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Caged Morpholino Oligonucleotide for Control of Gene Expression in Zebrafish

Chad Michael Jarreau
Louisiana State University and Agricultural and Mechanical College, cjarre6@tigers.lsu.edu

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CAGED MORPHOLINO OLIGONUCLEOTIDE FOR CONTROL OF GENE EXPRESSION IN ZEBRAFISH

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 Masters of Science in Biological and Agricultural Engineering

In

The Department of Biological and Agricultural Engineering

by

Chad M. Jarreau
B.S., Louisiana State University, 2007
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ABSTRACT

To control gene expression in vivo with spatial and temporal precision remains a significant hurdle in laboratory studies of development as well as clinical genetic therapies. Here we demonstrate such control over gene expression by use of photochemistry to reversibly inactivate the hybridization of a nucleic acid analog used for specific protein knockdown. A morpholino oligonucleotide, commonly used for knockdown of protein expression in developmental studies, was “caged” using carbodiimide conjugate chemistry which yielded photocleavable adducts that can be removed with light exposure. Photochemical inactivation approaches to produce caged molecules have been used to control the spatiotemporal activity of biomolecules such as nucleotides, neurotransmitters, proteins and nucleic acids. In this case, the morpholino oligonucleotide was caged through direct alkylation of exocyclic amines with a carboxylic acid-based nitrobenzyl cage compound to demonstrate blockade of hybridization. Due to the site of attachment on nucleobases, results indicate that presumably, any nucleic acid antisense molecule could be used in this reaction scheme and thus, effectively caged. The degree of cage alkylation was determined using absorbance spectrophotometry, and the light-induced control over hybridization was characterized with gel-shift and fluorescence-based melting temperature assays. Using a behavioral assay in the zebrafish embryonic model as an endpoint for synthetic molecule assessment, in vivo demonstration of light-induced protein knockdown was shown where caged morpholino oligonucleotides do not possess protein knockdown activity until exposed to near-UV light. Perfect binary on/off behavioral responses with light exposure were not observed in the in vivo studies, presumably due to the statistical, or random-style of cage attachment to the many suitable bases on the oligonucleotide. This investigation should act to expand caged morpholino oligonucleotide technologies, and more generally antisense
technologies as a whole due to the ease of synthesis required in caging these compounds, as well as further the understanding of molecular mechanisms governing embryonic development.
CHAPTER 1: INTRODUCTION

1.1 Caged Compounds – Unidirectional Mechanism for Control of Genetic Expression

A remaining challenge to developmental biology is the spatiotemporal study of genes with regard to cell proliferation, migration, and differentiation [Ouyang et al., 2009]. Shestopalov and Chen have explored several genetic approaches for conditional gene regulation that provided key insights to the molecular mechanisms of cell patterning and function [Shestopalov et al., 2008]. Aside from the biological approaches commonly employed, a class of chemical compounds coined “caged” molecules are also used as tools to aid in unveiling the mysteries of cellular activity. These compounds functionally encapsulate biomolecules and render them inactive. The inactivated, or silenced, compounds can later be liberated by photomanipulation via a specific wavelength, characteristic to the chromophore, which restores activity and allows perturbation of the targeted bioprocess [Ellis-Davies, 2007]. This chemical approach to studying biological function quickly gained popularity due to its prolific application and invaluableness in concern to the aforementioned classes of biological study.

“Caged compounds,” or this class of light activatable molecules, originated in 1978 with Kaplan et al.’s synthesis of phosphate caged adenosine triphosphate (Fig1A) [Kaplan et al., 1978]. One year earlier, Engels et al. synthesized a cyclic adenosine monophosphate derivative (Fig1B) containing a photolabile leaving group. The light activatable phenomenon of this molecule was not the focus of the paper, nor was the “caged/caging” terminology ever inducted into scientific vocabulary, so inception was postponed [Engels et al., 1977]. As Figure 1.1 depicts, “caging” is a rather ambiguous or liberally used term to describe the protection. More accurately, these can simply be described as light removable protecting groups. However, since
the jargon has been accepted and used for the past 30 years, we will continue to refer to these as caged molecules from here onward.

These compounds exhibit the most preliminary examples of caged molecules for bioactivity. That said, a full history of caged compounds can be found in cited reviews [Pelliccioli et al., 2002, Goeldner et al., 2005, Marriot, 1998, Morrison, 1993, Mayer and Heckle, 2006]. Furthermore, a summary of popular applications that caging groups have found will be presented later in this thesis.

**Figure 1.1 – Examples of the first synthesized light activated compounds.** A) Caged ATP by Kaplan et al., 1978. B) Adenosine monophosphate derivative by Engels et al., 1977

1.1.1 Desired Characteristics of Caged Molecules

Caging biomolecules begins with the synthesis of the coupled compound, that is, attachment of the caging compound to the biomolecule or molecule of interest. Typically, these forms are made using synthetic organic chemistry reactions, some of which are simple enough to be employed by biologist with little or no organic synthesis experience [Ellis-Davies, 2007]. Most syntheses are carried out via a multi-step reaction; however, there are also single coupling mechanisms that directly attach the caging chromophore to its proper location on the
biomolecule. There exist a number of general guidelines (listed in bold below), for design and/or use of caging molecules and caged compounds, which will greatly optimize the photochemical reaction. This convention should be verified and followed when introducing any caging compound to a cellular environment:

1.1.1.1 Quantum Yield

Generally, the photoreaction should occur with a high quantum yield, \( \phi \). A quantum is the most minimum unit of any physical entity that can be involved in an interaction and a photon is a single quantum of light. The quantum yield of a photochemical reaction can be expressed mathematically as the ratio of photons absorbed by the photoreceptive molecule over total photons emitted by the source.

\[
\phi = \frac{\text{rate of reaction induced by photon absorption}}{\text{flux of absorbed photons}}
\]

Experimentally, this specific molecule characteristic value, or functional quantum yield, can be determined by

\[
\phi = \frac{1}{I \varepsilon t_{90\%}}
\]

where \( I \) is the irradiation intensity in moles of photons in \( \text{cm}^{-1} \text{s}^{-1} \), \( \varepsilon \) (mentioned in the next section) is the logarithmic extinction coefficient of the particular light receptive molecule contained in \( \text{cm}^{2} \) per mole of substrate and \( t_{90\%} \) is the irradiation time in seconds it takes to achieve 90% conversion [Adams et al., 1988].

1.1.1.2 Extinction Coefficient

The light receiving molecule, or chromophore, should have a high extinction coefficient, \( \varepsilon \), (this is a measure of how strongly a chemical species absorbs or scatters light at a given
wavelength) at wavelengths above 300nm. Light at wavelengths below 300nm are particularly avoided because at these wavelengths, many biological samples themselves have high extinction coefficients, thus they can readily absorb high energy light that has a wide range of deleterious effects. Lower uncaging efficiencies have proven to be damaging to the cellular environment. The organism itself cannot tolerate the number or density of photons needed to uncage the compound without also receiving the deleterious effects of this bombardment.

The extinction coefficient can be determined experimentally beginning with Beer-Lambert’s Law, which relates the absorption of light to the properties of the material through which the light is traveling:

\[ A = \varepsilon cl \]

Where \( A \) is the absorbance, or optical density of the sample or material, \( \varepsilon \) is the extinction coefficient, \( c \) is the concentration of the sample, and \( l \) is the path length through the sample that the light travels. The absorbance \( A \) can be defined as

\[ A = -\log_{10} \frac{I}{I_0} \]

Where \( I \) is the intensity of light at a specific wavelength that has passed through the sample and \( I_0 \) is the initial intensity from the source before it enters the sample.

**1.1.1.3 Photorelease Properties**

The photochemical by-products, the compounds being photolyzed, should be nearly transparent at the photoactivating wavelength and not interfere with photorelease in order to avoid competitive absorption of photons. Furthermore, released products should be biocompatible. They should be non-toxic, not interact with any normal cellular processes, and avoid formation of free radicals.
1.1.1.4 Rate of Uncaging

In kinetic studies, the rate of uncaging essentially must be more rapid than the process being studied. The speed requirements will be fully dependent on the phenomenon being analyzed.

1.1.1.5 Off-Target Effects

The caged agent must be biologically inert before photolysis. It must not elicit any cellular response, nor act as an agonist or antagonist when applied at a useful/working concentration in the biological preparation. It should be soluble in the target (aqueous) media, and may be required to bypass biological membranes [reviewed in Pelliccioli et al., 2002].

Upon actualization of these design requirements, the compounds themselves can be induced to in vivo analysis. However, because of the time dependency of biological processes, a distinct understanding of the reaction mechanism, and specifically the release rate of the compound, is strongly recommended.

1.2 Reaction Mechanisms and Release Rates

To date, a variety of caging compounds have been characterized. Beginning with the pioneering work of Engels and Hoffman using nitrobenzyl caged ATP, photolabile chemicals have gained popularity as pertinent applications have been recognized. For the applications of particular interest to this work and due to ease of availability, the nitrobenzyl based caging compounds were chosen.

![Figure 1.2 – Nitrobenzyl Structure](image)

**Structure.** Substituents include: \( R_1 = H, R_2 = H \): \( o \)-nitrobenzyl (ONB);
\( R_1 = OCH_3, R_2 = H \): 4,5-dimethoxy-2-nitrobenzyl (DMNB);
\( R_1 = OCH_3, R_2 = CH_3 \): (4,5-dimethoxy-2-nitrophenyl)ethyl (DMNPE) [Young et al, 2006].
1.2.1 Ortho-Alkylated Nitrobenzyl Compounds

Of several very effective caging groups the 2–nitrobenzyl group was chosen due to the availability of this compound. In general, nitrobenzyl groups are by far the most commonly utilized caging agent due to their practical synthesis and, in most cases, relative ease of attachment to the intended active site. Furthermore, these compounds are readily and easily decorated with electron donating functional groups (CH$_3$O) that “red–shift” the absorption maximum. Particularly, the attachment of multiple groups produces a hyperchromatic shift in the absorbance, which enables the chromophore to absorb a longer, lower energy wavelength of light that is less photodamaging. The adverse consequence of these attachments are typically found in a reduction of photolysis efficiency [Aujard et al., 2006].

![Diagram of 2-Nitrotoluene and E-Z isomers](image)

**Figure 1.3 – Photochemistry of 2-nitrobenzyl compound.**
Electronic excitation of 2–nitrobenzyl compounds rapidly induces cis–trans, or E–Z stereoisomers through tautomerization [Figure 1.3 – Phototautomerization of 2-Nitrotoluene] [Pelliccioli et al., 2002]. Due to the resonance energy that the fundamental benzylic structure
offers, a photon is able to be absorbed by the nitrobenzyl compound. Upon absorption, an
electron, which originally existed at a vibrational level in the ground state, is excited to one of
many vibrational levels in the singlet or triplet excited states. The photoreaction is triggered by
intramolecular benzylic hydrogen atom transfer to the excited nitro group, subsequently forming
the *aci*–nitro stereoisomer, as well as others [Bley et al., 2008, Schupp et al., 1987, Gee et al.,
al., 2002, Gee et al., 1999]. Bley et al. reported that, specifically, 2-Nitrosobenzenes are the
products of the 2-nitrobenzyl photoreaction and that the *aci*-nitro forms created were the major
intermediates in the photoreaction, whereas the cyclic benzisoxazoline and hemiacyclal have not
been observed [Bley et al., 2008]. From these reported results, it is implied that the cyclic
benzisoxazoline and hemiacyclal forms are not immediately formed upon irradiation, but
generated at a later time point as the spontaneously resonating structure adjusts to a
thermodynamically favorable confirmation and ultimately leads to released biomolecule [Figure
1.4 – Bley 2008 Scheme 1]. Theoretically, due to the radiationless energy transfer in return to the
ground state, the *aci*–anions of the nitrobenzyl compound can find the cyclic benzisoxazole and
subsequent hemiacetal conformations. Hemiacetals are generally regarded as unstable
compounds and, in the presence of a basic solution or water, elimination rapidly occurs, leading
to liberation of caged compound and the left over nitrobenzyl structure containing a carbonyl
functional group [II’ichev et al., 2004].

II’ichev et al. proposed a revised mechanism for the light induced reactions that 2-
Nitrobenzyl esthers undergo. [Figure 1.5 – Revised photocleavage mechanism] In their proposed
mechanism, the phototautomerization reaction takes place, producing the E–Z sterioisomers of a
nitrobenzyl conjugated biomolecule. The *aci*-nitro intermediate formed is considered to be both
an acid, pK_{a,2} \approx 4, and a weak base. Due to this duality, equilibrium will establish three aci-transients; a protonated intermediate (A^+), the neutral resonating structure (A), and a negative ionic form (A^-). Hydration of the protonated intermediate will yield a nitroso hydrate (B'). The spontaneous dehydration produces an unstable hemiacetal that will quickly free the biomolecule. Variable transient formation is favored by different buffering conditions. The additional pathway that the protonated intermediate (A^+) in Figure 1.5 accounts for the effect of buffers on the aci-transients. The rates of reaction paths in Figure 1.5 may be strongly dependent on the reaction medium (the solvent, pH, buffer, and buffer concentration) and the biomolecule, or leaving group [II'ichev et al., 2004]. However, in spite of the longstanding interest in the photochemistry of nitrobenzyl compounds, only

Figure 1.4 – Bley 2008 Scheme 1 of the 2–nitrobenzyl photoreaction.
fragmentary information is available about the elementary steps that lead to deprotection [Pelliccioli et al., 2002].

1.3 Examples of Caged Compounds

To facilitate an appreciation of the large number of applications that caging chromophores have established, a modest overview of their function in the biological sciences
will be presented beginning with related studies of small molecules. Macromolecular caged compounds will then be briefly presented and, finally, transitioning to our applications, attachment to oligonucleotides (or short nucleic acid/nucleic acid analog polymer, typically 20 or fewer base pairs) and nucleic acids.

1.3.1 Small Molecules

The following sections are designed to give the reader an appreciation of applications that biomolecules find when they can be rendered inert. This is by no means an exhaustive list. Excellent reviews containing more examples and applications can be readily explored [Ellis-Davies, 2007, Pelliccioli et al., 2002, Mayer and Heckel, 2006].

1.3.1.1 Neurotransmitters - Caged Glutamate

Perturbation of sensory processing and manipulation of neural activity with single cell resolution is a long standing desire of neuroscientists for applications in disease treatment. Using caged glutamate, an action potential can be induced in only a small number of neurons which can disclose information on the neuronal circuitry that will, expectantly, lead biologist to their goal [Matsuzaki et al., 2008]. Caged glutamate is the most widely used caged neurotransmitter by biologist, and many approaches to caging this molecule have been effected using different chromophores and caging strategies [Ellis-Davies, in press]).

1.3.1.2 Nucleotides and Nucleosides

As previously mentioned, the Kaplan, Forbush, Hoffman group were the pioneers of the “caging” frontier. In their 1978 work, they successfully caged ATP using a five step synthesis reaction. Beginning with the nitrobenzyl caging compound, they created caged phosphate which was coupled to the terminal phosphate of adenosine diphosphate, ADP. Formation of the caged adenosine triphosphate followed. Demonstration of photolytic features of caged ATP lead to the
applicable objectives, which were the effects of ATP/caged ATP on sodium/potassium pump governing ATP-ase and its effects on sodium/potassium transport associated with its enzymatic activity in human red blood cells [Kaplan et al., 1978].

One year prior to the Kaplan 1978 publication, Engels et al., “caged” cAMP. Although their original objectives of adding a chemical moiety to the adenosine structure were to enhance lipophilicity, they were concerned with restoring the original activity of this important second messenger. While some of 6 different triesters of cAMP were synthesized with the intention of direct hydrolysis to free the biomolecule, 2 involved the nitrobenzyl moiety for release via photolysis. As a delivery vehicle for enhanced lipophilicity, the results were validated in rat glioma cells [Engels et al., 1977]. The caged ATP and cAMP were the first reported caged compounds to be synthesized, used, and uncaged in living cells. Since that time many nucleotides and their analogs have been caged for biological applications [reviewed in Pelliccioli et al., 2002].

1.3.2 Macromolecules

Similar to that of small molecules, blocking the function of macromolecules has been met with much success. It is generally accepted that significant differences lie in targeting the active site of the molecule as opposed to strategies of attachment to small molecules where the mere presence of the blocking compound can impose a steric hindrance, affecting action. Similar to the overview of small molecules, this section on macromolecules will generally describe the use of caged large molecules.

A considerable variety of caged peptides (short polymers formed from linking amino acids in a defined order) have been made, specifically in the form of enzymes and polypeptides. Caged enzymes usually serve as inhibitory peptides designed to disrupt protein–protein
interactions and caged polypeptides act as the larger counterparts to standard small molecule caged compounds. In this case, where direct attachment to the enzyme or protein is advanced, its catalytic function is blocked by the caging chromophore [Ellis-Davies, 2007]. The progress of macromolecular caging is often more difficult than that of small molecules due to the fact that caged peptides appear to be inherently unstable. However, development of these complex conjugates is a fundamental necessity because they provide an inactivation approach that is conceptually distinct from that offered by the small molecules. This idea is highlighted by the fact that many cellular processes are not regulated by cofactors, but by direct protein–protein interaction [Ellis-Davies, 2007].

1.3.2.1 Peptides and Proteins

Proteins and peptides have been caged by many commercially available reagents that modify specific amino acid residues. The ability to target particular locations on the biomolecule is especially important because blockage of the active site, usually through steric hindrance, will render it inert. Identifying and targeting certain locations, a crucial facet to this method that ultimately optimizes monetary and temporal resources, is achieved by predetermined bioconjugate techniques that are fully dependent on well known and characterized chemical reactions [Ellis-Davies, 2007]. There are several points to consider when planning caged protein synthesis: (i) due to its size, use of caged proteins will inherently introduce a percentage of residual activity effectively complicating outcomes in the biological system (in most situations, an equilibrium will establish, greatly reducing the probability of caging 100%, leaving the small percent of residual activity to potentially introduce ambiguous results), (ii) full recovery of function is problematic, especially when “shotgun” or “statistical” caging is the method of inactivation, and (iii) there is no especially efficient way to introduce these silenced compounds
to an *in vivo* environment [Ellis-Davies, 2007]. Additionally, proteins typically contain a large number of nucleophilic sites (sites that preferably attach to an electrophilic counterpart through the donation of electrons) which can be difficult to interact in a site-specific manner with exogenous caging agents, when the endogenous, competitive interactions with other proteins in the cellular environment is a much more favorable reaction [Curley et al., 1999]. Also, there may not be an appropriate nucleophilic residue necessary for interaction, at or near the desired site of modification. Fortunately, many of these problems have been circumvented in recent studies. The two primary mechanisms of passage are beyond the scope of this review [refer to Ellis-Davies paper 18, 49, 50, and 51] [Mendel et al., 1991, Petersson et al., 2003, Muralidharan et al., 2006, and Hahn et al., 2004]. In summary there is an exponential growth of complexity for caging reactions introduced by increasing molecular weight compounds.

### 1.3.2.2 Oligonucleotides and Nucleic Acids

Effective alteration of gene activity at precise times and locations is especially attractive for delineation of protein function in whole organisms, and where uncaging technology is being increasingly applied to accelerate this work. Genetic function can be efficiently controlled by caging deoxyribonucleic acids (DNA) and (messenger) ribonucleic acids (mRNA) fragments, as well as gene-regulating oligonucleotides. Thus far, the most efficient method of caging mRNAs or DNAs has proven to be the most useful biologically [Ellis-Davis, 2007]. “Direct, multi-site,” “shotgun,” or “statistical” caging, terms coined to describe a specific phenomena, of DNA with reactive nitrophenyl–diazo compounds, thoroughly binds and inactivates the molecule [Monroe et al., 1999] [Figure 1.6]. The elegance of the approach of statistical backbone caging clearly lies in its ease and simplicity of preparation [Mayer and Heckel, 2006]. However, at least with the modifying reactions and caging chromophores used to date, systematic binary (on/off) response
of genetic function has been unattainable. An alternative to backbone attachment is the introduction of caging groups at positions within nucleic acids [Mayer and Heckel, 2006], and specifically for our applications, direct attachment to the bases. In this context, a review of various approaches to caging oligonucleotides is appropriate.

1.4 General Oligonucleotide Caging Methods

![Diagram of backbone caging](image)

**Figure 1.6 – Backbone caging of DNA with a reactive nitrophenyl–diazo compound**

1.4.1 Statistical Backbone Caging

As mentioned in the previous section on oligonucleotide and nucleic acid caging strategies, one of the existing techniques used to prepare caged nucleic acids relies on what has been coined “statistical backbone caging” [Mayer and Heckel, 2006]. Monroe and Haselton conceptualized this idea by modifying DNA plasmids coding for GFP [Monroe et al., 1999]. Owing to the unselective mechanism of attachment during reactions under benzylating
conditions, the plasmids were modified with a statistical distribution of the altering agent. In this approach, probable locations of attachment are estimated and selected as likely sites that are dependent on the chemical species being exploited. Although this approach brings one as far as a hypothetical structure of the chemical species complex, the statistical analysis begins with calculations based on the extinction coefficient of the caging chromophore. Based on the extinction coefficient, the number of caging groups per oligonucleotide sequence can be quantified [Friedman 2006].

1.4.2 Site Specific Attachment

The appeal of the statistical backbone method stems from its simplicity of preparation. However its primary downfall, which calls for implementation of alternative strategies, is the crux that full restoration, or complete removal of attachment compounds, is not yet attainable. The most obvious alternative would be a direct, site-specific attachment scheme. The alternative to circumvent the disadvantageous properties introduced by statistical attachment is to accurately target locations at defined positions on nucleic acids.

This is exactly the approach that Chaulk and MacMillan took one year prior to Monroe and Haselton’s work. In their conception, a complementary approach was developed that allowed for the isolation of specific RNA structures or complex formations through transient blockings or caging of particular RNA functional groups involved in the transition between two different states, particularly the 2’ hydroxyl functionality [Chaulk et al., 1998]. The 2’ hydroxyl group was chosen as a blocking site due to its association with general RNA functionality, since specific 2’ hydroxyls act as nucleophiles in a number of biologically relevant transesterifications [Uhlenbeck, 1987, Branch et al., 1991, Buzayan et al., 1986, Guo et al., 1995, Peebles et al., 1986]. The 2’ modified RNA was used to control hammerhead ribozyme (ribonucleic acid
enzyme) activity with light. Ribozymes are RNA molecules that are able to catalyze a chemical reaction. Modification of the 2’ group with a nitrobenzyl blocking compound stopped catalytic activity of the enzyme whereas upon irradiation, full restoration of activity was restored. This is a hallmark example of site–specific, finely controlled, binary behavior prior to and post irradiation albeit at the demand of a markedly higher synthetic effort. Although different synthetic methods are employed and target areas are varied, the aforementioned techniques have commonality, namely, backbone caging which assumes the existence of the very nature of operation nucleic acids are still intact. This nature is the Watson–Crick interaction capability. A complete explanation of how backbone caging affects hybridization has not yet been offered. Alternatively, assuming labeling on the bases, one can clearly understand that the presence of the caging groups offers a steric hindrance that disables the hydrogen bonding ability of an oligonucleotide sequence.

### 1.4.3 Alternative Novel Approaches

Before proceeding, notable approaches have been employed that are acutely different from the somewhat expectable approaches mentioned until now. Met with success, the pioneers who crafted these techniques engaged diverse tools of molecular biology with a single specific design and function that fortunately operated in the hypothesized manner. Several of these tools that exercise nucleobase caging techniques, or direct Watson–Crick disruption, will now be discussed.

Dmochowski et al. employed a synthetic route for incorporating a photocleavable (PC) linker containing a DABSYL (fluorescence quencher) moiety and fluorescein at adjacent cytidines (cytosine nucleoside, formed when cytosine is attached to the ribose ring, or in the case of DNA deoxycytidine) in the middle of a 25mer oligonucleotide [Tang et al., 2004]. Fluorescent
molecules are characteristic compounds (similar to caging compounds in that they are polyaromatic hydrocarbons) that are able to absorb light at a specific wavelength and can subsequently re-emit or radiate energy at a different, and equally specific, wavelength. They are simply used as reporters that disclose location in this application. Quenchers are molecules that resemble fluorophores in that they are able to absorb energy, but the re-emission is a non-radiative process usually through heat loss. Fluorescent excitation of fluorescein is prompted by 491nm (near UV, but still within the visible range on the electromagnetic spectrum) wavelength light and emission is of 520nm wavelength light (which appears green in the electromagnetic spectrum). However, with the presence of the DABSYL quencher, absorption occurs and is followed by emission, but instead of the green hue being emitted, fluorescence quenching occurs where the energy is absorbed by the quencher and dissipated as heat. The novelty of this complex is the presence of the PC linker containing the quencher. At any time, irradiation with 355nm light will release the quencher and subsequent exposure to appropriate wavelengths (491nm) will enable fluorescence detection. One abiding goal of these studies is to generate caged molecules that bind to their complementary target and fluoresce.

Figure 1.7 - Schematic of Molecular Beacon analysis
when triggered with light [Tang et al., 2004]. In this way, one can determine exactly when the ligand/receptor complex formed and is present.

Analogous to the quenched fluorophore, a novel idea arising from this technology introduced and combined another set of molecular diagnostics, providing an additional degree of precise control. To begin, molecular beacons, first synthesized in 1996, are nucleic acid probes that recognize and report the presence of specific nucleic acids in a homogeneous solution or environment [Tyagi et al., 1996]. The beacons undergo a spontaneous structural change when a target nucleic acid sequence is identified that ultimately brings about a fluorogenic state [Figure 1.7]. The innovation that stemmed from this already remarkable DNA hybridization biosensor promised to release functional molecules, which the group specified as drugs, upon identification of target sequences and consequent exposure to an external stimulus, particularly, light [Okamoto et al., 2003]. The molecule releasing system used is controlled by intramolecular quenching. Photoresponse, and drug release, of the probe oligonucleotide sequence containing the photoactive chemical moiety and the quencher proved, in fact, to be an inefficient delivery

Figure 1.8 – MB Caging mechanism utilized by Okamoto et al., 2003
mechanism when the existing state was in the “closed loop” form, whereas, upon Watson–Crick coupling with its target and consecutive irradiation, rapid release of the functional molecule was observed [Okamoto et al., 2003] [Figure 1.8].

![Diagram](image)

**Figure 1.9 – Shestopalov et al., 2007 linker mechanism for morpholino caging**

The final unconventional caging strategy, and most similar to the work described herein, was the strategy developed by Chen’s group at Stanford. Studying zebrafish development, the group employed a synthetic DNA analog oligonucleotide, known as a morpholino, which will be described in greater detail subsequently, as an RNA blocking group. Caging this synthetic biomolecule effectively stopped its ability to hybridize target RNA, whereas ablation of the attached group with light fully restored activity. A method was devised for coupling an inhibitory oligomer to a distinct morpholino sequence that targeted a confirmed gene via a photocleavable (PC) linker [Shestopalov et al., 2007]. The inhibitory, or caged, sequence was synthesized in solid phase and functionalized at termini with a photolabile linker that permit simplistic connection, one of which was a photolabile compound that served as a single link in the chemical chain. Upon irradiation, the tether is broken and the entire inhibitory sequence is
released [Figure 1.9]. Functional studies in whole organisms exploit a seemingly infinite number of drugs. The constitutive activity of these reagents limit their experimental utility, ultimately creating a daunting enigma. Owing to this puzzle, caging compounds have been identified by their aptitude for elucidation of this fundamental dilemma.

### 1.5 Light Sources for Photo-Activation of Caged Compounds

A large number of photon sources are available for photo-activation. These variable devices offer advantageous and disadvantageous elements that are especially dependent on the applications involving the caged compound that require their use. Characteristics one should consider when choosing the appropriate light source for their application include: heat generation by the source, the spectra that is generated, and density of radiation incident. Without performing a full review of available light sources, which can be found at [Ellis-Davies, 2007, Casey et al., 2009], it is sufficient to mention that, currently, the majority of uncaging experiments exploit flash lamps and lasers [Rapp et al., 1989, Blidner et al., 2008, Mikat et al., 2007, Ando et al., 2004, Shah et al., 2005, Young et al., 2008, Shestopalov et al., 2007, Kaplan et al., 1978]. For our application, we apply a specific UV-light source for our studies. Distinguishing from others, we operate a device, commercially tagged, “Green–Spot” that uses industry standard pressurized 100 watt mercury lamp, mounted vertically, in a dichroic–coated elliptical reflector to generate an intense, 5mm spot of light in the UVA, UVB, UVC, and visible range [American Ultraviolet Co]. The device is marketed by its simplistic design, interface and reliability of operation. The optics system contains a removable quartz IR filter that allows UV light to pass from the 300-480 nm range [Forman, 2007]. The Green–Spot device uses a standard fiber optic cable, or light guide, designed to operate in the 320 – 500nm range. Confining spectral output to 365nm is
preferable, due to the fact that the methoxy-nitrobenzyl absorbs maximally at 355nm, as well as reducing the potential for undesirable heat accretion [Forman, 2007].

1.6 Gene-Silencing Oligonucleotides – Tools for Gene Knockdown

1.6.1 The Opportunity for Antisense Oligonucleotides

An ever increasing opportunity for antisense drugs is becoming more evident as the windings of molecular biology and disease diagnosis are being unraveled. This understanding at the molecular level validates the approach as a whole since biological process dissection enables and promotes rational drug design which almost entirely abolishing conventional drug synthesis methods. The conception of this technology derives from a sound understanding of nucleic acid structure and function that is ultimately dependent on Watson–Crick hybridization [Watson, 1953], and fundamentally promises gene-selective reagents and drugs [Crooke, 2007]. Thus, arguably, clear demonstration that nucleic acid hybridization is feasible and moderately controllable, and the advances of in situ hybridization and probe technology, establishes evidence for the most basic elements of the foundation supporting the antisense theory [Gillespie et al., 1965, Thompson et al., 1990].

The first use of antisense oligonucleotides as therapeutic agents was performed in the work of Zamecnik and Stevenson in 1978 [Zamecnik et al., 1978]. Here, a 13 base pair tridecamer was synthesized that was complementary to an equal length segment of the 3’ and 5’ reiterated terminal sequences of Rous sarcoma virus (RSV) 35SRNA. The RSV 35S RNA contains a 21 nucleotide sequence just internal to the 5’ cap that is exactly identical to the same length segment adjacent to the poly A terminus at the 3’ end of the molecule, approximately 10,000 base pairs away. The reiterating terminal sequences critically impact in circularization of viral DNA, prior to its integration into the host genome. Induction of the tridecamer, targeting
the 3’ and 5’ locations, into cell culture infected with RSV resulted in inhibition of virus production [Zamecnik et al., 1978]. This implied that the oligonucleotide displayed evidence of antiviral activity. However, the most impactful result that the authors reported was the possible sites for attachment via hybridization on the viral RNA and potential mechanism of action of the oligonucleotide.

1.6.2 The Challenges Faced by the Field

Essentially, the development of antisense technology is the creation of a new pharmacology, or more specifically, an entire new study of how these exogenous chemicals alter the normal biological function of the organisms with which they interact [Crooke, 2007]. The receptors for this drug are defined nucleic acid sequences in a target strand. Accordingly, perfectly analogous with enzymatic kinetic analysis, from a pharmacological perspective, a comprehension of the structure, function, and intermediary metabolism of RNA is essential.

Conceptually, the mechanisms of action that antisense molecules can assume is divided into three classes: pre– or non–hybridization, hybridization, and posthybridization. These hybridization properties must be considered owing to the basis that typical cellular levels of target mRNAs is less than 100 copies per cell, and at such a low concentration, the interaction that occurs usually has a negligible effect on the total antisense drug concentration. Consequently, these unaccounted for off–target effects, primarily with cellular and extracellular proteins, attribute to the pharmacokinetics and non–pharmacologically–based toxicological properties of antisense drugs. Finally, while impressive progress has been reported about understanding posthybridization processes, curiously, very little has been said about how hybridization to a specific target RNA occurs, in particular, the kinetics of intracellular hybridization events [Crooke, 2007].
1.7 Basic Principles – RNA Intermediary Metabolism

Oligonucleotides are molecules designed to alter RNA intermediary metabolism. They are designed to modulate the information transfer from gene to protein. Figure 1.10 depicts this mechanism. RNA metabolism is initiated with transcription. Specific sequences of DNA are recognized by transcription initiation complexes that act to locally denature DNA by separating the double stranded sequence into two independent identifiable strands. A member of the RNA polymerase family, a protein family that can assemble RNA polymers from genetic information in the form of DNA, can then complex with the single stranded DNA and serve its function to transcribe one strand of the DNA (the antisense strand) to its sense pre–mRNA polymer. Typically during transcription, the 5’ end of the pre–mRNA is “capped” by adding a methyl–guanosine, and also by methylation of one or two adjacent sugar residues. This process is vital to creating mature mRNA, which is able to undergo translation. Capping also ensures the mRNA polymer’s stability as it undergoes transcription in protein synthesis and may affect a number of valuable RNA processing events [Mizumoto et al., 1987]. Between the 5’ cap and the sight where translation occurs is a stretch of nucleotides that are untranslated, known as the 5’ untranslated region (5’ UTR), that also serve to affect mRNA half life and transitional efficiency [Ross, 1988]. Similar to the 5’end of the mRNA, the 3’ end also undergoes modification. On the 3’ side exists several hundred nucleotides beyond the translation termination signal. Much like the 5’ bit, this untranslated region too plays a role in determining the half life of the mRNA as a whole. Moreover, post transcriptional modifications in the form of a polyadenylated tail stabilize mRNA. This modifier is crucial for transporting mature mRNA out of the nucleus, and additionally, it may serve significant roles in the cytoplasm [Friedman et al., 1987, Manley, 1988].
Besides modifying the 3’ and 5’ ends of pre–mRNA, proper splicing is important in conventional mRNA metabolism. Because eukaryotic genes usually contain ancillary sequences (introns) embedded between active protein coding regions (exons), proper function entails excision of these superfluous regions with the remaining segments spliced together. Splicing reactions are complex, highly regulated, involve specific sequences, small molecular weight RNA species, and numerous proteins [Crooke, 2007]. Since RNA splicing involves withdrawal of delineated intronic sequences and coupling of exonic fragments, alternative splicing reactions, or removal of alternative sequences, will generate different mature mRNAs and therefore, different proteins. Although introns have been observed as waste, important sequences are conserved and have been recognized to play roles in coding for proteins, antisense transcripts, and noncoding RNAs [Black, 2003]. Once capping, polyadenylation, and splicing occurs, mature mRNAs are shuttled out of the nucleus and into the cytoplasm where they engage in translation. As previously maintained, the half life of these compounds range from minutes to hours, and are highly regulated.

1.7.1 Antisense Action

Simply stated, an antisense oligonucleotide is a short strand of deoxyribonucleotide or deoxyribonucleotide analogue that is complementary to a sense strand of mRNA. As a consequence of the complementation, the antisense strand can hybridize to the sense sequence by way of Watson–Crick base pairing.Formation of this heteroduplex induces a number of potential circumstances including: induction of RNase H activity which leads to mRNA destruction, steric hindrance and sequential translational arrest by blockage of the ribosomal units, and/or interference with mRNA maturation by inhibiting splicing or destabilization of pre–mRNA in the nucleus[Chan et al., 2006]. Depending on the mode of action of the particular oligonucleotide,
one of the aforementioned perturbations will be exemplified. In any case, the end effect is specific reduction of protein synthesis. Mechanisms of mediation will now be briefly illustrated to grant the reader a more appropriate understanding of activity of these protein knockdown oligos.

Figure 1.10 – Schematic of RNA Metabolism and Antisense Action
1.7.2 Potential Sites of Disruption

Traditionally, competitive antagonists are assumed to alter biological processes through binding to receptors, effectively preventing agonists from accessing and inducing normal biological process. Binding of oligonucleotides to endemic complementary sequences may inhibit RNA interaction with proteins, nucleic acids, or other intracellular machinery necessary for proper intermediary metabolism [Crook, 2007].

1.7.2.1 Splicing Transitioning

Splicing reactions, the excision of introns necessary in the intermediary metabolism of mRNA, are sequence specific and demand the concerted action of spliceosomes, therefore, oligonucleotides that append to mRNA that naturally undergo the excision process may prevent amalgamation of necessary factors (proteins) and prevent fragmentation [Crooke, 2007]. This would result in prevention of mature mRNA synthesis. A number of chemically modified antisense oligonucleotides have been shown to alter spicing in vitro and in vivo [Sazani et al., 2003]. Considering the necessity of oligonucleotide hybrids that are more robust and resistant to degradation, certain chemical alterations to structure are required. Specifically, chemically modified antisense products such as: s fully modified 2’–methoxy, 2’–MOE, peptide nucleic acid (PNA), morpholino (which will be described in more detail later), and fully modified locked nucleic acid (LNA) analogs [Sazani et al., 2003].

Alternative splicing, a subset of splicing reactions that allows for the assembly of necessary proteins but alters the output of the original intended reaction, is also a domain of focus because theoretical conception of alternative proteins can be envisioned.

1.7.2.2 Translational Arrest

Translational arrest, as hinted by the name, is a supposition that translation is inhibited. In this stage of RNA metabolism, some process denies the messenger RNA from being read and
construed to protein. Because polysomes are capable of “melting” or disassociating structures in RNA, translational inhibitors classically interact in the 5’ untranslated region (UTR), the translation initiation codon area, or an internal ribosomal entry (IRE) sequence [Crooke, 2001, Crooke, 1999]. However, inhibitors designed to bind to sites in coding sequences have also been shown to be active [Crooke, 2007].

1.7.2.3 5’ Capping Alterations

An imperative elementary step in RNA processing and metabolism is 5’ capping. The pervasiveness of this action is a testament to its importance and it serves a number of roles in the cell which are exemplified by stabilizing the pre–mRNA as well as the mature mRNA, essential binding to the nuclear matrix, and its presence plays a role in transport of mRNA out of the nucleus. Several oligonucleotides that bind near the cap site have shown favorable silencing activity, presumably by inhibiting standard protein binding required for attachment of the capping molecule [Saxena et al., 1990, Westermann et al., 1989]. Also, oligonucleotides have been designed to bind to the 5’ cap structure that selectively identify reagents that cleave the 5’ cap itself off of the mRNA. This demonstrated that addition and subsequent abstraction of the 5’ cap still serves as an effective means to inhibit the binding of translation initiation factors [Baker et al., 1992].

1.7.2.4 Inhibition of 3’ Polyadenylation

Similar to 5’ capping, in the 3’ UTR of pre-mRNA, there are sequences that necessitate posttranscriptional addition of hundreds of nucleotide long tracts of polyadenylate. Analogously, polyadenylation stabilizes mRNA and plays a multitude of other roles in RNA metabolism. Logically, using the same approach as disruption of 5’ capping protein binding, interactions in the 3’–terminal region could inhibit polyadenylation and destabilize the RNA species. Although
many oligonucleotides target the 3’ UTR and display antisense activity, only one study has
typified evidence for alterations in polyadenylation [Vickers et al., 2001]. Here, fully modified
2’–MOE antisense agents prompted polyadenylation to be redirected, effectively leading to RNA
stability increases and enhanced protein synthesis.

1.7.2.5 RNase H Enzymes

The RNase H knockdown mechanism has proven to be the most widely studied system to
date. In this pathway, RNase H enzymes hydrolyze RNA in RNA–DNA hybrids [Stein et al.,
1969]. In addition, there are RNases that recognize double stranded RNA, or RNA/RNA
duplexes, and denature these complexes. Although it has been accepted that ASOs (antisense
oligonucleotides) similar in structure to DNA cause target RNA reduction by binding RNA and
inducing indigenous RNase H enzymes to degrade the product (by creating duplexes that serve
as a substrate for the protein), definitive proof that this mechanism is responsible for the
observed knockdown effects in vivo has been lacking [Crooke, 1999, Crooke 2001]. However,
the addition of members of this protein family to DNA/ RNA heteroduplexes in cell free systems
results in degradation of target RNA [Wu et al., 1999, Denisov et al., 2001, Crooke et al., 1995].
The same has been displayed with DNA–like antisense oligonucleotides [Crooke et al., 2001].
Thanks to the substantial progress reported thus far in understanding RNase H, their roles served
with ASO complexes, and the factors that influence function, many groups feel that the
intellectual framework for designing optimized RNase H dependent ASOs exists, and they are
issuing vast resources to the cause as they see a potential for success [Crooke, 2007].

1.7.3 Factors Influencing Antisense Drug Selectivity

The following are examples of factors that influence antisense drug selectivity (a full list
can be found and reviewed by Crooke, 2007).
1.7.3.1 Affinity

The contingency for oligonucleotide binding is dependent on hybridization interactions with its receptor sequence. The two prominent determinants for augmentation of the free energy of coupling, also known as oligonucleotide affinity, are hydrogen bonding and base stacking within the double helix [Crooke, 2007]. These two characteristics bear such a substantial contribution that they can be fully accredited with governing the affinity; in other words, affinity increases as the length, or number of interacting bases, increases. Affiliation between two sequences also varies by the sequential composition. The nearest–neighbor model supports the prediction of the free energy of binding for DNA–DNA and RNA–RNA hybrids with relatively high fidelity [Breslauer et al., 1986, Freier et al., 1986].

As with other drug–receptor interactions, activity requires a threshold level of affinity to be exceeded. For many antisense oligonucleotides, the minimum length required to display any function is 12–14 nucleotides [Crooke, 2007].

Albeit theoretical bonding for single stranded oligonucleotide interactions are relatively large, pragmatically, association constants are substantially lower owing to a number of constituents. Undoubtedly, the most important facet contributing to this anomaly is that RNA can adopt a variety of secondary and tertiary structures [Chastain et al., 1993, Ecker 1993]. A concomitant circumstance that can potentially impact binding affinity, in a negative way, is the potential for oligonucleotides to form secondary and tertiary structures within and among themselves. As a general guideline, avoiding duplex formation entails choosing oligonucleotides that do not contain self–complementary regions. However, unforeseen structures such as tetrameric complexes that are not well understood have been reported. These complexes, consisting of guanosine quartets (found in oligonucleotides containing multiple guanosines) and
other base sequences can be highly stable, and can prevent antisense interaction as well as have a number of biological effects, but have confounded interpretation of experiments [Tuerk et al., 1990, Wyatt et al., 1994, Wang et al., 1993, Leroy et al., 1993, Gehring et al., 1993].

Additional forthcoming considerations for antisense lie in in vivo testing. Since RNA and oligonucleotide structures are dependent on the ionic milieu and interactions with proteins and polycations, the in vivo environment is considerably more complicated to anticipate. Proportionally, little is known about the interplay between these complexities and the effects on true affinities between oligonucleotides and target sequences [Crooke, 2007], so there is still work to be done before the full potential of clinical antisense therapies are realized.

1.7.3.2 Specificity for Nucleic Acid Sequence

Gene targeting using antisense oligonucleotides, appears to be orders of magnitude more specific compared to traditional drug design which targets a particular class of receptors, where nonspecific interactions with other receptors and proteins containing similar active site geometries introduce side effects. This specificity derives from the selectivity of Watson–Crick or other types of base pairings. Also of note is the fact that DNA/RNA molecules are not synthetic therapeutics, whose cellular targets may be numerous and unknown. The decrease in affinity associated with a mismatched base pair is astounding, and varies as a function of the mismatch itself, the positioning of the mismatch along the oligonucleotide hybridizing, and the sequences surrounding the mismatch [Crooke, 2007]. For example, in a conventional interaction between complementary 18–mers, the change in the Gibbs free energy of binding induced by a single mismatch varies from +0.2 to +0.4 kcal/mol/modification at 100mM NaCl, or relatively, impacts this length oligonucleotide by decreasing the affinity 500–fold [Freier et al., 1992].
At the genomic level, any given sequence of 17 residues is expected to occur only once [Thein et al., 1986]. Stepping down to RNA which is not as copious, and assuming a random distribution of sequences, any arrangement of 13 bases is expected to occur only once in the cellular RNA population; contrasting mammalian cells, an 11-mer or possibly smaller oligonucleotide could identify and bind to a unique sequence [Helene, 1993].

Additional determinants that conceivably alter specificity are RNA secondary and tertiary structures, whereby these formations practically assure that not all sequences are readily accessible. Particularized design of oligonucleotides to interact with portions of RNA involved in the maintenance of these structures can theoretically enhance specificity and, if the structure enhances stability or function of the polynucleotide, potency [Crooke, 2007]. Ensuing, in many cases, both RNA and DNA interact extensively with proteins. Due to this synergy, it is conceivable that far more diversity will be met in response to an antisense oligonucleotide addressing these protein interacting sequences than might be predicted solely on the basis of differences in nucleic acid sequence [Crooke, 2007].

1.7.3.3 Protein Binding

As previously mentioned, influencing the selectivity and potency of antisense drugs stems from RNA binding interactions with proteins. Although understanding RNA structure provides crucial information that enhances identification of optimal binding sites for antisense drugs, insufficiencies stem from the fact that RNA binds a multitude of proteins at various positions [Saunders et al., 2003]. Any protein participation at or near a target site may adversely affect the efficacy of the antisense drug. Despite the progress made in understanding antisense drug activity, relatively little is known about antisense/protein agonistic behavior [Crooke, 2007].
1.7.3.4 Levels of Target mRNA

While one may conceive that the concentration, transcription rate, and/or stability of RNA may influence the effectiveness of antisense drugs, experimental results prove that these factors do not have such a strong impact on antisense performance [Miraglia et al., 2000]. Miraglia et al.’s study varied concentrations of either exogenous or induced endogenous RNA from 1 to 400 copies per cell and showed that the deviated collection of nucleic acid had no effect on the potency or efficacy of the antisense drugs. They went on to show that transcription rate had no consequence either. This occurrence may be explained if we consider the simplistic equation governing drug action:

\[ D + R \rightleftharpoons DR \rightarrow \text{Effect} \]

Where \( D \) is the drug concentration, \( R \) is the receptor concentration, and \( DR \) is the drug–receptor complex. Given the low concentration of pre– and m–RNA in cells and the high intracellular concentrations of antisense drug, the receptor concentration can be neglected and the drug effect should be exclusively dependant on drug concentration. Transcription rate should have no effect because receptor concentrations are irrelevant [Crooke, 2007].

1.8 Morpholino Antisense Oligonucleotides – Robust Nucleic Acid Analogs for Gene Silencing

As evidence has been assembled, one can recognize the promises forged by antisense oligonucleotides in treating a broad range of diseases that involve specific genes. However, inefficiencies with this approach exist. While they have not been wholly depicted here and generally, a large margin of error is eminent. Most notable, inadequate specificity, ineffective delivery to cellular compartments, and unpredictable cellular interactions have stymied the antisense field [Summerton et al., 1997]. In fact, a dominant consideration involved in the design of most antisense oligonucleotides has been to devise a structure that is resistant to nucleases
while still resembling nucleic acids. In an effort to circumvent these dilemmas, multiple generations of antisense oligonucleotides have been generated, many of which aid, but cannot fully elude the problematic actions including physiological degradation, off-target effects, and other functional insufficiencies. Thus far, one of the seemingly most promising members of the second generation family of synthetic oligonucleotides that avoid these negative effects is the phosphorodiamidate morpholino oligomers.

The phosphorodiamidate morpholino oligomers (PMO) are comprised of (dimethylamino) phosphinylideneoxy–linked morpholino backbone moieties, which is where their informal designation, morpholino, is derived. [Figure 1.11] The morpholino moieties contain heterocyclic base recognizing subdivisions of DNA (A, C, G, and T) attached to a substituted morpholine ring system. When linked to each other via the (dimethylamino) phosphinylideneoxy function, the employable group formed by intersubunit linkage is commonly referred to as a phosphorodiamidate [Crooke, 2007].

As previously mentioned, morpholinos offer beneficial features more explicitly stated as; the characteristic that PMOs are highly resistant to degradation [Hudziak et al., 1996], PMOs mechanism of action eschews oligomers serving as cofactors for enzymatic cleavage of RNA, thus they appropriate an RNase H free pathway [Giles et al., 1993, Stein et al., 1997], morpholinos refrain from forming G–quartet structures (tetrameric complexes mentioned earlier) capable of off target gene regulation [Burgess et al., 1993, Hudziak et al., 2000], and PMOs do not bind to cellular and extracellular proteins [Stein et al., 1993, Stein, 1994, Shoeman et al., 1997].

The lack of iterated charge appears to eliminate non–targeted binding to cellular components other than RNA. The limited (negligible) coupling with proteins and weak
interactions with cell membranes imply that PMOs available for hybridization with RNA will be minimally competed for by nonspecific binding [Crooke, 2007]. This phenomenon forms the basis for the hypothesis that the sequence–dependent pharmacokinetics for PMOs is unique comparatively to the ionic oligomer chemistries [Arora et al., 2004].

Figure 1.11 – Morpholino Oligonucleotide Structure
Thus far, we have established a platform that has combined the subject material of caged compounds and antisense oligonucleotides with particular emphasis on the nitrobenzyl labeling compound and morpholino oligonucleotides. This data presented has served to embody the current progression in the fields of both photoactivatable compounds and antisense nucleic acids. With this information, we are now set to explain the thesis endeavor which is to manipulate the morpholino oligonucleotide, an entity that is distinctively designed to resist any sort of reaction other than with its target, to foster attachment or reaction of a caging “silencing” compound, and acquire unidirectional control over the morpholino oligonucleotide’s cellular activity.

Minor data is available about successfully caging PMOs. As previously introduced, Chen and colleagues used the tethering of a complementary strand which acted to impede activity and was subsequently removed with precisely controlled wavelength light. This approach, while very effective, is impractical for interlab usage by cause of the complex synthesis demanded.

Herein, we describe a statistical base-caging approach that effectively inhibits morpholino hybridization when the photosensitive labeling compound is coupled. Exposure to near-UV wavelengths of energy abolishes the coupling and restores hybridization activity. The encouragement that this approach offers is ease of synthesis, in that, commercially available PMOs can be inserted into the protocol and successfully altered in a batch style process, as opposed to the complexities offered in synthesis by the Chen group where solid phase synthesis is required for construction of the inhibitory sequence.

1.9 Bioconjugate Techniques – Chemical Coupling to Nucleic Acids

Bioconjugate techniques integrating nucleic acids have become one of the most eminent areas of crosslinking and modification chemistry. Without delving exhaustively into the field of chemistry and structure of nucleic acids and oligonucleotides, highlighted are the critical
functionalities necessary for transition to the appropriated chemical synthesis method employed in this study, namely, the lack of the existing phosphate backbone (found on DNA and absent on the PMO) which lead to alternative propositions.

1.9.1 **Zero Length Cross Linkers**

The smallest available reagent sets for bioconjugation are termed zero length cross linkers, which mediate the combination of two molecules by forming a covalent bond containing no additional atoms.

1.9.2 **Carbodiimides**

Carbodiimides are used to mediate the formation of amide linkages between carboxylates and amines or phosphoramidate linkages between phosphates and amines [Hoare and Koshland, 1966, Chu et al., 1986, Ghosh et al., 1990]. Carbodiimides are seemingly, the most accepted type of zero length cross linking agent in use being efficient in forming conjugates between two proteins, peptides and proteins, oligonucleotides and proteins, biomolecules and functionalized surfaces or particles, or any combination of these with small molecules [Bioconjugate Techniques 2nd Edition – find actual reference]. These compounds can be divided into two subsets: water soluble and water insoluble. For most bioconjugating applications, the water soluble carbodiimides find precedence, because most biological macromolecules are soluble in aqueous buffer solutions. Water insoluble carbodiimides, in contrast, are frequently exploited in peptide synthesis and other affixing reactions involving molecules soluble in organic solvents.

1.9.2.1 **EDC**

EDC (or EDAC; 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) is the most favored water soluble carbodiimide derivative used for establishing covalent bonds between carboxylates and amines. [Figure 1.12]
A variety of chemical conjugates may be formed using EDC, provided one of the molecules contains an amine and the other a carboxylate group [Chu et al., 1976, 1982, Chu and Ueno, 1977, Yamada et al., 1981, Chase et al., 1983]. N–substituted carbodiimides can react with carboxylic acids to form highly reactive, \( o \)-acylisourea intermediates. [Figure 1.13]. The active species can then react with a nucleophile such as a primary amine to form an amide bond [Williams and Ibrahim, 1981].

Figure 1.12 EDC structure

Figure 1.13 – EDC Chemical Synthesis Reaction
1.9.2.2 Sulfo–NHS

Sulfo–NHS may be used in combination with carbodiimides to form active ester functionalities with carboxylate groups. Sulfo–NHS esters are hydrophilic reactive groups that couple rapidly with amines on target molecules [Staros, 1982; Denney and Blobel, 1984; Kotite et al., 1984]. The advantage of adding sulfo–NHS to carbodiimide reactions is to increase the solubility and stability of the active intermediate, which reacts with the attacking amine [Bioconjugate Techniques 2nd Edition]. Sulfo coupled reactions are highly efficient and usually increase the yield of conjugation significantly over that obtained solely with the carbodiimide [Staros et al., 1986].

1.9.2.3 DCC

DCC (dicyclohexyl carbodiimide) is a frequently used coupling agent in organic synthesis reactions, when the water soluble EDC is not applicable. The activation efficiency of DCC is extraordinarily high, especially in anhydrous solutions that do not contain the competing hydrolysis problem. For our applications, the carboxylic acid containing compound is nonsoluble in aqueous solution, so DCC [Figure 1.14] is the choice material due to its compatibility with organic solvents.

![Structure of DCC]

DCC

N,N'-Dicyclohexyl carbodiimide

Figure 1.14 – Structure of DCC
1.10 *In vivo* Zebrafish Model

*In vitro* analyses are critical for any drug characterization scheme, but where the technology really finds applicability and true test of efficacy is in the *in vivo* system. We selected the organism *Danio rerio*, commonly called zebrafish, as our *in vivo* system. The zebrafish was chosen for reasons including ease of maintaining whole populations, its genome is completely sequenced and available, and that it is typically regarded as the model system to investigate fundamental principles of developmental biology and genetics [Lieschke et al., 2007]. However, of particular interest is the optical clarity of this organism throughout its lifetime from embryo to adult. Serving as a model organism, we chose an easily verifiable biological endpoint displayed by this organism, particularly, the nicotine induced behavior response. This behavior response stems from the binding of nicotine to nicotinic acetylcholine receptors (nAChRs) in the developing embryo. Nicotine and acetylcholine are both agonists for this family of receptors and both of these chemicals modulate embryonic motor output in acute exposure paradigms [Thomas et al., 2009]. The nAChRs are ligand–gated pentameric ion channels which have been well–characterized [Purves et al., 2008]. Particular interest is vested in the α2 subunit, as it plays a crucial role in functionality pertaining to nicotine response [Zirger et al., 2003]. The presence of α2 containing receptors allows nicotine to trigger channel opening, allowing sodium, calcium, and potassium flux, leading to rapid cell depolarization, and resulting in the firing of an action potential. The gross effect of acute exposure to nicotine results in increased muscular bend rates in zebrafish embryos which can be readily quantified. Incorporation of α2 splice blocking morpholinos in zebrafish embryos inhibits proper protein production and thus receptor function, resulting in no behavior response to nicotine exposure. Using our caging chemistry, we
synthesize caged α2 splice blocking morpholino oligonucleotides and utilize this biological endpoint for in vivo testing.

Caged morpholinos could provide the means to stop functional protein production in various stages of zebrafish embryonic development, a goal currently unattainable due to the requirement of delivering these antisense agents via microinjection at the 1–2 cell stage. In the case of studying genes such as the nAChRs that are directly involved in developmental processes, knockdown at these early stages could disrupt the biology to the point that endpoint analyses become impossible. A caged morpholino injected at the 1–2 cell stage could remain inactive and be carried through cell divisions such that when photoactivated at later times (and even specific locations, the biological response to gene suppression could be more accurately studied. In this study, we seek to demonstrate such control over commonly used morpholinos by caging them and subsequently photoactivating them to control hybridization and thus gene silencing in developing zebrafish embryos.

1.1 References


CHAPTER 2: SYNTHESIS AND ANALYSIS OF BASE-CAGED MORPHOLINO OLIGONUCLEOTIDES

2.1 Introduction

A remaining challenge to developmental biology is the spatiotemporal study of genes with regard to cell proliferation, migration, and differentiation [Ouyang et al., 2009]. Aside from commonly employed biological approaches, a class of chemical compounds coined “caged” molecules are also used as tools to aid in unveiling the mysteries of cellular activity. These compounds functionally encapsulate biomolecules and render them inactive. The inactivated, or silenced, biomolecules can later be liberated by photomanipulation of a specific wavelength, characteristic to the chromophore, which restores activity and allows perturbation of the targeted bioprocess [Ellis-Davies, 2007]. This externally triggered chemical approach to studying and regulating biological function has quickly garnered research interest due to its invaluable application in both laboratory and potentially, clinical settings. Effective alteration of gene activity at precise times and locations is especially attractive for delineation of gene function in whole organisms, and where caging technology is being increasingly applied. Antisense technology is the study of how these exogenous nucleic acid oligonucleotides alter normal biological function of the organisms with which they interact [Crooke, 2007]. The receptors for these drugs are defined nucleic acid sequences in an intracellular DNA or RNA target. Although antisense molecules are the subject of expansive studies in developmental biology and medicine, inadequate stability and specificity, off-target effects, susceptibility to enzymatic degradation, ineffective delivery to cellular compartments, and unpredictable cellular interactions have previously stymied the antisense field [Summerton et al., 1997]. One of the seemingly most promising members of the second generation family of synthetic oligonucleotides that surmount
these inadequacies is the phosphorodiamidate morpholino oligomers (PMOs) (Figure 2.1), comprised of (dimethylamino) phosphinylideneoxy–linked morpholino backbone moieties. The morpholino moieties contain heterocyclic base recognizing subdivisions of DNA (A, C, G, and T) attached to a substituted morpholine ring system. PMOs offer many beneficial features, including: a high resistance to enzymatic degradation [Hudziak et al., 1996], an RNAse H free pathway [Giles et al., 1993, Stein et al., 1997], reduced off-target gene regulation [Burgess et al., 1993, Hudziak et al., 2000], and an absence of binding affinity to cellular and extracellular proteins [Stein et al., 1993, Stein, 1994, Shoeman et al., 1997].

We demonstrate a unique manipulation of the morpholino oligonucleotide, an entity that is distinctively designed to resist any sort of reaction other than with its targeted complementary nucleic acid, to foster attachment or reaction of a caging “silencing” compound, resulting in unidirectional control over its gene-regulating activity. Caging biomolecules begins with synthesis of the coupling compound, followed by attachment of that caging compound to the biomolecule or molecule of interest. Typically, these bioconjugates are made using synthetic organic chemistry reactions, ranging from single coupling mechanisms to complex multi-step reactions [Ellis-Davies, 2007].

Through widespread use, antisense oligonucleotides have evolved through multiple generations of structural change in an attempt to evade previously listed off target effects encountered in the cellular environment. As the development of these compounds have removed many of the previously utilized constructs, notably the phosphate backbone, new tailored chemistries for targeted attachment of blocking groups to these new constructs have had to be incorporated. Thus far, the most common method of caging mRNAs or DNAs has proven to be the most useful biologically [Ellis-Davis, 2007]. “Direct, multi-site,” “shotgun,” or “statistical”
caging, multiple terms used to describe attachment schemes, of DNA with reactive nitrophenyl–
diazo alkylates, thoroughly binds and inactivates the molecule [Monroe et al., 1999]. The
elegance of the approach of statistical backbone caging clearly lies in its ease and simplicity of
preparation [Mayer and Heckel, 2006]. However, at least with the modifying reactions and
caging chromophores used to date, systematic binary (on/off) response of genetic function has
been unattainable. An alternative to backbone attachment is the introduction of caging groups at
positions within nucleic acids [Mayer and Heckel, 2006], and specifically for our applications,
direct attachment to the bases.

Figure 2.1 – Structure of DNA and Morpholino Oligonucleotides.
We report a statistical base-caging approach that effectively inhibits morpholino hybridization when the photosensitive labeling compound is coupled (Figure 2.2). Exposure to near-UV wavelengths of energy abolishes the attaching covalent bond and restores hybridization activity *in vitro* and in our *in vivo* zebrafish model.

![Base Caged Morpholino Oligonucleotide](image)

**Figure 2.2 – Schematic of PMO Base Caging/Uncaging**

The novelty that this approach offers is ease of synthesis, in that commercially available PMOs can be utilized and successfully altered in a batch style process, as opposed to the complexities offered in solid-phase synthesis methods to cage PMOs (Ouyang et al., 2009). Specifically, using carbodiimide chemistry, we attach a nitrophenyl photolabile compound to exocyclic amines on an intact oligonucleotide (Figure 2.3). This carbodiimide conjugation
scheme capitalizes on reacting a carboxylic acid caged compound with exocyclic amines in an intact oligonucleotide, and thus could be amenable to many different forms of nucleic acids (Figure 2.1). Sampling two structurally distinct oligonucleotides, we demonstrate successful caging of both DNA and PMOs with our novel chemical caging approach. Our synthetic scheme for caged PMOs fulfills the design requirements for caged biomolecules as described above. The use of carbodiimide chemistry for the successful caging of antisense oligonucleotides has relevance to the fields of caged compounds, antisense technology, gene therapy, and developmental biology.

*In vitro* analyses are critical for any drug characterization scheme, but where the technology really finds applicability and true test of efficacy is in the *in vivo* system. We selected the organism *Danio rerio*, commonly called zebrafish, as our *in vivo* system. The zebrafish was chosen for reasons including ease of maintaining whole populations, its genome is completely sequenced and available, and that it is typically regarded as the model system to investigate fundamental principles of developmental biology and genetics [Lieschke et al., 2007]. However, of particular interest is the optical clarity of this organism throughout its lifetime from embryo to adult. Serving as a model organism, we chose an easily verifiable biological endpoint displayed by this organism, particularly, the nicotine induced behavior response. This behavior response stems from the binding of nicotine to nicotinic acetylcholine receptors (nAChRs) in the developing embryo. Nicotine and acetylcholine are both agonists for this family of receptors and both of these chemicals modulate embryonic motor output in acute exposure paradigms [Thomas et al., 2009]. The nAChRs are ligand–gated pentameric ion channels which have been well–characterized [Purves et al., 2008]. Particular interest is vested in the α2 subunit, as it plays a crucial role in functionality pertaining to nicotine response [Zirger et al., 2003]. The presence of
$\alpha_2$ containing receptors allows nicotine to trigger channel opening, allowing sodium, calcium, and potassium flux, leading to rapid cell depolarization, and resulting in the firing of an action potential. The gross effect of acute exposure to nicotine results in increased muscular bend rates in zebrafish embryos which can be readily quantified. Incorporation of $\alpha_2$ splice blocking morpholinos in zebrafish embryos inhibits proper protein production and thus receptor function, resulting in no behavior response to nicotine exposure. Using our caging chemistry, we synthesize caged $\alpha_2$ splice blocking morpholino oligonucleotides and utilize this biological endpoint for in vivo testing.

Caged morpholinos could provide the means to stop functional protein production in various stages of zebrafish embryonic development, a goal currently unattainable due to the requirement of delivering these antisense agents via microinjection at the 1–2 cell stage. In the case of studying genes such as the nAChRs that are directly involved in developmental processes, knockdown at these early stages could disrupt the biology to the point that endpoint analyses become impossible. A caged morpholino injected at the 1–2 cell stage could remain inactive and be carried through cell divisions such that when photoactivated at later times (and even specific locations, the biological response to gene suppression could be more accurately studied. In this study, we seek to demonstrate such control over commonly used morpholinos by caging them and subsequently photoactivating them to control hybridization and thus gene silencing in developing zebrafish embryos.

2.2 Experimental Procedures

2.2.1 General Synthetic Procedures

All reactions were carried out in Seal–Rite® 1.5mL microcentrifuge tubes (USA Scientific, Ocala, FL) at RTP using commercially available reagents without further purification,
unless otherwise stated. Reactions were agitated using a Benchmark BlotBoy™ 3-D Rocker (Spectrum Scientifics, Philadelphia, PA) and monitored by spectroscopic analysis.

Figure 2.3 – Caged Morpholino synthesis using carbodiimide approach
2.2.1.1 DNA/Morpholino Oligonucleotide Conjugation Reaction

50mg of N,N’–Dicyclohexylcarbodiimide (DCC) (Thermo Scientific, Waltham, MA) and 5mg of (4,5–dimethoxy–2–nitrophenyl) acetic acid (DMNPAA) (Alfa Aesar, Ward Hill, MA) were dissolved into 500µL of dimethylformamide (DMF) (Sigma–Aldrich, St. Louis MO) and allowed to react at RTP for 15 minutes. After 15 minutes of reaction, 15mg of N–hydroxysulfosuccinimide (Sulfo–NHS) (Thermo Scientific, Waltham, MA) dissolved in 50µL distilled water was added to the reaction. The mixture was vortexed for a few seconds to ensure dispersion. Following vortexing, it was allowed to react for 15 minutes at RTP. After the second reaction sequence, 30µg of Morpholino oligonucleotide (sequence: 5’– ATG CAA AGT ATC AAC TTA CCA CA T C –3’, Gene–Tools LLC., Philomath, OR) was added to the vessel and allowed to react at RTP for 18 hours in dark conditions.

2.2.2 Caged Oligonucleotide Purification Scheme

Sephadex LH–20 was functionalized using water as the functionalizing agent as described by GE Healthcare, and prepared as a media slurry. The sephadex slurry (4ml) was added to a PD–10 desalting column (GE Healthcare, Waukesha, WI). For a given reaction, 6 1mL fractions were collected from the Sephadex column. Following fractionation, recovered product (verified through UV–Vis spectroscopy) was spin filtered using Amicon centrifugal filters (Amicon Ultra 3K – 0.5mL 3000MWCO Centrifugal Filters) until the eluent showed no absorbance at 355nm.

2.2.3 Spectroscopic Characterization of Native, Caged, and Flashed Oligonucleotides

DNA oligonucleotides, 4,5–dimethoxy–2–nitrophenyl acetic acid (DMNPAA) conjugated “caged” DNA oligonucleotides, morpholino oligonucleotides, and DMNPAA conjugated “caged” morpholino oligonucleotides were dissolved (70ng/µL) in 0.2µm filter
purified distilled water (Synergy – Millipore, Billerica, MA) and scanned from 200nm to 500nm for maximum absorption (ThermoSpectronic Genesys 6 UV–Vis Spectrophotometer). Relative absorbances at 260nm and 355nm were used to determine the average amount of cage moieties per oligonucleotide, similar to the approach used by Ghosn et al. (Ghosn et al., 2005)

### 2.2.4 UVA Irradiation

DMNPAA caged oligonucleotides were exposed to UVA light for 4 minutes in 0.5mL TempAssure PCR tubes (USA Scientific), where the light source output was located 3cm away from the sample. The Greenspot, a 100–watt, mercury lamp with a 5mm x 1000mm light guide, produces a peak spectral output at 365±8 nm (American Ultraviolet, Lebanon, IN) (Forman et al., 2007). The lamp has a fluence of 206 mW/cm² with short bandpass (1.5mm thick, 2.4mm diameter SWP–2502U–400, Lambda Research Optics, CA) and IR filters (818–ST–UV detector, Newport Corporation, Irvine, CA) in place.

### 2.2.5 Oligonucleotide In Vitro Hybridization Assay

Hybridization melting curve assays and gel electrophoresis techniques were employed to demonstrate functional attributes of native, caged, and flashed DNA and morpholino oligonucleotides. The oligonucleotide samples were designated as native sample (unreacted DNA or morpholino oligonucleotide, which was never introduced to the reaction conditions and served as a positive control), caged samples (oligonucleotides containing photolabile blocking group), and flashed sample (oligonucleotides exposed to 365nm light causing photolysis of the blocking group), 1µg of each oligonucleotide sample was hybridized with 1µg of its complementary DNA, along with 15µL of intercalating 100X SYBR Green I (Invitrogen – SYBR Green 1 nucleic acid gel stain; 10000X concentrate in DMSO), 20uL of 10X hybridization buffer (1M NaCl, 100mM NaP, 1mM EDTA, pH 7.0 – 7.5), and distilled water
was also added to the reaction tubes (Bio–Rad CLR PCR tube, Flat Cap Strips) to bring the final volume to 100µL. Triplicate samples were first held at 95°C for 5min in a thermal cycler (Bio–Rad MJ Mini 48–Well Personal Thermal Cycler) and followed by cooling to 5°C over 5min. Oligonucleotide melting curves were generated by measuring the fluorescence of SYBR Green I during a linear temperature transition from 5°C to 95°C at 0.1°C/s.

2.2.6 Oligonucleotide Gel Electrophoresis

For gel electrophoretic analysis, 500ng of morpholino oligonucleotide hybridized to its DNA complement was added to each well of a 15% TBE polyacrylamide gel (Bio–Rad) and run for 90 minutes at 85V. Gels were stained after electrophoresis with 1X SYBR–Gold (Invitrogen – SYBR Gold nucleic acid gel stain; 10000X concentrate in DMSO) in 1% TBE buffer for 15 minutes. Gel images were captured using Quantity One (Bio–Rad – 1–D Analysis Software).

2.2.7 Morpholino Microinjections

Morpholino solutions were prepared immediately prior to the microinjection procedure. A control morpholino (Sequence: 5’–CCT CTT ACC TCA GTT ACA ATT TAT A–3’) was injected at 0.25 mM and α2 splice blocking morpholinos (native, caged, and uncaged in vitro) were injected at 2mM (~17µg/µL). All morpholino solutions were prepared in 0.1% phenol red (Sigma) and 125ng/µL rhodamine-conjugated dextran (Sigma, St. Louis, Missouri, USA) to facilitate confirmation of successful injections. Each solution was loaded into a micropipette needle pulled on a Flaming/Brown P-97 micropipette puller (Sutter Instruments, Novato, CA) using fire-polished borosilicate glass capillaries with an internal filament (outer diameter of 1.2 mm, Sutter Instruments). All solutions were then injected in the yolk stream of 1-2 cell stage zebrafish embryos [Nasevicius et al., 2000]. At ~6 hours post fertilization (hpf), all injected zebrafish embryos were individually screened for fluorescence on a Zeiss Axiovert inverted
microscope equipped with epifluorescence using a 10x objective. Embryos with no fluorescence were removed and were not used for any subsequent experiments. At 12 hpf, half of embryos injected with the caged α2 morpholinos were placed in 3 mL of embryo media (15-20 embryos per petri dish) and were irradiated in vivo with the Green Spot UVA light source, at 24 J/cm² [Dong et al., 2007]. All zebrafish experiments were performed in the presence of minimal light to minimize photolysis of caged compounds. Embryos were raised at 28°C and protected from light until behavioral analysis.

2.2.8 Behavior

Behavioral analysis based on nicotine binding to nAChRs provided a biological endpoint for assessing functionality of the α2 splice block morpholino and caged α2 morpholino oligonucleotides. Embryos were placed in embryo media in 10mm Petri dishes and their baseline spontaneous activity in their chorions in the form of spinal musculature bends was videotaped for 3 minutes using a Kohu video camera mounted to a Zeiss SV6 dissecting microscope. The embryos were quickly transferred into a nicotine solution (60μM) (−)-nicotine, catalog # N3876-5ml, Sigma, St. Louis, Missouri, USA) and the musculature bends were recorded for 1-3 minutes. The motor output was quantified for each individual embryo as the number of bends in a one-minute epoch. Following behavioral examination embryos were placed in fresh embryo media and raised at 28°C. All behavior experiments were carried out at ~25-26°C with minimal light.

2.2.9 Zebrafish Husbandry, IACUC

Wild-type zebrafish purchased from EkkWill Waterlife resources were kept at standard laboratory conditions (28°C on a 14 hr light: 10 hr dark photo-period in a recirculating system).
Embryos were collected from group spawns, and rinsed several times in embryo medium (Westerfield 2000) prior to microinjection procedures.

2.3 Results and Discussion

2.3.1 Absorbance Spectroscopic Characterization of Native, Caged, and Flashed Oligonucleotides

The absorbance spectra of native DNA and morpholino oligonucleotides are shown in Figure 2.4. Spectral similarities result from absorption of the nitrogenous bases with the maximum occurring at 260nm and display no absorption characteristics beyond 300nm. The caging moiety, 4,5–dimethoxy–2–nitrophenyl acetic acid (DMNPAA) displays an absorption peak at 355nm, commonly found in dimethoxy–nitrophenyl cage compounds both independently, and when attached to nucleic acid moieties [Walker et al., 1988].

![Figure 2.4 – UV Vis spectrometry scans. Scans comparing DNA and morpholino absorbance characteristics.](image)

As Figures 2.5 and 2.6 show, attachment of this molecule to the DNA and morpholino oligonucleotides adds a peak beyond 260nm at 355nm. We use UV–Vis absorbance
spectroscopy as a quantitative assay to approximate the amount of attachment that can be achieved through the carbodiimide reaction. By recording the relative peak heights at 260nm, 355nm, and 500nm, percent of attachment can be calculated as previously described [Ghosn et al., 2005]. Interestingly, when comparing this novel base attachment scheme to backbone caging methods, knockdown of hybridization can be achieved with as little as 12 - 16% attachment (corresponding to 3 – 4 caged groups per 25mer oligo) to the required 70 – 80% [Ghosn et al., 2005] (corresponding to 14 – 16 caged groups per 20mer oligo), indicating a more efficient disruption of hybridization by base caging as compared to phosphate caging. Importantly, this assay is used as a quantitative means to determine reaction success.

Figure 2.5 – Spectroscopic Scans comparing Native, Caged, and Flashed DNA. 355nm peak (indicative of the caging compound) comparing native, caged, and flashed DNA samples. Inset shows an enlarged scale to better quantify 355nm absorbances associated with cage attachment and release.
2.3.2 Oligonucleotide In Vitro Hybridization Characterization

When double stranded oligonucleotide complexes are subjected to heating, they slowly dissociate in a series of steps where each step represents a discrete segment, or melting domain. In general, the temperature required to separate a melting domain increases with increasing GC content. The $T_m$ is defined as the melting temperature and is the temperature at which 50% of the oligonucleotide duplex is unhybridized [SantaLucia, J., 1998]. Manipulating melting curve data by taking the negative first derivative generates a curve where the $T_m$ is easily discernable as it produces a well defined peak at the sequence dependent temperature, as shown in the sample labeled Native DNA in Figure 2.7. Caged oligonucleotides can not hybridize to their complementary sequence. Photoexposure liberates the oligonucleotide allowing hybridization to

![Spectroscopic Scans comparing Native, Caged, and Flashed Morpholino.](image)

Figure 2.6 – Spectroscopic Scans comparing Native, Caged, and Flash Morpholino. 355nm peak (indicative of the caging compound) comparing native, caged, and flashed Morpholino samples. Inset shows an enlarged scale to better quantify 355nm absorbances associated with cage attachment and release.
be restored. Figure 2.7 depicts a first derivative melting curve analysis of a native, caged, and flashed DNA oligonucleotide. Normalizing this data based on the maximum signal of the Tm enables the percent knockdown, or efficiency of the caging reaction, to be easily calculated. Caged samples indicate a 42% reduction in activity as compared to the native sample. Exposing the caged DNA to 365±8nm light at 24 J/cm² fully restores hybridization activity of the oligo, indicated by the flashed DNA sample.

![Melting curve data plot of native, caged, and flashed DNA.](image)

Similar to the DNA oligonucleotide counterpart, melting curve analyses were conducted on the morpholino oligonucleotide as shown in Figure 2.8, where the SYBR green fluorophore, which undergoes an increase in quantum yield upon intercalating into dsDNA, was appropriate for the morpholino-DNA heteroduplex. The caged morpholino indicates a 58% reduction in
activity when compared to the native. Flashed samples indicate restoration of hybridization ability.

Figure 2.9 demonstrates the effects of increasing light doses on photorelease. As dosage increases, so too does fluorescence intensity which corresponds to greater hybridization activity. Native and caged samples were used as relative maxima and minima, respectively. Increase in activity occurs as light exposure is intensified from 1 J/cm$^2$ to 3 J/cm$^2$ to 6 J/cm$^2$, followed by a full restoration of activity, equal to that of the native sample, with 24 L/cm$^2$. Of particular interest is the emergence of a peak broadening phenomenon in the flashed samples as compared to the sharp point displayed by the native sample. This may be explained by the heterogeneity of the sample. First, due to the statistical attachment scheme that these oligonucleotides undergo, one may expect a bell curve response explained by varying degrees of caging and differing sites.

Figure 2.8 – Melting curve data plot of native, caged, and flashed morpholino.
of alkylation of the caged conjugate. Furthermore, impurities such as byproducts from the carbodiimide reaction synthesis may be present and partially interfere with duplex formation as compared to completely pure compounds found in the native sample. *In vitro* oligonucleotide melting curve analysis provides a qualitative means of assessing product efficacy. This assay indicates that the blocking group removal, indicated by hybridization restoration is a light induced process.

![Figure 2.9 – Caged morpholino light dose response curves.](image)

### 2.3.3 Electrophoretic Analysis of Hybridization Properties

Gel electrophoretic analysis of synthesized product provides spatial resolution between native hybridized complexes and caged single stranded moieties. Morpholino oligonucleotides are designed with a nonionic backbone to circumvent obstacles in the cellular environment normally faced by alternative antisense molecules, such as short half life and off target effects.
This structural type thus possesses little or no electrophoretic mobility. However, hybridization to its complementary DNA allows enough anionic contribution to pull these molecules through a gel. In this indirect way, gel electrophoresis too can be used as a tool to test efficacy through hybridization of caged morpholino products, similar to the approach of Ghosn et al. for caged DNA [Ghosn et al., 2005].

In Figure 2.10, duplex bands (top, broad bands) have a much larger size and molecular weight to charge ratio. These larger entities can be resolved from single stranded complementary DNA oligonucleotides, (bottom, narrow bands) as they have a much lower charge to weight ratio and can migrate more rapidly through the gel matrix. Single stranded DNA oligonucleotides were used as the control. Figure 2.10 confirms the effects of the caging group to alter hybridization ability, evident by the presence of single stranded DNA oligonucleotide bands in caged morpholino samples and little or no ssDNA band in native and flashed morpholino samples. Hybridization assays (displayed to the right of the gel) confirm the electrophoresis results of these samples. Noted was the decreased intensity of the duplex band and corresponding increase in intensity of the single stranded DNA band which indicated that the hybridization event was blocked by the caging moiety. Hybridization was completely restored in the flashed sample exposed to 24 J/cm² (Lane 5), indicated by the absence of the single stranded complementary DNA. The hybridization assay located at the top right of the Figure 2.10 shows melt curves for samples in lanes 3, 4, and 5. The hybridization melting curves reveal a 45% decrease in fluorescence intensity corresponding to reduced activity in caged vs native morpholino samples.
2.3.4 Zebrafish Behavior Response to Nicotine

Figure 2.11 contrasts the population behavior data between the α2 splice blocking morpholino and a control sequence morpholino that is non–specific to endogenous RNA sequences found in zebrafish. For this assay, the first three minutes are used to develop a baseline behavior for the embryos. At the end of minute 3 (24 hpf), the embryos are quickly transferred to a 60μm nicotine containing solution and behavior is recorded. As these results indicate, the control morpholino injected embryos exhibit a normal nicotine response quantified by the drastic increase in bend rate [Thomas et al., 2009]. However, embryos injected with the α2 splice blocking morpholino do not exhibit the nicotine response due to the absence of functional α2 subunits. This effect indicates that the α2 subunit is necessary in forming a
functional receptor. This also establishes biological endpoints between where caged and flashed samples are expected to operate.

**Figure 2.11 - Nicotine induced behavior response.** At 3 minutes, nicotine was introduced and bend rate was calculated for the control morpholino and α2 morpholino injected population.

Ultimately, UVA light will be used to liberate caged molecules. Thus, we had to determine if UVA doses affected the behavior response. Knowing behavioral endpoints set by the control morpholino and α2 splice blocking morpholino, effects of light on embryonic development and specifically nicotine induced behavior had to be assessed. Embryos were raised to 12 hpf and irradiated for 4 min and 10 min. Based on these times, the doses that the embryos were exposed to 24 J/cm² and 60 J/cm², respectively, from the Greenspot UVA light source. Figure 2.12 displays population data for the nicotine response. At min 5 (26 hpf), control and flashed embryos were exposed to 60µM nicotine and behavior was recorded. Consistent with the control population, photoexposed embryos display the same behavior pattern of increased bend
rate, averaging 5 bends per minute (bpm) compared to 45 bpm, when exposed to nicotine. These data are consistent with prior studies of nicotine–induced behavior [Thomas et al., 2009].

2.3.5 Light Dosing Effects on Behavior Response

Figure 2.13, panel A shows bend rates for individual embryos during sixth minute, the time when nicotine was introduced. Plot order was chosen for increasing bends in order to facilitate trending and threshold determination.

Control embryo bend rates range from 30 to 70 bpm. The nicotine response of the fish exposed to 4min and 10min UVA light was similar to that of the controls, indicating that light has little or no effect on the behavior response. Using a thresholding method to generate a bar graph, this data can be easily visualized. The embryo with the lowest number of bends from the control group in Figure 2.13 panel A was used to set a minimum threshold. All fish having bend rates are above this threshold are counted and the individuals that lie below the threshold are

Figure 2.12 – Embryonic population plot of behavior response to nicotine exposure. At minute 5, embryos are introduced to 60µM nicotine solution and immediately display increased bend rates.

2.3.5 Light Dosing Effects on Behavior Response

Figure 2.13, panel A shows bend rates for individual embryos during sixth minute, the time when nicotine was introduced. Plot order was chosen for increasing bends in order to facilitate trending and threshold determination.

Control embryo bend rates range from 30 to 70 bpm. The nicotine response of the fish exposed to 4min and 10min UVA light was similar to that of the controls, indicating that light has little or no effect on the behavior response. Using a thresholding method to generate a bar graph, this data can be easily visualized. The embryo with the lowest number of bends from the control group in Figure 2.13 panel A was used to set a minimum threshold. All fish having bend rates are above this threshold are counted and the individuals that lie below the threshold are
excluded as shown Figure 2.13 panel B. Similar techniques of analysis and visualization are common in the zebrafish community (Ouyang et al., 2009) and allow for easy discernment of data. All subsequent data is presented in this way and accompanied by individual behavior response.

**Figure 2.13 – Individual Embryo Thresholding.** Panel A – individual bend rates in ascending order for establishment of thresholding analysis method; Panel B – Using the thresholding analysis technique, this is the bar graph generated by scoring embryos above threshold.
Figure 2.13, Panel A shows bend rates for individual embryos when nicotine was introduced. Plot order was chosen for increasing bends in order to allow easy visualization of the data set.

2.3.6 *In vivo* Experimentation – Injecting Caged Morpholino Oligonucleotides into Zebrafish

Rhodamine dextran was injected with all morpholino samples and used as a marker to determine injection success, which was determined by a confluent distribution throughout the embryo blastoderm as shown in Figure 2.14.

![Figure 2.14 – Injection of α2 splice block morpholino with rhodamine dextran tracker](image)

Initially, verification that the synthesized cage morpholino was participating similarly to the non–mRNA specific control morpholino was required. *In vivo* behavioral studies were initially conducted to assess this phenomenon prior to flashing. As shown in Figure 2.15, we verified that the caged morpholino and the control morpholino participate *in vivo* similarly, indicating that the photolabile blocking groups are inhibiting the activity of the α2 splice blocking morpholino sequence.
Figure 2.15 – *In vivo* behavioral assay assessment prior to flashing. Panel A) shows individual bend rate behavior. Panel B) shows the bar graph of the data after the thresholding method of scoring is incorporated. As the figure shows, the α2 cage and control morpholino elicit similar behavior responses as compared to the active α2 splice blocking morpholino.
For *in vivo* experiments, control morpholino sequence, native α2 splice blocking sequence provided by Gene Tools, synthesized base caged morpholino α2, and *in vitro* flashed (24J/cm² 365±8nm UVA light source) base caged α2 sequence were injected into the embryo yolk at the 1–2 cell stage, the common time point for morpholino introduction [Nasevicius et al., 2000] and imaged 6–7 hpf to determine injection success (Figure 2.14) using rhodamine dextran to track the injected material’s location. The sample number of embryos injected with the base caged morpholino oligonucleotides was doubled so that half of that subpopulation could be flashed *in vivo*. At 24 hpf, injected embryos were analyzed using the techniques previously detailed for UVA effects on bend rate. Figure 2.16, Panel A shows individual bend rate data for each morpholino injected group. Using the embryo with the lowest bend rate of the control group, a threshold is set at 38 bpm. All fish above this threshold are counted for the sample set where all below the set threshold are excluded from the data set depicted in panel B. Panel B shows that α2 injected embryos have a marked decrease in individuals having bend rates above the set threshold, again indicating the absence of functional nicotine binding receptors in those embryos. Embryos injected with caged α2 MO display similar characteristics to that of the control sample population, indicating that the action of the α2 splice blocking morpholino was stopped or reduced. Half of the embryos designated as “α2 cage” were removed from that set and exposed to 24J/cm² UVA light (365±8nm). These embryos were designated as “uncaged *in vivo*” and as the graph shows, have a reduced number of bend rates when compared to the caged subpopulation. This indicates a restoration of activity of the splice block morpholino due to the removal of photolabile blocking groups. The *in vitro* flashed sample set includes embryos injected with caged morpholino that was exposed to 24J/cm² light prior to injection.
Figure 2.16 - Individual Embryo Thresholding. Panel A – individual bend rates in ascending order for establishment of thresholding analysis method; Panel B – Using the thresholding analysis technique, this is the bar graph generated by scoring embryos above threshold. Inset shows corresponding *in vitro* melting curve analysis.
The phenomenon of not being able to achieve efficient *in vitro* uncaging as compared to the *in vivo* uncaging was often observed throughout experimentation. This was thought to be contributed to by the concentration of the caged sample prior to and post injection, which has been shown to affect uncaging efficiencies [Ellis – Davies, 2007]. The inset on panel B is corresponding hybridization data. Interestingly, as the melting curve assay indicated, the *in vitro* flashed sample never regained hybridization activity. This chemical assay predicted biological function which translated to observable behavior.

### 2.4 Conclusions

We have shown a novel reaction scheme to cage exocyclic primary amines on intact DNA and morpholino oligonucleotides using a statistical attachment strategy that could be applied to any antisense oligonucleotide. Both *in vitro* and *in vivo* assays confirm the disruption of hybridization in the caged form, which can be recovered with light exposure. Using zebrafish nicotine induced behavior response for *in vivo* validation of the presence of α2 subunits located on nAChR pentameters, rendering a diminished response to nicotine treatment. Caged splice blocking morpholinos could not hybridize to α2 mRNA because of the steric inhibition introduced by the presence of photolabile blocking groups. This enabled fish injected with the caged morpholino to properly synthesize the α2 protein and undergo normal nicotine response. Having these two endpoints to operate with, we tested the functionality of our caged oligonucleotide. Utilizing the full potential of the caging technology, spatial and temporal activation of the morpholino’s splice blocking ability was within our control. At 12 hpf, we have shown the ability to effectively liberate the morpholino and stop α2 production, confirmed by the behavior assay. Normal efficacy of morpholino oligonucleotides last 3 days post injection [Sumanas et al., 2002]. Using delayed time points for photoactivation, it may be possible to
extend the half–life of the morpholino beyond what has been observed. Furthermore, we were able to accurately predict \textit{in vivo} efficacy based on the results obtained from \textit{in vitro} experimentation.

This is the first time that statistical caging has been actualized on the morpholino oligonucleotide through direct attachment to the bases. Initial attempts using DMNPE caging strategies developed for phosphodiester backbone nucleic acids proved unsuccessful due to the lack of available binding sites on the morpholino [Monroe et al., 1999, Blidner et al., 2008]. Additionally, pertaining to the morpholino, this effort was initially designed to make the moiety more electrophilic, but being incapable of this, ultimately was trying to force nucleophilic/nucleophilic interaction.

This interdisciplinary study combined applications from biological chemistry, biological engineering, rational drug design, and developmental biology. Based on results obtained through chemical analysis, we can accurately and reliably predict biological function in whole organisms through the use of a reliable and reproducible behavior assay. This investigation should act to expand caged morpholino oligonucleotide technologies, and more generally antisense technologies as a whole due to the ease of synthesis required in caging these compounds, as well as to facilitate further understanding of the molecular mechanisms governing embryonic development.

2.5 References


CHAPTER 3: CONCLUSIONS AND FUTURE DIRECTIONS

3.1 Conclusions

In this study, through direct alkylation of exocyclic amines on intact oligonucleotides, we have successfully devised a method for synthesis of antisense oligonucleotides. Specifically DNA antisense oligonucleotides and morpholino antisense oligonucleotides but, presumably any available structure could be inserted into the reaction scheme and successfully caged. The caged compounds that we synthesized were characterized by both in vitro and in vivo methods. Absorbance spectroscopy served as a first quantitative check for reaction success. Comparing relative absorbance values from 260nm, 355nm, and 500nm, caging efficiency was calculated. Once this was done, qualitative means of analysis was performed in the form of hybridization assays including melting curve analysis and electrophoretic gel mobility. Upon completion of these methods, we were confident in the product which allowed a transition to in vivo validation. Performed in the zebrafish model, a well characterized behavior response was used to assess the efficacy of the caged product. Behavior provides an exceptional means of genetic manipulation due to the fact that whole organisms are preserved and can be used to further confirm results in other assays. This interdisciplinary study combined applications from biological chemistry, biological engineering, rational drug design, and developmental biology. Based on results obtained through chemical analysis, we can accurately and reliably predict biological function in whole organisms through the use of a reliable and reproducible behavior assay. This investigation should act to expand caged morpholino oligonucleotide technologies, and more generally antisense technologies as a whole due to the ease of synthesis required in caging these compounds, as well as gain an understanding of the molecular mechanisms governing embryonic development.
3.2 Future Directions

Future directions of this work include the following:

- HPLC purification and analysis of caged products is a critical method of cleanup for synthesis reactions. The current purification scheme is rather intense. Incorporation of HPLC to not only purify, but separate caged products from unreacted species would aid in reducing variation in both in vitro and in vivo results.

- We only touched the surface of mass spectrometry analysis on the caged morpholino products. Considering MALDI and ESI as possible routes of validation, we tested both methods. MALDI proved to be unable to process samples in their current state. We hypothesized that this was due to the inability of the caged morpholino to crystallize on the matrix and that the caging reaction was somehow changing the chemistry of the oligonucleotide. ESI proved feasible but the signal was noisy, reconfirming the need to efficiently purify these products through HPLC analysis.

- A 9-ethylguanine conjugation reaction is essential to confirm that the caging compound is attaching to the exocyclic amine on this compound. However, due to the fact that the current purification scheme requires species with MW greater than 3000, it was not plausible at the time.

- NMR analysis of purified products would also confirm location of the caging group along the oligonucleotide.

- Also important is quantification of the lifetime efficacy of the morpholinos in vivo. It is currently known that morpholinos are active up to 3 days post-injection in zebrafish. It will be important to assess if the caging moieties extend the lifetime of morpholinos in in vivo environments.
• Because the α2 morpholino acts through splice blocking, RT-PCR would compliment behavioral analysis by confirming the mRNA splicing changes. This would be a final endpoint following nicotine-induced behavioral response assay.

• Similar to RT-PCR, immunohistochemistry would compliment results obtained via behavioral analysis, via antibody-antigen interactions on α2 receptors. Using fluorescent microscopy imaging techniques, we would confirm observations from in vivo studies. Both RT-PCR and immunohistochemical staining could analyze single fish to further confirm results.
VITA

Chad Michael Jarreau, son of Tim and Peggy Miller, was born in February, 1984, in Baton Rouge, Louisiana. He graduated from False River Academy in 2002. In May, 2007, he earned his Bachelor of Science in Biological and Agricultural Engineering degree from Louisiana State University from Baton Rouge, Louisiana. He plans to obtain his Master of Science in Biological and Agricultural Engineering degree in May 2010 under the direction of his primary advisor Dr. Todd Monroe, and committee members Dr. Kurt Svoboda and Dr. Marybeth Lima.