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DNA-Catalyzed Transfer of a Reporter Group

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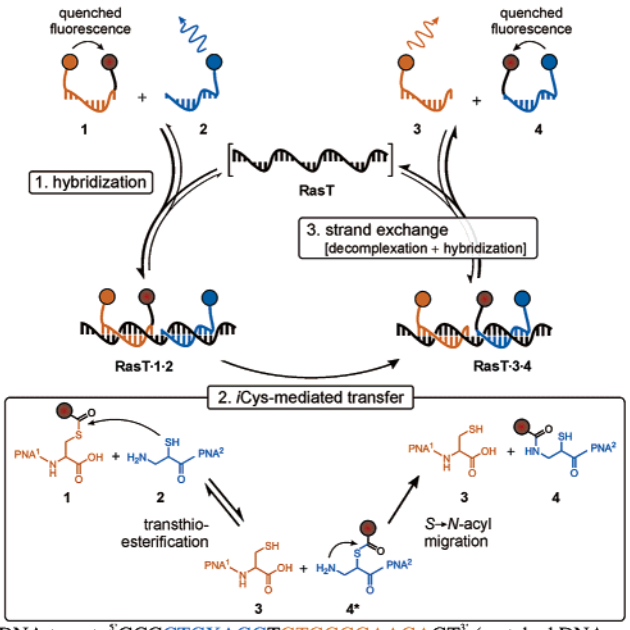
DNA-templated reactions are emerging as a general approach to control the reactivity of synthetic molecules by modulating the effective molarity.[1] DNA templates align the reactive groups of these molecules to allow fast and selective reactions at reactant concentrations that are much lower than those required for conventional synthesis. Among various types of chemistries, most DNA-directed reactions involve the covalent ligation of two modified oligonucleotide strands.[2] In general, ligation reactions suffer from an increased affinity of the product to the DNA template. This causes product inhibition and prevents high catalytic activity of the nucleic-acid template. However, catalysis would be a valuable asset, particularly in DNA and RNA detection chemistries. Catalytic turnover would provide for signal amplification and would, thus, enable highly sensitive DNA and RNA detection in homogeneous solution and in living cells. To reduce product inhibition, flexibility enhancing ligation reactions have been developed.[2i,m] However, ligation products still have higher template affinity than the reactant probes. Other approaches to prevent product inhibition involve DNA-directed O-deacylation of modified oligonucleotides by ester hydrolysis[3] or Staudinger reaction.[4] Even though these approaches yield product complexes with unchanged stability, they have not led to a large number of catalytic turnover so far. In this Communication we show that DNA-directed transfer reactions may present a general solution to the problem of product inhibition as evidenced by high turnover (TO) and signal amplification.

Our design[5] of a reaction in which the DNA analyte acts as catalyst involves an *iso*-cysteine(*i*Cys)-mediated transfer of a reporter group (Figure 1). A donating **1** and accepting **2** peptide nucleic acid (PNA) probe are designed to hybridize adjacently to the complementary DNA template **RasT**. The reporter group (dark red) in **1** is covalently bound to a PNA-peptide conjugate (orange) as thioester. Probe **2** (blue) bears an N-terminal *i*Cys capable of attacking the thioester in probe **1** in a native chemical ligation-like fashion.[2m,6] Adjacent hybridization of probe **1** and **2** in ternary complex **RasT·1·2** triggers transthioesterification to form **4***. This is followed by an irreversible S→N-acyl migration, yielding ternary complex **RasT·3·4**. As result, the reporter group is transferred from PNA **1** to PNA **2**. Catalysis requires that products **3** and **4** in ternary complex **RasT·3·4** can be replaced by nonreacted probes **1** and **2**. Reactant and product probes have been fashioned to have similar affinity to DNA **RasT** and, thus, strand exchange may proceed quickly.

We decided to develop a fluorescence-based read-out, which facilitates real-time measurements. Probe **1** was N-terminally modified with 6-carboxyfluorescein (FAM, orange) and **2** was labeled with 5-carboxytetramethylrhodamine (TAMRA, blue) on the α-amino group of a C-terminal lysine. In both cases [2-(2-aminoethoxy)ethoxy]acetic acid (AEEA) was used as spacer to minimize the influence of the fluorophores on the hybridization properties. 4-[4-Dimethylamino)phenylazo]benzoylglycine (Dabcyl-Gly), representing the reporter group (dark red), was attached as thioester to probe **1**. The Dabcyl group is capable of quenching both FAM and TAMRA fluorescence when in proximity.



DNA target: 5'GCGCTGXAGGTGTGGGGAAGAGT3' (matched DNA target **RasT**: X = T, single-base mismatched DNA target **RasG**: X = G);
1: FAM-AEEA-tcttccccac-Cys(S-Gly-Dabcyl)^{COOH}
2: *i*Cys-cctacag-Lys(N$^{\alpha}$H-AEEA-TAMRA)^{CONH_2}
3: FAM-AEEA-tcttccccac-Cys^{COOH}
4: Dabcyl-Gly-*i*Cys-cctacag-Lys(N$^{\alpha}$H-AEEA-TAMRA)^{CONH_2}

Figure 1. (top) Catalytic cycle of the DNA-catalyzed transfer of a reporter group (dark red) from the donating **1** to the accepting PNA probe **2**; (bottom) *i*Cys-mediated transfer reaction.

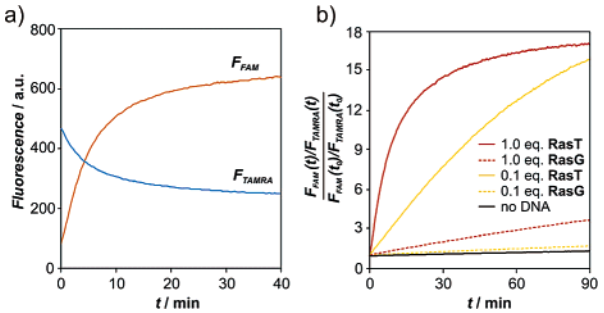


Figure 2. (a) FAM (λ_{ex} = 465 nm, λ_{em} = 525 nm) and TAMRA (λ_{ex} = 558 nm, λ_{em} = 593 nm) fluorescence of the DNA-directed transfer reaction (probe **1** and **2**) in the presence of 1 equiv **RasT**; (b) time courses of relative F_{FAM}/F_{TAMRA} ratio at different **RasT** and **RasG** concentrations (200 nM probes, 10 mM NaH_2PO_4, 200 mM NaCl, 0.2 mM TCEP, 0.1 mg/mL roche blocking reagent, pH 7.0, 37 °C).

Figure 2a shows the time-dependent intensities of FAM (orange line) and TAMRA (blue line) fluorescence, in a reaction of probe **1** and **2** in the presence of 1 equiv matched DNA **RasT**. Prior to transfer the FAM fluorescence of probe **1** is quenched by Dabcyl.

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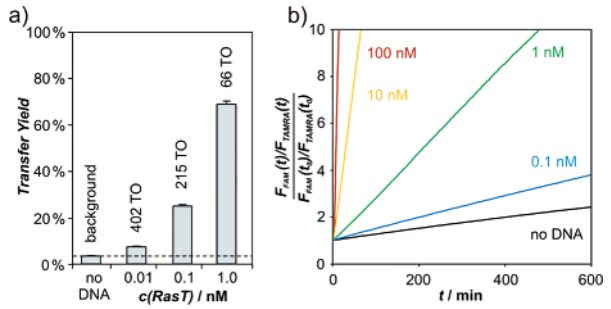

Figure 3. Transfer reaction of probe **1** and **2**: (a) transfer yields after 24 h and turnover numbers (TO); (b) time courses of relative F_{FAM}/F_{TAMRA} ratio at shown **RasT** concentrations (100 nM probes, 10 mM NaH_2PO_4, 200 mM NaCl, 1 mM TCEP, 0.1 mg/mL roche blocking reagent, pH 7.0, 32 °C).

Dabcyl-Gly transfer and formation of probe **3** results in an increase of FAM fluorescence. Simultaneously, the fluorescence intensity of TAMRA decreases, owing to the transfer of Dabcyl-Gly to probe **2**. This setup allows the specific detection of product formation. The transfer reaction can be further monitored by means of the ratio of fluorescence intensities F_{FAM}/F_{TAMRA} (Figure 2b). The time course of relative F_{FAM}/F_{TAMRA} ratio shows a rapid signal increase in the presence of 1 equiv matched DNA **RasT** (red line) reaching 17-fold enhancement after 90 min. Compared to the reaction in the absence of DNA (black line), the transfer reaction is accelerated by a factor of 1720. The useful 5-fold increase above background signal level is obtained already after 3.5 min. Substoichiometric amounts of **RasT** (0.1 equiv DNA, yellow line) proved also efficient in conferring a rapid signal increase and provided 7.0 TO after 1 h. The initial rates on single-base mismatched DNA **RasG** (dashed lines) are significantly reduced. At 200 nM probes and 20 nM target DNA, the transfer reaction on the mismatched **RasG** proceeds 44 times slower than on **RasT**.

The next aim was to specify the catalytic activity of the DNA template. With a load of 0.01 equiv (1 nM) DNA catalyst **RasT**, the transfer reaction furnished 69% of transfer product after 24 h (Figure 3a). In the absence of DNA, 100 nM probe **1** and **2** yielded 3.4% background transfer. It was concluded that 0.01 equiv DNA **RasT** conferred 66 TO. After 12 h we already observed 54 TO (see Supporting Information). A notable 25% yield (215 TO) was obtained with 0.001 equiv **RasT** (0.1 nM). Ligation reactions require 100 times higher DNA concentrations and 10 times more probe excess to obtain similar TO.[2i,m] The highest turnover number of 402 was achieved with 0.0001 equiv **RasT** (0.01 nM), surpassing TO reported for alternative sequence-specific methods.[2d,4] It has often been observed that the speed of the DNA-catalyzed reaction is reduced at very low DNA/probe ratios to an extent that reaction yields are dominated by the off-template background reaction. However, the 7.4% transfer product **4** obtained with 0.0001 equiv **RasT** (0.01 nM) demonstrate that the DNA-catalyzed transfer (4.0%) proceeds more efficiently than the background transfer (3.4%), a result not being reported yet.

The catalytic activity of the DNA analyte was considered useful for achieving signal amplification in fluorescence-based DNA detection. It is instructive to estimate fluorescence signaling provided by a conventional hybridization probe used in homogeneous DNA detection. A hybridization probe that yields 17-fold fluorescence enhancement upon quantitative hybridization will give only 2.6, 1.16, or 1.016-fold increases in the presence of 0.1, 0.01, and 0.001 equiv DNA, respectively. In contrast, our transfer reaction catalyzed by 0.1 equiv DNA (10 nM) provides for 10-fold enhancement of the F_{FAM}/F_{TAMRA} signal within 1 h (Figure 3b). Useful 7-fold (after 5 h) or 3-fold (after 7 h) enhancements were obtained in the presence of 0.01 equiv (1 nM) and 0.001 equiv (0.1 nM) DNA, respectively. At 0.1 nM **RasT** F_{FAM}/F_{TAMRA} signaling rate is 2-fold over background. Lower DNA template concentrations would require the use of longer transfer probes (>10 nt) to increase the template affinity. The predominant source of background is thioester hydrolysis (see Supporting Information). The use of less reactive thioesters and/or the transfer of fluorescent reporter groups should allow for further reductions of background.

Previous nonenzymatic DNA-catalyzed reactions based on ligation required high probe concentrations (10 μM) and large probe excess (10.000-fold) to drive displacement of the product from the product-template duplex, at the cost of low ligation rate and high ligation background.[2i,m] In addition, amplification of a fluorescence signal based on ligation has not been demonstrated. The potential for signal amplification has been shown in DNA-triggered cleavage reactions of esters in PNA-probes.[3] In these cases, turnover numbers were lower than 10, presumably because of relatively fast off-template reactions.

The DNA-directed transfer[7] of a reporter group developed by us is new and is the first reaction to combine high catalytic activity of the DNA analyte with useful reaction yields and low background under turnover conditions. The described Dabcyl transfer is restoring fluorescein emission while switching off rhodamine emission. This allowed monitoring of both transfer (FAM + TAMRA) and hydrolysis (FAM). Other reporter groups are possible as we expect a general applicability of the new transfer reaction in DNA catalysis. Future studies will be aimed at increasing signal output by applying this concept to other types of reactions, oligonucleotides, and readout systems.

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Supporting Information Available: Synthesis, experimental details, and HPLC data. This material is available free of charge via the Internet at http://pubs.acs.org.

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