Caged RNA

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# RNA Interference Controlled by Light of Variable Wavelength**

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***Abstract:*** *Known molecular, "caged" siRNAs are activated by UV light. Since the light of this type is toxic to cells, the uncaging can cause undesired side effects. A modular, molecular system for designing siRNAs is reported, which can be activated by non-toxic light in live cells. For example, siRNAs responsive to green and red light are described. The uncaging is mediated by $^1O_2$ photogenerated on a photosensitizer, which is attached to the 3′-terminus of the lagging strand. The 5′-terminus of the guide strand is alkylated ("caged") with a 9-anthracenyl residue. The latter fragment reacts with the $^1O_2$ with formation of the free (uncaged) 5′-OH terminus. Simultaneously with the uncaging the photosensitizer is bleached and no more $^1O_2$ is generated after this process is completed. The photoactivation of the siRNAs described here is not toxic to cells.*

Small interfering RNAs (siRNAs) are about 21–23 nucleotide-long double-stranded RNAs. They are used as inhibitors of gene expression acting via the RNA interference (RNAi) mechanism. The latter includes phosphorylation of the 5′-terminus of one of the strands (guide strand), siRNA dissociation accompanied by digestion of the second RNA strand (lagging strand) and insertion of the guide strand into the RNA-induced silencing complex (RISC). The latter associate binds specific mRNA sequences, followed by their cleavage.[1] Photoactivatable ("caged") siRNAs can be prepared by introducing photocleavable protecting groups into the parent siRNA duplex, which block either the phosphorylation (5′-modifiers) or the RNA recognition by RISC (internal modifiers). These reagents are valuable tools for the biochemical research, which allow for spatial and temporal control of gene expression. The first example of "caged" siRNAs has been described by Friedman,[2] who deactivated the GFP and RFP inhibitors by random alkylation of phosphodiester groups in their backbone with 1-(1-diazoethyl)-4,5-dimethoxy-2-nitrobenzene (DMNPE-$N_2$). The inhibitors obtained could be uncaged by UV light ($\lambda >$ 320 nm). The same group has later demonstrated that siRNAs can be caged by the attachment of the DMNPE group to the 5′-terminal phosphate monoester.[3–5] Heckel et al. have introduced O4-protected G and T deoxynucleotides into the guide strand of EGFP-targeting siRNAs, which could be uncaged by their exposure to UV light.[6] Other relevant examples have been described.[7] A common disadvantage of the known inhibitors is the need for using the UV light as a trigger. This trigger is not well compatible with live cells, since it causes DNA mutations and is cytotoxic.[8] Several photoswitchable groups responsive to the longer wavelength (up to 546 nm) light are known.[9–12] Their applicability towards caging siRNAs remains to be proven. The visible light at wavelengths shorter than 550 nm is also not ideally suitable for cellular applications, since it is strongly absorbed by cellular components and can excite endogeneous photosensitizer riboflavin causing both its bleaching and formation of toxic species for example, radicals and singlet oxygen.[13]

In contrast to molecular systems, hybrid organic–inorganic nanomaterials responsive to light of wavelengths longer than 600 nm are known. For example, siRNAs bound to nanoparticles Au-NPs[14] or Si-UCNPs[15] remain inactive in the dark. However, they release siRNAs into cytosol upon their exposure to the light from powerful IR lasers (for AuNPs: 800 nm, 3.1 W cm$^{-2}$ for 20 s). Although the NPs are functional in live cells, these systems are still disadvantageous. In particular, they are complex, the NPs remain in the cell after the uncaging, which can potentially cause undesired side effects, and the uncaging is conducted by using powerful light sources. All these factors can potentially limit broader applications of such NPs in photocontrolled RNAi.

We have recently adapted a singlet oxygen ($^1O_2$)-mediated system[16] for caging antisense oligonucleotides.[17–19] In this case the uncaging event includes the cleavage of a $^1O_2$-sensitive linker. The $^1O_2$ mediator is photogenerated in the presence of a photosensitizer (**P**). Though we have demonstrated that such reagents work in vitro,[17–19] they remain inactive in cells.

Herein we report on an application of a 9-alkoxyanthracenyl fragment as a $^1O_2$-sensitive moiety for caging siRNAs. It is attached directly to the 5′-terminus of a siRNA thereby preventing its phosphorylation and, correspondingly, inhibiting its activity (Figure 1). The 3′-terminus of the lagging strand is conjugated to a **P**. We selected red (**P1** and **P2**) and green (**P3**) light-absorbing **P**s to demonstrate the generality of the approach. The resulting "caged" siRNAs can be uncaged upon their irradiation with the light absorbed by the corresponding **P** in a $^1O_2$-mediated cleavage as it is shown in Figure 1. The potential advantage of this system is that the cleavable moiety has the source of electrons, which are stored in the C(10)–H bond (**2**, Figure 2 and Figure S10 in the Supporting Information). In particular, after the initial addition of $^1O_2$ to **2** the electrons of the C(10)–H bond can be transferred to one of the oxygen atoms of the endoperoxide moiety thereby facilitating cleavage of the O–O bond.

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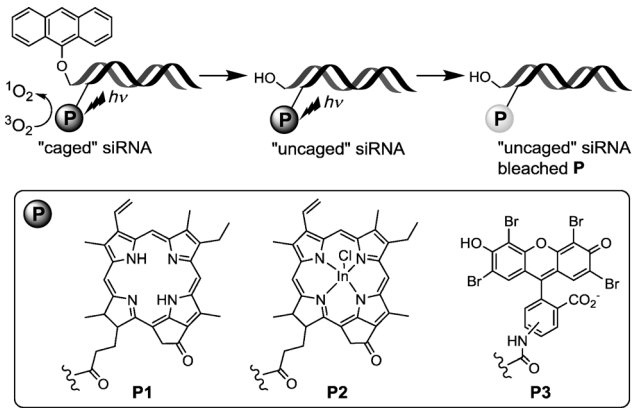


***Figure 1.*** A concept of "caged" siRNAs activated by the light of selectable wavelength. **P** = photosensitizer (**P1**–**P3**).

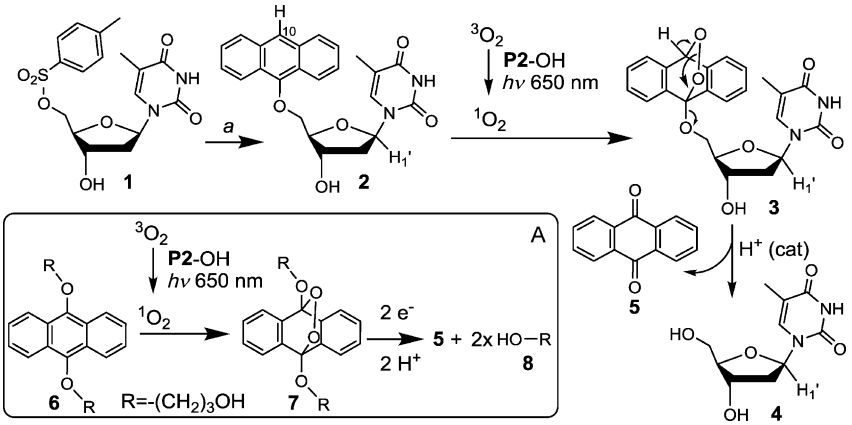


***Figure 2.*** Synthesis of **2** and the mechanism of its uncaging. A) The mechanism of cleavage of **6** by $^1O_2$.[19]

Since this reaction does not consume electrons, it is not expected to induce oxidation of the intracellular reductants and should be better suitable for applications in live cells than the reported systems.[17–20]

To evaluate this hypothesis we first prepared compound **2**. Upon its exposure to 650 nm light in the presence of **P2**-OH (0.1 equiv) absorbance as well as emission bands characteristic for the anthracene disappear (Figure S6). According to $^1$H NMR spectroscopy **2** is converted to **4** and **5** (Figure 3A). At the conditions of competitive cleavage of **2** and 9,10-dialkoxyanthracene **6**[19] the latter substance generates endoperoxide as a side product, whereas **2** is cleanly cleaved (Figure S9). Inspired by these positive results we prepared 5′-anthracenyl-modified guide strands g_RNA3 and g_RNA5 as well as the corresponding lagging strands carrying **P1**–**P3**. These RNAs were assembled in solution to form "caged" siRNAs targeting two representative genes AcGFP-mRNA and KIF11 (Figure 4). By using UV-melting experiments we confirmed that both modified and unmodified siRNAs are highly stable: $T_m = 74.1$–$76.4\,^\circ$C. Next, we tested in vitro activation of the inhibitors targeting AcGFP, which carry either red-light- (**P1**, **P2**) or green-light-responsive **P**s (**P3**). In particular, we observed that after exposure of g_RNA3/l_RNA6 and g_RNA3/l_RNA7 to 650 nm light (0.23 W) for only 2 min, less than 50% of the guide strand g_RNA3 is converted to its deprotected form g RNA2 (Figure 5B–D). Further irradiation for up to 30 min leads to the complete activation of the siRNAs. As expected, in the control duplex g_RNA3/l_RNA9 lacking **P**, the 5′-**ANdT** remains intact under the irradiation (open triangles in Figure 5C,D). Interestingly, both **P1** and **P2** are quickly bleached during the process of uncaging. The bleaching rate was found to be higher in "caged" siRNAs than that in the duplexes lacking the 5′-**ANdT** modifier (Figure 5C,D). We assume that the endoperoxide, which is initially formed in the uncaging process, can oxidize the **P**s and induce their bleaching. The quick deactivation of **P** here is a positive effect, since the photogeneration of toxic $^1O_2$ is stalled after the uncaging of the siRNA is complete that prevents further undesired side effects.

As expected, "caged" siRNA g_RNA3/l_RNA8 containing eosin **P3** was found to be efficiently uncaged by its exposure to green light (Figure S14). Thus, the design of "caged" siRNAs reported here is not restricted to one type of trigger.

For monitoring activity of siRNAs in live cells we used the dual fluorescence reporter assay.[21] In particular, plasmids expressing AcGFP and DsRed2 were introduced into cells (HeLa) together with a corresponding siRNA targeting AcGFP.[22] Since DsRed2 is not affected by inhibitors, it was used as a reference. In the untreated cells ratio of green (AcGFP) to red (DsRed2) fluorescence (AcGFP/DsRed2) was $1.4 \pm 0.1$. We observed that activities of siRNAs carrying either 5′-dT (g_RNA2/l_RNA9, AcGFP/DsRed2 $= 0.1 \pm 0.1$) or 5′-U (g_RNA1/l_RNA9, AcGFP/DsRed2 $= 0.2 \pm 0.1$) were practically the same, which is in agreement with the previous findings.[23] The 5′-dT-containing

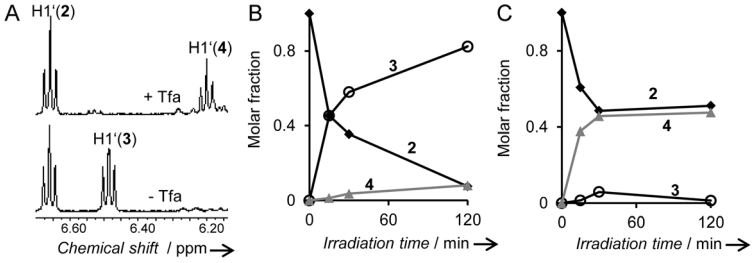


***Figure 3.*** A) $^1$H NMR spectra of a mixture of **2** (15 mM) and **P2**-OH (1.5 mM) in $CDCl_3$/[$D_6$]DMSO (100/1, v/v) irradiated for 15 min with 650 nm light (0.29 W) in the absence (−TFA) and presence (+TFA) of 1% (v/v) TFA. Kinetics of the uncaging of **2** at these conditions is shown in insets B (−TFA) and C (+TFA). Molar fractions of **2**, **3**, and **4** were determined by integrating signals of H1′ atoms.

  

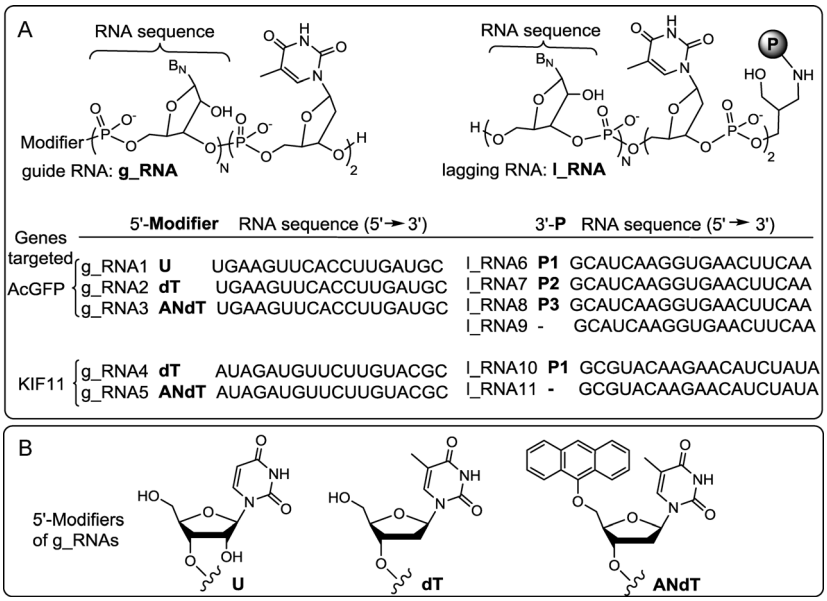


***Figure 4.*** A) Sequences and labeling scheme of RNA strands and B) structures of 5′-terminal modifiers of guide RNA strands used in this study are shown on the plot. Structures of 3′-terminal modifiers **P** of lagging RNA strands are provided in Figure 1.

siRNA was further used as a positive control, since "caged" siRNAs release the former RNA as a product in the result of their uncaging. As expected, 5′-**ANdT**-modified siRNA g_RNA3/l_RNA9 was practically inactive (AcGFP/DsRed2 = 1.5 ± 0.3, Figure 6A). In this case the 5′-modifier blocks intracellular 5′-phosphorylation of the siRNA required for its RNAi activity. Control siRNAs g_RNA2/l_RNA7 and g_RNA2/l_RNA8, which carry 3′-**P1** or 3′-**P2** correspondingly, exhibited the high inhibitory activity similar to that of the parent siRNA g_RNA2/l_RNA9 without any 3′-modifier (Figure 6A). These data indicate that 3′-modifications in the sense strand do not affect the activity of siRNA. We observed that "caged" siRNAs g_RNA3/l_RNA6 and g_RNA3/l_RNA7 exhibit significantly reduced inhibitory activity with respect to that of the unmodified siRNA (Figure 6A).

However, in comparison to g_RNA3/l_RNA9 some residual activity is still observed, which is especially pronounced for the siRNA containing 3′-**P1**. We believe that it is caused by partial activation of the siRNAs because of their limited exposure to light during the handling. We were pleased to observe that exposure of both "caged" siRNAs g_RNA3/l_RNA6 and g_RNA3/l_RNA7 to a short pulse of red light (650 nm, 0.23 W, 2 min) leads to significant enhancement of their inhibitory activity (entries 6 and 7, Figure 6A). In contrast, the activity of control siRNAs was found to be independent upon irradiation of red light (entries 1–5, Figure 6A).

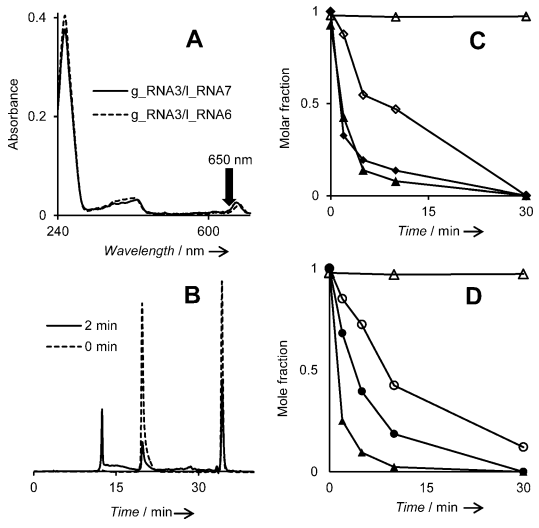


***Figure 5.*** A) UV/Vis spectra of g_RNA3/l_RNA6 (1 μM) and g_RNA3/l_RNA7 (1 μM) in phosphate buffer (20 mM, pH 7.4, NaCl 130 mM), 22 °C. B) HPLC profiles of g_RNA3/l_RNA6 (50 μM) obtained before (dotted trace) and after uncaging with 650 nm light for 2 min (0.23 W, solid trace). The peak observed at 34.2 min corresponds to l_RNA6, that at 19.7 min to g_RNA3 and that at 12.4 min to g_RNA2. C) Kinetics of uncaging of g_RNA3 in g_RNA3/l_RNA9 (open triangles) and g_RNA3/l_RNA7 (black triangles) and of photobleaching of l_RNA7 in g_RNA2/l_RNA7 (open diamonds) and g_RNA3/l_RNA7 (black diamonds); molar fraction of g_RNA3 = [g_RNA3]/([g_RNA3] + [g_RNA2]); molar fraction of l_RNA7 = [l_RNA7]/$[l\_RNA7]_{t=0}$. D) Kinetics of uncaging of g_RNA3 in g_RNA3/l_RNA9 (open triangles) and g_RNA3/l_RNA6 (black triangles) and of photobleaching of l_RNA6 in g_RNA2/l_RNA6 (open circles) and g_RNA3/l_RNA6 (black circles); molar fraction of l_RNA6 = [l_RNA6]/$[l\_RNA6]_{t=0}$.

Next, we aimed to prove that "caged" siRNAs described here are applicable for light-controlled regulation of endogeneous genes. We selected KIF11 (Eg5) as a representative target. KIF11 is a member of the kinesin-5 family and is an essential enzyme for mitosis in most organisms.[23] Inhibition of KIF11 is reflected in accumulation of cells in a G2 phase and increasing the ratio of cell numbers in G2 and G1 states (G2/G1) (Figure 6B,C). In particular, we observed that in the absence of any inhibitor G2/G1 is equal to 0.63 ± 0.04, whereas in the presence of a positive control g_RNA4/l_RNA11 (10 pmol) G2/G1 is equal to 2.0 ± 0.2. 3′-**P1**-modified siRNA was found to be slightly less potent with G2/G1 = 1.54 ± 0.31. As expected, "caging" the 5′-terminus of the guide strand (g_RNA5/l_RNA11) abandons the activity completely: G2/G1 = 0.59 ± 0.15. In comparison, the corresponding "caged" siRNA g_RNA5/l_RNA10 is only slightly more active in the dark: G2/G1 = 0.75 ± 0.03. Exposure of g_RNA5/l_RNA10 to red light (650 nm, 0.23 W, 2 min) leads to significant enhancement of its inhibitory activity up to G2/G1 = 1.47 ± 0.30. As expected, the activity of the control siRNAs was independent of the light exposure (Figure 6B,C).

In summary, we developed modular "caged" siRNAs that can be potentially activated by light of any selected wavelength. For example, we demonstrated that green and red light can act as efficient triggers of the activity of "caged" siRNAs. Red-light-sensitive siRNAs were applied to photocontrol expression of two representative genes (exogeneous: AcGFP and endogeneous: KIF11). In the process of uncaging

  

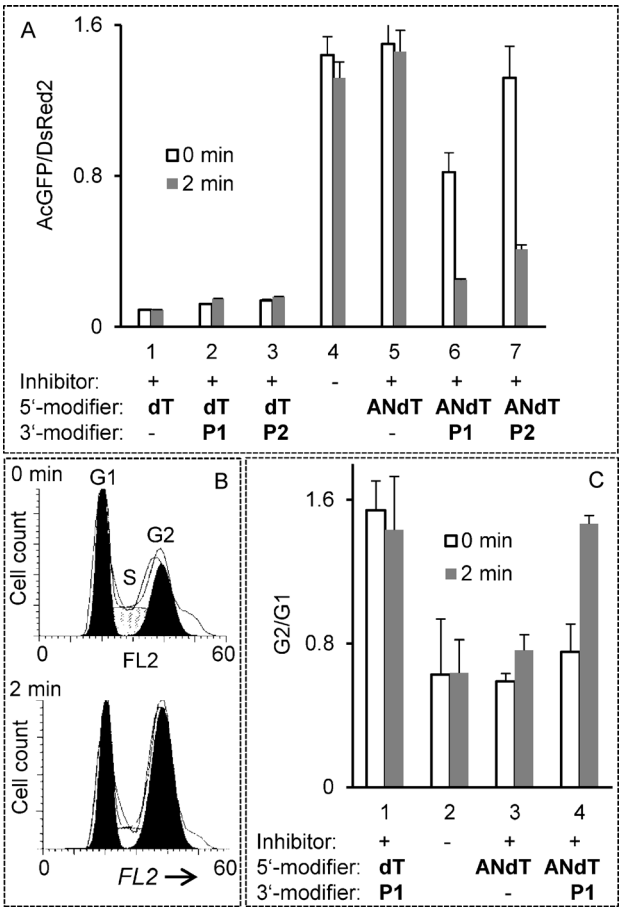


***Figure 6.*** A) HeLa cells expressing AcGFP and DsRed2 and loaded with siRNAs targeting AcGFP (10 pmol) were either left in the dark (open bars) or exposed to 650 nm light (0.23 W) for 2 min. After 24 h, the fluorescence ($\lambda_{ex}$ = 488 nm) of AcGFP ($\lambda_{em}$ = 495–555 nm) and DsRed2 ($\lambda_{em}$ = 557–609 nm) was quantified by flow cytometry and their ratios (AcGFP/DsRed2) were determined. B) Cell cycle analysis for HeLa cells treated with g_RNA5/l_RNA10 (10 pmol) and kept in the dark (0 min) or exposed to 650 nm light for 2 min (2 min). C) Ratios of numbers of cells in G2 and G1 phases: G2/G1. Data for irradiated cells are shown as gray bars, those for non-irradiated ones as open bars.

the photocatalyst is quickly bleached. Therefore, it does not produce toxic $^1O_2$ after the activation of the siRNAs is complete. Moreover, the 9-anthracenyl moiety, used as a blocker of siRNA-phosphorylation, is cleaved in a process, which does not require any additional electrons, in contrast to the previously used 9,10-dialkoxyanthracene-based fragment. Due to these favorable properties uncaging of the reported siRNAs does not produce any toxic response (Figure S18). This is the first molecular system, which can be activated by non-toxic and low-power (0.23 W) light at a wavelength longer than 600 nm in live cells. Apart from being modular and tunable, our "caged" siRNAs can be easily prepared from cheap and accessible starting materials and are brought into cells by the standard transfection reagent (Lipofectamine 2000). Therefore, these siRNAs are expected to be well accessible to the broad community of researchers working on biological problems.

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