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Caged siRNAs for Spatiotemporal Control of Gene Silencing

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Abstract: Various strategies have been employed to achieve control over delivery of siRNA molecules to intended target cells. Photocaging is one specific class of modifications for silencing oligonucleotides that block their bioactivity until exposure to near-ultraviolet light. These caged RNAi effectors enable both spatial and temporal targeting of a dosed release of gene silencing agents by directed light exposure that photocleaves the cage moieties. Herein we compare the photochemical properties of cage compounds and strategies for their use, attached either randomly or site-specifically, to demonstrate various forms of gene expression regulation *in vitro* and *in vivo*. This light-controllable strategy has potential applications for precisely probing developing biological systems and eventually enabling targeted gene-silencing therapeutics.

Keywords: Caged; siRNA; photoactivation; UVA light; targeted gene silencing

Introduction

Since its discovery a decade ago, the use of RNA molecules to silence gene expression has spawned a revolution in genetic manipulation that is poised to have significant clinical impact. RNAi has gained attention in laboratory and therapeutic settings because the small molecule effectors, such as small interfering RNAs (siRNAs), are generally more potent regulators of gene expression than traditional antisense compounds.[1] Many nucleic acid chemical modifications have been attempted to improve the pharmacological properties of siRNAs (reviewed in ref 2). In order to serve as a useful therapeutic, the siRNA must be efficiently delivered either systemically or directly to the intended tissue, exhibit specific knockdown of the target gene, and maximize the duration of silencing.[3] In the common effort of expanding the utility of siRNAs, many have explored the use of chemical modifications that optimize RNA lipophilicity, increase duplex stability, or impart nuclease resistance to achieve these aims. Although improving pharmacological efficacy through chemical modification is important for the realization of RNAi therapeutics, less progress has been made with respect to both spatial and temporal control over gene silencing. Some techniques shown to control RNAi use chemically inducible endogenous expression of hairpin RNA.[4,5] While these approaches do add a level of dose and temporal control via delivery of the inducer, light-based triggers are potentially much faster and may allow for spatial resolution on the nanometer scale.[6] Because light can be delivered in step function fashion, and in the absence of light, no further photoactivation occurs, there is lesser activity following removal of the light trigger, whereas residual concentrations

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of chemical inducers may cause slower returns to the uninduced biological state.

One way to achieve photocontrol is to introduce a temporary chemical modification which blocks silencing activity and can be removed with an external light trigger. Here we review the photocaging spatiotemporal strategy, which involves the covalent attachment of photolabile compounds to effector nucleic acid species that blocks bioactivity until triggered with near-ultraviolet light. Various cage groups are compared, including classic nitrobenzyl and other newer cages attached to oligonucleotides to control siRNA activity. Cage attachment to siRNA molecules has evolved from random attachment to site-specific incorporation. Knowledge of the RNAi process and how the components of this process interact with one another allows for the directed attachment of cage compounds to improve photoactivation efficiency. In the future, this strategy may also be used to couple efficient caging with the incorporation of delivery agents functionalized to noncritical locations of the RNAi effectors. Although this field is still progressing, photocontrol of knockdown oligonucleotide activity has been demonstrated with a variety of approaches, where caging also afforded additional protection against nuclease digestion. The beneficial properties of these caged gene silencing effectors may allow their use for spatial and temporal targeting in a controlled dose release manner, which has potential applications for precisely probing developing biological systems and as a targeting strategy for gene-silencing therapies.

Caged Compounds for Control of Bioactivity

Caging is a specific term which describes the use of a photolytic chromophore for the rapid release of a biologically active substrate.[7] The covalent attachment of a photosensitive compound produces a photocaged compound when the attachment of this group blocks native biochemical or biological activity and the blocked activity can be restored by light treatment. Therefore, the term caged, in this context, is descriptive of the photoactivation property and does not refer to physical trapping of the inactivated substance within a crystal lattice or shell. Caging an effector should abolish its normal biological activity, yet allow complete restoration with photoactivation (Figure 1). This cessation of activity in the caged form is particularly relevant in many siRNA studies where small amounts of "leak", or activity in the uninduced state, could lead to a blurring of kinetic study results or silencing

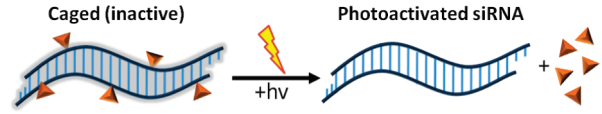

Figure 1. Caged siRNA is inactive until exposed to light, enabling initiation of targeted gene silencing.

from a potent sequence in unintended tissues. Attachment of the cage and inactivation of the biomolecule must be transient and reversible in the presence of the appropriate incident light. This restriction eliminates stable photoresponsive elements, such as biological photoswitches (reviewed in ref 8). Although the definition of caging requires that the photochemistry be used to release a biochemically active substrate, the initial findings by Kaplan and colleagues were inspired by earlier reports in which photolabile substituents were used as protecting groups in synthetic chemistry for carboxylic acids, alcohols, amines, ketones, or phosphates (reviewed in ref 9).

Classically, caged biomolecules have been used to study the time course of cellular responses induced by a millisecond step increase in the intracellular concentration of the analyte of interest. As aforementioned, Kaplan *et al.*[7] were the first to apply this type of photochemistry to release an inducer in a biological system; in this case, caged ATP controlled the Na:K ion pump in erythrocytes. Time course experiments using caged compounds have been used to study a wide range of biological processes *in vitro* and *in vivo*, including studies of cell motility, muscle fibers, active transport proteins, biological membranes and other intracellular responses (reviewed in refs 8, 10−13). Applications of cage groups have expanded beyond their beginnings in organic synthesis and step increases in concentration of biological inducers to play an important role in the inactivation of macrobiomolecules. While the early biochemical studies were limited to evaluating the time course of brief biological responses *in vitro* and *in situ*, caged macromolecules afford control to a broad range of laboratory and potentially therapeutic applications. Photoprotected species can be dispersed throughout the biological target without eliciting the normal bioactivity, which is a major advantage over conventional methods

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of delivery. Additional benefits of this strategy include control over local concentration and spatial distribution, and that the temporal release of the effector can be varied from seconds to nanoseconds.[14]

In order to maximize efficacy in biological systems, cage compounds must optimize the induced response while avoiding unwanted effects. The chromatic properties of the caged species must allow for efficient and nontoxic substrate release. Therefore the cage should have a high extinction coefficient at wavelengths that do not damage biological systems. Additionally, high quantum yield of the attached chromophore is desired to minimize the light dose required for photoactivation. For applications in which a rapid biological response is being measured, the rate at which the product is released through dark-reaction intermediates may also be a significant factor. The caged molecule should be water-soluble and resistant to hydrolysis in order to be properly utilized under aqueous conditions in physiological pH ranges. Additionally, the caged molecule and the released byproduct should be physiologically inert to avoid potentially toxic side-responses. Lastly, in order to progress beyond proof-of-concept studies, the mode of covalent attachment between the cage and biomolecule should be relatively simple, reproducible, and cost-efficient. Although many caging systems adequately adhere to these characteristics, no single caged nucleic acid exhibits the ideal properties for all categories. Many types of caging groups have been developed in efforts to improve the aforementioned properties. However, most of these are simple conjugations to existing photoprotecting groups. For instance, nitrobenzyl derivatives are often adorned with electron-donating functional moieties to achieve a red-shift of absorbance into the near-UV-light range.[15] A review of caging groups for nucleic acids is presented in the following section.

In the case of controlling siRNA activity, there are several steps in the RNAi pathway that could be disrupted with caged compounds. While each of these process control points is discussed in more detail later in this review, a graphical overview for context is provided in Figure 2. Following delivery to the cytosol by various means, both 5′ ends of these 19−21pb RNAs are phosphorylated before assembly into the RNA-induced silencing complex (RISC). A cage group on the 5′ hydroxyl moiety may prevent kinase activity and render these siRNAs inert until photolysis. Likewise, siRNAs with caged terminal phosphates may similarly disrupt downstream processing.[16] Internal phosphate backbone caging of siRNAs may also disrupt the RNA-binding affinities

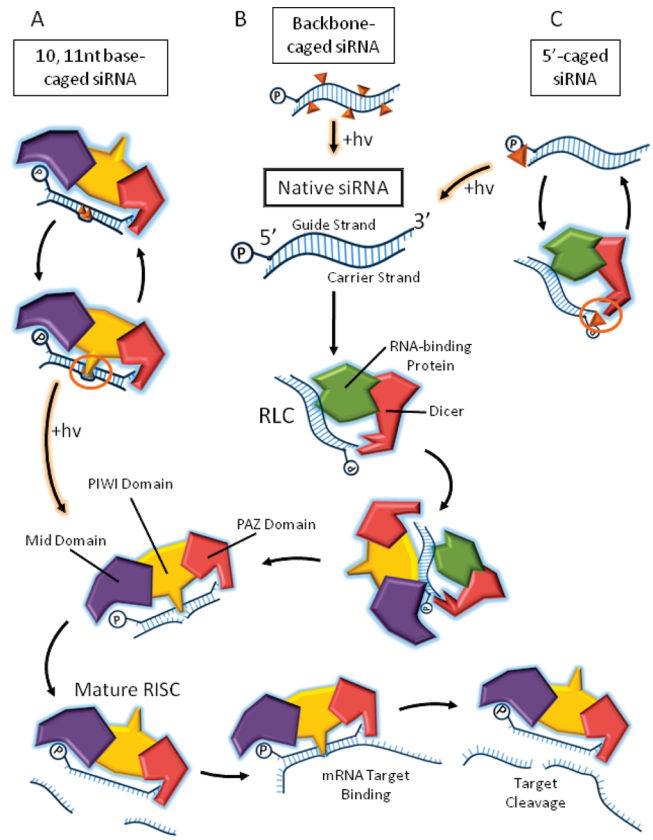

Figure 2. Overview of caged siRNA processing control points in RNAi. Boxed labels refer to caged siRNAs in various forms that may be used to control gene silencing with light exposure, and orange ovals indicate inhibition of processing by cage substituents: (A) Caging internal (e.g., 10th, 11th) nucleotides of siRNAs may prevent cleavage and removal of the carrier strand. (B) Statistically backbone-caged siRNAs may not fully hybridize or associate with RISC components due to steric blockade. (C) Caged 5′-phosphates of siRNAs may disrupt interactions with the RISC-loading complex (RLC). Caged siRNAs that are photoactivated continue through the RNAi process to mRNA cleavage and recycling as would native (noncaged) siRNAs.

of the proteins in RISC, preventing its activation.[17] Following these initial steps, cage groups on the antisense (guide) strand may offer greater control rather than on the sense (carrier) strand, as they are separated, leaving the antisense strand associated with a mature RISC that hybridizes to the target mRNA. Nucleobase cage groups on the siRNA may disrupt the duplex, preventing hybridization, or blocking catalytic

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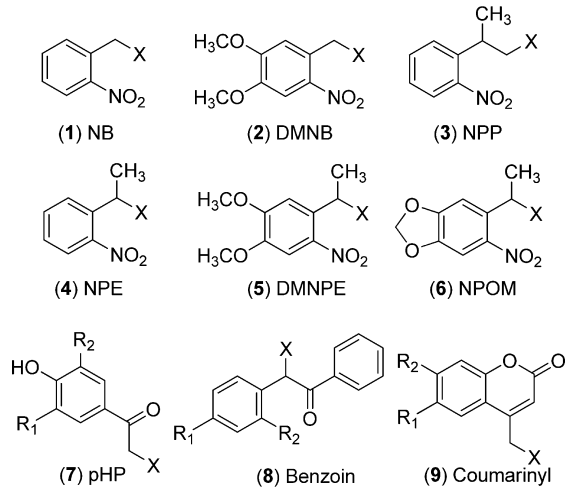

Figure 3. Common cage compounds amenable for use with siRNAs (also see Table 1): NB (nitrobenzyl), DMNB (dimethoxy-nitrobenzyl), NPP nitrophenylpropyl, NPE (nitrophenylethyl), DMNPE (dimethoxy-nitrophenylethyl), NPOM (6-nitropiperonyloxymethyl), *p*HP (*p*-hydroxyphenacyl), benzoin, and coumarinyl.

cleavage of mRNA. Cleavage occurs between nucleotides 10 and 11 (from the 5′ end), so a cage group in this vicinity of the antisense strand may maximally disrupt silencing activity.[18] Due to the recycling of the antisense strand to act on multiple mRNA targets,[19] and in some biological systems amplification of silencing agents by RNA-dependent RNA polymerases,[20] a single photoactivation event at any of these steps can initiate a cascade of silencing activity, enabling a powerful control technique.

Cage Compounds To Control siRNA Function

The number of incidences in which photoprotection groups have been used to cage biomolecules has been rapidly expanding since ATP was first caged by Kaplan in 1978. For the purposes of this review, emphasis is placed on various *categories* of cage compounds and examples of their use in controlling siRNAs and other knockdown oligonucleotides. Chemical structures of the cages, with X denoting the effector, are shown in Figure 3, with relevant optical parameters, such as absorption maxima, quantum yields, and extinction coefficients, compared in Table 1.

2-Nitrobenzyl Caging Group. The 2-nitrobenzyl (2-NB) caging group and its derivatives are the most widely utilized cage molecules to date, and therefore are the best characterized.[21] The pervasiveness of 2-NBs in the literature is not necessarily an indicator that this caging group has ideal photochemical properties or is the easiest to use via synthetic or biochemical methods, but rather of its widespread use as the first type of cage compound applied to biological systems. Hoffman and colleagues' pioneering work using caged ATP for controlling the NA:K pump utilized 2-NB cages from this category.[7] These 2-NB cages were also the first used to study millisecond time-scale induction of the actin−myosin response to photolyzed caged ATP.[22,23] The basic structure that defines the group is a benzyl frame with a nitro substitution ortho-located to the effector. Variants of this structure commonly include substitutions directly on the aromatic ring and/or alternative attachment chemistry between it and the effector.

Table 1. Photonic Characteristics of Common Cage Compound Candidates for Use with siRNAs[a]

cage	λ_{max} (nm)	ε (1/M × cm)	Φ	effector
NB[7]	320	430	0.63	ATP
DMNB[24]	350	5000	0.07	ATP
NPP[29]	355	400	0.3	thymidinyl carbonate
NPE[27]	320	9100	0.63	ATP
DMNPE[27]	365	4795	0.07	ATP
NPOM[96]	356	6887	0.094	DNA
*p*HP[14]	286	14600	0.3	ATP
benzoin[38]	350		0.39	cAMP
Bhc[137]	368	17470	0.019	glutamate

[a] Absorption spectrum maximum, extinction coefficient, and quantum yield of commonly used cages. Bhc: 6-bromo-7-hydroxycoumarin-4-ylmethyl.

Cage compounds in the 2-NB family can be further subdivided based on different substitutions at positions 4 and 5 as well as the exocyclic linker, as illustrated in Figure 3. Common subdivisions include the following: (**1**) 2-nitrobenzyl (NB), (**2**) 4,5-dimethoxy-2-nitrobenzyl (DMNB), (**3**) (1-(2-nitrophenyl)propyl (NPP), (**4**) (1-(2-nitrophenyl)ethyl (NPE), (**5**) 1-(4,5-dimethoxy-2-nitrophenyl)ethyl (DMNPE), and (**6**) 6-nitropiperonyloxymethyl (NPOM). The driving motivation for developing derivatized forms of the cage compound is to improve either the rate or efficiency of photolysis. Generally, electron-donating ring substitutions result in a hyperchromatic absorbance shift, allowing photolysis at longer wavelengths that are less damaging to

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biological systems. However, these substitutions often significantly reduce the efficiency of photolysis.[15,21,24] Benzylic carbon α-substitutions presumably affect the C−H bond at the site of attachment in the excited state, which may affect the rate of hydrogen transfer that forms the *aci*-nitro intermediate.[21] However, the decay process of 2-NB photocleavage is complex, and the rate constants at each stage depend heavily on pH, other metabolites present, and the nature of the substrate, as well as derivations to the cage compound.[25] The currently theorized mechanism of 2-NB photolysis begins with the delivery of a photon that can be absorbed by the chromophore and induces an excited state. Conversion to the hemiacetyl intermediate is then further hydrolyzed to the 2-nitrosobenzylcarbonyl and the released biomolecule.[21,25−27] The nitroso byproduct has historically been theorized to be potentially reactive and toxic to biological systems. However, there are only a few incidences of complications related to this byproduct reported in the literature,[7,28] and subsequent work suggests that these concerns may have been overstated.[21] Newer nitrobenzyl compounds such as NPP generate a less reactive α-methylnitrostyrene rather than the nitroso compound photoproducts from the other 2-NB cage groups.[29]

***p*-Hydroxyphenacyl Caging Group.** The *p*-hydroxyphenacyl group (*p*HP) (**7**, Figure 3) is a relatively new category of caging compounds. Although it was first utilized in photoreactions in 1962[30] and identified as a potential photolabile protecting group by releasing glycine in 1973,[31] it has only been a decade since this family of compounds was first utilized as a caging group for biological applications. Much like the original 2-nitrobenzyl caging of ATP by Kaplan, this moiety was also exploited by Givens and Park to generate photoreleased ATP.[32,33] Despite their recent entry into the caging field, these compounds have been met with enthusiasm and are often cited as promising candidates for wider use.[8,12]

As denoted in the name, the general structure of the *p*HP group consists of a phenyl ring with an acyl attachment chemistry and a hydroxyl in the para-position. The acyl functional group attachment allows for several attachment schemes such as carbonyls, acyl halides, esters, and more. Other substitutions can be made at the meta-positions on the phenyl ring. Electron-donating groups at these positions cause a hyperchromatic shift, but they also drastically reduce the quantum yield of the photoreaction.[34] In contrast, electron-withdrawing ring substitutions tend to increase the quantum yield.[21] Interestingly, the withdrawing substitutions have little effect on the absorption spectra. The photorelease mechanism of the *p*HP cage is not as well understood as the 2-NB group, but a general chemical pathway has been proposed.[21,34]

Biomolecules caged by *p*HP include, but are not limited to, ATP,[32,33] other phosphates,[33,34] and various amino acid-based structures.[35] These studies indicate that the *p*HP cage group is capable of desirable quantum yields, rapid photorelease, acceptable solubility and stability in aqueous conditions, and nontoxic photo products. In addition to the favorable aspects of these compounds, several drawbacks were also identified. Most notably, these compounds do not absorb well for wavelengths above 320 nm, which reduces enthusiasm for their use in cell and tissue systems.[12] As aforementioned, derivations on the ring structure can improve the absorption properties, but generally result in a decrease in quantum yield.

Benzoin Caging Group. The benzoin group was first reported in 1971, when 3′,5′-dimethoxybenzoin-caged acetate was released upon photoexposure.[31] This study reported a clean photolysis reaction yielding acetate and dimethoxybenzofuran at an exceptionally high quantum efficiency ($\Phi = 0.64$). Although various other derivatives have been tested, the two most common forms include the unsubstituted and the 3′,5′-dimethoxy benzoins shown in **8**, Figure 3 (reviewed in ref 12). The high degree of disagreement in several of the proposed mechanisms of photolysis is likely due to varying substrates and the attachment chemistries which have a profound effect on the mode of photorelease.[31,36]

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Benzoin cage compounds typically exhibit high quantum yields and fast release kinetics.[37−39] However, there are several drawbacks to this cage group. It has a strong absorption coefficient, but this occurs in the 300 nm range.[12] Radiation of this wavelength is not well-tolerated by biological systems. Additionally, these compounds exhibit strong fluorescence, limiting their use for some fluorescence-based *in vitro* assays.[40] Finally, these compounds are highly hydrophobic and reduce the solubility of the bound substrate in physiological conditions. An attempt to develop a water-soluble derivative using bis(carboxymethoxy) substitutions reduced the photolytic yield.[41] While the hydrophobicity of the benzoin substituents may hinder studies of some caged small molecules, these characteristics might be of use in siRNAs. For instance, a localized hydrophobic region at a critical site on the antisense strand created by a bulky nonpolar cage group might disrupt interactions with more polar residues of RISC proteins. Additionally, overall charge reduction on siRNAs and substitutions to increase lipophilicity are desirable for cellular delivery. For example, conjugation of cholesterol moieties to siRNAs can promote cell uptake and increase *in vivo* delivery.[42] The concept of caging to promote cellular delivery has been demonstrated with small molecules such as luciferin,[43] but has not been applied to siRNAs; thus nonpolar groups like benzoins may have utility in such applications.

Coumarinyl Caging Group. The coumarinyl group was first recognized as a potential photoprotection group in 1984, when a (coumarin-4-yl)phosphate ester was found to be photosensitive.[44] It was nearly a decade later before this chemical group was evaluated as an alternative cage compound for the 2-NB group.[45] These compounds typically possess high absorption coefficients, fast photorelease kinetics, and large two-photon excitation cross sections. The strong absorption properties of these compounds (ε_{max} typically from 4,000 to 20,000 M^{-1} cm^{-1}) make up for relatively poor quantum yields of photorelease.[21] Another property of these cage compounds is a strong fluorescence signal. The emission spectra may overlap with common fluorescent probes, which limits the use of coumarin derivatives in certain biochemical assays. Despite this minor limitation, the coumarinyl group has demonstrated practical photochemical properties along with a wide diversity of derivatization possibilities.

The base structure of this group is a derivatized coumarin, as the name indicates. Coumarins consist of two fused six-membered rings, one of which is an oxygen-containing heterocyclic ring with an adjacent carbonyl group (**9**, Figure 3). Attachment is in the meta-position in reference to the ring-bound oxygen. A methyl functionality lies between the bound substrate and the coumarin. Ring substitutions at C6 and C7 are commonly used to red-shift the absorption maxima. Ring derivatizations can also be used to improve the caged species' membrane permeability and water solubility. In addition to these substitutions, the nature of the bound substrate can have a significant effect on the photochemical properties of cage release. The coumarinyl group can be subdivided into several groups consisting of 7-alkoxy, 6,7-dialkoxy, 6-bromo-7-alkoxy, and 7-dialkylamino (reviewed in ref 21).

A photorelease mechanism was proposed from the studies of various coumarinyl-caged phosphates.[46−48] In this scheme, photoactivation results in an excited singlet state, followed by heterolysis or homolysis and subsequent electron transfer at the site of substrate attachment. The unstable cationic released cage is then trapped by the solvent. Alternatively, studies of coumarinyl-caged amines suggested a slightly

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different mode of photorelease.[49−51] Photoexcitation results in electron-donor-dependent homolytic cleavage and the formation of a radical anion intermediate.

The coumarinyl group has successfully been used to cage phosphates, carboxylates, carbonyls, alcohols, amines, and sulfonates (reviewed in ref 21). Generally, the photokinetic properties of these compounds and the flexibility for creating various coumarin derivatives make them desirable cage groups. Despite the success of this group when used for *in vitro* assays, only a few examples in biological systems exist. However, these studies have yielded positive results. One of the first examples of a coumarinyl-caged macromolecule used in a biological system was Bhc-caged mRNA for photoinduction of gene expression in zebrafish embryos, detailed below.[52,53]

Caging of siRNAs

Examples of caged nucleic acids are much less abundant and more recent when compared to other categories of caged compounds, such as small molecule metabolites or proteins. This is surprising, given that the original model for caged biomolecules was based on nucleotides such as caged ATP.[7] Additionally, the potential utility of caging NAs is readily apparent since they participate in a variety of biochemical reactions that are inherently dependent on structure. Caging has been used for microarray oligonucleotide synthesis[54] and as an alternative nucleobase protection for solid-phase synthesis.[55] This technique has also been used to control the self-annealing of a hairpin[56] and the hybridization of an oligonucleotide to its complement.[57] Caging compounds have also been employed by Meldrum *et al.* to study DNA repair kinetics.[58] This study was an extension of their earlier work in which radiolabeled caged ATP was used to study the same phenomenon.[59]

Although earlier examples of caged nucleic acids exist, the first study in which a caged nucleic acid was used *in vitro* or *in vivo* for control of gene expression did not occur until 1999.[60] In this work, plasmids encoding Green Fluorescent Protein (GFP) or luciferase were caged with DMNPE groups in bulk, based on diazo attachment to nucleic acid backbone phosphates. Using this strategy, the authors were able to demonstrate photoinduced transgene expression. Despite this three-decade gap from caged ATP to caged DNA use in biological systems, this study ignited interest in the field by demonstrating the utility of caging nucleic acids to control gene expression. Since that time there have been two classifications of caged nucleic acid molecules: those that were caged using this random method, and more recent site-specific approaches to dictate the exact location of the cage molecule on the effector; both of these categories are detailed below.

Statistical Backbone Caging of siRNAs. Monroe *et al.* used a batch-style reaction to achieve random attachment of DMNPE to the phosphate backbone based on diazo attachment chemistry developed by Walker *et al.*[60,61] A hydrazone precursor to DMNPE can be oxidized to react with phosphates, sulfates or carboyxlates on effectors, so the likely target on nucleic acids is the phosphate backbone. This approach was later adopted to cage mRNA and plasmid DNA using a derivatized coumarin caging group.[52,53] The strategy of bombardment with excess cage has since been termed "statistical caging" by Mayer and Heckel.[8] Through statistical Bhc caging, Ando *et al.* were able to control transcription of GFP and eng2a mRNA in a zebrafish model, inducing spatially constrained fluorescence and inhibition of eye development, respectively.[53] The Bhc-caged mRNA appeared to exhibit longer lifetimes than uncaged mRNA *in vivo*. This could be the result of a conformational change that prevented nuclease access, as caged mRNAs exhibited substantially different electrophoretic mobility than native sequences.

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The first report of caged siRNAs came from Friedman's group, who used a similar scheme of diazo attachment of DMNPE to double-stranded siRNAs for silencing of transient GFP expression in cell cultures.[62] In this study, comparison of uncaged siRNA targeting GFP versus expression level of cotransfected RFP showed moderate photocontrol of GFP expression with light exposure. They were able to reduce GFP fluorescence with photolysis of the caged siRNA by an amount equaling about 80% of that of native siRNA, with 35% residual knockdown activity in nonirradiated caged samples ("leakage" activity).

The stability of these RNAs contrasts with other published results,[17] in which caging of double-stranded RNA (dsRNA) resulted in fragmentation thereof, presumably through a 2′-hydroxyl attack at the phosphotriester.[63] However, the conflicting results regarding backbone-caged dsRNA stability are likely due to differences of scale: in Shah and Friedman's work, for example, an average of only 1.4 cage groups per double-stranded siRNA (42 nucleotides in total) was used. Presumably, lesser adduct present resulted in a decreased chance of hydrolysis of these smaller RNA species. Additionally, with an average of one cage compound per siRNA, the cage could have frequently been bound to the terminal phosphate of the oligonucleotide, precluding hydrolysis activity. When Shah attempted to decrease leakage GFP expression by increasing the percentage of nucleotides caged to 4.8%, 10.8%, and 15.2%, higher proportions of cage compound severely reduced the amount of activity recovered after irradiation.[62] While the group attributed this phenomenon largely to incomplete photolysis, hydrolysis of the siRNA species could have been a significant factor as well.

Caging of 2′-Protected siRNAs. One potential means to prevent phosphotriester hydrolysis of caged RNAs is to replace the catalytic 2′-hydroxyl with a stabilizing modification. In a related effort to increase the efficiency of siRNAs through prolongation of lifetimes following delivery, chemically modified nucleic acids have been explored for their ability to mimic the activity of small RNAs through the RNAi pathway. To be practical candidates for RNAi caging studies, these chemical modifications should (1) be incorporated at enough nucleotide locations to protect the RNA oligonucleotide, (2) demonstrate similar silencing activity relative to the analogous small RNAs, and (3) exhibit limited toxicity. Chiu and Rana completed a particularly thorough investigation of which RNA modifications can be tolerated by the RNAi pathway.[64] Evaluation of the 2′ modifications such as *O*-methyls, locked nucleic acids (LNAs), or other bulky substitutions recommends minimal substitution, as they can protrude into the minor groove and disrupt RISC recognition of the duplex.[65−67] Complete 2′-deoxy substitution results in a conformational change of the duplex to a B-type helix and does not exhibit RNAi activity. 2′-Deoxy-2′-fluoro modifications, both in the trans and arabino (inverted) form, seem to be the best-tolerated alteration with respect to maintaining silencing activity.[68−70]

Recent work has shown that siRNAs containing 2′-fluoro modifications at all purine and pyrimidine nucleotides elicit RNAi.[71] These fully-2′-fluorinated nucleic acids (FNAs) were generated for RNAi studies through either custom solid-phase synthesis or *in vitro* transcription using a mutated polymerase and 2′-fluorinated nucleoside triphosphates. This work also demonstrated that FNAs are highly resistant to sugar-specific enzymatic digestion, which may improve lifetimes following delivery and improve *in vivo* efficacy. Because melt curve analysis of FNAs demonstrates an increase in the thermal stability of the synthetic FNA duplexes, this could offset potential decreases in thermal stability due to cage attachment. In addition to improving resistance to enzymatic degradation and increased thermal stability, it has been demonstrated that 2′-fluoro modifications do not result in a loss of hybridization sequence specificity.[72] Although the observed dramatic increase in melting temperature of these 2′-fluoro modifications might suggest a possibility of silencing of closely matching target sequences, these modifications have been shown to reduce off-target effects normally observed following siRNA delivery.[73−75] In fact, siRNAs

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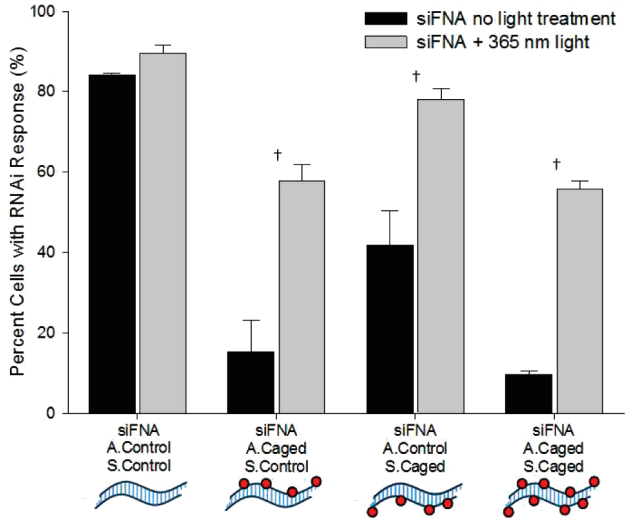

Figure 4. DMNPE-caged siFNA silencing of GFP in BHK cells. Black bars represent treatments that were protected from light. A significant difference exists between all 365 nm photoexposed (gray bars) and non-photoexposed (black bars) samples (denoted with †, $p < 0.05$). Note differences in siRNA inactivation when the antisense, sense or both strands were caged. Reprinted from ref 17. Copyright 2008 Royal Society of Chemistry.

containing 2′-fluoro modifications have shown an ability to evade the cellular immune response by avoiding activation through Toll-like receptors (TLRs) in the endosome.[74] This suggests that 2′-modified siRNAs may be inherently less immunostimulatory than unmodified siRNAs.

In a series of studies, Monroe and colleagues evaluated a RNAi system targeting a cotransfected GFP reporter gene with siRNAs containing fully 2′-fluoro-modified nucleosides (siFNAs) followed by the phototriggering of this system through DMNPE caging.[17] FNA strands were internally caged through the same DMNPE diazo attachment schemes described above to yield an average of 8 and 9 cage groups per 21-mer oligonucleotide strand for the sense and antisense FNA strands, respectively. The resulting DMNPE-caged FNAs were resistant to a sugar nonspecific nuclease, demonstrating improved enzymatic stability relative to the uncaged species. Characteristic of studies employing this type of caging strategy, RNAi effectiveness was reduced in all caged siFNAs, and exposure to 365 nm light partially restored the silencing activity in all cases (Figure 4). Since both the antisense and sense strands were caged separately, four combinations of the combined siFNAs were possible

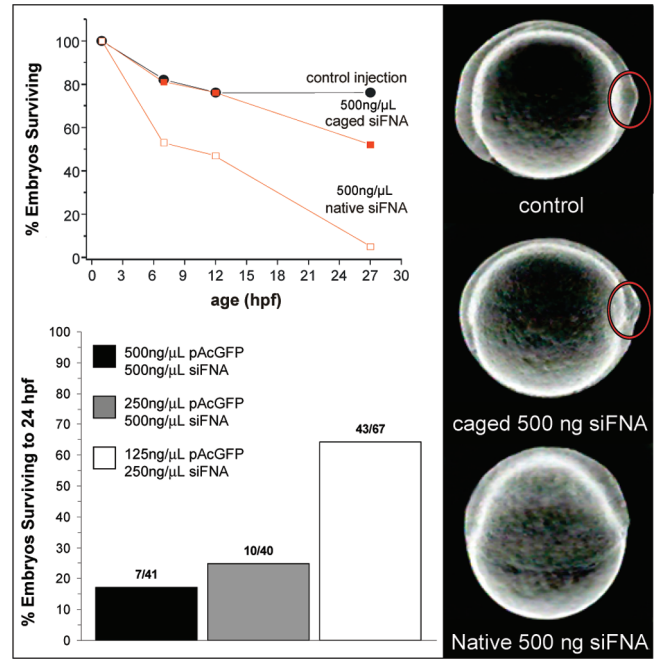

Figure 5. *In vivo* demonstration of caged siRNAs acting to prevent toxicity of these effectors at similar concentrations injected into zebrafish embryos. Bottom left: siFNA concentrations above 250 ng/μL resulted in increased mortality. Top left: Caging of siFNAs protects the developing embryo from the siFNA induced toxicity (at 500 ng/μL). Right: Developmental delay at 12 h postfertilization in response to siFNA toxicity at a concentration of 500 ng/μL. Control (no siFNA) and caged siFNA injected embryos exhibit a developing tail bud, encircled. Reprinted with permission from ref 17. Copyright 2008 Royal Society of Chemistry.

(uncaged antisense uncaged sense, caged antisense uncaged sense, uncaged antisense caged sense, caged antisense caged sense). The antisense strand was predictably more sensitive to inactivation through caging, which also led to a reduced photoactivation response.[17]

Microinjected zebrafish embryos were used as a model platform by Monroe and colleagues to demonstrate photoactivated RNAi *in vivo*.[17] In addition to supporting the results of their cellular system, they demonstrated that caging of siFNAs protected the biological system from toxic doses of the effector (Figure 5). In this study, embryo development stalled when extreme levels of RNAi effectors were delivered to the system. It is possible that the high concentration of siFNA competitively sequestered the available RISC proteins, halting endogenous miRNA developmental regulation of gene expression (Figure 5, right panel). Although the mode of toxicity was not verified, the combined results of this work suggest that photocaging may offer control over RNAi therapeutics for spatially and temporally directed activation, while improving enzymatic stability and potentially enabling therapeutic dosing via photoexposure intensity.[71] Since photorelease of active RNAi effectors from caged precursors is inherently a function of incident light delivery, partial photorelease of active siFNA could be achieved and thera-

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Table 2. Summary of Caged Knockdown Oligonucleotide Studies[a]

cage	oligo type	cage location	target	system	max knockdown (%)	leak	oligo modification	light source	dose	λ_{max} (nm)	ref
Bhc	mRNA	backbone	GFP, eng2a	Zf	67	0		100 W mercury lamp	200 mJ/cm^2	365	53
DMNPE	siRNA	backbone	GFP/RFP	HeLa	79	35		Blak-Ray UV lamp			62
DMNPE	siRNA	backbone	GFP	BHK, Zf	63	11	2′-Fluoro	Greenspot	40 J/cm^2	365	17
NB	RNAzyme	2′-OH			100	0		Lamda Physik EMG 201 MSC laser	11.7 J/cm^2	308	81
NPE	siRNA	5′ phosphate	GADPH	HeLa	108	8		UCOM microplate photoactivator	2 J/cm^2	365	16
NPE PL	PNA	5′, 3′ termini	bozozok	Zf	94	6	2′-OMe	UV transilluminator	4.32 J/cm^2	365	100
NPE PL	PNA	5′, 3′ termini	chordin	Zf	85	16	2′-OMe	UV Transilluminator	4.32 J/cm^2	365	100
NPE PL	antisense	5′, 3′ termini	c-MYB	k562	52	0	PS	Spectroline UV transilluminator	2.7 J/cm^2	365	101
NPE PL	RNA bandage	5′, 3′ termini	GFP		95*	30*	2′-OMe	UV transilluminator	2.7 J/cm^2	365	102
NPOM	antisense	base (dT)	Luciferase	3T3	95	0	PS	Spectroline UV lamp		365	95
NPOM	DNAzyme	base (dT)	RNA		91	0		UV transilluminator		365	96
NPP	siRNA	base (dG, dT)	EGFP	HeLa	95	13	D	MinUvis Hg lamp	2.88 J/cm^2	366	18
PL	morpholino	5′, 3′ termini	no tail	Zf				mercury lamp		360	105

[a] Column headings and abbreviations are as follows. Cage compounds: photolytic molecule used (PL, photolinker). Oligo type: variant of antisense/knockdown agent. Systems: HeLa, immortal HeLa cancer cell line; k562, immortalized leukemia cell line; 3T3, fibroblast cell line; BHK, baby hamster kidney cells; Zf, zebrafish. Max knockdown: (knockdown of target by photoactivated oligonucleotide)/(knockdown by positive control). Leak: (knockdown of target by caged oligonucleotides)/(knockdown by positive controls). Asterisk indicates those RNA bandage studies where an increase in expression was activated with light rather than gene silencing. Modifications: D, deoxynucleotide insertions; PS, phosphorothioate; 2′-OMe, methoxy insertions at 2′-hydroxyl; 2′-fluoro, fluorination at 2′-hydroxyl.

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

Site-Specific Caging. Each incidence of statistical RNA backbone caging described above found that the caged effectors demonstrated reduced activity relative to their respective controls, which was partially restored by exposure to 350−365 nm light. However, no statistically caged product has shown perfect binary behavior with respect to both suppression and restoration of activity. A summary of these performance criteria, as well as the effectors and the model systems in which they were evaluated, is shown in Table 2. Significant progress has been made in recent years toward site-specific caging of nucleic acids. Consideration of the studies above suggests that, in order to achieve superior, "on-off behavior" for caged siRNAs, control over positioning of the caging group is necessary. In many studies, the statistical caging method is a limiting factor in further understanding mechanistic interactions of caged siRNAs. While an average number of cage groups for each oligonucleotide can be determined, the number on each individual strand is left to chance and can likely vary substantially from the mean. In addition, it is believed that certain positions along an oligonucleotide may be more effective in inactivating the strand than others,[64] and statistical caging leaves the investigator no opportunity to guide the cage compounds to proposed advantageous sites. Accordingly, recent foundational work has focused on attaching photocages to predetermined sites on knockdown oligonucleotides. Other studies have used photocaging to investigate the folding dynamics of RNA strands, further developing the methods for site-specific caging.[8,76−78] More recently, short DNA and RNA oligonucleotides have been inactivated with site-specific cage chemistry reviewed in this section.

Heckel and Mayer used NPP cage groups on one or two of the six thymidine residues of a 15-mer DNA aptamer which inactivates thrombin to investigate the activity of the aptamer as a function of cage position.[79] It was determined that caged thymidine acted as a temporary base mismatch, preventing interaction with thrombin (thus decreasing blood clotting time) until UV irradiation, after which near-native binding kinetics were restored. Krock and Heckel also used 2-nitrobenzyl-caged thymidine residues to disrupt transcription activity through inhibition of T7 RNA polymerase.[80] The group synthesized a 68-nucleotide-long strand of DNA with a T7 promoter region and caged a shorter initiation strand complementary to the promoter region, preventing T7 recognition and processing until photolysis availed the

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complementary strand for duplex formation. Building on these diverse caging studies, subsequent work has expanded the possibilities for light-modulated genetic expression by attaching photolabile compounds to an assortment of carefully determined locations upon siRNAs and other antisense nucleic acids. In the case of siRNAs, several moieties present possible sites for cage attachment, including 2′-hydroxyls, nucleobases, photolinkers, and terminal or internal phosphates, each of which is discussed below.

2′ Hydroxyl Caging. The first example of a nucleic acid with a caged compound inserted at a precise location was at the 2′-hydroxl of RNA, demonstrated by Chaulk and Macmillian.[81] In this study, the authors engineered the hammerhead ribozyme reaction to be photoresponsive by using 2′-*O*-(2-nitrobenzyl)-caged adenosines at the active site of substrate RNAs; this was also the first incidence of RNA caging. They prepared a ribozyme target that was caged at the site of catalytic cleavage and demonstrated protection of the RNA substrate until photoexposure: no ribozyme cleavage was detected with caged substrates prior to 308 nm irradiation, but activity equal to that of native ribozymes (70−80% cleavage) was detected after photorelease. Notably, the authors showed that attaching a nitrobenzyl to the 2′-hydroxyl did not disrupt complexing between the ribozyme and substrate; the cage compound merely prevented the hydroxyl attack. Although highly efficient, this technique required extensive synthetic procedures to produce the protected oligonucleotide. These authors expanded on this original work to disrupt spliceosome and polymerase activity using the site-specifically caged RNA strategy and have more recently published detailed protocols for their technique.[82,83] This 2′-hydroxyl caging strategy was later adopted by Pitsch for the development of alternative conditions for synthesizing RNA.[84] In addition to these early studies, there is a large body of literature relating to photoinduction of strand breaks using photosensitive adducts (reviewed in ref 8). To date, there have been no reports of 2′-caged siRNAs, although the site may be appropriate for photocontrol of RNA-RISC interactions. While Chaulk and MacMillan's work showed full tolerance for ribozymes to bind 2′-modified substrates, Chiu and Rana have shown that even the mildly bulky methoxy group, when placed at each 2′ hydroxyl of an siRNA duplex, completely abolishes siRNA activity.[85] It would seem, therefore, that the 2′-hydroxyl location on siRNAs has potential to effectively control bioactivity.

5′-Phosphate Caging. In an effort to reduce the leak activity, improve the efficiency of photoinduction, and avoid RNA hydrolysis, two research teams have separately expanded on caged siRNA by incorporating a single photolabile group onto the 5′ terminal phosphate of the siRNA antisense strand.[16,86] Each exploited a commercially available NPE-derived linker on the 5′-terminal nucleotide during 3′ to 5′ RNA solid phase synthesis. Based on previous work, this phosphate is thought to be critical for siRNA incorporation into RISC.[66,85,87] However, the results of these studies yielded mixed performance (compared in Table 2). Nguyen *et al.* demonstrated that these caged siRNAs had very little activity and that silencing was efficiently restored with light doses that were required to remove all cage linkers, which was determined *in vitro*. Observations of minute leak activities were explained by N-1 contaminants from incomplete RNA synthesis that had silencing activity due to the absence of the final caged linker. Shah and Friedman disputed this interpretation since their data shows significant leak RNAi activity in the caged uninduced state. Additionally, Friedman and colleagues tested highly purified antisense RNA strands with non-photolabile linkers at the 5′-phosphate to verify their results. Their interpretation was that the RISC components have the ability to interact with the remaining nonbridging oxygen, allowing some RNAi to proceed. Although the 5′-phosphate on the antisense strand is known to be required for RNAi activity,[88−90] it has also been shown that various linker modifications to this terminal location are tolerated if the 5′-phosphoester bond is intact.[90,91] To avoid this residual activity, light-activated RNAi might require an alternate site of cage attachment or caging on multiple sites for each siRNA. However, depending on the

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system studied and the necessity for complete abolishment and restoration of activity between the uninduced and induced state, these 5′-phosphate-caged siRNAs may be as effective as any of the other strategies presented (compared in Table 2).

Nucleobase-Caged siRNAs. The original site-specific caging work by Chaulk and MacMillian was limited to adenosine residues with the photolabile compounds attached to the 2′-hydroxyl.[81] However, the design of a caged siRNA for various mRNA target sequences will require more flexibility and include the other nucleobases as potential disruption sites. Pitsch and colleagues expanded the 2′-O-caging to include uracil, cytosine, and guanine.[84] However, additional work has attempted to react the caging species with nucleobases. The first example of a caged nucleobase appeared in 1992, where a caged adenosine derivative was used for self-replication but was not incorporated into an oligonucleotide.[92] The first example of a caged base within an oligonucleotide was also an adenosine derivative in 2004, whereby a nontraditional photoactive C8 thioether-linkage controlled DNAzyme activity.[93] Shortly after this study, a 2-nitrobenzyl group was utilized to cage the O4-position of thymine and the resulting nucleic acids were used to disrupt T7 polymerase[80] and control aptamer activity.[79] This same team also caged the C6 carbonyl of guanine in order to control aptamer activity by controlling G-quadruplex structure formation.[94] Another group concurrently presented NPE-caged guanine to study tertiary folding kinetics of RNA.[78] Immediately following these studies, Höbartner and Silverman produced a complete set of NPE base-caged RNA residues.[76]

These developments were soon incorporated into RNAi studies, and Mikat and Heckel built on the research done by Höbartner and Silverman to control siRNAs with the 2-(2-nitrophenyl)propyl (NPP) group.[18] Deoxyguanosine and deoxythymine residues were modified at the O6 and O4 positions, respectively, with the NPP moiety: dG^C and dT^C (dT^C structure shown in Figure 6). It has previously been shown that partial inclusion of deoxynucleotides does not significantly inhibit siRNA activity,[85] and this tolerance for deoxy-substitutions was confirmed by Mikat and Heckel with their sequences and reporter assay. The group generated a variety of combinations of caged siRNAs targeting GFP transfected into HeLa cells by placing a single caged nucleotide at locations 9, 10, 11, or two caged nucleotides at sites 10 and 11. Placing a single dT^C group at position 10 was found to be the most effective manner of modulating siRNA activity: leakage was found to be approximately 8%, while knockdown activity following induction with light exposure was 92% of positive controls (Figure 6). After analyzing additional combinations of caged sense and antisense strands, Mikat and Heckel concluded that caging central nucleotides of the antisense siRNA strand inhibits the RNAi cleavage step, which cleaves complementary mRNA at a position opposite to nucleotides 9−11 of the siRNA.[85] Inserting caged nucleotides away from the center of the antisense strand or at any location along the sense strand exhibited a drastically reduced ability to inhibit RNAi prior to photolysis.[18]

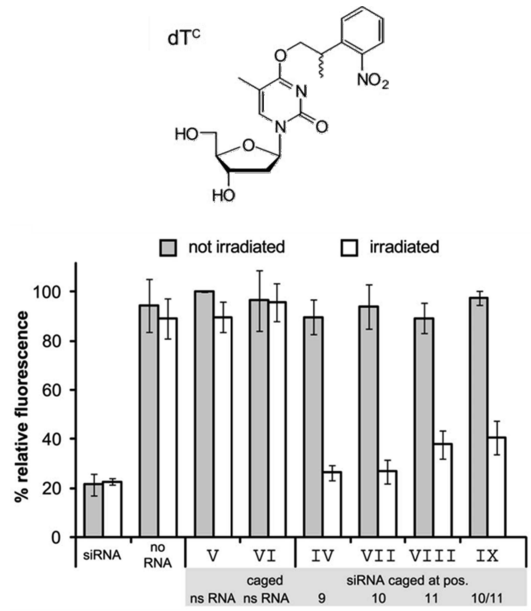

Figure 6. Nucleobase caging at cleavage site leads to reversible inactivation of siRNA. Fluorescence intensity of HeLa cells transfected with different siRNAs with (gray bars) and without (white bars) irradiation for 40 min 4 h past transfection. As a positive control, indicating full activity, a commercially available siRNA was transfected. The various negative controls were a sample not treated with any siRNA (no RNA), a sample transfected with a nonsilencing RNA (V) whose fluorescence intensity was defined as 100%, and the same nonsilencing RNA bearing photolabile protection groups (VI). The different caged siRNA sequences, with the NPP-caged T shown above inserted into the antisense strand at specified locations (IV, VII, VIII, and IX), are all inactive within error limits. For sequences IV and VII, a reactivation that can be assumed to be complete could be achieved, while sequences VIII and IX, even after irradiation, display a residual inactivation. Adapted with permission from ref 130. Copyright 2007 Cold Spring Harbor Laboratory Press.

Other Nucleobase-Caged Knockdown Oligonucleotides. Comparable site-specific nucleobase caging has been demonstrated in controlling other forms of gene-silencing oligonucleotides such as DNAzymes and phosphorothioate (PS) antisense nucleotides by Deiters's group. Utilizing standard

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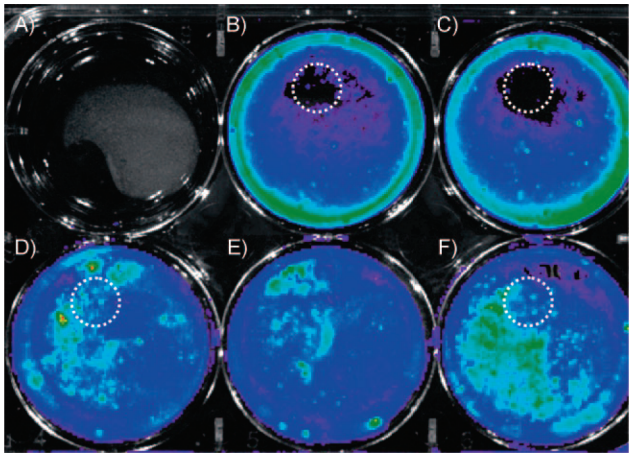

Figure 7. Spatial control of luciferase expression in cell cultures with site-specific NPOM caged antisense oligonucleotides. The cellular monolayer was only irradiated inside the white dashed circle (365 nm, 5 min, 23 W). (A) Negative control without luciferase plasmid. (B) Transfection with luciferase plasmid and PS DNA containing three caged thymidines. (C) Transfection with luciferase plasmid and PS DNA containing four caged thymidines. (D) Positive control without PS DNA. (E) Positive control without PS DNA and without irradiation. (F) Transfection with luciferase plasmid and inactive control PS DNA. Reprinted with permission from ref 95. Copyright 2008 Wiley-VCH Verlag GmbH & Co. KGaA.

DNA synthesis, NPOM-caged thymidine residues were introduced into antisense PS oligodeoxynucleotides targeting *Renilla* luciferase in 3T3 cell cultures.[95] Chemiluminescence from caged transfectants showed no observable leak activity, with an impressive photorestoration to 95% of control antisense treatments. This work also demonstrated the ability of the photocage assay to spatially restrain antisense knockdown activity to irradiated areas of cells in a culture dish (Figure 7).[95] In addition, in prior work, a NPOM-caged thymidine was shown to fully inactivate a DNAzyme, where the restoration to 91% cleavage activity of RNA was achieved when compared to native (noncaged) controls.[96]

These more recent site-specific demonstrations of nucleobase modification offer a great deal of design flexibility in developing caged siRNA effectors. However, these compounds have yet to be adopted by the broader biological and biomedical community studying gene silencing for several reasons. First, generation of the caged precursors requires considerable chemical expertise and is a limiting factor for many laboratories that are not focused on modified nucleobase synthesis. Second, some of the caging groups, such as nonderivatized 2-nitrobenzyl groups, require exposure to UV wavelengths for photorelease which can cause damage to biological systems. Finally, the scope of these caged RNAi effector studies is limited to date, leaving the effectiveness of this strategy for controlling the complex RNAi system open to scrutiny. However, with the advent of nucleobase-caged siRNAs and other related nucleotides, control of these systems more closely resembles the on−off binary photoreactivity that will be required for routine use. The development of commercially available nucleobase-caged phosphoramidites (discussed in the section Conclusions and Future Directions) will also allow more laboratories to investigate these techniques and allow more rapid development of caged siRNA applications.

Photolinkers. One final category of site-specific caging to control silencing oligonucleotide activity utilizes photocleavable linkers that tether a blocking moiety to the nucleic acid until photolysis. In these examples, knockdown is prevented by a complementary oligonucleotide strand tethered to the antisense oligonucleotide. Photocleavage of the linker separates the two strands, availing the antisense sequence for hybridization to target. This approach differs from those previously mentioned, as it requires no direct cage attachment to the silencing oligonucleotide, and may achieve total blockade of hybridization with the single photocleavable linker (reviewed in ref 97).

Dmochowski and colleagues prepared a 25-mer oligonucleotide containing a nitrobenzyl linker that connects a cytosine to a fluorescence quencher.[98] An adjacent nucleotide contains a fluorophore which emits a fluorescent signal upon separation from the quencher, thereby confirming photoactivation. This cage attachment chemistry was used to control RNase H-mediated digestion of an mRNA target through hairpin loop caging.[99] Irradiation breaks the photocleavable link at the bend of the hairpin, drastically reducing T_m and allowing hybridization of the antisense side of the hairpin to the target. A similar photolinker was used to tether a 2′-methoxy RNA oligonucleotide to a negatively charged peptide nucleic acid (ncPNA) for gene silencing in zebrafish embryos.[100] Upon photoactivation, the release of the photolinker significantly lowers the melting temperature for the duplex, allowing the antisense ncPNA to hybridize with target mRNA and induce RNase H degradation of the target (Figure 8).[100] With this strategy, restoration to 94% of RNase H silencing activity targeting the bozozok gene in zebrafish was achieved, with only 6% leakage in the nonirradiated, caged state. Similarly, the group silenced 85% of chordin gene expression, relative to positive RNase H controls in zebrafish embryos, with only 16% leakage. Tang and Dmochowski also used phosphorothioate antisense oligodeoxynucleotides (ODNs) with the NPE-photolinkers to exert photocontrol over c-myb mRNA in K562 cells.[101] After optimizing the PS DNA/photolinker conjugates, the group was able to achieve reduction in protein expression equivalent

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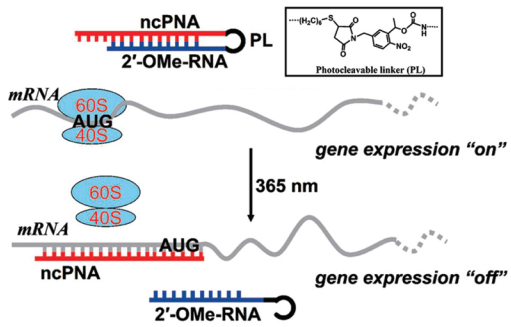

Figure 8. Control of gene expression using a photocleavable linker (PL). Photolysis released 2′-OMe RNA and allowed ncPNA hybridization to mRNA, thereby blocking protein synthesis in zebrafish embryos. Adapted with permission from ref 100. Copyright 2007 American Chemical Society.

to 52% that of positive antisense controls, with no detectable leak activity. The group concluded that the length of the loop section of the complex should be minimized so that it consists of only the photolinker in order to increase molecular stability and decrease background (leak) activity; however, it was noted that further optimization of the leakage/photoactivation balance may be necessary.[101]

An interesting demonstration of this NPE-photolinker system is the tethering of two noncomplementary ~10-mer RNA oligonucleotides in which light is used to activate genetic expression, as opposed to knockdown.[102] When tethered together, the two oligonucleotides form ~20 nucleotides that are complementary to an mRNA target. When the linker is photocleaved, the separated short strands are no longer stable enough to remain hybridized with the target, allowing translation to proceed. 2′-O-Methyl groups were used to stabilize the complexes, both for nuclease resistance and increased strand melting temperature.[102,103] The nonirradiated complex bound to start codons and Kozak sequences within untranslated regions of GFP mRNA inhibited 70% of expression; after photolysis, the GFP reached 95% of positive control expression. Notably, the group found no correlation between the complex's change in melting temperature with its target after irradiation and its ability to restore genetic expression. It was noted that targeting long mRNAs proved especially difficult due to challenges in predicting mRNA secondary structure, and that the strategy may work better when targeting shorter strands such as siRNAs.[102]

Chen and colleagues used a photolinker system to modulate morpholino knockdown oligonucleotide activity in zebrafish.[104,105] Using a photocleavable linker to tether an antisense morpholino oligonucleotide to a complementary 10-mer "inhibitor", the group targeted no-tail (ntl) mRNA critical for embryonic development. The embryos transfected with the caged antisense sequences developed normally unless they were irradiated with 360 nm light, in which case the tails showed significant shortening and a notable loss of anatomical organization. Wild-type embryos similarly irradiated showed no defects. Embryos were transfected while still unicellular, irradiated four hours postfertilization (or kept in the dark), and imaged one day postfertilization. The defects resulting from photoactivated morpholinos were spatially restricted to the areas of irradiation, demonstrating effective spatial targeting of gene silencing.

One final mention of recent development in this category of photolabile tethers is the demonstration of a singlet oxygen-sensitive linker that, when combined with a photosensitizer, demonstrated cleavage *in vitro* with red wavelengths of light. While this strategy has yet to be employed in cells to control gene expression, and may be inherently more involved due to co-delivery of the photosensitizer, the red wavelengths used are promising due to their lack of photodamage and increased tissue penetration depths.[106] In recent work by Ohtsuki and colleagues, photoinduction of RNAi was observed after red light exposure to cells transfected with siRNAs conjugated to fluorophore-labeled peptides, thought to generate reactive oxygen species that promoted endosomal escape of siRNAs and subsequent gene silencing.[107]

Overall, the photocleavable linker strategy toward genetic modulation represents a significant augmentation of the efficiency of photolysis, as the photoactivity is consolidated into one functional group and a single photorelease event. This contrasts with strategies requiring multiple photoactive molecules per strand and therefore potentially requiring greater irradiation doses for complete photoactivation that would scale geometrically with the number of attached cages. However, the boost in efficiency of the photolytic step comes at a cost of decreasing the simplicity of deprotection, as a

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successfully photolyzed complex still relies upon statistical changes in the hybridization of an inhibitory strand to a target. This, then, is the balance which will need to be met in order to achieve binary activity: minimum leakage, with oligonucleotide sequences fully hybridized in the photolinked state, versus maximum recovery of activity, with sequences which fully disassociate following irradiation.

Photoactivating Light Sources and their Biological Effects. A variety of light sources are available with UVA (315−400 nm) output capable of photoactivating caged siRNAs in cells and tissues. Considerations of light source include irradiance, spectrum, heat generation, temporal and spatial resolution, and cost. The mercury arc lamp used in Kaplan *et al.*'s original caged ATP work was a 1000 W lamp attenuated with heat and UV filters.[108] Mercury lamps are still among the most common light sources used for uncaging.[17,18,52,62,95,105,108] Of the work focused on photoactivating caged oligonucleotides, those that used lamps reported doses from 200 mJ/cm^2 to 40 J/cm^2 to irradiate cell cultures and zebrafish, though the median dose was approximately 2 J/cm^2 (Table 2). Xenon light sources have also been used for photolysis, yet they have been handicapped by weaker irradiances in the UVA range.[109] Flash lamps have limited timing capabilities and can generate unnecessarily large and complicating electromagnetic fields, particularly disruptive to electrophysiological recordings, although not as critical for gene expression quantification.[110]

Laser systems, also commonly used for rapid photolysis, can produce high intensity UV pulse-beams with fine temporal and spatial resolution. However, such systems are generally bulky and have high initial and maintenance costs.[109] Yttrium aluminum garnet (YAG), ruby, nitrogen, and argon lasers are all common laser lines used for photoactivation.[110] Recent developments have also enabled light-emitting diodes, LEDs, to serve as a source of near-ultraviolet light in a variety of sub-400 nm wavelengths.[111−113] While still too recent to have been used extensively, UVA-emitting LEDs have been tested with biological systems and proven to be a less expensive and sufficiently powerful option for inducing photolysis.[110,114,115] Potential benefits of LED sources are straightforward coupling into fiber optics for delivery, and duty lifetimes reaching 100,000 h.[116] As the sophistication and breadth of use of these LED systems increases, they should provide exciting new possibilities for two-dimensional control or patterning of irradiation, single-cell studies, and eventually submicrosecond irradiation durations.

The mechanisms involved in UVA-induced cell damage are complex and vary depending on factors such as cell line, viability/damage assay, source spectra, and photoexposure conditions. It should be noted that individual UVA sources have been shown to induce cell necrosis or apoptosis at widely varying doses of energy.[117] This is likely due to UVB wavelengths (280−315 nm) that are often emitted from so-called pure UVA lamp sources.[118] Even if less than 1% of the total emittance contains UVB wavelengths, this light can be one hundred times more damaging to biological targets than UVA photons by causing direct DNA lesions.[119] In these types of caged nucleic acid studies reviewed here, rapid arc-photolysis is often not necessary due to typical kinetics on the order of hours and days, but large irradiances can be required to achieve complete photoactivation when multiple cage compounds are present on a single effector. In addition, cell health must be maintained throughout the experimental period, which can last several days rather than minutes as seen in some neuroscience applications. Thus it is necessary to characterize the cellular responses to such UVA photoexposures. For example, differences in the intensity of photoexposure can result in different levels of cell damage. Similar doses of UVA from a high intensity mercury lamp were applied to cell cultures, comparing a 20 min exposure at a distance of 11.5 cm (intensity 5 mW/cm^2) to a 3 min exposure at 4.2 cm (intensity 40 mW/cm^2). A significant difference in the percentage of apoptotic and necrotic cells was found between the 20 min exposure (62 ± 5%) and 3

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min exposure ($46 \pm 2.4\%$).[117] These results suggest that the intensity of the light source and duration of photoexposure can affect cellular response to UVA. This is contrary to the law of reciprocity thought to govern 1-photon events where the number of photons delivered determines outcome rather than the intensity or duration of the dose.[120] These facts warrant further exploration into the mode of photoexposure, comparing similar cumulative doses at varying intensities and even shorter durations of exposure separated by periods of no irradiation. One interesting aspect of photobiology is that zebrafish embryos show UVA tolerances greater than 100 times that seen in cell cultures, highlighting their use as a model for photoactivation studies.[121]

One of the newest frontiers in caging technology is the application of the two-photon excitation (TPE) strategy, first applied by Webb and colleagues.[122] The time required to produce an excited electron state when a chromophore absorbs a single photon is on the order of 10^{-15} s^{-1}.[10] TPE occurs when two photons of half the necessary energy are absorbed within this time frame, or "simultaneous" absorption. The probability for the simultaneous absorption of two photons is proportional to the square of the incident light intensity and the two-photon absorption cross-section, measured in units of Göppert-Mayer ($1\ GM = 10^{-50}\ cm^4 \cdot s \cdot photon^{-1}$).[12,123] There have been caged compounds synthesized, such as the bromohydroxycoumarins and a newer nitrodibenzofuran, that have useful TPE cross sections and may be amenable to caging RNAs.[124−126] The TPE strategy has several advantages over single-photon uncaging. Since the excitation is exponentially dependent on the incident light intensity, much greater three-dimensional control over substrate release can be achieved. Due to the photon densities required for TPE, its activation volume is confined to a very small region near the focal point of the excitation laser, compared to a much larger depth seen in single photon excitation, even when paired with confocal optics. It has been demonstrated that, with appropriate optics, the spatial resolution of TPE can reach sub-femtoliter volumes.[127,128] Another benefit of TPE is that it relies on near-infrared radiation for excitation of caged compounds. Light in this wavelength range is capable of deeper tissue penetration and is less likely to cause damage to biological systems such as those described above. Nevertheless, phototoxicity in response to TPE has been observed in biological systems when the power of the incident laser exceeds 5 to 10 mW.[129] To date, there has not been a demonstration of TPE in uncaging siRNAs. However, the technique holds great promise for highly localized silencing, which may elucidate the mechanisms of many position-dependent genes such as those involved in regulating developmental pathways.

Conclusions and Future Directions

A variety of means of attaching photocage compounds to oligonucleotides have been demonstrated, with widely varied efficacies. Similar to the many studies demonstrating chemical modifications for improved siRNA performance, types of caging have been singular and have not, for instance, combined nucleobase with phosphate caging. Due to the sequence dependence shown for many chemical modifications, thorough investigation of caged siRNAs for many targets is warranted. Greater precision over the photocaging process, both attachment and photorelease, will likely be required for the advancement of the field to more functional technologies and widespread application. Following the successful demonstration of site-specifically caged RNA generation, an analysis of RNAi with point cage insertions should be initiated, such as those shown with DNA aptamers.[79] Mikat and Heckel's work has most closely approached this level of analysis by examining an assortment of siRNAs with caged thymines or guanines.[130] Most recently, Deiters's group's NPOM-caged dT phosphoramidite has become commercially available; presumably this will be followed by RNA phosphoramidites that would allow siRNAs to be purchased with intended nucleotides caged.[131] This will allow probing into the siRNA of a given sequence to determine the optimal site(s) for photocaging. Ideally a single cage compound could be inserted into the siRNA molecule to completely ablate gene silencing activity. However, certain

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locations along siRNAs have been identified as structurally significant to the RNAi process, and these can be exploited now to generate a reasonable hypothesis for caged siRNA activity. It is likely that two locations will prove sensitive enough for single-caged siRNA photoactivation. The first of these is at the 5′-OH on the antisense strand (Figure 2). Caging at this location should abolish RNAi activity by preventing phosphorylation in the cytoplasm. It is known that a 5′-phosphate on the antisense, or guide strand, is required for siRNA recognition and RISC formation both from crystallography studies of argonaute proteins and from incorporating modifications on siRNAs to disrupt the RNAi pathway.[88−90,132] The other ideal location for caging is the between the 10th or 11th nucleobase from the 5′-end of the antisense strand, as demonstrated by Mikat and Heckel.[130] Caging at this location should abolish RNAi activity by creating a temporary base-pair mismatch and bulge at the scissile site on the target mRNA. Similar to chemical modification strategies, there is a large dependence on particular sequence and system of study. Once an optimal caging strategy has been identified, this site-specific approach could be used in conjunction with other useful siRNA modifications. For example, caging the 10th or 11th nucleobase of a fully-2′-fluorinated nucleic acid will likely result in a enzymatically- and thermally-stable siFNA with a nearly binary on−off activity via photoactivation. A third chemical modification could also be incorporated at a noncritical location for general or targeted cellular delivery as described in other contributions of this featured topic issue. It is important to note that caged siRNAs will still rely on effective delivery agents to reach intended cellular targets. A likely location for a delivery modification would be the 5′-end of the sense strand, which would not interfere with caging, and could be performed prior to siRNA hybridization. Since the sense strand is discarded early in the RNAi process, this modification should have no effect on target recognition or cleavage. Increased siRNA delivery has been accomplished by conjugation with compounds that promote cellular uptake or can permeate the cell membrane.[42] By combining and optimizing these three technologies, siRNA effectors can be designed for biological stability, improved delivery, and targeted activity and/or dosing of the therapeutic agent.

To date, caged siRNAs have only been demonstrated on reporter genes, except for the few photolinker studies on specific targets in zebrafish embryos. In addition to laboratory applications of this technology, there are clinical arenas that would benefit from photoactivated nucleic acids. Several companies have ongoing clinical trials of RNAi-based treatments for age-related macular degeneration. These siRNAs silence vascular endothelial growth factor receptor-1 (VEGF), halting angiogenesis and preventing choroidal neovascularization.[133] However this therapy requires direct subretinal injection for choroidal delivery, which is potentially uncomfortable for the patient and carries a risk of subretinal hemorrhage. A strategy for these and potentially other intravitreal applications (such as the existing antisense drug Vitravene) would deliver the inactive caged siRNA through standard iv injection (e.g., into the branch of the carotid that feeds the ophthalmic artery) followed by pinpointed photoactivation applied through the pupil of the patient. A similar approach has shown UVA uncaging of thrombin in retinas of rabbits after iv injection.[134] Other sites of application are those which are optically available, such as dermatological, wound healing and directed tissue regeneration approaches utilizing siRNAs (reviewed in ref 135). Additional clinical applications could come at sites accessible through standard catheterization techniques, where photoactivating light could be delivered via fiber optics, as has been demonstrated with caged small molecule metabolites in the laboratory.[136] In summary, siRNA photocage technology is currently developing to a state of maturity that is ready for more applied systems with clinically relevant targets. These strategies for spatiotemporal control will help elucidate mechanistic studies as well as contribute to the realization of siRNA therapeutics.

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