Bioconjugate Chemistry

Article

pubs.acs.org/bc

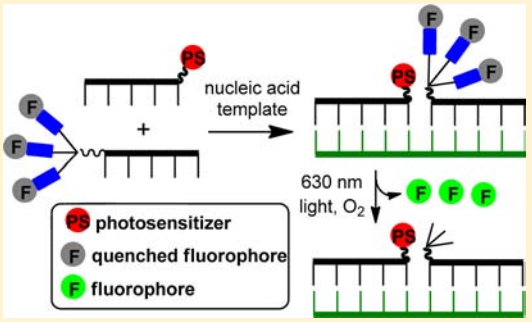
Fluorogenic, Catalytic, Photochemical Reaction for Amplified Detection of Nucleic Acids

Subrata Dutta, Annabelle Fülöp, and Andriy Mokhir*

Institute of Organic Chemistry II, Friedrich-Alexander-Universität Erlangen-Nürnberg, Henkestr. 42, 91054 Erlangen, Germany

Supporting Information

ABSTRACT: Photochemical, nucleic acid-induced reactions, which are controlled by nontoxic red light, are well-suited for detection of nucleic acids in live cells, since they do not require any additives and can be spatially and temporally regulated. We have recently described the first reaction of this type, in which a phenylselenyl derivative of thymidine (5′-PhSeT-ODNa) is cleaved in the presence of singlet oxygen (Fülöp, A., Peng, X., Greenberg, M. M., Mokhir, A. (2010) A nucleic acid directed, red light-induced chemical reaction. *Chem. Commun. 46*, 5659−5661). The latter reagent is produced upon exposure of a photosensitizer 3′-PS-ODNb (PS = Indium(III)-pyropheophorbide-a-chloride: InPPa) to >630 nm light. In 2012 we reported on a fluorogenic version of this reaction (Dutta, S., Flottmann, B., Heilemann, M., Mokhir, A. (2012) Hybridization and reaction-based, fluorogenic nucleic acid probes. *Chem. Commun. 47*, 9664−9666), which is potentially applicable for the detection of nucleic acids in cells. Unfortunately, its yield does not exceed 25% and no catalytic turnover could be observed in the presence of substrate excess. This problem occurs due to the efficient, competing oxidation of the substrate containing an electron rich carbon−carbon double bonds (SCH═CHS) in the presence of singlet oxygen with formation of a noncleavable product (SCH═CHSO). Herein we describe a related, but substantially improved photochemical, catalytic transformation of a fluorogenic, organic substrate, which consists of 9,10-dialkoxyanthracene linked to fluorescein, with formation of a bright fluorescent dye. In highly dilute solution this reaction occurs only in the presence of a nucleic acid template. We developed three types of such a reaction and demonstrated that they are high yielding and generate over 7.7 catalytic turnovers, are sensitive to single mismatches in nucleic acid targets, and can be applied for determination of both the amount of nucleic acids and potentially their localization.



INTRODUCTION

Templated chemical reactions have been explored for detection of nucleic acids in vitro and in live cells, in nanotechnology, organic synthesis, for discovery of new chemical reactions,[1−4] and in studies of origin of life.[5,6] They occur with either formation (ligation) or cleavage of a chemical bond. In most of these reactions, reacting groups X and Y are placed on the 5′-terminus of one oligonucleotide (ODN) probe (5′-X-ODNa) and the 3′-terminus of another one (3′-Y-ODNb). In place of ODN's one can also use their chemical analogues, e.g., peptide nucleic acids (PNAs). The probes bind to the neighboring sequences of the template nucleic acid, which are complementary to ODNa and ODNb, that brings the reacting groups X and Y in proximity to each other thereby facilitating the interaction between them. In related templated reactions Y is selected to be a catalyst and X its substrate. For example, we have developed a nucleic acid templated ester hydrolysis, which is catalyzed by Cu^{2+}-complexes,[7−9] and $^1O_2$-mediated cleavage of organic substrates, which is catalyzed by metal complexes of pyropheophorbide-a (MPPa, M = In^{3+}, Pd^{2+}, Zn^{2+}).[10−13]

Photochemical, templated reactions are especially well suited for detection of nucleic acids in live cells, since they do not require any additives and the light dose and the illumination area can be easily controlled allowing spatial and temporal control of the reactions. Though UV-light-induced, templated, photochemical processes are most common,[14−17] their application in live cells and in vivo is problematic, since UV-light induces nucleobase transformations in the genomic DNA and activates intracellular photosensitizers, e.g., flavonoids.[18] To avoid the damage of biomolecules, application of visible light in place of UV-light is desirable. In 2010 we described the first templated, photocatalytic reaction induced by nontoxic visible light (>630 nm), in which a phenylselenyl derivative of thymidine (5′-PhSeT-ODNa) is cleaved in the presence of $^1O_2$.[11] The latter reagent is produced in the presence of a photosensitizer 3′-PS-ODNb (where PS is InPPa). We observed that 4.5 catalytic turnovers could be generated in this process in the presence of a complementary nucleic acid target. Unfortunately, the PhSe cleavage cannot be directly applied for detection of nucleic acids in cells, since neither the substrate nor the product formed are fluorescent. Substitution of the PhSeT for a substrate L-F, where L is a $^1O_2$-sensitive

Received: March 26, 2013
Revised: August 5, 2013
Published: August 21, 2013

dx.doi.org/10.1021/bc400152n | *Bioconjugate Chem.* 2013, 24, 1533−1542

linker containing a SCH═CHS moiety[19] and F is a fluorophore (e.g., *N,N,N′,N′*-tetramethylrhodamine or fluorescein), partially solves this problem.[10] In particular, red light-exposure of the mixture of 5′-L-F-ODNa, 3′-InPPa-ODNb, and a template nucleic acid causes release of the free dye F, whose fluorescence quantum yield is 2- to 5-fold higher than that of the same dye in the intact conjugate 5′-F-L-ODNa. Unfortunately yield of this reaction does not exceed 25% and no catalytic turnover could be observed in the presence of substrate excess. This problem occurs due to the efficient, competing oxidation of SCH═CHS in the presence of $^1O_2$ with formation of the noncleavable S(O)CH═CHS fragment.[10,20,21]

Herein we report on a further improvement of the substrate of this reaction by the replacement of the SCH═CHS-containing moiety for 9,10-alkoxyanthracene. The latter linker was selected based on our previous studies of its $^1O_2$-mediated photocleavage.[22] In contrast to SCH═CHS, it reacts with $^1O_2$ quickly and cleanly and acts as a quencher of the fluorescence of dyes (Figure 1). Using the resulting substrates we designed a

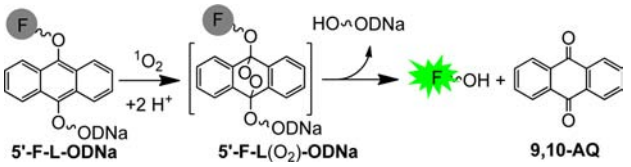


Figure 1. Mechanism of $^1O_2$-mediated cleavage of 9,10-dialkoxyanthracene linker in ODN_L-F conjugate, which occurs with release of a fluorescent dye F.[22]

fluorogenic reaction templated by nucleic acids, which generates over 7.7 catalytic turnovers and produces stronger template-induced fluorescence enhancement. We also designed reactions, which are suitable either for ratiometric nucleic acid detection or for determination of localization of nucleic acids. No red light dependent, templated reaction, which is fluorogenic and able to generate catalytic turnover, has been reported before this study. In particular, photochemical reactions reported by our group earlier either are not fluorogenic[11] or do not exhibit catalytic turnover.[10] In 2011 the group of D. R. Liu has described a Ru(bipy)$_3$ complex-catalyzed azide photoreduction, which is compatible with aqueous buffers.[23] Based on these findings N. Winssinger and co-workers have developed the templated reaction of this type, which is controlled by 455 nm light and which generates a fluorescent product catalytically.[24] Potentially, this reaction can be controlled by >600 nm light, providing that a red-light absorbing Ru-complex is used as a catalyst. However, this remains to be confirmed experimentally.

■ EXPERIMENTAL PROCEDURES

Commercially available chemicals of the best quality from Aldrich/Sigma/Fluka (Germany) were obtained and used without purification. Unmodified DNAs and ODN1_FAM conjugate (Scheme 1) were purchased from Iba GmbH (Germany). DNA synthesis was conducted on a K&A H-8 DNA/RNA synthesizer. MALDI-TOF mass spectra were recorded on a Bruker MicroTOF mass spectrometer. The matrix mixture (2:1 v/v) was prepared from 6-aza-2-thiothymine (ATT, saturated solution in acetonitrile) and diammonium citrate (0.1 M in water). Samples for mass spectrometry were prepared by the dried-droplet method by using a 1:2 probe/matrix ratio. Mass accuracy with external calibration was 0.1% of the peak mass, that is, + 7.0 at *m/z* 7000. HPLC was performed at 22 °C on a Shimadzu liquid chromatograph equipped with a UV detector and either a Macherey−Nagel Nucleosil C4 250 × 4.6 mm column or a Macherey−Nagel Nucleosil C18 250 × 4.6 mm. Gradient of solution B (CH_3CN) in solution A (0.1 M aqueous (NEt_3H)(OAc). In cases of hydrophobic conjugates 5% CH_3CN in 0.1 M aqueous (NEt_3H)(OAc) was used as solution A. UV/vis spectra were measured on a Varian Cary 100 Bio UV/vis spectrophotometer by using 1 cm optical path black-wall absorption semimicrocuvettes (Hellma GmbH, Germany) with a sample volume of 0.7 mL. These cuvettes were also used for photochemical experiments. Fluorescence spectra were acquired on a Varian Cary Eclipse fluorescence spectrophotometer by using fluorescence cuvettes (Hellma GmbH, Germany) with a sample volume of 1 mL.

Synthesis. Conjugates were synthesized on the Bz-A-SynBase CPG 1000/110 solid support (Link Technologies, UK) using a DNA/RNA synthesizer and commercially available phosphoramidites from Link Technologies, UK. In particular, for assembly of oligonucleotide strands dmf-dG-CE-, Bz-dA-CE-, Bz-dC-CE-, and dT-CEphosphoramitites were applied. Fluorophores were coupled to 5′-termini of ODNs using 5′-fluorescein-CE (6-FAM) or 5′-tetrachloro-fluorescein-CE (TET). 5′-Terminal OH-groups of ODNs were converted into NH_2-groups with help of 5′-amino modifier C6 CE. Coupling of all these reagents was conducted in accordance with recommendations of the manufacturer. Linker L (Scheme 1) was coupled using 9-(3-DMT-oxypropyl)-10-(3-oxypropyl)anthracene [(cyanoethyl)(*N,N*-diisopropyl)]-phosphoramidite as described earlier. Oxidation of P(III) derivatives (I_2, pyridine) and removal of 4,4′-dimethoxytrityl group (CCl_3CO_2H) were conducted at standard conditions usually applied in DNA synthesis. For cleavage of the conjugates from the solid support and their deprotection they were treated with aqueous ammonia solution (25%) for 8 h at 22 °C. Then, the CPG was filtered off and the filtrate was evaporated to dryness. Water (150 μL) was added to the crude conjugates and the solutions obtained were purified by HPLC using a gradient of solution B (CH_3CN) either in solution A (0.1 M aqueous (NEt_3H)(OAc)) or in solution A* (0.1 M aqueous (NEt_3H)(OAc) with 5% CH_3CN). The fractions containing pure conjugates were combined, lyophilized, redissolved in water, and analyzed by HPLC (puritiy control) and MALDI-TOF mass spectrometry (identification, Supporting Information).

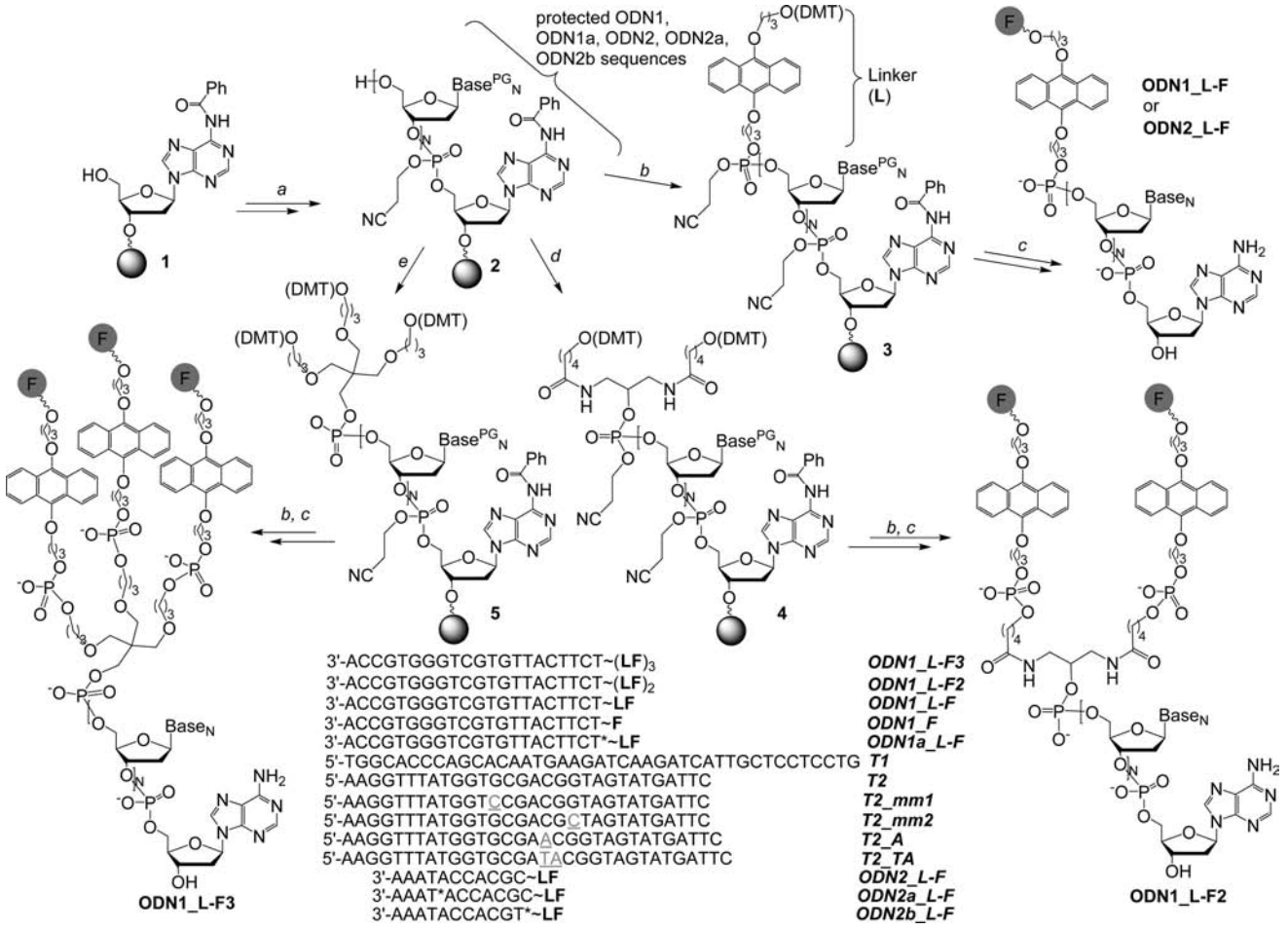
ODN1_L-FAM. Yield: 71%, HPLC (Nucleosil C18 column; gradient: in 32 min from 0 to 30% solution B in solution A, in 10 min to 90% solution B): R_t = 29.0 min; MALDI-TOF MS, negative mode, calculated for $C_{252}H_{305}N_{72}O_{145}P_{22}$ ([M-H]$^-$): *m/z* 7342, found 7335.

ODN1a_L-FAM. Yield: 4%, HPLC (Nucleosil C18 column; gradient: in 5 min from 0 to 2% solution B in solution A, in 27 min to 40% solution B, in 10 min to 90% solution B): R_t = 28.8 min; MALDI-TOF MS, negative mode, calculated for $C_{285}H_{341}N_{76}O_{149}P_{22}$ ([M-H]$^-$): *m/z* 7893, found 7895.

ODN2_L-FAM. Yield 24%; HPLC (Nucleosil C4 column; gradient: 5 min at 0% solution B in solution A, in 30 min to 50% B, in 10 min to 70% B): R_t = 23.2 min; MALDI-TOF MS, negative mode, calculated for $C_{153}H_{179}N_{45}O_{75}P_{12}$ [M-H]$^-$: *m/z* 4219, found 4220.

dx.doi.org/10.1021/bc400152n | *Bioconjugate Chem.* 2013, 24, 1533−1542

Scheme 1. Synthesis of Probes I (Figure 1) and Sequences of DNA Templates Used in This Study



Base is a residue containing a nucleobase: adenine, guanine, cytosine, and thymine. PG (in compounds **2**, **3**, **4**, and **5**) are protecting groups of nucleobases: N-benzoyl for adenine and cytosine, N-dimethylformamidine for guanine. DMT is a dimethoxytrityl group. F's are fragments containing fluorescent dyes: (5-carboxyfluorescein (FAM), 6-carboxy-2′,4,7,7′-tetrachlorofluorescein (TET), 6-carboxy-*N,N,N′,N′*-tetramethylrhodamine (TAMRA), Alexa Fluor350, Alexa Fluor647, and eosin. ODNs are oligo-2′-deoxyribonucleotides. T* is TAMRA-dT. Mismatched nucleobases and bulges in templates T2_mm1, T2_mm2, T2_A, and T2_TA are underscored and indicated with red color. Synthesis conditions: (a) solid phase DNA synthesis; (b) 9-(3-dimethoxytrityloxypropyl)-10-(3-oxypropyl)anthracene [(cyanoethyl)(*N,N*-diisopropyl)]-phosphoramidite, 1*H*-tetrazole; (c) (1) 1% CCl_3CO_2H, CH_2Cl_2; for conjugation of FAM and TET: (2) 1*H*-tetrazole and either 5′-fluorescein-CE phosphoramidite or 5′-tetrachloro-fluorescein-CE phosphoramidite (Link Technologies, UK); for conjugation of Alexa Fluor350, TAMRA, Alexa Fluor647 and eosin: (2) 5′-MMT-amino modifier-CE phosphoramidite, 1*H*-tetrazole, (3) 1 % CF_3CO_2H, CH_2Cl_2, (4) either Alexa Fluor 350 carboxylic acid succinimidyl ester or Alexa Fluor 647 carboxylic acid succinimidyl ester (Life Technologies) or eosin-5-isothiocyanate (R. J. Cherry, A. Cogoli, M. Oppliger, G. Schneider, G. Semenza, *Biochemistry*, **1976**, *15*, 3653) or 6-carboxy-*N,N,N′,N′*-tetramethylrhodamine activated by a mixture of *O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxy-1*H*-benzotriazole (HOBT), and *N,N*-diisopropylethylamine (DIEA); (d) symmetric doubler phosphoramidite (Glen Research, USA), 1*H*-tetrazole; (e) trebler phosphoramidite (Glen Research, USA).

ODN2a_L-FAM. Yield: 15%; HPLC (Nucleosil C4 column; gradient: 5 min at 0% solution B in solution A, in 30 min to 50% B, in 10 min to 70% B): R_t = 24.5 min; MALDI-TOF MS, negative mode, calculated for $C_{186}H_{214}N_{49}O_{80}P_{12}$ $[M\text{-}H]^-$: *m/z* 4787, found 4789.

ODN2b_L-FAM. Yield: 14%; HPLC (Nucleosil C4 column; gradient: 5 min at 0% solution B in solution A, in 30 min to 50% B, in 10 min to 70% B): R_t = 24.7 min; MALDI-TOF MS, negative mode, calculated for $C_{196}H_{227}N_{51}O_{87}P_{13}$ $[M\text{-}H]^-$: *m/z* 5092, found 5099.

ODN1-L-FAM2. Yield: 51%, HPLC (Nucleosil C4 column; gradient: in 32 min from 0 to 40% solution B in solution A*, in 10 min to 90% solution B): R_t = 27.1 min; MALDI-TOF MS, negative mode, calculated for $C_{312}H_{375}N_{75}O_{167}P_{25}$ ($[M\text{-}H]^-$): *m/z* 8618, found 8614.

ODN1-L-FAM3. Yield: 15%, HPLC (Nucleosil C4 column; gradient: in 32 min from 0 to 40% solution B in solution A*, in 10 min to 90% solution B): R_t = 29.2 min; MALDI-TOF MS, negative mode, calculated for $C_{360}H_{427}N_{74}O_{184}P_{27}$ ($[M\text{-}H]^-$): *m/z* 9566, found 9557.

ODN1-L-TET. Yield: 61%, HPLC (Nucleosil C4 column; gradient: in 32 min from 0 to 40% solution B in solution A*, in 10 min to 90% solution B): R_t = 24.0 min; MALDI-TOF MS, negative mode, calculated for $C_{251}H_{299}N_{72}Cl_4O_{145}P_{22}$ ($[M\text{-}H]^-$): *m/z* 7462, found 7469.

ODN3. Yield: 13%, HPLC (Nucleosil C18 column; gradient: in 32 min from 1 to 70% solution B in solution A*, in 10 min to 90% solution B): R_t = 27.1 min; MALDI-TOF MS, negative mode, calculated for $C_{247}H_{303}N_{92}O_{121}P_{21}In$ ($[M\text{-}H]^-$): *m/z* 7354, found 7360.

dx.doi.org/10.1021/bc400152n | *Bioconjugate Chem.* 2013, 24, 1533−1542

ODN4. Yield: 42%, HPLC (Nucleosil C4 column; gradient: in 32 min from 0 to 70% solution B in solution A, in 10 min to 90% solution B): R_t = 29.1 min; MALDI-TOF MS, negative mode, calculated for $C_{178}H_{221}N_{59}O_{87}P_{14}In$ ($[M-H]^-$): m/z 5125, found 5122.

ODN5. Yield: 15%; HPLC (Nucleosil C4 column; gradient: 5 min at 0% solution B in solution A, in 30 min to 50% B, in 10 min to 70% B): R_t = 28.6 min; MALDI-TOF MS, negative mode, calculated for $C_{157}H_{189}N_{47}O_{73}P_{12}$ $[M-Cl-2H]^-$: m/z 4114, found 4111.

Protocols for synthesis of those conjugates, which were not obtained by the standard methods outlined above:

ODN1-L-TAMRA. ODN1 sequence was synthesized on the Bz-A-SynBase CPG 1000/110 solid support under standard conditions using a DNA/RNA synthesizer. Next, 9-(3-DMT-oxypropyl)-10-(3-oxypropyl)anthracene [(cyanoethyl)(*N,N*-diisopropyl)]phosphoramidite9 was coupled to the 5′-terminus of ODN1 in the presence of 1*H*-tetrazole. The DMT-group of the modified solid support was cleaved off and the 5′-hydroxyl group obtained was converted to an amino group by coupling 5′-amino modifier C6 CE phosphoramidite, followed by the deprotection of the MMT group in the presence of 1% CF_3CO_2H in CH_2Cl_2. Next, a mixture of 6-carboxy-*N,N,N′N′*-tetramethylrhodamine (5 mg, 11.6 μmol), *O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU; 4 mg, 10.5 μmol), and 1-hydroxy-1*H*-benzotriazole (HOBT; 1.8 mg, 11.6 μmol) were dissolved in DMF (116 μL), and *N,N*-diisopropylethylamine (DIEA; 2 μL, 23.2 μmol) was added. This solution was vortexed and immediately added to the CPG-bound nucleic acid strand (5′-amino modified ODN1-L). The slurry obtained was vigorously stirred for 2 h. Afterward the CPG was filtered, washed with DMF (2 × 1 mL) and CH_3CN (2 × 1 mL) and dried in vacuum (0.01 mbar). The conjugates was deprotected and cleaved from the solid support as described above and purified by HPLC. Yield: 1%, HPLC (Nucleosil C4 column; gradient: in 32 min from 0 to 30% solution B in solution A, in 10 min to 90% solution B): R_t = 27.7 min; MALDI-TOF MS, negative mode, calculated for $C_{256}H_{317}N_{74}O_{143}P_{22}$ ($[M-H]^-$): m/z 7396, found 7392.

ODN1-L-Eosin. Eosin-5-isothiocyanate (7.1 mg, 10 μmol) was dissolved in DMF (200 μL) and DIEA (3.8 μL, 22 μmol) was added. The resulting mixture was added to 5′-amino group modified ODN1-L (3 mg, 100 nmol of NH_2 groups, the synthesis was described above). The slurry obtained was shaken for 4 h, then filtered, washed with DMF (2 × 1 mL) and CH_3CN (2 × 1 mL), and dried in vacuum (0.01 mbar) for 2 h. The conjugates was deprotected and cleaved from the solid support as described above and purified by HPLC. Yield: 5%, HPLC (Nucleosil C4 column; gradient: in 32 min from 0 to 30% solution B in solution A, in 10 min to 90% solution B): R_t = 27.2 min; MALDI-TOF MS, negative mode, calculated for $C_{252}H_{302}N_{73}Br_4O_{144}P_{22}S$ ($[M-H]^-$): m/z 7683, found 7685.

ODN1-L-Alexa Fluor647. Alexa Fluor647 succinimide (0.1 mg, 0.1 μmol) was dissolved in dry DMF (15 μL) and DIEA (0.3 μL, 1.7 μmol) was added. The resulting mixture was added to 5′-amino group modified ODN1-L (3 mg, 100 nmol of NH_2 groups). The slurry obtained was shaken for 4 h at 37 °C, then filtered, washed with DMF (2 × 1 mL) and CH_3CN (2 × 1 mL), and dried in vacuo (0.01 mbar) for 2 h. The conjugates was deprotected and cleaved from the solid support as described above and purified by HPLC. Yield: 28%, HPLC (Nucleosil C4 column; gradient: in 32 min from 0 to 40% solution B in solution A (0.1 M aqueous $(NEt_3H)(OAc)$ with 5% CH_3CN), in 10 min to 90% solution B): R_t = 24.8 min; MALDI-TOF MS, negative mode, calculated for $C_{267}H_{339}N_{74}O_{152}P_{22}S_4$ ($[M-H]^-$): m/z 7822, found 7820.

ODN1-L-Alexa Fluor350. Alexa Fluor350 conjugate was synthesized analogously except that Alexa Fluor350 succinimide (1 mg, 2.4 μmol) was used in place of Alexa Fluor647 succinimide. Yield 35%, HPLC (Nucleosil C18 column; gradient: in 32 min from 0 to 35% solution B in solution A*, in 10 min to 90% solution B): R_t = 25.8 min; MALDI-TOF MS, negative mode, calculated for $C_{243}H_{304}N_{73}O_{145}P_{22}S$ ($[M-H]^-$): m/z 7277, found 7275.

Photogeneration of $^1O_2$. $^1O_2$ was generated by irradiation of solutions of PS-containing oligonucleotides (ODN3, ODN4, or ODN5) placed in black-walled fluorescence cuvettes with the monochromatic light (635 nm, 2 mW), which was applied from the top of the cuvette via an optical fiber.

Determination of Turnover Number in the Photochemical Reaction of Cleavage of ODN1_L-F3. Turnover number (TN) in the photocatalytic reaction of decomposition of ODN1_L-F3 (50 nM) in the presence of ODN4 (50 nM) and T1 (2 nM) with formation of 3 equiv fluorescein dye F was determined using fluorescence spectroscopy. In this reaction intermediate products containing 2 and 1 fluoresceins attached to the ODN1 are also formed. Their fluorescence quantum yield is similar to that of the starting material (Figure 5, -T). We assumed that the effect of their generation on the overall fluorescence of the mixture is negligible. Correspondingly, the fluorescence of the reaction mixture (F) could be expressed as $F = (1 - x)*F_0 + x*F_{fluorescein}$, where F_0 is the fluorescence intensity of pure ODN1_L-F3 (50 nM) and $F_{fluorescein}$ is that of pure fluorescein (50 nM); x is the fraction of fluorescein in the mixture, whereas $1 - x$ is the fraction of the starting material and intermediates containing two and one fluoresceins. Using experimental data (F) a fraction of fluorescein in the mixture (x) was calculated and converted to the turnover number (TN) according to the formulas: $TN = [ODN1\_L\text{-}F3]_0*x/[T1]$, where $[ODN1\_L\text{-}F3]_0$ is the initial concentration of ODN1_L-F3 (50 nM) and [T1] is the concentration of template T1 (2 nM).

RESULTS AND DISCUSSION

Design of Nucleic Acid Probes. Three designs of the red-light-controlled, templated reaction (A-C) were realized based on the new 9,10-dialkoxyanthracene-containing substrate. In all of them probe I is cleaved by $^1O_2$ generated on probe II (Figure 2). Probes I and II bind to the neighboring sites on a nucleic acid template that facilitates the cleavage reaction, since it brings the substrate (L-F) and the catalyst (PS = InPPa) in proximity to each other (Figure 2). The effect is especially strong in highly dilute solutions (≤100 nM), in which the template-free reaction is practically negligible. Due to the strong dependence of the rate of probe I cleavage from the concentration of nucleic acid templates, reactions A-C can be used for detection of specific nucleic acid sequences. Designs A-C differ from each other only in probe I composition. In particular, probe I for reaction A carries a 5′-terminal L-F substrate as well as a second fluorophore, *N,N,N′,N′*-tetramethylrhodamine (T), which is attached far away from the reaction center (Figure 2A). In this reaction the fluorophore F is cleaved from probe I and dequenched, whereas the fluorophore T remains attached and its fluorescence is not significantly affected. This process can be monitored by detecting ratio of fluorescence intensities

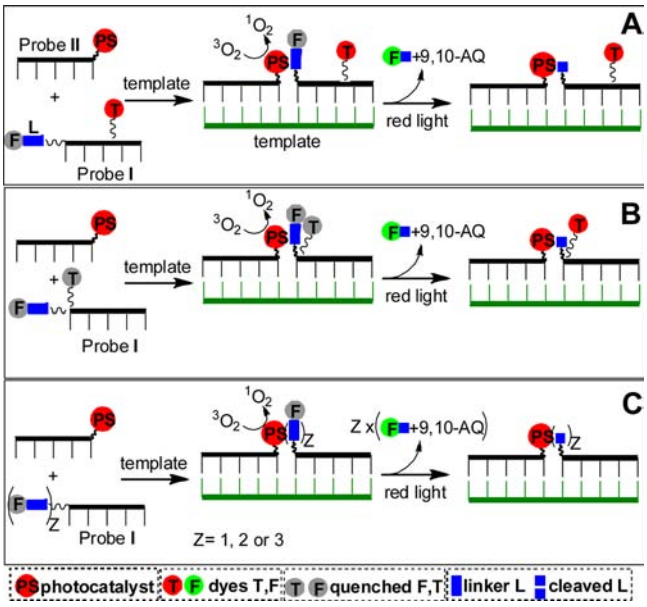
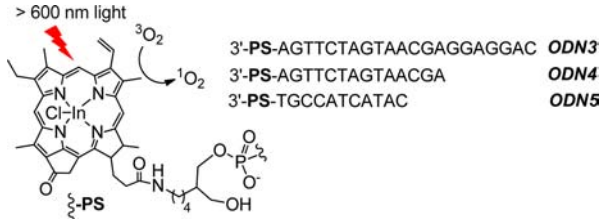


Figure 2. Designs (A-C) of a fluorogenic, photochemical, templated reaction; in all cases probe II is modified with a photocatalyst PS, which catalyzes conversion of $^3O_2$ into $^1O_2$ upon its exposure to >630 nm light. Design A: probe I contains a fluorogenic substrate L-F (L = 9,10-dialkoxyanthracene linker, F = fluorescein) and an internal reference fluorophore T (T = *N,N,N′,N′*-tetramethylrhodamine). In the reaction the F is released, whereas the fluorescence of the T is not affected. Design B: probe I contains a fluorogenic substrate L-F and a closely positioned fluorophore T, which is quenched. The fluorescent F (released from the duplex) and the T (attached to the duplex) are formed as a result of the templated reaction. Design C: probe I contains *Z* fluorogenic substrates (*Z* = 1, 2, 3). These substrates are converted to *Z* equiv bright fluorophores F and 9,10-anthraquinone (9,10-AQ)9 during the reaction.

characteristic of fluorophores F and T: F(F)/F(T).[25] Since monitoring such ratiometric signals is prone to less false positive and negative results than that of the single fluorescence intensity, the suggested reaction should be potentially well suited for detection of nucleic acids in complex systems, e.g., cell lysates and live cells. Probe I for reaction B carries a dye T, which is attached in close proximity to the 5′-terminal L-F (Figure 2B). In this conjugate strong contact interaction between the fluorophores and the linker leads to quenching of both dyes. Upon photocleavage of the linker both fluorophores are dequenched. Since dye T remains bound to probe I/probe II/template duplex, its fluorescence can be used to monitor localization of the nucleic acid template by, e.g., fluorescent microscopy or single molecule imaging. Finally, probe I for reaction C contains several (*Z*) fluorogenic substrates L-F (Figure 2C). In this reaction *Z* equiv fluorescent dye F (in this paper conjugates with *Z* = 1, 2, or 3 were described) can be potentially released from the duplex probe I/probe II/template. Therefore, it is potentially suitable for the amplified detection of nucleic acids.

Synthesis of Probes I and II as Well as Control Compounds (Schemes 1, 2). Probes I, which contain one (ODN1_L-F, ODN2_L-F), two (ODN1_L-F2), and three (ODN1_L-F3) fluorogenic substrates, were synthesized in accordance with Scheme 1. In the first step (a) the protected and bound to the controlled pore glass (CPG) sequences ODN1, ODN1a, ODN2, ODN2a, or ODN2b were assembled on the commercially available CPG solid support carrying 5′-

Scheme 2. Structure of a Photocatalyst PS and Sequences of Probes II



dimethoxytrityl (DMT) protected *N*-benzoyl adenosine (1) by using solid phase synthesis under standard conditions (solid support 2). Next, the fluorogenic substrate was attached in several steps (b and c). In particular, 9-(3-DMT-oxypropyl)-10-(3-oxypropyl)anthracene [(cyanoethyl)(*N,N*-diisopropyl)]-phosphoramidite was first coupled in the presence of 1*H*-tetrazole on the 5′-terminus of solid support 2 to attach linker L (solid support 3). This step was followed by the cleavage of the DMT group in the presence of 1% CCl_3CO_2H in CH_2Cl_2. Further, fluorophores available as phosphoramidites (5-carboxyfluorescein (FAM) or 6-carboxy-2′,4,7,7′-tetrachloro-fluorescein (TET) were coupled on the 5′-terminal hydroxyl group to obtain ODN_L-F conjugates. For the fluorophores available as succiniimides of carboxylic acids (Alexa Fluor350, Alexa Fluor647), free carboxylic acids (*N,N,N′N′*-tetramethylrhodamine, TAMRA) or isothiocyanates (eosin) the synthesis was slightly modified. In particular, after the cleavage of the DMT-group of solid support 3 the 5′-hydroxyl group obtained was converted to an amino group by coupling of 5′-MMT-amino modifier-CE phosphoramidite (Link Technolgies, UK), followed by the deprotection of the MMT group in the presence of 1% CF_3CO_2H in CH_2Cl_2. Finally, the corresponding fluorophore-containing succinimide, carboxylic acid, or isothiocyanate was coupled. A control probe I lacking the linker (ODN1_F) was purchased from commercially sources. For two representative fluorophores (Alexa Fluo350 and FAM) conjugates containing two or three fluorogenic substrates (ODN1_L-F2 and ODN1_L-F3) were also synthesized (Scheme 1). In particular, either symmetric doubler or trebler phosphoramidite (Glen research, USA) was first coupled to solid support 2 according to manufacturer recommendations. Then, steps b and c were conducted to conjugate *Z* fluorogenic substrates (*Z* = 2 or 3). Finally, the conjugates were cleaved from the solid support and deprotected by using aqueous ammonia (25%) solution and purified by HPLC. Purity of the conjugates discussed in this paper was confirmed by analytical HPLC and their identity by MALDI-TOF mass spectrometry (Supporting Information). As a photocatalyst PS for probes II we selected InPPa.[12,13] We observed earlier that this complex is an efficient photosensitizer. In particular, it can photocatalyze $^1O_2$-mediated bleaching of over 6000 equiv 2,5-di-(4-carboxyphenyl)-isobenzofurane. We attached this coordination compound to 3′-termini of 21- (ODN3), 14- (ODN4), and 11-mer (ODN5) oligodeoxyribonucleotide strands as previously described (Scheme 2).[12]

Monofunctionalized Probes I. We designed two probes I and II targeting a selected sequence of *β*-actin gene: T1. This sequence is a 42-mer with 50% GC content (Scheme 1). A corresponding DNA T1 was synthesized and used as a model template. As probe I we applied 5′-terminally modified ODNs containing a variety of fluorophores (F) and a 9,10-dialkoxysubstituted anthracene linker (L) (ODN_L-F, Table

dx.doi.org/10.1021/bc400152n | *Bioconjugate Chem.* 2013, 24, 1533−1542

1). Fluorescent spectra of a representative monofunctionalized conjugate ODN1_L-F (F = FAM, further ODN1_L-FAM), a control ODN1_F (F = FAM, further ODN1_FAM) and an unconjugated FAM dye in buffered at pH 7 aqueous solution are shown in Figure 3A. The fluorescence quantum yield of FAM (λ_{ex} = 495 nm, λ_{em} = 520 nm) was found to be reduced by 10.1-fold in ODN1_L-FAM and by 2.1-fold in ODN1_FAM in comparison to the free dye that indicate that both the linker L and nucleobases of the ODN1 strand are responsible for the quenching of the FAM in ODN1_L-FAM. In contrast, we observed only 5-fold fluorescence quenching in the analogous conjugate containing the SCH=CHS fragment as a linker.[10] Assembly of 21-mer substrate ODN1_L-FAM, 21-mer catalyst ODN3, and template T1 in aqueous buffer solution (pH 7) leads to formation of the duplex structure that causes further 1.6-fold quenching of the fluorescence of the FAM. The quenching is caused by the interaction of the dye and the PS, which are positioned in close proximity in the duplex ODN1_L FAM/ODN3/T1 (Figure 1). The overall quenching of the fluorescence of the FAM relative to the free dye is equal to 16-fold. Irradiation of the duplex with red light (635 nm, 2.6 mW) leads to the PS-induced generation of $^1O_2$ from $^3O_2$. The latter reagent reacts with the linker of ODN1_L-FAM inducing its cleavage and the release of free FAM. At the beginning of this reaction the reactive intermediate ($^1O_2$) is photogenerated on the PS. At the same time a competing reaction of $^1O_2$ quenching by water reduces amount of singlet oxygen in the mixture.[26] Before the steady state is achieved the reaction of the cleavage of the linker in ODN1_L-FAM is very slow as observed in the kinetic curves as an induction period (Figure 3B). Initial rates of the fluorescence intensity increase (dF/dt, where F is the fluorescence intensity at the time t) discussed in this paper were determined in the kinetic region coming immediately after the induction period. This period is indicated in Figure 3B with a straight, solid line. For example, dF/dt observed for the duplex ODN1_L-FAM/ODN3/T1 (100 nM) irradiated with the red light ($(dF/dt)_{+T}$) is equal to 3.4, whereas in the absence of the template the reaction is substantially slower: $(dF/dt)_{-T}$ = 0.2. Ratio of the rates of the templated to the background reaction ($(dF/dt)_{+T}/(dF/dt)_{-T}$) is equal to 14.7. For the SCH=CHS containing analogue of probe I ratio $(dF/dt)_{+T}/(dF/dt)_{-T}$ was found to be equal to 7.3 that indicates that the 9,10-dialkoxyanthracene linker is cleaved 2 times quicker at the initial phase of the templated reaction than the SCH=CHS linker. The reaction is not saturated for a long time, which allows generating 77.2% of the product within 150 min. In the absence of the template only 7.3% of the product is formed at the same conditions (Table 1). In contrast, 25% and 8% of the product are formed correspondingly in the presence and absence of the template when probe I contains SCH=CHS in place of 9,10-dialkoxyanthracene.[10] In the presence of sodium azide (0–200 mM) the templated cleavage reaction is inhibited in a concentration dependent fashion, which confirms that singlet oxygen acts as its mediator (Figure S16, Supporting Information).

Since the mismatch discrimination with >20-mer probes is usually poor, we tested whether the templated reaction can also be conducted with shorter oligonucleotides. In particular, we substituted the 21-mer catalyst ODN3 for the 14-mer catalyst ODN4 in the duplex ODN1_L-FAM/catalyst/T1. We were pleased to observe that the cleavage of the substrate in ODN1_L-FAM/ODN3/T1 occurs with the same rate as that in ODN1_L-FAM/ODN4/T1 (Supporting Information, Figure S17).

For all fluorophores F studied, we observed the template-induced acceleration of the linker cleavage in the range between 4.1 and 117.8 (Table 1). However, some of the F's were found to be less suitable for practical applications. For example, the initial very fast increase of the fluorescence intensity of ODN1_L-AlexaFluor 647 is quickly saturated, which is indicated by low F_{150}/F_0 ratio of 1.5 (Table 1). This behavior can be explained by bleaching of the fluorophore in the presence of $^1O_2$. Furthermore, the yield of the templated reaction with conjugate ODN1_L-AlexaFluor 370 is only 3.9 times better than that of the background reaction: 46.0% vs 11.8% (Table 1). The conjugate with F= eosin exhibits low template induced cleavage acceleration of only 4.1, whereas that with F= TAMRA is cleaved very slowly in the presence of the template: only 25% product yield after 150 min of the reaction

Table 1. Kinetic Parameters of Cleavage of ODN1_L-F Conjugates in the Photochemical, Templated Reaction Catalyzed by ODN3[a]

Dye F in ODN1_L-F	$(dF/dt)_{+T}/(dF/dt)_{-T}$[b]	$[F_{150}/F_0]$[c]	yield (%)[d] +T1	yield (%)[d] −T1
AlexaFluor370	20.2 (16.3)		46.0	11.8
FAM	14.7 (5.6)		77.2	7.3
TET	13.8 (5.6)		75.1	7.0
Eosin	4.1 (2.0)		-	-
TAMRA-dT	25.0 (3.2)		25.7	0.3
AlexaFluor647	117.8 (1.5)		-	-

[a][ODN1_L-F] = [ODN3] = [T1] = 100 nM; buffer MOPS 10 mM, pH 7, NaCl 150 mM, 37 °C. [b]$(dF/dt)_{+T}$ fluorescence change in the mixture of ODN1_L-F, ODN3, T1 irradiated with red light; $(dF/dt)_{-T}$ fluorescence change in the mixture of ODN1_L-F, ODN3 irradiated with red light. [c]$(F_{150}/F_0)_{max}$ −Increase of the fluorescence intensity obtained after 150 min of the templated, photochemical reaction; F_{150} − fluorescence intensity obtained after 150 min of the reaction; F_0 − initial fluorescence intensity. [d]Yield was determined after 150 min of irradiation of stoichiometric amounts of the substrate, the catalyst, and the template.

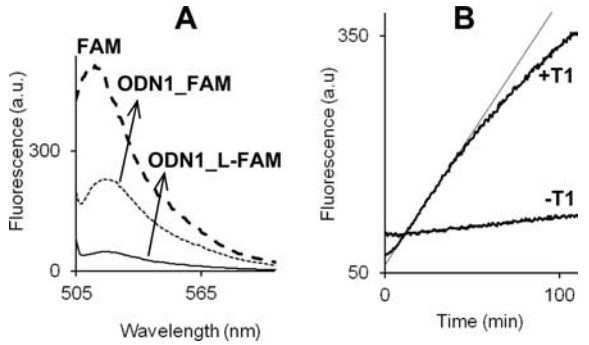


Figure 3. (A) Fluorescence spectra (λ_{ex} = 495 nm) of fluorescein (FAM) and conjugates ODN1_FAM and ODN1_L-FAM (concentration of each compound is 100 nM) in the buffer consisting of 3-(*N*-morpholino)propansulfonic acid (MOPS), 10 mM, pH 7 and NaCl (150 mM); (B) A mixture of ODN1_L-FAM (100 nM) and ODN3 (1 equiv) in the presence (+T1) or absence (−T1) of the template T1 (1 equiv) was irradiated with red light (635 nm, 2 mW) and fluorescence at 520 nm (λ_{ex} = 495 nm) was monitored as a function of time; the region of the constant reaction rate is indicated with a straight, solid light; buffer: the same as in A; the experiment was done at 37 °C.

dx.doi.org/10.1021/bc400152n | *Bioconjugate Chem.* 2013, 24, 1533–1542

(Table 1). In contrast, ODN_L-F conjugates with F = TET and FAM are cleaved quickly and generate high product yields (Table 1). The conugates containing these dyes were found to be equally well suited for the photochemical, templated cleavage reaction. For further studies we selected the FAM dye, since commercially available starting materials for synthesis of FAM-containing conjugates are cheaper.

To confirm the generality and to study the mismatch discrimination ability of the assay based on the photochemical templated reaction, we designed 11-mer probes I (ODN2_L-FAM) and II (ODN5) targeting another nucleic acid sequence T2, which is a 30-mer with 43% GC-content (Scheme 1). The short length of the probes was selected to achieve better mismatch discrimination.[27,28] We observed that the cleavage of ODN2_L-FAM in the presence of catalyst ODN5 is accelerated by 28.7-fold in the presence of the template T2. Single mismatches in T2 both on the substrate side (T2_mm1) and on the catalyst side (T2_mm2) led to practically complete inhibition of the photochemical reaction (Table 2). Thus, the assays based on this reaction can be potentially applied for detection of single nucleotide polymorphism (SNP).

Table 2. Kinetic Parameters of Cleavage of ODN2_L-FAM Conjugate in the Photochemical Templated Reaction Catalyzed by ODN5 and in the Presence of Different Templates[a]

template	$(dF/dt)_{+T}/(dF/dt)_{-T}$[b]	yield (%)[c]
-	-	3.2
T2	28.7	49.6
T2_mm1	1.2	3.9
T2_mm2	1.2	4.3
T2_A	19.5	32.0
T2_TA	11.9	26.1

[a][ODN2_L-FAM] = [ODN5] = [templates] = 100 nM; buffer MOPS 10 mM, pH 7, NaCl 150 mM, 25 °C. [b]$(dF/dt)_{+T}$ fluorescence change in the mixture of ODN2_L-FMA, ODN5, and a corresponding template irradiated with red light; $(dF/dt)_{-T}$ fluorescence change in the same mixture lacking the template irradiated with red light. [c]Yield was determined after 60 min of irradiation of stoichiometric amounts of the substrate, the catalyst, and the template.

Interestingly, one (T2_A) or two (T2_TA) bulged nucleotides in the template, which are positioned between the substrate and the catalyst, slow down the reaction by 1.5- and 2.4-fold, correspondingly (Table 2). This indicates that the reaction occurs more efficiently when the substrate and the catalyst are positioned at the closest possible proximity to each other. This experimental observation can be explained by the short lifetime of singlet oxygen, which is a mediator of the templated reaction described here, in aqueous buffers.[26]

Designs A and B (Figure 2). Next, we designed two 11-mer probes I, which carry an internal TAMRA dye additionally to the 5′-L-FAM modification: conjugates ODN2a_L-FAM and ODN2b_L-FAM (Scheme 1). In the former conjugate the TAMRA dye is positioned 7 nucleotides away from the 5′-terminal modification. We found that in in this case the effect of the TAMRA dye on the FAM dye and vice versa is minimal. For example, template T2 accelerated the cleavage of ODN2a_L-FAM by 23.5-fold, which was comparable to the acceleration observed for ODN2_L-FAM: 28.7-fold. Moreover, practically no fluorescence resonance energy transfer (FRET) was observed between TAMRA and FAM dyes and the fluorescence of the TAMRA was only weakly affected during the templated cleavage of the linker (Figure 4A). Therefore, the

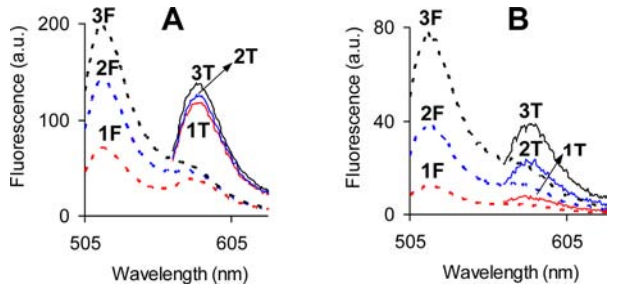


Figure 4. (A) Fluorescent spectra of a mixture containing ODN2a_L-FAM (100 nM), ODN5 (1 equiv), and T2 (1 equiv) in MOPS buffer (10 mM, pH 7), NaCl 150 mM, 25 °C: traces labeled with F were obtained by using λ_{ex} = 495 nm and those labeled with T were obtained by using λ_{ex} = 556 nm; initial spectra are labeled with 1, spectra of mixtures irradiated with 635 nm light (2 mW) for 90 min are labeled with 2, and those irradiated for 180 min with 3. (B) Same as in A except that ODN2b_L-FAM was used in place of ODN2a_L-FAM.

second dye (TAMRA) in ODN2a_L-FAM can be considered as an internal standard. In this case the concentration of a nucleic acid target correlates with the ratio of green to red fluorescence intensities (design A, Figures 2, 3A) rather than with the fluorescence intensity only that should eliminate the possibility of false negative or positive results. The latter problem is especially pronounced when nucleic acids are detected in complex mixtures, e.g., in live cells.

In another conjugate (ODN2b-L-FAM) the second dye is positioned next to the L-FAM moiety. Therefore, the fluorescence of this dye is strongly quenched by the contact interaction with the L-FAM (Figure 3B). In contrast to the previously described system, here the template-induced cleavage acceleration was somewhat lower (12.3-fold). We believe that it is a reflection of the lower affinity of ODN2b to T2 (10 basepair − duplex is formed) than that of ODN2a to T2 (11 basepair − duplex is formed). Moreover, the distance between the substrate (L-FAM) and the catalyst (InPPa) in the duplex ODN2b_L-FAM/ODN5/T2 is larger than that in the duplex ODN2a_L-FAM/ODN5/T2 (Scheme 1). As expected, the cleavage of the L in ODN2b_L-FAM results in substantial (5.3-fold) increase of the fluorescence intensity of the TAMRA dye. In contrast to the previously described reactions based on ODN1_L-FAM and ODN2a_L-FAM, in this case the switched-on fluorophore is retained on the nucleic acid target rather than dissociated from it. Such probes are useful not only for detection of nucleic acids, but also for monitoring their localization (design B, Figure 2).

Earlier we demonstrated that the photochemical cleavage reaction based on ODN~SCH═CHS~TAMRA probes is compatible with the single molecule (SM) fluorescence imaging.[10] However, in this initial design the fluorescence intensity of the system is decreased as a result of the templated reaction. In this case false positive results are possible that complicate the interpretation of the data. In contrast, in design B, the fluorescence intenisty of the TAMRA is increased in the presence of nucleic acids, which makes these substrates potentially better suited for the SM fluorescent imaging. We and our cooperation partners are currently testing the

dx.doi.org/10.1021/bc400152n | Bioconjugate Chem. 2013, 24, 1533−1542

applicability of these probes toward SM fluorescence imaging and the results of this study will be published in due course.

Design C (Figure 2): A Strand-Exchange Independent, Catalytic Templated Reaction. Few known chemical, templated reactions provide for amplification of nucleic acid targets.[1−4] In these reactions a substrate conjugated to an ODN or its analogues is first converted on the template to a product. Next, the product containing the ODN is exchanged for the substrate−ODN and the next reaction cycle can take place. The rate of the latter exchange step is dependent upon the ODN sequence and usually occurs within several minutes.[29] For quick chemical reactions this step is rate limiting. Therefore, all known catalytic templated reactions are slow and provide for low substrate conversion.[1−4,30,31] To solve the problem of the intrinsically slow strand exchange, we designed probes I, which carry multiple (*Z*) fluorogenic substrates L-FAM (Figure 2, design C). In this case *Z* catalytic cycles can occur without the strand exchange, which was expected to improve the substrate conversion and the reaction rate. The related idea has been previously applied by Kool et al. to achieve strong nucleic acid dependent fluorescence enhancement in the templated Staudinger reactions.[32] In particular, they prepared a double substrate (ODNa-F-X2, where X = azidoether linker conjugated to a quencher and F is a fluorescent dye) and a double reagent (Y2-ODNb, where Y = a triphenylphosphine-containing fragment). In the conjugate ODNa-F-X2 fragment F is not fluorescent, whereas in the presence of the template reagents Y cleave two substrates Xs thereby restoring the fluorescence of the dye F. In contrast to the previously described Staudinger reactions, where only one quencher was cleaved, the fluorescence enhancement was observed to be stronger.

Though FAM is known to be a weak catalyst for photogeneration of $^1O_2$, one could expect that *Z* dyes in multiply modified probes I can generate a substantial amount of this reagent, which could lead to the undesired template-free activation of the probe ODN1_L-F*Z* (*Z* > 1). However, we observed that multiple substrates quench each other efficiently in ODN1_L-F*Z*. In particular, the fluorescence quantum yield of both doubly and triply modified conjugates ODN1_L-F2 and ODN1_L-F3 (Scheme 1) was found to be 77% of that of the monofunctional conjugate ODN1_L-F. Therefore, the background cleavage of all mono and multiply modified probes I is slow (Figure 5, right plot). In the presence of template T1 the multiply modified probes are cleaved substantially more quickly than the monofunctionalized probe, which can be explained by the higher effective concentration of the substrates in proximity to the photocatalyst in the former case (Figure 5, left plot). Correspondingly, stronger amplification of a nucleic acid target is achieved with this probe than with a monofunctional probe.

Furthermore, we tested the sensitivity in the detection of nucleic acids using the templated, photochemical cleavage reaction with the most efficient probe prepared: ODN1_L-F3 (Figure 6). We observed that the reaction rate correlates with the concentration of the nucleic acid target T1 (Figure 6). The lowest detectable concentration of T1 was found to be 2 nM, for which $((dF/dt)_{+T}/(dF/dt)_{-T}$ was still >3.0. At these conditions 7.7 turnovers were generated in 134.5 min, which corresponds to formation of 15 nM fluorescein in the mixture. Since in the absence of strand-exchange only 6 nM fluorescein (3 TN's) could be generated, we conclude that the strand exchange also plays a role in this case. The reaction of

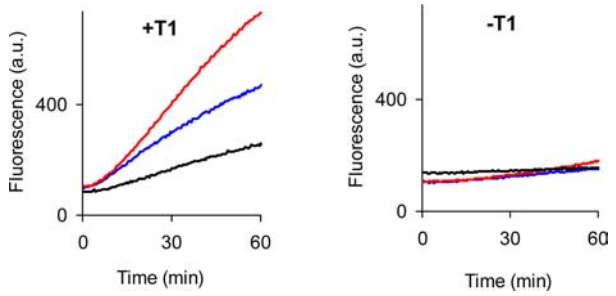


Figure 5. Left plot (+T1): fluorescence intensity increase (λ_{ex} = 495 nm, λ_{em} = 520 nm) of solutions containing ODN4 (100 nM), T1 (1 equiv), and either ODN1_L-F (1 equiv, black trace), or ODN1_L-F2 (1 equiv, blue trace) or ODN1_L-F3 (1 equiv, red trace) observed upon irradiation with red light (635 nm, 2 mW) for the time specified on the O*X*-axis; right plot (−T1): the same solutions were irradiated in the absence of T1; buffer: MOPS (10 mM, pH 7), NaCl (150 mM), 37 °C.

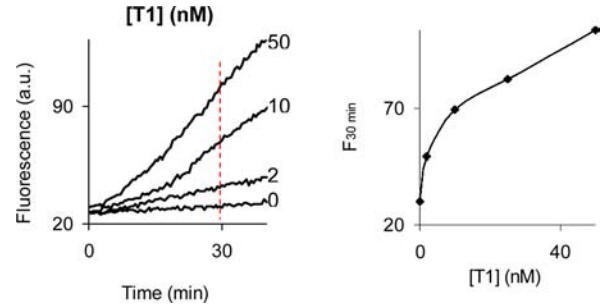


Figure 6. Left plot: fluorescence intensity changes (λ_{ex} = 495 nm, λ_{em} = 520 nm) of solutions containing ODN1_L-F3 (50 nM), ODN4 (1 equiv), and T1 at concentrations shown on the plot observed upon irradiation with red light (635 nm, 2 mW) for the specified on the O*X*-axis time; fluorescence intensity readings indicated with red, dotted line is plotted on the right plot as a function of the template concentration; buffer: MOPS (10 mM, pH 7), NaCl (150 mM), 37 °C.

templated cleavage of a PhSeT fragment, which we described earlier, generates only 4.5 turnovers of the product.[11]

CONCLUSIONS

Substitution of a SCH═CHS-containing linker (L) in conjugates 5′-FAM-L-ODNa for a 9,10-alkoxyanthracene fragment leads to substantial improvement of the templated photocleavage of this compound in the presence of 3′-PS-ODNb. In particular, the initial reaction rate is accelerated by 2.0−3.9-fold in comparison with the SCH═CHS-based system. The yield of the reaction in the presence of stoichiometric amounts of the probes and the template is improved from 25% to 77.2%. Moreover, 7.7 catalytic turnovers were observed at the optimized conditions. In contrast, in the SCH═CHS-based fluorogenic cleavage reaction no catalytic turnover could be observed, whereas the nonfluorogenic, templated PhSeT-cleavage generates 4.5 turnovers. Since the anthracene-containing linker is a quencher of the fluorescence of FAM, the ratio of fluorescence quantum yields of the product (FAM) and the substrate (5′-FAM-L-ODNa) were also improved from 5- to 10.1-fold in comparison to the previously described, SCH═CHS-based reaction. We demonstrated that the formation of fluorescent dyes, that was mediated by singlet oxygen and templated by nucleic acids,

dx.doi.org/10.1021/bc400152n | *Bioconjugate Chem.* 2013, 24, 1533−1542

can be used as a flexible tool for detection of nucleic acids in variable formats. For example, we realized reactions (a) suitable for detection of single nucleotide polymorphism, (b) generating ratiometric fluorescent signal, (c) occurring with formation of either a free fluorescent dye or a fluorescent dye attached to the nucleic acid target, and (d) suitable for the amplified detection of nucleic acids. Moreover, the reaction based on design B (Figure 2), where the TAMRA dye conjugated to the nucleic acid probe is dequenched upon exposure to light, is potentially compatible with single molecule fluorescent imaging.

■ ASSOCIATED CONTENT

Supporting Information

MALDI-TOF mass spectra of all new conjugates discussed in the paper; kinetics of FAM-photorelease from ODN1_L-FAM in the presence of T1 and either ODN3 or ODN4. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: Andriy.Mokhir@fau.de, Tel: 09131-85-22554.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

A.M. thanks Boehringer Ingelheim Foundation for funding within the PLUS3-Programm.

■ ABBREVIATIONS

9,10-AQ, 9,10-anthraquinone; DMT, dimethoxytrityl; DIEA, *N,N*-diisopropylethylamine; FAM, 5-carboxyfluorescein; FRET, fluorescence resonance energy transfer; InPPa, indium(III)-(pyropheophor-bide-a)chloride; HBTU, *O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyl-uronium hexafluorophosphate; HOBT, 1-hydroxy-1*H*-benzotriazole; MMT, monomethoxytrityl; MOPS, (*N*-morpholino)-propansulfonic acid; ODN, oligo-2′-deoxyribonucleotide; PPa, pyropheophorbide-a; PS, photosensitizer; TAMRA, 6-carboxy-*N,N,N′,N′*-tetramethylrhodamine; TET, 6-carboxy-2′,4,7,7′-tetra-chlorofluorescein.

■ REFERENCES

(1) Shibata, A., Abe, H., and Ito, Y. (2012) Oligonucleotide-templated reactions for sensing nucleic acids. *Molecules 17*, 2446–2463.

(2) Grossmann, T. N., Strohbach, A., and Seitz, O. (2008) Achieving turnover in DNA-templated reactions. *ChemBioChem 9*, 2185–2192.

(3) Jacobsen, M. F., Cló, E., Mokhir, A., and Gothelf, K. V. (2007) Model systems for activation of nucleic acid encoded prodrugs. *ChemMedChem 2*, 793–799.

(4) Silvermann, A. P., and Kool, E. T. (2006) Detecting RNA and DNA with templated chemical reactions. *Chem. Rev. 106*, 3775–3789.

(5) Bag, B. G., and von Kiedrowski, G. (1996) Templates, autocatalysis and molecular replication. *Pure Appl. Chem. 68*, 2145–2152.

(6) Orgel, L. E. (1995) Unnatural selection in chemical systems. *Acc. Chem. Res. 28*, 109–118.

(7) Boll, I., Jentzsch, E., Krämer, R., and Mokhir, A. (2006) Metal complex catalysis on a double stranded DNA template. *Chem. Commun.*, 3447–3449.

(8) Boll, I., Krämer, R., Brunner, J., and Mokhir, A. (2005) Templated metal catalysis for single nucleotide specific DNA sequence detection. *J. Am. Chem. Soc. 127*, 7849–7856.

(9) Brunner, J., Mokhir, A., and Krämer, R. (2003) DNA-templated metal catalysis. *J. Am. Chem. Soc. 125*, 12410–12411.

(10) Dutta, S., Flottmann, B., Heilemann, M., and Mokhir, A. (2012) Hybridization and reaction-based, fluorogenic nucleic acid probes. *Chem. Commun. 47*, 9664–9666.

(11) Fülöp, A., Peng, X., Greenberg, M. M., and Mokhir, A. (2010) A nucleic acid directed, red light-induced chemical reaction. *Chem. Commun. 46*, 5659–5661.

(12) Arian, D., Cló, E., Gothelf, K. V., and Mokhir, A. (2010) A nucleic acid dependent chemical photocatalysis in live cells. *Chem.—Eur. J. 16*, 288–295.

(13) Arian, D., Kovbasyuk, L., and Mokhir, A. (2011) Control of the photocatalytic activity of bimetallic complexes of pyropheophorbide-a by nucleic acids. *Inorg. Chem. 50*, 12010–12017.

(14) Mukae, M., Ihara, T., Tabara, M., Arslan, P., and Jyo, A. (2009) Photodimerisation of anthracenes using a DNA template and its analytical applications. *Supramol. Chem. 21*, 292–295.

(15) Ami, T., Yoshimura, Y., Matsuzaki, T., and Fujimoto, K. (2009) Photochemical ligation of DNA probe prepared in click chemistry. *J. Photopolym. Sci. Technol. 22*, 267–272.

(16) Yoshimura, Y., Okamura, D., Ogino, M., and Fujimoto, K. (2006) Highly selective and sensitive template-directed photoligation of DNA via 5-carbamoylvinyl-2′-deoxycytidine. *Org. Lett. 8*, 5049–5051.

(17) Liu, J., and Taylor, J. -S. (1998) Template-directed photoligation of oligodeoxyribonucleotides via 4-thiothymidine. *Nucleic Acids Res. 26*, 3300–3304.

(18) Meunier, J. -R, Sarasin, A., and Marrot, L. (2002) Photogenotoxicity of mammalian cells: a review of the different assays for in vitro testing. *Photochem. Photobiolog. 75*, 437–447.

(19) Baugh, S. D. P., Yang, Z., Leung, D. K., Wilson, D. M., and Breslow, R. (2001) Cyclodextrin dimers as cleavable carriers of photodynamic sensitizers. *J. Am. Chem. Soc. 123*, 12488–12494.

(20) Rotaru, A., and Mokhir, A. (2007) Nucleic acid binders activated by light of selectable wavelength. *Angew. Chem., Int. Ed. 46*, 6180–6183.

(21) Rotaru, A., Kovács, J., and Mokhir, A. (2008) Red light activated phosphorothioate oligodeoxyribonucleotides. *Bioorg. Med. Chem. Lett. 18*, 4336–4338.

(22) Arian, D., Kovbasyuk, L., and Mokhir, A. (2011) 1,9-Di(alcoxy)anthracene as a singlet oxygen-sensitive linker. *J. Am. Chem. Soc. 133*, 3972–3980.

(23) Chen, Y., Kamlet, A. S., Steinman, J. B., and Liu, D. R. (2011) A biomolecule-compatible visible-light-induced azide reduction from a DNA-encoded reaction-discovery system. *Nat. Chem. 3*, 146–153.

(24) Röthlingshöfer, M., Gorska, K., and Winssinger, N. (2012) Nucleic acid templated uncaging of fluorophores using Ru-catalyzed photoreduction with visible light. *Org. Lett. 14*, 482–485.

(25) Cardullo, R. A., Agrawal, S., Flores, C., Zamecnik, P. C., and Wolf, D. E. (1988) Detection of nucleic acid hybridization by nonradiative fluorescence resonance energy transfer. *Proc. Natl. Acad. Sci. U.S.A. 85*, 8790–8794.

(26) Schweitzer, C., and Schmidt, R. (2003) Physical mechanisms of generation and deactivation of singlet oxygen. *Chem. Rev. 103*, 1685–1757.

(27) Dose, C., Ficht, S., and Seitz, O. (2006) Reducing product inhibition in DNA-template-controlled ligation reactions. *Angew. Chem., Int. Ed. 45*, 5369–5373.

(28) Kolpashchikov, D. M (2010) Binary probes for nucleic acid analysis. *Chem. Rev. 110*, 4709–4723.

(29) Plum, G. E., Breslauer, K. J., and Roberts, R. W. (1999) Thermodynamics and kinetics of nucleic acid association/dissociation and folding processes. *Comprehensive natural products chemistry* (Sir Barton, D., and Nakanishi, K., Eds.) pp 15–53, Chapter 2, Vol. 2 (DNA and aspects of molecular biology, Kool, E. T., Ed.), Elsevier Science Ltd., Oxford, UK.

(30) Abe, H., and Kool, E. T. (2004) Destabilizing universal linkers for signal amplification in self-ligating probes for RNA. *J. Am. Chem. Soc. 126*, 13980–13986.

(31) Grossmann, T. N., and Seitz, O. (2006) DNA-catalyzed transfer of a reporter group. *J. Am. Chem. Soc. 128*, 15596−15597.
(32) Franzini, R. M., and Kool, E. T. (2011) Two successive reactions on a DNA template: a strategy for improving background fluorescence and specificity in nucleic acid detection. *Chem.—Eur. J. 17*, 2168−2175.