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'Caged' peptide nucleic acids activated by red light in a singlet oxygen mediated process

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ABSTRACT

Common 'caged' nucleic acid binders, which can be applied for temporal and spatial control of gene expression, are activated by high energy light (<450 nm). The light of this type is damaging to cells and is strongly absorbed by cellular components. Therefore, shifting the triggering light to the visible region (>550 nm) is highly desirable. Herein we report on a cyclic peptide nucleic acid (PNA), whose backbone contains a 9,10-dialkoxy-substituted anthracene linker. The sequence of this compound was selected to be complementary to a representative microRNA (miR-92). We demonstrated that the cyclic PNA does not bind complementary nucleic acids and is, correspondingly, 'caged'. Its uncaging can be conducted by its exposure to red light (635 nm) in the presence of pyropheophorbide-a. The latter process is mediated by singlet oxygen ($^1O_2$), which cleaves the 9,10-dialcoxyanthracene linker within the PNA with formation of a linear PNA, an efficient binder of the complementary ribonucleic acid. This is the first example of a red light-activated, 'caged' peptide nucleic acid.

Gene expression can be inhibited by oligonucleotides or their chemically modified analogues via antisense mechanism[1] or by small interfering RNA duplexes (siRNA) via RNAi mechanism.[2] In both cases messenger RNA (mRNA) of a gene of interest is targeted. Antisense reagents and siRNAs were found to exhibit comparable activity and specificity.[1b] However, selection of siRNAs for modulation of gene expression in cell cultures can be in most cases conducted in silico, whereas both careful design and experimental pre-tests are usually required to find a corresponding antisense inhibitor. Therefore, siRNAs are nowadays more often used in biochemical studies The field of antisense inhibitors has been recently revived by the finding that they can be used to suppress activity of micro RNAs (miRNAs).[3] miRNAs are 20–24 nucleotide long duplexes, which participate in post-transcriptional control of gene expression and are responsible for regulation of numerous cellular events, such as apoptosis, proliferation or differentiation.[4] Their activity relies on hybridization of miRNA with mRNAs that is accompanied by inhibition of expression of the corresponding gene. It has been demonstrated that miRNAs can be inhibited by 2′-*O*-methyl (2′-OMe) and 2′-*O*-methoxyethyl (2′-MOE) RNAs, morpholino ODNs, peptide nucleic acids (PNAs) and locked nucleic acids (LNAs) as well as their hybrids with ODNs.[4b]

To obtain temporally and spatially resolved information on biological processes light-responsive ('caged') probes are necessary. Both 'caged' antisense inhibitors (including e.g., DNAs, 2′-OMe RNAs and PNAs) and siRNAs activated by UV-light are known.[5] However, since UV light is rather toxic to cells,[6] it would be desirable to develop reagents responsive to light of longer wavelength, ideally red light. Apart from being non-toxic, red light is also less absorbed by the biological material. Therefore, it can penetrate through tissue substantially deeper. In 2007 we have reported on 'caged' DNA-hairpins, whose hybridization to nucleic acids is controlled by >630 nm light.[7] The loop in these hairpins contains a ~SCH=CHS~ linker. Upon their exposure to red light in the presence of pyropheophorbide-a or another red light absorbing photosensitizer singlet oxygen ($^1O_2$) is formed. Its following reaction with the linker leads to cleavage of the latter and release of a single stranded DNA, which can bind nucleic acid targets. We have applied the related idea to prepare 'caged' phosphorothioate DNAs.[8] Moreover, we have shown that substitution of the ~SCH=CHS~ linker for 9,10-dialkoxy-anthracene improves dramatically the photoactivation reaction.[9] Unfortunately, the secondary structure of 'caged' hairpins is destabilized in the presence of transfection reagents, which are required to bring these reagents into cells. Therefore, these reagents cause light-independent uncaging of the hairpins.[10] The possible solution of this problem would be to explore methods of caging of inhibitors, which are not based on formation of non-covalent duplex structures.

In our earlier studies we have demonstrated that PNA deactivation towards hybridization can be conducted by its cyclization. The cyclic PNA can be activated back by cleaving its backbone at one specific position. Based on this principle we have designed cyclic,

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Cu^{2+}-controlled PNAs.[11] The activity of these molecules relies only on their primary covalent structure, therefore, they are potentially compatible with transfection reagents. UV-light sensitive, cyclic morpholino oligomers, where the same principle has been applied, have been reported later on by the group of Tang.[12] Inspired by these findings, we designed red light-responsive, cyclic PNAs, which are complementary to a non-coding micro RNA hsa-miR-92 (further miR-92, Fig. 1). The latter biomolecule is a member of oncogenic miR-17-92 cluster, which is expressed endogenously in a number of cancer cells, including, for example, grandular cancer (HeLa), leukemia and hepatocellular carcinoma, but is practically undetectable in at least some normal cells, for example, normal blasts.[13] Therefore, 'caged' inhibitors targeting miR-92 can potentially be used in cancer research. According to results of our previous study on cyclic, Cu^{2+}-dependent peptide nucleic acids,[11] short linkers between N- and C-termini of PNA preclude binding of these compounds to complementary nucleic acids. Based on these earlier findings, we prepared two cyclic PNAs (c_PNA12, c_PNA14) with a shortest possible linker, which consists of a chain of 15 atoms between N- and C-termini of PNA (red colored in Fig. 2) and includes our most reactive $^1O_2$-sensitive fragment: 9,10-dialkoxy-substituted anthracene (AN, Fig. 2).

Synthesis of the cyclic PNAs was started from preparation of required building blocks **2** and its mono-allylated analogue **3** (Fig. 2A). In the first step 9,10-anthraquinone was reduced by sodium dithionite and alkylated in situ with *t*-butyl bromoacetate in the immiscible mixture of water and CH_2Cl_2 in the presence of a phase transfer catalyst Adogen 464. The resulting *t*-butyl protected **2** was treated with trifluoroacetic acid to obtain acid **2** with 55% yield over two steps. Then compound **2** was reacted with 1 equiv allyl alcohol in the presence of activator *N*,*N*-1-ethyl-3-dimethylaminopropylcarbodiimide (EDC). The resulting mixture containing starting materials as well as mono- and bis-allyl esters of **2** was separated by column chromatography on silica gel to obtain pure **3** in 21% yield. The latter compound was prepared to avoid possible side reactions of oligomerization, which could potentially occur at the conditions of coupling of unprotected diacid **2** to PNA. The synthesis of the PNA started from the attachment of Fmoc-Lys(Boc)-OH amino acids to TGR-resin containing a Rink linker (Merck, 2 μmol of amino groups on the resin surface) in the presence of activator 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU). Then, the α-amino group was deprotected in the presence of piperidine. Using the same reaction sequence of coupling and deprotection another Fmoc-Lys(Boc)-OH and Fmoc-Lys(Alloc)-OH were attached to the solid support. Next, the α-amino group of the terminal aminoacid was deprotected and acylated with acetic acid anhydride. We introduced this last amino acid to be able to attach photosensitizers or fluorophores (in place of the acetyl group) to the 'caged' PNAs in future. In the next reaction steps, the ε-N-Alloc was cleaved in the presence of $Pd(PPh_3)_4$ complex and (α-N-Fmoc, γ-N-Alloc)-protected diaminobutyric acid was coupled and deprotected with piperidine. The following step included compilation of the PNA strand (either 12-mer or 14-mer) on the available α-amino-group of the 2,4-diaminobutyric acid residue using Fmoc and Bhoc-protected PNA building blocks (PNA **5**, Fig. 2). The solid support obtained was split into two equal portions. The first one was deprotected with trifluoroacetic acid/*m*-cresol mixture to obtain linear PNA 12-mer (PNA12) and 14-mer (PNA14), whereas the second one was further modified. In particular, the N-terminal amino group of the PNA was acylated with either **2** or **3** in the presence of HBTU to obtain PNAs **6a** and **6b** correspondingly. We observed that both **2** and **3** are coupled with the same efficiency indicating that the mono-protection of the di-acid **2** does not bring any advantages. Therefore, further synthesis was conducted with the product of coupling of **2** (Fig. 2A). In the next step, γ-N-Alloc of the C-terminal diaminobutyric acid residue was cleaved in the presence of $Pd(PPh_3)_4$ complex and the cyclization was induced by adding diisopropycarbodiimide (DIC), N-hydroxybenzotriazole (HOBT) and 4-dimethylaminopyridine (DMAP) and incubating the resulting suspension for 12 h. The compound obtained was deprotected and cleaved from the solid support by using trifluoroacetic acid/*m*-cresol mixture and, finally, purified by HPLC to obtain c_PNA12 and c_PNA14 (Fig. 3). New PNA conjugates were identified by MALDI-TOF mass spectrometry. Their purity was found to be better than 90% according to HPLC analysis. An HPLC trace of a representative, analytically pure conjugate c_PNA12 is shown in Figure 3A. HPLC traces and MALDI-TOF mass spectra of all new conjugates discussed in this manuscript are given in the Supplementary data (Figs. S1–S8).

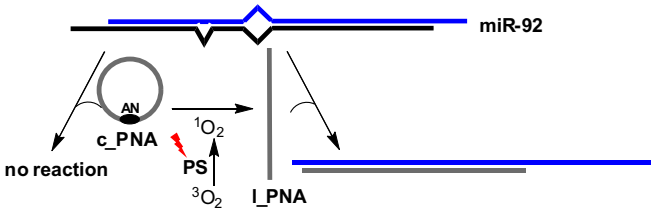

Figure 1. Red-light induced activation of cyclic PNA (c_PNA, grey cycle in the figure) with formation of linear PNA (l_PNA, grey stick). l_PNA is capable of binding the leading strand (blue colored stick) of the miR-92 duplex; c_PNA contains a $^1O_2$ sensitive linker (black ellipse labeled with AN) in the backbone; PS is a red-light absorbing photosensitizer (pyropheophorbide-a was used in this work); few mismatched base pairs are present in natural miR-92 duplex as it is indicated in this figure.

Earlier we have demonstrated that 9,10-dialkoxyanthracenes, which are introduced within the DNA backbone or at its termini, are efficiently cleaved in the presence of photogenerated singlet oxygen.[9] Therefore, it was sensible to assume that such a reactivity will be also retained when 9,10-dialkoxyanthracene will inserted within the PNA structure. To confirm this assumption experimentally, photocleavage of c_PNA12 (2 μM) in the presence of pyropheophorbide-a (10 equiv) and upon exposure to red light (635 nm) was studied by using MALDI-TOF mass spectrometry (Fig. 3). It has been earlier shown that this method can be used for semi-quantitative monitoring chemical reactions of oligonucleotides[14] and peptide nucleic acids.[11,15] HPLC was less suitable in this case, since the retention time of the cyclic PNA (c_PNA12, R_t = 24.6 min) was very close to that of the linear product (l_PNA12, R_t = 24.4 min). We found that c_PNA12 (observed *m*/*z* 4117.4, calculated 4116.8) is converted into a single product with *m*/*z* 3941.7, which corresponds to a loss of 176.1 mass units from the cyclic PNA (Fig. 3). This mass difference corresponds to a loss of an anthracene fragment (−178.1 Da) and protonation of two alcohol groups left (+2 Da). Therefore, the product formed could be identified as a linear PNA (l_PNA12, Fig. 2). If the reaction mixture is allowed to stand in the dark for 45 min, c_PNA12 remains intact (Fig. 3B), thereby confirming that the uncaging reaction is induced by red light. HPLC profile of the irradiated for 30 min c_PNA12/pyropheophorbide-a (1/10, [c_PNA12] = 8 μM) mixture contains a single peptide nucleic acid peak with the retention time of 24.4 min (data not shown). The product eluted at this time was analyzed by MALDI-TOF spectrometry and it was confirmed that it corresponds to l_PNA12. In the HPLC of the same, but non-irradiated mixture a peak with the retention time of 24.6 min was observed. According to the mass spectrometric analysis, it corresponds to the intact cyclic PNA. Thus, both MALDI-TOF mass spectrometric and HPLC data are in agreement with the clean linearization of c_PNA12 upon its exposure to red light in the presence of the photosensitizer.