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Design and Application of Photoactivatable Oligonucleotides

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Design and Application of Photoactivatable Oligonucleotides

Abstract
Photoactivatable oligonucleotides are DNA or RNA molecules whose function is temporarily blocked or "caged" by a chemical moiety that can be activated by light. By using light to turn on activity, high spatial and temporal control of oligonucleotide function can be attained. We have developed novel techniques to cage oligonucleotides using light-activatable groups that introduce a strand break upon photolysis. One method of inhibiting gene expression involves the use of catalytic DNA strands known as DNAzymes, specifically ones with the ability to cleave RNA. We have demonstrated efficient photomodulation of a biotinylated split DNAzyme by employing streptavidin to disrupt the active conformation. Light-controlled split DNAzymes could potentially be a tool for studying gene expression in vitro. Additionally, a novel photochemical approach for isolating mRNA from single neurons was developed by creating hairpin-bandage hybrids. This design utilizes a photoactivatable oligonucleotide hairpin that targets the poly(A)+ tails of mRNA. The antisense strand of the hairpin is linked via a photocleavable spacer to the blocking strand, which is divided into two parts by a second photocleavable spacer. Experiments in single neurons have confirmed the utility of hairpin-bandage hybrids for fluorescently monitoring oligonucleotide dissociation in vivo and for isolating of mRNA from single cells.

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DESIGN AND APPLICATION OF PHOTOACTIVATABLE OLIGONUCLEOTIDES

Brittani K. Ruble

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ABSTRACT

DESIGN AND APPLICATION OF PHOTOACTIVATABLE OLIGONUCLEOTIDES

Brittani K. Ruble

Ivan J. Dmochowski

Photoactivatable oligonucleotides are DNA or RNA molecules whose function is temporarily blocked or “caged” by a chemical moiety that can be activated by light. By using light to turn on activity, high spatial and temporal control of oligonucleotide function can be attained. We have developed novel techniques to cage oligonucleotides using light-activatable groups that introduce a strand break upon photolysis. One method of inhibiting gene expression involves the use of catalytic DNA strands known as DNAzymes, specifically ones with the ability to cleave RNA. We have demonstrated efficient photomodulation of a biotinylated split DNAzyme by employing streptavidin to disrupt the active conformation. Light-controlled split DNAzymes could potentially be a tool for studying gene expression in vitro. Additionally, a novel photochemical approach for isolating mRNA from single neurons was developed by creating hairpin-bandage hybrids. This design utilizes a photoactivatable oligonucleotide hairpin that targets the poly(A)$^+$ tails of mRNA. The antisense strand of the hairpin is linked via a photocleavable spacer to the blocking strand, which is divided into two parts by a second photocleavable spacer. Experiments in single neurons have confirmed the utility of hairpin-bandage hybrids for fluorescently monitoring oligonucleotide dissociation in vivo and for isolating of mRNA from single cells.
**TABLE OF CONTENTS**

Chapter 1: Introduction to light-activatable oligonucleotides

I. Overview of caged compounds ..................................................... 2
II. Examples of caging groups ......................................................... 2
III. Photoactivatable oligonucleotides ............................................. 4
   a. Caged plasmids and messenger RNA ..................................... 5
   b. Light-regulatable catalytic oligonucleotides ............................. 7
   c. Caged antisense oligonucleotides ......................................... 10
   d. Photoregulatable aptamers .................................................. 14
   e. Other photoregulatable oligonucleotides ................................. 15
   f. Indirectly photomodulated oligonucleotides ............................. 18
IV. Improving photoactivatable oligonucleotides ............................. 20
V. Research aims ........................................................................... 23
VI. References ................................................................................. 24

Chapter 2: Mismatch discrimination and efficient photomodulation of split 10-23 DNAzymes

I. Introduction .................................................................................... 35
II. Experimental procedures ............................................................ 38
   a. RNA cleavage by DNAzymes ............................................... 38
   b. Thermal denaturation of DNAzyme-RNA hybrids ..................... 39
   c. Circular dichroism spectroscopy of DNAzyme-RNA hybrids ......... 40
   d. Photoactivation of biotinylated split DNAzymes ....................... 40
Chapter 3: Caged hairpin-bandage hybrids for transcriptome in vivo analysis

I. Introduction ........................................................................................................... 82
   a. Background ...................................................................................................... 82
   b. Current techniques for isolating neurons ...................................................... 84
   c. Caged compounds for studying the central nervous system ....................... 86
   d. Hairpin-bandage hybrid design strategy ....................................................... 87

II. Experimental procedures .................................................................................. 89
   a. Oligonucleotide synthesis and purification .................................................. 89
   b. Conjugation of TIVA-tags to cell-penetrating peptides ............................... 90
   c. Mass analysis of TIVA-tags .......................................................................... 91
   d. Melting point determinations ....................................................................... 91
   e. FRET analysis of Cy3/Cy5-labeled TIVA-tags ............................................. 92
   f. Polyacrylamide gel electrophoresis analysis of TIVA-tags ............................ 92

III. Results and discussion .................................................................................... 93
   a. TIVA-tag characterization ............................................................................ 93
b. In vitro FRET monitoring of TIVA-tag dissociation..........................95

c. Gel analysis of TIVA-tag uncaging........................................96

d. Preparation of TIVA-tags for cellular experiments.........................97

e. In vivo studies...........................................................................98

IV. Conclusions..............................................................................100

V. References..................................................................................104

Appendix: Supplemental information for caging siRNA

I. Caged siRNA design strategy.........................................................133

II. Experimental procedures............................................................134
   a. Oligonucleotide synthesis and purification...............................134
   b. Mass analysis of caged siRNA.................................................134
   c. Oligonucleotide ligation...........................................................135
   d. Gel analysis of caged siRNA....................................................135

III. References.................................................................................137
LIST OF TABLES

Table 2.1 Split DNAzyme mismatch sequences and cleavage efficiencies…………… 76

Table 3.1 Mass analysis of TIVA-tags……………………………………………….. 108

Table 3.2 TIVA-tag melting temperatures…………………………………………… 109
LIST OF FIGURES

Figure 1.1  Caged ATP structure and photolysis.............................................. 28

Figure 1.2  Commonly used caging groups...................................................... 29

Figure 1.3  Photoregulation of a DNA polymerase.......................................... 30

Figure 1.4  Light-activatable DNA hairpin scheme.......................................... 31

Figure 1.5  Photoactivatable RNA bandage strategy......................................... 32

Figure 1.6  Design for photoregulating DNAzymes........................................... 33

Figure 2.1  Structures and sequences of DNAzymes......................................... 50

Figure 2.2  Melting curves of DNAzymes......................................................... 51

Figure 2.3  CD spectroscopy of DNAzymes...................................................... 71

Figure 2.4  PAGE analysis of RNA cleavage by half enzymes............................ 72

Figure 2.5  DNAzyme cleavage with different metal ions .................................. 73

Figure 2.6  DNAzyme cleavage assay varying [Mg$^{2+}$].................................... 74

Figure 2.7  PAGE analysis of DNAzyme cleavage............................................. 75

Figure 2.8  PAGE analysis of RNA cleavage by mismatch DNAzymes............... 77

Figure 2.9  Biotinylated split DNAzyme photomodulation................................. 78

Figure 2.10 Photocleavable biotin DNAzyme cleavage assay.............................. 79
Figure 2.11  Photocleavable biotin-streptavidin mismatch DNAzyme assay...........80

Figure 3.1  TIVA-tag design strategy..............................................................106

Figure 3.2  Structures of TIVA-tags...............................................................107

Figure 3.3  TIVA-tag melting curves..............................................................110

Figure 3.4  Melting curves of new TIVA-tags................................................112

Figure 3.5  Emission spectra of TIVA-tags......................................................118

Figure 3.6  PAGE analysis of TIVA-tag uncaging...........................................121

Figure 3.7  HPLC traces of CPP-conjugated TIVA-tags....................................122

Figure 3.8  MALDI spectra of CPP-conjugated TIVA-tags..............................124

Figure 3.9  TIVA-tag uncaging in a cultured neuron......................................126

Figure 3.10  Activation of TIVA-tag in a single dendrite................................127

Figure 3.11  Thermal denaturation curves of TIVA-tag with poly(A)^+ RNA.......128

Figure 3.12  Nanochip analysis of TIVA-tag isolated RNA.............................129

Figure 3.13  Structure of folate-conjugated TIVA-tag...................................130

Figure 3.14  Structure of hairpin-bandage hybrid targeting let-7 microRNA....131

Figure S1.1  Caged siRNA structure and ligation scheme..............................138

Figure S1.2  Mass analysis of caged siRNA..................................................139
Figure S1.3  PAGE analysis of caged siRNA ligation................................. 140
Chapter 1

Introduction to light-activatable oligonucleotides
I. Overview of caged compounds

A “caged” compound can be defined as one whose activity is temporarily blocked by a chemical moiety until it undergoes photolysis. Caged compounds have a chemical group that is covalently attached and rapidly cleaved upon irradiation with light. The most famous caged compound is light-activatable ATP, in which the gamma phosphate is caged with a 2-nitrobenzyl group or 1-(2-nitro)phenylethyl group. In the caged state, ATP cannot be hydrolyzed by ATP-dependent proteins. After near-UV irradiation at 340 nm, the nitrobenzyl group photoisomerizes, causing a bond break with the gamma phosphate and restoring ATP function (Figure 1.1)[1].

The use of caged compounds can allow reactions to be spatially localized, for instance, to single cells, and the time of initiation can also be controlled. In one example, caged ATP was used to study reaction kinetics of contraction and relaxation in muscle fibers[2]. This study highlights the key advantages of light-activatable groups, namely providing higher spatial and temporal resolution for studying complex biological systems than many traditional methods. To date, several small molecules have been caged for use in biological studies; these include Ca$^{2+}$, fluorophores, neurotransmitters, and other nucleotide triphosphates. Caging groups have also been utilized in a vast array of biological studies, such as those examining cell motility and other cellular processes [3].

II. Examples of caging groups

The three most commonly used caging groups are nitrobenzyl, coumarin, and azobenzene (Figure 1.2). Since near-UV light is less invasive and orthogonal to most biological processes, it is important to find groups that can be uncaged at longer
wavelengths. Nitrobenzyl groups used in caged ATP studies absorb at wavelengths below 350 nm, but modifications have been made to improve its uncaging efficiency at longer wavelengths. An improved nitrobenzyl derivative that absorbs at wavelengths greater than 350 nm is the 1-(4,5-dimethoxy-2-nitrophenyl)ethyl compound.

The coumarin caging group is another highly used moiety. Brominated 7-hydroxycoumarin-4-ylmethyl esters and carbamates have been used to make two-photon caged compounds that have led to the first three-dimensionally resolved maps of neuron glutamate sensitivity in intact mouse brain slices\(^4\). For two-photon uncaging, a compound absorbs a pair of photons at IR wavelengths almost simultaneously, as opposed to the one UV photon required for other caging moieties. The longer wavelengths required are less affected by scattering, less damaging to cells and biological samples, and can penetrate tissue more deeply\(^5\). However, there are disadvantages to two-photon uncaging. First, it requires pulsed lasers that can be tuned to the wavelengths required. Additionally, this technique is more synthetically challenging, and the chemistry involved in synthesizing these compounds is not as well-developed.

For reversibly controlling function, azobenzene groups have been utilized\(^6\). At visible wavelengths, azobenzene is in the extended trans conformation, but after UV irradiation, it adopts the cis conformation. While the utility of early azobenzene derivatives suffered because azobenzene cis $\rightarrow$ trans isomerizes rapidly at 37 °C, requiring continuous UV irradiation to maintain the cis conformation, variants with improved thermal stability have been developed recently\(^7\).
More recently, caging techniques have been expanded to develop light-activated oligonucleotides for spatiotemporally regulating gene expression to elucidate complex biological processes. By using photolabile moieties that transiently block DNA or RNA function, it is possible to control the activity of oligonucleotides in a variety of applications. For instance, controlling when or where proteins are expressed will facilitate the study of how they affect cellular development. Furthermore, the ability to isolate total mRNA populations from a single cell at a given point in time will aid in elucidating the link between RNA and cell phenotype.

III. Photoactivatable oligonucleotides

Caging techniques have greatly expanded over the last twenty years going from small molecules to biologically relevant compounds, such as oligonucleotides and proteins. However, caging oligonucleotides is often more challenging than small molecules due to their larger size and complexity. There are currently a few methods to incorporate caging groups. First, if the oligonucleotide is short, the photoactivatable group can be made into a phosphoramidite and incorporated during solid-phase synthesis. Phosphoramidites, in which a base has been modified with a photocleavable group (such as diethylaminocoumarin) at a position on a nucleoside ring\(^8\), have been synthesized, as have linkers that can be added between the bases (resulting in strand breaks post-photolysis)\(^9\). Photoactivatable groups can also be incorporated after oligonucleotide synthesis. Bifunctional linkers that are reactive with nucleophiles, such as thiols or amines, have been successfully added after solid-phase synthesis\(^{10}\). Photocleavable groups that are reactive to the phosphate backbone of the oligonucleotide have also been
Examples of this type include diazoethane derivatives of coumarin and nitrobenzyl groups and 1-(4,5-dimethoxy-2-nitrophenyl)ethyl.

Even though the term “caging” can be a bit of a misnomer in the case of oligonucleotides because caging groups can be incorporated to turn oligonucleotide function “off” or “on” post-photolysis, the term “caged” continues to be used because their function is controlled by light. Photoactivatable oligonucleotides have several applications, which will be discussed in the sections below. These include caging plasmids and messenger RNA to block transcription or translation, caging atoms required for the catalytic activity of nucleic acid enzymes, photomodulating antisense oligonucleotides to control oligonucleotide binding to complementary sequences, photoregulating oligonucleotides for other uses, and indirectly regulating oligonucleotide activity by caging small molecules and cofactors.

a. Caged plasmids and messenger RNA

The first example of a caged plasmid used 1-(4,5-dimethoxy-2-nitrophenyl)diazoethane groups to react with oxygens of the phosphate backbone in a GFP plasmid\[12\]. In this study there were ~270 caging groups per plasmid, and this suppressed most transcription of the DNA in HeLa cells. Unfortunately, the bioactivity of the plasmid was not fully restored after uncaging with 365 nm light. This was likely due to the large number of caging groups present, which required a high dose of light for photo-deprotection, but this led to cell death. Additionally, there was some background GFP expression because the caging reaction is random, resulting in different plasmid structures, with some having no caging groups and others having a large number of them.
In another example, Ando et al. utilized 6-bromo-4-diazomethyl-7-hydroxycoumarin moieties, which form a covalent bond with the phosphate backbone of RNA and label ~30 sites per 1 kb of DNA or RNA\textsuperscript{[13]}. Here, a GFP plasmid was caged, as was GFP messenger RNA. The zebrafish was utilized as a model system because of its transparency, and after transfection with the GFP plasmid, irradiation with UV light resulted in GFP expression, with expression levels increasing with longer irradiation times. They expanded work with this caging group to investigate the role of the \textit{lhx2} gene in zebrafish\textsuperscript{[14]}. While \textit{lhx2} is known to be involved in brain and eye development, its function had previously been difficult to study because zebrafish embryos would become extremely dorsalized with enlarged heads when the gene was overexpressed using traditional methods. This is because overexpression occurs in the whole embryo early in development. By caging \textit{lhx2} mRNA, they successfully overexpressed Lhx2 only in the head at a specific time in development. By controlling overexpression of \textit{lhx2}, the authors were able to rescue knockdown of another gene, \textit{six3}, in the head region at 11 hours post-fertilization. This study is a prime example of how useful caged oligonucleotides are for studying problems in developmental biology.

Since the presence of multiple caging groups requires more irradiation to uncage than would be necessary for a single caging moiety, more recent strategies have aimed to incorporate only one or two caging groups. Recently, a plasmid with only a single caging group was developed\textsuperscript{[15]}. In this design, a DNA primer with a 5’-amine modification was synthesized and incorporated into a GFP plasmid a few bases upstream of the cytomegalovirus (CMV) promoter via PCR. After reacting the amine with a photocleavable biotin, streptavidin was added to sterically block the promoter region of
the plasmid, thus blocking transcription factors from binding. The caging moiety used for this study is the 6-bromo-7-hydroxycoumarin-4-ylmethyl group. In cell studies, enhanced GFP (EGFP) expression increased from 65% of normal levels (i.e. that of non-caged plasmid) prior to irradiation up to 92% after irradiation.

b. Light-regulatable catalytic oligonucleotides

DNAzymes and ribozymes are nucleic acids that are capable of catalyzing chemical reactions. While some ribozymes, such as the hammerhead ribozyme, occur naturally, all known DNAzymes have been evolved by \textit{in vitro} selection. The first example of photoregulating a nucleic acid enzyme incorporated a caged adenosine within an RNA cleaving ribozyme\textsuperscript{[16]}. An adenosine with a caged 2′ hydroxyl was synthesized as a phosphoramidite to allow it to be incorporated during solid-phase synthesis. The 2′-nitrobenzyl cage at this position blocked the ribozyme’s function because the 2′ hydroxyl is required for magnesium dependent nucleophilic attack on the phosphodiester linkage in RNA.

Since the first caged ribozyme, other caged nucleic acid enzymes have been developed. Strategies for caging DNAzymes have included blocking their function by caging their bases because the catalytic mechanisms of DNAzymes are not fully understood. Two of the most studied DNAzymes are the 8-17 DNAzyme and the 10-23 DNAzyme, both of which have been caged by site-specifically incorporating photoactivatable bases at important positions within the enzyme using phosphoramidite chemistry\textsuperscript{[17]}. In both of these studies, DNAzyme function was blocked until uncaging with UV irradiation resulted in RNA degradation, which turned gene expression “off.” In
the case of the 8-17 DNAzyme, an adenosine analogue was incorporated at a position in
the stem loop that is required for activity\textsuperscript{[17a]}. The caged DNAzyme was completely
inactive, but after irradiation, \textasciitilde30\% of activity was restored. Lusic et al. developed a
thymidine phosphoramidite caged with the 6-nitropiperonyloxymethyl (NPOM) group to
introduce into the catalytic core of a 10-23 DNAzyme\textsuperscript{[17b]}. They discovered that
disrupting a single hydrogen bond could completely block enzymatic activity if
strategically placed, and irradiation with 365 nm light could activate the enzyme with
greater than 50\% of the original activity restored. More recently, the Deiters lab has
successfully photoregulated DNAzyme function is mammalian cells\textsuperscript{[18]}. Incorporating
three caged bases into a DNA decoy strand that is complementary to the catalytic loop of
the DNAzyme prevented the decoy strand from binding the DNAzyme until after
irradiation with UV light. After irradiation, the \textit{trans} acting decoy strand hybridizes to
the DNAzyme, which prevents RNA digestion. They also demonstrated that the
complementary strand could be added before the 5′ binding arm of the DNAzyme rather
than as a separate strand.

Additionally, reversible 8-17 and 10-23 DNAzymes with azobenzene have been
developed. This approach can be challenging because the oligonucleotide must function
normally when the azobenzene is in one conformation, but its function must be blocked
when the azobenzene is in the other conformation. However, the ability of azobenzene to
photoisomerize reversibly can be advantageous in some instances since light-activation of
other caging groups irreversibly removes the photoactivatable moiety. Because of this,
reversibly photoregulatable DNAzymes have been developed by replacing important
bases with a nucleotide that is modified with an azobenzene group\textsuperscript{[19]}. Interestingly, the
8-17 DNAzyme displayed higher catalytic activity with azobenzene in the *trans* form, but the 10-23 DNAzyme was more active with the azobenzene in the *cis* conformation. When two azobenzene groups were substituted for nucleotides in the binding arms of an 8-17 DNAzyme, the catalytic rate of the enzyme was approximately five times higher in the *trans* conformation than in the *cis* form\(^{[19a]}\). This is a result of the increased stability of the enzyme-substrate complex due to the ability of *trans*-azobenzene to stack within the DNA double helix, thereby stabilizing it. In the case of the 10-23 DNAzyme, a single nonconserved nucleotide within the catalytic core of the enzyme was replaced with a 2′-deoxyuridylate analogue linked to an azobenzene moiety\(^{[19b]}\). When the enzyme-azobenzene conjugate is in *trans* form, the activity is decreased by nine-fold compared to the wild-type enzyme. After UV irradiation, when the azobenzene is in the *cis* conformation, the enzyme’s cleavage rate approaches that of the unmodified DNAzyme.

More recently, a photoregulatable 10-23 DNAzyme nanomachine was created by adding complementary hairpin sequences incorporating three or four azobenzenes on either side of the DNAzyme’s binding arms\(^{[20]}\). Since the azobenzenes are in the *trans* conformation in visible light, a stable duplex hairpin-like structure forms, and the DNAzyme cannot cleave RNA. However, in UV light, the azobenzenes isomerize, causing the duplex to dissociate. The enzyme is then able to bind and digest its RNA target. Photoregulation of this DNA nanomachine was highly efficient, allowing digestion to be turned completely off after UV irradiation.
c. Caged antisense oligonucleotides

A more recent, widely used caging approach aims to control the activity of antisense oligonucleotides. These are short oligonucleotides comprised of 15-25 nucleotides that hybridize to an mRNA transcript to cause it to be degraded or prevent translation by sterically blocking the ribosome. For example, when a short DNA strand hybridizes to its target mRNA in cells, RNase H is recruited and degrades the mRNA. Modified oligonucleotides, such as RNA with 2’ modifications, morpholinos, peptide nucleic acids, and phosphorothioate DNA, can bind to an mRNA of interest to prevent translation by blocking the ribosome. The technique most commonly used for antisense caging is to block it transiently from hybridizing to its target mRNA.

As discussed previously with caged plasmids, hybridization of antisense oligonucleotides to target mRNA can be prevented by caging the phosphate backbone. Ghosn et al. randomly caged the phosphate backbone oxygens of a 20mer DNA using 1-(4,5-dimethoxy-2-nitrophenyl)ethane\textsuperscript{[21]}. The resulting oligonucleotide was labeled at an average of 14-16 sites, and this was sufficient increase disruption of hybridization to a complementary molecular beacon from 14% of non-caged control to 80% after irradiation with UV light. To achieve more specific backbone labeling, an iodoacetamide derivative of azobenzene has been utilized to cage phosphorothioate DNA\textsuperscript{[22]}. In this example, one base per 16mer was caged, and this resulted in a 7 °C decrease in melting temperature post-photolysis.

Photoactivatable modifications within the nucleotides of caged antisense oligonucleotides can also be used to control activity. For example, incorporation of three
nitrophenyl-modified thymidine phosphoramidites in a 25-nucleotide DNA strand resulted in significant decrease in melting temperature after photocleavage, lowering it by 21.5 °C\textsuperscript{[23]}. Azobenzene modifications have also been created as phosphoramidites. By incorporating three of these moieties in a 22-nucleotide DNA strand, a three-fold increase in RNase H digestion of the target RNA was achieved after UV irradiation\textsuperscript{[24]}. More recently, the Deiters group utilized thymidine phosphoramidites in the synthesis of phosphorothioate DNA\textsuperscript{[25]}. In this study, incorporation of three or four caged residues per 18mer oligonucleotide led to successful gene regulation in cell culture. This group also developed light-activatable morpholino oligonucleotides for controlling EGFP expression in cell culture and in live zebrafish embryos\textsuperscript{[26]}. In this study, four morpholino monomers caged with the 6-nitropiperonyloxymethyl (NPOM) group were included in a 25mer sequence. This design was successful in silencing EGFP expression post-photolysis in cells, zebrafish, and Xenopus embryos.

Another method for using antisense oligonucleotides to achieve gene knockdown is by caging siRNA to control RNA interference (RNAi)\textsuperscript{[27]}. siRNAs are short RNA duplexes comprised of 21-23 base pairs, which can be transfected into cells to achieve mRNA degradation. After transfection, the strands bind to the RNA-induced silencing complex (RISC), which removes the sense strand, aids in hybridization of target mRNA to the antisense strand, and finally degrades the mRNA. In one of the earliest examples of caged siRNA, the phosphate backbone of a siRNA duplex was labeled with the 1-(4,5-dimethoxy-2-nitrophenyl)ethyl group; the duplexes were labeled with an average of 1.4 caging groups to block sterically the siRNA-RISC interaction before photolysis\textsuperscript{[28]}. When the caged siRNA duplexes were co-transfected with a GFP plasmid into HeLa
cells, a two-fold decrease in GFP fluorescence was observed post-photolysis. Unfortunately, this technique of caging siRNA did not completely inhibit RNA interference. This is likely because the caging is not site-specific, so some duplexes are not modified, allowing them to bind the RISC. Another potential negative is that increasing the number of caging groups (which may be required to ensure all duplexes are caged) requires longer irradiation times, and this can lead to phototoxicity. Longer irradiation times also lead to loss of spatial and temporal resolution, which is relevant for many biological applications. Still, this approach was used to label 2'-fluoro modified siRNA duplexes with 7.9 caging groups per 21mer sense strand and nine caging groups per 21mer antisense strand[29]. The 2'-fluorinated residues were used to increase the enzymatic stability of the oligonucleotide in vivo. This is because 2'-fluoro RNA maintains the C3'-endo sugar pucker to preserve the A-form duplex required for RNAi activity. In this example, only ~55% of GFP expression was silenced in cell culture after photolysis, but this was an increase from the ~10% silencing observed prior to irradiation. Additionally, the authors demonstrated that the caged 2'-fluoro modified siRNA could decrease GFP expression after UV irradiation in zebrafish.

Another method developed to cage siRNA involves incorporation of a single caging moiety at the 5' terminal phosphate of the antisense strand because it has been shown to be necessary for binding to the RISC complex[30]. In one example, a nitrophenyl-ethyl (NPE) group was introduced at the 5'-phosphate, reducing siRNA activity to 40% and allowing activity to be fully restored after UV irradiation[31]. Attempting to improve upon this approach, Shah et al. chose to incorporate 1-(4,5-dimethoxy-2-nitrophenyl) ethyl (DMNPE) groups, but they discovered that this caging
group preferentially labels the terminal phosphate groups of oligonucleotides, suggesting that those groups were indeed modified in previous studies that sought to cage the phosphate backbone\textsuperscript{[32]}. In fact, their work suggests that the commonly employed DMNPE group actually reacts poorly with backbone phosphates. By labeling the phosphate groups at the four termini of a siRNA duplex with DMNPE, they achieved up to a six-fold decrease in gene expression. They hypothesize that the incomplete blocking they observed before irradiation is due to Dicer developing a tolerance to the modifications or to a cellular process that can remove the photocleavable groups.

To improve the nuclease stability of siRNA, phosphorothioate residues have been incorporated while also using the DMNPE caging group\textsuperscript{[33]}. With two phosphorothioate linkages per strand at the termini of the duplex, the authors were able to decrease the level of RNAi activity prior to uncaging. In this case, the phosphorothioate groups help prevent nucleases from cleaving the DMNPE group from the oligo, keeping the duplex blocked until near-UV irradiation.

Recent work by the Friedman group has focused on fully blocking RNAi activity prior to photolysis\textsuperscript{[34]}. With this aim, they developed a new photocleavable moiety, cyclo-dodecyl (dimethoxy-2-nitrophenyl)ethyl (CD-DMNPE), which was designed to completely block RNAi machinery from interacting with the siRNA. CD-DMNPE labels the terminal phosphates just like the commonly used DMNPE, but this caging group is bulkier due to the addition of a carboxyl synthetic handle and amine groups. The added bulk at all four termini should sterically block Dicer or nucleases that could remove the caging group. When modified siRNA duplexes targeting GFP were tested in cell
monolayers, they were completely blocked from participating in the RNAi pathway prior to irradiation, but after photolysis, native activity was fully restored.

Additional work with other photocleavable groups, such as biotin, amines, or linkage to the sense strand at the 5’-end of the antisense strand, have shown that these approaches reduce siRNA activity, but they do not completely abolish it. This could perhaps be because the caging groups only block one of the charges on the phosphate, leaving another oxygen unmodified and potentially capable of binding to the RISC\textsuperscript{[35]}. As demonstrated with other antisense oligonucleotide approaches, caged nucleobases have been utilized to photomodulate siRNA activity. For instance, incorporation of caged guanosine or thymidine at positions 9-11 of the antisense strand has led to complete photomodulation of activity\textsuperscript{[36]}. Because RNA cleavage occurs opposite the tenth and eleventh residues of the antisense strand, this design results in a bulge that inhibits RNAi, turning it completely off prior to irradiation. Activity is fully restored post-photolysis.

d. Photoregulatable aptamers

Spatiotemporal control of aptamers is another application of light-switchable oligonucleotides that has been developed. Aptamers are short, single-stranded oligonucleotides that fold into well-defined shapes upon binding to their target small molecules. Heckel et al. employed caged thymidine nucleobases to photoregulate an anti-thrombin aptamer\textsuperscript{[37]}. They chose this 15mer ssDNA because it is one of the most extensively studied aptamers to date, and it has a unique, stable G-quartet fold comprised of six thymidine and nine guanosine nucleotides. By incorporating a single NPP-caged
thymidine in different locations, it was discovered that thrombin did not interact with any of the caged aptamers before irradiation, but binding was almost completely restored after uncaging.

In another study, a coumarin derivative, 6-bromo-4-diazomethyl7-hydroxycoumarin (Bhc-diazo) was used to cage the phosphate backbone to control the previously studied anti-thrombin aptamer\cite{38}. With an average of 6.8 Bhc caging groups per 15mer aptamer strand, the caged aptamer still had approximately 14\% affinity for thrombin; uncaging only restored up to 60\% affinity compared to the unmodified aptamer. While the aptamer's activity was not fully restored post-photolysis, this caging group does offer the advantages of synthetic ease of and two-photon uncaging potential.

In yet another study, a caged aptamer targeting cytohesin was designed\cite{39}. This study was interesting in that the authors were able to generate a light-controlled aptamer without any prior knowledge of the structure or interactions of the aptamer with its target protein. Cytohesin-1 was chosen as the target because \textit{in vitro} selection methods aimed at discovering an RNA aptamer towards it had already proven successful. After successful aptamer development, it was discovered that a single nucleotide caged with either the NPP or NPE groups was sufficient to prevent the aptamer from binding cytohesin-1.

e. \textbf{Other photoactivatable oligonucleotides}

Early photomodulation of oligonucleotides was developed to assist with purification or characterization. Introduction of a nitrobenzyl moiety during solid-phase synthesis allowed for the creation of light-induced strand breaks. This design allowed for
tags, such as fluorophores and biotin, to be removed from the oligonucleotide by photolysis when they are no longer necessary. The photocleavable groups can also be incorporated as protecting groups during solid-phase synthesis\textsuperscript{[40]}. Rather than using the standard treatment of ammonium hydroxide to cleave oligonucleotides from solid support and remove the protecting groups, irradiation can be used. In 1996, Olejnik et al. developed a photocleavable biotin (PCB) phosphoramidite to aid in purification and phosphorylation of oligonucleotides\textsuperscript{[41]}. The PCB was incorporated on the 5′-terminal phosphate during solid-phase synthesis, allowing the full-length oligonucleotide to be isolated via incubation with immobilized streptavidin. To attain the pure desired product, the oligonucleotide was then irradiated with 300-350 nm light, which cleaved the PCB moiety. In another study, a photocleavable spacer comprised of a 2-nitrobenzyl group was introduced to connect a DNA strand to a fluorescein at the 5′-end\textsuperscript{[42]}. By also incorporating a 3′-biotin, the oligonucleotide was first immobilized on a streptavidin-coated glass surface. After irradiating the sample with UV light and washing thoroughly, the change in fluorescence before and after photolysis could be measured. This study found an almost 80% decrease in fluorescence after photolysis and laid the groundwork for utilizing a dye-labeled photocleavable nucleotide for DNA sequencing\textsuperscript{[43]}.  

Strand breaks such as these have also been utilized to identify single base mismatches. Since single nucleotide polymorphisms (SNPs) may be responsible for some types of genetic diseases, a rapid and inexpensive detection method would be highly useful. In one technique, a fluorescence detection assay was developed to identify single-base mismatches by exploiting the lower melting temperatures of strands with mismatches after photolysis\textsuperscript{[44]}. A DNA strand with a centrally-located photocleavable
group (5′-o-nitrophenyl thymidine) and a Disperse Red 1 detection tag at the 5′-end was immobilized on agarose so that the target strand did not require modification in any way. After hybridization of the target strands and UV irradiation, the tag remained bound to the solid support in the case of a fully matched strand, but the red tag was released into solution in the mismatched case. This assay was effective in detecting even a single mismatch after a two hour hybridization and subsequent ten minute irradiation time.

Recently, caged oligonucleotides have been utilized in the development of nanodevices. For example, an azobenzene-modified reversible DNA nanotweezer has been developed\cite{45}. When the azobenzene groups were in the trans configuration, the tweezers were closed. Upon UV irradiation, the azobenzene groups isomerized, opening the tweezers. DNA nanoscissors have also been brought under the control of light\cite{46}. Work by Tsai et al. combined a nanoparticle core with a monolayer of hydrazone-modified triplex-forming oligonucleotides, which are able to capture and cleave their target DNA duplex after irradiation at 460 nm.

Other caged oligonucleotides have been utilized to control PCR product generation. The Deiters lab incorporated caged thymidine residues into DNA primers, disrupting DNA hybridization until after photoactivation\cite{47}. This allowed them to temporally control activation of the PCR reaction. Additional work by Tanaka et al. also controlled PCR with light via incorporation of a caged thymidine into a PCR primer\cite{48}. The caged nucleotide was incorporated site-specifically to block DNA polymerase under PCR conditions, so the elongation products of the PCR cycles before irradiation were terminated at the caged residue. After UV irradiation to remove the NPP caging groups,
sticky ends were created at the end of the PCR products. These products were then ligated into an expression plasmid following digestion with restriction enzymes.

Additionally, photomodulation of nucleic acid folding has been achieved. Better understanding of DNA and RNA folding can be useful for discovering important tertiary contacts and for differentiating between local and global structure formation. Controlling this folding with light may lead to real-time monitoring of folding kinetics. As a proof-of-concept study, the Silverman lab incorporated 2-nitrophenyl ethyl (NPE) caged RNA nucleotides to study the folding of *Tetrahymena* group I intron RNA, whose tertiary structure is already well-defined\(^{[49]}\). Each of the four RNA nucleotides was caged, and one caged base per RNA strand was incorporated Strategically based upon the molecular X-ray crystal structure. The caging groups were successful in disrupting global folding of the RNA, and folding was restored upon photolysis. The Heckel group opted to probe the G-quadruplex formation of a common sequence used for studying telomeric DNA\(^{[50]}\). Different dG nucleotides were replaced with NPP-caged ones, and it was discovered that substitution of just one residue in the core of the sequence was enough to prevent G-quadruplex formation.

**f. Indirectly photomodulated oligonucleotides**

Toyocamycin, a small molecule inhibitor of a self-cleaving ribozyme, was caged to control expression of a luciferase mRNA transcript\(^{[51]}\). In this design, a self-cleaving ribozyme sequence was cloned into the 5′-UTR of a luciferase mRNA to test the efficacy of the caged small molecule. In the presence of toyocamycin, the correct folding of the ribozyme is inhibited. When the caged toyocamycin was co-transfected with the plasmid
in cell culture, the self-cleaving ribozyme was able to cleave itself prior to irradiation, resulting in degradation of the transcript and no luciferase expression. After irradiation, toyocamycin was able to inhibit the ribozyme’s function, so the transcript was intact, allowing for luciferase expression.

Another example is the light-activation of a riboswitch regulatory cofactor. Riboswitches are non-coding RNAs that are usually located in the 5′-UTR of bacterial genes. When a riboswitch binds to its cofactor, usually a metabolite, it undergoes a conformational change, which results in control of gene expression. Recently, glucosamine-6-phosphate (GlcN6P), the cofactor for the glucosamine-6-phosphate synthetase (glmS) riboswitch, was caged\(^{[52]}\). When the glmS riboswitch was cloned into the 3′-UTR of an EGFP plasmid, the riboswitch retained its self-cleaveage activity in the presence of caged GlcN6P. However, after irradiation, the uncaged cofactor reduced the amount of EGFP translated.

Photoregulatable cellular delivery is yet another example of indirect caging of oligonucleotides. An example of this is the modification of cationic gold nanoparticles with a nitrobenzyl group modification\(^{[53]}\). Prior to irradiation, DNA associates with positively-charged nanoparticles with photoactive groups present. After UV irradiation, the nitrobenzyl linkage is cleaved, leaving a negatively charged carboxylate group on the nanoparticle. When this occurs, the negatively changed backbone of DNA is released from the nanoparticle, resulting in gene expression being turned “on.”

In another report, Young et al. used spiropyran, as their light-sensitive small molecule\(^{[54]}\). This chromophore is able to undergo rapid conformational changes to
isomers of different colors. More specifically, in visible light conditions, spiropyran is colorless; after irradiation with UV light, it switches to its purple merocyanine form. Utilizing in vitro selection methods, an RNA aptamer capable of binding to one form of spiropyran was engineered. Upon binding spiropyran, the conformation of the RNA can be reversibly switched by applying UV light, resulting in release of the aptamer.

As these diverse examples and new advances (such as light-controlled molecular beacons\textsuperscript{55} and microRNA inhibitors\textsuperscript{56}) demonstrate, there are multitudes of uses for caged oligonucleotides. However, there are also a number of improvements that are still necessary. Next, the contribution our lab has made in advancing strategies for photomodulating oligonucleotides will be discussed.

IV. Improving photoactivatable oligonucleotides

In some of the first examples of caging oligonucleotides, large excesses of caging groups were used to non-specifically label many sites on plasmids and mRNA. In addition to increasing the cost of synthesis, multiple caging groups are inefficient to uncage and require longer irradiation times. Our group aims to improve upon the existing methods by developing new designs that only require one or two caging moieties. We also incorporate them site-specifically to control oligonucleotide function. Furthermore, we have introduced fluorescent reporters to verify photolysis.

One of our lab’s earliest approaches involved the synthesis of a DNA primer strand with an adjacent photocleavable fluorophore-quencher pair which prevented extension of the primer by DNA polymerase\textsuperscript{57}. In this design, the quencher was removed after photolysis (which was confirmed by an increase in fluorescence) and the
primer was extended (Figure 1.3). In additional work by our lab, Dr. XinJing Tang developed a novel method for controlling oligonucleotide hybridization\cite{note58}. Using a single photocleavable linker to join an antisense DNA to its complementary strand, he was able to block the DNA from binding to its normal RNA target. The photocleavable linker includes a maleimide for reaction with thiols and an NHS ester for reaction with amines. The DNA and blocking strands were synthesized with amine and thiol modifications to facilitate reaction with the linker after synthesis (Figure 1.4). Before photolysis, the complementary strand has a high melting temperature because it is covalently attached to the DNA, but after UV irradiation, the strand is no longer attached and can dissociate. This allows the DNA to bind its target RNA, which is significantly longer than the complementary strand. These photoactivatable DNA hairpins were then used to regulate RNase H-mediated RNA digestion in cells\cite{note59}. This work was further extended to another antisense oligonucleotide, negatively charged peptide nucleic acid, to photoregulate the expression of *bozozok* and *chordin* genes in zebrafish embryos\cite{note60}. The DNA hairpin design necessitates a balance between a high melting temperature between the DNA and blocking strand before photolysis and a low melting temperature afterwards so that the DNA will hybridize preferentially to the RNA and not to the blocking strand. Therefore, optimization was required to determine the length and position of the blocking strand.

While DNA hairpins are useful for turning gene expression “off” after photolysis, Dr. Julia Richards, a previous graduate student in our lab, thought it would be equally useful to turn gene expression “on.” Based on this premise, she sought to develop RNA bandages which are comprised of two 2′-O-methyl (2′-OMe) RNA strands joined by the
aforementioned photocleavable linker\textsuperscript{[61]}. In this design (Figure 1.5), 2′-OMe RNA was employed because previous work studying structural modifications to antisense oligonucleotides that would improve mRNA binding affinity and specificity discovered that this modification of the RNA ribose ring improves both nuclease resistance and mRNA hybridization\textsuperscript{[62]}. The design of RNA bandages enabled them to bind their mRNA target at the start codon and Kozak sequence in the 5′-UTR to block translation until uncaging. Because the timing and location of protein expression in cells are important, controlling these factors could shed light on the function of genes of interest. In this design, the melting temperatures of the two short, tandem oligonucleotides are significantly lowered after photolysis when compared to the caged oligonucleotide. This decreased affinity for the target allows them to melt off the mRNA, allowing the ribosome to bind and translate the mRNA. While the best bandage design resulted in a three-fold increase in translation after photolysis, there were drawbacks to this design. During bandage optimization, it was discovered that there was not a simple correlation between the change in melting temperature and a bandage’s ability to block translation. This could make it difficult to design bandages for sequences with poorly understood secondary structures.

Additional work by Dr. Richards centered around photoregulating the 10-23 DNAzyme\textsuperscript{[63]}. While the 10-23 DNAzyme had been site-specifically regulated previously using caged bases, our lab aimed to turn its activity on and off using a photocleavable spacer. The advantage of this approach is that the photocleavable spacer is commercially available as a phosphoramidite, eliminating the need to perform a lengthy synthesis of caging an individual nucleotide phosphoramidite. This also allows
for the photocleavable group to be incorporated at any desired position within the oligonucleotide. In order to turn the DNAzyme’s activity “off,” two photocleavable spacers were incorporated within the DNAzyme, one in a binding arm and one within the catalytic core (Figure 1.6). To create a DNAzyme that can be turned “on,” a circular DNAzyme linked to a complementary strand with two photocleavable spacers was made. After UV irradiation, the blocking strand melts off the DNAzyme, leaving the enzyme free to bind and cleave its target RNA. These strategies should facilitate the development of DNAzymes for numerous uses in biotechnology.

V. Research Aims

Work presented in this dissertation demonstrates novel ways of caging oligonucleotides. In chapter two, a new method for photoregulating a 10-23 DNAzyme and characterization of its catalytic loop are discussed. The third chapter presents a new design for improving caged hairpins with the addition of a second photocleavable linker and a FRET pair (to monitor dissociation post-photolysis) along with results from its application in neurobiology. Finally, a new technique for caging siRNA by enzymatically circularizing it is proposed, and the initial synthesis, purification, and ligation schemes are presented.
VI. References


**Figure 1.1** Caged ATP structure and photolysis.
Figure 1.2 Structures of caging moieties commonly used for oligonucleotides. Wavy lines indicate the point at which the oligonucleotide is attached. A) Nitrobenzyl group and common derivatives, B) Coumarin group with derivatives, and C) Azobenzene.

A) \[
\begin{align*}
\text{NO}_2 \quad \text{R}_1 \\
\text{R}_2 \\
\end{align*}
\]

\[\text{R}_1 = \text{H, Me} \]
\[\text{R}_2 = \text{H, OMe, -CH}_2\text{OCH}_2\text{-} \]

B) \[
\begin{align*}
\text{R}_1 & \quad \text{R}_2 \\
\text{R}_1 & \quad \text{R}_2 \\
\text{O} & \quad \text{O} \\
\text{R}_1 & \quad \text{R}_2 \\
\text{OH} & \quad \text{OH} \\
\text{O} & \quad \text{O} \\
\end{align*}
\]

\[\text{R}_1 = \text{H, Br} \]
\[\text{R}_2 = \text{OH, OMe, NMe}_2 \]

C) \[
\begin{align*}
\text{N} & \quad \text{N} \\
\text{N} & \quad \text{N} \\
\text{N} & \quad \text{N} \\
\text{N} & \quad \text{N} \\
\end{align*}
\]

\[\text{UV} \quad \text{Vis} \]
Figure 1.3  A) Strategy for photoregulation of primer extension using Klenow fragment (KF). Adjacent cytidines were modified with fluorescein (green) and a photocleavable dabsyl (red) prevent DNA polymerase from extending the primer. UV irradiation removes dabsyl, which allows KF to elongate the primer and fluorescein to fluoresce green. B) Structure and photolysis products of photocleavable dabsyl. 

*This figure was adapted from X. Tang et al. Bioorg. Med. Chem. Lett., 2005, 15, 5303-5306.*
Figure 1.4  A) Scheme for the design of light-activated DNA hairpins. An antisense oligonucleotide (red) is blocked from binding mRNA (purple) by a complementary strand (black) joined by a photocleavable linker (green). The length and position of the complementary blocking strand can be varied to optimize hybridization. After photolysis, the antisense oligonucleotide is liberated, allowing it to bind its mRNA target. B) Structure of the heterobifunctional linker.

*This figure was adapted from X. Tang et al. Angew. Chem. Int. Ed.,** 2006, 45, 3523-3526.
Figure 1.5 General strategy for making caged RNA bandages. Bandages comprised of two short 2′-OMe RNA strands complementary to an mRNA target are linked via a photocleavable linker. Before irradiation, the bandages blocked translation. After photolysis, the strands melt off, restoring translation. *This figure was adapted from J. Richards et al. Bioorg. Med. Chem. Lett., 2008, 18, 6255-6258.*
Figure 1.6  Design for photoregulating the 10-23 DNAlzyme. A) Two photocleavable groups (gray box) placed in the binding arm and catalytic core of a DNAlzyme prevent the enzyme from cleaving RNA after UV irradiation. B) A circular DNAlzyme linked to a complementary strand via two photocleavable spacers cannot cleave its RNA substrate until activity is restored with UV light.

This figure was adapted from J. Richards et al. ChemBioChem., 2010, 11, 320-324.
Chapter 2

Mismatch discrimination and efficient photomodulation of split 10-23 DNAymes

I. Introduction

Since Breaker and Joyce’s report of an RNA-cleaving DNAzyme in 1994\cite{1}, DNAzymes have become important biochemical tools\cite{2} and have been utilized for applications as diverse as RNA ligation\cite{1} and Diels-Alder reaction catalysis\cite{3}. Additionally, recent advances have succeeded at creating DNAzymes that are promising biosensors\cite{4} and potential therapeutic agents for gene suppression\cite{5}. Furthermore, by cloning the 10-23 DNAzyme into a plasmid, progress has been made in creating DNAzymes that do not require exogenous delivery\cite{6}.

One of the most widely studied DNAzymes is the 10-23 DNAzyme, which can cleave RNA at a junction between purine and pyrimidine bases, preferentially A–U\cite{7}. The 10-23 DNAzyme structural motif consists of a 15-nucleotide (nt) catalytic loop flanked by two 6-12-nt binding arms that are complementary to the RNA target (Figure 2.1). Enzyme activity is dependent on divalent metal ion binding to the catalytic loop\cite{1,8}. The catalytic loop has been studied by deletion\cite{9} and mutation\cite{10} of residues, and by substitution of the backbone phosphates with phosphorothioates\cite{11}. However, the structure of the loop and its catalytic mechanism require further characterization. The existing crystal structures are not believed to represent the active conformation of the loop, which is likely very flexible\cite{12}. Our lab’s recent discovery that the 10-23 DNAzyme can work as two separate ‘a’ and ‘b’ strands (Figure 2.1) exemplifies this flexibility\cite{13}. Here, we sought to expand our understanding of the structural requirements of the split 10-23 DNAzyme and investigate its potential for mismatch discrimination and photoregulation.
The ability to function in two parts is not unique to the 10-23 DNAzyme, as it has also been demonstrated with peroxidase-like DNA enzyme\textsuperscript{[14]}, DNAzyme E6\textsuperscript{[15]}, and deoxyribozyme ligase\textsuperscript{[16]}. Furthermore, multi-component nucleic acid enzymes (MNAzymes), which become catalytically active when an assembly facilitator is added to the partial enzymes, have been developed\textsuperscript{[17]}. Additionally, two-component DNAzymes consisting of 2’-O-methylribonucleotide effectors and 10-23 DNAzymes with shortened 3’ binding domains have been engineered\textsuperscript{[18]}. These notable examples motivated us to investigate further the structural requirements of the 10-23 DNAzyme.

We chose a DNAzyme with binding arms that target VEGFR2 RNA, which was shown previously by Zhang et al. to achieve gene knockdown in cell culture and mice\textsuperscript{[5a]}. We determined that this full-length DNAzyme exhibits activity even at low concentrations of the requisite divalent metal ion. This favors its use for \textit{in vivo} studies, and also provides a useful platform for designing split DNAzymes. We explored the 10-23 DNAzyme’s functionality as separate strands by dividing the enzyme between different residues of the catalytic loop to generate fourteen split DNAzymes, Dz1-Dz14 (Figure 2.1). The split DNAzymes cleaved RNA with efficiencies that depended on where the loop was divided. Split DNAzymes divided at the middle of the catalytic loop (Dz7, Dz8) showed activity comparable to the full-length enzyme (Dz0). Because of the short length of the binding arms (9-nt), the split enzymes were more sensitive than Dz0 to single base mismatches with the target RNA. Much higher metal ion concentrations were required to maintain activity than observed with the full-length DNAzyme.

After determining the best cleaving half enzymes, we aimed to design a photoregulatable DNAzyme that could be turned “on” upon irradiation. RNA-cleaving
DNAzymes, such as the 10-23 DNAzyme, have been demonstrated to be promising gene silencing agents\cite{5a, 19}, motivating the development of light-controlled DNAzymes for possible biological applications\cite{5b}. In a pioneering example\cite{20}, Lusic et al. disrupted DNAzyme function by incorporating photoresponsive bases within the 8-17 and 10-23 DNAzymes using phosphoramidite chemistry. When the DNAzymes were photolyzed, the caging groups were removed, which activated enzyme activity and turned gene expression “off”. Additionally, DNAzymes that can be activated and deactivated with light in mammalian cells have been developed\cite{19}. To achieve this, caged thymidine residues were site-specifically incorporated within the DNAzyme or a DNA decoy strand. In order to turn DNAzyme function from “on” to “off,” caged DNA decoys or hairpin DNAzymes with thymidines in positions that prevent hairpin formation prior to irradiation were employed. To activate the DNAzyme, caged residues were incorporated within the catalytic loop to prevent the DNAzyme from cleaving RNA or within the binding arms to prevent hybridization to the target.

While several techniques for turning DNAzyme function from “on” to “off” have been developed\cite{13, 19}, there are fewer that allow DNAzyme function to be turned “on”\cite{21} using a single photoactive group\cite{21a, 21c}, as we have shown can achieve very efficient photomodulation of oligonucleotide function\cite{22}. Ting et al. pioneered the design of a photoregulatable DNAzyme with a single caging moiety, however the 280 nm light required to activate this enzyme is undesirable for most biological studies\cite{21c}. The photoswitchable DNAzyme designed by Keiper et al. incorporated a single azobenzene-modified nucleotide in the catalytic loop\cite{21a}. We previously discovered that the activity of the full-length 10-23 DNAzyme can be turned from “off” to “on” upon 365 nm
irradiation by attaching a complementary strand via two photocleavable linkers and circularizing the conjugate using Circligase\cite{13}. Similar approaches employing a single photocleavable linker joining an oligonucleotide to a complementary blocking strand have been designed\cite{22c, 23}. In our previous approach, the DNAzyme was no longer covalently bound to the blocking strand after near-UV irradiation, allowing it to cleave its RNA target. However, native activity was not fully restored post-photolysis, based on lingering hybridization of the DNAzyme to the sense strand. This motivated the search for alternate photoregulation approaches. Previous work by Stojanovic et al. showed that incorporating a biotinylated dT in deoxyribozyme E could block activity in the presence of streptavidin\cite{24}. Similarly, a biotinylated caging agent was used to recruit streptavidin to block transcription factors from binding to a promoter sequence of an expression plasmid\cite{25}. In our study, efficient photoregulation of a biotinylated split 10-23 DNAzyme was achieved by employing streptavidin to disrupt the active conformation. Unlike in previous strategies, the DNAzyme with photocleavable biotin can be constructed entirely from commercially available reagents in high yield using solid-phase DNA synthesis.

II. Experimental procedures

a. RNA cleavage by DNAzymes

Single turnover reactions (total volume 24 µL) were performed at 37 °C with 50-fold excess of DNAzyme to RNA substrate. DNAzyme (4.2 µM) and 0.083 µM P-32 labeled 28-nt RNA substrate (5′-GCGCGAGGUGCAGG AU GGAGAGCAAGGC-3′, A–U cleavage site highlighted in bold) were reacted in standard buffer, defined here as 10
mM Tris buffer (pH 7.5) with 10 mM MgCl₂ and 83 mM NaCl. Oligonucleotides were obtained from Integrated DNA Technologies. Aliquots (4 µL) were removed after one hour, at which point the reaction was quenched with 6 µL RNA loading buffer II (Ambion). The aliquots were analyzed by polyacrylamide gel electrophoresis (PAGE) using a 7 M urea, 20% polyacrylamide gel at 300 V for 40 minutes. Gels of RNA digestion were imaged using an Amersham Biosciences Storm 860 phosphorimager. Gels were subsequently analyzed using TotalLab Software (Nonlinear Dynamics) to detect the band intensities and correct for background using the rubber band subtraction function. Finally, the ratio of intensities of the uncleaved and cleaved RNA bands was calculated.

b. Thermal denaturation of DNAzyme-RNA hybrids

Thermal denaturation studies were conducted in standard buffer with a 19-nt RNA sense strand (5′-AGGUGCAGGAUGGAGAGCA-3′) (1 µM) incorporating a non-cleavable 2′-O-methylated (2′-OMe) adenosine (highlighted in bold) at the typical scission site. To hybridize the split DNAzyme (1 µM) to this RNA target, solutions were heated to 95 °C for five minutes in a water bath and then allowed to cool to room temperature. Melting studies were conducted on a Beckman Coulter DU800 UV-Vis spectrophotometer equipped with a programmable Peltier temperature controller. Samples were monitored at 260 nm while being heated or cooled at a rate of 1.0 °C min⁻¹, with a one minute hold per degree Celsius. Melting temperatures were determined from the peak of the first derivative plot of A₂₆₀ versus temperature.
c. **Circular dichroism spectroscopy of DNAzyme-RNA hybrids**

Circular dichroism (CD) spectroscopy was performed using an Applied Photophysics Chirascan instrument. Samples of 2 µM DNAzyme and 2 µM 19-nt non-cleavable 2′-OMe RNA target were prepared in standard buffer. Samples were pre-annealed by heating to 95 °C for five minutes and cooling slowly to room temperature.

d. **Photoactivation of biotinylated split DNAzymes**

The RNA cleavage assay (total volume 24 µL) was performed in standard buffer with equimolar DNAzyme halves Dz7a or Dz8a (4.2 µM) and photocleavable 5′-biotinylated Dz8b. The solution was irradiated by UV transilluminator (9 mWcm^{-2} at 365 nm) for 20 minutes. Non-irradiated control reactions were also prepared. Next, streptavidin (100 pmol, equimolar with DNAzyme) was incubated in the reaction mixture at 37 °C for 30 minutes, P-32 labeled RNA substrate (0.083 µM) was added, and the reaction was allowed to proceed for one hour at 37 °C. Aliquots (4 µL) were analyzed by PAGE as described above.

III. Results and discussion

a. **Split DNAzyme characterization**

The full-length 10-23 DNAzyme in this work (Dz0) has 18 bases complementary to the target RNA, while the split DNAzymes have separate binding arm regions that are each just nine nucleotides long. Thermal denaturation experiments (Figure 2.2) confirmed that the split DNAzymes produce lower melting temperatures, relative to Dz0, when hybridized to a 19-nt non-cleavable RNA target. Melting temperatures ($T_m$) for
Dz1-Dz14 ranged from 54 °C to 57 °C, compared to 65 °C for Dz0. We also performed circular dichroism (CD) spectroscopy of Dz8, Dz0 and DNAzyme halves Dz8a or Dz8b, hybridized to the same non-cleavable RNA target (Figure 2.3). CD spectra of the DNAzyme-RNA hybrids (Figure 2.3) agreed with the previously published CD spectrum of Dz0 by Cieslak et al\textsuperscript{[26]}. The asymmetric peak at 270 nm, the negative effect at 244 nm, and the peak at 223 nm are characteristic of DNAzyme-RNA hybrids\textsuperscript{[26]}, and are also consistent with our previous characterization of Dz0\textsuperscript{[13]}.

b. Activity of split DNAzymes

We tested mixtures of split DNAzymes lacking the internal phosphate group, where the DNA was divided into hydroxyl-terminated ‘a’ and ‘b’ strands at every residue of the catalytic core (Figure 2.1C). The reaction was initiated upon addition of RNA to the DNA ‘a’ and ‘b’ strands, without pre-incubation. The large excess of DNAzyme was used to find reaction conditions with even low-level activity, in order to guide enzyme optimization.

Dividing the catalytic loop between nucleotides 7 and 8 (Dz7), 8 and 9 (Dz8), 11 and 12 (Dz11), and 12 and 13 (Dz12) produced very functional split DNAzymes. Dz11 and Dz12 cleaved greater than 25% of the RNA target after one hour incubation, whereas the most efficient split enzymes (Dz7, Dz8) cleaved approximately 50% (Figure 2.4). Under the same conditions, the full-length enzyme (Dz0) cleaved 75% of the RNA target. Split DNAzymes divided between nucleotides 6 and 7 (Dz6), 9 and 10 (Dz9), 13 and 14 (Dz13), and 14 and 15 (Dz14) cleaved 4-10% of the RNA. Dividing the DNAzyme at other loop positions did not produce measurable activity.
c. Metal ion requirements of split DNAzymes

To investigate further the functionality of the split catalytic loop, we tested the activity of Dz0 and Dz8 in the presence of various divalent metal ions. While the 10-23 DNAzyme was originally discovered to be Mg$^{2+}$ dependent, studies have shown a T8 mutant has comparable activity with Ca$^{2+}$[9]. We found that Dz0 and Dz8 were able to cleave the RNA target in the presence of Mg$^{2+}$, Mn$^{2+}$, and Ca$^{2+}$, with Dz8 somewhat less active in each case (Figure 2.5). Furthermore, the split enzymes were found to have similar melting temperatures with each of these metal ions (Figure 2.2). We also tested DNAzyme activity with Na$^{+}$ ion to rule out any contribution of this monovalent cation to RNA cleavage. Finally, Zn$^{2+}$ was found to elicit no activity in Dz0 or Dz8, as has been seen previously with the 10-23 DNAzyme[8]. Next, we varied the concentration of Mg$^{2+}$ ion to determine the optimal RNA cleavage conditions. Reactions with Dz0 resulted in significant cleavage with Mg$^{2+}$ ion concentrations as low as 0.01 mM. Neither Dz0 nor Dz8 were active when no Mg$^{2+}$ was added to the buffer (Figure 2.5). However, Dz8 showed modest activity at 3 mM Mg$^{2+}$ and showed high activity only at concentrations above 5 mM Mg$^{2+}$ (Figure 2.6). The requirement for much higher magnesium ion concentration (in the background of high ionic strength standard buffer) suggests that the split DNAzymes have lower affinity for Mg$^{2+}$ ions that are critical for catalytic activity.

The finding that the full-length DNAzyme is still active at 0.01 mM Mg$^{2+}$ was initially surprising, as Cieslak et al. previously discovered that the 10-23 DNAzyme targeting β3 Integrin mRNA is only active at Mg$^{2+}$ concentrations at or above 0.5 mM[26]. Concerns that residual Mg$^{2+}$ ions may remain after the T4 polynucleotide kinase reaction to P-32 label the RNA substrate led us to test the ability of the DNAzyme to cleave a
non-labeled RNA substrate (Figure 2.7). This experiment confirmed our previous findings, as the full-length enzyme was still able to cleave the RNA substrate. While this activity at low Mg\(^{2+}\) concentration was unexpected, it is noteworthy that successful cellular experiments by Zhang et al. were performed with the same DNAzyme employed in our work\[^{5a}\]. Since this DNAzyme was able to function at intracellular Mg\(^{2+}\) concentrations, which are approximately 0.5 mM for free Mg\(^{2+}\), it is conceivable that it could exhibit catalytic activity at the low concentration demonstrated here. The difference in Mg\(^{2+}\) requirements for these different 10-23 DNAzymes may be due to differences in binding arm sequences. This hypothesis is supported by a previous study which discovered that two DNAzymes that both target telomerase mRNA have large differences in their optimal Mg\(^{2+}\) concentrations\[^{27}\]. One enzyme is most efficient at 1 mM and the other at 20 mM. Upon further investigation, the enzymes showed little difference in Mg\(^{2+}\) requirements once the DNAzymes had hybridized to their targets.

**d. Split DNAzyme mismatch detection**

To assess mismatch discrimination, we tested both the full-length DNAzyme (Dz0) and the split DNAzyme with the highest activity (Dz8) by introducing one noncomplementary base at different positions within the binding arms of both enzymes (Table 2.1). Gel electrophoresis showed that the intact 10-23 DNAzymes were able to cleave the 28-nt RNA target efficiently in all cases, with the original, full-length DNAzyme sequence (Dz0, Table 2.1) yielding very similar cleavage efficiencies to the seven full-length mismatch sequences (m1-m7, Table 2.1). Yields of RNA cleavage ranged from 70-80% for most sequences tested, but decreased to 50-60% when the mismatch was closest to the catalytic loop, in m3 and m4 (Table 2.1, Figure 2.8A).
By comparison, considerably higher mismatch discrimination was observed for the split DNAzymes. The seven split DNAzymes with mismatch sequences (m1-m7) demonstrated significantly less RNA cleavage relative to the comparable full-length DNAzymes in all cases, with greater than 20% cleavage observed only for split DNAzymes m1, m6, and m7 (Table 2.1, Figure 2.8B).

e. Photoactivatable split DNAzyme

Having determined that Dz7 and Dz8 give the highest activity among the split DNAzymes, we hypothesized that split DNAzyme activity could be inhibited by placing a biotin moiety near the center of the catalytic loop (Figure 2.9A). We incorporated a photocleavable biotin at the 5' end of Dz8b and performed the standard cleavage reaction by adding Dz8a and the 28-nt RNA target. A small amount of DNAzyme activity was retained when biotinylated Dz8b was paired with Dz8a (Figure 2.10). However, when the biotinylated split DNAzymes (Dz7a + Dz8b or Dz8a + Dz8b) were incubated with streptavidin, all catalytic activity was lost (Figure 2.9C). We tested Dz7a because our previous study demonstrated that substituting the thymine in position 8 in the catalytic loop with a photocleavable spacer resulted in a highly active enzyme upon photolysis\textsuperscript{[13]}. Activity was restored by irradiating at 365 nm, which cleaved the biotin moiety and allowed separation from the streptavidin blocking agent. Interestingly, DNAzyme activity was restored to 60% RNA cleavage for Dz7a and 39% RNA cleavage for Dz8a upon photolysis (Figure 2.9C). This increased activity with Dz7a and Dz8b has been seen previously when residues within the full-length 10-23 DNAzyme were replaced with a photocleavable spacer and then irradiated\textsuperscript{[13]}. Notably, the 60% activity observed post-
photolysis for Dz7a + Dz8b equates to complete restoration of native activity for this split DNAzyme. In this example, we succeeded in turning the DNAzyme from completely “off” (< 1% activity) to completely “on”, with no apparent interference from the biotin-streptavidin pair. The recovery of activity when Dz8b was no longer covalently linked to biotin-streptavidin suggests a lack of non-specific binding of streptavidin to the oligonucleotides or metal ions in solution. An added benefit of regulating a split DNAzyme rather than Dz0 is that the split enzymes retain their sensitivity to single base mismatches post-photolysis (Figure 2.11).

IV. Conclusions

In conclusion, we have demonstrated that the best split DNAzymes (Dz7, Dz8) are nearly as active as the full-length DNAzyme (Dz0) (Figure 2.4). Our discovery that the most active split enzymes are those divided after the 7th or 8th nucleotide is consistent with earlier observations that the full-length DNAzyme best tolerates mutations and deletions closest to the center of the catalytic core\cite{10}. This flexibility of the catalytic loop is not unique to the 10-23 DNAzyme: studies of the 8-17 DNAzyme have demonstrated that its catalytic domain can be modified to contain only guanosine and cytidine residues while still retaining detectable enzymatic activity\cite{28}, and an extensive mutational analysis of its catalytic core has shown the ability to retain activity\cite{29}. Additionally, we have established that the full-length 10-23 DNAzyme targeting VEGFR2 mRNA is capable of retaining enzymatic activity at Mg\(^{2+}\) concentrations as low as 0.01 mM, providing support for the use of DNAzymes in cellular studies. We further demonstrated that the recently discovered ability of DNAzymes to function as split enzymes imparts greater selectivity towards the RNA target. Tuning the affinity of the short ‘a’ and ‘b’ strands provides a
route for achieving even higher single-base mismatch selectivity. In addition to our experiments with the split 10-23 DNAzyme, Kolpashchikov developed a split peroxidase-like DNAzyme to be used for colorimetric detection of SNPs\textsuperscript{[15a]}. Finally, because photochemical approaches for modulating gene expression have many potential uses in biological systems\textsuperscript{[30]}, we were interested in exploring the utility of photocleavable biotin. Our approach incorporates a photocleavable biotin moiety at a position within the DNAzyme that can tolerate substitution. In the case of the Dz7a + Dz8b split DNAzyme, we showed complete blockage by streptavidin and then complete restoration of activity upon biotin photo-release. The ability of the 10-23 DNAzyme to function in two parts creates flexibility, with new avenues for controlling its function.
V. References


Figure 2.1  A) General scheme for the formation of active DNAzyme from two separate DNA strands. B) Sequences of RNA target (red), full-length DNAzyme (Dz0, with sticky arms in black and catalytic loop in blue). C) Sequences of Dz0 and split DNAzymes (Dz1-Dz14), with loop residues indicated in blue.
Figure 2.2  Melting temperature data for DNAzymes hybridized with 19-nt RNA target with a 2′-OMe adenosine at the cleavage site. Melting temperature studies were performed in standard DNAzyme buffer (10 mM Tris buffer, pH 7.5, with 10 mM MgCl₂ and 83 mM NaCl). For studies testing different metal ions, the 10 mM MgCl₂ was replaced with 10 mM CaCl₂, MnCl₂, ZnCl₂, or NaCl. Melting temperatures could not be determined with MnCl₂ or ZnCl₂.
Dz3

Absorbance

Temperature (°C)

Dz4

Absorbance

Temperature (°C)
Dz0 in Ca\(^{2+}\) buffer

Dz0 in Na\(^{+}\) buffer
Dz0 mismatch 1

Dz0 mismatch 2
Dz8 mismatch 4

Temperature (°C)

Absorbance

Dz8 mismatch 5

Temperature (°C)

Absorbance
Figure 2.3  Circular dichroism spectroscopy was performed in standard DNAzyme buffer with 2 µM DNAzyme and 2 µM 19-nt RNA target with a 2′-OMe adenosine at the cleavage site. Samples were pre-annealed by heating to 90 °C and cooling slowly to room temperature. The asymmetric peak at 270 nm, the negative effect at 244 nm, and the peak at 223 nm are characteristic of DNAzyme / RNA hybrids.
Figure 2.4  P-32 labeled RNA visualized by 7 M urea, 20% PAGE after one hour reaction with full-length Dz0 and split DNAzymes, Dz 1-Dz14. Dz7, Dz8, Dz11, and Dz12 cleaved greater than 25% of target RNA. Data are averages of three independent trials, with bars indicating the range of values.
Figure 2.5  Cleavage of the 28-nt RNA substrate by Dz0 or Dz8 was investigated in the presence of various metal ions. P-32 labeled RNA was imaged on 7 M urea, 20% polyacrylamide gel after 1 h reaction with DNAzymes. Single turnover assays were performed at 37 °C with 4.2 µM DNAzyme, 0.083 µM RNA in standard buffer with 10 mM MgCl$_2$, MnCl$_2$, CaCl$_2$, NaCl, or ZnCl$_2$. 

<table>
<thead>
<tr>
<th>DNAzyme</th>
<th>Mg$^{2+}$</th>
<th>Mn$^{2+}$</th>
<th>Ca$^{2+}$</th>
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Figure 2.6  P-32 labeled RNA substrate on denaturing polyacrylamide gel electrophoresis (20% PAGE / 7 M urea). Cleavage reactions were performed as described previously. Standard DNAzyme buffer was substituted with buffers of various Mg$^{2+}$ concentrations.
Figure 2.7  Full-length 10-23 DNAzyme and RNA substrate on denaturing polyacrylamide gel electrophoresis (20% PAGE / 7 M urea). Single turnover assays were performed at 37 °C with a 50-fold excess of DNAzyme to RNA (1000 pmol : 20 pmol) in standard buffer with 10 mM or 0.01 mM MgCl₂. After one hour, the reaction was quenched with 10 µL RNA loading buffer II (Ambion). The gel was stained with ethidium bromide for analysis.
Table 2.1  Ratio of RNA target cleaved by the intact (Dz0) and split (Dz8) DNAzymes, which also incorporated single base mismatches within the binding arms. These seven new sequences are labeled m1-m7, with mismatch sites underlined and shown in bold font. The gap in the middle of the sequences reflects where the split DNAzymes were divided.

<table>
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<th>DNAzyme Sequences</th>
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</tr>
<tr>
<td>Dz8: tgcetccaGGCTAGCTACAACGAcctgcacct</td>
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</tr>
<tr>
<td>m1: tgcetccaGGCTAGCTACAACGAcctgcacc</td>
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<tr>
<td>m2: tgcetccaGGCTAGCTACAACGAcctgcacca</td>
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</tr>
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</tr>
<tr>
<td>m7: agetctccaGGCTAGCTACAACGAcctgcacct</td>
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</tr>
</tbody>
</table>
Figure 2.8  A) Full-length DNAzymes with single-base mismatches (m1-m7) show comparable activity to Dz0, which is fully complementary to the RNA target. B) Split DNAzymes with single-base mismatches (m1-m7) cleave greater than 20% of RNA only when the mismatch minimally perturbs hybridization, at either the 5′ or 3′ end. Sequences of enzymes with single base mismatches are provided in Table 1. Data are averages of three independent trials, with bars indicating the range of values.
Figure 2.9  A) Schematic of general structure and function of photoactivatable DNAzyme controlled by biotin-streptavidin interaction. B) Structure of biotinylated photocleavable spacer. C) Photocleavable DNAzyme assay. Photocleavable biotinylated Dz8b and streptavidin were added to either Dz7a or Dz8a. Samples were irradiated by UV transilluminator (9 mWcm$^{-2}$ at 365 nm) for 20 minutes prior to addition of RNA. Reactions were incubated at 37 °C for one hour.
Figure 2.10 P-32 labeled RNA substrate on denaturing polyacrylamide gel electrophoresis (20% PAGE / 7 M urea). Cleavage assay was performed as described previously. The Dz8b half with a 5'-photocleavable biotin was used instead of the normal Dz8b.
Figure 2.11 P-32 labeled RNA substrate on denaturing polyacrylamide gel electrophoresis (20% PAGE / 7 M urea). Cleavage assay was performed as described previously. The Dz8b half with a 5'-photocleavable biotin was used instead of the normal Dz8b. Dz7a or Dz8a with single base mismatches incorporated (Table 1) were tested. All samples were irradiated by UV transilluminator (9 mWcm$^{-2}$ at 365 nm) for 20 minutes. Streptavidin (100 pmol, equimolar with DNAzyme) was incubated in the reaction mixture at 37 °C for 30 minutes, P-32 labeled RNA substrate was added, and the reaction was allowed to proceed for one hour at 37 °C.

A)

<table>
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B)

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Chapter 3

Caged hairpin-bandage hybrids for transcriptome *in vivo* analysis
I. Introduction

a. Background

The caged hairpins and bandages previously developed by our lab accomplished their basic purposes, but they both required a good deal of optimization to achieve high melting temperatures before photolysis and lower ones afterwards. Unfortunately, there were also a number of other factors that determined how well each of these designs controlled gene expression. For instance, there was no direct correlation between the change in melting temperature of the DNA hairpin and its ability to photo-regulate RNase H-mediated digestion of RNA\[^1\]. Specifically, a variety of hairpins all comprised of the same 20mer antisense sequence but different lengths of blocking strands and different numbers of mismatched bases were synthesized. When the hairpins were tested \textit{in vitro}, it was discovered that the size of the loop formed by the photocleavable linker in joining the DNA to its blocking strand was a more important factor. A similar problem was encountered with the RNA bandages; the bandages with the largest changes in melting temperature before and after photolysis were not the most effective in regulating gene expression. The targeted region of mRNA was found to be more significant than thermodynamics\[^2\].

Due to the complexity of the biological systems we wish to study and the above-mentioned factors that require optimization, it is difficult to determine the effectiveness of a given caged oligonucleotide. This led us to combine the hairpin and bandage techniques to develop hairpin-bandage hybrids that would not require as much optimization of the length and sequence of the blocking strands. By combining these two
approaches, we designed an antisense strand that is linked via a photocleavable spacer to a blocking strand that is divided into two parts by a second photocleavable spacer. After UV irradiation, the blocking strand is broken into two shorter oligonucleotides, which can more easily dissociate from the antisense strand. This design allows the blocking strand to be as long as possible before uncaging, efficiently blocking the antisense strand from binding to its mRNA target.

Previous work by our lab has concentrated on utilizing caged oligonucleotides to gain control of gene expression. However, the hairpin-bandage hybrids are being utilized to isolate mRNA from single neurons in culture and in mouse hippocampal slices. For this work, we have been collaborating with the Eberwine lab in the Department of Pharmacology at the University of Pennsylvania.

Research in the Eberwine lab focuses on studying RNA populations in neurons to gain a better understanding of central nervous system function and to learn how neurons respond to stimuli at the transcriptional level. Studying RNA populations from single cells is important because, even though all cells contain the same DNA, their function and morphology differ greatly. It has been shown that these phenotypic differences are associated with which RNAs are present and the level at which they are expressed. Unfortunately, it is very difficult to transfect single cells in a large population of cells using existing methods. Therefore, a photoregulatable antisense oligonucleotide that could be transfected into an entire populations of cells but uncaged site-specifically in a single cell would be a valuable tool.
b. Current techniques for isolating neurons

To date, there are a number of approaches for studying complex brain tissue. These include fluorescence activated cell sorting\(^4\), immunopanning\(^5\), translating ribosome affinity purification\(^6\), manual sorting\(^7\), laser capture microdissection\(^8\), atomic force microscopy nanoprobe extraction\(^9\), and isolation via patch pipette\(^10\). Fluorescence activated cell sorting (FACS) was utilized to purify a genetically labelled population of neurons\(^4\). A transgenic mouse line, in which the mice contained an EGFP-expressing cell-type-specific regulatory element, was developed. Hippocampal slices from these mice were then enzymatically dissociated, and the neurons were subjected to FACS purification. This process separated out only the neuronal population expressing EGFP, allowing for isolation of RNA from only the neuronal subtype of interest.

Another method used to better understand brain development and function is immunopanning\(^5\). With this technique, brain cells suspended in buffer are added to an antibody-coated panning plate to remove cell types that adhere to the selected antibody. Sequential panning with additional antibodies is performed until the desired neural cell population, the oligodendrocyte lineage in this example\(^5\), is isolated.

Translating ribosome affinity purification (TRAP) is another process for purifying RNA from neurons. Heiman et al. developed the TRAP methodology, which initially involves the development of a mouse line that expresses a transgene encoding EGFP fused to a ribosomal protein\(^6\). The transgene was designed to be under the control of a receptor expressed in a specific cell type, resulting in expression of affinity-tagged translating ribosomes in that cell population. After dissection and homogenization of
hippocampal tissue, affinity purification of the ribosomes and their mRNA can be achieved using anti-GFP antibody-coated beads. In an additional study, the TRAP method was expanded to profile 24 different CNS cell populations.[11]

An alternative technique involves manual sorting of reporter-labelled cells in dissociated cell culture.[7] By dissecting slices from the forebrains of transgenic mouse lines expressing fluorescent proteins in subsets of neurons, the authors suspended the cells in solution and successfully isolated fluorescent neurons under a dissecting microscope. The purified sample was then lysed to attain RNA for analysis.

Laser capture microdissection is a procedure that can be used for isolating RNA from a single cell.[8] In this technique, a transparent thermoplastic film is applied to tissue or cell cultures on a glass slide. Then, a laser pulse is applied, melting the film to “capture” the cell(s) of interest, which can then be lysed and examined.

Recently, atomic force microscopy (AFM) nanoprobes have been employed to extract RNA from single cells.[9] The single cell nanoprobe is inserted into the cell of interest to extract the cellular ingredients. Unfortunately, limited access to an atomic force microscope has prevented this technique from gaining wide-spread use at this point.

Finally, the most commonly used approach is RNA isolation via patch pipette, which was developed by the Eberwine lab.[10] A patch pipette penetrates the cell membrane to microinject reverse transcriptase and an oligo(dT) primer with a T7 RNA polymerase promoter site into a single, live cultured primary hippocampal neuron.[10a] The oligo(dT) primer is used to prime the poly(A)^+ mRNA for cDNA synthesis by reverse transcriptase. Then, the cellular contents are aspirated from the cell into the patch
pipette for the cDNA to be amplified using T7 RNA polymerase to transcribe RNA copies.

Unfortunately, each of these techniques has disadvantages. FACS and manual sorting can isolate single cells, but they are limited in that they do not provide any insight into the location of the cell in tissue. LCM, the AFM nanoprobe method, and TRAP often suffer from RNA contamination from nearby cells, and TRAP only collects RNA that is associated with ribosomes. The only approaches that can achieve single cell RNA isolation while still collecting information about the cellular physiological properties are LCM, AFM nanoprobe extraction, and patch-pipette isolation. However, the small amount of mRNA that can be absorbed by the AFM probe makes this technique difficult to use, even when coupled with PCR. Finally, patch-pipette mediated isolation is difficult to perform and it mechanically injures the surrounding tissue. These drawbacks motivate the design of an approach that will isolate mRNA from single cells in vivo.

c. Caged compounds for studying the central nervous system

As discussed in chapter one, there are a number of advantages to utilizing light in biochemical studies. Since photochemical processes are well-understood, light is orthogonal to biological processes, and both UV and visible light are minimally invasive, these compounds can be ideal for studying biological systems. Furthermore, the high degree of spatiotemporal control these compounds afford can allow for time-resolved studies and for spatial resolution to study dendrites, synapses, and individual nuclei.

These advantages have led other groups to apply light-activatable compounds to the study of the central nervous system (CNS). For example, Lima et al. microinjected
DMNPE-caged ATP into the CNS of *Drosophila* to study the role of dopaminergic neurons in the control of movement; a pulse of laser light photoreleased the ATP which was then free to act as an agonist for an ion channel expressed in a set of neurons[12].

Another approach to photocontrolling a neuronal ion channel was developed by the Kramer lab[13]. Their caged compound is comprised of a maleimide functional group (for cysteine tethering to the ion channel) and a quaternary ammonium group linked via a photoisomerizable azobenzene moiety. In the *trans* form, the ammonium group can block the channel, but UV irradiation converts azobenzene to its *cis* form, rendering the compound too short to block the channel. In other work, a caged AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor antagonist was developed to monitor surface-exposed AMPA receptors in individual *Xenopus* oocytes[14] and in single cells in rat hippocampal cell cultures[15].

**d. Hairpin-bandage hybrid design strategy**

Considering the many advantages of caged oligonucleotides and the success in utilizing light-activatable moieties to probe the CNS, we sought to design hairpin-bandage hybrids to isolate mRNA from single neurons, a technique we have coined Transcriptome *In Vivo* Analysis (TIVA). Since the caged hairpin-bandage hybrids discussed in the remainder of this chapter are being used for TIVA, they will be referred to as “TIVA-tags” for the rest of this chapter.

In designing TIVA-tags to isolate mRNA from individual neurons, we first chose to utilize a probe strand that could target the poly(A)$^+$ tails of mRNA (Figure 3.1). Since messenger RNAs (mRNAs) have 3’-polyadenine tails that aid in translation, we designed
an antisense strand consisting of uracils that can hybridize to this region of mRNA. In previous work, the Eberwine lab developed a technique that employs peptide nucleic acids (PNAs) to isolate RNA strands associated with RNA-binding proteins\cite{16}. The PNA hybridizes to RNA and then photo-crosslinks to the associated proteins. Instead of using an antisense strand comprised of PNA, we chose a well-characterized, commercially available RNA analog with increased thermal stability, 2′-fluoro RNA. Substitution of a fluoro group at the 2′-position locks the ribose ring into the 3′-endo conformation, which causes duplexes of the RNA to favor A-form helices and increases the melting temperature of the duplex 2 °C per base relative to DNA\cite{17}.

To develop a hairpin-bandage hybrid, two photocleavable spacers are incorporated to join the probe strand to two blocking strands (Figure 3.1). Both blocking strands are comprised of 2′-OMe RNA, which also increases the thermal stability and nuclease resistance of the duplex. Additionally a 3′-biotin tag and a 5′-thiol were added to the oligonucleotide. Cy3 and Cy5 fluorophores were included to monitor dissociation of the oligonucleotide after photolysis. These dyes are ideal because they do not absorb at wavelengths below 400 nm where the photocleavable spacer is activated.

After successfully monitoring oligonucleotide dissociation in vitro, a cell-penetrating peptide (CPP) was conjugated to the oligonucleotide via a disulfide linkage in order to transport the oligonucleotide into cells. Upon entering the cell, the disulfide bond is reduced, allowing the peptide to dissociate from the hairpin. In vivo FRET experiments have been performed in primary cultured neurons and mouse hippocampal slices. Additionally, mRNA has been isolated from single neurons utilizing the TIVA-
tag; by lysing the cells and adding magnetic streptavidin beads, it is possible to isolate the antisense strand bound to mRNA. This allows the mRNA to be eluted by heating and amplified by the inverse transcription method developed by the Eberwine lab\cite{18}.

The studies presented in this chapter detail the \textit{in vitro} characterization of TIVA-tags and their \textit{in vivo} application to isolate mRNA from single neurons both in primary culture and in mouse hippocampal slices. Future, more extensive, biological analyses will allow for gene expression in single cells to be quantified, providing insight into how mRNA abundances vary temporally as well as spatially (e.g. in dendrites versus synapses).

II. Experimental procedures

\textbf{a. Oligonucleotide synthesis and purification}

Oligonucleotides were synthesized by standard phosphoramidite chemistry using an ABI DNA/RNA 394 nucleic acid synthesis system on 1.0 \textmu mole or 10.0 \textmu mole scales. Reagents obtained from Glen Research included 2‘-Fluoro U (10-3430), 2‘-OMe A (10-3100), 5‘-thiol C6 spacer (10-1936), Cy3 (10-5913), Cy5 (10-5915), and photocleavable spacer (10-4913) phosphoramidites, and a 3‘-biotin TEG CPG (20-2955). Coupling times were adjusted to manufacturer’s recommendations, and 0.02 M iodine was used for oxidation steps.

After cleavage and deprotection using ammonium hydroxide at room temperature for 24 hours, oligonucleotides were purified on an Agilent 1100S reverse-phase HPLC (C18 column) with eluents of 0.05 M triethylammonium acetate (A) and acetonitrile (B);
gradient, 0-40 minutes, 10-60% B, then 60-80% B in 40-50 minutes in A+B; flow rate, 1 mL/min; 40 °C. The retention time of the purified TIVA-tags with 5′-thiol modification was ~ 50 minutes. Removal of the 4,4′-dimethoxytrityl group was performed by treating the purified oligonucleotides with 80% acetic acid for 20 minutes at room temperature. The TIVA-tag was desalted on a Nap-5 column (GE Healthcare) and dried under vacuum.

b. Conjugation of TIVA-tags to cell-penetrating peptides

The method of conjugating oligonucleotides to CPPs through disulfide bonds was modified from Turner et al. Briefly, 5 nmol of oligonucleotide with 5′-thiol modification was deprotected using 50 mM TCEP for two hours. The TCEP was removed by desalting on a NAP-5 column (GE Healthcare), and the oligonucleotide was dried under vacuum. After drying, the oligonucleotide was redissolved in 50 µL of 0.33 M TEAA, 150 µL of formamide was added, and the sample was vortexed. Two CPPs, (D-Arg)$_9$ and Tat (YGRKKRRQRRR-NH$_2$), each with a cysteine activated with a 3-nitro-2-pyridinesulfenyl group (Npys) on the C-terminus, were obtained from Anaspec. CPPs were dissolved at a concentration of 1 mM in water. To the dissolved oligonucleotide, a four-fold excess of CPP was added, and the reaction was allowed to proceed overnight at room temperature.

The conjugation product was purified by anion exchange on an Agilent 1100S HPLC using a 1 mL Resource Q column with a flow rate of 1 mL/min and a gradient of 0-100% buffer B in 30 minutes (buffer A: 20 mM Tris-HCl (pH 6.8), 50% formamide; buffer B: 20 mM Tris-HCl (pH 6.8), 50% formamide, 400 mM NaClO$_4$). Finally, the product was
desalted on a NAP-5 column, dried under vacuum, and characterized by MALDI-TOF mass spectrometry at the Wistar Institute Proteomics Facility.

c. Mass analysis of TIVA-tags

Matrix-assisted laser desorption/ionization (MALDI) of TIVA-tags was performed at the Wistar Institute Proteomics Facility at the University of Pennsylvania. Mass spectra were recorded on an Applied Biosystems Voyager System 6030 MALDI-TOF mass spectrometer operated in negative mode with 3-hydroxypicolinic acid matrix. In addition to peaks that correspond to full-length products, peaks consistent with masses of fragments from photocleavage reactions are present in the MALDI spectra. This is due to UV pulse laser irradiation required for MALDI-TOF and has been observed previously when characterizing light-activatable oligonucleotides[20].

d. Melting point determinations

Melting point studies were conducted on a Beckman Coulter DU800 UV-Vis spectrophotometer equipped with a programmable Peltier temperature controller. Samples were monitored at 260 nm while heating or cooling at a rate of 1.0 °C/min, with a one minute hold per degree Celsius. Melting temperatures were determined from the peak of the first derivative plot of Abs$_{260}$ vs. temperature.

TIVA-tags were prepared at 1 µM concentration in 10 mM Tris pH 7.5 with 300 mM NaCl and 10 mM MgCl$_2$. To ensure the oligonucleotides were properly annealed, the samples were heated to 90 °C in a water bath for five minutes, then slowly cooled to room temperature over approximately three hours. For samples that were photolyzed, irradiation was carried out using a UV transilluminator (Spectronics Corporation TL-
365R) at wavelengths centered on 365 nm (9 mW/cm² at peak intensity) for 15 minutes with the samples in open 200 µL microcentrifuge tubes.

e. FRET analysis of Cy3/Cy5 labeled TIVA-tags

TIVA-tags with Cy3/Cy5 FRET fluorophores and TIVA-tags with only Cy3 or only Cy5 were prepared at 1 µM concentration in the same buffer as for the melting point determination experiments, and photolysis was carried out as discussed above. The samples were pre-hybridized, and measurements were made in a sub-micro cuvette that was incubated at 37 °C during emission collection. The fluorescence emissions of Cy3 at 565 nm and Cy5 at 667 nm, upon excitation at 552 nm, were monitored by a Varian Eclipse fluorimeter (scanning rate of 120 nm/min, and averaging time of 0.5000 sec).

f. Polyacrylamide gel electrophoresis analysis of TIVA-tags

Caged and uncaged TIVA-tag samples (200 pmol in 5 µL water and 5 µL formamide) were loaded onto 20 % polyacrylamide, 7 M urea gel. The following irradiation conditions were tested: 405 nm laser for 100 µsec per pixel for an image of 512 x 512 pixels; UV laser (351 nm and 364 nm laser lines) for 100 msec, 5 sec, or 10 sec; and UV transilluminator for 15 minutes. Gels were electrophoresed at 300V for 45 minutes, and staining was performed with ethidium bromide for ten minutes. Gels were imaged on a Bio-Rad Gel Doc 2000 system or a GE Typhoon imaging system.
a. TIVA-tag characterization

In a preliminary experiment, an 18mer 2′-F strand of all uracils with a biotin tag was capable of isolating RNA in pull-down assays (unpublished data, Eberwine lab). To choose an initial blocking strand length, the melting temperature of an 18mer 2′-F RNA strand of all uracils against different strand lengths of all adenes found the melting temperature of the poly(U) strand against a 7mer 2′-OMe strand of adenes to be approximately 24 °C, which is much lower than the temperature used in cell experiments (Julia Richards, 2010, Development of photoactivatable oligonucleotides, Doctoral dissertation). Therefore, the first TIVA-tag without dyes was synthesized with an 18mer uracil antisense strand and two 7mer blocking strands (Figure 3.2A), and it was characterized by MALDI mass spectrometry at the Wistar Proteomics Facility (Table 3.1). Thermal denaturation experiments demonstrated that the TIVA-tag has a pre-photolysis melting temperature of 53.9 °C and a post-photolysis melting temperature of 30.2 °C (Table 3.2, Figure 3.3A). However, the post-photolysis melting temperature is difficult to assign since the curve is not sigmoidal in shape. This was also the case with the 18mer 2′-F RNA strand of all uracils against the 7mer 2′-OMe strand of adenes tested by Dr. Richards (Julia Richards, 2010, Development of photoactivatable oligonucleotides, Doctoral dissertation). Since the same experiment performed with a 9mer 2′-OMe strand instead of a 7mer resulted in a sigmoidal shaped curve and a melting temperature of 31 °C, we determined that the post-photolysis melting temperature of the TIVA-tag is too low to measure accurately. This is because the spectrophotometer’s
Peltier temperature controller could not cool samples below 15 °C. The short lengths of oligonucleotides produced after photocleavage should have low melting temperatures, making it difficult to measure them using this apparatus. While this limitation should be noted, we are actually most interested in the pre-photolysis melting temperature since all of the post-photolysis melting temperatures are lower than the 37 °C required for cellular experiments.

Since there did seem to be a large change in melting temperature (ΔT_m) for the TIVA-tag without dyes, a fluorescently-labeled version of the TIVA-tag (with Cy3 and Cy5 dyes) was synthesized. This TIVA-tag also had a seemingly large ΔT_m with a calculated melting temperature of 59.0 °C before photolysis and 29.0 °C after uncaging (Table 3.2, Figure 3.3B), but the post-photolysis melting curve was also not sigmoidal in shape. However, since we were confident that the post-photolysis melting temperature is below 37 °C, we chose to fully characterize the dye-labeled TIVA-tag.

While testing the original fluorescently-labeled TIVA-tag, we also synthesized and characterized (Figure 3.2, Table 3.1) TIVA-tags with various lengths of antisense and blocking strands to see if we could generate larger pre-photolysis melting temperatures since some of our lab’s previously designed hairpins had melting temperatures of approximately 70 °C. We also introduced a C6 spacer between the 2’-F antisense strand and the first photocleavable linker to see if the increased flexibility in the loop of the hairpin would result in a change in melting temperature, but it had little effect (<1 °C) on the melting temperature (Table 3.2, Figure 3.4A). For the original 18mer antisense strand, the pre-photolysis melting temperature increases ~7 °C if the blocking strand lengths are increased to eight adenines each (Table 3.2, Figure 3.4B). On the other hand,
decreasing the length of the blocking strands or using two blocking strands of different lengths resulted in a decrease in melting temperature before photolysis (Table 3.2, Figure 3.4C-3.4F).

TIVA-tags with longer antisense strand lengths were also synthesized. When a TIVA-tag with a 20mer antisense strand and two 7mer blocking strands was examined, it was found to have a lower pre-photolysis melting temperature than the original TIVA-tag (Table 3.2, Figure 3.4G). Increasing the number of uracils to 20 or 22 increased the pre-photolysis melting temperature when 9mer blocking strands were used (Table 3.2, Figure 3.4H-3.4I). Both of these uncaged constructs had a melting temperature of ~65 °C.

Finally, we performed thermal denaturation studies on TIVA-tags comprised of all 2′-F RNA bases or all 2′-OMe RNA bases. When the original TIVA-tag was designed, 2′-F RNA phosphoramidites were more expensive to purchase, so 2′-F uracils were only used to synthesize the antisense strand. We chose to synthesize the blocking strands with 2′-OMe RNA. Now, however, the analogues are similar in cost, so we synthesized TIVA-tags to determine if it would be preferable to use one analogue over the other. Since these TIVA-tags had lower pre-photolysis melting temperatures than the original TIVA-tag (Table 3.2, Figure 3.4J-3.4K), we determined that it is not preferable to use one only one analogue.

b. In vitro FRET monitoring of TIVA-tag dissociation

To monitor the dissociation of the Cy3/Cy5-labeled TIVA-tag after photolysis, we measured the fluorescence spectrum and calculated the FRET efficiency (Figure 3.5A). The FRET efficiency was defined as: \( I_a / ((\gamma^* I_a) + I_a) \), where \( I_a \) is the intensity of the
acceptor (Cy5) fluorescence, $I_d$ is the intensity of the donor (Cy3) fluorescence, and $\gamma$ is the correction factor for the difference in donor and acceptor quantum yields (the quantum yields for Cy3 and Cy5 are 0.15 and 0.3, respectively). In addition to collecting the emission spectra for the Cy3/Cy5 TIVA-tag, fluorescence experiments were performed with a Cy5-only TIVA-tag (Figure 3.5B) and a Cy3-only TIVA-tag (Figure 3.5C) to measure any decrease in fluorescence intensity due to irradiation at 365 nm for uncaging. By subtracting the Cy5 emission spectrum upon excitation at 552 nm from that of the Cy3/Cy5 TIVA-tag, any contribution of Cy5 emission by direct excitation, rather than energy transfer, was eliminated. To calculate the FRET efficiency, the average of three independent trials with the Cy5-only TIVA-tag was subtracted from the average of three trials with the Cy3/Cy5 TIVA-tag, and the corrected emission spectra were graphed and analyzed (Figure 3.5A). The FRET efficiency was found to be 0.8307 before photolysis and 0.08851 afterwards. This is a 74 % change in FRET efficiency, indicating a conformational change in the TIVA-tag, i.e. a lysis of the oligonucleotide into separate parts. Emission spectra of both the Cy3/Cy5 TIVA-tag and the Cy5-only TIVA-tag excited at 643 nm were also collected to monitor the direct excitation of Cy5 dye in each case (Figure 3.5D-3.5E).

c. Gel analysis of TIVA-tag uncaging

We also verified oligonucleotide dissociation post-photolysis by denaturing polyacrylamide gel electrophoresis (PAGE) (Figure 3.6A). TIVA-tags before or after photolysis were loaded in a 20% PAGE, 7M urea gel, which was electrophoresed at 300V for 45 minutes. Staining with ethidium bromide for ten minutes revealed bands corresponding to intact TIVA-tag and photolysis products. Only one band was present
for the caged TIVA-tag sample. Samples irradiated with a 405 nm laser or for five or ten seconds with a UV laser displayed two photolysis bands that ran faster on the gel than the caged oligonucleotide, but there was also some caged TIVA-tag present. Irradiation for 100 msec with the UV laser uncaged very little TIVA-tag. Finally, irradiating for 15 minutes with a UV transilluminator completely uncaged the TIVA-tag, as can be determined by the presence of only two photolysis product bands on the gel. The two photolysis products seen on the gel correspond to the 3’ end of the TIVA-tag with either one photocleavable group (the highest photolysis band) or two photocleavable groups (the lowest photolysis band) cleaved. This was confirmed by a denaturing polyacrylamide gel imaged with a GE Typhoon Imager using the fluorescence setting (Figure 3.6B). In this gel, both photolysis products fluoresce green, indicating the presence of the Cy3 dye. The band for the uncaged TIVA-tag appears yellow due to the overlap of the Cy3 and Cy5 (shown in red) dyes (Figure 3.6B).

d. Preparation of TIVA-tags for cellular experiments

To facilitate the cellular uptake of TIVA-tags we conjugated them to cell-penetrating peptides (CPPs). Preliminary experiments performed by Dr. Ditte Lovatt in the Eberwine lab discovered that fluorescently-labeled (specifically carboxytetramethylrhodamine-labeled) Tat and (D-Arg)₉ CPPs at a concentration of 10 μM efficiently transduced the dye into cortical cultured neurons (Unpublished results, Eberwine lab). Therefore, we chose to conjugate TIVA-tags to these CPPs via disulfide bonds following a protocol modified from work done by Turner et al[19].
Following the conjugation reaction, TIVA-tag conjugates were purified by anion exchange chromatography using highly denaturing conditions (50% formamide) in order to prevent excess cationic CPP from remaining bound to the negatively charged oligonucleotide and to prevent precipitation. Typical HPLC chromatograms show that compared to the reduced oligonucleotide alone (Figure 3.7A), both the (D-Arg)₉-conjugated TIVA-tag (Figure 3.7B) and the Tat-conjugated TIVA-tag (Figure 3.7C) eluted a couple of minutes earlier at approximately 22 minutes. These results are consistent with the earlier elution time for conjugates observed by Turner et al. for their CPP-oligonucleotide conjugates¹⁹. After desalting and drying the conjugates under vacuum, they could be redissolved in water, that their masses were confirmed by MALDI-TOF mass spectrometry. The masses of (D-Arg)₉-TIVA-tag and Tat-TIVA-tag were found to be 14,430.31 and 14,432.46, respectively (compared to expected masses of 14,412.7 and 14,547.8, respectively) (Figure 3.8).

e. **In vivo studies**

Our collaboration with the Eberwine lab has enabled us to test TIVA-tags in dispersed primary neuron cell culture and acute mouse hippocampal slices. The initial aim was to monitor the change in FRET in cultured neurons. By incubating cultured cortical neurons (prepared for 7-12 days *in vitro*), with 10 µM (D-Arg)₉-TIVA-tag for ten minutes, a decrease in Cy5 intensity and increase in Cy3 intensity in a single neuron was observed after irradiation with a 405 nm laser (Figure 3.9, in collaboration with Dr. Ditte Lovatt).
Next, we moved on to investigate whether TIVA-tag dissociation could be monitored in hippocampal tissue. While cultured neurons are very useful for single cell studies, there are significant differences between cultured cells and intact tissues. For instance, cultured cells are grown on 2-D plates, so they lack the inherent 3-D morphology of tissue. Additionally, cellular organization and neuronal connections are maintained in tissue but not cultured cells. Due to these inherent differences and the fact that live tissue slices are more similar to the intact brain, it would be highly beneficial to be able to isolate mRNA from single neurons in live tissue. Therefore, we incubated live hippocampal slices from a 60-day old mouse for 40 minutes with 10 µM (D-Arg)$_9$-TIVA-tag. After uncaging the TIVA-tag with a 405 nm laser, there is a significant increase in Cy3 fluorescence and a decrease in Cy5 fluorescence intensity, indicating dissociation of the TIVA-tag (Figure 3.10, in collaboration with Dr. Jai-Yoon Sul).

In addition to monitoring TIVA-tag photolysis in vivo, mRNA has been isolated successfully from a single neuron from a live slice. After incubating a slice from a 60-day old mouse with TIVA-tag and uncaging the TIVA-tag in a single neuron, the cells were lysed, and magnetic streptavidin beads were added to pull-down the antisense strand still bound to mRNA. Next, the oligonucleotides were heated to greater than 50 °C to elute the mRNA since the melting temperature of an 18mer 2′-F uracil oligonucleotide tagged with Cy3 and 3′-biotin against an 18mer poly(A)$^+$ RNA strand was found to be 50 °C (Figure 3.11). Finally, the RNA was amplified using the antisense RNA amplification procedure$^{[10]}$, the RNA was cleaved into smaller fragments, and the material was loaded onto a nanochip gel and electrophoresed in a nanochip bioanalyzer. As shown in Figure 3.12, the uncaged TIVA-tag was successful in isolating mRNA from a single neuron, and
there was minimal RNA background from an uncaged neuron (Figure 3.12, in collaboration with Dr. Peter Buckley). Ongoing sequencing analysis of the isolated RNA will enable us to qualitatively compare what RNA sequences the TIVA-tag is isolating, but preliminary results indicate that the TIVA-tags are pulling down RNA comparable to the patch pipette method and that the isolated RNA population is not rich in poly(A)$^+$ sequences. However, the real strength of the TIVA-tag is that it is capable of isolating RNA from live tissue, which is a limitation of current RNA isolation approaches.

IV. Conclusions

The goal of combining the photoactivatable hairpins and bandages previously developed by our lab was to develop a hairpin with a longer blocking strand but that retains a low melting temperature after photolysis. Thus far, this approach has been successful in the case of our original TIVA-tag. In addition to the large change in melting temperature, the first generation TIVA-tag has been used successfully in vivo. In our studies with cultured neurons and live hippocampal slices, there appears to be minimal background prior to photolysis, suggesting that the TIVA-tag is stable in cells.

To investigate whether the basic TIVA-tag design could be improved upon, particularly as other biological uses are developed, we synthesized and characterized TIVA-tags with various lengths of antisense and blocking strands. Since the first generation TIVA-tag has a lower pre-photolysis melting temperature (60 °C) compared to our lab’s successful caged hairpins (~70 °C), it is possible that the TIVA-tag design may need to be optimized, focusing on increasing its stability before uncaging to ensure that no RNA is collected before photolysis. Upon investigating other designs, it was
determined that new TIVA-tags with the largest pre-photolysis melting temperatures are the 18mer antisense strand with 8mer blocking strands and the 20mer probe with 9mer blocking strands.

Also, we have started to consider more extensive modifications that can be made in the development of second generation TIVA-tags. These include introducing new targeting moieties for optimizing specificity for particular cell types or cellular locations, changing to caging moieties like those with two-photon capabilities to optimize spatiotemporal control, and attempting to increase the thermal stability of the TIVA-tag by adding G-C residues to the 3′ or 5′-end of the poly(U)/poly(A) + strands.

Thus far, work has only begun on considering other methods for targeted delivery of TIVA-tags. An obvious possibility for new targeting moieties, due to the increased commercial availability of CPPs, would be to conjugate other CPPs to the TIVA-tag to target specific cell types. Another option we have started to explore is folate receptor delivery. Folate receptors are highly selective tumor markers that are commonly overexpressed in cancer cells, particularly ovarian cancer cells\textsuperscript{[21]}. Folate receptors have a high-affinity for folic acid, which has been utilized as a receptor mediated delivery agent for cargo, such as antibodies\textsuperscript{[21]}, contrast agents\textsuperscript{[21]}, and oligonucleotides \textsuperscript{[22]}. This is because folate is able to retain its receptor binding properties when conjugated to a biomolecule via its γ-carboxyl. Therefore, we synthesized a folate-conjugated TIVA-tag (Figure 3.13), which may open the door for utilizing TIVA-tags to study mRNA specifically from cancer cells, if its \textit{in vivo} use can be optimized.
Another obvious extension is to apply the hairpin-bandage hybrid approach to traditional antisense applications. One such possibility is to inhibit microRNA activity. microRNAs (miRNAs) are short, double stranded RNA molecules comprised of 21-23 nucleotides with two nucleotide 3′ overhangs which regulate gene expression. Let-7 was the first known miRNA; it was discovered in *C. elegans* and is evolutionarily conserved. It is an interesting target because it has been shown to target several known oncogenes, making it an interesting subject for cancer research\[^{23}\]. Preliminary work in our group has focused on photomodulating let-7 miRNA activity using the hairpin-bandage hybrid design. To this end, we have caged a 2′-OMe strand that is antisense to the let-7 miRNA sequence using two photocleavable groups to divide the blocking strand into two parts (Figure 3.14). A FRET pair was also incorporated in order to monitor dissociation. The field of photoregulating microRNA has only recently been explored. The Li lab has developed an oligonucleotide design similar to our caged hairpins where they have a 2′-OMe RNA antisense strand that blocks a miRNA of interest linked to a blocking strand via a bifunctional caged linker\[^{24}\]. Since their antisense strand was not fully blocked, a small amount of background activity was observed before photolysis. If the change in melting temperature for the caged miRNA inhibitor can be optimized, the hybrid design should be a better approach because there should not be any background activity due to complete blockage of the antisense strand. Future work in our lab will include characterizing and testing this caged miRNA in zebrafish embryos.

In conclusion, we have developed a new method for utilizing two photocleavable linkers to lower the melting temperature of oligonucleotides after photolysis. We have also monitored oligonucleotide dissociation, both *in vitro* and *in vivo*, using a FRET pair.
Finally, we have successfully employed the first generation of TIVA-tags to pull-down mRNA from single neurons. Future work will focus on further developing the next generation TIVA-tags in addition to creating other hairpin-bandage hybrids for a variety of applications.
V. References


**Figure 3.1** Schematic representation of TIVA-tag before and after photolysis. A 2′-F RNA strand (blue) is caged via a photocleavable linker (red) to two shorter complementary 2′-OMe RNA strands (green) which are joined by a second photocleavable linker (red). The 2′-F RNA strand is labeled with a biotin moiety, and a cell-penetrating peptide (CPP) is connected to the 5′-end of the 2′-OMe RNA strand by a disulfide bond. After photolysis, the 2′-OMe RNA strand breaks into two shorter oligonucleotides, which then dissociate from the 2′-F RNA. Dissociation can be monitored by means of the Cy3 (pink) and Cy5 (purple) dyes on the 2′-F RNA and 2′-OMe RNA strands, respectively. Finally, the 2′-F RNA can hybridize to the poly(A)⁺ tail of mRNA (gray) in the cell.
Figure 3.2  Structures of TIVA-tags:  A) General structure of TIVA-tags with various probe and blocking strand lengths, B) TIVA-tag with Cy3/Cy5 dyes, which was utilized for *in vitro* and *in vivo* experiments.
Table 3.1  Table of TIVA-tags and masses determined by MALDI-TOF mass spectrometry.

<table>
<thead>
<tr>
<th>TIVA-tag</th>
<th>Expected Mass (g/mol)</th>
<th>Measured Mass (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIVA (no dyes)</td>
<td>11,862.7</td>
<td>11,851.49</td>
</tr>
<tr>
<td>Cy-TIVA</td>
<td>13,019.9</td>
<td>13,047.06</td>
</tr>
<tr>
<td>Arg-Cy-TIVA</td>
<td>14,412.7</td>
<td>14,430.31</td>
</tr>
<tr>
<td>Tat-Cy-TIVA</td>
<td>14,547.8</td>
<td>14,432.46</td>
</tr>
<tr>
<td>Folate-Cy-TIVA</td>
<td>13,165.7</td>
<td>13,209.02</td>
</tr>
<tr>
<td>Cy5-TIVA</td>
<td>12,499.9</td>
<td>12,604.22</td>
</tr>
<tr>
<td>Arg-Cy5-TIVA</td>
<td>13,892.7</td>
<td>13,952.62</td>
</tr>
<tr>
<td>All 2’-F TIVA</td>
<td>11,708.2</td>
<td>11,673.49</td>
</tr>
<tr>
<td>All 2’-OMe TIVA</td>
<td>12,093.4</td>
<td>12,086.65</td>
</tr>
<tr>
<td>18U/6A/6A TIVA</td>
<td>11,058.2</td>
<td>11,134.18</td>
</tr>
<tr>
<td>18U/8A/8A TIVA</td>
<td>12,431.2</td>
<td>12,489.78</td>
</tr>
<tr>
<td>20U/7A/7A TIVA</td>
<td>12,493.0</td>
<td>12,602.78</td>
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<tr>
<td>20U/9A/9A TIVA</td>
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<td>13,800.85</td>
</tr>
<tr>
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<td>14,487.06</td>
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<td>11,218.53</td>
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<tr>
<td>18U/5A/7A TIVA</td>
<td>11,190.2</td>
<td>11,211.94</td>
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<td>18U/7A/9A TIVA</td>
<td>12,563.2</td>
<td>12,569.06</td>
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<tr>
<td>C6 Spacer TIVA</td>
<td>12,152.8</td>
<td>12,178.03</td>
</tr>
</tbody>
</table>
Table 3.2 Melting temperatures of TIVA-tags. Oligonucleotide concentration of 1 µM in 10 mM Tris pH 7.5 buffer with 300 mM NaCl and 10 mM MgCl₂ was used for all experiments.

<table>
<thead>
<tr>
<th>TIVA-tag</th>
<th>Pre-photolysis Melting Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIVA (no dyes)</td>
<td>53.9</td>
</tr>
<tr>
<td>Cy-TIVA</td>
<td>59.0</td>
</tr>
<tr>
<td>Arg-Cy-TIVA</td>
<td>52.0</td>
</tr>
<tr>
<td>Tat-Cy-TIVA</td>
<td>49.9</td>
</tr>
<tr>
<td>Folate-Cy-TIVA</td>
<td>56.8</td>
</tr>
<tr>
<td>Cy3-TIVA</td>
<td>58.1</td>
</tr>
<tr>
<td>Cy5-TIVA</td>
<td>59.1</td>
</tr>
<tr>
<td>All 2‘-F TIVA</td>
<td>54.0</td>
</tr>
<tr>
<td>All 2‘-OMe TIVA</td>
<td>53.1</td>
</tr>
<tr>
<td>18U/6A/6A TIVA</td>
<td>56.4</td>
</tr>
<tr>
<td>18U/8A/8A TIVA</td>
<td>61.0</td>
</tr>
<tr>
<td>20U/7A/7A TIVA</td>
<td>51.5</td>
</tr>
<tr>
<td>20U/9A/9A TIVA</td>
<td>64.8</td>
</tr>
<tr>
<td>22U/9A/9A TIVA</td>
<td>64.8</td>
</tr>
<tr>
<td>18U/7A/5A TIVA</td>
<td>41.2</td>
</tr>
<tr>
<td>18U/5A/7A TIVA</td>
<td>44.6</td>
</tr>
<tr>
<td>18U/7A/9A TIVA</td>
<td>55.5</td>
</tr>
<tr>
<td>C6 Spacer TIVA</td>
<td>54.9</td>
</tr>
</tbody>
</table>

Thermal denaturation curves for TIVA-tags after photolysis were not sigmoidal in shape. Therefore, it was not possible to assign melting temperatures to the TIVA-tags after photolysis. This is likely due to the fact that the Peltier temperature controller was unable to cool the samples to temperatures lower than 15 °C, and the short oligonucleotides produced after photolysis should have melting temperatures of less than 30 °C.
Figure 3.3 Thermal denaturation curves for TIVA-tag A) without dyes, B) with Cy3/Cy5 dyes, and C) with only Cy3 or only Cy5 dyes before and after photolysis at 365 nm. TIVA-tags were hybridized in standard buffer, and melting temperatures were determined from the peak of the first derivative plot of Abs$_{260}$ versus temperature. The melting temperature for the TIVA-tag without dyes were found to be 53.9 °C pre-photolysis. For the TIVA-tag with dyes, the before photolysis melting temperature was 59.0 °C.
C)

Absorbance (a.u.) vs Temperature (°C) for Cy3 TIVA-tag, Cy3 TIVA-tag + UV, Cy5 TIVA-tag, Cy5 TIVA-tag + UV.
Figure 3.4  Thermal denaturation curves of TIVA-tags with A) C6 spacer, B-I) various sense and antisense strand lengths, J) all 2’-F residues, and K) all 2’-OMe residues before and after photolysis at 365 nm. TIVA-tags were hybridized in standard buffer, and melting temperatures were determined from the peak of the first derivative plot of Abs$_{260}$ versus temperature.
E)

F)
Figure 3.5 Emission spectra of TIVA-tags before and after photocleavage. TIVA-tags were prepared in standard buffer and hybridized prior to FRET analysis. Fluorescence emission spectra were collected at 37 °C. A) The emission spectra of the Cy5-only TIVA-tag upon excitation at 552 nm were subtracted from the original wavelength scans of the Cy3/Cy5 TIVA-tag also excited at 552 nm to correct for any direct excitation of Cy5 dye (not due to energy transfer). The FRET efficiency was found to be 83.1 % before photolysis and 8.9 % post-photolysis, which is a 9.4-fold decrease in FRET efficiency. B) Emission spectra of Cy5-only TIVA-tag upon excitation at 552 nm. C) Emission spectra of Cy3-only TIVA-tag upon excitation at 552 nm. D) Emission spectra of Cy3/Cy5 TIVA-tag excited at 643 nm. E) Emission spectra of Cy5-only TIVA-tag excited at 643 nm. Each spectrum (full lines) is the average of three independent trials, and each graph also includes a spectrum of the minimum (dotted lines) and maximum values (dashed lines) of the three trials.

A)
Figure 3.6  Denaturing PAGE (20%) analysis of TIVA-tags before and after uncaging. A) 200 pmol of oligonucleotide were loaded per well, gel was electrophoresed at 300 V for 45 minutes, and staining was performed with ethidium bromide for ten minutes. (1) Non-irradiated TIVA-tag; (2) TIVA-tag + irradiation with 405 nm laser; (3) TIVA-tag + 100 msec irradiation with UV laser; (4) TIVA-tag + 5 sec irradiation with UV laser; (5) TIVA-tag + 10 sec irradiation with UV laser; (6) TIVA-tag + 15 min irradiation with UV transilluminator. B) 200 pmol of oligonucleotide were loaded per well, gel was electrophoresed at 300 V for 45 minutes, and gel was imaged with a GE Typhoon Imager using the fluorescence setting. (1) Non-irradiated TIVA-tag; (2) TIVA-tag + irradiation with 405 nm laser. Green indicates Cy3 dye, and red represents Cy5 dye.
Figure 3.7 Representative HPLC traces showing purification of Cy3/Cy5 TIVA-tag conjugation reaction to CPPs. A) TIVA-tag deprotected with TCEP has a retention time of ~24 minutes. B) TIVA-tag conjugated to (D-Arg)$_9$ has ~22 minute retention time. C) TIVA-tag conjugated to Tat has retention time of ~22 minutes. Absorbances at 552 nm and 643 nm are shown (rather than oligonucleotide absorbance at 254 nm) due to the inherent absorbance of formamide (which is present in the running buffer) at 254 nm.

A)

B)
Figure 3.8 MALDI-TOF analysis of CPP-conjugated TIVA-tags. A) Mass spectrum of (D-Arg)$_9$-TIVA-tag. The peak at 14,430.31 corresponds to the intact (D-Arg)$_9$-TIVA-tag (expected mass is 14,412.7). B) Mass spectrum of Tat-TIVA-tag. The peak at 14,432.46 corresponds to the intact Tat-TIVA-tag (expected mass is 14,547.8). In both spectra, lower mass peaks are indicative of photolysis products resulting from oligonucleotide cleavage due to MALDI laser exposure.
Figure 3.9  Uncaging of TIVA-tag in the body of a single cultured neuron. Cultured cortical neurons (7-12 days in vitro) were incubated with 10 μM (D-Arg)$_9$-TIVA-tag. Irradiation was performed at 405 nm to uncage the oligonucleotide. Emission from 532 nm to 707 nm was collected after excitation at 514 nm.
Figure 3.10 Activation of TIVA-tag in a single dendrite and an individual cell soma in the live brain slice preparation from a 60-day old mouse. Slices were incubated with 10 µM (D-Arg)₉-TIVA-tag. The arrow points to the dendrite in which the TIVA-tag was uncaged with 405 nm light. The inset shows a single cell soma in which TIVA-tag is irradiated at 405 nm. Both the dendrite and the cell soma were excited at 514 nm, and emission from 532 nm to 707 nm was collected. Cy5 fluorescence is indicated by red, and Cy3 fluorescence is shown in green.
Figure 3.11  Thermal denaturation curve of an 18mer 2′F-U strand with Cy3 dye and biotin at the 3′-end hybridized to 18mer poly(A)+ RNA strand. Oligonucleotides were hybridized in standard buffer, and melting temperatures were determined from the peak of the first derivative plot of $A_{260}$ versus temperature. The melting temperature was found to be 50.2 °C.
Figure 3.12  Representative nanochip analysis of mRNA isolated from a single neuron. After TIVA-tag isolation from a single neuron in the live slice preparation, the mRNA was amplified using the antisense RNA amplification procedure. Then, the RNA samples were then cleaved into smaller fragments and loaded on a nanochip gel and electrophoresed in a bioanalyzer. The left lane is the RNA ladder. The sample in the caged lane was isolated from a cell that was not irradiated, so it serves as the control. The uncaged sample represents RNA isolated from a single neuron.
Figure 3.13 Structure of folate-conjugated TIVA-tag with Cy3 and Cy5 dyes.
Figure 3.14 Structure of hairpin-bandage hybrid targeting *let-7* microRNA.
Appendix

Supplemental information for caging siRNA
**I. Design strategy**

As discussed in chapter one, there is a demand for the development of light-activatable approaches for controlling gene expression, and many recent advances have been made in controlling RNA interference (RNAi) with light. This is a desirable approach because it is not challenging to design siRNA sequences for any gene of interest, and very small amounts of material are required (on the order of pmol). Unfortunately, many of the early caged siRNA designs were unable to completely block siRNA activity prior to irradiation. A more recent advance has ameliorated RNAi inhibition pre-photolysis using a new photocleavable group, cyclo-dodecyl DMNPE (CD-DMNPE). Incorporation of this photolabile group at both the 3′ and 5′ ends of the sense and antisense strands of an siRNA duplex prevents any RNAi prior to irradiation. However, this caging strategy requires a lengthy synthesis. Therefore, we sought to design a siRNA duplex that is fully blocked the 3′ and 5′ ends, but that involved a simpler synthesis strategy.

In our design, a siRNA that incorporates two photoactivatable groups linking a 2′-fluoro (2′-F) antisense strand targeting EGFP and a 2′-F/DNA chimera blocking strand was synthesized entirely by solid-phase synthesis (Figure S1.1) and characterized by MALDI-TOF (Figure S1.2). Previous studies have demonstrated that 2′-F siRNA has increased nuclease resistance *in vitro* and *in vivo* (compared to unmodified RNA) and is equally effective at gene suppression. Blidner et al. have demonstrated that a fully 2′-fluoronated siRNA can elicit RNAi with similar activity to unmodified RNA when they photoregulated a siRNA. However, since they were using the DMNPE group, their
approach also suffered from siRNA activity prior to photolysis. To fully block any activity from the caged construct, we circularized the duplex by ligation with Circligase enzyme to fully block the 3’ and 5’ ends (Figure S1.3).

II. Experimental procedures

a. Oligonucleotide synthesis and purification

Oligonucleotides were synthesized by standard phosphoramidite chemistry using an ABI DNA/RNA 394 nucleic acid synthesis system on a 1.0 µmol scale. Reagents were obtained from Glen Research, coupling times were adjusted to manufacturer’s recommendations, and 0.02 M iodine was used for oxidation steps. After cleavage and deprotection with ammonium hydroxide at room temperature for 24 hours, oligonucleotides were purified on an Agilent 1100S reverse-phase HPLC (C18 column) with eluents of 0.05 M triethylammonium acetate (A) and acetonitrile (B); gradient 0-40 minutes, 10-60% B in A+B; flow rate, 1 mL/min. The retention time for the purified product is ~30 minutes. Removal of the 4,4’-dimethoxytrityl group was performed by treating the purified oligonucleotides with 80% acetic acid for 20 minutes at room temperature and drying under vacuum.

b. Mass analysis of caged siRNA

Matrix-assisted laser desorption/ionization (MALDI) was performed at the Wistar Institute Proteomics Facility at the University of Pennsylvania. Mass spectra were recorded on an Applied Biosystems Voyager System 6030 MALDI-TOF mass spectrometer operated in negative mode with 3-hydroxypicolinic acid matrix. In addition to the peak that corresponds to full-length product, peaks consistent with masses of
fragments from photocleavage reactions are present in the MALDI spectra. This is due to UV pulse laser irradiation required for MALDI-TOF and has been observed previously when characterizing light-activatable oligonucleotides\cite{4}.

c. **Oligonucleotide ligation**

Purified oligonucleotides were ligated using Circligase II (Epicentre). Briefly, 40 pmol of oligonucleotide was reacted in a total reaction volume of 20 µL according to manufacturer’s recommendations. The reaction was performed at 60 °C for one hour. Then, the enzyme was heat inactivated, and the reaction mixture was treated with exonucleases I and III (NEB, following manufacturer’s protocols) to cleave any oligonucleotide that was not circularized. For oligonucleotide purification, the reaction mixture was extracted with phenol/chloroform and precipitated with sodium acetate. Finally, the circularized oligonucleotide was applied to an Amicon 10,000 Da molecular weight cut-off centrifugal filter unit to ensure the full-length oligonucleotide was isolated from any short, linear nucleotides produced during the exonuclease reaction.

d. **Polyacrylamide gel electrophoresis analysis of caged siRNA**

Samples (40 pmol of unligated caged siRNA, 40 pmol of caged siRNA after Circligase reaction, 20 pmol of purified caged siRNA after Circligase and exonuclease reactions, and 20 pmol of purified product after irradiation at 365 nm for 15 minutes) were brought to a total volume of 10 µL. Then, 10 µL of loading buffer II (Ambion) was added to each sample, and they were loaded onto 20% polyacrylamide, 7 M urea gel. The gel was electrophoresed at 300 V for 45 minutes, and staining was performed with ethidium bromide for 10 minutes. Gels were imaged on a Bio-Rad Gel Doc 2000 system.
Samples that were uncaged were irradiated with a UV transilluminator (Spectronics Corporation TL-365R) at 365 nm (9 mW/cm$^2$ at peak intensity) for 15 minutes.
III. References


**Figure S1.1** Caged siRNA structure and general ligation scheme. 2’-F bases are indicated in blue, DNA bases are black, and photocleavable spacer groups are red.
**Figure S1.2** MALDI-TOF analysis of caged siRNA. The peak at 14,310 corresponds to the intact caged siRNA (expected mass is 14,136). The lower mass peaks are indicative of photolysis products resulting from oligonucleotide cleavage due to MALDI laser exposure.
**Figure S1.3** Denaturing PAGE (20%) analysis confirming formation of circularized siRNA by ligation. 40 pmol of (1) unligated caged siRNA and (2) caged siRNA after Circligase reaction were loaded on gel. 20 pmol of (3) purified caged siRNA after Circligase and exonuclease reactions and (4) purified product after irradiation at 365 nm for 15 minutes were loaded on the gel. The gel was electrophoresed at 300 V for 45 minutes, and staining was performed with ethidium bromide for 10 minutes.