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SITE-SPECIFIC DEOXYRIBONUCLEIC ACID MODIFICATIONS FOR USE WITH CHEMICAL CAGES

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Site-Specific Deoxyribonucleic Acid Modifications for Use with Chemical Cages

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Contents

Abstract

The RNAi process has great potential as an engineering mechanism for genetic control. It provides a highly specific platform for knocking down target genes with substantial efficiencies. However, many potential applications of the process may require greater precision, either temporally (proteomics), spatially (therapeutics) or both. Photocage molecules offer a method of such spatiotemporal control, as they render target molecules (such as siRNAs) biologically inert until ultraviolet irradiation, at which point such targets should regain near-native activity. The caging process is one which has been forced to strike a balance between the degree of inactivation and the potential to regain activity at biologically acceptable levels of ultraviolet light intensity. In this work, the convertible nucleotide approach to site-specific caging is explored, whereby strands of nucleic acids are modified with convertible nucleotides at predetermined strategic locations. These convertible molecules can then undergo an induced reaction to place a desired molecule at those chosen sites; in this case, an aminated photocage molecule is attempted to be ligated to strands at the predetermined sites of convertible nucleotides. Such a reaction would expedite the development of photocage chemistry by providing a means for determining and utilizing the optimal location of a single cage compound or cage duo, thereby minimizing the amount of light required for reactivation. A Texas Red Cadaverine dye is first characterized using an EDC reaction at oligodeoxynucleotide 5'-phosphates, and then the dye is inserted with the convertible nucleotide chemistry at nucleotides 6 and 13 (from 5' antisense terminus) of ISIS 2302. Following this, the photocage molecule nitrobenzyl amine is inserted at sites 6 and 13.

Chapter 1: Introduction

The newly discovered process of Ribonucleic Acid (RNA) interference (RNAi) has immense possibilities for the control of destructive genes. When double-stranded RNA is injected into a cell with a homologous genetic sequence, the expression of the gene coded by the sequence is silenced (Fire, Xu et al. 1998). The process is sequence-specific, so one can easily control which genes are silenced (Paddison, Caudy et al. 2002). This has enormous implications, as it offers the ability to effectively turn on or off an organism's expression of certain traits. As the process is in fact a natural mechanism, the utilization of RNAi as a new therapy would be minimally intrusive (Volpe, Kidner et al. 2002). Since the discovery of RNAi in the late 1990's, interest in its potential has grown exponentially, and the Nobel Prize committee recognized its promise of revolutionary medicines by awarding Fire and Mello a 2006 Nobel Prize (Fire, Xu et al. 1998).

If the science community determines which genes are responsible for a given disease, they could use RNAi to limit the expression of those genes. RNAi is believed to have evolved from a viral protection mechanism, so protection from HIV and other viruses is a strong possibility, as well (Carthew 2001). In general, all diseases with living components, whether they are a part of the diseased organism or an infectious organism, could be subject to control by RNAi techniques as they are developed. However, RNAi is an extremely new mode of genetic control. Accordingly, there is much that remains unknown about the intricacies of this phenomenon. As this dearth of information about trends and attributes still exists, long-term capabilities and effects, and the precise details of involved molecules and mechanisms, have a vast degree of uncertainty. Thus both quantification and qualification remain before RNA interference becomes available for therapeutic uses.

One method of studying and controlling the RNAi process is through cage chemistry, which inactivates targets through prohibitive binding. When bound, cage molecules prevent the target from interacting with enzymes, ligands, or other necessary chemistries. When the cages are released, however, the target – for example, an oligonucleotide – is restored to full functionality (Figure 1). Photolabile cage molecules have been developed which can be affixed to oligonucleotides, inactivating them, and then released – "photocages." These have been shown to work with oligonucleotides used in the RNAi process, inactivating the process for a gene sequence and reactivating RNAi upon photo-release (Richard A. Blidner 2007). At the moment, little is known about where on a deoxyribonucleic acid (DNA) or RNA strand a photocage binds; the approach has been to simply bombard nucleic acids with photocage until noticeable inactivation is observed – "statistical" caging. This wastes expensive chemicals, as the vast majority of the cages are unused, and makes the light activation process inefficient, as a number of cage bonds must be lysed.

The ability to attach a photocage compound to a specific, pre-determined site on an oligonucleotide could drastically increase the efficiency and efficacy of both caging and photolysis reactions. Sites – individual nucleotides along a strand – could be chosen which are most effective at preventing RNAi when caged (Figure 2). If inactivation can be induced by

Figure 1 - Caged siRNA is inactive until exposed to light, enabling initiation of targeted gene silencing.

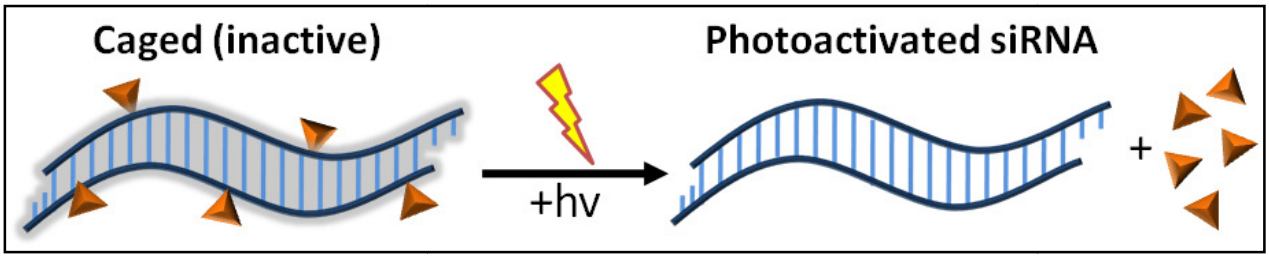
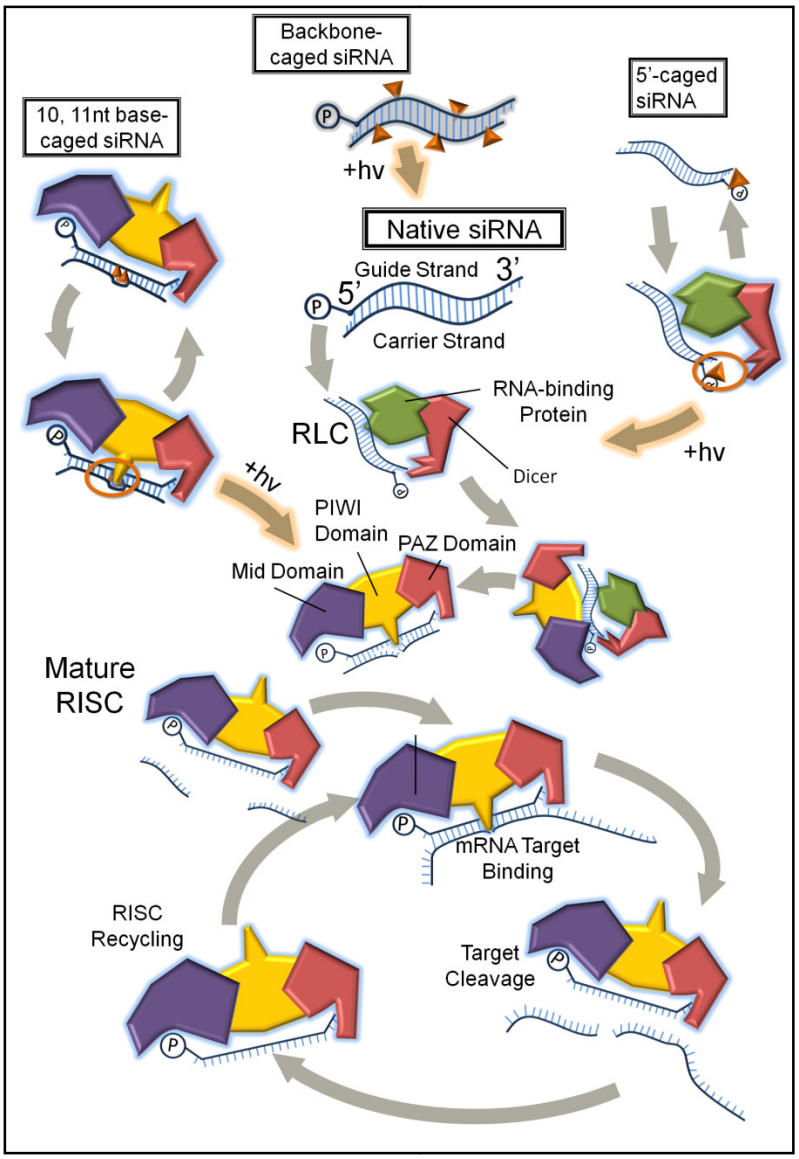


Figure 2 - Overview of caged siRNA processing in RNAi. Boxed labels refer to caged siRNAs in various forms that may be used to control gene silencing with light exposure, and orange ovals indicate inhibition of processing by a cage compound. a.) Caging central nucleotides of the dsRNA complex can prevent cleavage and removal of the carrier strand; irradiation ("+hv") will allow carrier strand removal and RISC maturation. b.) Statistically backbone-caged dsRNAs are believed to be inert, while irradiation will restore caged siRNA to its native state and allow RNAi processing. c.) 5'-phosphate caged siRNAs are believed to be prevented from interacting with the RISC-Loading Complex (RLC), and irradiation will restore the siRNA to its native state. Irradiated caged samples will continue through the RNAi process to target cleavage and be recycled as native samples.



merely two well-placed cages, the photolysis reaction will restore a greater proportion of the caged oligonucleotides.

Objectives

The key aim of the project is to optimize photocage chemistry with oligodeoxynucleotides, using short strands of DNA as a model for siRNAs. The investigator attempts to site-specifically attach photocage groups to critical locations on a 20nt DNA strand. More specifically, objectives are: a.) to verifiably attach the photocage compounds to a oligodeoxynucleotide (ODN); b.) to develop an assay with which to separate caged

oligonucleotides from those unsuccessfully caged; c.) to characterize the caged oligonucleotide in terms of its ability to hybridize with complimentary strands and the efficiency with which the cage compounds can be removed with ultraviolet (UV) light; d.) to cage, separate, and characterize a homologous strand of RNA; and e.) to transfect mammalian cell cultures with the caged species of siRNA and evaluate the inactivation of RNAi and subsequent knockdown of gene expression.

Chapter 2: Background

RNA Interference

The science community first recognized the RNAi concept in eukaryotes in a 1998 paper by Fire, *et al* (Fire, Xu et al. 1998). Unusual activity following the use transposons or alien genes as well as an interesting mobility of silencing across plant grafts foreshadowed this key paper. The paper itself was extremely important because it was the first to show a significant silencing effect following the insertion of double-stranded RNA into a cell, and also that the effects spread both to subsequent cellular generations and rapidly to contiguous cells. In 1999, scientists Cogoni and Macino determined that fungal quelling (RNAi in fungi) needed a specific gene which codes for a protein complex to fabricate dsRNA in the RNAi process (Cogoni and Macino 1999). This protein complex, RNA-dependent RNA Polymerase (RdRP), is now known to be a part of nearly all organisms' processes of RNAi. Also in 1999, Voinnet et al. recognized that the functioning piece of RNA was a twenty-five-nucleotide long antisense strand (Voinnet, Pinto et al. 1999). In 2001, Bernstein et al. published a paper which asserted the existence of a protein they called "Dicer" which cleaved RNA strands into these small pieces (Bernstein, Caudy

et al. 2001). Many of these studies were replicated to show parallel processes in plants, animals, and fungi (Galun 2003).

RNAi can be either triggered naturally by an overabundance of the RNA sequence in a cell or induced by transfecting the cell with the appropriate transgenetic RNA. The process therefore is a reaction to a significant presence of RNA material analogous to a sequence within an organism's genome. Accordingly, it likely developed evolutionarily as a method to prevent the over-expression of potentially hazardous genes with mercurial expression (Balbas and Argelia 2004). To induce RNA interference by introducing the relevant dsRNA sequences to the cell, scientists can use a variety of methods. Injection with micro-needles is a standard, traditional mode of accomplishing this. Also, electroporation, in which electric currents running through a solution open pores in the cell's membrane, is a common way of transfecting the cells (Gibson and Muse 2004). The RNA molecules flow into the cell through the membrane's openings. Additionally, scientists can transfect cells by attaching the RNA molecules to lipid complexes which are then taken in by the cell. A final, and significant, means for transfecting the cells is bacterial transgenesis (Gibson and Muse 2004). When the RNA sequences are inserted into a bacterial cell that already has such sequences in its genome, that bacteria will not only exhibit the RNAi phenomenon but also will pass it on to any organisms it lives off of. Accordingly, scientists can transfect bacteria with RNA molecules and then infect an organism with the bacteria in order to induce the RNAi process (Galun 2003).

The post-transcriptional aspect of RNAi is significant for characterization and utilization of the process. Usually, the control of gene expression is divided into two categories – transcriptional

and post-transcriptional. Transcriptional control prevents the appropriate nucleic acid sequence from ever being transcribed into a messenger RNA (mRNA) sequence, the first step in expressing a gene. This includes a variety of processes, like spatial storage of DNA such that the nucleotides are not exposed to the appropriate transcriptional proteins. Post-transcriptional control impedes the process after the sequences have been thus transcribed, stopping the mRNA from reaching the transfer RNA (tRNA) which would otherwise begin converting the sequence information into proteins (Campbell and Reece 2002).

When initiated, PTGS essentially becomes a cycle of protein complexes within a cell. Once dsRNA is present in significant amounts in the cell, the protein Dicer begins dividing, or "dicing," the long strands into short interfering RNA, or siRNA, which are usually twenty-one to twenty-six base pairs long and are characterized by two-nucleotide long overhangs on each side (Gupta and Raghav 2004). siRNA then binds to another complex and forms RNA-Induced Silencing Complex, or RISC – sometimes called "Slicer," as well. The sense side of the double helix is then removed, leaving the nucleotides from the antisense strand exposed. This macromolecule will bind to a nucleotide sequence complimentary to that bound to RISC, i.e. the mRNA sense strand for the gene. Slicer then cleaves the mRNA strand at the center of the sequence bound to it. This effectively silences the gene's expression, as it cannot be transcribed if it is no longer continuous. However, after Slicer cleaves the mRNA, nucleases recognize the split strands and further degrade them (Nature 2005). It is important to remember that mRNA produced from an anti-sense DNA strand is being degraded, not the original DNA itself.

This process is not only cyclical, but also possesses the appropriate infrastructure to facilitate amplification. If an RNA-dependent RNA Polymerase molecule binds to the cleaved sections of mRNA before nucleases degrade it, the RdRP will construct a dsRNA molecule out of it (2003). This dsRNA would then be diced into siRNA. These siRNA molecules can be once more divided into sense and antisense strands, and can serve as primers for an RNA polymerase: if they find a complimentary sequence, the siRNA will bind to it and will be recognized by this protein. The complex subsequently proceeds down the RNA strand, creating the appropriate complimentary nucleotides to append to the siRNA. Once more, the long strand of dsRNA is cleaved by Dicer. This time, the siRNA can either form RISC molecules or serve once more as primers for RNA polymerase (Nature 2005). The process is further amplified by the mobility of RNAi effects; by means as yet unknown PTGS spreads not only from cell to cell but can even spread from bacteria to host (Galun 2003).

Photocage Chemistry

Photolabile “cage” compounds are inactivating molecules which release from that which they inactivate in response to light. They were first utilized and defined by Kaplan, Forbush, and Hoffman in 1978, when they synthesized nitrobenzyl-caged adenosine triphosphate (ATP) and analyzed its interaction before and after photolysis with ATPases (Kaplan, Forbush et al. 1978). Generally, cage compounds should be stable, react with wavelengths greater than 300nm and in high yields, and photorelease at a faster rate than subsequent reactions with the uncaged molecule. Such compounds allow spatiotemporal control of biological activity via light-initiated chain reactions (Mayer and Heckel 2006).

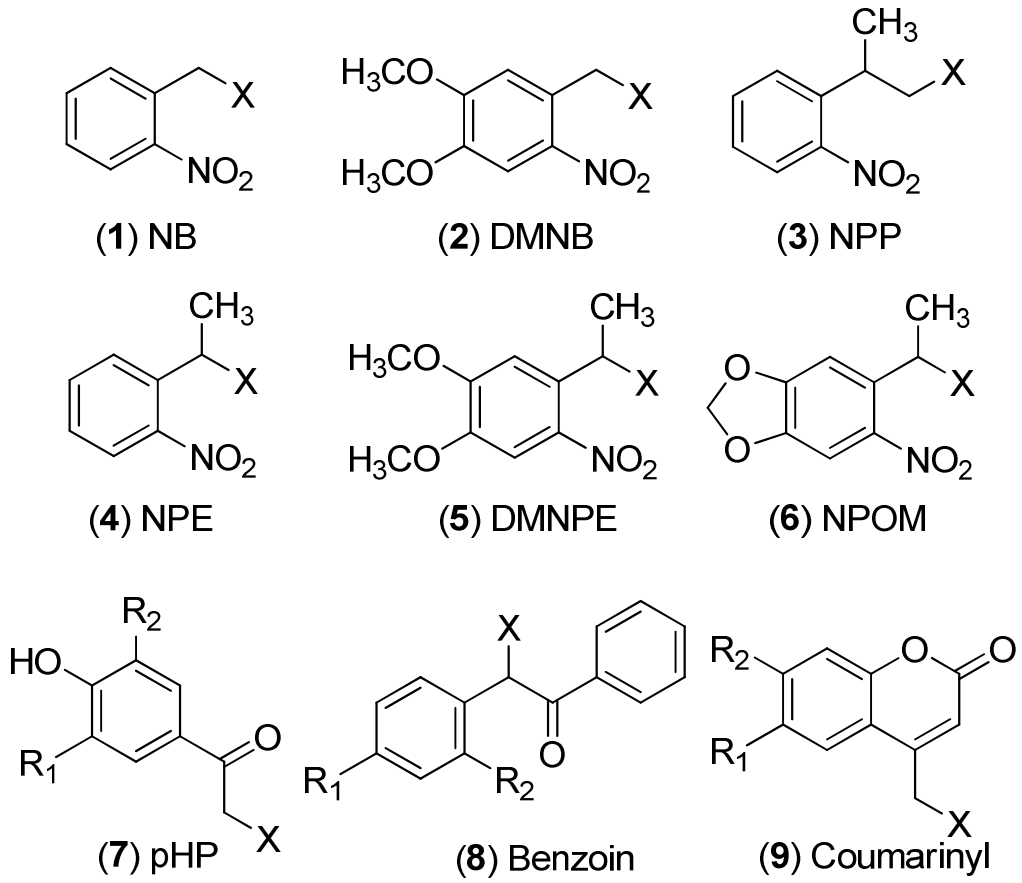


Figure 3- Common cage compounds amenable for use with siRNAs (also see Table 1). NB (nitrobenzyl), DMNB (dimethoxy-nitrobenzyl), NPP nitrophenylpropyl, NPE (nitrophenylethyl), DMNPE (dimethoxy-nitrophenyl-ethyl), NPOM (6-nitropiperonyloxymethyl), pHP (p-hydroxyp henacyl), benzoin, and coumarinyl.

Since Hoffman's original phosphate-caged ATP work, a variety of cage compounds have been developed and utilized (Table 1). However, the 2-nitrobenzyl moiety remains the most common, and most others are variants of nitrobenzyl or nitrophenyl groups (Figure 3). The NB group has a 6-carbon aromatic ring with a nitrate at the 2' position and a single methyl linkage to the effector molecule. This structure absorbs maximally at ~340 nm, with an extinction coefficient ε of 430 and quantum yield of 0.63 when complexed to ATP (Kaplan 1978, Momotake 2006). While the *ortho*-nitrobenzyl (NB) group generates potentially toxic molecules upon photolysis, nitrophenylethyl (NPE) groups react more quickly and produce less harmful products (Mayer

and Heckel 2006), and can also be modified to optimize the deprotection wavelength (Adams, Kao et al. 1989). These molecules have an additional methyl group stemming from the carbon linking the effector molecule to the nitrobenzyl ring. At 320 nm, ligated to ATP, NPE's extinction coefficient is 9100 and its quantum yield 0.63 (Walker, Reid 1988). The cage utilized for this study to inactivate ODNs is 1-(4,5-dimethoxy-2-nitrophenyl)ethyl (DMNPE). This compound is an NPE with methoxy groups opposite the nitrobenzyl ring from the effector molecule and nitro group. DMNPE's extinction coefficient when ligated to ATP, 4795 $M^{-1}cm^{-1}$ with 365 nm light, in combination with its quantum yield, 0.07, indicate a relatively efficient photorelease mechanism (Ghosn, Haselton, et al. 2005). Furthermore, its blue-shifted 365 nm absorption peak qualifies it as a suitable compound for use in biological studies.

Table 1 - Photonic characteristics of common cage compound candidates for use with siRNAs. Absorption spectrum maximum, extinction coefficient, and quantum yield of commonly used cages (BHC – 6-bromo-7-hydroxycoumarin-4-ylmethyl).

Cage(Reference)	λ_{max} (nm)	ε (1/M*cm)	Φ	Effector
NB(Kaplan, Forbush et al. 1978)	350	430	0.63	ATP
DMNB[24]	350	5000	0.07	ATP
NPP(Woll, Walbert et al. 2004)	366	247	0.3	Thymidine
NPE[27]	320	9100	0.63	ATP
DMNPE[27]	365	4795	0.07	ATP
NPOM(Lusic, Young et al. 2007)	356	6887	0.094	DNA
pHP(Givens, Weber et al. 1998)	286	14600	0.3	ATP
Benzoin(Givens, Athey et al. 1993)	350	-	0.39	cAMP
BHC(Furuta, Wang et al. 1999)	368	17470	0.019	Glutamate

More recent cage compounds have been developed with other advantageous properties – nitrodibenzofuran (NDBF) has a high extinction coefficient (18,400 $M^{-1}cm^{-1}$, as compared to ~5000 $M^{-1}cm^{-1}$ for NPEs) and quantum yield (Momotake, Lindegger et al. 2006), and 2-(2-nitrophenyl)propyl (NPP) groups produce highly innocuous nitrostyril groups upon photolysis (Mikat and Heckel 2007). Other cages used in recent studies are based on coumarin compounds, with adjacent six-member rings(Ando, Furuta 2004) (Mayer and Heckel 2006). However, many of these molecules are either insufficiently characterized, photolytically inefficient, or not widely available.

Caged Oligonucleotides

Examples of caged nucleic acids are much less abundant and more recent when compared to other categories of caged compounds, such as small molecule metabolites or proteins. This is surprising, given that the original model for caged biomolecules was based on nucleotides such as caged-ATP(Kaplan, Forbush et al. 1978). Additionally, the potential utility of caging NAs is readily apparent since they participate in a variety of biochemical reactions that are inherently dependent on structure. Caging has been used for microarray oligonucleotide synthesis(McGall, Labadie et al. 1996) and as an alternative nucleobase protection for solid-phase synthesis(Alvarez, Vasseur et al. 1999). This technique has also been used to control the self-annealing of a hairpin(Ordoukhanian and Taylor 1995) and the hybridization of an oligonucleotide to its complement(Ghosn, Haselton et al. 2005). Caging compounds have also been employed by Meldrum *et al*. to study DNA repair kinetics(Meldrum, Chittock et al. 1998). This study was an extension of their earlier work in which radiolabeled caged-ATP was used to study the same phenomenon(Meldrum, Shall et al. 1990).

Although earlier examples of caged nucleic acids exist, the first study in which a caged nucleic acid was used *in vitro* or *in vivo* for control of gene expression did not occur until 1999(Monroe, McQuain et al. 1999). In this work, plasmids encoding Green Fluorescent Protein (GFP) or luciferase were caged with DMNPE-groups in bulk, based on diazo attachment to nucleic acid backbone phosphates. Using this strategy, the authors were able to demonstrate photo-induced transgene expression. Despite this three-decade gap from caged ATP to caged DNA use in biological systems, this study ignited interest in the field by demonstrating the utility of caging nucleic acids to control gene expression. Since that time there have been two classifications of caged nucleic acid molecules: those that were caged using this random method, and more recent site-specific approaches to dictate the exact location of the cage molecule on the effector; both of these categories are detailed below.

Statistical Backbone Caging of siRNAs

Monroe *et al*. used a batch-style reaction to achieve random attachment of DMNPE to the phosphate backbone based on diazo attachment chemistry developed by Walker et al.(Walker, Reid et al. 1988; Monroe, McQuain et al. 1999). A hydrazone precursor to DMNPE can be oxidized to react with phosphates, sulfates or carboyxlates on effectors, so the likely target on nucleic acids is the phosphate backbone. This approach was later adopted to cage mRNA and plasmid DNA using a derivatized coumarin caging group(Ando, Furuta et al. 2001; Ando, Furuta et al. 2004). The strategy of bombardment with excess cage has since been termed “statistical caging” by Mayer and Heckel(Mayer and Heckel 2006). Through statistical BHC caging, Ando *et al*. were able to control transcription of GFP and eng2a mRNA in a zebrafish model, inducing spatially constrained fluorescence and inhibition of eye development, respectively. The BHC-

caged mRNA appeared to exhibit longer lifetimes than uncaged mRNA i*n vivo*. This could be the result of a conformational change that prevented nuclease access, as caged mRNAs exhibited substantially different electrophoretic mobility than native sequences.

The first report of caged siRNAs came from Friedman's group, who used a similar scheme of diazo attachment of DMNPE to double-stranded siRNAs for silencing of transient GFP expression in cell cultures(Shah, Rangarajan et al. 2005). In this study, comparison of uncaged siRNA targeting GFP versus expression level of co-transfected RFP showed moderate photo-control of GFP expression with light exposure. They were able to reduce GFP fluorescence with photolysis of the caged siRNA by an amount equaling about 80% of that of native siRNA, with 35% residual knockdown activity in non-irradiated caged samples ("leakage" activity).

The stability of these RNAs contrasts with other published results(Blidner 2008), in which caging of double-stranded RNA (dsRNA) resulted in fragmentation thereof, presumably through a 2'-hydroxyl attack at the phosphotriester(Breslow and Xu 1993). However, the conflicting results regarding backbone-caged dsRNA stability are likely due to differences of scale: in Shah and Friedman's work, for example, an average of only 1.4 cage groups per double-stranded siRNA (42 nucleotides in total) was used. Presumably, lesser adduct present resulted in a decreased chance of hydrolysis of these smaller RNA species. Additionally, with an average of one cage compound per siRNA, the cage could have frequently been bound to the terminal phosphate of the oligonucleotide, precluding hydrolysis activity. When Shah attempted to decrease leakage GFP expression by increasing the percentage of nucleotides caged to 4.8%, 10.8%, and 15.2%, higher proportions of cage compound severely reduced the amount of activity recovered after

irradiation(Shah, Rangarajan et al. 2005). While the group attributed this phenomenon largely to incomplete photolysis, hydrolysis of the siRNA species could have been a significant factor as well.

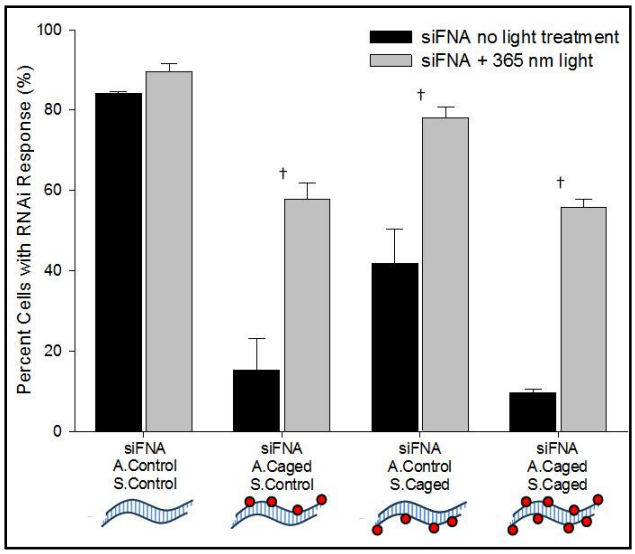
Caging of 2'-Protected Oligonucleotides

One potential means to prevent phosphotriester hydrolysis of caged RNAs is to replace the catalytic 2'-hydroxyl with a stabilizing modification. In a related effort to increase the efficiency of siRNAs through prolongation of lifetimes following delivery, chemically modified nucleic acids have been explored for their ability to mimic the activity of small RNAs through the RNAi pathway. To be practical candidates for RNAi caging studies, these chemical modifications should: 1) be incorporated at enough nucleotide locations to protect the RNA oligonucleotide, 2) demonstrate similar silencing activity relative to the analogous small RNAs, and 3) exhibit limited toxicity. Chiu and Rana completed a particularly thorough investigation of which RNA modifications can be tolerated by the RNAi pathway(Chiu and Rana 2002). Evaluation of the 2' modifications such as O-methyls, locked nucleic acids (LNAs), or other bulky substitutions recommends minimal substitution, as they can protrude into the minor groove and disrupt RISC recognition of the duplex(Braasch, Jensen et al. 2003; Chiu and Rana 2003; Czauderna, Fechtner et al. 2003). Complete 2'-deoxy substitution results in a conformational change of the duplex to a B-type helix and does not exhibit RNAi activity. 2'-Deoxy-2'-fluoro modifications, both in the trans and arabino (inverted) form, seem to be the best-tolerated alteration with respect to maintaining silencing activity(Manoharan 2003; Manoharan 2004; Dowler, Bergeron et al. 2006).

Recent work has shown that siRNAs containing 2'-fluoro modifications at all purine and pyrimidine nucleotides elicit RNAi(Blidner, Hammer et al. 2007). These fully-2'-fluorinated nucleic acids (FNAs) were generated for RNAi studies through either custom solid-phase synthesis or *in vitro* transcription using a mutated polymerase and 2'-fluorinated nucleoside triphosphates. This work also demonstrated that FNAs are highly resistant to sugar-specific enzymatic digestion, which may improve lifetimes following delivery and improve *in vivo* efficacy. Because melt curve analysis of FNAs demonstrates an increase in the thermal stability of the synthetic FNA duplexes, this could offset potential decreases in thermal stability due to cage attachment. In addition to improving resistance to enzymatic degradation and increased thermal stability, it has been demonstrated that 2'-fluoro modifications do not result in a loss of hybridization sequence specificity(Kawasaki, Casper et al. 1993). Although the observed dramatic increase in melting temperature of these 2'-fluoro modifications might suggest a possibility of silencing of closely matching target sequences, these modifications have been shown to reduce off-target effects normally observed following siRNA delivery(Jackson, Burchard et al. 2006; Cekaite, Furset et al. 2007; Furset and Sioud 2007). In fact, siRNA containing 2'-fluoro modifications have shown an ability to evade the cellular immune response by avoiding activation through Toll-like receptors (TLRs) in the endosome(Cekaite, Furset et al. 2007). This suggests that they may be inherently less toxic than extracellular siRNAs.

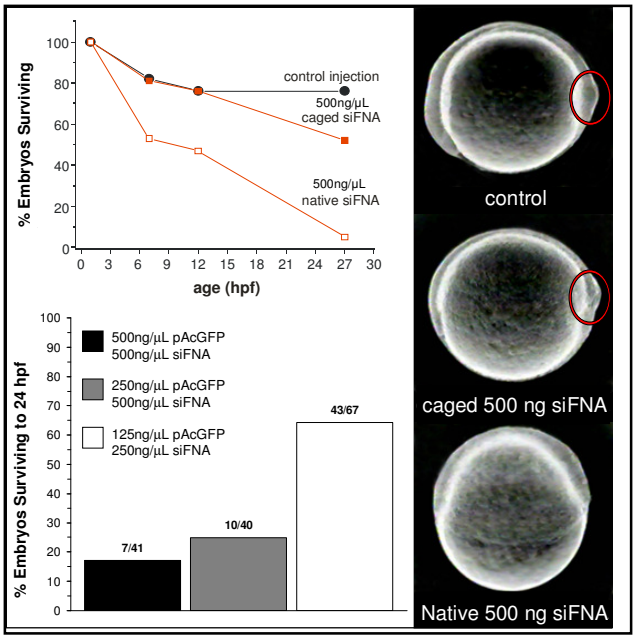
In a series of studies, Monroe and colleagues evaluated a RNAi system targeting a co-transfected GFP reporter gene with siRNAs containing fully 2'-fluoro-modified nucleosides (siFNAs) followed by the photo-triggering of this system through DMNPE caging(Blidner 2008). FNA strands were internally caged through the same DMNPE diazo attachment schemes described above to yield an average of 8 and 9 cage groups per 21mer oligonucleotide strand for the sense and antisense FNA strands, respectively. The resulting DMNPE-caged FNAs were resistant to a sugar non-specific nuclease, demonstrating improved enzymatic stability relative to the uncaged species. Characteristic of studies employing this type of caging strategy, RNAi effectiveness was reduced in all caged siFNAs, and exposure to 365 nm light partially restored the silencing activity in all cases (Figure 4). Since both the antisense and sense strands were caged separately, four combinations of the combined siFNAs were possible (uncaged antisense uncaged sense, caged antisense uncaged sense, uncaged antisense caged sense, caged antisense caged sense). The antisense strand was predictably more sensitive to inactivation through caging, which also led to a reduced photoactivation response(Blidner 2008).

Figure 4 - DMNPE-caged siFNA silencing of GFP in BHK cells. Black bars represent treatments that were protected from light. A significant difference exists between all 365nm-photoexposed (grey bars) and non-photoexposed (black bars) samples (denoted with †, $p < 0.05$). Note differences in siRNA inactivation when the antisense, sense or both strands were caged. Reproduced from (Blidner 2008) by permission of the Royal Society of Chemistry.



Microinjected zebrafish embryos were used as a model platform by Monroe and colleagues to demonstrate photoactivated RNAi *in vivo*(Blidner 2008). In addition to supporting the results of their cellular system, they demonstrated that caging of siFNAs protected the biological system from toxic doses of the effector (Figure 5). In this study, embryo development stalled when extreme levels of RNAi effectors were delivered to the system. It is possible that the high concentration of siFNA competitively sequestered the available RISC proteins, halting endogenous miRNA developmental regulation of gene expression (Figure 5, right panel). Although the mode of toxicity was not verified, the combined results of this work suggest that photocaging may offer control over RNAi therapeutics for spatially and temporally directed activation, while improving enzymatic stability and potentially enabling therapeutic dosing via photoexposure intensity(Blidner, Hammer et al. 2007). Since photorelease of active RNAi effectors from caged precursors is inherently a function of incident light delivery, partial

Figure 5 - In vivo demonstration of caged siRNAs acting to prevent toxicity of these effectors at similar concentrations injected into zebrafish embryos. Top left: siFNA concentrations above 250 ng/µL resulted in increased mortality. Bottom left: Caging of siFNAs protects the developing embryo from the siFNA induced toxicity (at 500ng/µL). Right: developmental delay at 12h post-fertilization in response to siFNA toxicity at a concentration of 500 ng/µL. Control (no siFNA) and caged siFNA injected embryos exhibit a developing tail bud, encircled. Reproduced from (Blidner 2008) by permission of the Royal Society of Chemistry.



photorelease of active siFNA could be achieved and therapeutic dosing carried out by controlling the incident light intensity and duration. Due to the fact that the caged siFNAs are enzymatically resistant and prevent toxicity of the large initially delivered dose, they represent a promising means to achieve extended duration of silencing with a timecourse of light triggers applied post-delivery. This approach would thus be advantageous over repeated injections or transfections of siRNAs, particularly in cases where delivery is disruptive to the system studied. However, further studies are necessary to determine lifetimes of the caged products *in vivo* and their photo-reactivity at long times following delivery.

Site-Specific Caging

Each incidence of statistical RNA backbone caging described above found that the caged effectors demonstrated reduced activity relative to their respective controls, which was partially restored by exposure to 350-365 nm light. However, no statistically caged product has shown perfect binary behavior with respect to both suppression and restoration of activity. A summary of these performance criteria, as well as the effectors and the model systems in which they were evaluated, is shown in Table 2. Significant progress has been made in recent years toward site-specific caging of nucleic acids. Consideration of the studies above suggests that in order to achieve superior, “on-off behavior” for caged siRNAs, control over positioning of the caging group is necessary. In many studies, the statistical caging method is a limiting factor in further understanding mechanistic interactions of caged siRNAs. While an average number of cage groups for each oligonucleotide can be determined, the number on each individual strand is left to chance and can likely vary substantially from the mean. In addition, it is believed that certain positions along an oligonucleotide may be more effective in inactivating the strand than

others(Chiu and Rana 2002), and statistical caging leaves the investigator no opportunity to guide the cage compounds to proposed advantageous sites. Accordingly, recent foundational work has focused on attaching photocages to predetermined sites on knockdown oligonucleotides. Other studies have used photocaging to investigate the folding dynamics of RNA strands, further developing the methods for site-specific caging(Hobartner and Silverman 2005; Mayer, Kröck et al. 2005; Wenter, Furtig et al. 2005; Mayer and Heckel 2006). More recently, short DNA and RNA oligonucleotides have been inactivated with site-specific cage chemistry reviewed in this section.

Heckel and Mayer used NPP cage groups on one or two of the six thymidine residues of a 15-mer DNA aptamer which inactivates thrombin to investigate the activity of the aptamer as a function of cage position(Heckel and Mayer 2005). It was determined that caged thymidine acted as a temporary base mismatch, preventing interaction with thrombin (thus decreasing blood clotting time) until UV irradiation, after which near-native binding kinetics were restored. Krock and Heckel also used 2-nitrobenzyl-caged thymidine residues to disrupt transcription activity through inhibition of T7 RNA polymerase(Krock and Heckel 2005). The group synthesized a 68-nucleotide-long strand of DNA with a T7 promoter region and caged a shorter initiation strand complementary to the promoter region, preventing T7 recognition and processing until photolysis availed the complementary strand for duplex formation. Building on these diverse caging studies, subsequent work has expanded the possibilities for light-modulated genetic expression by attaching photolabile compounds to an assortment of carefully determined locations upon siRNAs and other antisense nucleic acids. In the case of siRNAs, several moieties present possible sites for cage attachment, including 2’-hydroxyls,

nucleobases, photolinkers, and terminal or internal phosphates, each of which is discussed below.

2' Hydroxyl Caging

The first example of a nucleic acid with a caged compound inserted at a precise location was at the 2'-hydroxl of RNA, demonstrated by Chaulk and Macmillian(Chaulk and MacMillan 1998). In this study, the authors engineered the hammerhead ribozyme reaction to be photoresponsive by using 2'-O-(2-nitrobenzyl)-caged adenosines at the active site of substrate RNAs; this was also the first incidence of RNA caging. They prepared a ribozyme target that was caged at the site of catalytic cleavage and demonstrated protection of the RNA substrate until photoexposure: no ribozyme cleavage was detected with caged substrates prior to 308 nm irradiation, but activity equal to that of native ribozymes (70-80% cleavage) was detected after photorelease. Notably, the authors showed that attaching a nitrobenzyl to the 2'-hydroxyl did not disrupt complexing between the ribozyme and substrate; the cage compound merely prevented the hydroxyl attack. Although highly efficient, this technique required extensive synthetic procedures to produce the protected oligonucleotide. These authors expanded on this original work to disrupt spliceosome and polymerase activity using the site-specifically caged RNA strategy and have more recently published detailed protocols for their technique(Chaulk and MacMillan 2001; Chaulk and MacMillan 2007). This 2'-hydroxyl caging strategy was later adopted by Pitsch for the development of alternative conditions for synthesizing RNA(Pitsch, Weiss et al. 1999). In addition to these early studies, there is a large body of literature relating to photo-induction of strand breaks using photosensitive adducts (reviewed in(Mayer and Heckel 2006)). To date, there have been no reports of 2'-caged siRNAs,

although the site may be appropriate for photocontrol of RNA-RISC interactions. While Chaulk and MacMillan's work showed full tolerance for ribozymes to bind 2'-modified substrates, Chiu and Rana have shown that even the mildly bulky methoxy group, when placed at each 2' hydroxyl of an siRNA duplex, completely abolishes siRNA activity(Chiu and Rana 2003). It would seem, therefore, that the 2'-hydroxyl location on siRNAs has potential to effectively control bioactivity.

5'-Phosphate Caging

In an effort to reduce the leak activity, improve the efficiency of photo-induction, and avoid RNA hydrolysis, two research teams have separately expanded on caged siRNA by incorporating a single photolabile group onto the 5' terminal phosphate of the siRNA antisense strand(Nguyen, Chavli et al. 2006; Shah and Friedman 2007). Each exploited a commercially-available NPE-derived linker on the 5'-terminal nucleotide during 3' to 5' RNA solid phase synthesis. Based on previous work, this phosphate is thought to be critical for siRNA incorporation into RISC(Chiu and Rana 2003; Czauderna, Fechtner et al. 2003; Ma, Yuan et al. 2005). However, the results of these studies yielded mixed performance (compared in Table 2). Nguyen et al. demonstrated that these caged siRNAs had very little activity and that silencing was efficiently restored with light doses that were required to remove all cage linkers, which was determined *in vitro*. Observations of minute leak activities were explained by N-1 contaminants from incomplete RNA synthesis that had silencing activity due to the absence of the final caged linker. Shah and Friedman disputed this interpretation since their data shows significant leak RNAi activity in the caged un-induced state. Additionally, Friedman and colleagues tested highly purified antisense RNA strands with non-photolabile linkers at the 5'-

phosphate to verify their results. Their interpretation was that the RISC components have the ability to interact with the remaining non-bridging oxygen, allowing some RNAi to proceed. Although the 5'-phsophate on the antisense strand is known to be required for RNAi activity(Nykanen, Haley et al. 2001; Martinez, Patkaniowska et al. 2002; Schwarz, Hutvagner et al. 2002), it has also been shown that various linker modifications to this terminal location are tolerated if the 5'-phosphoester bond is intact(Schwarz, Hutvagner et al. 2002; Harborth, Elbashir et al. 2003). To avoid this residual activity, light-activated RNAi might require an alternate site of cage attachment or caging on multiple sites for each siRNA. However, depending on the system studied and the necessity for complete abolishment and restoration of activity between the un-induced and induced state, these 5'-phosphate caged siRNAs may be as effective as any of the other strategies presented (compared in Table 2).

Nucleobase-Caged siRNAs

The original site-specific caging work by Chaulk and MacMillian was limited to adenosine residues with the photolabile compounds attached to the 2'-hydroxyl(Chaulk and MacMillan 1998). However, the design of a caged siRNA for various mRNA target sequences will require more flexibility and include the other nucleobases as potential disruption sites. Pitsch and colleagues expanded the 2'-O-caging to include uracil, cytosine, and guanine(Pitsch, Weiss et al. 1999). However, additional work has attempted to react the caging species with nucleobases. The first example of a caged nucleobase appeared in 1992, where a caged adenosine derivative was used for self-replication but was not incorporated into an oligonucleotide(Hong, Feng et al. 1992). The first example of a caged base within an oligonucleotide was also an adenosine derivative in 2004, whereby a non-traditional photoactive C8 thioether-linkage controlled

DNAzyme activity(Ting, Lermer et al. 2004). Shortly after this study, a 2-nitrobenzyl group was utilized to cage the O4-position of thymine and the resulting nucleic acids were used to disrupt T7 polymerase(Krock and Heckel 2005) and control aptamer activity(Heckel and Mayer 2005). This same team also caged the C6 carbonyl of guanine in order to control aptamer activity by controlling G-quadruplex structure formation(Mayer, Krock et al. 2005). Another group concurrently presented NPE-caged guanine to study tertiary folding kinetics of RNA(Wenter, Furtig et al. 2005). Immediately following these studies, Höbartner and Silverman produced a complete set of NPE base-caged RNA residues(Hobartner and Silverman 2005).

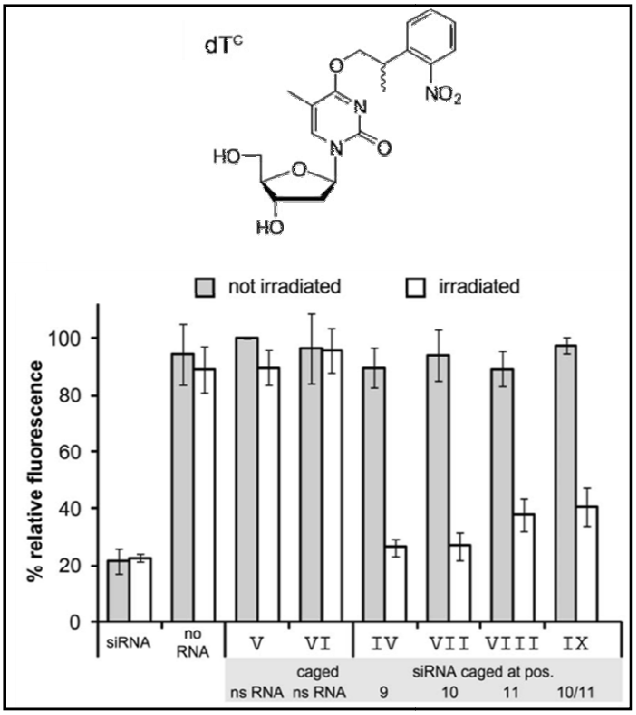


Figure 6 - Nucleobase caging at cleavage site leads to reversible inactivation of siRNA. (A) Fluorescence intensity of HeLa cells transfected with different siRNAs with (gray bars) and without (white bars) irradiation for 40 min 4 h past transfection. As a positive control, indicating full activity, a commercially available siRNA was transfected. The various negative controls were a sample not treated with any siRNA (no RNA), a sample transfected with a nonsilencing RNA (V) whose fluorescence intensity was defined as 100%, and the same nonsilencing RNA bearing photolabile protection groups (VI). The different caged siRNA sequences (IV, VII, VIII, and IX) are all inactive within error limits. For sequences IV and VII, a reactivation that can be assumed to be complete could be achieved, while sequences VIII and IX, even after irradiation, display a residual inactivation. Adapted with permission from (Mikat and Heckel 2007). Copyright Cold Spring Harbor Laboratory Press.

These developments were soon incorporated into RNAi studies, and Mikat and Heckel built on the research done by Höbartner and Silverman to control siRNAs with the 2-(2-nitrophenyl)propyl (NPP) group(Mikat and Heckel 2007).

Deoxyguanosine and deoxythymine residues were modified at the O6 and O4 positions, respectively, with the NPP moiety – dG^C and dT^C (dT^C structure shown in Figure 6). It has previously been shown that partial inclusion of deoxynucleotides do not significantly inhibit siRNA activity(Chiu and Rana 2003), and this tolerance for deoxy-substitutions was confirmed by Mikat and Heckel with their sequences and reporter assay. The group generated a variety of combinations of caged siRNAs targeting GFP transfected into HeLa cells by placing a single caged nucleotide at locations 9, 10, 11, or two caged nucleotides at sites 10 and 11. Placing a single dT^C group at position 10 was found to be the most effective manner of modulating siRNA activity: leakage was found to be approximately 8%, while knockdown activity following induction with light exposure was 92% of positive controls (Figure 6). After analyzing additional combinations of caged sense and antisense strands, Mikat and Heckel concluded that caging central nucleotides of the antisense siRNA strand inhibits the RNAi cleavage step, which cleaves complementary mRNA at a position opposite to nucleotides 9-11 of the siRNA(Chiu and Rana 2003). Inserting caged nucleotides away from the center of the sense strand or at any location along the antisense strand exhibited a drastically reduced ability to inhibit RNAi prior to photolysis(Mikat and Heckel 2007).

Other Nucleobase-caged Knockdown Oligonucleotides

Comparable site-specific nucleobase caging has been demonstrated in controlling other forms of gene-silencing oligonucleotides such as DNAzymes and phosphorothioate (PS) antisense nucleotides by Deiters' group. Utilizing standard DNA synthesis, NPOM-caged thymidine residues were introduced into antisense PS oligodeoxynucleotides targeting *Renilla* luciferase in 3T3 cell cultures. Chemiluminescence from caged transfectants showed no observable leak

activity, with an impressive photorestoration to 95% of control antisense treatments. This work also demonstrated the ability of the photocage assay to spatially restrain antisense knockdown activity to irradiated areas of cells in a culture dish (Figure 7)(Young, Lusic et al. 2008). In addition, in prior work, a NPOM-caged thymidine was shown to fully inactivate a DNAzyme, where the restoration to 91% cleavage activity of RNA was achieved when compared to native (non-caged) controls(Lusic, Young et al. 2007).

These more recent site-specific demonstrations of nucleobase modification offer a great deal of design flexibility in developing caged siRNA effectors. However, these compounds have yet to be adopted by the broader biological and biomedical community studying gene silencing for several reasons. First, generation of the caged precursors requires considerable chemical expertise and is a limiting factor for many laboratories that are not focused on modified nucleobase synthesis. Secondly, some of the caging groups, such as nonderivatized 2-nitrobenzyl groups, require exposure to UV wavelengths for photorelease, which can cause damage to biological systems. Finally, the scope of these caged RNAi effector studies is limited to date,

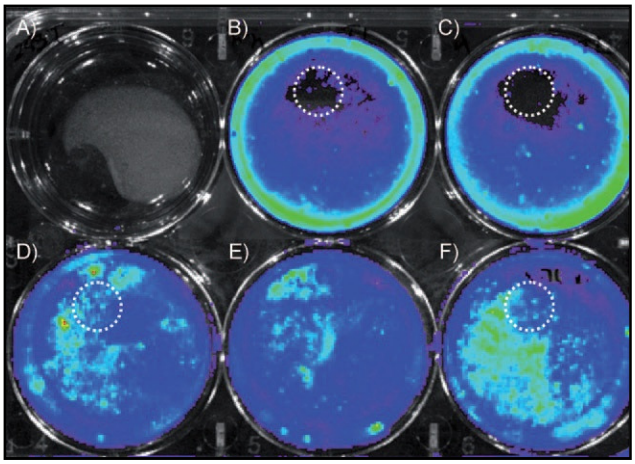


Figure 7 - Spatial control of luciferase expression in cell cultures with site-specific NPOM caged antisense oligonucleotides. The cellular monolayer was only irradiated inside the white dashed circle (365 nm, 5 min, 23 W). A) Negative control without luciferase plasmid. B) Transfection with luciferase plasmid and PS DNA caged on three separate nucleotides. C) Transfection with luciferase plasmid and PS DNA caged on four separate nucleotides. D) Positive control without PS DNA. E) Positive control without PS DNA and without irradiation. F) Transfection with luciferase plasmid and inactive control PS DNA. Reproduced with permission from (Lusic, Young et al. 2007). Copyright Wiley-VCH Verlag GmbH & Co. KGaA.

leaving the effectiveness of this strategy for controlling the complex RNAi system open to scrutiny. However, with the advent of nucleobase-caged siRNAs and other related nucleotides, control of these systems more closely resembles the on-off binary photo-reactivity that will be required for routine use. The development of commercially-available nucleobase-caged phosphoramidites (discussed in Future Directions below) will also allow more laboratories to investigate these techniques and allow more rapid development of caged siRNA applications.

Photolinkers

One final category of site-specific caging to control silencing oligonucleotide activity utilizes photocleavable linkers that tether a blocking moiety to the nucleic acid until photolysis. In these examples, knockdown is prevented by a complementary oligonucleotide strand tethered to the antisense oligonucleotide. Photocleavage of the linker separates the two strands, availing the antisense sequence for hybridization to target. This approach differs from those previously mentioned, as it requires no direct cage attachment to the silencing oligonucleotide, and may achieve total blockade of hybridization with the single photocleavable linker (reviewed in (Tang and Dmochowski 2007)).

Dmochowski and colleagues prepared a 25mer oligonucleotide containing a nitrobenzyl linker that connects a

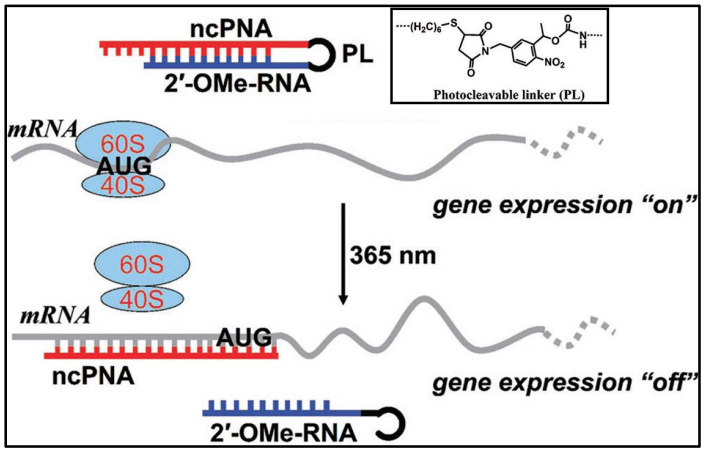


Figure 8 - Control of gene expression using a photocleavable linker (PL). Photolysis released 2'-OMe RNA and allowed ncPNA hybridization to mRNA, thereby blocking protein synthesis in zebrafish embryos. Adapted with permission from (Tang and Dmochowski 2007). Copyright 2007 American Chemical Society.

cytosine to a fluorescence quencher(Tang and Dmochowski 2005). An adjacent nucleotide contains a fluorophore which emits a fluorescent signal upon separation from the quencher, thereby confirming photoactivation. This cage attachment chemistry was used to control RNase H-mediated digestion of an mRNA target through hairpin loop caging(Tang and Dmochowski 2006). Irradiation breaks the photocleavable link at the bend of the hairpin, drastically reducing T_m and allowing hybridization of the antisense side of the hairpin to the target. A similar photolinker was used to tether a 2'-methoxy RNA oligonucleotide to a negatively charged peptide nucleic acid (ncPNA) for gene silencing in zebrafish embryos(Tang, Maegawa et al. 2007). Upon photoactivation, the release of the photolinker significantly lowers the melting temperature for the duplex, allowing the antisense ncPNA to hybridize with target mRNA and induce RNase H degradation of the target (Figure 8)(Tang, Maegawa et al. 2007). With this strategy, restoration to 94% of RNase H silencing activity targeting the Bozozok gene in zebrafish was achieved, with only 6% leakage in the non-irradiated, caged state. Similarly, the group silenced 85% of chordin gene expression, relative to positive RNase H controls in zebrafish embryos, with only 16% leakage. Tang and Dmochowski also used phosphorothioate antisense oligodeoxynucleotides (ODNs) with the NPE-photolinkers to exert photo-control over c-myb mRNA in K562 cells(Tang, Swaminathan et al. 2008). After optimizing the PS DNA/photolinker conjugates, the group was able to achieve reduction in protein expression equivalent to 52% that of positive antisense controls, with no detectable leak activity. The group concluded that the length of the loop section of the complex should be minimized so that it consists of only the photolinker in order to increase molecular stability and decrease

background (leak) activity; however, it was noted that further optimization of the leakage/photoactivation balance may be necessary(Tang, Swaminathan et al. 2008).

An interesting demonstration of this NPE-photolinker system is the tethering of two non-complementary ~10mer RNA oligonucleotides in which light is used to activate genetic expression, as opposed to knockdown(Richards, Tang et al. 2008). When tethered together, the two oligonucleotides form ~20 nucleotides that are complementary to an mRNA target. When the linker is photocleaved, the separated short strands are no longer stable enough to remain hybridized with the target, allowing translation to proceed. 2'-O-methyl groups were used to stabilize the complexes, both for nuclease resistance as well as increased strand melting temperature(Braasch and Corey 2002; Richards, Tang et al. 2008). The non-irradiated complex bound to start codons and Kozak sequences within untranslated regions of GFP mRNA inhibited 70% of expression; after photolysis, the GFP reached 95% of positive control expression. Notably, the group found no correlation between the complex's change in melting temperature with its target after irradiation and its ability to restore genetic expression. It was noted that targeting long mRNAs proved especially difficult due to challenges in predicting mRNA secondary structure, and that the strategy may work better when targeting shorter strands such as siRNAs(Richards, Tang et al. 2008).

Chen and colleagues used a photolinker system to modulate morpholino knockdown oligonucleotide activity in zebrafish(Shestopalov, Sinha et al. 2007; Shestopalov and Chen 2008). Using a photocleavable linker to tether an antisense morpholino oligonucleotide to a complementary 10mer "inhibitor," the group targeted no-tail (ntl) mRNA critical for embryonic

development. The embryos transfected with the caged antisense sequences developed normally unless they were irradiated with 360 nm light, in which case the tails showed significant shortening and a notable loss of anatomical organization. Wild-type embryos similarly irradiated showed no defects. Embryos were transfected while still unicellular, irradiated four hours post-fertilization (or kept in the dark), and imaged one day post-fertilization. The defects resulting from photoactivated morpholinos were spatially restricted to the areas of irradiation, demonstrating effective spatial targeting of gene silencing.

One final mention of recent development in this category of photolabile tethers is the demonstration of a singlet oxygen-sensitive linker that, when combined with a photosensitizer, demonstrated cleavage *in vitro* with red wavelengths of light. While this strategy has yet to be employed in cells to control gene expression, and may be inherently more involved due to co-delivery of the photosensitizer, the red wavelengths used are promising due to their lack of photodamage and increased tissue penetration depths(Rotaru, Kovacs et al. 2008). In recent work by Ohtsuki and colleagues, photoinduction of RNAi was observed after red light exposure to cells transfected with siRNAs conjugated to fluorophore-labeled peptides, thought to generate reactive oxygen species that promoted endosomal escape of siRNAs and subsequent gene silencing(Endoh, Sisido et al. 2008).

Overall, the photocleavable linker strategy towards genetic modulation represents a significant augmentation of the efficiency of photolysis, as the photoactivity is consolidated into one functional group and a single photorelease event. This contrasts with strategies requiring multiple photoactive molecules per strand and therefore potentially requiring greater

irradiation doses for complete photoactivation that would scale geometrically with the number of attached cages. However, the boost in efficiency of the photolytic step comes at a cost of decreasing the simplicity of deprotection, as a successfully photolyzed complex still relies upon statistical changes in the hybridization of an inhibitory strand to a target. This, then, is the balance which will need to be met in order to achieve binary activity – minimum leakage, with oligonucleotide sequences fully hybridized in the photolinked state, versus maximum recovery of activity, with sequences which fully disassociate following irradiation.

Modified Nucleobases

Currently, investigators seeking to utilize nucleobase-caged oligonucleotides must perform complex synthetic chemistry in order to develop caged nucleosides for phosphoramidite synthesis. The present lack of commercially available caged oligonucleotides limits the availability of this promising approach and significantly slows the work of those seeking to use or expand upon the technique. However, the development of relatively simple chemical techniques to react convertible nucleosides with cage compounds could make the use of nucleobase-caged oligonucleotides substantially more feasible. In such a case, commercially available convertible nucleosides could be inserted into a standard short antisense DNA or RNA strand and reacted with a cage compound to yield nucleobase-caged knockdown strands.

The Verdine group produced and characterized a series of such convertible nucleosides for site-specific modification of nucleic acids in the late 1990's (Allerson 1996; Allerson, Chen et al. 1997). While intended for use in studying the structure and conformation of nucleic acids, the nucleosides were designed for solid-phase synthesis and appear to be extremely useful for site-

specific caging studies. They each possess a chlorophenyl- or nitrobenzyl-based leaving group which can be removed by treatment with an appropriate amine-terminated nucleophile. Two of these nucleosides – the convertible adenosine and convertible guanine – were available at the start of this work through Berry & Associates and so were considered for use with this study.

The convertible adenosine molecule is 6-O-(4-chlorophenyl)inosine: an adenosine residue with an O-chlorophenyl leaving group replacing the amine on the base. Upon substitution with an alkyl amine nucleophile, the inosine will convert to an adenosine molecule with the given alkyl group bound to the base's amine group. As a free nucleoside in solution, the molecule was found to react very poorly and slowly with pure ammonia, while ethylenediamine and 1,4-diaminobutane were found to be much more quickly reacting nucleophiles. As the center nucleotide in an 11-nucleotide-long strand of RNA, the convertible adenosine underwent the nucleophilic substitution reaction in highest yields with methylamine, ethylenediamine, and ethanolamine. Notably, the benzylamine – the molecule in the study closest to nitrobenzyl – reacted to 67% yield relative to the methylamine reaction (considered 100% yield) (Allerson, Chen et al. 1997).

The convertible guanine molecule is 2-fluoro-6-O-(4-nitrophenethyl)inosine. Upon reaction with an alkyl amine, this molecule converts to 2-N-alkyl guanine residues. This moiety reacted much faster than the convertible adenosine, as measured by half times for substitution reactions on the free nucleosides. As the center nucleotide in an 11-nt oligonucleotide strand, the convertible guanine reacted in highest yields with pure ammonia and ethanolamine. The

reaction with the convertible guanine and benzylamine proceeded to 65% of the yield of the reaction with ammonia (considered 100% yield) (Allerson, Chen et al. 1997).

For our study, the convertible adenosine was chosen for substitution with an aminated photocage compound because the strand of interest – ISIS 2302 – has adenosine residues at more strategic locations than those of guanine (see below).

Chapter 3: Cadaverine Labeling

Introduction

In order to optimize the chemistry and separation of the site-specifically modified oligonucleotides, it was decided to first use an aminated dye which could be easily seen throughout different procedures. Texas Red Cadaverine (TRC), a large, bulky dye which is excited at 591nm and emits at 612nm and has an amine terminus, was chosen for use in this study. The dye's absorbance and emission spectra are ideal, as they are far from the typical absorbance of DNA and RNA. The compound is also readily available. The dye was first attached to the 5'-phosphate of an ISIS 2302 strand in a reaction utilizing *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDAC). After this reaction was shown, the dye was reacted with convertible nucleotides at positions 6 and 13 of ISIS 2302. Of the adenosine residues on ISIS 2302 (5' – GCC CAA GCT GGC ATC CGT CA – 3'), these nucleotides are believed to be ideal sites for disrupting hybridization, as they minimize the length of any contiguously complementary section of a duplex to 5, 6, and 7nt. The amine-terminated hydrocarbon chain

extending from the TRC molecule is expected to react as a nucleophile in a substitution reaction in a manner comparable to that discussed by Allerson, et al (Allerson, Chen et al. 1997).

Attachment of Texas Red Cadaverine to 5'-Phosphate of Oligodeoxynucleotides

Protocol

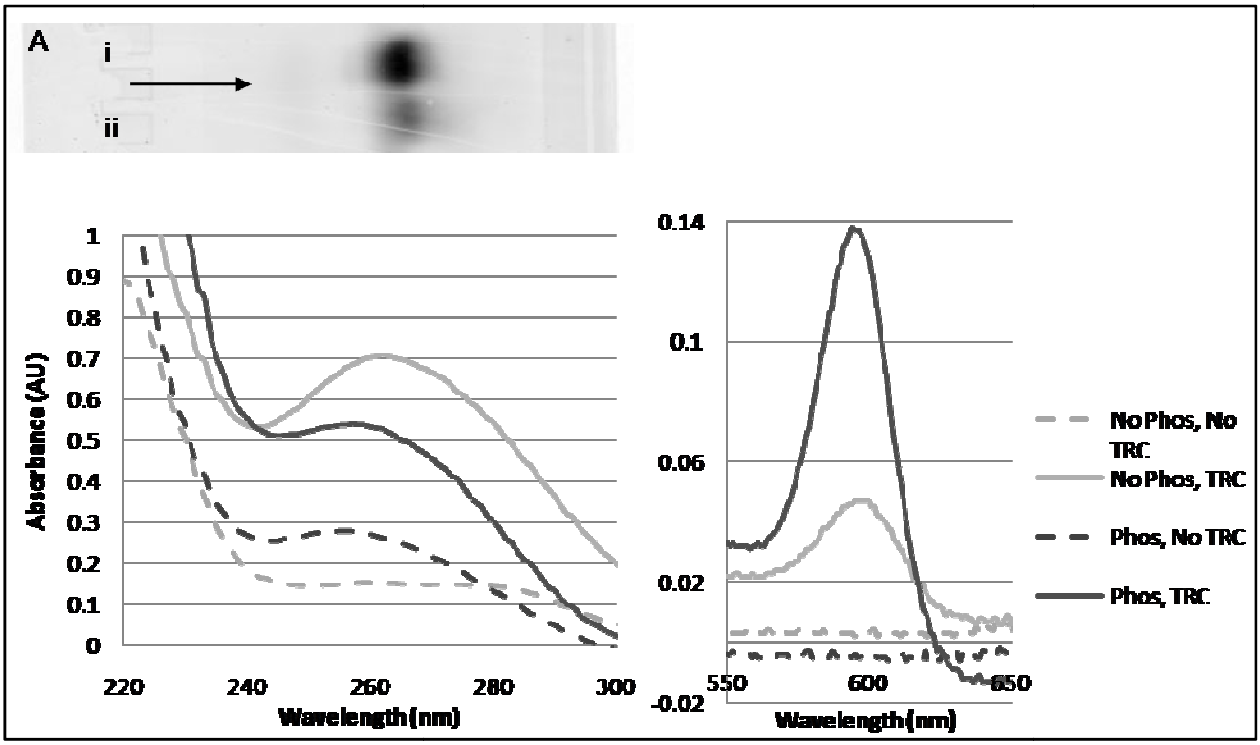
For the 5'-phosphate reaction, a protocol available from invitrogen.com was adapted. A 2X labeling buffer was first created with 900µL deionized water (dH_2O), 23µL of 1-methylimidazole, and 10uL 2M NaOH. 74.7mg of EDAC was added to the vial. After allowing the EDAC to settle, the vial was filled to 1.2mL with dH_2O. The buffer was then mixed to fully dissolve the EDAC. This was divided into ~100µL aliquots, which were then dried down and frozen. After re-suspending a dried aliquot in the original solution, 10µL of this labeling buffer was added to 10µL of dH_2O with 2µg of TRC and 20µg of DNA. The solution was vortexed for 4.5 hours, then left to react at room temperature overnight. The resulting solution was passed through 2mL of Sephadex LH20 (1.1mL bed height) in pure dH_2O, and fractions collected every 0.5mL up to 5mL. Negative controls were: non-phosphorylated ISIS 2302 without dye, non-phosphorylated ISIS 2302 with dye, and phosphorylated 2302 without dye. Each was run in duplicate. The fractions were analyzed via spectrophotometry and the fractions with the highest amount of DNA were run through a 15% polyacrylamide gel in TBE.

Results and Discussion

Spectrophotometric analysis showed the samples with 5'-phosphorylated DNA retained more TRC per gram of DNA than non-phosphorylated DNA, indicating that the reaction was successful

(Figure 9, B, C). The 260nm absorption for the TRC-complexed phosphorylated and non-phosphorylated samples was 0.70 and 0.53, respectively. With an extinction coefficient, ε_{260}, of 184,100$M^{-1}cm^{-1}$(manufacturer specifications), this corresponds to concentrations of 3.81µM and 2.89µM. The absorbance at 591nm of these samples (ε_{591} of TRC is 85,000$M^{-1}cm^{-1}$, manufacturer specifications) was 0.04 and 0.13, corresponding to concentrations of 0.52µM and 1.53µM, respectively. Accordingly, it can be deduced that the non-phosphorylated samples had TRC on approximately 13.7% of DNA strands, while phosphorylated samples had TRC on approximately 52.9% of strands. This ability to differentially label 5'-phosphorylated and non-5'-phosphorylated DNA was confirmed with gel electrophoresis, whereby phosphorylated samples registered a much stronger signal on a fluorescent scanner (30nm band pass emission filter

Figure 9 - A, gel electrophoresis of TRC-labeled phosphorylated samples (i) and non-phosphorylated samples (ii) imaged with fluorescent scanner; B and C, absorption spectra of samples at relevant wavelengths.



centered at 610nm) (Figure 9). Note that the electrophoresis assay shows the samples retained no free dye, but that the dye seen through spectrophotometry was confirmed to be complexed to oligonucleotides. This preliminary work evinced an ability to utilize an amine-terminated molecule in site-specific nucleic acid chemistry and to subsequently clean reaction products of free dye.

Site-Specific Attachment of Texas Red Cadaverine to Oligodeoxynucleotide Nucleobases

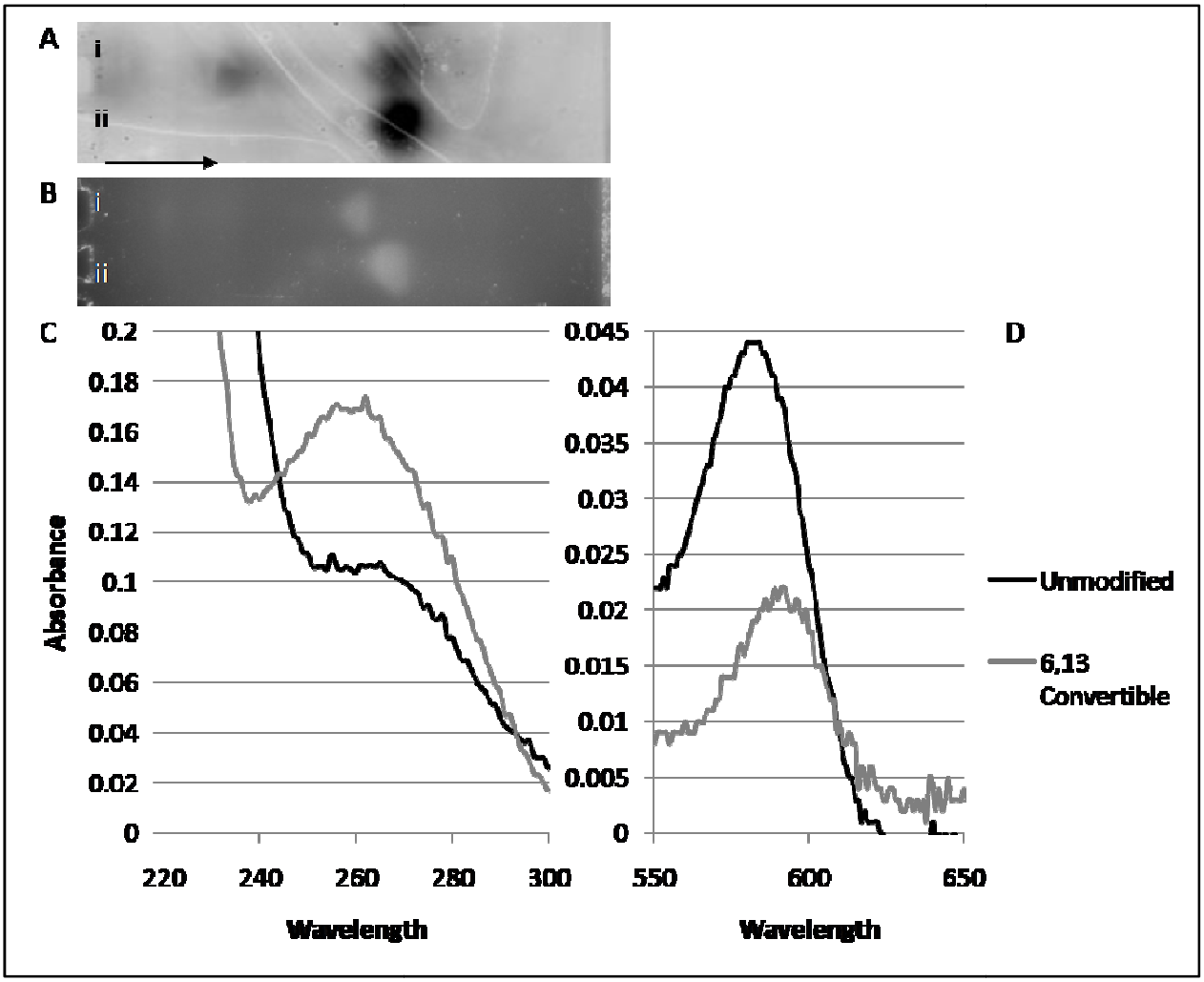
Protocol

Two samples were prepared for this reaction: an unmodified ISIS 2302 sample and an ISIS 2302 sample with convertible adenosines (6-O-(4-chlorophenyl)inosine) replacing standard adenosines at nucleotides 6 and 13 from the 5' end of ISIS 2302. For each sample, 350µg (8.4mM) of TRC and 20µg of DNA (14µM) were added to a 60µL solution: 34.1µL methanol, 5µL dH_2O, and 20.9µL (3M) triethylamine. These samples were placed in a water bath maintained at 42^{o}C for 18 hours with periodic vortexing. The resulting solution was passed through 2mL of Sephadex (1.1mL bed height) in pure dH_2O, and fractions collected every 1.5mL up to 7.5mL.

Results

Spectrophotometric analysis shows the modified oligonucleotide sample to have a 260nm absorbance of 0.164, corresponding to a DNA concentration of 0.89µM. The absorbance at 591nm is .017, corresponding to a TRC concentration of 0.20µM (Figure 10 C, D). This indicates an average of approximately two TRC molecules per nine oligonucleotide strands, or a reaction at 22.5% of available sites. The unmodified ISIS 2302 sample had negligible absorption at 260nm and 591nm until the final separation fraction, when it is believed that mostly free TRC

Figure 10 - A, gel electrophoresis of unmodified DNA (i) and DNA with two convertible nucleotides (ii) following TRC reaction, imaged with a fluorescent scanner; B, the same samples imaged via SYBR Gold intercalating dye; C and D, absorption spectra of samples at relevant wavelengths.



and free DNA were washing through the column. The variance of reaction yield is corroborated by a gel electrophoresis assay which shows a strong red fluorescent signal (30nm band pass filter centered at 610nm) for the modified oligonucleotide but weaker, dispersed signals from the unmodified oligonucleotide sample (Figure 10, A). While the identity of each band is unclear at present, it may be that the middle band is unreacted TRC and the lower band is DNA which reacted with the dye at extremely low yields. A further assay to assess the location of DNA on the same gel was performed using SYBR Gold dye (Figure 10, B). This shows bands of DNA at approximately the same location as the fluorescent gel scan, but slightly upstream. This may indicate that the TRC molecule's attachment to the oligonucleotide alters its conformation in such a way that expedites its ability to progress through the gel, and the strongest signals on the SYBR Gold scan were bands from unreacted DNA. In any case, the fluorescence signal indicates the reaction of TRC with convertible nucleotides in ISIS 2302 proceeded to a fuller extent than the negative control without convertible nucleotides.

Finally, a hybridization assay was conducted using a molecular beacon to determine the melting temperature of a sequence complimentary to the convertible oligonucleotide strand after the TRC reaction (Figure 11). The samples were run in duplicate, and the results displayed are the average thereof. Here, one can see that the oligonucleotides which reacted with the TRC had a substantial drop in melting temperature when compared to the un-reacted ISIS 2302. While the un-reacted, unmodified oligonucleotide strand remained hybridized (50% or greater fluorescence) to the molecular beacon up to 65^{o}C, the TRC-reacted convertible oligonucleotide melted (dropped below 50% fluorescence) at 51^{o}C. This 14^{o}C drop is presumed to be due to steric impedances caused by the presence of TRC at nucleotides 6 and 13, and therefore

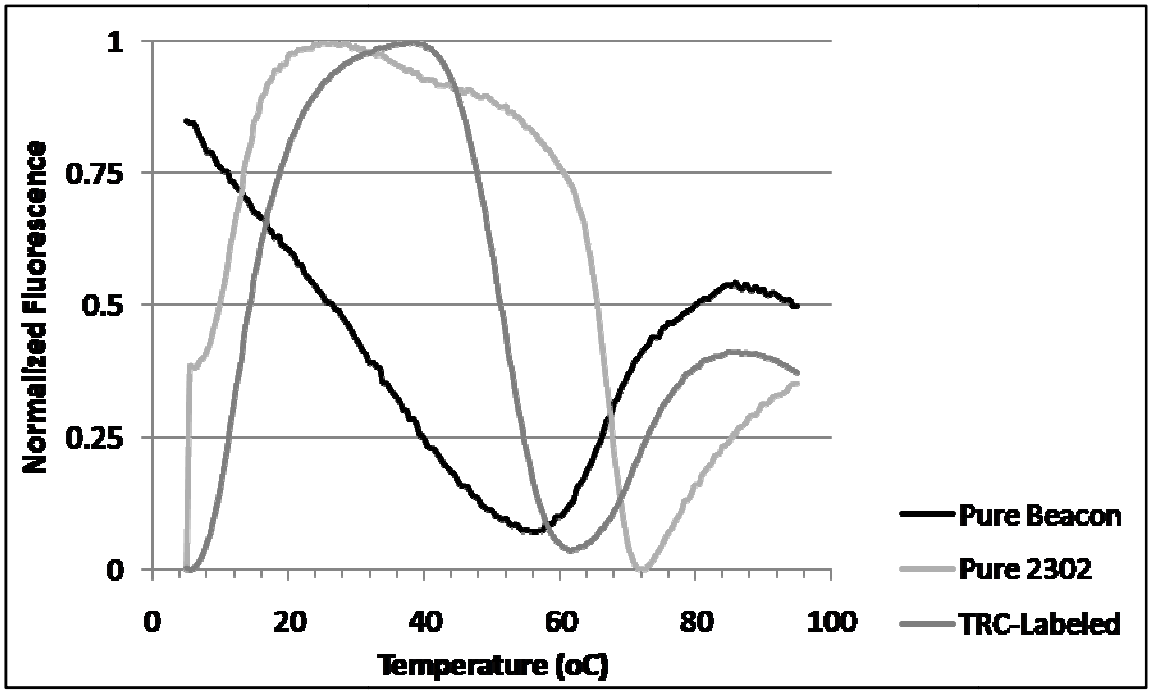


Figure 11 - Molecular beacon assay showing melting temperatures for pure molecular beacon (negative control), unmodified DNA and molecular beacon (positive control), and TRC-reacted modified ISIS 2302.

believed to be an indication that the reaction proceeded as designed. This reaction supports the amine substitution chemistry and reaction conditions as a viable method of placing small molecules at specific nucleobases along an oligonucleotide strand.

Chapter 4: Site-Specific Nucleobase Caging of Oligodeoxynucleotides

Introduction

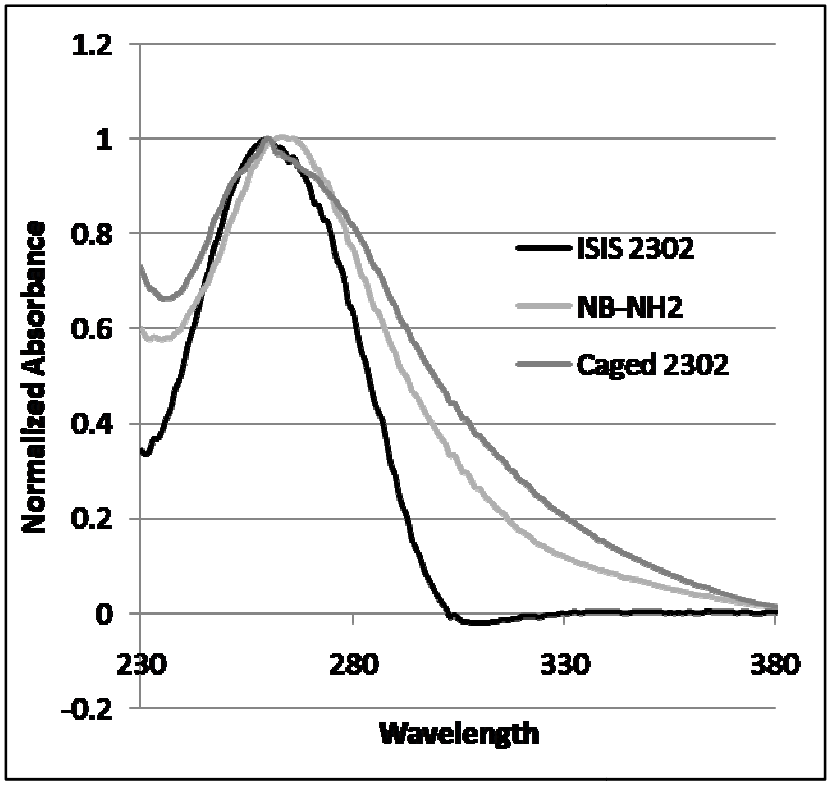
While reacting the convertible adenosine-modified ISIS 2302 strand with the Texas Red Cadaverine compound showed a strong ability to disrupt hybridization activity, the purpose of the project is the attachment of photoremovable adducts. The final stage of this project, therefore, is the use of 2-nitrobenzyl-amine (NB-NH_2) with convertible nucleotides to inactivate an oligonucleotide in a light-reversible manner. The modified strand is, again, ISIS 2302 with

insertions of convertible adenosine nucleotides at positions 6 and 13 from the 5' terminus. The nitrobenzyl-amine molecule is expected to undergo nucleophilic attack on the leaving group of the convertible nucleotides as discussed in (Allerson, Chen et al. 1997).

Protocol

The protocol used for attaching nitrobenzyl to ISIS 2302 is similar to that used for attaching TRC to the same convertible nucleobases. 40µg (15µM) of convertible DNA (diluted in 10µL of dH_2O) and 1.6mg of NB-NH_2 were mixed into 41.8µL of triethylamine and 58.2µL of methanol. The reaction was placed in a 42°C water bath for 18 hours and vortexed periodically to prevent separation. Following the reaction, half of the product was dried down (45°C for 10min) to near dryness and resuspended in 50µL of dH_2O. The other half of the product was stored at 4°C for future use. The resuspended product was purified by running through 5.5mL (3.1cm) of Sephadex LH20 in pure dH_2O and collecting fractions every 1mL for 10mL. Fractions found to contain significant amounts of DNA were further purified by running through Amicon centrifuge filters for three hours. Flashed samples

Figure 12 - Normalized absorption spectrum of modified ISIS 2302 reacted with nitrobenzyl-amine compared to unreacted ISIS 2302 and pure nitrobenzyl-amine.

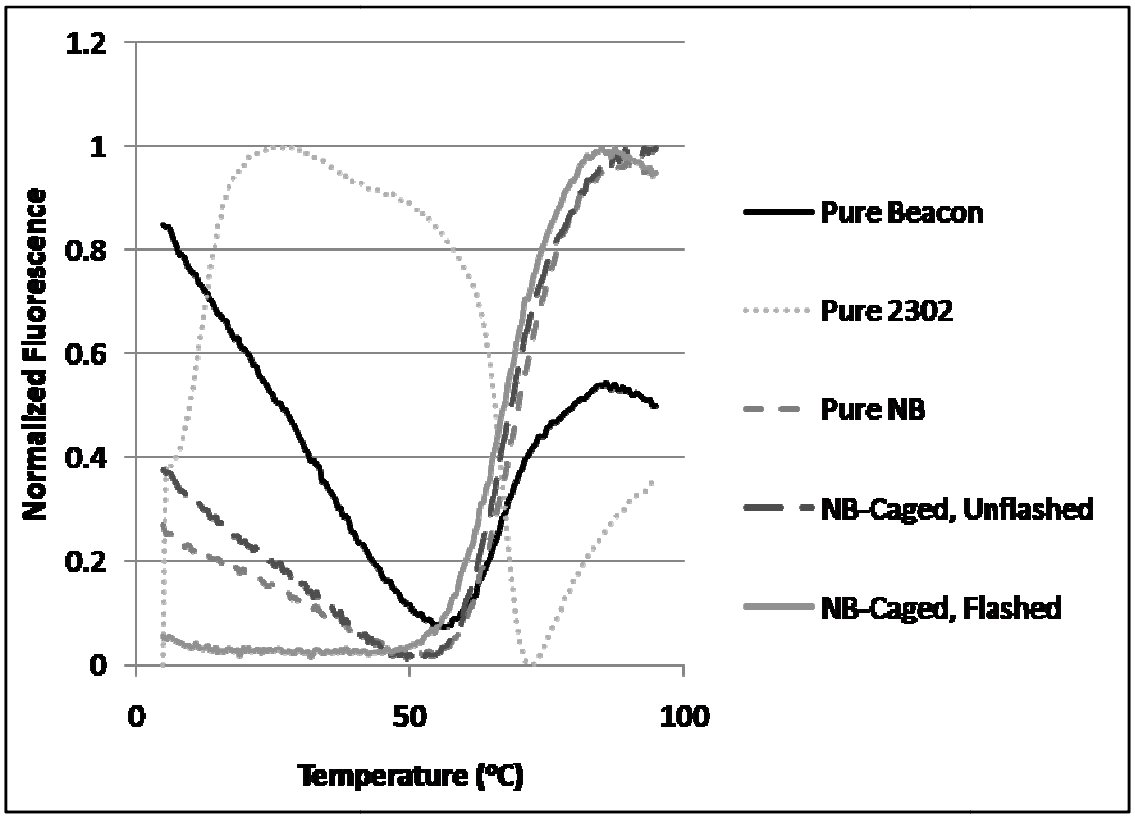


were irradiated for five minutes on a gel box with 310nm light output.

Results

Spectrophotometric analysis of the nitrobenzyl-caged samples is not as simple as with the TRC-labeled samples, due to differences in absorbance and emission spectra. The TRC molecule has exclusive absorbance and emission wavelengths in the red side of the spectrum which makes the extent of the reaction easy to determine. The spectra for nitrobenzyl, though, resemble that of DNA much more closely, complicating comparisons. However, one can see from the absorbance spectra (Figure 12) that the normalized absorbance of the reacted sample has a nitrobenzyl-esque pattern well above that of pure DNA, indicating the presence of nitrobenzyl

Figure 13 - Normalized fluorescence results of a molecular beacon assay to show melting temperatures for flashed and unflashed modified ISIS 2302 after reaction with nitrobenzyl amine; compared to positive control (pure 2302) and two negative controls (pure beacon, pure NB).



in the samples.

While the spectra show nitrobenzyl to be present, this analysis does not indicate how much, if any, of the nitrobenzyl compound may be free, unreacted nitrobenzyl-amine. In order to further characterize the samples, a molecular beacon assay was run (Figure 13). The modified 2302 samples show a negligible ability to hybridize to the beacon and induce fluorescence. Instead, they behave much as pure molecular beacon, the negative control, does. Even following irradiation of samples, the caged sample does not behave as the native oligonucleotide sample does. It is therefore uncertain whether and how far the reaction proceeded with the presented modes of analysis.

Chapter 5: Conclusion and Future Directions

The ability to use commercially available convertible phosphoramidites to cage specific nucleotides along an oligonucleotide strand would represent a very powerful new technique, both for therapeutics and for basic research studies of nucleic acid chemistry and genetic behavior. Such a strategy would make caging studies more facile and allow investigators to probe locations along a strand for increased understanding of conformations and interactions. This study develops a potential method for achieving such a strategy.

The first part of the project investigated the utility and reactivity of the dye Texas Red Cadaverine with various nucleic acid chemistries. The work indicates an ability to specifically attach this molecule both to the 5’-phosphate of oligodeoxynucleotide strands and, through separate chemistry, to convertible adenosine residues within an oligonucleotide. These results

are promising, as they corroborate the feasibility of the chemical approach used to substitute an amine-terminated molecule for a leaving group. They furthermore show the ability to disrupt hybridization through such chemistry, another promising outcome.

However, the success of the latter part of the project, the attachment of nitrobenzyl-amine to convertible nucleotides, is as yet undetermined. While samples exhibit absorbance spectra indicative of the presence of nitrobenzyl and nearly abolish hybridization to complimentary strands, the absorption spectra could be the result of free nitrobenzyl and the hybridization inhibition could stem from convertible nucleotides acting as base mismatches. This possibility is strengthened by the inability of the five minutes of ultraviolet irradiation to restore much hybridization in flashed samples.

Accordingly, the progression of the project will require greater and more sophisticated characterization of the samples. A gel electrophoresis assay will be run to determine how much free nitrobenzyl may be lingering in DNA samples. The convertible, modified DNA strand will be tested in a hybridization assay to determine its ability to disrupt hybridization without undergoing a reaction, and reaction products will be irradiated for longer to ensure sufficient opportunity for photocleavage. Using high-pressure liquid chromatography to further purify samples will likely yield clearer results, as well. It is believed that with cleaned reaction chemistry and increased methods of analysis, this site-specific caging strategy will become a useful approach for photo-modulating genetic expression.

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Table 2. Summary of caged knockdown oligonucleotide studies. Column headings and abbreviations: cage compounds: photolytic molecule used (PL – photolinker); oligo type: variant of antisense/knockdown agent; systems: HeLa – immortal HeLa cancer cell line, k562 – immortalized leukemia cell line, 3T3 – fibroblast cell line, BHK – baby hamster kidney cells, Zf – zebrafish; max knockdown: (knockdown of target by uncaged oligonucleotide)/(knockdown by positive control); leak: (knockdown of target by caged oligonucleotides)/(knockdown by positive controls); modifications: D – deoxynucleotide insertions, PS – phosphorothioate, 2'-OMe – methoxy insertions at 2'-hydroxyl, 2'-Fluoro – fluorination at 2'-hydroxyl.