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Red light-controlled polymerase chain reaction†

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A 23-mer DNA "caged" at its 3′-terminus with a 9-anthracenyl moiety was prepared. It can be uncaged in the presence of photosensitizer (In(pyropheophorbide-*a*)chloride)-containing DNAs (9–12 mers) and upon irradiation with red light. This mixture of DNAs was used to design red-light controlled polymerase chain reaction.

Polymerase chain reaction (PCR) is highly sequence specific and sensitive. It is routinely used in biological applications for amplification and quantification of DNAs and, in combination with reverse transcription, of RNAs.[1] Moreover, PCR is a key process in nucleic acid sequencing, cDNA cloning, detection of single nucleotide polymorphism (SNP)[2] and in nanotechnology.[3]

The development of light-sensitive PCR can further broaden the scope of its applications. For example, Dmochowski and co-workers[4] and later on Deiters,[5] Kuzuya and Komiyama with colleagues[6,7] have achieved the photochemical control of PCR by using switchable primers containing caged nucleotide units. Upon irradiation of these compounds with UV light, they release activated primers, which support PCR. Furthermore, Turro and Ju with co-workers have used 3′-caged nucleoside-5-triphosphates as photoresponsive substrates for PCR in light-mediated pyrosequencing. In this system, the 3′-OH groups could be deprotected by irradiation with 355 nm-light.[8] Finally, Deiters and co-workers have developed "caged" Taq polymerase by introducing a 365 nm-light-responsive tyrosine derivative into the enzyme.[9] In all these approaches 2-nitrobenzyl-based caging moieties were applied, which are responsive to UV-light. The latter trigger can potentially induce undesired side reactions during PCR including *e.g.* modification of nucleic acids.[10] More-over, UV-light is harmful for the human eye and UV-light sources are in general more expensive than visible light sources.

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Recently, groups of Heckel[11] and Klán and Wirz[12] have described a range of protecting groups sensitive to visible light at $\lambda < 528$ nm. However, they have not yet been applied to control PCR.

Herein we report on the strategy for caging of primers at the 3′-OH group for the PCR controlled by low power red light (650 nm, 0.29 W) lacking the above mentioned disadvantages of UV-light.

We tested two approaches (A and B, Fig. 1). In both cases the 3′-OH group of one of the primers was alkylated with a 9-anthracenyl fragment (ON1-AN), whereas another remained unmodified (ON3). In approach A conjugate PS-ON2a (PS = In(pyropheophorbide-*a*)chloride) was designed to bind to the DNA target in proximity to the modified primer ON1-AN as is shown in Fig. 1. Upon irradiation of this construct with red light, which is absorbed by the PS, singlet oxygen ($^1O_2$) is generated in proximity to the 9-anthracenyl moiety (AN). The $^1O_2$ reacts with the AN inducing its cleavage and release of functional primer ON1. The rate of the reaction shown in inset A (Fig. 1) is dependent upon the concentration of the nucleic acid target.[13] In cases when the target is present in small quantities, the uncaging is expected to be incomplete. In contrast, in the second approach B, PS-ON2b was designed to
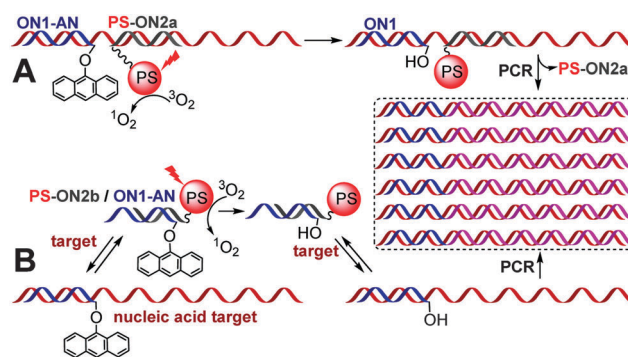

Fig. 1 Two designs (A and B) of red-light activated primers for polymerase chain reaction (PCR). PS = photosensitizer, primer: ON1, "caged" primer: ON1-AN, and amplified nucleic acid: nucleic acid target.

bind to ON1-AN. Here the uncaging reaction is expected to be practically independent of the target concentration providing that the concentration of primers is substantially higher than that of the target.

We have used earlier an AN for "caging" siRNAs.[14] However, in this known case a primary alcohol group (5′-OH) was protected. In contrast, in ON1-AN the AN fragment protects a secondary alcohol group (3′-OH). To test if "uncaging" can in this case also be cleanly induced by $^1O_2$ we prepared and studied the reactivity of a model compound, **3** (Fig. 2).

In particular, in the first step compound **1**[15] was alkylated in the presence of anthrone and NaH (step a), which was followed by cleavage of the 4,4′-dimethoxytrityl (DMT) group under acidic conditions (step b). We observed that the absorbance and emission intensity characteristics for an anthracene fluorophore (Fig. S7 and S8, ESI†) are decreased upon irradiation of the **3**/In(pyropheophorbide-*a*)Cl[16] (0.1 eq., PS) mixture with red light (650 nm, 0.29 W, Fig. S7 and S8, ESI†). By using ^1H NMR spectroscopy we confirmed that under these conditions **3** is converted to anthraquinone **6** and thymidine: 65% conversion upon irradiation of **3** (5 mM in CDCl$_3$), PS (0.1 eq.), and CF$_3$CO$_2$H (1%) mixture for 30 min (Fig. S9, ESI†). In the absence of the acid the reactivity of **3** was reduced by 1.3 fold, which may indicate the involvement of H$^+$ as a catalyst in the conversion of intermediate **5** to **6** (Fig. 2B). Under the competitive conditions the initial rate of uncaging of compound **3** is 1.7 times faster than that of previously reported 5′-*O*-(anthracenyl)-thymidine **7** (Fig. S10 and S11, ESI†).[14]

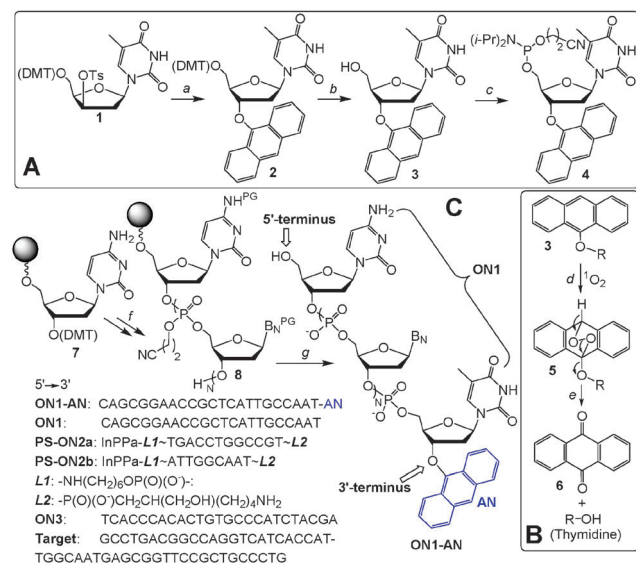Next, **3** was converted to phosphoramidite **4** (Fig. 2A). The latter compound was coupled to the controlled-pore-glass (CPG)-bound ON1 oligonucleotide, which was assembled by using commercially available phosphoramidites for reverse (5′ → 3′) DNA synthesis as outlined in Fig. 2. Final cleavage and deprotection of this conjugate was followed by its HPLC purification and MALDI-TOF mass spectrometric identification.† PS-modified conjugates PS-ON2a and PS-ON2b were prepared as previously described.[14] All conjugates used in this work were >90% pure according to analytical HPLC (Fig. S12 and S13).†

In tests of photoresponsive primers we used a synthetically prepared 50-mer DNA as a target (Fig. 2). The sequence of this DNA matches a part of the sequence of β-actin–mRNA. At 22 °C formation of a stable assembly ON1-AN/target/PS-ON2a was expected in the buffered aqueous solution (pH 7.4) of ON1-AN, PS-ON2a and the target (design A, Fig. 1). This suggestion is based on high UV-melting points of duplexes ON1/target (T_m = 73.9 ± 0.6 °C) and target/PS-ON2a (T_m = 62 ± 1 °C) under these conditions. We observed that irradiation of ON1-AN/target/PS-ON2a with red light (650 nm, 0.29 W) for 10 min leads to conversion of 73% of ON1-AN into the uncaged primer ON1 (Fig. 3A). Further irradiation does not improve the reaction yield substantially, since the photocatalyst PS-ON2a is bleached simultaneously with the uncaging. A similar effect has been observed earlier during uncaging of red light activated siRNAs.[14] In contrast to design A, in design B the photosensitizer-containing conjugate PS-ON2b was selected to be complementary to ON1 rather than to the template. Since ON2b is rather short (9-mer), the ON1-AN/PS-ON2b duplex was expected to be only partially formed at 22 °C (T_m[ON1/PS-ON2b] = 28 ± 1 °C) thereby enabling quick strand exchange between the duplex and the target (Fig. 1B). Despite the low stability of the duplex, the residing time of the PS in proximity to the AN seemed to be sufficient to induce efficient uncaging of the latter fragment upon irradiation with red light. In particular, even after 5 min of irradiation of the ON1-AN/PS-ON2b 89% of ON1-AN was found to be converted to ON1 (Fig. 3B). These data indicate that both designs A and B are suitable for primer caging.

Finally, we explored the applicability of "caged" primer ON1-AN in PCR. In the control experiment, a 295-nucleotide-long stretch of cDNA, which was obtained by reverse transcription (RT) from β-actin mRNA present in the total RNA isolated from HeLa cells (15–150 ng), was amplified by using ON1 and ON3

Fig. 2 (A) Synthesis of phosphoramidite **4**: (a) anthrone, NaH, and DMSO; (b) CCl$_3$CO$_2$H and CH$_3$CN; and (c) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, (i-Pr)$_2$NEt, and CH$_2$Cl$_2$. (B) The mechanism of the reaction of **3** with $^1O_2$ (steps d–e). (C) A list of conjugates described in this communication and an outline of the synthesis of ON1-AN: (f) reverse DNA synthesis; (g) (1) **4** and 1*H*-tetrazole, (2) Ac$_2$O, pyridine, and *N*-methylimidazole, (3) I$_2$, pyridine, H$_2$O, and THF, and (4) CCl$_3$CO$_2$H and CH$_2$Cl$_2$.
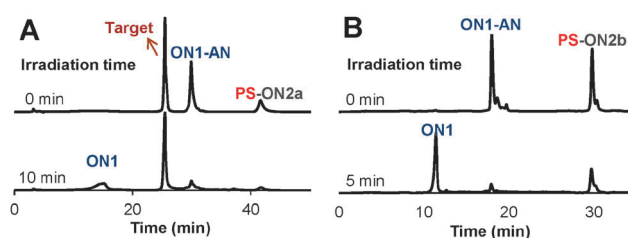

Fig. 3 (A) HPLC traces (monitored by detecting absorbance at 260 nm) of a mixture of ON1-AN (25 μM), PS-ON2a (1 eq.) and target DNA (1 eq.) in annealing solution (pH 7.4, HEPES 6 mM, KOH 5 mM, KCl 20 mM, and MgCl$_2$ 0.4 mM) obtained before and after its 10 min-long irradiation with red light (LED array, 650 nm, 0.29 W). (B) HPLC traces of a mixture of ON1-AN (20 μM) and PS-ON2b (1 eq.) in annealing solution obtained before and after its 5 min-long irradiation with red light.
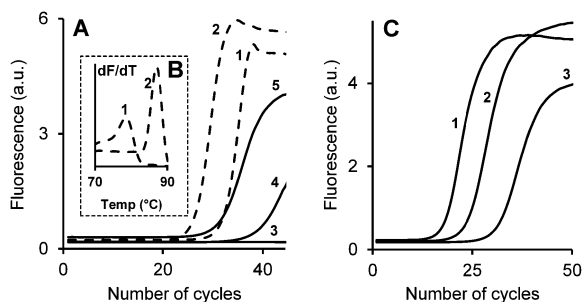
Fig. 4 (A) Amplification of cDNA derived from β-actin–mRNA by using (RT)-PCR; SYBR Green I fluorescence (λ_{em} = 521 nm, λ_{ex} = 494 nm) was monitored: (1) ON1 (1 eq.), PS-ON2a (1 eq.), ON3 (1 eq., C_p = 29); (2) the same as (1) except that total RNA (15 ng) was added (C_p = 25); (3) ON1-AN (1 eq.), PS-ON2a (1 eq.), ON3 (1 eq.), and total RNA (150 ng, no amplification detected), the same result was obtained with 15 ng of total RNA; (4) the same as (3) except that total RNA (15 ng) was added and the mixture was irradiated for 10 min with an LED light source (650 nm, 0.29 W) in the annealing buffer (C_p = 40); (5) the same as (4) except that more total RNA (150 ng) was added (C_p = 31). (B) Fluorescence melting profiles (dF/dT versus T) of products obtained in reactions (1) and (2) from (A). (C) RT-PCR of β-actin–cDNA: (1) ON1 (1 eq.), PS-ON2b (2 eq.), ON3 (1 eq.), total RNA (150 ng, C_p = 17); (2) ON1-AN (1 eq.); PS-ON2b (2 eq.), ON3 (1 eq.); total RNA (150 ng) and 10 min irradiation in the annealing buffer before the reverse transcription (RT) (C_p = 23); (3) the same as (2) except that the irradiation was conducted in the master mix (C_p = 31).

as primers, both in the absence and presence of PS-containing conjugates PS-ON2a (design A) and PS-ON2b (B). We observed that the amplification was practically unaffected by the latter compounds (Fig. S16 and S17, ESI†). Moreover, both in the absence (trace 1, C_p = 29) and presence of the target (present in 15 ng of total RNA, trace 2, C_p = 25) amplification of nucleic acids occurred (Fig. 4A). Melting point analysis of the resulting mixtures (Fig. 4B) allowed us to conclude that in the absence of RNA the so called primer–dimer product was formed, which melts at a substantially lower temperature (T_m = 78 ± 1 °C) than the desired cDNA duplex (T_m = 87 ± 2 °C). Formation of primer–dimers is an often occurring problem in the analysis of samples containing low concentrations of nucleic acids. It can be solved by optimization of primer sequences, conditions of PCR or using hot start PCR. All of these solutions are time consuming and costly. We were pleased to observe that in the presence of "caged" primer ON1-AN, catalyst PS-ON2a and primer ON3 (design A, Fig. 1) no amplification product both in the absence and presence of the target (15 ng and 150 ng of total RNA) was generated in the dark (trace 3, Fig. 4A). In contrast, after irradiation of this mixture with red light for 10 min in the annealing buffer before RT-PCR the amplified product corresponding to the cDNA duplex (T_m = 87 ± 2 °C) was formed in a concentration dependent manner. In particular, in the presence of 15 ng of total RNA the curve with C_p = 40 was observed (trace 4), whereas in the presence of 150 ng of total RNA that with C_p = 31 was observed (trace 5). It should be mentioned that the C_p values obtained for uncaged primers are substantially smaller than those for the unmodified ones: C_p = 40 versus 25 for 15 ng of RNA and C_p = 31 versus 14 for 150 ng of RNA. These data indicate that the uncaging process in this case was not complete. Analogous

effects were observed in the previously reported PCR-system controlled by UV-light.[5] As discussed earlier the photochemical reaction outlined in design A is stoichiometric with respect to the template (Fig. 1 and 3).[13] Since the template (cDNA) concentration is substantially lower than that of the primers in the mixtures for PCR, only a small portion of the primer can be uncaged. To solve this problem, we used primers, whose uncaging is not [cDNA]-dependent (design B, Fig. 1). In particular, we observed that the amplification of cDNA in the mixture of ON1-AN, PS-ON2b, ON3 and the target (C_p = 23) after 10 min of irradiation was substantially (ΔC_p = 8) more efficient than that observed for the previous system under the same conditions (trace 2, Fig. 4C). Therefore, we conclude that design B is substantially better suitable for the red light-dependent PCR than design A. For practical reasons it is preferable to prepare a complete mixture containing all components required for the PCR and then start the reaction by its irradiation with red light. We observed that in this case the efficiency of the amplification is reduced from C_p = 23 to 31 (ΔC_p = +8, trace 3, Fig. 4C) with respect to the case when primers are first uncaged and then the master mix is added (trace 2). These data may indicate that components of the master mix inhibit to some extent the uncaging. Further studies are required to better understand and ultimately alleviate this effect.

In summary, we developed for the first time "caged" primers for PCR, which can be efficiently activated by red light (650 nm). These reagents do not generate primer–dimer side products and exhibit target amplification after uncaging, which depends upon the concentration of the target present in the mixture.

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