectrometer, Ocean Optics, Inc., Dunedin, FL). Gels were stained after electrophoresis with 1X SYBR-Gold nucleic acid gel stain (Molecular Probes, Eugene, OR) in TBE buffer for 30 minutes. A polyacrylimide non-denaturing gel was also run identically in order to characterize the resulting samples of caged and caged-flashed phosphorothioate ODNs.

Hybridization of ODNs to Complementary Molecular Beacons

In order to determine the role that DMNPE adduction plays in the disruption of hybridization of the ODN to complementary DNA or RNA, a hybridization assay using molecular beacons was developed. Molecular beacons are fluorogenic probes that signal hybridization with a complementary nucleic acid target (Tyagi 1996). These DNA oligonucleotides contain a 5' fluorophore, a 3' quenching group, and 4-6 complementary bases on the 3' and 5' "stem" ends, which cause the beacon to form a hairpin structure. Unless the inner "loop" region hybridizes to a complementary nucleic acid, the fluorescence of the beacon in its hairpin configuration is quenched (Figure 2.1). When hybridized with a complementary ODN, the hairpin structure linearizes, distancing the fluorophore from the quencher generating fluorescence (Bonnet 1999).

A molecular beacon (MB1) was designed to hybridize with the specified ODN used in this experiment (Monroe 2003). The sequence of MB1 was designed with the aid of *mfold*, an RNA/DNA folding analysis program (Zuker 2000) and synthesized by Biosearch Technologies (Novato, CA). The sequence of molecular beacon 1 (MB1) is 5'-FAM-gtgcgTGACGGATGCCAGCTTGGGCcgcac-BHQ1-3', where capital letters indicate bases complementary to the caged and native ODN, and lowercase letters

indicate bases forming the stem region of the beacon. The quenching group in this beacon is BlackHoleQuencher-1 (BHQ1) and the fluorescent label is [(3',6'-

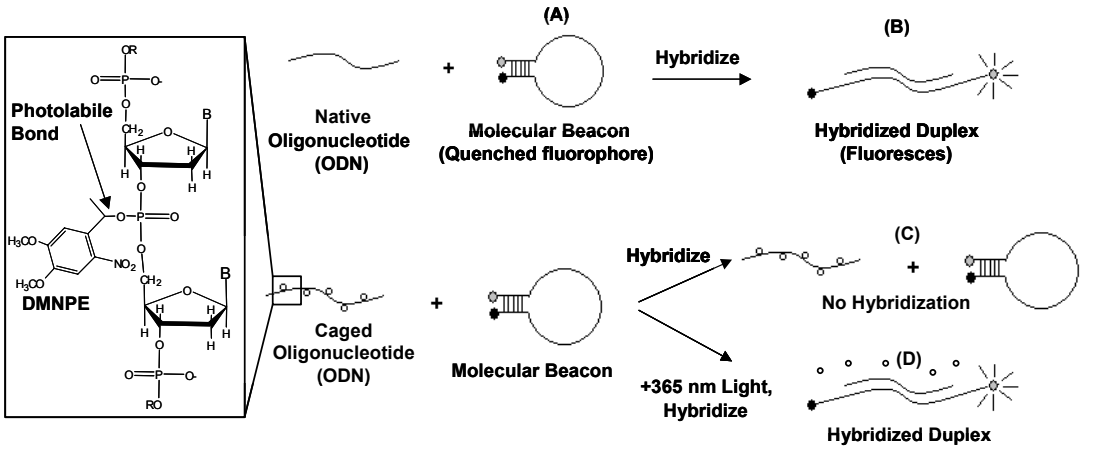

Figure 2.1. Hybridization assay of caged probe and complementary molecular beacon. Non-hybridized molecular beacon assumes the stem-loop configuration (A) and does not show fluorescence. When hybridized to a complementary nucleic acid target, the beacon fluoresces (B). Caged oligonucleotide does not hybridize with molecular beacon (C) until photoactivated with 365nm light (D). Inset shows theoretical location of adduction of DMNPE cage molecule at phosphate backbone of the ODN.

dipivaloylfluoresceinyl)-6-carboxamidohexyl]-1-O-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite (FAM).

Hybridization of caged and native ODNs with molecular beacon was performed as follows: 630 ng of native (non-caged), caged, or caged-flashed ODN were mixed with 300 ng of complementary molecular beacon (3.6:1 ODN:Beacon molar ratio) in 100ul solutions of 100 mM NaCl, 1mM EDTA, pH 7.5. The mixtures were denatured at 90°C for 5 min and allowed to slowly cool to 25°C over 60 min.

A similar hybridization experiment was also run in order to determine the effects of caging on the phosphorothioate ODNs. Some changes were noted as only a 1x ratio as run in order to determine the effects. Samples were otherwise prepared identically to

the procedure described above.

Fluorescence Measurements of Molecular Beacons

Fluorescence of hybridization solutions was used to quantify the hybridization of molecular beacons with ODNs. Each hybridization mixtures (100 μL) was excited at 492nm and emitted fluorescence quantified at 515nm in triplicate with a LS55B Luminescence Spectrophotometer (Perkin Elmer, Boston, MA).

A functional quantum yield (Φ) was calculated, based on the increases in fluorescence from the molecular beacon to signal conversion of caged ODNs, based on Equation 4:

$$\Phi = \frac{1}{I\ \varepsilon\ t_{90\%}} \tag{4}$$

where I is the irradiation intensity in moles of photons cm^{-2} s^{-1}, ε is the decadic extinction coefficient of DMNPE in cm^2 per mole of substrate, and $t_{90\%}$ is the irradiation time in seconds for 90% conversion (Adams 1988). A value of 4795 $M^{-1}cm^2$ was used for the 355 nm molar extinction coefficient for DMNPE that was calculated as described in “spectral scanning protocol”.

Electrophoresis of Caged ODNs and Molecular Beacons

Standard gel electrophoresis was used to confirm hybridization of caged ODNs with complementary molecular beacons. 210 ng of the ODN-molecular beacon hybridization mixture was run at 70V in 15% non-denaturing polyacrylamide in TE buffer (4 mM tris-borate, 0.1 mM EDTA, pH 8.5) for 90 min. Gels were stained in 1x TBE buffer for 30 minutes as previously described in the non-denaturing gel electrophoresis

assay with the nucleic acid stain of Sybr Gold. Samples were then visualized under UV light.

Results

Absorbance Spectrophotometry

Figure 2.2 shows the absorbance spectra of DMNPE-caged ODN, caged-flashed (caged-light exposed) ODN, caged-flashed-filtered, and native (non-caged) ODN. Unlike native ODN, DMNPE-caged ODN has a characteristic absorbance peak at 355 nm, consistent with the attachment of the DMNPE cage compound (Walker 1988). Based on the extinction coefficient of attached DMNPE, an average number of DMNPE caging groups per ODN was calculated as described previously, but here we also account for the 260nm absorbance of DMNPE due to higher adduction rates of DMNPE to ODNs than required to inactivate DNA plasmids (Monroe 1999). Absorbance at 355 nm indicates that caged ODN has approximately 14-16 DMNPE cage groups per 20mer ODN. Once flashed, a shift can be noted in the 355 nm peak that broadens towards longer wavelengths. The absorbance of the

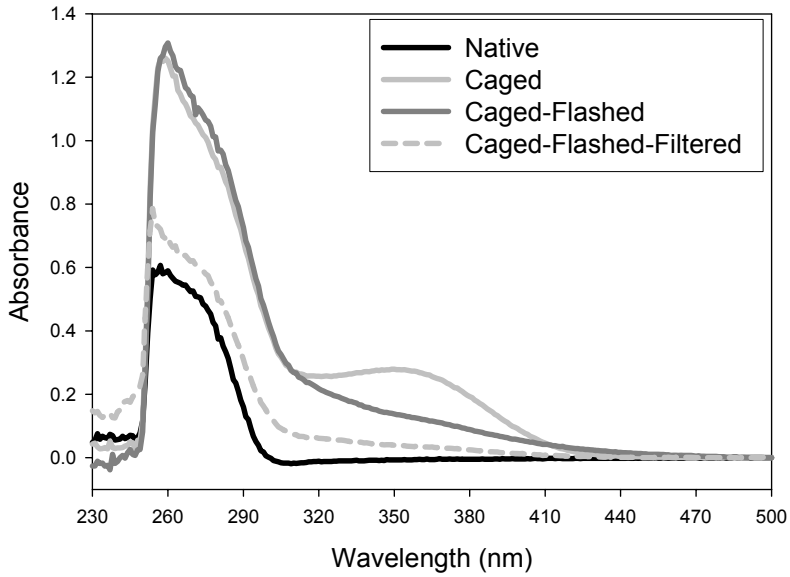

Figure 2.2. Spectral scans of DMNPE-caged, caged-Flashed, caged-flashed-filtered, and native PS-ODN. All three samples were dissolved in 33% DMF in separate cuvettes and scanned for absorbance from 230 to 500 nm.

released cage group prevents estimation of photoactivation by spectrophotometry, so some samples were filtered to remove the released cage and then scanned again. The caged-flashed-filtered ODN product results in an even lower absorbance with a similar trend in the range of 350 to 370 nm. The caged-flashed-filtered ODN has approximately 2-4 DMNPE cage groups per ODN.

Non-denaturing Gel Electrophoresis

Electrophoresis of caged ODNs shows characteristic changes in mobility corresponding to the addition and removal of the DMNPE caging groups (Figure 2.3). The DMNPE-caged oligonucleotides have reduced mobility compared to that of native oligonucleotides. A light-induced change is seen between the caged and caged-flashed samples, with the caged-flashed band having mobility more comparable to the native ODN subjected to caging conditions and processes of the caging reaction, but without the addition of DMNPE. The intensity of the band corresponding to the caged ODN was also less than the caged-light-exposed and native ODNs, suggesting an alteration of the ODN that interferes with its staining.

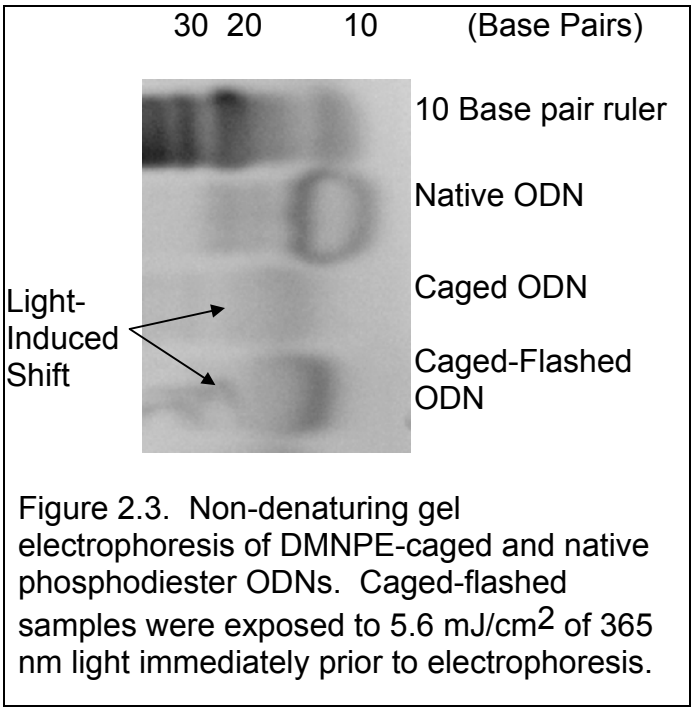

Figure 2.3. Non-denaturing gel electrophoresis of DMNPE-caged and native phosphodiester ODNs. Caged-flashed samples were exposed to 5.6 mJ/cm^2 of 365 nm light immediately prior to electrophoresis.

In Figure 2.4, the native phosphorothioate ODN does however electrophorese much closer to the 20 base pair marker in the molecular ruler, thus showing a reduced

mobility. Also, very little staining is seen in the caged phosphorothioate ODN compared to native phosphorothioate ODN, corresponding to a greater amount of cage compound attached to the ODNs. Restoration of the ODNs to mobility and staining characteristics closer resembling that of the native ODN can be seen with the light induced shift between the caged and caged-flashed samples.

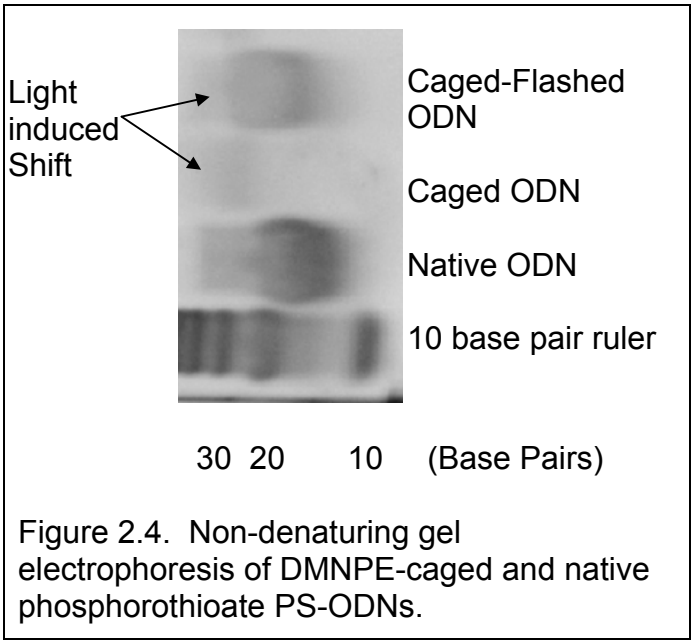

Figure 2.4. Non-denaturing gel electrophoresis of DMNPE-caged and native phosphorothioate PS-ODNs.

Fluorescence Measurements of Hybridization Products

Fluorescence emissions from a complementary molecular beacon indicate differing amounts of hybridization for caged and native ODNs (Figure 2.5). The relative fluorescence of molecular beacon in solution alone was 34.6 $\pm$ 9.4 (mean $\pm$ SD, n=3) relative fluorescence units (RFUs) and 414 $\pm$ 34 RFUs when native compliment was added. Fluorescent emission of the hybridization mixture of caged ODN and molecular beacon is 89.2 $\pm$ 8.3 RFUs, which is 14.4% of the relative fluorescence of the native probe hybridization mixture when background signal of molecular beacon alone in solution is removed, indicating a low level of hybridization. However, the caged-light-

exposed sample shows an increase in fluorescence to 336 ± 8.0 RFUs, which is 79.5% of the native solution, indicating an increase in hybridization of the photoactivated ODNs. Significant difference from beacon alone is denoted by the cross symbol, while asterisks denote significant difference from both caged ODN and beacon alone (n = 3, $p<0.05$, Bonferroni's t-test).

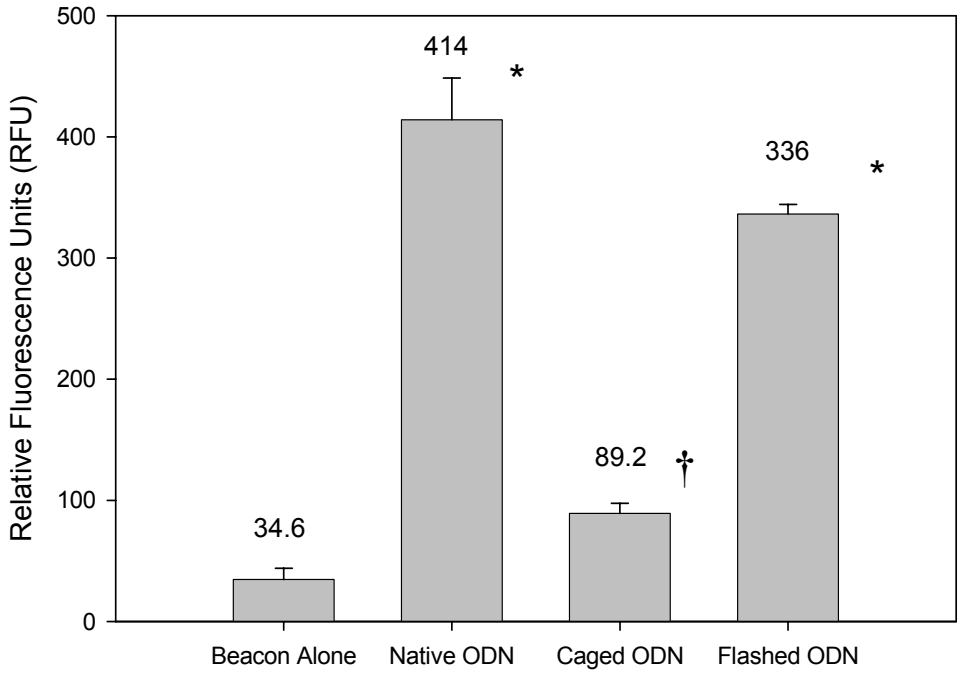

Figure 2.5. Relative fluorescence intensities from solutions of molecular beacon hybridized with phosphodiester native, caged or caged-light-exposed ODNs. 630 ng of native, caged, or caged-light-exposed ODNs were mixed with 300ng of molecular beacon (3.6:1 target:beacon molar ratio) and tested for hybridization in a fluorescence spectrofluorimeter. Significant difference from beacon alone is denoted by cross symbol, while asterisks denote significant difference from caged ODN also (n = 3, $p<0.05$, Bonferroni's t-test).

In the case of the phosphorothioate caged ODNs in comparison, restriction of hybridization by the cage compound was much greater than that of the phosphodiester ODNs. As seen in Figure 2.6, the relative fluorescence for the molecular beacon alone was 53.4 ± 0.9, while the native ODN had a fluorescence of 974 ± 18. The fluorescence

emission of the caged ODNs in this set of samples was 61.4 ± 0.7, or 0.88% of the native solution when the background noise is removed as stated above. Once again, when exposed to UV-light of 365 nm, the activity is restored, although not as greatly as with the phosphodiester samples. The caged-flashed ODN in this experiment had a fluorescence of 217 ± 6.7. This is equivalent to a restoration of the ability to hybridize of the sample to 17.8% from 0.88%, which is over a 20-fold increase of hybridization from caged to caged-flashed ODNs.

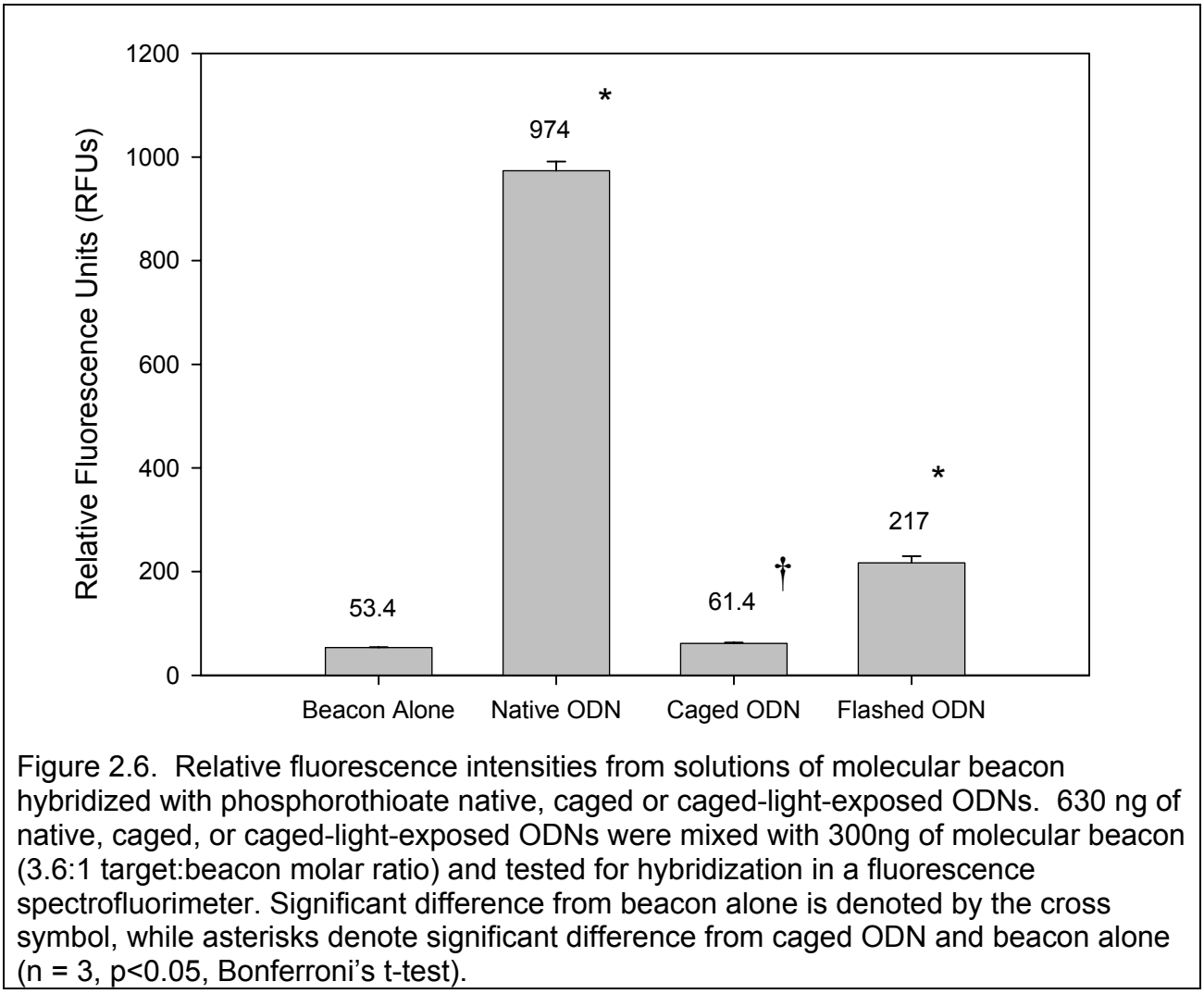

Figure 2.6. Relative fluorescence intensities from solutions of molecular beacon hybridized with phosphorothioate native, caged or caged-light-exposed ODNs. 630 ng of native, caged, or caged-light-exposed ODNs were mixed with 300ng of molecular beacon (3.6:1 target:beacon molar ratio) and tested for hybridization in a fluorescence spectrofluorimeter. Significant difference from beacon alone is denoted by the cross symbol, while asterisks denote significant difference from caged ODN and beacon alone ($n = 3$, $p<0.05$, Bonferroni's t-test).

Non-denaturing Gel Electrophoresis of Hybridization Products

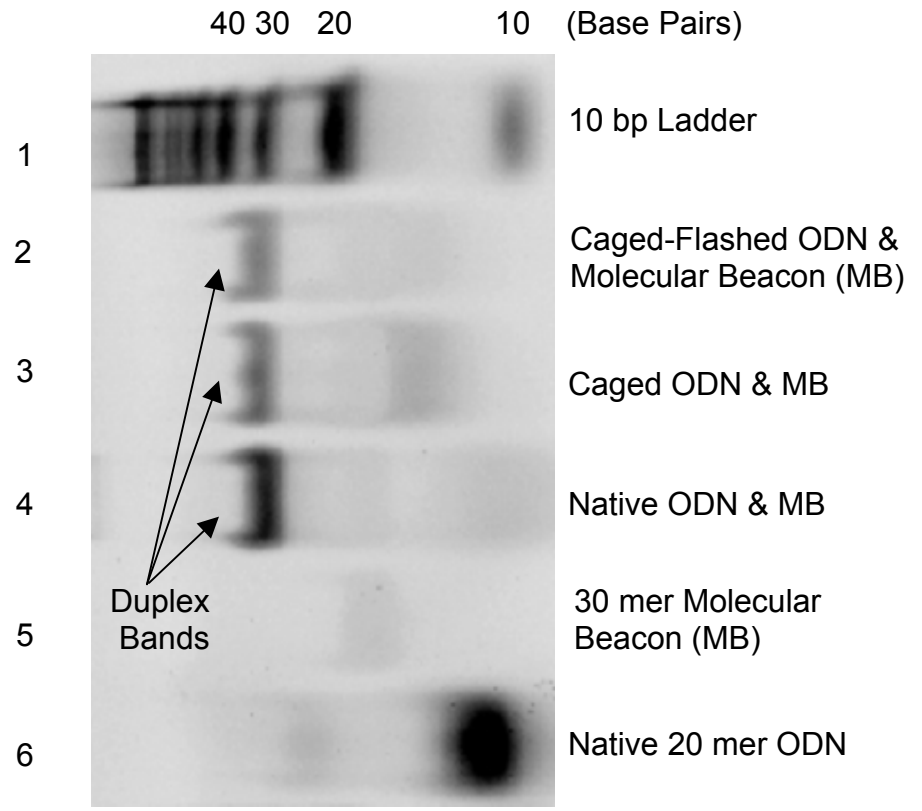

Figure 2.7. Inverse image of non-denaturing gel of hybridization products from caged and caged-flashed samples of hybridized oligonucleotides. Changes in hybridization of native, caged and caged-flashed mixtures of probe ODNs and molecular beacon were analyzed for changes in intensity of the band corresponding to the duplex conformation.

To confirm the results from the solution measurements of molecular beacon hybridization, non-denaturing polyacrylamide electrophoresis was used to show gel mobility shifts upon hybridization with caged and caged-light-exposed ODNs. Note the changes in the hybridized duplex and non-hybridized ODN band intensities between caged and caged-flashed samples (Figure 2.7, lanes 2 & 3). Caged ODN does not completely hybridize with its complementary sequence on the molecular beacon. However the sample that was exposed to light shows more ODN in the duplex form, resembling that of the non-caged native probe hybridization (shown for comparison in

Lane 4). The non-hybridized molecular beacon does not stain well when run in a gel at these concentrations, possibly due to the beacon's quenching of the nucleic acid stain used (lane 5).

Light dose-response of caged ODNs and Molecular Beacons

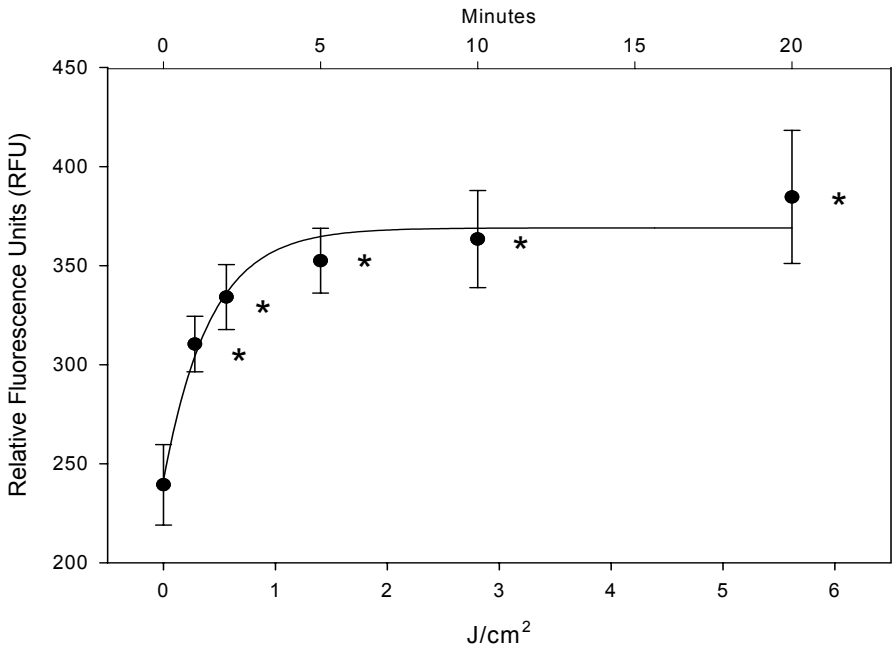

Figure 2.8. Effect of cumulative light exposure time on solution fluorescence of caged ODN-molecular beacon hybridization mixtures, with times of 1, 2, 5, 10, and 20 minutes. Significant difference from caged ODN (0 minutes) denoted by asterisks (n = 3, $p<0.05$, Bonferroni's t-test).

To determine the proper dose of light exposure required for photoactivation of caged ODNs, hybridization mixtures were exposed to increasing durations of light before denaturating and fluorescence measurement (Figure 2.8). Fluorescence emission increased with increasing light exposure, from 230 RFU with no light exposure, to values of 302, 325, 344, 372 and 380 RFU with light exposures of 1, 2, 5, 10, and 20 minutes, respectively. Significant difference from caged ODN (0 minutes)

denoted by asterisks (n = 3, $p<0.05$, Bonferroni's t-test). All of the samples showed a significant difference from the control sample. The experimental data retrieved from this section was utilized to determine the functional quantum yield for the caging of ODNs with DMNPE (see Equation 4).

Discussion

Our data suggests that the adduction of photocleavable cage compounds to DNA oligonucleotides (ODNs) can reversibly block hybridization. This strategy relies on the covalent attachment of a cage compound, which disrupts DNA bioactivity until photocleavage restores DNA to its native and bioactive form. We have partially blocked ODN bioactivity with the cage compound DMNPE, the same compound that has been shown to reversibly control transcriptional activity of plasmid DNA (Monroe 1999).

Absorbance data and gel shifts indicate that DMNPE adducts to ODNs and is photocleaved with 365 nm light. Spectral scans show characteristic absorbance at 355 nm (Figure 2.2). Similar to DMNPE-caged plasmid DNA and DMNPE-caged ATP, absorption at this wavelength is consistent with the presence of DMNPE caging groups, as native DNA does not absorb in this region. Calculations based on the extinction coefficient of attached DMNPE indicate that there is an average of 14 to 16 cage molecules present per 20-mer ODN. The spectral scans in Figure 2.2 also indicate that photo-cleavage of the caged ODNs was achieved. The filtration of the flashed products (caged-flashed-filtered) confirms this as the absorbance at the 355 to 390 nm range decreases once the sample was filtered. By filtering the caged-flashed product, released cage could be removed, and thus a more effective characterization of the flashed product was possible. In gel electrophoresis, caged ODNs have lower

electrophoretic mobility than native (non-caged) ODNs (Figures 2.3 & 2.4). This observation is consistent with the attachment of the non-polar DMNPE cage groups that retard ODN mobility by neutralizing otherwise negative charges on the phosphodiester backbone or base structures. It was originally hypothesized that the DMNPE attaches to the phosphate backbone of DNA, similar to its demonstrated attachment to phosphates of nucleotides (Walker 1988). While structural studies of the DMNPE-caged ODNs have not been completed to date, alterations in staining intensity between caged and native ODNs (lanes 2 and 3) suggest that attachment of the DMNPE may also block some reported base-associated labeling of the SYBR-Gold nucleic acid stain used to visualize these ODNs in gels (Tuma 1999). Even if the majority of DMNPE adduction occurs at the phosphate backbone of the ODN, this conformation may still disrupt hybridization, as shown with other phosphate modifications (Jeong 1999).

Two assays utilizing molecular beacons demonstrate that caging ODNs modified their hybridization activity. The switch-like fluorescence properties of the molecular beacon allow it to act as a direct measure of hybridization, so that solutions of hybridized ODN and beacon can be assayed for hybridization directly in a spectrofluorimeter. Fluorescence emission of a hybridization mixture of caged ODNs and complementary beacon is much lower than that of the native hybridization mixture (Figures 2.5 & 2.6). When exposed to 5.6 J/cm^2 of 365 nm light prior to hybridization, fluorescence emission increases, indicating a restoration of hybridization activity. When the background of native probe alone is subtracted, the level of activity between the caged and caged-light-exposed ODN-beacon hybridization increases from 15% to 79% of the native ODN's activity. This was also the case in the hybridizations run with the

phosphorothioate caged ODNs. However in this case, a much higher suppression of the ODN was achieved at 0.88% and restored to 17.8%, or over a 20-fold restoration. To corroborate the results found in solution, molecular beacons were also used in a non-denaturing PAGE assay, to show shifts in gel bands upon hybridization (Figure 2.7). Decreased intensity of the band representing the duplex hybridization of 20mer ODN and 30-mer molecular beacon shows that caged ODN does not completely hybridize with a complementary beacon. When exposed to 365 nm light prior to hybridization and electrophoresis, cage groups are photocleaved from the ODN allowing it to hybridize. The caged-light-exposed sample showed a stronger band in the duplex conformation resembling that of the non-caged native probe hybridization. The presence of a stronger band of the 20-mer non-hybridized ODN in the caged sample that disappears in the flashed sample also confirms the alteration of hybridization activity seen in solution.

Restoration of hybridization of caged ODNs is a light-dependent process as seen in the 365 nm light dose response of hybridization activity in Figure 2.8. An exponential relationship is seen between the amount of fluorescence from a molecular beacon and increasing light exposure, with a $dose_{1/2}$ of 0.288 J/cm^2. It is difficult to compare this dose of light with other photolysis studies because most caged compounds are directly synthesized with only one cage moiety per effector molecule. However, the doses for functional restoration of DMNPE-caged ODNs do appear to be within the range of other published values of photolysis for similar cage compounds and light delivery systems (Rossi 1997; Rinnova 2000). The caging group used here, DMNPE, in general has relatively low quantum yields when compared to other caged groups. For instance, the

reported Q_p for DMNPE-caged ATP is 0.07, much lower than the 0.63 Q_p of NPE-caged ATP (Walker 1988; Wooton 1989). An attempt to estimate the functional quantum yield of DMNPE-caged ODNs was made based on the increase in functional activity with light exposure in the beacon assay in Figure 2.8. The measured increase in hybridization was used to determine complete conversion rather than the traditional method of quantum yield determination from absorption increases. The fact that each caged ODN has multiple DMNPE adducts precludes the simple use of an absorption increase because restoration of hybridization may not correlate with the photoconversion of less than all the attached cages. Our calculations indicate that DMNPE-caged ODNs have a functional quantum yield of at least 0.09. This number assumes a 100% response from the molecular beacon to indicate hybridization of a photoreleased ODN, which may not be the case. Lower efficiencies of the molecular beacon system in detecting hybridization of a photoactivated ODN would impart increases to the resulting quantum yield.

Modifications of the strategy could lead to improvements in blockade and subsequent restoration of hybridization of ODNs. Since our initial report of caging plasmid DNA with DMNPE, other promising cage groups have been identified. For example, brominated 7-hydroxycoumarin-4-ylmethyls (BHC), a modification of Tsien's bromocoumarin cage, has recently been used to randomly cage mRNA, allowing it to be activated at selected sites for translation in zebrafish embryo's, facilitating the elucidation of several genes involved in development (Ando 2001). BHC reportedly has better quantum efficiencies than DMNPE, which would require less light for complete photocleavage after delivery to cells (Furuta 1999). In addition to a lower light dose, the

architecture of light exposure to tissue could be engineered to minimize cellular-induced responses, while maximizing total dose of light delivered to the caged ODNs. Two-photon excitation could be an alternative technique for photoactivation of caged ODNs (Piston 1999). Longer wavelengths utilized in the two-photon uncaging have lower energy than the 365 nm light, causing less photodamage to cells and tissues (Denk 1995). The longer wavelengths also have deeper tissue penetration and would allow targeting at greater depths. This technique, combined with newer cage molecules having biologically useful 2-photon cross-sections, could minimize cellular damage while increasing targeting precision (Furuta 2004).

A significant improvement in this strategy would be to cage a single nucleotide and incorporate it into ODN synthesis. This approach offers the advantage of controlling precisely the number and attachment site of cage groups to each ODN. It is also possible that different attachment sites of the caging moiety on the ODN will have different rates of photorelease. If the caging sites which give the highest efficiency of photorelease could be identified, caging conditions or synthetic strategies to incorporate the cage in a site specific manner can be developed to target those positions which are most readily released upon irradiation.

In summary, our data indicate that light-activated hybridization can be achieved with the use of photocleavable cage compounds. Spectrophotometric, gel-shift, and molecular beacon fluorescence data indicate that caged compounds can be used to reversibly alter the hybridization activity of single stranded DNA. Attachment of DMNPE to phosphodiester ODNs presents a strategy for the temporal and spatial control of hybridization. This strategy has application in controlling DNA hybridization activity

such as primer activity in PCR, FISH, microarrays, molecular computers, nanomachines, DNA-biosensors and targeting of antisense ODNs to specific locations in tissues. Extension of this technique to phosphorothioate ODNs and RNAi species could be applicable in controlling gene silencing and protection of these compounds from enzymatic degradation.

Chapter 3

Optimal Solvent Selection and Purification Techniques for DNA Oligonucleotide Caging

Introduction

The aim of this study is to determine an optimal solvent and purification technique combination to allow for effective and efficient characterization of DMNPE-caged ODNs. Selection of an appropriate solvent is necessary to allow for a consistent and potent reaction, while also allowing for proper characterization of the caged products. Determination of the optimal purification technique is an important step in providing a valid assessment of the cage compounds abilities to control oligonucleotide hybridization activity. Presence of excess cage does not allow for proper assessment of the purification method, and may have negative effects in future *in vivo* studies.

In an effort to improve efficiency of the caging reaction and characterization of its products, we chose to look at the effects of different solvents in the caging reaction of DMNPE with DNA oligonucleotides (ODNs) as previously described in chapter 2. Proper solvent selection was necessary to promote cage attachment, avoid denaturing and irreversible alterations of the DNA, provide separation of products from unattached cage compound, and to facilitate accurate characterization. Study of the effects of solvents such as N,N-dimethylformamide (DMF) or dimethyl sulfoxide (DMSO) on nucleic acids has shown denaturing through activity of endonuclease S1 (Wetmur 1968). DMSO has also recently been used for the reaction of cage compounds with nucleic acids in an effort to develop a photo-control method for hybridization (Monroe 1999; Ando 2001).

DMNPE has minimal solubility in water (Walker 1988), and thus is problematic when working with ODNs aqueous solutions. In studies on caging of DNA plasmids, 33% dimethyl sulfoxide was used in mixture with a 10mM Bis-Tris solution, allowing for solubility levels that were adequate to keep the caged DNA plasmids in solution (Monroe 1999). However, more heavily caged samples are more likely to precipitate in an aqueous solution, due to higher amounts of the hydrophobic cage compound. It was also noted that DMSO would crystallize at 4ºC in the reaction, thus preventing a full reaction from taking place when used with DNA ODNs. DMF was initially chosen to replace DMSO, as they share very similar properties and DMF does not crystallize at 4ºC. One drawback of using DMF, however, is the high extinction coefficient of the solvent at wavelengths of 200 to 260 nm, thus providing possible difficulty in accurately characterizing the ODNs. Four organic solvents, chosen based on previous use or compatibility with materials used, were evaluated to determine the optimal choice for caging DNA ODNs with DMNPE: N,N-dimethylformamide (DMF), isopropanol (IPA), methanol (MeOH), and acetonitrile (MeCN). Structures of these solvents can be seen in Figure 3.1.

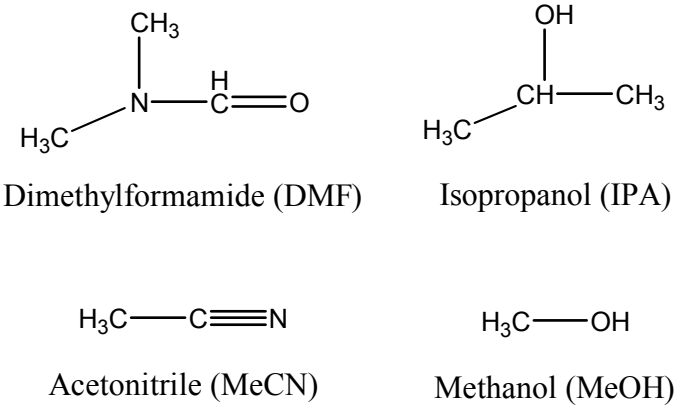

Figure 3.1. Structures of Solvents tested.

Purification of the caged ODN products upon completion of the chemical reaction was also studied. The presence of excess cage compound required separation of the target caged DNA ODNs from the reaction volume. Initially performed chloroform

extractions and ethanol precipitations by Monroe (1999) with DNA plasmids, this technique was not efficient at separation of the caged and native DNA ODNs from the excess cage compound due to the greater degree of DMNPE adduction with the more heavily caged products portioning into the organic phase. This was most notable with the phosphorothioate DNA ODNs, which were more reactive with the cage compound when compared to the phosphodiester DNA ODNs. In order to account for this issue, a study was run to determine an efficient method for purifying the reaction volumes. The three methods studied were the use of Slide-a-Lyzer dialysis (Pierce Biotechnology, Rockford, IL), Sephadex G-25 Oligo Quick Spin columns (Roche, Basel, Switzerland) and Microcon YM-3 molecular weight cut-off filters (Millipore, Bilerica, MA). Upon completion of this study, optimal caging reactions could be performed and characterized, thus allowing for an efficient investigation of the ability to control DNA ODN hybridization with DMNPE.

Materials and Methods

DMNPE Cage Compound Solubility

DMF, isopropanol, methanol, and acetonitrile were tested to determine the solubility of DNA oligonucleotides (ISIS 2302) and DMNPE cage compound. In order to determine the solubility of the cage compound in the organic solvents, a 5 mg sample of 1-(4,5-dimethoxy)-2-nitroacetophenone hydrazone (DMNPE hydrazone) was dissolved in 1 mL of DMF, and aliqouted into 50 µL volumes that were then dried down to 5 µL and resuspended in 300 µL of MeCN, DMF, IPA, or MeOH. Spectrophometric scans of these samples were then performed from 200 nm to 500 nm (baselined in their respective solvent) in order to determine solubility.

To determine whether activated DMNPE has differing solubility in these solvents, the DMNPE hydrazone solution used above was activated using 35 mg of MnO_2, followed by covering the samples in aluminum foil. The samples were then vortexed for 15 seconds followed by agitation at 4 ºC for 20 minutes. The samples were then placed in a microcentrifuge for 30 seconds and filtered through 100 mg of Celite supported by glass wool to remove the MnO_2. After filtration, a second set of aliquots was made as described in the initial DMNPE solubility study from the activated solution. These samples were then dried and resuspended to a volume of 300 µL in one of the solvents and scanned in the spectrophotometer as previously described.

DNA Oligonucleotides in Various Solvents

DNA ODNs were dissolved in either pure solvents or 33%, 50%, 66% mixtures of each with HPLC grade water in order to determine solubility. Previously made solutions of both phosphorothioate and phosphodiester 20-mer ODN (ISIS 2302) were initially diluted in water to 50 µL at a concentration of 0.05 µg/uL and dried down in a vacufuge at 30 ºC to a final volume of 5 µL. Samples were then resuspended, one of each type of ODN in the four aforementioned pure solvents up to a volume of 300 µL and scanned in a spectrophotometer as previously described.

DNA Oligonucleotides in Mixed Solvents

Based on the results of the initial experiment, further samples were prepared with acetonitrile or methanol in mixture with water as solvents. 100 µL samples of the DNA ODNs (0.05 µg/uL) were each brought up to 300 µL in final volume by adding one of the solvents and water to make solutions of 33%, 50%, and 66% of each organic solvent in

water. These samples were then scanned in the spectrophotometer as in the previous experiment to determine sample solubility.

Purification Technique Evaluation

To determine the most efficient method of purification, two caging reactions (300 µg of DNA ODN, 300 µL of activated DMNPE) were run following the protocol located in Appendix B, one with phosphodiester DNA ODNs and the other with phosphorothioate DNA ODNs. Three 300 µL aliquots of each completed reaction were then prepared. An additional standard reaction was also run with phosphodiester DNA ODNs for comparing a second protocol used with the Microcon YM-3 filter with additional washes to help improve removal of the unattached DMNPE. Samples were then scanned in a spectrophotometer and characterized quantitatively. Working stocks were prepared for each reaction at a concentration of 50 µg/µL, and then analyzed in molecular beacon hybridization and gel electrophoresis assays following the same methods found in chapter 2. Samples were compared and analyzed to determine which purification technique was the most promising in several categories, such as volume, duration, ODN recovery, and cost.

Variable Solvent Caging Reaction

Three caging reactions were performed as stated in chapter 2 with various solvents in place of DMF. This reaction was run in DMF, MeOH, and DMSO. Samples of DMNPE hydrazone were activated in each of the solvents, and then the reaction was performed accordingly. The cage sample was then reacted with both phosphodiester and phosphorothioate ODNs. Samples were only reacted with 100 µL of active DMNPE. In all steps of the reaction and subsequent purification, DMF was replaced

with the solvent of choice. Upon completion of the reaction and purification, spectrophometric scans were taken and samples were assessed in molecular beacon hybridization identical to protocol in chapter 2.

Results

DNA Solubility in Solvents

Figure 3.2 shows the absorbance spectra of phosphodiester DNA ODNs (PD ODNs) in each of the four organic solvents. These four solvents were N,N-dimethylformamide (DMF), acetonitrile, isopropanol, and methanol. It was noted that very little to no absorbance is seen at a wavelength 260 nm, the characteristic peak for DNA. The predicted absorbance in water for this concentration would have been between 0.3 and 0.4 with a concentration of 0.01

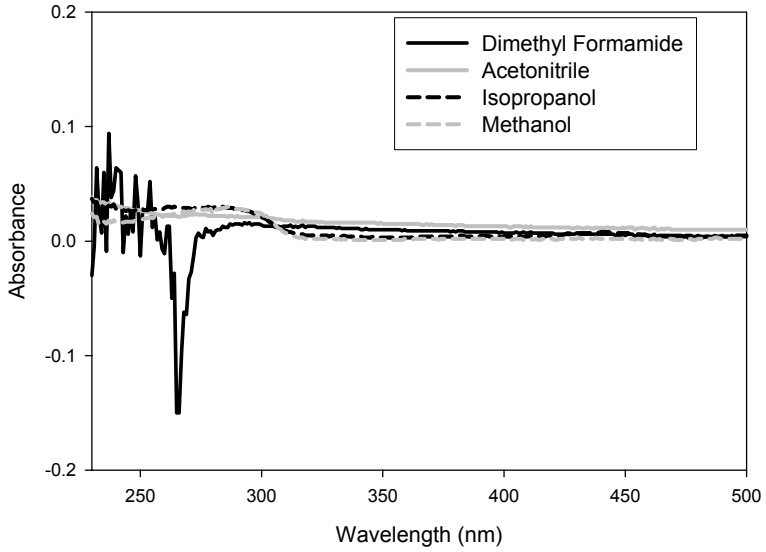

Figure 3.2. Spectrophotometer scans of Phosphodiester DNA ODNs in several organic solvents.

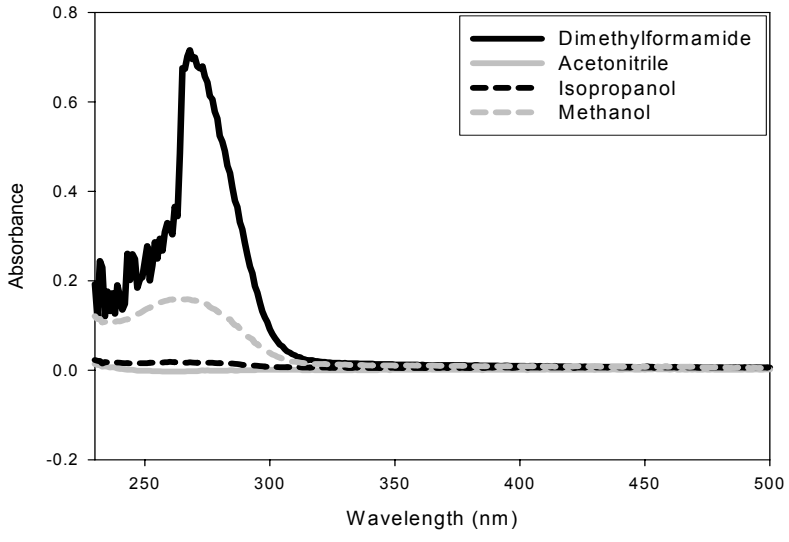

Figure 3.3. Spectrophotometer scans of Phosphorothioate DNA ODNs in several organic solvents.

μg/μL. In Figure 3.3, the same experiment showed the absorbance spectra of phosphorothioate DNA ODNs (PS ODNs). In this case, a confirmed peak at 260 nm for the DMF sample was observed, while a visible peak was noted for the methanol sample. This however was not the case in the acetonitrile and isopropanol samples.

The next step in the study looked at the solubility of the two types of DNA ODNs in solvents of a mixed nature. The two solvents studied here were acetonitrile and methanol. DMF was not tested as it is shown that the ODN is soluble in the solvent in Figure 3.3. In Figure 3.4, samples of PD ODNs were in mixtures of methanol, and all remained similar to the spectrum in water. The PD ODNs behaved similarly in acetonitrile, however these samples were slightly more variable in their absorbance near 260 nm as can be seen in Table 3.1. There was also a slight shift in the peak, however this was very minimal and within one to two nm. The PD-ODNs in DMF also behaved similarly.

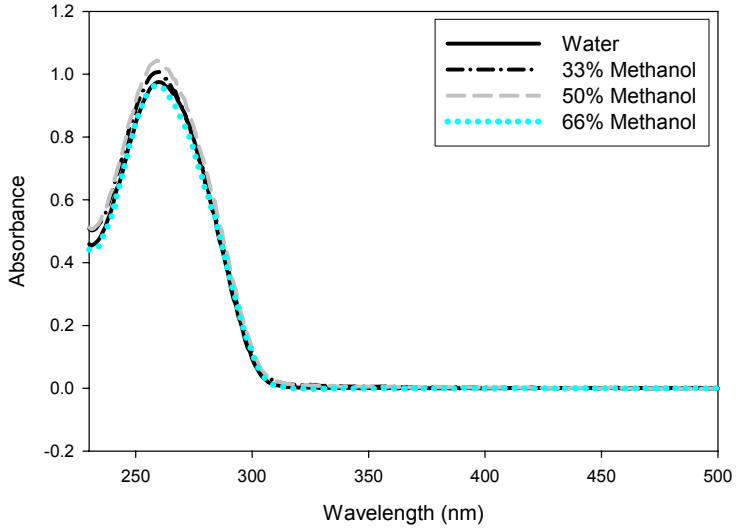

Figure 3.4. Spectrophotometer Scans of Phosphodiester DNA Oligonucleotide Solubility in Methanol Mixtures.

Looking at Table 3.1, phosphorothioate ODNs followed a similar spectra to that of the phosphodiester ODNs although there was more variation between the peaks the. Some unique trends in the phosphorothioate samples were seen with acetonitrile mixtures. The 33% acetonitrile sample had a higher peak than the 50% sample, however the 66% sample is higher than both, but all are within the error of the spectrophotometer. This was the same case for the DMF samples.

Table 3.1. 260 nm absorbance for ODNs in DMF, methanol and acetonitrile mixtures.

Solvent Mixture	*Phopsphodiester ODN 260 nm Absorbance*	*Phosphorothioate ODN 260 nm Absorbance*
Water	0.98	1.14
33% Methanol	1.01	1.28
50% Methanol	1.04	1.34
66% Methanol	0.96	1.37
33% Acetonitrile	1.12	1.31
50% Acetonitrile	1.05	1.24
66% Acetonitrile	1.03	1.45
33% DMF	1.08	1.11
50% DMF	1.23	1.30
66% DMF	1.07	1.27

DMNPE Solubility in Solvents

In Figure 3.5, the spectrophotometer scans of the DMNPE hydrazone are shown for each solvent. In this case, it was important to look at the absorbance at 355 nm as the key indicator of DMNPE solubility. Figure 3.5 showed that all solvents provide

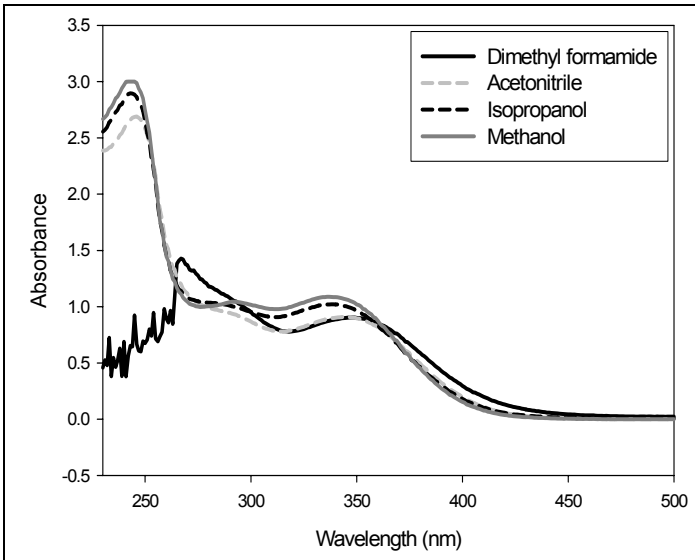

Figure 3.5. Spectrophotometer scans of DMNPE hydrazone in organic solvents.

relatively the same peaks at 355 nm and 260 nm for the inactive DMNPE hydrazone. This was also the case for the activated DMNPE compound (Figure 3.6), where there was little difference from that of the inactive DMNPE hydrazone. All samples had very similar peaks and shapes at the 260 nm and 355 nm wavelength regions.

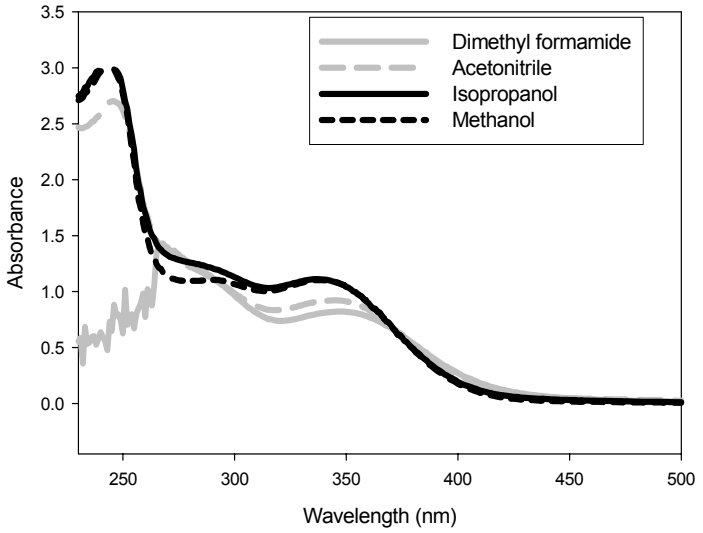

Figure 3.6. Spectrophotometer scans of active DMNPE cage compound in organic solvents.

Spectrophometric Scans of Purification Technique Reactions

The spectrophotometer scans in Figures 3.7 (PD ODN) and 3.8 (PS ODN) below provided initial characterization of the efficiency to purify caged DNA oligonucleotides. Peaks for DNA were at approximately 260 nm, while DMNPE peaks were accounted for at both 355 nm and 260 nm. In Figure 3.7, the highest 260 nm was achieved by the Microcon YM-3 filter, while it also

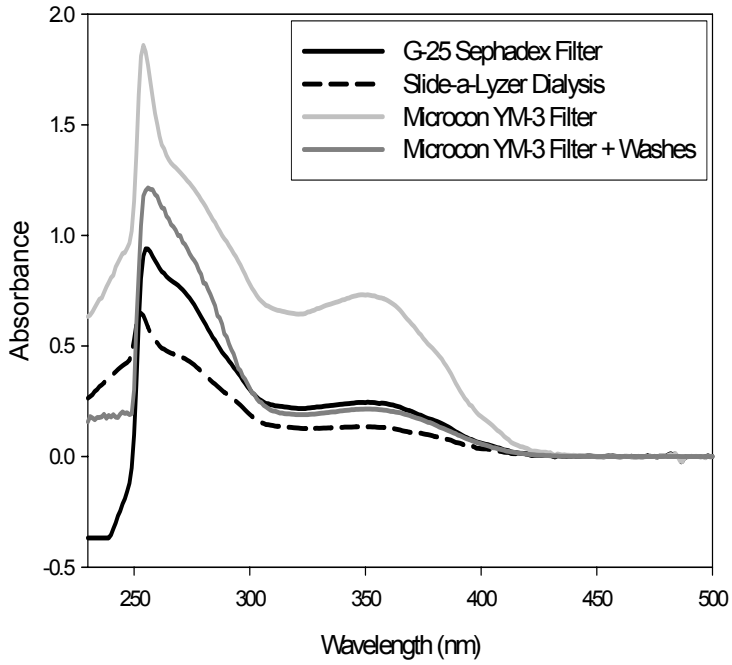

Figure 3.7. Spectrophotometer scans of caged phosphodiester DNA ODN after various filtration techniques.

had the highest 355 nm peak. The Microcon YM-3 filter's 355 nm absorbance dropped dramatically when the extra washes were performed, while it also maintained most of its 260 nm peak.

The caged phosphorothioate ODNs behaved slightly different as is shown in Figure 3.8. Here, the 260 nm peak for the Microcon YM-3 filter was once again the highest. The 260 nm peak of the G-25 Sephadex filter showed very little PS-ODN present, and also had a relatively low 355 nm peak. The 355 nm peaks of the other two filters however varied greatly showing a much lower peak for the Slide-a-Lyzer.

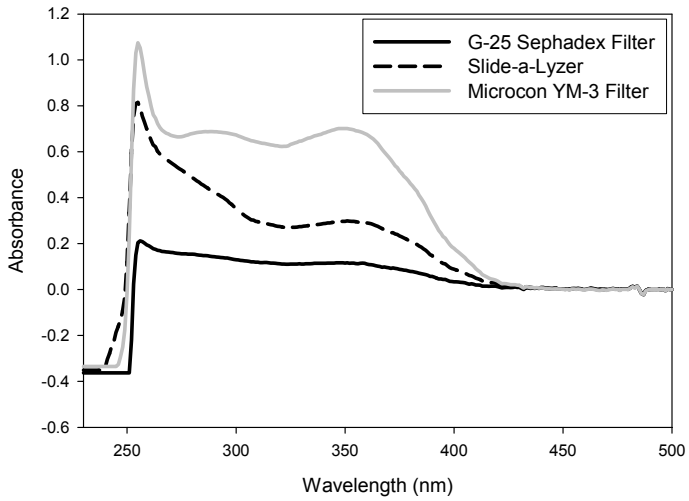

Figure 3.8. Spectrophotometer scans of caged phosphorothioate DNA oligonucleotide after various filtration techniques.

Table 3.2. Caging reaction characteristics for various filtration techniques.

ODN Type & Purification Technique	*Perceived Caging Efficiency*	*% Yield of ODN*
PD Sephadex	65.38%	56.0%
PD Dialysis	77.44%	22.4%
PD Amicon	113.7%	106.5%
PD Amicon with washes^	38.11%	93.4%
PS Sephadex	85.8%	22.0%
PS Dialysis	106.6%	37.2%
PS Amicon	169.3%	82.0%

ODN yields and perceived caging efficiencies for each purification technique were calculated from the spectrophotometer scans identically to those performed in the previous chapter and displayed in Table 3.2. Since aliquots from the same reaction were used with each technique, the actual caging efficiency remained the same for all. Variations are entirely based on the presence of unattached DMNPE and the DNA retained in the purified samples. The total amount of oligonucleotide was out of an initial 100 µg of DNA oligonucleotide. Caging efficiency was determined by the amount of cage present and number of possible binding sites available in the solution as described in chapter 2. Values for caging efficiency greater than 100% indicated an excess of unattached DMNPE cage compound not filtered from the sample. This could, however, also have been due to the cage compound binding to sites other than the predicted sites along the phosphate backbone. Higher caging efficiencies with phosphorothioate ODNS could possibly be due to a difference in extinction coefficient when attached to the sulfur atom as well as possible complications with purification methods. It was noted that as the reaction was visibly more active for phosphorothioate ODNS with greater N_2 gas production and a much quicker color change of the reaction volume from red to yellow.

Gel Electrophoresis of Purification Technique Reactions

Gel electrophoresis of the caged ODNs showed the normal characteristic changes in mobility and staining normally attributed to adduction and photo-release of the DMNPE cage compound for all of the techniques. PD caged samples (Figure 3.9) all behaved similarly with reduced staining and mobility. Upon exposure to light, the staining and mobility of all samples were partially restored.

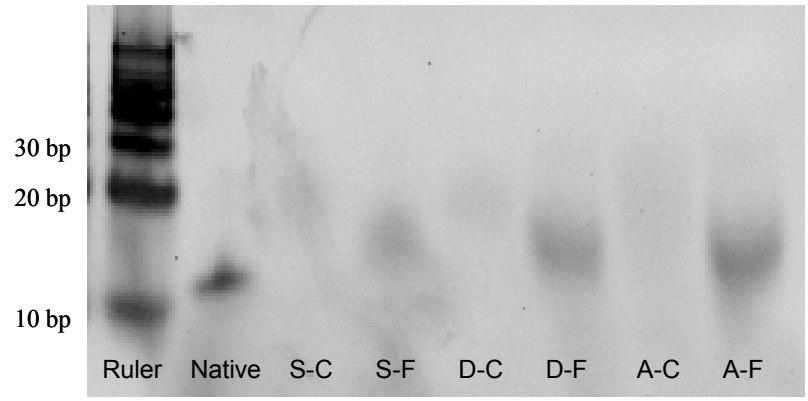

Figure 3.9. Non-Denaturing Gel Electrophoresis of DMNPE-caged and native PD-ODNs
C = caged sample, F = Caged-flashed Sample.
S = G-25 Sephadex filter, D = Slide-A-Lyzer Dialysis, and A = Microcon YM-3 Filtered samples.

The same trend was seen in the gel electrophoresis results for the PS ODNs displayed in Figure 3.10. In this gel, the most improved restoration of staining ability was attained from the samples run through the G-25 Sephadex filter. It should be noted that very little of the actual DNA was recovered during the G-25 Sephadex purification, and so required a greater amount of the sample to bring it to the desired concentration.

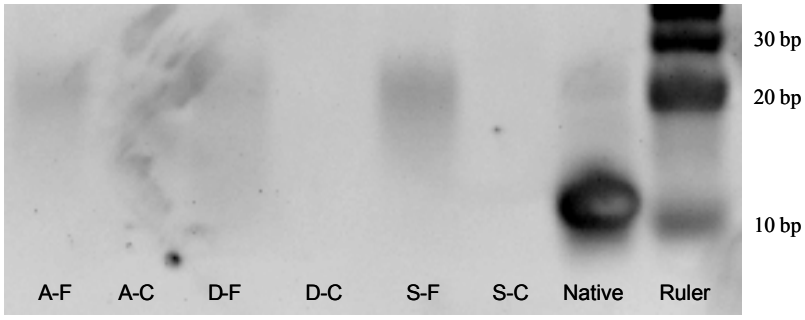

Figure 3.10. Non-Denaturing Gel Electrophoresis of DMNPE-caged and native PS ODNs.
C = caged sample, while F = Caged-flashed Sample.
S = G-25 Sephadex filter, D = Slide-A-Lyzer Dialysis, and A = Microcon YM-3 Filtered samples.

Fluorescence Measurements of Molecular Beacon Hybridization Products

Fluorescence emissions of samples hybridized with the molecular beacon provide evidence as to the ability of the DMNPE cage compound to control hybridization. As seen in Figure 3.11, the purified caged PD ODNs for each technique showed reduction in hybridization with the molecular beacon. A background noise value of the molecular beacon alone was of 32.4 relative fluorescence units (RFUs), while the solution containing the native PD ODN and the molecular beacon had a very high value of 490 RFUs. The caged samples all ranged from a low value of 62.9 to a high of 69.4 RFUs. Once exposed to UV-light of 365 nm in wavelength, all three samples recovered some ability to hybridize with the molecular beacon ranging from values of 220 to 268 RFUs.

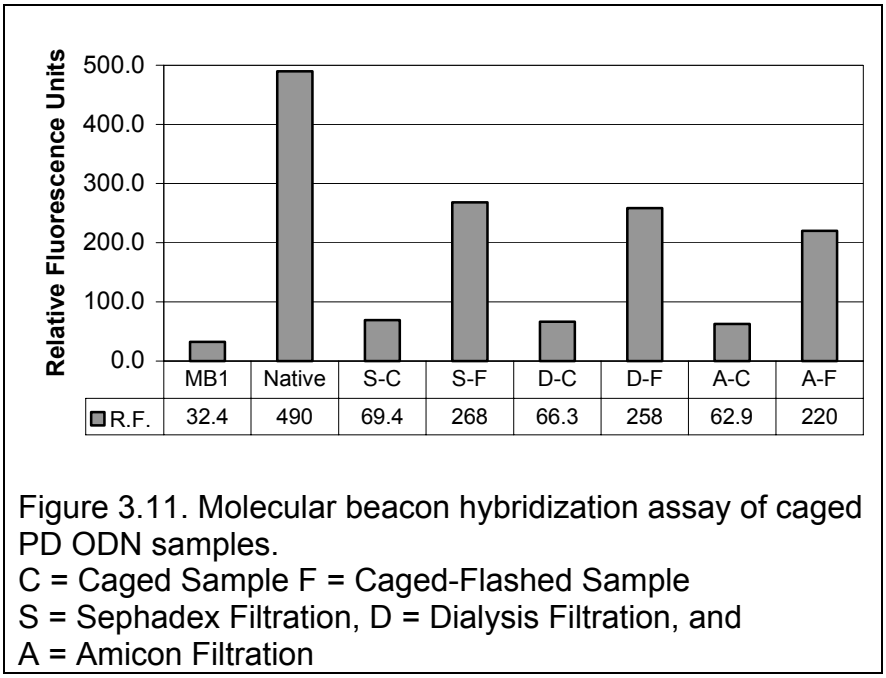

Figure 3.11. Molecular beacon hybridization assay of caged PD ODN samples.
C = Caged Sample F = Caged-Flashed Sample
S = Sephadex Filtration, D = Dialysis Filtration, and
A = Amicon Filtration

This was not the case in the run containing the phosphorothioate ODNs. As can be seen in Figure 3.12, restoration of the capacity to hybridize was negligible, however the cage compounds prevention of hybridization was greater in the case of the PS

ODNs. Previous experiments as shown in Figure 2.6 of chapter 2 have provided some evidence that PS ODNs can be successfully caged and then restored with exposure to light. The difference between the two sets of experiments was the use of follow up washes during the purification process.

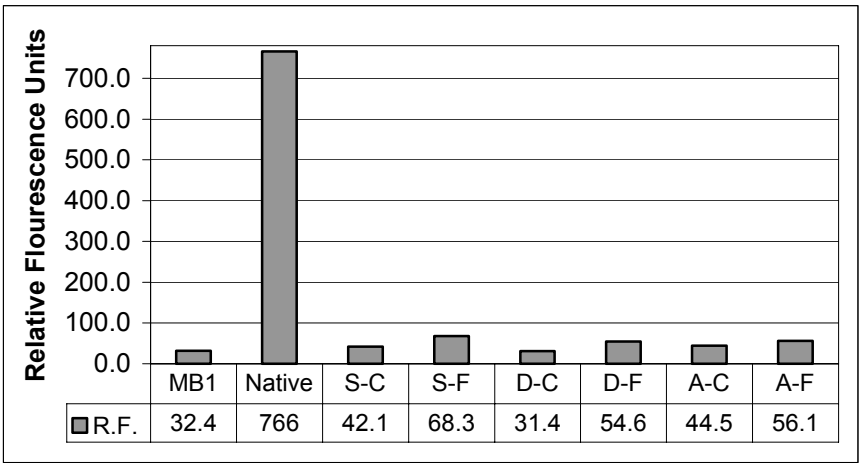

Figure 3.12. Molecular beacon hybridization assay of caged PS ODN samples.
C = Caged Sample F = Caged-Flashed Sample
S = Sephadex Filtration, D = Dialysis Filtration, and
A = Amicon Filtration

Various Solvent Caging Reactions: Fluorescence Measurements of Molecular Beacon Hybridizations

Fluorescence emissions from a complementary molecular beacon indicate differing amounts of hybridization for caged and native PD-ODNs in various solvents (Figure 3.13). The relative fluorescence of molecular beacon in solution alone was 27.0 $\pm$ 0.7 (mean $\pm$ SD, n=3) relative fluorescence units (RFUs) and 440 $\pm$ 15 RFUs when native compliment was added. Fluorescent emission of the hybridization mixture of caged ODN (DMF) and molecular beacon is 90.7 $\pm$ 5.1 RFUs, which is 15.4% of the relative fluorescence of the native probe hybridization mixture when background signal of molecular beacon alone in solution is removed, indicating a low level of hybridization.

The caged-light-exposed ODN (in DMF) showed an increase in fluorescence to 231 ± 4.2 RFUs, which is 49.4% of the native solution, indicating an increase in hybridization of the photoactivated ODNs. In the case of ODNs in DMSO, the caged ODN showed a fluorescence of 77.0 ± 1.1 RFUs or 12.1% the activity of native ODN, while caged-light-exposed ODNs had a fluorescence value of 164 ± 8.1 RFUs or 33.2% of native activity. The last samples were studied with methanol as a solvent. The caged-ODN in this case had a relative fluorescence of 365 ± 6.8 RFUs or 81.8% activity of native, where as the caged-light-exposed ODNs had an increased fluorescence of 397 ± 13.4 RFUs or 89.6%. Significant difference from beacon alone denoted by crosses asterisks, while asterisks also denote significant difference from caged ODN (n = 3, $p<0.05$, Bonferroni's t-test).

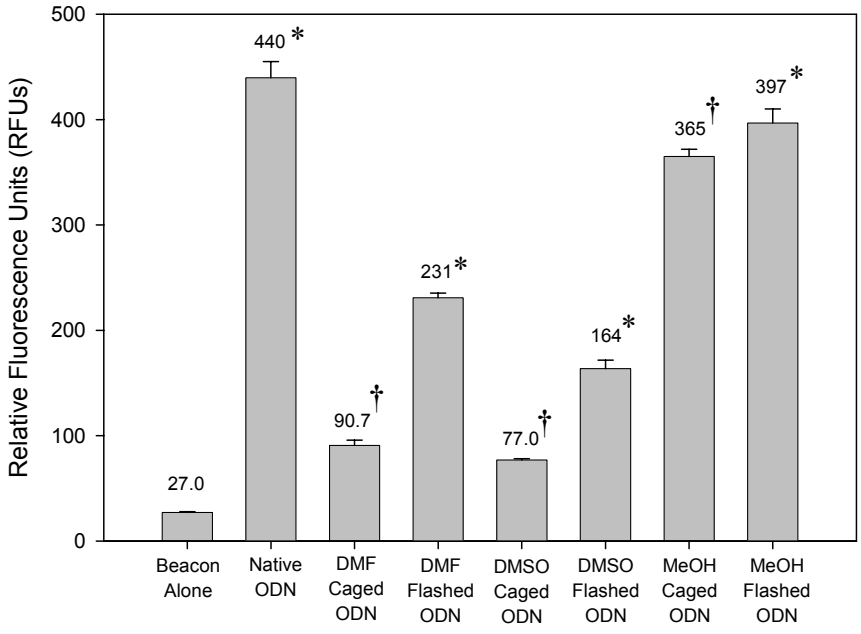

Figure 3.13. Molecular beacon hybridization assay of caged PD-ODN samples in various solvents.

In the case of the phosphorothioate caged ODNs in comparison, restriction of hybridization by the cage compound was much greater than that of the phosphodiester ODNs. As seen in Figure 3.14, the relative fluorescence for the molecular beacon alone was 27.0 ± 0.7, while the native ODN had a fluorescence of 391 ± 9.8. The fluorescence emission of the caged PS-ODNs in DMF was 31.3 ± 0.5, or 1.18% of the native solution when the background noise is removed as stated above, while the caged-light-exposed PS-ODNs had a relative fluorescence of 68.7 ± 3.5 or a value of 11.5% activity. Samples in DMSO faired similarly with a fluorescence of 39.0 ± 2.0 RFUs (3.30% activity) for caged PS-ODNs and 67.4 ± 2.2 RFUs (12.5% activity) for caged-light-exposed ODNs. Phosphorothioate samples tested in methanol (MeOH) behaved differently than PD-ODNs in methanol with a caged-ODN fluorescence of 49.5 ± 0.6 RFUs (6.18% activity) and a caged-light-exposed ODN relative fluorescence value of 86.8 ± 3.4 RFUs (16.4% activity).

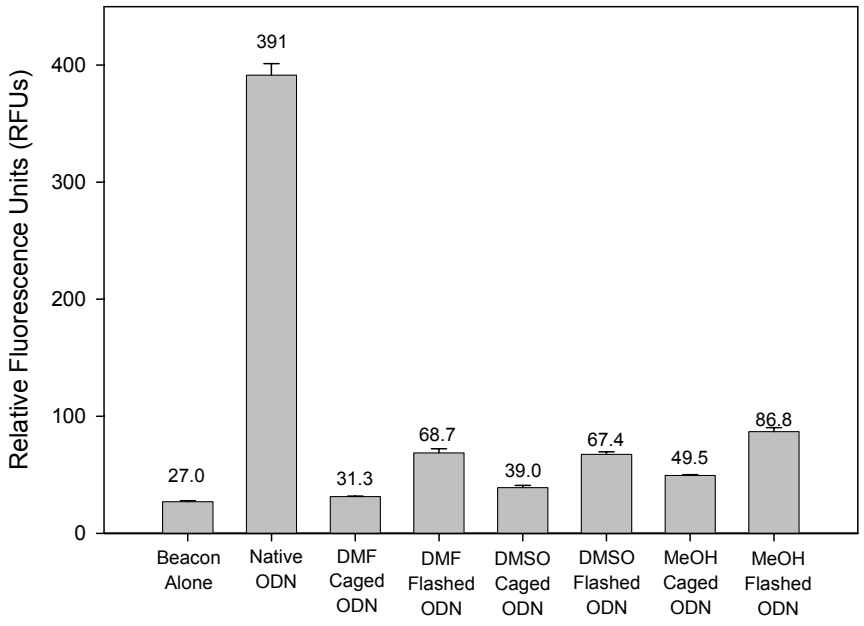

Figure 3.14. Molecular beacon hybridization assay of caged PS-ODN samples in various solvents. Significant difference from beacon alone denoted by crosses asterisks, while asterisks denote significant difference from caged ODN and beacon alone (n = 3, $p<0.05$, Bonferroni's t-test). PS-ODN caged in DMF showed insignificant difference when compared to beacon alone with the same test.

Discussion

Data indicates that solvent choice did have an effect on the efficiency and ability to characterize the ODN caging reactions. In the chapter 2, we demonstrated that use of the DMNPE cage compound allowed for temporal control of the ODN's hybridization activity. By optimizing the solvents, we were able to ensure the most promising cage attachment, purification, and performance of light-activated hybridization. The initial step of this study was to identify the solvent that could replace dimethyl sulfoxide (DMSO), which was previously used with plasmid DNA caging (Monroe 1999). Due to its relatively high freezing point, and the preferable reaction of DMNPE with the DNA ODNs at 4 ºC, DMSO was limiting in the efficiency of the caging reaction.

The initial focus of this study was to determine the solubility of the cage compound (DMNPE) and ODNs in several solvents. It was also necessary to determine whether the ODNs and cage compound could be accurately characterized in the solvents. Figure 3.5 shows that the inactive DMNPE hydrazone is soluble in all of the solvents tested. The 355 nm peaks does show similar solubility in the different solvents. Similar results were noted when the activated samples of DMNPE were scanned as can be seen in Figure 3.6, and so this leads us to determine that DMNPE is adequately soluble in all of the solvents studied.

Next, it was necessary to determine whether the DNA oligonucleotides would be soluble in these solvents. The results of phosphodiester ODN samples are shown in Figure 3.2. They demonstrated very little solubility in any of the solvents including DMF. In Figure 3.3, it was observed that again very little solubility was noticeable in the solvents, with the exceptions of DMF and slightly in methanol. At this point, methanol

and acetonitrile were evaluated with mixtures of 33%, 50%, and 66% of each with water and made from native phosphodiester and phosphorothioate ODN stocks. DMF mixtures were also showed adequate solubility of the DNA oligonucleotides.

As shown in Figure 3.4 and Table 3.1, phosphodiester DNA ODNs showed solubility in all of these mixtures for both solvents. This was also the case with the phosphorothioate DNA ODN samples demonstrated in Table 3.1 as absorbance at 260 nm was very close for all samples. In the case of acetonitrile mixtures, the solvent was extremely volatile, and thus some higher peaks at 260 nm for the higher percentage acetonitrile solutions could be attributed to reduction in volumes by evaporation, which lead to higher concentration solutions. Quick evaporation of the acetonitrile samples was noted in more than one of the samples. However, a fairly consistent region of peaks was observed in all samples, providing a solid starting ground for utilizing these solvents to improve characterization of the caged oligonucleotides. The spectral interference found with DMF is not found with any of the other solvents. Due to its high absorbance of light at 260 nm, there is a reduction in the accuracy of the spectral scans taken for the ODNs. This was not noted as an issue with the other solvents studied. Table 3.3 below displays the solvents and their characteristics in several categories including solubility of DNA in mixtures of solvents, solubility of cage compounds, compatibility with filters, and spectral interference they may have.

One focal point for these solvents was their compatibility with the purification techniques used. Isopropanol was initially eliminated with no solubility of DNA as expected. Unfortunately, the high volatility of acetonitrile made it unsuitable with many of the filtration systems. DMF in low percentages was compatible with the filters. Methanol,

although seemingly compatible, was unable to adequately activate the DMNPE when suspended entirely in methanol (Figures 3.13 and 3.14), nor was the caging reaction itself very efficient when compared to that of DMF and DMSO. Figures 3.13 and 3.14 also demonstrate a greater restoration of activity for DMF when compared to DMSO. DMF has a restoration of activity by 3.2 and 9.7-fold for PD-ODNs and PS-ODNs, respectively, as compared to 2.7 and 3.8-fold restoration in DMSO samples. This limitation lead to us to chose DMF as the solvent of choice with high reactivity of the cage compound and ODNs in the solvent.

Table 3.3. Solvent Compatibility Comparison.

Solvent	*DNA Solubility*	*Cage Solubility*	*Solubility of caged DNA**	*Compatibility with filters*	*260 nm Spectral Interference*
Water	+++	X	+	+++	+++
DMF*	+++	+++	+++	+	+
MeCN*	X	+++	+++	X	+++
MeOH*	+	+++	+++	+	+++
100% IPA	X	+++	X	X	+++

- * = Mixtures of solvent and water
- +++ = Compatible ++ = Moderate + = Slight X = Incompatible

We next focused on determining the optimum technique for purifying the samples upon completion of the reaction. It is important to recall that prior to filtration, the samples used were completely identical. Looking at the results of the purification technique study, Figures 3.7 and 3.8 showed the spectral analysis of the samples after they were purified. Figure 3.7 shows the spectral scan for the phosphodiester ODN samples, with the highest absorbance at 260 nm attributed to the Microcon YM-3 molecular weight cut-off filters. This technique also, however, had the highest 365 nm peak, which suggested the presence of excess unattached cage compound. This was due to the lack of secondary washes, which were later performed. Upon completion of

four additional washes of 33% DMF, excess caging compound was removed. The next highest peak in this case was attributed to the sample run through the Sephadex G-25 column, while the lowest peak was ascribed to the dialysis sample. Complications with the organic solvent mixture of DMF caused corrosion of the membrane of the Slide-a-Lyzer cassettes used to dialyze the sample. Compatibility of the DMF reaction with this technique is unlikely to be very effective when used regularly.

In Figure 3.8, it was observed that in the case of phosphorothioate DNA ODNs, the highest peak was again found with the Microcon YM-3 filters, however the Slide-a-Lyzer nearly matched it. The Sephadex filter had a very minute peak, and thus showed a low ability to filter out this reaction when phosphorothioate DNA ODN is used. This may be due to swelling or other alteration of the Sephadex matrix in the presence of DMF. Looking at Table 3.2, the efficiencies of the samples follow a similar pattern. The highest yield with phosphodiester DNA could be noted in the Microcon YM-3 filters. Although it had the highest caging efficiency of the three methods, this was improved by the multiple washes. Phosphorothioate samples presented the Slide-a-Lyzer unit as the most efficient in DNA recovery and excess cage removal. This would likely have differed if secondary washes were performed with the Microcon YM-3 filters. Cost also became an issue when presented with the dialysis technique, as was the time required. The cost of the Slide-a-lyzer technique cost more than four times that of the Microcon YM-3 filters, which also require approximately four hours to complete purification. This is much shorter than the overnight dialysis process. The gel electrophoresis run for each set of samples, seen in Figures 3.9 and 3.10, provided evidence that hybridization control was attained through the caging reaction. The samples shown provided an

insight into reduced mobility for caged samples along with reduced stain attachment. Once exposed to UV-light of 365 nm in wavelength, increased mobility as well as improved staining provides evidence of the control of hybridization. The molecular beacon hybridizations in Figures 3.11 and 3.12 confirm the results attained from the gel electrophoresis. Although hybridization was not very well restored in the phosphorothioate samples, these samples were characterized as more heavily caged, and so may have required longer exposure with the 365 nm light. Restoration of the ability to hybridization is shown in Figure 3.11, where caged samples remained at low levels, and were restored to nearly 4 times the relative fluorescence of the caged ODNs once exposed to the 365 nm light.

Table 3.4. Purification Technique Comparison.

Purification Technique	*Solvent Compatibility*	*Volume of Reaction filter can hold*	*Cost*	*Efficiency of Recovery*
Microcon YM-3	Yes	500 µL	Low	High
G-25 Sephadex	Yes	50 µL	High	Mid
Slide-a-Lyzer	No	500 µL	Mid	Mid

Based on cost and the other factors, we can see in Table 3.4 below that the Microcon YM-3 filters showed the most overall promise in the majority of the areas that were rated of importance, even though they were not the best in the experiment with the phosphorothioate samples. Recovery of the ODNs becomes of great importance when phosphorothioate ODNs are used, as cost of the modified oligonucleotides is greatly increased in comparison to the phosphodiester ODNs, and the most consistent DNA recovery was shown in the Microcon YM-3 filters. The time factor was also important, as the dialysis technique was run overnight in order to efficiently purify the samples. Also, the Micron YM-3 filters had the capacity to work under the highest concentration of DMF.

The overall results of this study provide clarity in choosing a solvent in which to run and characterize caging reactions. DMF has proven to be the most appropriate solvent, and adequately oxidizes the DMNPE hydrazone precursor into the activate diazoethane. Of the solvents, DMF was the most consistent at compatibility with the purification methods, and allows for adequate spectral characterization. DMF also seemed to have little complication with the chosen purification method of the Microcon YM-3 filters. These filters provided adequate volume to purify an entire caging reaction, as well as to be completed rapidly when compared to dialysis. Due to its low cost in comparison to the other two techniques, the Microcon YM-3 filter also gives the highest DNA recovery. This combination of solvent and purification technique was suitable for this reaction and the subsequent molecular assays.

Chapter 4

Conclusions and Future Considerations

Conclusions

The control of oligonucleotides has become an important focus in the development of gene therapies. By controlling the ability for oligonucleotides to hybridize, a more effective approach to using oligonucleotides for a multitude of purposes can be developed. The purpose of this research was to develop a method to control DNA oligonucleotides through the use of photo-cleavable cage compounds, thus allowing for a spatial and temporal management of DNA. The presence of the attached cage compound has been shown to block hybridization. The strategy was to provide a simple, efficient method through which light could be used as a trigger for hybridization activation. While the use of such control methods has been studied with mRNA and DNA plasmids reacted with cage compounds, little in depth study has been placed on DNA oligonucleotides (Ando 2001). We have demonstrated in several bioassays that caging DNA oligonucleotides, both modified and unmodified, can block the DNA's capacity to hybridize. We were also able to show that once exposed to near UV-light, restoration of hybridization is partially restored to the DNA oligonucleotides.

DNA oligonucleotides were reacted with the cage compound 1-(4,5-dimethoxy-2-nitrophenyl)diazoethane (DMNPE) in order to temporarily block hybridization activity that could later be restored with exposure to light. Spectrophotometry data demonstrated that cage attachment had occurred during the reaction, and this was also confirmed through the use of non-denaturing gel electrophoresis. Through the use of molecular beacon hybridization assays, we were able to demonstrate that control of the

caged DNA oligonucleotides functionality was evident. In our studies, the caged oligonucleotides demonstrated less than 15% of the activity shown by the native (non-caged) oligonucleotides. Once exposed to light, these samples restored much of their hybridization activity, reaching nearly 80% of that of the native oligonucleotide. Qualitative analysis of these molecular beacon hybridizations were performed using non-denaturing gel electrophoresis, and provided confirmation of the results attained from the molecular beacon assays.

The utility of the cage compounds in controlling the hybridization activity of DNA oligonucleotides was not limited to standard phosphodiester DNA oligonucleotides. The caging method was shown to be more reactive with the modified phosphorothioate DNA oligonucleotides, and reduced hybridization activity with even greater consistency than with the phosphodiester oligonucleotides. Once again, this reaction was demonstrated through the use of spectrophotometry, molecular beacon hybridization, and gel electrophoresis assays. The more reactive phosphorothioate oligonucleotides demonstrated much greater reduction in hybridization activity than that of the phosphodiester oligonucleotides, although restoration of activity proved much less efficient. This restoration did recover activity from caged values as low 2% to light exposed values of 35% of the hybridization activity of the native oligonucleotide. This was most likely attributed to the heavier caging, and thus may require exposure to light for longer amounts of time or under more intense energy, as well as to a lower quantum yield for DMNPE caging of a target when sulfur modifications are present (Walker 1988).

Solvent and purification technique studies provided optimization of the caging

reaction and the purifications of samples. N,N-dimethylformamide (DMF) was retained as the solvent used in the caging reactions of the ODN. It provided the most effective caging, while also allowing for adequate and proper purification. Although the spectral interference caused by the absorbance of light by DMF from 200 to 260 nm provided some concern, the benefits provided from using the solvent greatly outweighed this drawback. Use of Microcon YM-3 filters provided the optimal purification of the ODNs in comparison to the other techniques studied. With the addition of secondary washes to the filtration process, optimal purification of the caged ODNs was achieved.

In an effort to develop greater sensitivity in the molecular beacon assays, experiments were run to help provide a more accurate understanding of the hybridization ability of caged and caged-flashed oligonucleotides at various temperatures. Applications of this study could help to provide a better understanding of the likelihood of improper hybridization *in vivo*. These oligonucleotides were also run in comparison to base mismatched oligonucleotides, which have previously been shown to have differing hybridization activity at varying temperatures (Bonnet 1999; Tsourkas 2002; Tsourkas 2003). The study demonstrated that there is little effect of temperature on the hybridization activity of caged and caged-flashed oligonucleotides when compared to the native and base mismatched samples.

In conclusion, the data presented has shown that the use of cage compounds can provide a photo-cleavable method for spatial and temporal control the hybridization activity of oligonucleotides. This also provides a strategy for future application of this strategy as a tool in providing targeted antisense and RNAi drug delivery and application.

Future Considerations

Findings of this project showing the ability to photo-control DNA oligonucleotide hybridization activity through the use of the cage compound DMNPE provide great promise for future development of cage compounds with respect to genetic therapy. The use of cage compounds can differ for each individual study, however several other cage compounds other than DMNPE have been used with DNA and RNA. 6-bromo-7-hydroxycoumarin-4-ylmethly (BHC) was used by Ando to cage mRNA in zebrafish embryos (Ando 2001). Use of other caging compounds such as 4,5-Dimethoxy-2-nitrobenzyl bromide (DMNB) may provide a less expensive technique for achieving similar results to those seen with DMNPE. DMNB has previously been demonstrated to cage hydroxyls on β-ecdysone and estradiol as a means to control gene expression (Cruz 2000; Lin 2002). It may provide another, possibly more efficient, method for achieving spatial and temporal control of DNA oligonucleotides.

Further studies into purification techniques of the samples may also prove beneficial to future research. The issue of solvent compatibility has lead to much concern when dealing with cage compounds that are minimally soluble in water, and thus can cause issues. By developing more improved purification techniques and protocols, better knowledge of the caging method can be achieved. Initial studies shown in chapter showed that filtration of caged samples once exposed to light, helped to remove the released cage molecule and prevent any interference with the assays. Scavenger compounds such as dithriothreitol (DTT) are another possible technique that could bind the released nitrosoketone and prevent it from causing any interaction problems for hybridization. DTT is also permeable to cell membranes, and thus could

be an important tool when caging is used with antisense technologies. Functional separation of the caged oligonucleotides from the non-caged oligonucleotides remaining in the samples could also help improve efficiency as well as reduce leakage in the case of antisense oligonucleotides. One technique that could be used would be attachment of ODNs complementary to our caged ODN. By hybridizing with the non-caged ODNs in the solution, the ODNs attached to the beads may functionally separate the caged oligonucleotides, thus reducing the risk of non-caged oligonucleotides interfering with assays or possibly causing leakage in antisense activity prior to light exposure if *in vivo*.

Currently, the caging reaction is performed with random attachment of DMNPE to the target oligonucleotides, and thus leads to increased and varied amounts of cage compound attached to the target, and requiring greater light-exposure in order to restore activity. Studies of base mismatch location provide insight onto a method to account for this, and improve the efficiency of the caging of oligonucleotides, and minimizing the light exposure required. Studies by Bonnet indicated that the location of base mismatches along an oligonucleotide affected the ability for the oligonucleotide to hybridize, especially noted at 37ºC, which is the standard temperature within a healthy cell (Bonnet 1999). By synthesizing the oligonucleotides with cage compounds in specific sites along the sequence, the effectiveness of this control method could be greatly augmented. By determining the most appropriate attachment sites for the cage compound along the specific sequence in question, improved photo-restoration of hybridization can be achieved. This can become extremely important if used for antisense therapies, as less exposure of cells and tissue to UV-light reduce the risks of

cell damage. Use of two-photon photolysis as described in chapter 1 can also be used as a means to reduce risk of cellular damage.

Improved molecular assays for determining the effects of cage compounds on oligonucleotides would also be of great benefit to future studies of the use of cage compounds to control ODNs. By improving molecular beacon assays through temperature variation, greater sensitivity can be used to verify the effectiveness of a cage compound in controlling hybridization activity. Studies focusing on other phosphate backbone and base modifications along an oligonucleotide could provide an insight on ways to improve this technology. This would also help to possibly provide further understanding and confirmation of the location of attachment of the cage compound when reacted with nucleic acids. It could also confirm the effectiveness of the temperature varying molecular beacon hybridization to properly represent the abilities of the cage compounds.

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Appendix A: Molecular Beacon Temperature Assays

Introduction

The sensitivity of molecular beacons to their targets has been a new area of interest in the field. Several studies have demonstrated how accurately the molecular beacons would be able to distinguish their target from other similar sequences, including those with only a single base alteration (Tyagi 1998). Shorter loop regions show more interference with a single base mismatch on hybridization (Aboul-ela 1985). This specificity is affected by several conditions including pH, salt concentration, and temperature. Looking at temperature, several studies have demonstrated an increase in mismatch discrimination of the molecular when temperature is increased to certain levels (Bonnet 1999; Tsourkas 2002; Tsourkas 2003). These studies showed significant decreases in the hybridization of beacon with targets with as little as a single base mismatch, leading to the belief that other modifications to the oligonucleotide may have a similar result.

In this study we focus on the effects of temperature on caged and caged-flashed ODNs and their ability to hybridize with molecular beacons. This would also be in comparison with the perfect or native target, as well as two other modified ODNs, one with a single base mismatch and the other with two base mismatches. In order to determine the variable specificity of the molecular beacon assay with respect to caged and caged flashed ODNs, it was important to look at effects of temperature on the caged samples themselves, and compare the results to those retained through standard beacon hybridizations.

Materials & Methods

Sample Preparation

As was described in chapter 2, samples were prepared similarly in this study. Samples of caged ODNs (ISIS 2302) were prepared using the same protocol as found in Appendix A, and purified using the Microcon YM-3 filters as shown in Appendix B with 4 secondary washes. After characterization, the caged samples, along with native 20mer ODNs (GCCCAAGCTGGCATCCGTCA), a 20-mer single mismatch ODN (T-MM: GCCCAAGCT**T**GCATCCGTCA) and a 20-mer two mismatch ODN (TT-MM: GCCCAAGCT**TT**CATCCGTCA) were all diluted to 400 μL working stocks with a concentration of 0.05 μg/μL. 200 μL of the caged sample was then exposed to UV-light (365 nm) for 20 minutes and filtered with the Microcon YM-3 filter protocol and resuspended back to μL.

Once prepared the working stocks were then run through molecular beacon hybridization. For each sample, the ODNs were prepared as performed with a 5:1 molar ratio of target to molecular beacon as follows: 0.305 μg of native (non-caged), caged, caged-flashed, T-MM, TT-MM target ODN were each mixed with 0.105 μg of complementary molecular beacon in 500 μL solutions of 100 mM NaCl, 1mM EDTA, pH 7.5. One sample containing only 0.105 μg of molecular beacon was also prepared in identical buffers and brought up to 500 μL. A seventh solution containing 0.085 μg of a 5'FAM modified 20-mer ODN identical in sequence to the native was also prepared following the same protocol and used as a control.

Hybridization Reaction and Fluorescence Detection

100 μL of each of the mixtures was then denatured at 90°C for 5 min and allowed to

slowly cool to 25°C over 60 min. Fluorescence of hybridization solutions was used to quantify the hybridization of molecular beacons with ODNs. Each of the 100 μL hybridization mixtures was excited at 492nm and emitted fluorescence quantified at 515nm in triplicate with a LS55B Luminescence Spectrophotometer (Perkin Elmer, Wellesley, MA).

300 μL from each of the stocks was then placed in 100 μL aliquots into separate wells of a 96-well plate and run on an iCycler thermal cycling unit IQ Optical System (Bio-Rad Laboratories, Hercules, CA). Samples were initially brought up to 95 °C and held there for 5 minutes, at which point the temperature was decreased 1°C every minute until the final temperature of 25 °C was achieved. As temperature decreased, fluorescence readings of all samples were taken for each one °C. The wavelengths of light used by the optical system were 488 nm for excitation and 515 nm for emission detection. Samples were initially calibrated dynamically by the iCycler system at 95 °C. For dynamic calibration, samples were initially cycled between 60 °C and 95 °C several times to ensure that any duplexes would be denatured, at which point the system remained at 95 °C and took readings from each sample and normalized their fluorescence at 95 °C.

Results

Looking at the fluorescence readings shown in Figure A.1, differing amounts of hybridization are seen between the several target ODNs. The relative fluorescence for the molecular beacon alone in this experiment has a relatively high fluorescence at 38.9 RFUs. It is not very distinct from that of the native as was the case in the experiment shown in chapters 2 and 3. The native ODN has a relative fluorescence of 92.0 RFUs,

while the single mismatch ODN (T-MM) has a nearly identical reading at 90.4 RFUs or 97.0% of native. This does slightly decrease for the two mismatch ODN (TT-MM) with a value of 74.5 RFUs or 67.2% of native ODN when background noise is subtracted. The caged sample had a value of 40.2 RFUs (9.18% of native) that is indicative of effective caging, and was restored to 62.1 RFUs (43.7% of native). The FAM-labeled ODN showed the highest fluorescence at 144 RFUs, which is expected since no quencher is present in the solution.

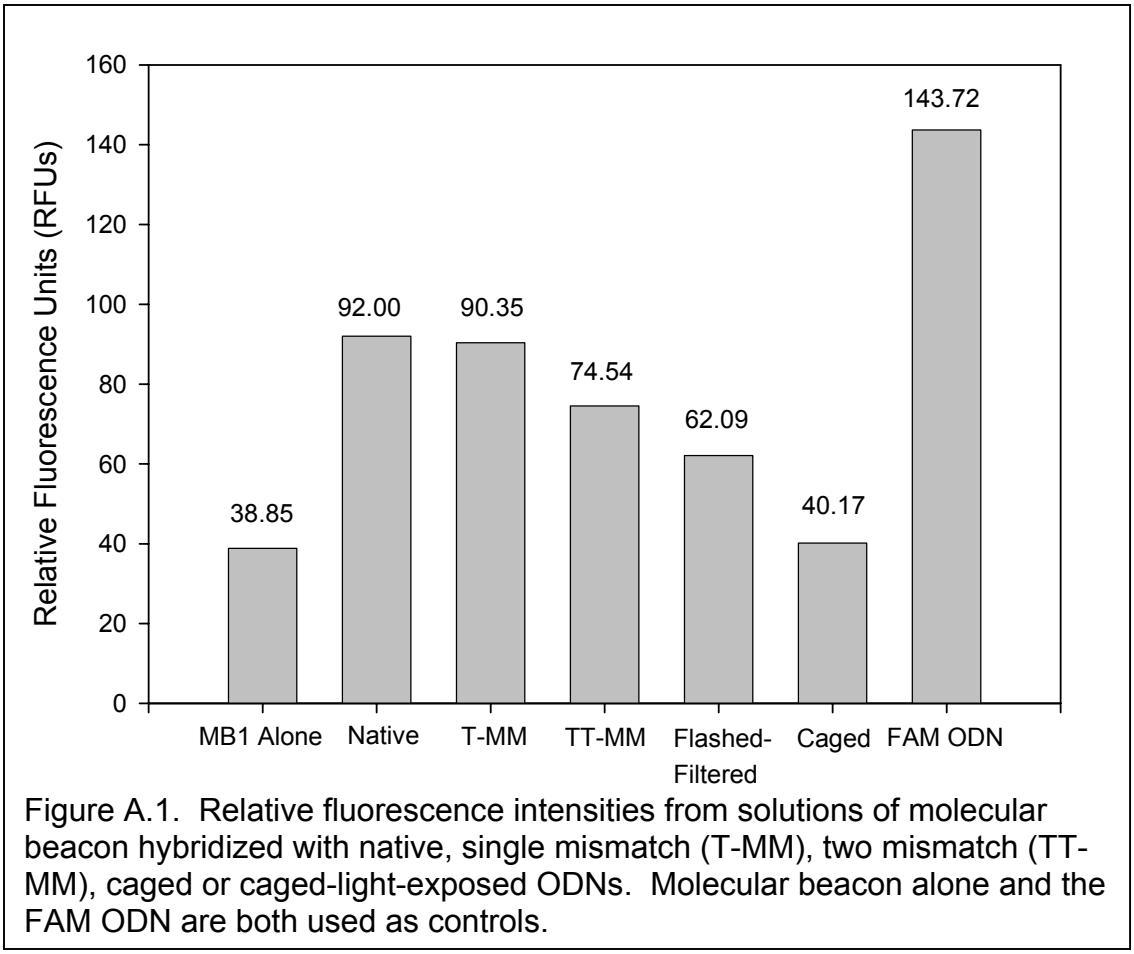

Figure A.1. Relative fluorescence intensities from solutions of molecular beacon hybridized with native, single mismatch (T-MM), two mismatch (TT-MM), caged or caged-light-exposed ODNs. Molecular beacon alone and the FAM ODN are both used as controls.

Relative fluorescence readings are shown in Figure C.2 for the samples at various temperatures and the effects of temperature change on the target ODNs. Molecular beacon alone follows its standard path at a value of 1449 RFUs at 25°C and

slowly increasing to 3198 RFUs at 95°C. The native ODN resulted in the highest fluorescence slowly increasing until peaked at 4014 RFUs at 54°C, and then returning to a similar 95°C value as that of molecular beacon alone. Similarly the single base mismatch ODN (T-MM) a slight increase from 25°C peaking at 3078 RFUs at 41°C, while the two base mismatch ODN (TT-MM) began to immediately decline from its 25°C fluorescence value of 2002 RFUs. For the caged and caged-flashed samples little change occurred from the 25°C values of 1163 and 2010 RFUs, respectively and then both following the molecular beacon alone trend after 45°C. The FAM ODN had a unique trend of steadily escalating in fluorescence as temperature increases from 25°C (2458 RFUs) to 95°C (3323 RFUs). All of the samples showed a similar amount of

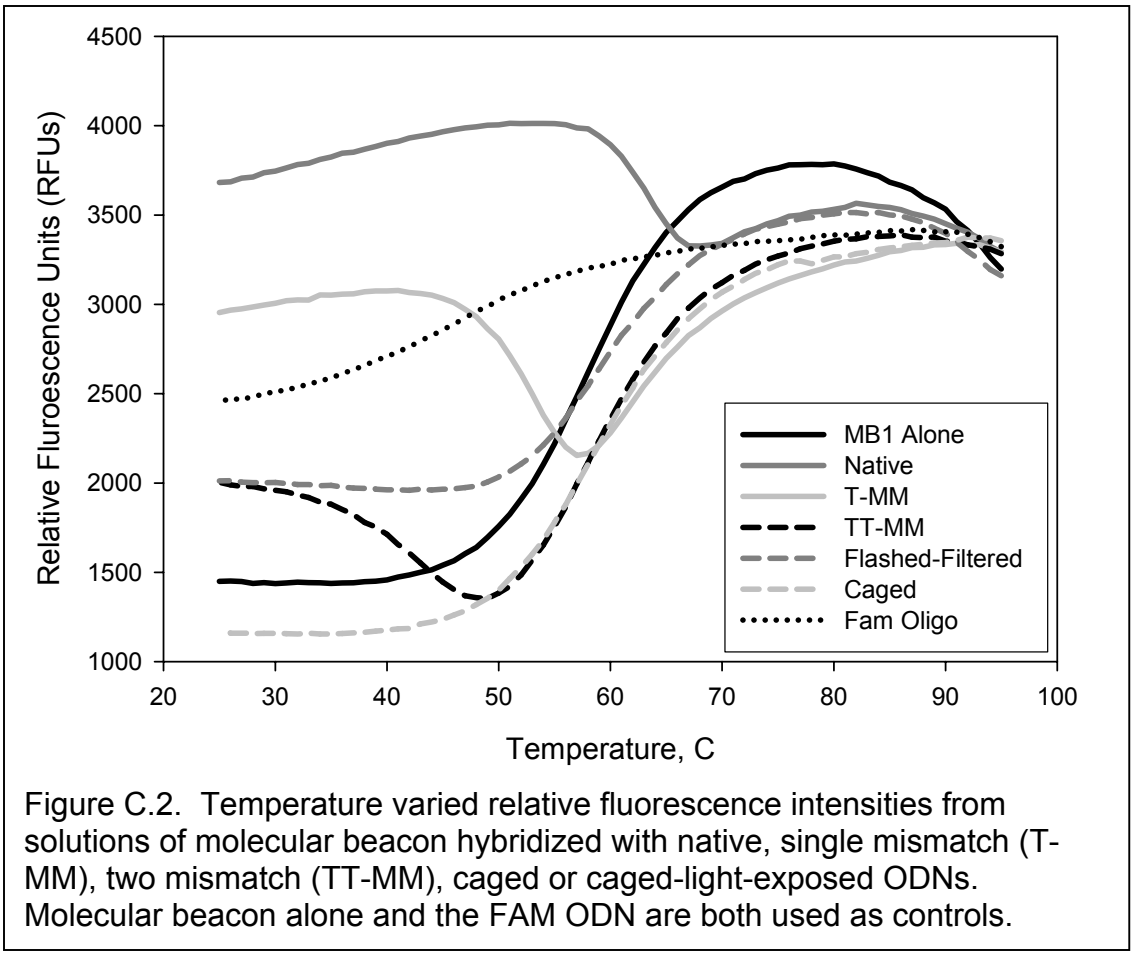

Figure C.2. Temperature varied relative fluorescence intensities from solutions of molecular beacon hybridized with native, single mismatch (T-MM), two mismatch (TT-MM), caged or caged-light-exposed ODNs. Molecular beacon alone and the FAM ODN are both used as controls.

fluorescence at 95°C due to the dynamic normalization run by the iCycler system prior to the performance of readings.

Discussion

The data from this study suggest that the effects of temperature on the discriminating ability of molecular beacons are dependent on the type of modification of the native ODN. In the case of the room temperature assays seen in Figure A.1, very little difference existed between native ODN and the single base mismatch ODN. The fluorescence slightly lowers as the number of base matches increased to two, as seen in the two base mismatch ODN, dropping from 96.97% of native in the single mismatch (T-MM) ODN to 67.15% of native for the double mismatch (TT-MM) ODN. The caged ODNs followed expected patterns by reducing hybridization to 9.18% of native, and once photo-exposed returning up to 43.73% of native fluorescence. This demonstrates that caging has a much greater affect on reducing hybridization ability than does a one or two base mismatch.

Temperature effects on the ability of these ODNs to hybridize followed previously reported findings. Effects of temperature on the mismatched ODNs versus that of the native ODN showed very similar results to studies performed by Tsourkas and Bonnet (Bonnet 1999; Tsourkas 2002; Tsourkas 2003). As the temperature increased from 25°C, characteristic decreases in the base mismatch ODNs could be seen. Initially, TT-MM ODN began to reduce as soon as the temperature was greater than 25°C. The T-MM ODN similarly followed this pattern, however its peak was noted at 41°C, prior to its decrease in fluorescence. In comparison to the native ODN, increases in the number of base mismatches lead to lower melting temperatures for the hybridized duplex of the

ODN and molecular beacon, as was seen in previous studies (Bonnet 1999). The native ODN reached its peak fluorescence at 54°C, thus providing an optimum temperature range of 52°C to 57°C at which to take readings for the most favorable discrimination between samples containing perfect target and single base mismatched target.

In the case of the caged and caged-flashed ODNs, very little change was noticed based on temperature outside of the behavior of the beacon alone. The caged sample remained below that of the molecular beacon alone at room temperature, and did not increase in fluorescence substantially until the temperature was greater that 45°C. The case was similar for the caged-flashed ODN, which although much higher at room temperature than the molecular beacon alone, there was little increase until this 45°C point. In comparison to native, these two samples were much lower in fluorescence, and increases in temperature showed little increase in the sensitivity of the molecular beacon with respect to caged samples. This is possibly due to the modification of the backbone theorized to take place in caging, as opposed to the base modifications seen in base mismatched ODNs. In modifying the backbone, increased suppression may be attainable, however hybridization of these caged samples may not be affected by temperature as is the case with the FAM ODN, which has much less fluctuation with varying temperature showing little effect of temperature on the fluorophore itself.

Appendix B: Caging Protocol for ODN DNA

a. Activate DMNPE:
In a plastic weigh boat, weigh approximately:

5 mg 4,5-dimethoxy-2-nitroacetophenone hydrazone (yellow crystals, stored at –20 °C)

50 mg Manganese (IV) oxide (black pellets, stored at 25 °C)

Transfer to a 1.5 ml eppendorf and wash in 1 ml DMSO (clear viscous liquid, stored at 25 °C). Protect the eppendorf from light from this point onwards by shielding it with aluminum foil. Vortex the solution for 15 sec., then rock on a Nutator™ for 20 min.

Before filtering, microfuge the solution for 30 sec. to draw large particles of MnO_2 to the bottom of the tube to prevent filter clogging. Solution should look murky and dark reddish-brown.

b. Filter MnO_2 from activated DMNPE:

a) Pack a 1 cc syringe with glass wool

b) Add 100 mg of Celite™ diatomaceous earth (use a syringe w/ stopper removed to poke the glass wool & Celite to the bottom.)

c) Pre-wet the filter with 1 ml DMSO

d) Remove the top 900 µl of microfuged cage solution and gently push through filter. Effluent should be a clearer red solution.

e) Microfuge this solution as well for 30 sec to ensure that any MnO_2 that possibly passed through the filter will not be added to the caging reaction (MnO_2 will oxidize DNA as well.)

c. Prepare DNA and add activated cage:

a) ODNs were kept in either 10 mM Bis-Tris or HPLC Grade water. For a reaction, 100 µg of DNA is brought up to 200 µl of 10 mM Bis-Tris in a 1.5 ml eppendorf. 100 µl of the activated cage solution is then added, vortexed briefly, and then put in an agitator for 24 hrs at 4 °C.

Appendix C: Purification Techniques

I. Sephadex Spin Columns

b. Dry down reaction volume(s) in vacufuge until precipitate forms (can take several hours, however DMF is volatile enough to be removed.

c. Resuspend the sample into 100 µL of 0.2 µm-filtered water.

d. Column preparation

   i. Flick tube or vortex for a few moments to try and spread buffer through out column.

   ii. To prevent a vacuum, first remove cap of column, then snap off bottom tip.

   iii. Place column(s) into clean, empty 1.5 mL Eppendorf tubes and place in microcentrifuge for 75 seconds at 1000 x g. Ensure that chipped edge of columns all face towards center of microfuge.

   iv. Discard buffer solution in eppendorf and run 300 µL of 0.2 µm filtered water through the column and place in micro centrifuge for 3 minutes at 1000 x g.

   v. Remove tube and place column into a new, sterile 1.5 mL Eppendorf tube.

II. Slide-a-Lyzer Dialysis

a. Hydrate Membrane

   i. Remove Slide-A-Lyzer® Cassette from its pouch and slip into the groove of an appropriate size buoy.

   ii. Immerse cassette in dialysis buffer for 30 seconds.

   iii. Remove cassette from buffer and remove excess liquid by tapping the edge of the cassette gently on paper towels.

b. Add Sample

   i. fill the syringe with the sample, leaving a small amount of air in the syringe.

ii. With the bevel sideways, insert the tip of the needle through one of the syringe ports located at a top corner of the cassette.

iii. Inject sample slowly. Withdraw air by pulling up on the syringe piston.

iv. Remove the syringe needle from the cassette while retaining air in the syringe.

c. Dialyze

i. Slip the cassette into the groove of the buoy and float this assembly in 300 mL of the dialysis solution of 33% DMF.

ii. Leave in Dialysis solution at room temperature at room temperature for 4 hours.

iii. Change the dialysis buffer solution and let dialyze for another 4 hours at room temperature.

iv. Change the dialysis buffer solution and let dialyze overnight at 4 °C.

d. Remove Sample

i. Fill the syringe with a volume of air equal to the sample size and, with the bevel sideways, insert the tip of the needle through another syringe port located at a corner of the cassette.

ii. Inject air slowly into the cassette to separate the membranes.

iii. Turn the unit so that needle is on the bottom and allow the sample to collect near the port. Withdraw the sample into the syringe.

III. **Microcon YM-3 Molecular Weight Cut-off Filters**

a. Remove a filter and accompanying 1.5 mL centrifuge tube. Place filter properly in tube.

b. Place up to 500 uL of sample into the filter, seal cap, and place into microcentrifuge.

i. Be sure to counterbalance the weight of the tube, filter, and sample once in the centrifuge.

c. Spin sample at maximum speed (13,400 RPMS, approximately 12,000 g's) for 90 minutes at room temperature.

d. Once complete, add 100 uL of 33% DMF solution to wash sample, and spin again for 30 minutes.

e. Repeat step "d" three more times.

f. Once complete, flip over the filter and place into a new fresh centrifuge tube, and spin in centrifuge at 1000 g's for 3 minutes.

g. Once complete, remove filter and discard. Keep centrifuge tube with newly filtered product solution.

Vita

Bilal Ghosn was born on the 7th of February, 1980, in Baton Rouge, Louisiana to Dr. A. H. and Soumaya Ghosn. He attended high school at Baton Rouge Magnet High School, where he graduated with honors in 1998. Following high school, Bilal enrolled at Louisiana State University in Baton Rouge, Louisiana, where he graduated with honors with a Bachelor of Science in Biological Engineering. Upon completion of his degree, he spent one semester of graduate study at the Georgia Institute of Technology studying for a master of science in bioengineering. He returned to Louisiana State University in the summer of 2003 and is currently enrolled as a candidate for the degree of Master of Science in Biological and Agricultural Engineering, which will be awarded in December 2004.