DNA-Templated Reactions

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Consecutive Signal Amplification for DNA Detection Based on De Novo Fluorophore Synthesis and Host–Guest Chemistry**

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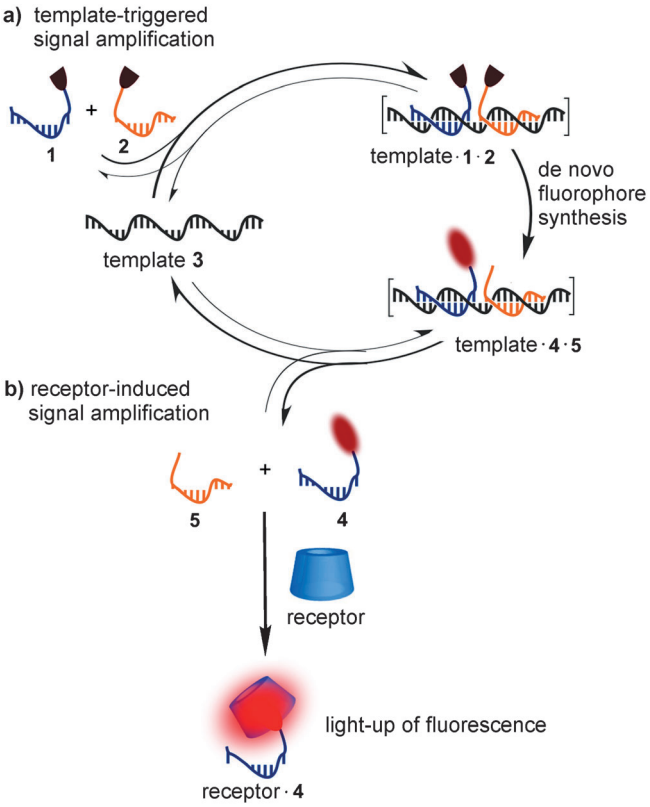
Chemical reactions templated by nucleic acids provide fascinating opportunities for both materials science and life science. Nucleic acid directed chemistry has recently been explored with an aim to construct replicable DNA nanoarchitectures, to facilitate drug screening, to detect DNA and RNA even within live cells, or to release or form druglike molecules.[1] Typically, stoichiometric amounts of the DNA template are required to drive reactions. However, many applications would benefit from turnover in the template.[1a] For example, significant efforts have been invested in the development of template-catalyzed reactions that facilitate the detection of minute amounts of DNA or RNA.[2–4] In these methods, one template molecule (= analyte) is envisioned to trigger the formation of many signal molecules. Accordingly, turnover in the template would provide a mechanism for the amplification of the detected signal. Of the typically measured signals, fluorescence is the most commonly used read-out.[3,4] Methods that lead to increases in fluorescence intensity upon exposure to the template allow real-time measurements and provide opportunities for RNA imaging in live cells and/or PCR-free detection of DNA.

Previous studies in this field focused on DNA-directed reactions between labeled oligonucleotides that contained quenched fluorophores or photosensitizers.[3,4] The fluorophore systems already existed and the template-controlled reactions served the sole purpose of removing any quenching moieties. Detection formats that rely on quenched fluorophores often have the disadvantage of a relatively high background signal originating from incomplete quenching and/or potential hydrolytic cleavage of ester-linked chromophores. Recently, aldol-type chemistry was used to create cyanine dyes by a DNA-directed ligation reaction,[5] wherein fluorescence occurred through formation rather than separation of the chromophores. However, ligation reactions suffer from product inhibition, which prevents turnover in the template and, thus, signal amplification. DNA-templated cleavage reactions allow a reduction in the product inhibition. For example, templated versions of the Staudinger reaction have been used to induce the release of nitrogen from azido-quenched fluorophores or α-azido-ether-linked quenchers.[4] These reactions enable turnover in the template and provide for large enhancements in the fluorescence.

Herein we report a new approach which involves an olefination reaction that proceeds by group transfer (rather than ligation or cleavage) and leads to the de novo synthesis of a fluorophore (Scheme 1 a). This fluorophore is recognized in a second step by a receptor molecule (Scheme 1 b). A gain in the signal is provided by two consecutive amplification mechanisms: a) a transfer reaction which facilitates turnover, because the probes after the reaction offer the same number of nucleotides for base pairing with the template as the probes before the reaction, and b) selective binding of the newly formed fluorophore to a receptor can be used to intensify the fluorescence by closing nonradiative decay channels. It is shown that this consecutive approach leads to very low background, delivers a more than 100-fold enhancement in the fluorescence, and results in a more than 300-fold gain in the signal compared to reactions in the absence of amplification.



Scheme 1. Consecutive signal amplification for DNA detection.

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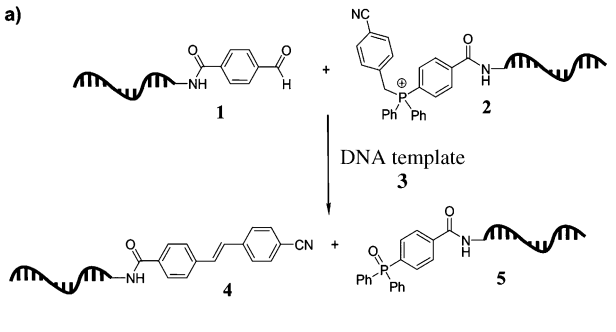
For the DNA-controlled fluorophore synthesis, we envisioned a reaction format where a benzylidene group of a DNA-tethered phosphonium salt is transferred to a DNA-tethered benzaldehyde.[6] The resulting stilbene would show fluorescence when suitable substituents were present. We focused on the reaction design shown in Scheme 2. The use of



b) probes

1a, OHC-C_6H_4-CONH-C_6H_{12}-OPO_2^--5'O-CCTACAGCGC-3'
1b, OHC-C_6H_4-CONH-C_2H_4OCONH-C_6H_{12}-OPO_2^--5'O-CACACCTACAGCGCC-3'
2a, 5'-ACTCTTCCCCAC-3'O-PO_2^--OC_6H_{12}-NHCO-C_6H_4-$P^+(Ph)_2$-CH_2-C_6H_4-CN
2b, 5'-CAGGGCACTCTTCCC-3'O-PO_2^--OC_6H_{12}-NHCO-C_6H_4-$P^+(Ph)_2$-CH_2-C_6H_4-CN

templates

3a, 5'-GCGCTGTAGGTGTGGGGAAGAGT-3'
3b, 5'-GGCGCTG**T**AGGTGTGGGGAAGAGTGCCCTG-3' (mutant)
3c, 5'-GGCGCTG**G**AGGTGTGGGGAAGAGTGCC CTG-3' (wild-type)

Scheme 2. a) DNA-templated Wittig reaction. b) Sequence of DNA probes and templates.

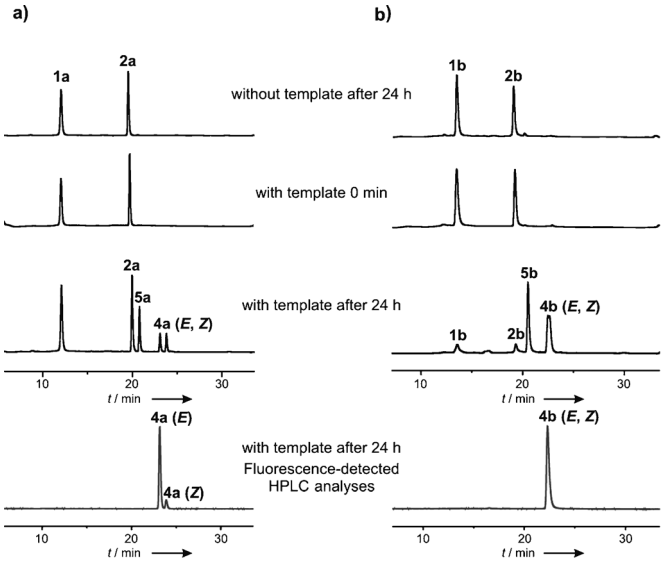


Figure 1. HPLC analysis of the reaction of probes **1** and **2** in the presence or absence of template **3**. Conditions: a) 100 mM NaH_2PO_4/Na_2HPO_4, 100 mM NaCl, pH 8.5, 50 °C, 5 μM **1a**, **2a**, and **3a** (1 equiv, when added); b) under optimized reaction conditions, 100 mM sodium bicarbonate, 1 M NaCl, pH 8.5, 60 °C, 500 nM **1b** and **2b**, 500 nM template **3b** (1 equiv, when added).

the phosphonium salt **2** allowed ylide formation at pH < 9. Benzaldehyde **1** offers a suitable compromise between stability and reactivity, and leads to the formation of a stilbene that fluoresces in buffer at 380 nm. In the event of a templated reaction, the targeted DNA template will align the DNA–aldehyde and DNA–phosphonium conjugates in immediate vicinity (Scheme 1a). The accompanying increase in the effective molarity will accelerate the Wittig reaction, which proceeds at very low rates when performed in the absence of a template. Turnover in the template is anticipated at conditions of dynamic strand exchange (near the melting temperature T_M), because the probes bind the template with a similar affinity before and after the reaction. The formation of the stilbene triggers a second recognition event: the interaction with a suitable receptor. On the basis of availability, water solubility, and ease of handling, we chose α-cyclodextrin as the receptor. The hydrophobic cavity of α-cyclodextrin offers a tight binding pocket which will prevent chemical and photochemical conversions of the bound *trans*-stilbene unit.[7] This results in enhancements in the stilbene emission, thus providing a second means of signal amplification.

We chose a sequence around the known carcinogenic G12V mutation of the *Ras* gene as template **3**. We first analyzed the DNA-templated Wittig reaction between modified oligonucleotides **1** and **2** by HPLC (Figure 1). The reaction proceeded smoothly in an aqueous buffer at pH 8.5 and 50 °C (Figure 1a), and led to the occurrence of three new signals, which were assigned through MALDI-TOF/MS and on-line fluorescence analysis. This analysis revealed that the *trans*-stilbene **4a** (*E*) and *cis*-stilbene **4a** (*Z*) were formed in an approximate 1:1 ratio along with the phosphine oxide **5a**. The reaction in the absence of template proved inefficient.

A survey of different templates revealed that the effect on yield was modest at least when there were 0–3 unpaired template bases between the two duplex segments in ternary complex **Target·1·2** (Scheme S1 in the Supporting Information). The DNA-templated reaction tolerated a range of reaction conditions (Tables S1–S3). The reaction proceeded rapidly at elevated temperatures required that the T_M value of the probe–template complex was not exceeded. We, therefore, increased the length of the oligonucleotide probes. Under the optimized conditions (Figure 1b), the templated reaction between oligonucleotide probes **1b** and **2b** (T_M = 65 °C and 64 °C, respectively, on template **3b**) provided stilbene **4b** in 83% yield after 24 h. The background was low: less than 0.8% product was formed after 24 h in the absence of the template. The reaction is sufficiently fast to allow measurements after short incubation times: Stilbene **4b** was obtained in almost 50% and 26% yield after only 2 h and 30 min, respectively (Table S3). We were pleased to see that the reaction allowed turnover in the template (Table 1 and Figure S1). The HPLC analysis revealed 84 turnovers when 0.0002 equivalents of template **3b** was included. The 1.7% yield of stilbene under these conditions exceeded the background by a factor of three.

We next examined the fluorescence read-out. The stilbene–DNA conjugate **4** formed from the DNA-templated reaction showed a broad fluorescence emission with a maximum at λ = 380 nm (Figure 2a). The addition of 0.1 equivalent template led to the synthesis of stilbene and a 12-fold enhancement in the fluorescence signal at 380 nm. Of note,

Table 1: Fluorescence intensities and signal gains.

Entry	Target [equiv]	Turn-over	*F.I.*[a] without α-CD	*F.I.*[a] with α-CD	Signal gain by α-CD	Total signal gain[b]
1	0.1	4	10.1	306	30	120
2	0.01	13	3.8	70	18	234
3	0.001	28	1.2	9.6	8	224
4	0.0002	84	1.0	4.2	4	336
5	0	–	0.88	2.7	3	3

[a] *F.I.* = fluorescence intensity from Figure 2a and b at 380 nm. [b] Total signal gain = turnover × signal gain by α-CD.

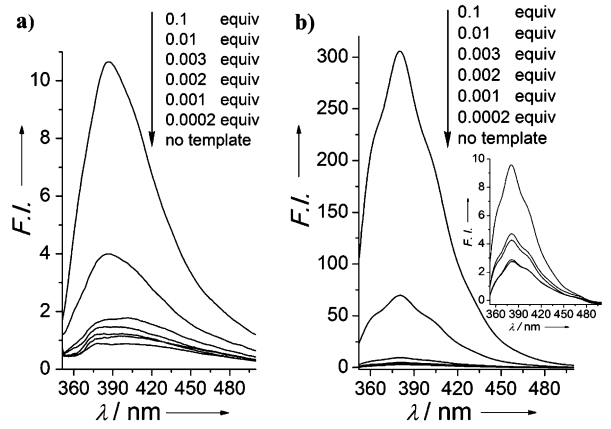


Figure 2. Fluorescence spectra measured after the template-promoted Wittig reaction of probes **1b** and **2b** in the presence of varied amounts of template **3b** in the a) absence and b) presence of 100 mM α-cyclodextrin (after 30 min incubation with 100 mM α-cyclodextrin at 25 °C). *F.I.* = fluorescence intensity. Conditions: 100 mM sodium bicarbonate, 1 M NaCl, pH 8.5, 200 nM **1b**, 500 nM **2b**, 60 °C for 24 h, λ_{ex} = 330 nm. Insert: amplified view.

a fourfold enhancement in the fluorescence over the background was obtained when the reaction was performed in the presence of 0.01 equivalent template. Inspired by the analyte–receptor model for signal amplification,[7,8] we explored the use of α-cyclodextrin (Figures S2–S7). This host molecule can encapsulate the newly formed stilbene, close reaction channels (such as photoisomerization) that lead to depletion of the excited state, and thereby increase the intensity of the fluorescence emission.[7] When 100 mM α-cyclodextrin was added to the reaction products formed in the presence of 0.1 equivalent template, the fluorescence increased by a factor of 30 compared to that in the absence of α-cyclodextrin (Figure 2b versus Figure 2a, entry 1 of Table 1).[9] The fluorescence was more than 100-fold greater than the background (= nontemplated reaction, Table 1: entry 1 versus entry 5). A 26-fold signal enhancement was obtained when the reaction was performed in the presence of 0.01 equivalent template (Table 1, entry 2 versus entry 5). This compares favorably with previously reported templated Staudinger reductions, which resulted in a $\leq$10-fold fluorescence enhancement under these conditions.[4e,f] Remarkably, the reaction in the presence of 0.001 equivalents of template still conferred a 3.5-fold enhancement in the fluorescence intensity (Table 1, entry 3 versus entry 5). This level of sensitivity has not been reported previously with templated fluorescence activation methods.[10] The limit of detection with the probes used and the conditions chosen was reached at 0.0002 equivalent template (40 pM).

Table 1 shows the total signal gain, which is the result of two amplification mechanisms. First, turnover in the template enables the formation of many signal molecules (stilbenes) per target molecule. For example, the transfer reaction in the presence of 0.0002 equivalent (40 pM) template will provide the 84-fold amount (about 3.4 nM) of signal molecules (= stilbenes). This amplification mechanism becomes less important at high template/probe ratios, and 0.1 equivalent (20 nM) template leads to a fourfold higher amount (80 nM) of stilbene. On the other hand, the amplification provided by the addition of α-cyclodextrin is more effective at high template loads than at low template loads. For example, the reaction on 0.1 equivalent template (20 nM) led to a 30-fold enhancement of the stilbene emission when α-cyclodextrin was added after the reaction. In contrast, a fourfold enhancement in the fluorescence was obtained in reactions triggered by 0.0002 equivalent template. This result is plausible, because quantitative encapsulation of stilbene will become increasingly difficult as the template amount, and thus the concentration of the formed stilbene, decreases. Two conclusions can be drawn from this analysis. First, the two amplification mechanisms have opposite directions and the resulting gain in the signal remained, regardless of the probe/template ratio applied, on the order of 10^2-fold compared to the theoretical signal in the absence of amplification/turnover. Secondly, the use of receptors that have a higher affinity than α-cyclodextrin ($K_a < 1 \times 10^3\,M^{-1}$)[11] for the formed stilbene should enable even higher signal amplification.[12]

We then investigated the sequence specificity and evaluated reactions on matched and single-base-mismatched DNA which spanned a sequence around the carcinogenic G12V mutation of the *ras* gene. We wanted to detect the mutant target **3b** but not the wild-type target **3c**, and for this purpose, substrates **1b**/**2b** are exactly matched to mutant target **3b**, whereas **1b**/**2b** have a single-base mismatch with wild-type target **3c**. The reaction proved sequence-specific. The reaction in the presence of matched target **3b** was accompanied by stronger fluorescence signaling than the reaction with single mismatched target **3c**. It is interesting to note that low template loads lead to higher match/mismatch selectivity (6-fold with 0.01 equivalents target) than high template loads (6-fold with 0.01 equivalents target versus 2-fold with 1 equivalents target; Figure 3). This can be attributed to the rather high T_M value of the involved 15 nt long oligonucleotides and the rather small temperature window in which hybridization is sequence-specific (**1b**·**3b** (matched), T_M = 65 °C; **1b**·**3c** (mismatched), T_M = 61 °C, measured at 1:1 ratio and 0.5 μM). Lowering the strand concentration will shift this window of discrimination to lower temperatures.

Several templated fluorogenic reaction systems have been reported to work within complex environments. For example, considerable efforts have been invested in the detection of RNA targets in cells. It is our long-term objective to develop a chemical method that facilitates the detection of DNA in biological samples. However, the double-stranded nature of

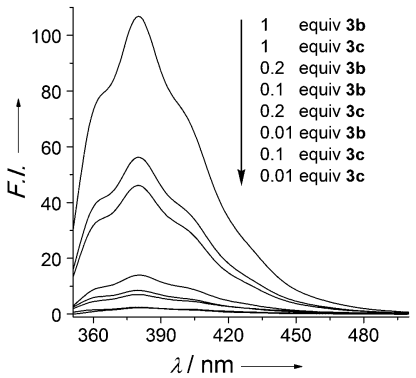


Figure 3. Sequence specificity of the background-corrected fluorescence measured after the template-promoted Wittig reaction between **1b** and **2b** in the presence of matched target **3b** or mismatched target **3c** at various loadings, *F.I.* = fluorescence intensity. Conditions: 200 nM **1b**, 200 nM **2b**, 100 mM sodium bicarbonate, 1 M NaCl, pH 8.5, 65 °C (60 °C when the reaction was performed on 0.01 equiv target), 2 h reaction time, 30 min incubation with 100 mM α-cyclodextrin at 25 °C.

DNA presents a formidable challenge to template chemistry and, as yet, there are only a few reactions that have been demonstrated to proceed with DNA from biotechnological sources. We chose an 81 nucleotide long DNA strand as the template and used the asymmetric polymerase chain reaction (PCR) to produce mostly double-stranded but also a small amount of single-stranded DNA (Figure S10).[13] After only 30 cycles, the crude PCR mixture was examined by means of the templated Wittig reaction (Figure 4a). The reaction proceeded smoothly despite the presence of nucleotides, oligonucleotides, and enzymes. A reaction time of 3 h with 2 pM starting template was sufficient to provide a 100-fold enhancement in the fluorescence (compared to the control in the absence of template). Most other sequence-specific DNA-detection methods used in PCR analysis provide $\leq$10-fold fluorescence enhancements. Remarkably, a starting concentration of the template of 20 fM still conferred a fivefold increase in the signal.

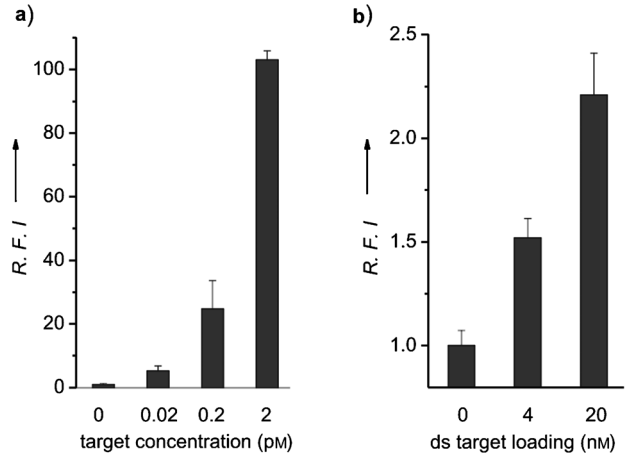


Figure 4. Relative fluorescence intensity (*R.F.I.*) at 380 nm determined after the reaction of probes **1b** and **2b** in the presence of a) DNA targets produced by asymmetric PCR or b) an 81 bp duplex DNA target. Conditions: a) 1. 30 cycles (95 °C for 10 s; 58 °C for 30 s; 72 °C for 20 s) at various concentrations of target, 800 nM forward primer, 100 nM reverse primer, Taq-DNA polymerase (1 u); 2. PCR product diluted (1/3, v/v) with buffer (100 mM sodium bicarbonate, 1 M NaCl, pH 8.5), 200 nM **1b**, 500 nM **2b**, 60 °C, 3 h prior; 3. 100 mM α-cyclodextrin, 25 °C, 30 min. b) 200 nM **1b**, 200 nM **2b**, 400 nM forward primer and reverse primer in buffer, 81 bp duplex DNA at various loadings, 3 h thermal cycling (95 °C for 10 s; 60 °C for 50 s).

Our results indicated that the DNA-triggered Wittig reaction tolerates the use of a variety of buffers. There was little background reaction at elevated temperatures. These properties facilitate applications where thermal denaturation is required to gain access to target segments. We used the 81-mer DNA double strand as the template[13] and examined whether the templated Wittig reaction proceeds under thermal cycling conditions. We envisioned that the reactive DNA probes **1b** and **2b** would bind to the single-stranded target segments formed transiently during thermocycling between 95 °C (denaturation) and 60 °C (annealing→templated Wittig reaction). An excess of oligonucleotide primers was used to slow down the reannealing of the DNA template.[14] Indeed, after temperature cycling (95 °C for 10 s, 60 °C for 50 s) for 3 h in the presence of 20 nM (0.1 equiv) dsDNA template, the reaction of the DNA probes furnished a 2.2-fold stronger fluorescence signal than the control with no template (Figure 4b). The reaction failed to proceed without thermal cycling (Figure S11). This finding together with a positive control, which involved the deliberate addition of single-strand target, proved that the template strand was quantitatively hybridized. A notable 50% increase in fluorescence was observed when 4 nM (0.02 equiv) dsDNA template was used. These results demonstrate that the DNA-triggered Wittig reaction is among the very few fluorogenic chemical reactions that allow the direct analysis of double-stranded DNA.[15] We assume that this method is not restricted to the analysis of purine-rich or AT-rich target sequences, which are required in reactions that involve triplex formation.

The consecutive signal amplification system based on the formation of double bonds and host–guest chemistry for the detection of DNA is a conceptually new approach. This reaction is, to the best of our knowledge, the first DNA-controlled reaction that provides turnover in the de novo synthesis of a fluorescent dye. The fluorescence enhancement achieved by the reaction system is in the order of 10^2-fold in the presence of 0.1 equivalent target. This exceeds the signal gain achieved by most other fluorogenic reactive probes.[16] The templated Wittig reaction proceeds with only a low background. Fluorescence signals can be detected within minutes. A further advantage is that elevated temperatures are tolerated. We have shown that this facilitates applications where thermal denaturation is required to unfold DNA targets. The reaction setup described by us may be only one of many reaction systems that allow signal amplification through consecutive reactions. Wittig and Horner reactions can be used to establish many other fluorophores, and a variety of receptors (e.g. cucurbiturils, calixarenes, antibodies) may be available for the subsequent recognition of the formed fluorophore. Potential applications may extend beyond the

detection of nucleic acids. For example, templated Wittig transfer reactions may provide turnover in the DNA- or RNA-encoded synthesis of druglike molecules. In future work we will widen the scope of the approach by examining other aldehydes, phosphorus-based ylides, and receptors.

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