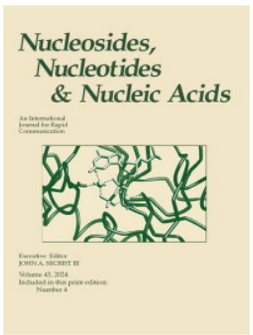

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Light-regulated RNA interference induced by p-hydroxyphenacyl-modified siRNA in mammalian cells

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ABSTRACT

siRNA is an important tool for modulating gene expression in current biomedical research. It would be highly desirable for siRNA to respond to an external stimulus. In this paper, we report a convenient, photolabile caging agent to regulate siRNA functions. 2-bromo-4′-hydroxyacetophenone (BHAP) can readily modify phosphorothioate backbones and inhibit siRNAs. Mild UV irradiation will cleave the modifying moiety to generate natural nucleic acid backbones, thus activating siRNA functions. Such modification is conveniently conducted in an aqueous solution with high efficiency and is cost-effective and scalable. This approach provides a convenient tool for the controlled regulation of gene expression by deploying minimal usage of complex organic synthesis for site-specific installation of the caging group to siRNA unlike previous reported works that required a series of intricate organic synthesis and cumbersome purification techniques to achieve similar aims. This study will open new doors for optochemical regulation of a variety of genes by pHP caging group in mammalian cell culture.

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KEYWORDS
siRNA; light-regulated gene expression; phosphorothioate groups; TEEP-OH

1. Introduction

RNA interference (RNAi) is a process of gene knockdown mediated by short-interfering RNAs (siRNAs).[1] An interesting development in RNAi is light-activated RNA interference (LARI) .[2–5] This strategy imparts an additional handle for controlling the spacing, timing, and amount of gene expression by fine-tuning the spacing, timing, and amplitude of irradiation.[6–10] In addition, different chemical modifications on siRNAs for LARI have enhanced the siRNA stability, imparted nuclease resistance, and prolonged the effect of gene silencing *in vitro* and *in vivo*.[10,11]

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Exploiting photocleavable organic groups as modification reagents renders a way of triggering a particular gene response by light. A variety of caging groups has been brought to attention so far and each one of them has pros and cons associated with them.[12,13] They have been extensively used to modify proteins to study cell signaling or for changing the conformation and activity of nucleic acids.[14–19] Analogously covalent attachment of photoactive groups on siRNA can arrest its activity and further exposure to brief UV can detach the caging group rendering the siRNA functional.[2,6]

Previously, Friedman et al. used 4,5-di-methoxy-2-nitrophenylethyl (DMNPE), cyclo-dodecyl DMNPE groups, etc. to cage the dsRNA.[2,20,21] Modified dsRNA with DMNPE group was found to be less stable than the unmodified version due to enhanced interaction of DMNPE modified RNA with nucleases and was unable to completely abolish RNAi.[20] It was hypothesized that maybe it couldn't block the interaction of dsRNA with dicer or RISC or might have been cleaved by cellular degradative processes prior to radiation.[2] Although cyclo-dodecyl DMNPE due to steric clashes was able to inactivate dsRNA before photolysis, however the introduction of cyclo-dodecyl DMNPE into dsRNA required complex and time-consuming synthetic reactions.

The p-hydroxyphenacyl (pHP) group has been deployed as a viable alternative to by far the most used o-nitrobenzyl photoremovable protecting group (PPG) .[22] Its assorted properties including hydrophilicity, hydrolytic stability, cleavage at nontoxic wavelengths, benign products after photo-cleavage and efficient release of the caged substrate due to the non-competing nature of rearranged photo product for incident radiation makes it an enticing PPG.[13,22,23] Researchers have demonstrated it to be effective in caging of steroids, hormones, amino acid residues cysteine, and tyrosine in proteins.[24,25] Recently pHP has been used to photocage guanosine to provide a new insight into light-activated regulation of gene expression by non-coding RNAs.[26] Xiang and coworkers, site-specifically photocaged DNAzyme by pHP for specific and precise control of target sensing and gene expression.[27] Upon light irradiation, the photocaged DNAzyme was restored to its native form.

Here, we exploit pHP to investigate and quantify light-dependent control of eGFP gene expression in mammalian cells and compare the eGFP expression before and after the site-specific incorporation of the caging group to siRNA, respectively (Figure 1). Figure 1 illustrates the covalent modification of the phosphorothioate backbone by 2-bromo-4′-hydroxyacetophenone and its photocleavage to generate functional siRNA. Our goal is to understand the potential of pHP as a caging group to phototrigger gene expression and if siRNA photocaged with pHP will exhibit a significant activity "off-on" switch.

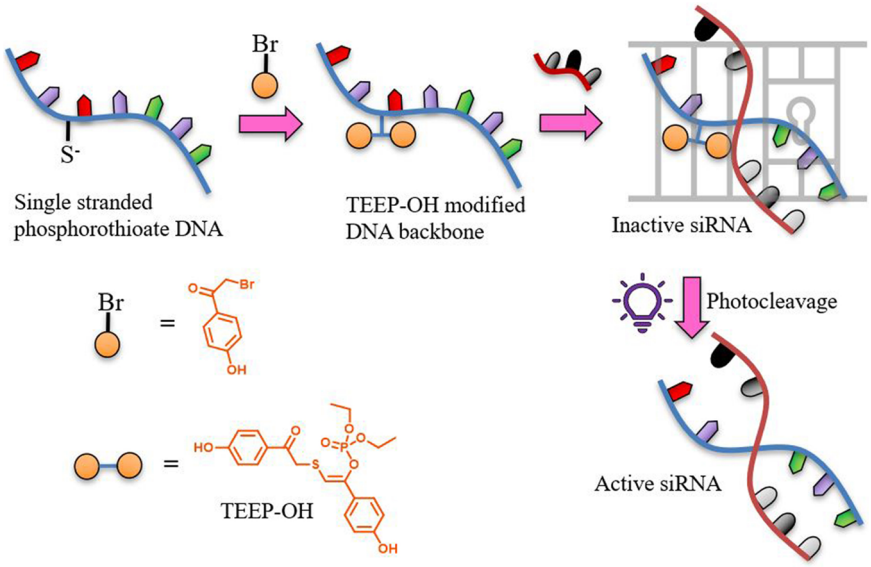

Figure 1. siRNA modification by thioether-enol phosphotriester (TEEP-OH) caging group and its subsequent photocleavage to generate active siRNA.

To this end, fluorescence images were collected for qualitative analysis, and fluorescence-activated cell sorting was performed for quantitative analysis of eGFP expression in mammalian cells. Interestingly, through the incorporation of the pHP caging group in the antisense strand, the siRNA reagent was executed inactive, presumably due to inhibition of the cleavage mechanism within the RISC complex. However, following irradiation, the caging group was cleaved, restoring siRNA function, and demonstrating optochemical regulation of genes in mammalian cells. The present study will provide exciting new avenues for optochemical regulation of a comprehensive range of genes in mammalian cell culture by the pHP caging group.

2. Materials and methods

All materials were directly used as such unless otherwise mentioned. Diethylpyrocarbonate (DEPC), 2-bromo-4′-hydroxyacetophenone, N, N-Dimethylformamide (> 99.9%), boric acid, Tris base, and Stains-All were purchased from Sigma Aldrich. Amicon Ultra centrifugal filters of 3 kDa MWCO (Molecular weight cut-offs) pore size were purchased from EMD Millipore. HeLa-eGFP cells were purchased from Cell Bio Labs, Inc.-AKR213. DMEM (Dulbecco's Modified Eagle's Medium with 4.5 g/L glucose and sodium pyruvate without L-glutamine), penicillin, streptomycin, L-glutamine, Phosphate Buffered saline, and trypsin-EDTA were purchased from Cellgro-Mediatech. Fetal Bovine Serum (FBS) was purchased

from Atlanta Biologicals. Urea, EDTA, Blasticidin, GibcoTM Opti-MEM Reduced Serum Medium, and LipofectamineTM 2000 were purchased from Thermo Fisher Scientific. 30% acrylamide/bisacrylamide solution was purchased from Bio-Rad. Cell-culture plates from CellStar and cover slips from Neuvitro Corporation were purchased.

2.1. Sense and anti-sense strands

All RNA and DNA strands were purchased from Integrated DNA Technologies, Inc. and were used without further purification. All strands were dissolved in 0.1% diethylpyrocarbonate (DEPC, Sigma Aldrich) - treated water.

The sequence of the designed siRNA and DNA strands is shown in Table 1.

In the sequences, RNA residues are represented as small letters, and DNA residues as capital letters. The underlined residue has a phosphothiolate bond at its 5′ position.

2.2. Modification of phosphorothioate backbone with 2-Bromo-4′-hydroxyacetophenone

TEEP-OH-modified phosphorothioate backbone was synthesized according to a previously reported procedure.[27] Briefly, 3 nmole (20 µg, 62.4 µL) of the DNA sense strand (P_2) was dissolved in 200 µL of DEPC water in an Eppendorf tube under buffered conditions in 100 µL of 100 mM PBS at pH 6.0. A solution of 2-bromo-4′-hydroxyacetophenone (5 µmoles, 50 mM) in 100 µL DMF (DNA: reagent = 1:1800) was added dropwise to the same Eppendorf tube. The Eppendorf tube was protected from light, and the reaction was allowed to stir undisturbed for 72 h at room temperature on a shaker at 2000 rpm. The antisense strand (A_1) was TEEP-OH modified

Table 1. Sequence of the designed sense (S) and antisense strand (as) investigated in the present study.

Strand	**Sequence***
J_1 (16 nt)	5′- GCACGAGTCCTAACGC-3′
Marker (32 nt)	5′-TATCACCGAATCGCATAATAGCGTCGAACGA-3′
Marker (18 nt)	5′-ATTGTGGCTACATACTAA-3′
Marker (16 nt)	5′-CATGAAGCTTCATGGC-3′
P_0 (21 nt)	5′- AGCTGACCCTGAAGTTCATCT-3′
P_2 (21 nt)	5′- A̲GCTGACCCTGAAGTTCATC̲T-3′
P_3 (21 nt)	5′- A̲GCTGA̲CCCTGAAGTTCATC̲T-3′
P_4 (21 nt)	5′ -A̲GCTGA̲CCCTGA̲AGTTCATC̲T-3′
A_0 (21 nt)	5′- ATGAACTTCagggucagcuug-3′
A_1 (21 nt)	5′- ATGAACTTCagggu̲carrgcuug-3′

*Small letters represent RNA residues, capital letters represent DNA residues, and underlined residue has a phosphothiolate bond at its 5′ position.

similarly. However, its modification needed prolonged stirring for 96 h at 37 °C.

The modification reaction of each of the sense and antisense strands was further confirmed by running a 20% native PAGE gel (prepared with 19:1 acrylamide/bisacrylamide solution and TBE buffer (89 mM Tris Base (pH 8.0), 89 mM boric acid, 2 mM EDTA) at 300 V on Hoefer SE 600 electrophoresis system for 4 h at room temperature. The electrophoretic gel with the original and the modified DNA strands was further stained with Stains-All (Sigma Aldrich) and scanned by an HP scanner (Scanjet 4070 Photosmart).

2.3. Purification of the modified strands

The resulting modified strands were further purified before siRNA transfection in mammalian cells. Each of the TEEP-OH modified DNA sense (P_2 (M)) and RNA/DNA antisense strand (A_1(M)) was purified using Amicon Ultra-0.5 mL Centrifugal Filters of 3 kDa MWCO (Molecular weight cut-offs) pore size (EMD Millipore) at a speed of 2000 rcf. The modified strands were then sequentially washed six times with DEPC water and DMF mixture (4:1) to ensure complete removal of organic reagent followed by six washes with DEPC water to remove traces of any residual DMF. The resulting purified TEEP-OH modified strands in DEPC water were then stored at −20 °C and protected from light as a safety precaution to ensure the reproducibility of the data in cell transfection experiments and gel electrophoresis analysis.

2.4. Preparation of the siRNA duplexes

The DNA sense strand was annealed to the corresponding DNA/RNA antisense strand to form the duplex respectively. The annealing reaction was performed under buffered conditions in 1X TBE in an Eppendorf tube in DNA-thermocycler. Quick annealing of the reaction mixture was conducted from 80 °C to 22 °C through the following time-temperature cycle: 80 °C/2mins, 50 °C/5mins, 37 °C/5mins, 22 °C/5mins. The duplex formation of the TEEP-OH-modified strand with the complementary strand was also performed under same conditions and we did not observe any difference in duplex formation efficiency when performed in TAE/Mg^{2+} buffer.

The duplex formation in each case was further confirmed by running a 15 or 20% native PAGE gel (prepared with 19:1 acrylamide/bisacrylamide solution and TBE buffer (89 mM Tris Base (pH 8.0), 89 mM boric acid, 2 mM EDTA) at 300 V on Hoefer SE 600 electrophoresis system for 4 h at room temperature. The electrophoretic gel with original single strands,

modified strands, and duplexes (original and unmodified) was further stained with Stains-All (Sigma Aldrich) and scanned by HP scanner (Scanjet 4070 Photosmart).

2.5. Photolysis of the TEEP-OH caging group from phosphorothioate backbone

The photolysis of the TEEP-OH caging group from either of the purified sense (P_2(M)) and antisense (A_1(M)) strands was performed under identical conditions. Briefly, each of the 40 µM modified strands under buffered conditions in 1X TBE in an Eppendorf tube were exposed to two different irradiation conditions at room temperature; one being variable UV of short-wavelength from the bench-top UV illuminator (Fisher Scientific) with an exposure time of 30 min and long-wavelength UV 365 nm achieved using bandpass filter 5860 (Jonathan Amy Facility for Chemical instrumentation at Purdue University) alongside bench-top UV illuminator with an exposure time of 1 h. Photocleavage of the caging group from the duplexes was performed exactly under identical conditions to those of single strands.

Photolysis of the caging group from resulting single strands/duplexes was further confirmed by running a 20% native PAGE gel (prepared with 19:1 acrylamide/bisacrylamide solution and TBE buffer (89 mM Tris Base (pH 8.0), 89 mM boric acid, 2 mM EDTA) at 300 V on Hoefer SE 600 electrophoresis system for 4 h at room temperature. The electrophoretic gel with the original, modified, and UV-irradiated single strands/duplexes was further stained with Stains-All (Sigma Aldrich) and scanned by an HP scanner (Scanjet 4070 Photosmart).

2.6. Cell culture of HeLa-eGFP cell line

HeLa-eGFP cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (with 4.5 g/L glucose and sodium pyruvate without L-glutamine, Cellgro) supplemented with 10% heat-inactivated (60 °C for 30 min) fetal bovine serum (FBS, Atlanta Biological), 50 units/ml penicillin, 50 µg/ml streptomycin (Cellgro), 2 mM L-glutamine (Cellgro) and 10 µg/ml Blasticidin (Thermo Fisher Scientific) at 37 °C in a 5% CO2/95% air humidified atmosphere. Cells were regularly sub-cultured when they grew to 80% confluence to maintain exponential growth and were given a maximum of 10 passages as fluorescence activated cell sorting experiments showed that HeLa-GFP cells maintained their GFP expression uniformly until 10 passages. However, transfection experiments were always performed with the cells in their 4^{th} to 8^{th} and cells in their 10^{th} passage were used if reproducibility of the data had to be ensured.

2.7. Transfection of HeLa-eGFP cells with siRNA using Lipofectamine™2000

Newly thawed HeLa-eGFP cells were always sub-cultured a minimum of three times before transfection experiments could be performed. Experiments were performed in triplicates and 18-24 h prior to transfection experiments, cells were seeded at a density of $5*10^4$ cells/ml in a 24-well cell culture plate (CellStar, surface area 1.9 cm^2) with 8 mm coverslips (neuVitro-GG8) in antibiotics-free (no penicillin, streptomycin, and Blasticidin) DMEM such that they attain a confluence of 30-50% at the time of transfection. Right before transfection, HeLa-GFP cells were washed with 1 mL phosphate-buffered saline (PBS, pH 7.4, Cellgro) per well followed by the addition of 500 µL of Opti-MEM reduced serum medium (Gibco by life technologies) into each well. Cells were incubated for 20 mins in Opti-MEM at 37 °C in a 5% CO2/95% air-humidified atmosphere before the addition of transfection complexes.

Meanwhile, 20 pmol of each siRNA to be transfected was diluted and well mixed in 50 µL Opti-MEM (final concentration, 33 nM; total volume, 600 µL). 3 µL of Lipofectamine™ 2000 was diluted and gently mixed in 50 µL Opti-MEM. After 5 mins incubation at room temperature, the two solutions were combined, gently mixed, and further incubated for 20 mins at room temperature to form siRNA: lipofectamine 2000 complexes. Complexes were then added into each well and the cell culture plate was gently rocked to mix the complexes in the medium. The cell culture plate was further placed undisturbed at 37 °C in a 5% CO_2/95% air-humidified atmosphere for 4 h. After 4 h of transfection, the transfection medium was removed, and the cells were washed twice with PBS (1 mL each time). The cells were further cultured for 72 h in DMEM without antibiotics at 37 °C in a 5% CO_2/95% air-humidified atmosphere.

For UV experiments, after 4 h of transfection, the transfection medium was removed, and the cells were washed twice with PBS (1 mL each time). 250 µL of 1X PBS (pH 7.4) was then added into each well and cells were irradiated for 1 h on a bench-top UV illuminator using a bandpass filter (5860) for the wavelength of 365 nm at 8 cm. (Distance between cells and the UV lamp). PBS solution was removed and fresh DMEM without antibiotics was added. Cells were further incubated for 72 h at 37 °C in a 5% CO_2/95% air-humidified atmosphere. Cells were assayed for eGFP fluorescence by Fluorescence Microscopy and Flow Cytometry.

2.8. Assay for eGFP expression in HeLa cells

2.8.1. Microscopy

After 72 h of incubation at 37 °C in a 5% CO_2/95% air-humidified atmosphere, HeLa-eGFP cells were washed with 1X PBS and then 500 µL of

1X PBS was then added into each well. Cells were then carefully observed under the microscope. Optical and fluorescence images of the cells were taken on the microscope (Olympus, BX51 Optical Microscope) with an attached CCD camera. For fluorescence imaging, an excitation filter of 460-490 nm and an emission filter of 520IF were used. For qualitative eGFP expression, fluorescence images were collected using a constant exposure time constant of 1/6 s and all images were taken using the 10x objective lens.

2.8.2. Flow cytometry analysis

Following fluorescence images, HeLa-eGFP cells were washed with 1 mL PBS and trypsinized for 2 mins at 37 °C with 100 µL of 0.25% trypsin-2.21 mM EDTA (Cellgro). 400 µL of cold 2% FBS in PBS was then added to quench trypsin followed by vigorous trituration of cell solution. The entire cell solution was further placed on ice for quantitative estimation of eGFP expression in HeLa cells by Flow Cytometry (FACSCalibur, Becton Dickinson) instrument, using a 488-nm excitation laser with a 530-nm band pass filter for GFP. The mean fluorescence of cells was measured for 10,000 cells using the Cellquest Pro software.

3. Results

3.1. SiRNA modification with 2-Bromo-4′-hydroxyacetophenone

The sequence of individual strands and designed siRNAs against eGFP investigated in this study is shown in Table 1 and Figure 2. The corresponding nomenclature for each of the sense and the antisense strand is described in Table 2. Figure 3 shows the synthetic modification of the siRNA phosphorothioate backbone with pHP to generate an interesting

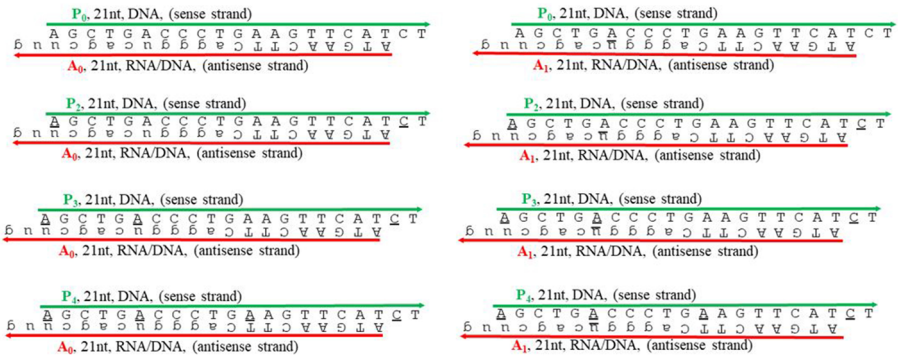

Figure 2. Sequence of siRNA duplexes investigated in the present study. RNA residues are represented as small letters and DNA residues as capital letters. The underlined residue has a phosphothiolate bond at its 5′ position.

Table 2. Nomenclature of the designed sense (S) and antisense strand (as) with the corresponding number of P-S linkages investigated in the current study.

Strand	Sense (S) /Antisense (AS) Strand	No. of P-S linkages
A_0	AS	0
A_1	AS	1
P_0	S	0
P_2	S	2
P_3	S	3
P_4	S	4

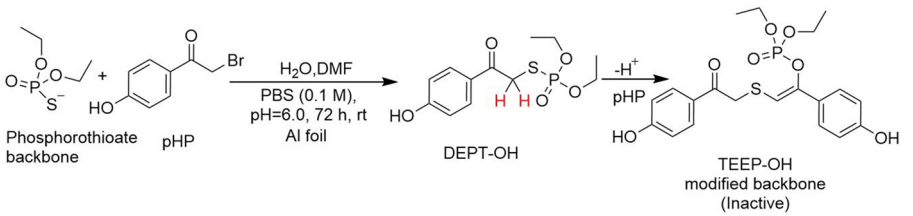

Figure 3. Synthetic modification of phosphorothioate to form TEEP-OH in the presence of two equivalence of pHP.

thioether-enol phosphotriester (TEEP-OH) modified siRNA backbone.[27,28] The modification reaction proceeds *via* a simple, facile nucleophilic substitution reaction that is accomplished in appreciable yields under mild reaction conditions without employing complex synthetic reactions and purification techniques.[27] The synthesis was performed in DMF as a solvent under buffered conditions at pH 6.0 at rt with protection from light. The modified strands with the photolabile group were purified using Amicon Ultra-0.5 mL Centrifugal Filters of 3 kDa MWCO (Molecular weight cut-offs) pore size before further experiments.

As shown in Figure 3, there is a concomitant attachment of two equivalence of pHP for one corresponding P-S linkage forming, TEEP-OH. ESI-MS and NMR studies by Xiang and coworkers confirmed the formation of TEEP-OH *via* the modification of diethyl thiophosphate (model compound of DNA phosphorothioate backbone) with 2-equivalence of 2-bromo-4′-hydroxycetophenone.[27] They deciphered the highly acidic nature of methylene proton adjacent to electron-withdrawing groups in DEPT-OH intermediate (protons in red) primarily accounts for the intramolecular rearrangement reaction leading to the formation of TEEP-OH.

The reaction was readily monitored by gel electrophoresis analysis due to the differential mobility of the original and the modified strand (M), the modified strand (M) being less mobile than the original strand as shown in Figure 4. Figure 4 shows the successful modification of the antisense strand (A_1(M)) and sense strand with two P-S linkages (P_2(M)). This demonstrates the high efficacy of the modification with the pHP

Figure 4. 20% native PAGE showing the modification of phosphorothioate backbone of the antisense strand (A_1) and sense strand (P_2) with TEEP-OH, and its subsequent photocleavage at two different wavelengths. The source of variable wavelength is the benchtop UV illuminator and 365 nm wavelength radiation was achieved using a 5860 filter with the benchtop UV illuminator.

caging group irrespective of the presence of P-S linkages in the sense or the antisense strand without complex organic synthesis and tedious purification techniques. Further, the formed TEEP-OH modification is highly stable in basic environment and harsh conditions of elevated temperatures. This observation points us to a highly robust nature and ease of scalability to the post synthetic modification reaction.[27]

3.2. Preparation of siRNA duplexes

The antisense strands (A_0, A_1, (A_1(M)) were successfully annealed to each of the sense strands (P_0, P_2, and P_2(M)) to form DNA-RNA/DNA duplex (Figure 2) in a buffered solution in DNA thermocycler through a temperature gradient cycle from 80 °C to 22 °C. The duplex formation was further confirmed by gel electrophoresis analysis due to the substantial difference between electrophoretic mobility of single sense/antisense strands and siRNA duplexes.

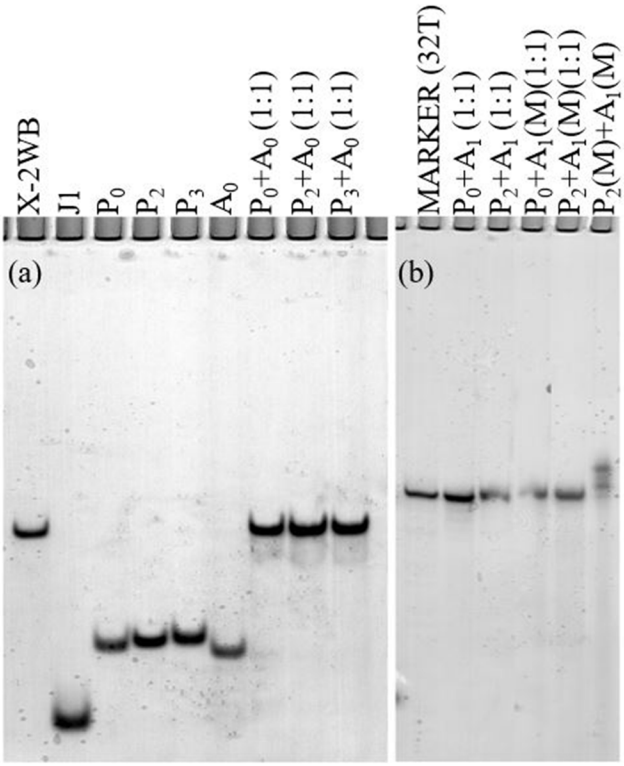

Figure 5. (a) 15% native PAGE showing the duplex formation between the sense strand and complementary antisense strand (A_0). (b) 20% native PAGE showing duplex formation between the unmodified sense strand and the complementary unmodified/modified antisense strand (A_1 and A_1(M)).

Figure 5 shows the successful duplex formation between the sense and complementary antisense strands. Further absence of any single strands in the electrophoresis gel confirms the high efficiency of duplex formation. As expected, single sense/antisense strands move quickly relative to duplexes (Figure 5a). Notably, when the photolabile TEEP-OH group is covalently attached to both the sense and antisense strand, the duplex P_2(M)/A_1(M) moves slower in contrast to the duplex where the TEEP-OH group is only attached to the antisense strand, P_2/A_1(M) (Figure 5b).

3.3. Photolability of the TEEP-OH caging group

After successful covalent attachment of the caging group, we investigated the cleavage of the TEEP-OH group on the phosphorothioate backbone under UV. Figure 6 shows the photolysis of caging group to generate native active siRNA with a phosphodiester backbone after light irradiation.[27,28]

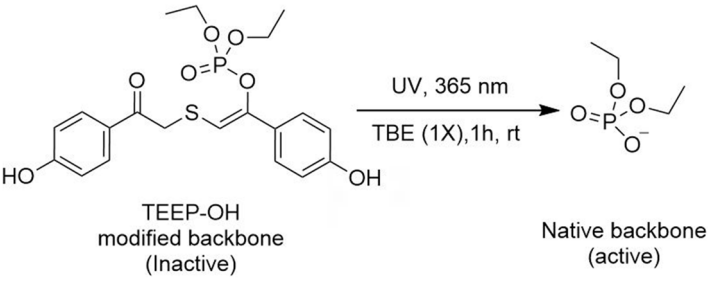

Figure 6. Photolysis of TEEP-OH caging group from modified phosphorothioate backbone generating native phosphodiester backbone.

Photocleavage was performed on the purified modified sense/antisense strands and the siRNA duplexes at room temperature for 30 mins under buffered conditions in 1X TBE in Eppendorf tubes ensuring complete UV transmission from Benchtop UV transilluminator to the sample under investigation. Photolysis of the caging group from both the single strand and the duplex was further confirmed by gel electrophoresis analysis due to differential mobility between the unmodified and modified strands. Figure 7a and b show the successful cleavage of the caging group from each of the modified sense strand (P_2(M)) and siRNA duplex with modified sense strand (P_2(M)/A_0) using a benchtop UV transilluminator.

Interestingly, the photolysis of the caging group from the modified antisense strand (A_1(M)) did not proceed as predicted under identical conditions as shown in Figure 7c. We don't fully understand this observation as to why the caging group when covalently attached to the antisense strand is not effectively removed in contrast to when attached to the sense strand. However, we speculate that the Favorskii rearrangement mechanism that uncages the pHP group is strongly dependent on the stereoelectronic effects and the chemical environment close to the photolabile group, which isn't readily accessible in the antisense strand.[22]

Following modification with TEEP-OH caging group, modified siRNA had to be eventually administered in mammalian cells to inspect the behavior of TEEP-OH in LARI. Researchers have previously demonstrated UV-mediated cellular toxicity by UV of wavelength less than 365 nm which is corroborated by the decrease in GFP expression in stably expressing GFP cell lines.[2] Considering the toxicity of UV radiation varied with wavelengths, we investigated the photolysis of the caging group using a band pass filter 5860 for 365 nm wavelength at room temperature for 1 h under buffered conditions in 1X TBE in Eppendorf tubes.[29]

As shown in Figure 7, electrophoretic gel analysis did not indicate any evidence of photocleavage both from the modified sense strand (P_2 (M)) and the modified antisense strand (A_1(M)) at longer wavelengths under these conditions in our hands. Further using a direct UV lamp of

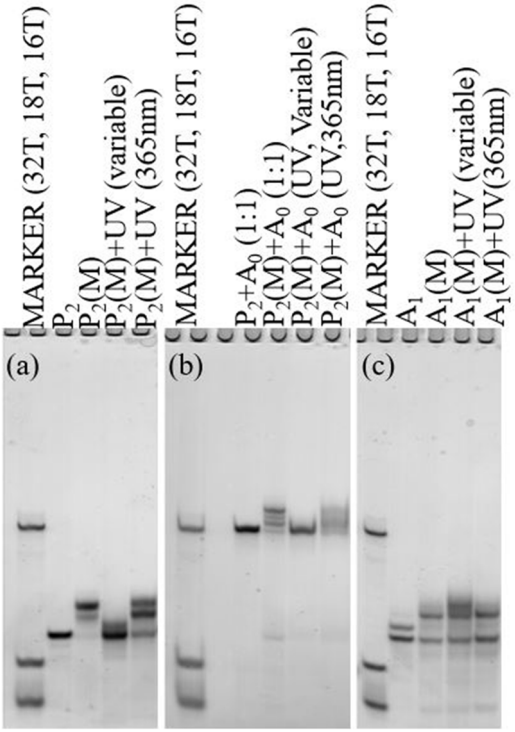

Figure 7. (a) 20% native PAGE showing the photolysis of TEEP-OH from modified sense strand (P_2(M)) at two different wavelengths (b) 20% native PAGE showing the photolysis of TEEP-OH from the dsRNA with modified sense strand (P_2 (M)). (c) 20% native PAGE showing the photolysis of TEEP-OH from modified antisense strand (A_1(M)) at two different wavelengths. The source of variable wavelength is the benchtop UV illuminator and 365 nm wavelength radiation was achieved using a 5860 filter with the benchtop UV illuminator.

wavelength 365 nm instead of the band pass filter 5860 did not make any difference to our observations. These results contrast with the previous study where TEEP-OH caged DNAzyme was efficiently activated upon light irradiation at 365 nm.[27]

Considering cellular studies are generally performed at the nanomolar range of siRNA and photocleavage reaction of caging group is concentration dependent, we decided to examine the photoregulation of GFP expression in mammalian cells and decipher if 365 nm wavelength would photolyze the TEEP-OH caging group attached to the nanomolar concentration of siRNA administered to mammalian cells.

3.4. Transfection of siRNA duplexes in eGFP expressing stable cell line

Results from Wang et al. work demonstrate the compatibility of the TEEP-OH caging group with cellular machinery and cellular components such as metabolites and proteins.[27] Further, it is also possible to rule out

its premature loss before irradiation, as it is immune to cellular degradative processes.[27] Encouraged by this reported work and our above results showing an efficient modification of sense (P_2) and antisense strand (A_1) by the TEEP-OH group, we transfected siRNA duplexes in mammalian cells to toggle the gene expression.

HeLa cells being the most robust cell line are the backbone of many cellular and biological experiments.[30] We, therefore, investigated the efficacy of pHP as a caging group to phototrigger eGFP expression in HeLa cells stably expressing eGFP. Transfection experiments were performed where siRNA duplexes, unmodified and modified with TEEP-OH caging group were transfected for 4 h at a concentration of 33 nM (20 pmol) using lipofectamine (L2K) 2000 as the transfection reagent in a 24-well cell culture plate. Cell transfection experiments performed with 50 nM siRNA and 5 µL L2K2000 led to severe toxicity in HeLa cells and consequently we observed decrease in GFP expression due to cellular toxicity and not as a result of siRNA action. Therefore, we decided to pursue these experiments with 33 nM siRNA and 3 µL L2K2000. As shown in Figure S1, proper controls were used to inspect for any nonspecific knockdown and ensure accurate interpretation of eGFP knockdown results.

eGFP expression was monitored both qualitatively by fluorescence microscopy and quantitatively by Fluorescence-activated cell sorting (FACS) after 72 h of incubation. To examine the influence of P-S vs. the P-O linkages in the antisense strand of the siRNA on eGFP expression, HeLa-eGFP cells were transfected with each of the siRNA under investigation. As observed qualitatively in the fluorescence image in Figures S2a and b, each of the designed siRNA with P-S linkages in either the sense strand, antisense strand, or in both strands work efficiently in inhibiting the eGFP expression.

Quantitative analysis from FACS data in Figure S3 and Table S1 shows that across the board, siRNA with P-S linkage in the antisense strand (A_1) performs better than the siRNA with P-O linkage in the antisense strand (A_0) with either of the sense strands. These observations are congruent with previous studies where siRNA duplexes with P-S linkages execute efficient gene knockdown due to enhanced nuclease stability evading degradation by serum enzymes.[2] In rationale to these results, we pursued antisense strand, A_1 for further a set of experiments.

Dieters and coworkers have previously investigated light-toggled gene expression by covalently attaching the caging group to the antisense strand of siRNA and found it to be a promising strategy to turn on/off the gene expression on-command. They site-specifically installed NPOM-caged nucleotides and effectively silenced an endogenous gene Eg5.[19,31] Motivated by these results, we then investigated the effect of incorporating TEEP-OH on the antisense strand (A_1(M)) of siRNA on eGFP expression in HeLa cells.

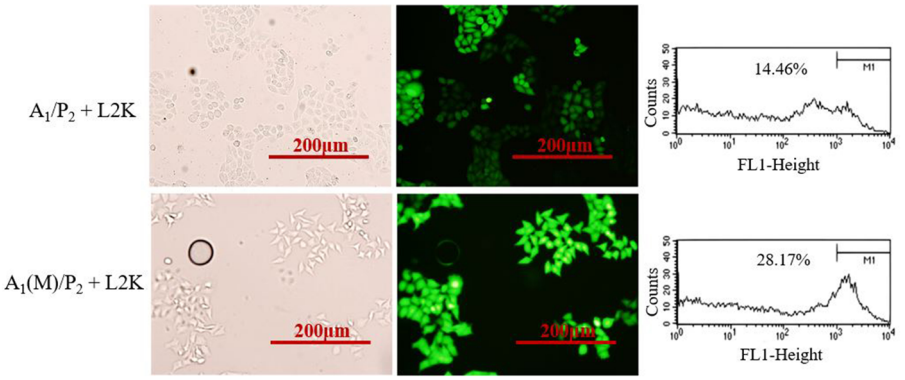

Figure 8. Fluorescence images (left) and flow cytometry analysis (right) showing RNA interference in HeLa-eGFP cells transfected with control siRNA duplex (A_1/P_2) and TEEP-OH modified siRNA on the antisense strand ($A_1(M)$).

Figures 8 and S4 show the eGFP expression in HeLa cells after 72 h of incubation with siRNA modified with TEEP-OH on the antisense strand ($A_1(M)$ and has 2 P-S linkages in the sense strand (P_2). Qualitatively, as observed in fluorescence images, HeLa cells transfected with siRNA covalently attached to TEEP-OH modified antisense strand ($A_1(M)/P_2$) show higher eGFP expression and thus lower knockdown % in contrast to HeLa cells transfected with unmodified corresponding siRNA counterpart (A_1/P_2). These results demonstrate that siRNA modified with TEEP-OH caging group on the antisense strand is capable of effectively obstructing siRNA activity which is corroborated by increased eGFP expression and is consistent with our hypothesis.

Further as observed in Figure S5, TEEP-OH modified siRNA gave similar eGFP expression to that of unmodified siRNA when transfected in HeLa-eGFP cells in the absence of L2K2000. These results indicate that modified siRNA transfected at the experimental concentration was nontoxic to cells.

We then examined the influence on the caging potential of TEEP-OH with the increasing number of P-S linkages in the sense strand. Quantitative FACS analysis data in Figures S6, S7, and Table S2, indicates that the difference between caged and uncaged (A_1/P_2) siRNA-eGFP knockdown activity is the most when the cells were transfected with siRNA ($A_1(M)/P_2$) in which the TEEP-OH modified antisense strand ($A_1(M)$) was annealed to sense strand with 2 P-S linkages (P_2);

(Δ_{KD} eGFP (A_1/P_2-$A_1(M)/P_2$) = 29.28% > (Δ_{KD} eGFP (A_1/P_3-$A_1(M)/P_3$) = 24.96% > (Δ_{KD} eGFP (A_1/P_4-$A_1(M)/P_4$) = 15.46%. Thus, the efficiency of obstruction of siRNA activity by caging decreases with an increase in the number of P-S linkages in the sense strand. According to previous studies, an increase in the number of P-S linkages in the siRNA duplex

could enable Dicer to process the siRNA duplex into an active specie, surpassing the caging group's blocking capability.[20] Our observed experimental results are consistent with this speculation.

We were further intrigued to decipher the effect of caging siRNA on eGFP expression by TEEP-OH modified sense strand containing 2 P-S linkages (P_2(M)) in siRNA against eGFP. Figures S8 and S4 show the eGFP expression in HeLa cells after 72 h of incubation with the siRNA under investigation. Qualitatively, as observed in each of these fluorescence images, HeLa cells transfected with siRNA covalently attached to TEEP-OH modified sense strand (A_1/P_2 (M)) show higher eGFP expression in contrast to HeLa cells transfected with unmodified corresponding siRNA counterpart (A_1/P_2). These results demonstrate that siRNA modified with TEEP-OH caging group on the sense strand (P_2(M)) hampers the siRNA activity which is in accordance with our hypothesis.

Notably, FACS analysis data in Figure S4 and Table S3 show that TEEP-OH modification in the sense strand is not as effective in abrogating RNAi contrary to when modification is performed in the antisense strand. Quantitative FACS analysis data in Table S3 shows the difference in eGFP knockdown percentage (Δ_{KD}) between caged and uncaged siRNA and is greater for siRNA caged on antisense strand (A_1(M)) in contrast to siRNA caged on sense strand (P_2(M)); (Δ_{KD} eGFP (A_1/P_2-A_1(M)/P_2) = 29.28% > (Δ_{KD} eGFP (A_1/P_2 -A_1/P_2(M) = 10.93%). These results confirm TEEP-OH caging of siRNA on the antisense strand is an efficient strategy for blocking RNAi in contrast to caging the siRNA on the sense strand.

With these promising results in our hands, we transfected HeLa eGFP cells with siRNA modified on both sense and antisense strands (A_1(M)/P_2(M)). As shown in Figures S4, S8, and Table S3, modification in both the sense and antisense strand also effectively hampers RNAi in contrast to when modification is performed only in the sense strand (A_1/P_2(M)). The quantitative FACS analysis data in Table S3 indicates the difference in eGFP knockdown percentage between caged and uncaged siRNA for siRNA modified on both the strands (A_1(M)/P_2(M)) is similar to siRNA caged only on antisense strand (A_1(M)/P_2) but greater in contrast to siRNA caged only on the sense (A_1/P_2(M)); Δ_{KD} eGFP (A_1/P_2 -A_1(M)/P_2(M) = 22.36%, Δ_{KD} eGFP (A_1/P_2-A_1(M)/P_2) = 29.28% > Δ_{KD} eGFP (A_1/P_2 -A_1/P_2(M) = 10.93%. With these results in hands, we decided not to investigate LARI with A_1(M)/P_3(M) or A_1(M)/P_4(M)) and conducted the subsequent experiments with only P_2(M).

Taken together these results reflect that TEEP-OH caging group effectively obstructs siRNA activity when present either on the antisense strand or on both the sense and antisense strands.

3.5. Photoactivation of TEEP-OH-modified siRNA in HeLa cells

Electrophoresis gel analysis as shown in Figure 7, indicates incomplete photolysis of caging group at 365 nm UV radiation at room temperature for 1 h under buffered conditions. However, to photoactivate TEEP-OH modified siRNA against eGFP, we irradiated HeLa cells transfected with modified siRNA to UV of 365 nm using a band-pass filter for 1 h at room temperature under buffered conditions in 1X PBS buffer at an 8 cm distance between the cells and the source of UV. A band-pass filter was used to prevent the exposure of short-wavelength UV to mammalian cells and thus any UV-mediated cellular toxicity.[32]

We first investigated the influence of the number of P-S linkages in the sense strand on the photolysis of the TEEP-OH caging group from the antisense strand to render siRNA functional. Gratifyingly, as observed from the Fluorescence image in Figure S9 and FACS analysis data in Figure S7 and Table S4, TEEP-OH caging group from the antisense strand (A_1(M)) was released efficiently from TEEP-OH-modified siRNA (A_1(M)/P_2) to generate active siRNA against eGFP and thus we observed eGFP knockdown. The uncaged siRNA (A_1/P_2) gave similar eGFP knockdown with and without light in the presence of L2K2000 thus eliminating light-induced damage of RISC as shown in Figures 8 and.9 Further as observed in Figure S10, TEEP-OH-modified siRNA gave similar eGFP expression to that of unmodified siRNA when transfected in HeLa-eGFP cells in the absence of L2K2000 in the presence of UV. These results indicate that modified siRNA transfected at the experimental concentration in the presence of UV was nontoxic to cells.

Further, as shown in Figures S7, S9, and Table S4, following irradiation, photolysis of the TEEP-OH caging group from the antisense strand (A_1(M)) takes place more effectively in the presence of P-S linkages in comparison to P-O linkages in the sense strand (P_0 vs. P_2, P_3, and P_4). On the other hand, irrespective of the number of P-S linkages in the sense strand (P_2, P_3, P_4), siRNA activity is significantly turned "on" as evinced by the decrease in eGFP expression in HeLa cells.

Finally, Figures 9, S4, and Table S5 show the photolysis of the TEEP-OH caging group from either the antisense strand (A_1(M) or the sense strand (P_2(M) or both the strands (A_1(M)/P_2(M)) to activate siRNA against eGFP. As illustrated in Table S5, across the board, the RNAi activity of the generated siRNA after the photolysis of the caging group is comparable to that of native siRNA irrespective of the attachment of the caging group to the sense strand, antisense strand, or both the strands. The quantitative FACS analysis data in Table S5, further indicates the difference in eGFP knockdown percentage between caged and uncaged siRNA after irradiation for siRNA modified on both the strands (A_1(M)/P_2(M)) is similar to

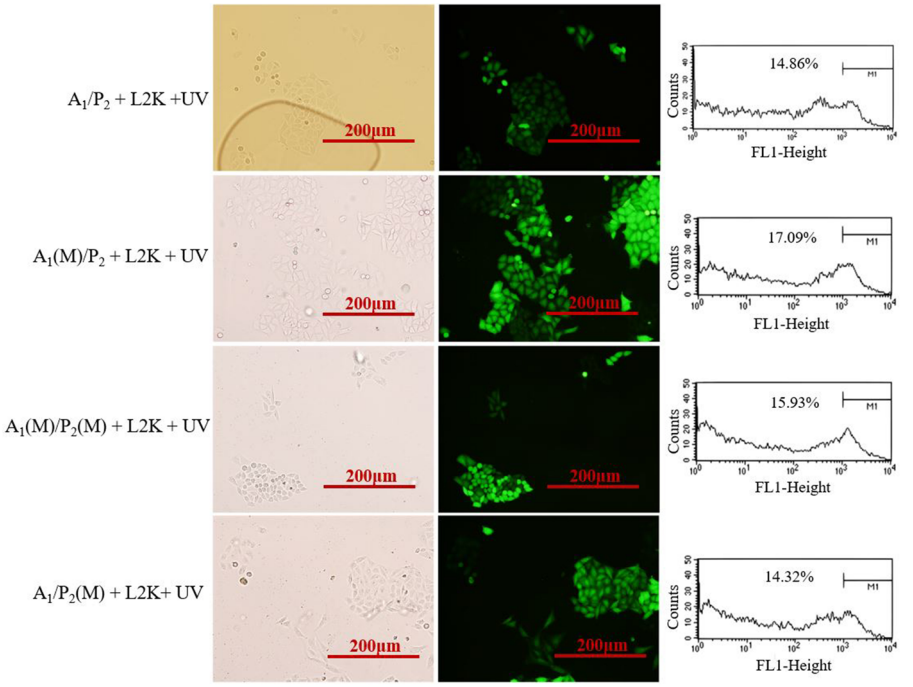

Figure 9. Fluorescence images (left) and flow cytometry analysis (right) showing photoactivated RNA interference in HeLa-eGFP cells transfected with siRNA duplexes with either no modification (A_1/P_2) or modification in either the antisense strand ($A_1(M)/P_2$), sense strand ($A_1/P_2(M)$), or both ($A_1(M)/P_2(M)$).

siRNA caged only on antisense strand ($A_1(M)/P_2$) but greater in contrast to siRNA caged only on sense strand; (Δ_{KD} eGFP ($A_1(M)/P_2$+UV)- ($A_1(M)/P_2$)) = 22.79%, (Δ_{KD} eGFP ($A_1(M)/P_2(M)$+UV)- ($A_1(M)/P_2(M)$)) = 18.47% > (Δ_{KD} eGFP ($A_1/P_2(M)$+UV)- ($A_1/P_2(M)$)) = 10.56%, indicating it is facile to generate siRNA following the photolysis of the caging group from the antisense strand (A_1) in comparison to the sense strand (P_2).

It is worth mentioning that although electrophoresis gel analysis showed incomplete cleavage of the caging at UV of 365 nm, however, cellular studies depicted the generation of functional siRNA after UV and thus efficient photoactivation of siRNA. Varied concentrations of modified siRNA duplexes with caging groups are strongly influenced by the intensity or strength of the radiation being used as demonstrated in previous studies and thus account for the discrepancy between the gel analysis and cellular studies in the present work.[14] Cellular studies were performed with 33 nM of siRNA duplexes comparable to 40 µM used in gel analysis and thus UV irradiation of 365 nm for 1 h was sufficient to photoactivate siRNA in mammalian cells at this lower administered concentration of siRNA. In summary, UV-irradiation-induced photolysis of TEEP-OH in a dose-dependent fashion renders siRNA active upon irradiation.

Figure S11 gives a complete quantitative analysis of eGFP knockdown percentage in HeLa cells under all investigated experimental conditions in this paper including transfection with modified and unmodified siRNA duplexes in the presence and absence of both lipofectamine 2000 and UV.

4. Discussion

Optochemical regulation of gene expression entails a comprehensive understanding of diverse biological processes. Using light as an external stimulus enables spatiotemporal regulation of gene expression. To realize this regulation of gene expression light-sensitive functional groups must be incorporated in siRNA and thus explains the need of such chemical modifications on siRNA. Interestingly, some of these modifications have prolonged the half-life of siRNA duplexes and improved the pharmacokinetic properties without jeopardizing the biological activity. Modifying siRNA with light-sensitive reagents can help realize a true turn "off-on" switch for probing disciplines like developmental biology. However, the incorporation of these light-responsive chemical modifications to siRNA sometime requires complex organic synthetic reaction and involves a number of purification techniques that makes it complicated to realize such systems in a simple and a cost-effective manner.

Here, we set out to achieve light toggled gene expression in mammalian cells by covalent attachment of the TEEP-OH caging group to the siRNA P-S backbone, executing siRNA nonfunctional and following brief UV, siRNA activity was restored by photolysis of the caging group.

To accomplish this objective, we designed siRNA against the green fluorescent protein (eGFP) guided by the established RNAi mechanism. The siRNA reagent was composed of 21 nucleotides with the complementarity of 19 bases between sense and antisense strands and 2 nucleotides overhangs at the 3′-end of each strand as shown in Figure 2. The sense strand constituted of DNA bases whereas nine RNA bases and twelve DNA bases made the antisense strand of the siRNA duplex. With DNA, as a sense strand diminishes the competition to which strand will be incorporated in RISC, considering DNA cannot adopt A-helix conformation, which is a prerequisite for siRNA functioning.[1,33]

Previous studies have shown that siRNA duplexes with phosphorothioate (P-S) linkages display enhanced nuclear resistance.[20,34] This motivated us to design the antisense strand (A_1) of siRNA with one P-S linkage where the S atom would provide us a nucleophilic center for a highly favorable and controllable reaction with -Br electrophile in pHP caging group.[27] P-S linkages were also installed in the sense strand with locations close to 5'end, 3′ end, and in the region complementary to the seed region (2-8 nucleotides) of the antisense strand as shown in Figure 2.[6,34,35] Each of

the sense and antisense strands were designated depending on the number of P-S linkages as shown in Table 1.

Considering the direct interaction of the antisense strand with the Dicer, we envisaged the eGFP gene expression would be more effectively toggled when the pHP caging group is covalently attached to the antisense strand in contrast to the sense strand of the siRNA duplex.[6] The caging group in the antisense strand would perturb A-form helix conformation, and interfere with the Dicer activity and argonaute cleavage mechanism. Further, the sterically demanding caging group will inhibit the effective placement of antisense strand in RISC, hampering protein function within RISC and thus suppressing the recognition of the target mRNA and obstructing eGFP knockdown.[19]

To validate our hypothesis, siRNA with a P-S backbone was modified by site-specific installation of a TEEP-OH caging group using a robust nucleophilic substitution reaction. Simple electrophoresis gel analysis evinced the efficiency of the modification reaction and the photolysis of the caging group. Its compatibility with the intracellular environment and nuclease resistance encouraged us to probe its efficiency in modulating gene expression in mammalian cells. We used a reporter gene, GFP to assess the activity and photoregulation of TEEP-OH caged siRNA.

Transfection experiments illustrate that the administration of TEEP-OH-modified siRNA blocks RNA interference effectively prior to irradiation but is able to deliver native-like knockdown when irradiated. This capability to toggle the gene expression is most pronounced when the caging group is tethered to the antisense strand of siRNA which is complementary to the sense strand with two P-S linkages (A_1(M)/P_2). The direct placement of the antisense strand of siRNA in the RISC complex is the rationale behind this observation.

Our results paint a clear picture reinforcing the potential of TEEP-OH in LARI to turn "off" and turn "on" the gene expression as desired. However, ambiguity about the insufficient blockage in RNAi points us toward the limitation of the designed strategy and further necessitates rigorous studies to develop TEEP-OH as a promising caging group in LARI, highlighting the scope of improvement in our designed strategy. While the precise determination of the mechanism for the observed insufficient abrogation in RNAi by TEEP-OH is difficult, it appears there is significant scope for improvement in its caging potential. Although we cannot definitively determine how it can completely abrogate RNAi, we suspect that the desired goal can be achieved if it can strongly affect the interaction of siRNA with the Dicer and RISC in an adverse fashion. We anticipate exploiting sterically hindered and bulky caging group as a precursor to TEEP-OH moiety instead of pHP group using the same nucleophilic substitution chemistry will hinder the effective placement of

antisense strand in RISC for the RNAi process thus completely abolishing RNAi.[14,34]

5. Conclusion

In summary, we demonstrated TEEP-OH as a potential caging group to toggle the gene expression in mammalian cells using light as an external stimulus. We have synthesized TEEP-OH modified siRNA by minimal operation of complex organic synthetic reactions and purification techniques. Further, the intracellular compatibility of the TEEP-OH caging group provided facile activation of caged siRNA upon light irradiation inside mammalian cells. Our transfection experiments in mammalian cells provide further insight into the significance of the position of covalent attachment of caging group on siRNA. These results are informative as they reflect TEEP-OH ability in abrogating RNAi is enhanced when present on the antisense strand in contrast to the sense strand. Likewise, its photolysis to yield native siRNA is efficient when present on an antisense strand. Furthermore, the obstruction in siRNA activity by TEEP-OH caging on the antisense strand decreases with an increasing number of P-S linkages in the sense strand.

Taken together, these results illuminate our understanding of TEPP-OH as a caging group for siRNA to trigger "on-command" gene expression. Further exploration of positional and steric factors controlling the LARI phenomenon by the TEEP-OH caging group should help guide the design of siRNA for applications requiring a true turn "off-on" switch for gene expression in disciplines such as developmental biology. This work will stimulate advances in studies investigating light modulation of gene expression.

Disclosure statement

No potential conflict of interest was reported by the authors.

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