Gene Regulation

Deutsche Ausgabe: DOI: 10.1002/ange.201510921
Internationale Ausgabe: DOI: 10.1002/anie.201510921

# Photochemical Regulation of Gene Expression Using Caged siRNAs with Single Terminal Vitamin E Modification

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***Abstract:*** *Caged siRNAs with a single photolabile linker and/or vitamin E (vitE) modification at the 5′ terminal were rationally designed and synthesized. These virtually inactive caged siRNAs were successfully used to photoregulate both firefly luciferase and GFP gene expression in cells with up to an 18.6-fold enhancement of gene silencing activity, which represents one of the best reported photomodulation of gene silencing efficiencies to date. siRNA tracking and vitE competition experiments indicated that the inactivity of vitE-modified siRNAs was not due to the bulky moiety of vitE; rather, the involvement of vitE-binding proteins has a large contribution to caged siRNA inactivation by preventing the dissociation of siRNA/lipo complexes and/or siRNA release. Further patterning experiments revealed the ability to spatially regulate gene expression through simple light irradiation.*

RNAi has been widely applied as a powerful tool for gene silencing in research and as a therapeutic reagent in drug development because of its sequence-specific degradation of target mRNA.[1] Currently, chemical modifications of siRNAs at multiple positions[2] including the nucleobases, sugar ring, backbone, and RNA terminals have been used to increase the effectiveness of siRNA by improving their stability, specificity, and cell permeability. However, conditional regulation of siRNA function is highly useful, particularly when specific gene expression regulation based on spatiotemporal resolution and amplitude is desired. Photoirradiation may be one of the best methods to achieve this goal.[2c,e,3] The development of photo-induced RNAi for controlling protein expression has become a topic of growing interest for the past couple of years, and different photocaging strategies[4] have been developed, such as caged nucleobases (thymidine, uridine, and guanosine),[5] caged internal phosphate,[6] and caged terminals of siRNAs.[7] Deiters[5a] and Heckel[5b] et al. reported that siRNAs with site-specific caged nucleotides could photomodulate siRNA activity. Two or more caged nucleobases may be needed to efficiently mask siRNA activity. Friedman[7b] and Monroe[6a] et al. applied another statistical caging strategy to mask the phosphate backbone with NPP or DMNPP; unfortunately, only partial inhibition of gene silencing was observed. Heavily caged siRNAs may also have problems with incomplete restoration of siRNA activity following photoirradiation. All four terminals of dsRNAs have been reported with large bulky cyclo-dodecyl DMNPE to block Dicer or nuclease processing because of a steric clash of the caging group.[7a] We aimed to develop caged siRNA with a single photolabile group without affecting siRNA duplex formation and to achieve the complete blocking of siRNA activity that could fully recover upon light irradiation. McMaster[7d] et al. reported the development of caged siRNAs with attachment of a single photolabile biotin molecule at the 5′ terminal that showed only moderate photomodulation of gene silencing. We previously developed four phosphine-caged nucleobase phosphoroamidites and site-specifically incorporated them in any position of both the sense and guide strands of siRNA duplexes.[6c] Only single phosphate-caged siRNAs at the 6th and 16th positions and the 5′ terminal phosphate showed moderate photomodulation of gene silencing activity. Labeling of all of these key positions achieved complete blocking and photo-induced recovery of siRNA activity.

Based on our preliminary experimental results, single modification of vitamin E (vitE) at the 5′ terminal of siRNA caused almost complete loss of siRNA activity. This observation inspired us to develop a series of photolabile vitE-modified siRNAs (vitE-siRNAs). In this work, we rationally designed and easily synthesized a series of single vitE-modified photolabile siRNAs (vitE-*p*-siRNA) by inserting a photolabile linker between the vitE moiety and 5′ terminus of a siRNA, masking the gene silencing function of siRNA (Scheme 1). We demonstrated that caged vitE-siRNAs with vitE modification at either the 5′ terminal of the antisense strand and/or the 5′ terminal of the sense strand showed excellent photomodulation of siRNA gene silencing activity. By using our caged vitE-siRNA, we successfully achieved efficient photomodulation of both firefly luciferase and GFP gene expression by global and/or patterned irradiation.

A photolabile linker (*N*,*N*-diisopropylamino-[1-(*o*-nitrophenyl)-2-dimethoxytrityloxy] ethoxy-2-cyanoethoxy-phosphine) was synthesized and inserted between vitE and the 5′ terminal of RNA strands by solid phase synthesis. The synthesis of photolinker and vitE phosphoroamidites was straightforward (Supporting Information). The RNA oligonucleotides with the attached vitE and/or a photolinker at the 5′ terminal were then cleaved and deprotected with ammonium hydroxide. Because of the hydrophobic vitE moiety, these RNA oligonucleotides are easily purified with reverse-phase HPLC column.

The photolysis of the photolabile single-stranded vitE-modified RNAs (vitE-RNAs) and their duplexes were inves-

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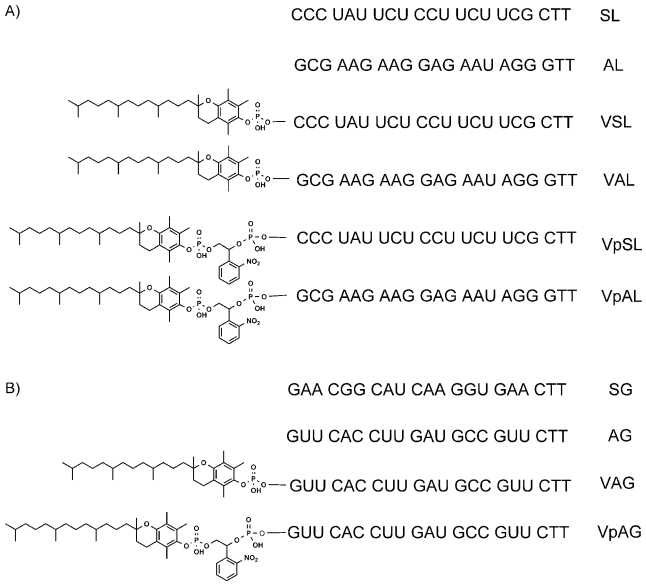
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201510921.




***Scheme 1.*** Structure and sequences of unmodified and modified siRNA of firefly luciferase (A) and GFP (B). Abbreviation list: "S," sense strand; "A," antisense strand; "V," Vit E; "p," photolinker; "L", firefly luciferase; "G," GFP.

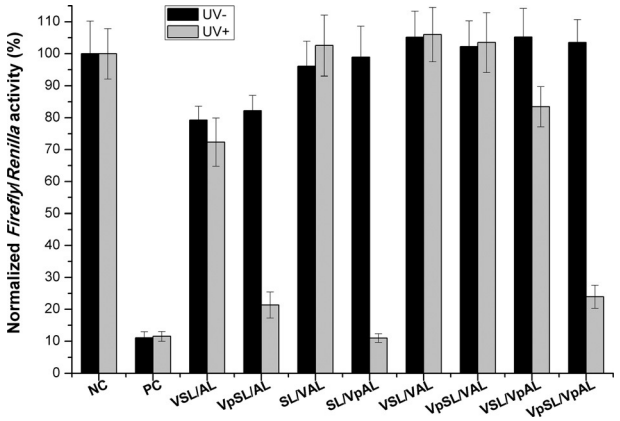



***Figure 1.*** Photomodulation of firefly luciferase activity (*Renilla* luciferase as the internal control) with different combinations of vitE-siRNAs.

tigated with PAGE gel analysis. For single-stranded RNA shown in Figure S1A, photolabile vitE-RNAs (VpAL and VpAG) showed similar mobility as their non-photolabile versions (VAL and VAG), but exhibited slightly reduced mobility compared to their native RNAs (AL and AG; Figure S1A). However, upon light irradiation, the cleavage of the photolinker caused removal of the caged group and vitE moiety and restored their mobility to that of native RNA strands (AL and AG). For double-stranded vitE-siRNAs, photolabile vitE-siRNAs could also be photo-uncaged and transformed to native siRNAs (Figure S1B). Furthermore, for siRNA formed by both vitE-RNAs (VSL/VAL, VSL/VpAL, and VpSL/VpAl; Figure S1B, lanes 3, 6, and 8, respectively), their mobility in PAGE gels was further retarded. When photolabile double vitE-siRNAs (VpSL/VpAL) were irradiated under the same conditions, we observed an extra band that corresponded to an siRNA duplex with the cleavage of one photolabile vitE moiety compared with the mobility of uncaged VSL/VpAL in the same gel (Figure S1B, lane 7). Therefore, prolonged irradiation may be needed to promote further cleavage of the photolabile vitE moiety.

To show the effect of vitE-modified caged and non-caged siRNAs (vitE-caged and non-caged siRNAs, respectively) on gene expression, we first applied a dual reporter firefly/renilla luciferase assay with siQuant vectors. siRNA sequences (AL and SL) were optimized to knockdown firefly luciferase expression with renilla luciferase as an internal control. Both sense and antisense strands of siRNA were modified with vitE or vitE/photolinker at 5′ terminals. Each of the vitE-RNAs was then hybridized with the corresponding complementary native RNAs or vitE-RNAs to form a series of siRNA duplexes (Figure 1, *x* axis).

These caged or non-caged siRNAs were then cotransfected into HEK293 cells with luciferase reporter vectors for 6 hours. After transfection, each set of cells was subjected to 3 min of light irradiation or kept in the dark; this was followed by further incubation for another 30 hours. The cells were then collected, and luciferase activity was analyzed according to standard methods with *Renilla* luciferase activity as the internal control. As shown in Figure 1, transfection with native siRNA (PC or AL/SL) at a 5 nM concentration substantially down-regulated firefly luciferase activity, with only 11% remaining. However, if the antisense strand of non-caged siRNA (SL/VAL) was modified with only a single vitE modification at the 5′ terminal, no knockdown of gene silencing was observed, as evidenced by the identical levels as the negative control (NC). Only single vitE modification at the 5′ terminal of the sense strand of siRNA (VSL/AL) maintained up to 80% firefly luciferase activity. Gene silencing activity was not observed if the 5′ terminals of both the sense and antisense strands of siRNA (VSL/VAL) were attached to a vitE moiety. Compared with the non-caged version of the corresponding siRNAs, insertion of a photolinker between the vitE moiety and 5′ terminal of RNA (sense or antisense strand) had no effect on firefly luciferase expression in the absence of light irradiation, indicating that these caged siRNAs were inactive because of the presence of a vitE moiety at the 5′ terminal of RNA. However, brief light irradiation of cells cotransfected with vitE-caged siRNA (SL/VpAL) removed the photolabile linker and vitE moiety, and restored siRNA activity; Indeed, photomodulation showed an approximately 9-fold efficiency with downregulation of firefly luciferase activity from 99% to 11% and to the levels that were identical to the positive control (PC). Our caging strategy is one of the most efficient caged siRNA-based gene silencing methods that has been reported.

Interestingly, light activation of siRNA (VpSL/AL, only on caged sense strands) also triggered up to 3.8-fold increase in gene silencing. This is due to the vitE modification at the 5′ terminal of the sense strand of siRNA. However, many previous reports have demonstrated that modification of the 5′ terminal of the sense strand of siRNA should not substantially inhibit siRNA activity. Here, we still observed approximately 80% luciferase activity for either the caged or non-caged siRNAs with vitE modification. We proposed that

there might be another way to prevent siRNA loading or activation through the RNA interference silencing complex. We then tracked the location of native siRNA (SL/AL) and vitE-siRNA (SL/VAL) after lipo-transfection. A Cy3-labeled sense strand RNA analog (Cy3-SL) was used for hybridization with AL or VAL. After co-transfection, the cells were washed and cultured for an additional 6 and 12 hours. The cells were then imaged (Figure S2). After 6 hours, cells transfected with Cy3-SL/AL or Cy3-SL/VAL contained bright red fluorescence spots of siRNA/lipo complexes. After 12 hours, a significant difference was observed since cells with Cy3-SL/AL had few red fluorescence spots left, while cells co-transfected with vitE-siRNA (Cy3-SL/VAL) still contained many red fluorescence spots in the cytoplasm. These data indicate that siRNA was released from the siRNA/lipo complexes in Cy3-SL/AL-transfected cells, yet vitE-siRNA/lipo complexes were defective in this process and vitE-siRNA was not released. According to other reports, vitE can interact with proteins such as α-tocopherol transport protein (α-TTP).[8] To confirm this, we first tested different inhibitors based on different mechanisms of cellular uptake. As shown in Table S1 and Figure S3, chlorpromazine, amiloride, methyl-β-cyclodextrin, and genestein almost did not inhibit the entrance of vitE-siRNA/lipo complexes into cells, while vitE and low temperature displayed clear blocking of vitE-siRNA entrance. These results indicated that the cellular uptake of vitE-siRNA/lipo complexes was most possibly mediated by vitE receptor proteins. Further vitE concentration dependence experiments indicated that the competition of vitE binding caused the less efficient delivery of vitE-siRNA/lipo complexes with increasing vitE concentrations (Figure S4). Protein binding at siRNA terminals would introduce much bulkier groups, which might lead to difficulty in complex dissociation and/or the interaction of vitE-siRNA duplexes with the RNAi cellular machinery.

Photomodulation of firefly luciferase activity of siRNA duplexes with one caged vitE-RNA and one caged or non-caged complementary vitE-RNA was also investigated. Light activation of VpSL/VAL and VSL/VpAL produced SL/VAL and VSL/AL siRNA duplexes. As expected, the non-caged VpSL/VAL and VSL/VpAL in cells showed the same levels of firefly luciferase activity as that of direct co-transfection of SL/VAL and VSL/AL in cells (Figure 1). For the caged siRNA (VpSL/VpAL), light irradiation partially recovered its knockdown of firefly luciferase with only a 4.3-fold enhancement of siRNA gene silencing activity. As indicated in Figure S1, brief irradiation may remove one vitE moiety from VpSL/VpAL to form VpSL/VAL or VSL/VpAL, which showed almost no gene silencing activity. Complete removal of both vitE moieties could fully restore siRNA function. Different light exposure times (1 to 7 min) were then applied to cells co-transfected with the caged VpSL/VpAL duplex. As shown in Figure S5, prolonged irradiation time recovered the full silencing ability of the caged VpSL/VpAL duplex. Maximum photomodulation (up to 10.5-fold) of siRNA activity was achieved with 6 min of light exposure.

Because siRNAs (SL/VpAL and VpSL/VpAL) were fully inactive at 5 nM without light activation, we further evaluated the dose dependency of the above caged vitE-siRNAs on photocontrol using firefly luciferase activity as a read-out (Figure 2 and Figure S6). The same dual reporter firefly/renilla luciferase assay in HEK293 cells was used with the following doses of caged siRNAs: 2.5 nM, 5.0 nM, 10.0 nM, and 20.0 nM. For cells treated with caged siRNA duplexes, slight inhibition of firefly luciferase expression was observed for only 20 nM siRNAs (approximately 80% for SL/VpAL and 92% for VpSL/VpAL).

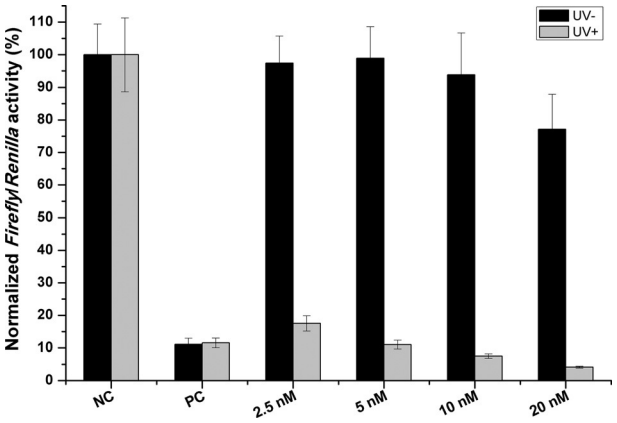



***Figure 2.*** Dose effect on photomodulation of luciferase activity with caged vitE-siRNAs (SL/VpAL). The concentration of PC siRNA was fixed at 5 nM.

Light activation restored siRNA activity, and a dose dependency for gene silencing was observed. The photomodulation ratios for different concentrations of SL/VpAL siRNA duplexes were up to 12.5-fold for 10 nM and 18.6-fold for 20 nM, which represent the best known photomodulation of caged siRNAs to date. Similar results were also achieved with a photomodulation efficiency of up to 7.3-fold for 20-nM VpSL/VpAL (Figure S5).

Based on the observations above, photolabile siRNA with only a single vitE modification at the 5′ terminal of antisense strand RNA could be used to efficiently photoregulate gene expression. Another gene, *GFP*, was then tested to confirm the generality of gene silencing with our caged vitE-siRNAs (Figure 3A). Antisense strands of GFP-targeting siRNA labeled with only a vitE moiety or vitE and a photolinker at the 5′ terminal were then synthesized using similar protocols. HEK293 cells were then cotransfected for 6 hours with pEGFP-N1 and pDsRed2-N2 (RFP, as the internal control), as well as SG/AG, SG/VAG, or SG/VpAG. Two sets of experiments were conducted with or without brief light activation. After another 42 hours of incubation, the cells were imaged, and GFP/RFP expression was quantified by flow cytometry (Figure 3B). The amount of cells with both GFP and RFP expression was then normalized to cells with RFP expression. As expected, light irradiation had no effect on GFP and RFP expression in both negative and positive control experiments. Cells treated with GFP siRNA (SG/AG) showed 91% knockdown of GFP expression. Although cells treated with non-caged vitE-siRNA (SG/VAG) still displayed strong GFP fluorescence, light irradiation had little effect on GFP expression, which is similar to gene silencing of firefly luciferase with non-caged siRNA (SL/VAL). In the presence

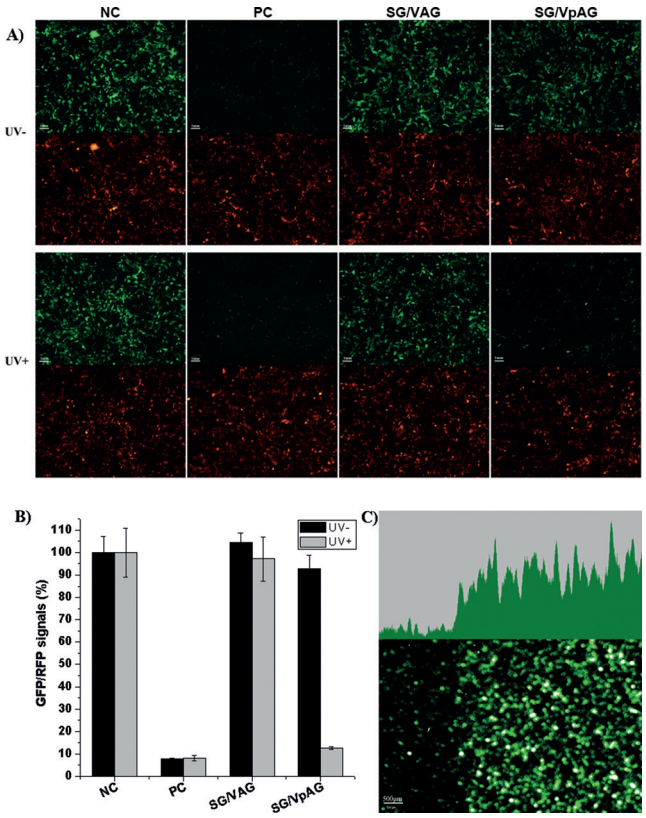



***Figure 3.*** Photomodulation of GFP expression cotransfected with pEGFP-N1, pDsRed-N1, and vitE-caged, or -non-caged siRNAs (SG/VpAG and SG/VAG). Cells were irradiated for 3 min (365 m, 7 $W cm^{-2}$) or kept in the dark. A) Cells were imaged using fluorescence microscopy with GFP and RFP channels, scale bar = 1 mm; B) Quantification of cell numbers with GFP expression divided by cell numbers with RFP expression using flow cytometry. Standard deviations were calculated from three individual tests; C) spatial regulation of GFP expression with patterned irradiation (left side of view), scale bar = 500 μm.

of vitE-caged siRNA (SG/VpAG), over 93% of GFP expression was still observed. This indicates the inactivity of caged vitE-*p*-siRNA in the cells. Upon brief light irradiation, the vitE moiety with a photolinker was removed, siRNA was subsequently released from the siRNA/lipo complexes, and the RNAi cellular machinery was activated. Under these conditions, GFP expression was down-regulated to 13% of the negative control and to levels that were similar to native siRNA data (SG/AG).

Additionally, we also tested the spatial control of GFP expression using caged vitE-siRNA (SG/VpAG) by masked light irradiation. Cells were first co-transfected with SG/VpAG and GFP/RFP plasmids, and then a section of plate of the cultured cells was irradiated to activate the siRNA. The cells were continued in culture for another 42 hours and were then imaged with a high content analyzer. As shown in Figure 3C, no effect on RFP expression was observed in either irradiated or non-irradiated regions. However, GFP in irradiated cell regions was virtually silenced, whereas the non-irradiated cell retained normal GFP levels. These results indicate that spatial control of gene expression with the Ve-caged siRNAs is possible.

In summary, we developed photolabile siRNAs with a single caging group as a tool to efficiently photomodulate gene silencing activity with spatial resolution. In the past, both our lab and others have shown that the attachment of an NPE[6c] or biotin-NPE[7b] group at terminal or internal phosphate groups had minimal to no effect on siRNA activity regulation. In this work, a series of caged and non-caged siRNAs with vitE modification at the 5′ terminal of RNAs was rationally designed and synthesized to target both firefly luciferase and GFP expression. Caged or non-caged siRNAs with vitE-modified antisense strands were inactive in target gene knockdown. Light activation of these caged vitE-siRNAs fully recovered gene silencing activity to levels identical to that of native siRNA, but not of corresponding non-caged vitE-modified siRNAs. The photomodulation efficiency was up to 18.5-fold greater with a single vitE-caged antisense strand, which represents the best known photomodulation of siRNA activity to date.

vitE modification at the sense strand of caged and non-caged siRNAs made them virtually inactive, a result which is inconsistent with previous observations. Our results could not be explained solely by a bulky vitE moiety. By tracking the siRNA/lipo complexes in cells, we found that native siRNA was readily released and interacted with the RNAi cellular machinery, whereas vitE-siRNA/lipo complexes may associate with vitE-binding proteins and remain inactive in the cytoplasm. Upon light irradiation, the complexes dissociated from the vitE-binding proteins and active siRNAs were released to silence target gene expression. This observation was further confirmed using double vitE-caged or non-caged siRNAs that showed complete inert gene silencing activity and partially restored function (24% reduction of firefly luciferase activity). Furthermore, siRNA with photolabile vitE modification of both sense and antisense strands also fully restored its gene silencing activity relative to native siRNA levels but required longer light irradiation time, indicating that prolonged light irradiation is required for multiple caging of siRNAs.

## Acknowledgements

This work was supported by the National Natural Science Foundation of China (21422201, 21372018, and 21302008), and the National Basic Research Program of China ("973" Program (2012CB720600).

**Keywords:** caged oligonucleotides · gene regulation · photoactivation · siRNA · vitamin E modification

**How to cite:** *Angew. Chem. Int. Ed.* **2016**, *55*, 2152–2156
*Angew. Chem.* **2016**, *128*, 2192–2196

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Received: November 24, 2015
Published online: December 28, 2015

  