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Two Successive Reactions on a DNA Template: A Strategy for Improving Background Fluorescence and Specificity in Nucleic Acid Detection

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Abstract: We report a new strategy for template-mediated fluorogenic chemistry that results in enhanced performance for the fluorescence detection of nucleic acids. In this approach, two successive templated reactions are required to induce a fluorescence signal, rather than only one. These novel fluorescein-labeled oligonucleotide probes, termed 2-STAR (STAR = Staudinger-triggered α-azidoether release) probes, contain two quencher groups tethered by separate reductively cleavable linkers. When a 2-STAR quenched probe successively binds adjacent to two mono-triphenylphosphine-(TPP)-DNAs or one dual-TPP-DNA, the two quenchers are released, resulting in a fluorescence signal. Because of the requirement for two consecutive reactions, 2-STAR probes display an unprecedented level of sequence specificity for template-mediated probe designs. At the same time, background emission generated by off-template reactions or incomplete quenching is among the lowest of any fluorogenic reactive probes for the detection of DNA or RNA.

Keywords: fluorescence · nucleic acids · quenched probes · Staudinger reaction · template synthesis

Introduction

Hybridization-based fluorogenic probes enable the sequence-specific detection of nucleic acids in solution phase and are of increasing interest for applications in biology and medicine. Dual-probe detection schemes are especially appealing with this respect because the requirement of two simultaneous recognition events for the generation of a readout signal engenders the possibility of greater sequence specificity and reduced background fluorescence relative to single probe approaches.[1,2] Template-mediated fluorogenic reactions have been established as a promising class of dual-probe nucleic acid detection schemes.[3] In this approach, adjacent hybridization of two modified oligonucleotide probes to the target nucleic acid initiates a chemical reaction,[4] eliciting a fluorescence turn-on signal. Templated reactive probes can provide isothermal signal amplification by a catalytic cycle of target hybridization, reaction, and dissociation.[5,6] Such probes can further be responsive to single-nucleotide polymorphisms and have been employed in the discrimination of bacteria species by single-nucleotide differences in rRNA.[7] However, for the detection of targets that occur in low copy numbers it is essential to minimize background fluorescence, which can obscure the detection signal in fluorogenic probes. For example, early templated reactive probes exhibited substantial background fluorescence, resulting from reactions of the fluorogenic probe with water or cellular components.

To improve the characteristics of template-mediated fluorescence activation schemes, several groups have investigated varied chemical reactions and fluorescence turn-on strategies for the detection of nucleic acids in vitro[8] and in cells.[7,9] One recent probe design that has displayed promise in engendering low background and high sequence specificity is that of quenched Staudinger-triggered α-azidoether release probes (Q-STAR probes).[10] These probes consist of a DNA hybridization sequence labeled with fluorescein, which is quenched by a dabsyl group attached to the DNA through an α-azidoether linker. A triphenylphosphine-(TPP)-labeled DNA probe reductively cleaves the linker, releasing the quencher and eliciting a fluorescence turn-on signal; adjacent binding of the two probes on the target greatly accelerates this reaction. Recent probe designs in general, and Q-STAR probes in particular, have significantly improved key aspects of DNA-/RNA-template-mediated fluorescence activation.[8,10] For example, nonspecific cleavage of the α-azidoether linker occurs slowly under cellular conditions, providing very low background fluorescence. Furthermore, Q-STAR probes provide an amplified detection signal, resulting from probe turnover on the target nucleic acid. Because of these favorable properties, Q-STAR probes are under further investigation for applications in cellular and medical diagnostics. However, optimizing the templated chemistry cannot alleviate all sources of background fluorescence. For example, bimolecular collisions of the probes engender off-template reactions and incomplete quenching results in residual fluorescence in the quenched probes. Furthermore, as for virtually all hybridization

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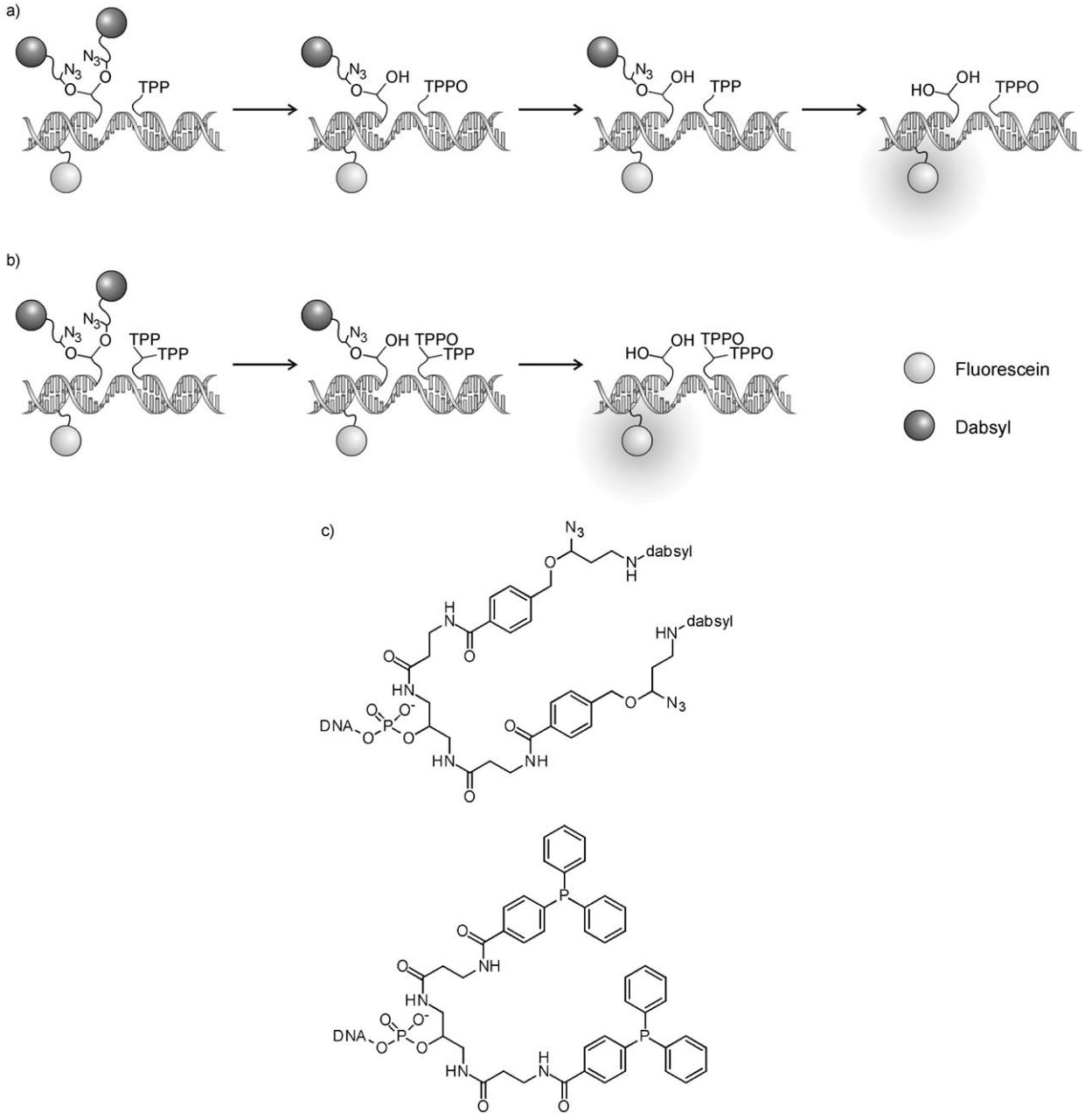
probes, discrimination with single-base sensitivity can be challenging for weakly destabilizing mismatches such as G–T or G–A, resulting in selectivity that is lower than ideal.

Here, we describe a new design of reactive probes, aimed at further lowering background signal and improving sequence specificity. This strategy, which may be generalizable to many kinds of templated chemistries, requires two successive templated reactions to generate a fluorescence signal. The reactive (2-STAR) probes are fluorescein-labeled oligonucleotides, containing two 5′-terminal α-azidoether linkers, each carrying a dabsyl quencher (Scheme 1), in contrast to previous probe designs that contained a single linker and quencher. The 2-STAR probes can be activated by two quencher release reactions upon templated reductive linker cleavage with two TPP–DNA probes, involving exchange of the TPP–DNA conjugates (Scheme 1a). Alternatively, a single activating probe, containing two TPP groups, can induce 2-STAR activation in a single binding event



Scheme 1. Illustration of the template-mediated fluorescence activation scheme based on probes, containing two releasable quenchers (2-STAR probes). a) Template-mediated reaction of a 2-STAR probe with a DNA probe that contains a single triphenylphosphine (TPP) moiety cleaves the α-azidoether linker, releasing the quencher, and forming triphenylphosphine oxide (TPPO). Exchange of two reacting TPP–DNA probes enables the consecutive release of both quenchers and fluorescence turn-on. b) Templated reaction of 2-STAR probe with a DNA probe, carrying two TPP groups, which cleaves both linkers in one binding event. c) Molecular structures of 2-STAR probe (top) and dual-TPP modifications (bottom)

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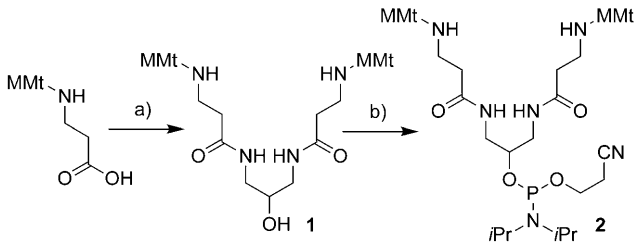
(Scheme 1b). Importantly, the release of both quenchers is required for the occurrence of a fluorescence signal and mono-quencher intermediates generated by nonspecific reactions are non-fluorescent.

Two examples of bifunctional fluorogenic probes for templated nucleic acid detection have been previously reported. Furukawa et al. have described probes based on a fluorescein profluorophore derivatized with two azidomethyl groups.[9d] However, in that design a single templated reaction, removing one azidomethyl group, generated a substantial turn-on signal, which obviates some of the possible benefits of doubly reactive probes. In a second report, our laboratory has described fluorescent probes containing two quenchers, but only one of the quenchers was displaced by a templated reaction, whereas the second quencher dissociated by nonspecific hydrolysis.[11] The present probe design requires for the first time two template-mediated reactions to induce a fluorescence turn-on event. This condition greatly lowers the probability of background-generating reactions that can occur off-template and with mismatched targets.

The described 2-STAR probes provide a strong fluorescence turn-on signal by a rapid templated reaction with TPP–DNAs and enable amplified detection of nucleic acid targets by probe turnover. Moreover, the requirement for two reactions greatly lowers the fluorescence signal arising from off-template reactions and allows for an unprecedented degree of single-nucleotide specificity for templated reactive probes.

Results and Discussion

Preparation of the reactive probes: The 2-STAR probes were obtained by post-synthetic solid-phase conjugation of two dabsyl-modified α-azidoether linkers to fluorescein-labeled DNAs with two terminal amine functionalities, following a previously described protocol.[10] A 5′-bis-amino-modifier was required for the attachment of two quencher release linkers to the DNA probe. For this purpose, we designed the monomethoxytrityl-(MMt)-protected 5′-bis-amino-modifier **2**, which was introduced as the terminal phosphoramidite during DNA solid-phase synthesis and deprotected on solid support by repeated washes with 2% trichloroacetic acid in dichloromethane. Compound **2** was conveniently prepared in two steps and 83% overall yield by coupling two molecules of *N*-MMt-3-aminopropionic acid[12] to 1,3-diamino-2-propanol followed by the formation of the phosphoramidite (Scheme 2). The same 5′-bis-amino-modifier **2** was also used for the preparation of the DNA probes with two TPP groups (dual-TPP–DNAs, Scheme 1c) by post-synthetic conjugation of 4-(diphenylphosphino)benzoic acid to 3′-amine-modified DNA synthesized in the reverse (5′→3′) direction. Probes that contained a single releasable quencher (Q-STAR probes, prepared for comparison to the new design) or a single TPP moiety (mono-TPP–DNAs) were synthesized as previously described.[10] Probes were purified by semi-preparative reverse-phase HPLC and analyzed by



Scheme 2. Synthesis of monomethoxytrityl-(MMt)-protected 5′-bis-amino-modifier phosphoramidite **2**. a) 1,3-diamino-2-propanol, dicyclohexylcarbodiimide (DCC), cat. dimethylaminopyridine (DMAP), 86% yield; b) 2-cyanoethyl-*N*,*N*-diisopropyl chlorophosphoramidite, diisopropylethylamine (DIEA), 97% yield.

MALDI-TOF mass spectrometry (see the Supporting Information, Table S1).

To evaluate the performance of 2-STAR probes in a DNA-templated reaction scheme, we selected two 27-nt DNA targets, differing by a single nucleotide. The sequences correspond to a ribosomal RNA site that has close homology (a single-nucleotide difference) in *Escherichia coli* and *Salmonella enterica* (Table 1, EC-DNA and SE-DNA).[7a]

Table 1. Sequences of probes and templates.

Probe	DNA sequence[a]
2-STAR EC	5′-(DabAzL)$_2$-AGT^{Fl}CGACA-3′
2-STAR SE	5′-(DabAzL)$_2$-AGT^{Fl}AGACA-3′
mono-quencher SE	5′-DabAzl-AGT^{Fl}AGACA-3′
9m TPP–DNA	5′-CAACCTCCA-TPP-3′
15m TPP–DNA	5′-AGGGCACAACCTCCA-TPP-3′
9d TPP–DNA	5′-CAACCTCCA-(TPP)$_2$-3′
15d TPP–DNA	5′-AGGGCACAACCTCCA-(TPP)$_2$-3′
EC-DNA	5′-GATGTCGACTTGGAGGTTGTGCCCTTG-3′
SE-DNA	5′-GATGTCTACTTGGAGGTTGTGCCCTTG-3′

[a] T^{Fl} = fluorescein-labeled deoxythymidine, DabAzL = dabsyl containing α-azidoether linker, EC = *E. coli*, SE = *S. enterica*.

The TPP–DNA probes were designed to bind adjacent to the 2-STAR probe on the same target site. Two types of TPP–DNAs were prepared, containing either one or two 3′-terminal TPP groups. We prepared both short and long versions of TPP–DNA probes: the shorter probes (DNA 9-mers) were designed to rapidly bind to and dissociate from the target under the reaction conditions, whereas the longer TPP–DNA probes (DNA 15-mers) were expected to bind tightly to the target and dissociate slowly (or not at all) under the same conditions. The following nomenclature is used throughout the text: mono-TPP–DNA short probes (9m); dual-TPP–DNA short probes (9d); mono-TPP–DNA long probes (15m); dual-TPP–DNA long probes (15d).

Fluorescence activation of 2-STAR probes with mono-TPP–DNAs: We initially tested the reactivity of the dual-quencher (2-STAR) probes. The 2-STAR probe (sequence EC, 100 nM) was co-incubated with the 9-mer (9m) TPP–DNA (600 nM) in the presence of the complementary template

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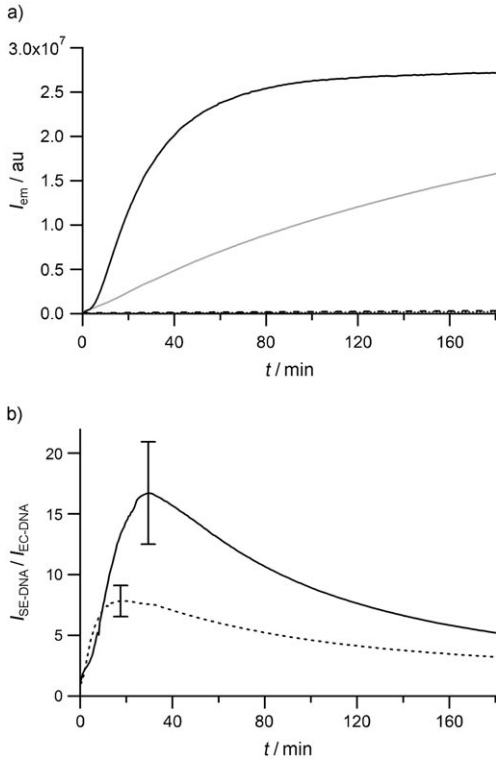

Figure 1. Templated fluorescence activation of 2-STAR probes with reactive probes containing a single TPP moiety. a) Kinetic analysis of template-mediated fluorescence activation of 2-STAR EC by mono-TPP–DNAs. Two TPP–DNA probes of different sequence length were investigated at 37 °C: 9m TPP–DNA (black trace) and 15m TPP–DNA (grey trace). Template dependence was assessed by incubating the probes with the matched template EC-DNA (——), the mismatched template SE-DNA (-----) and without template (•••••). (For a Figure with increased *y* axis see the Supporting Information). b) Comparison of single-nucleotide discrimination of 2-STAR SE (——) and mono-quencher SE (-----) for a weakly destabilizing A–G mismatch. Sequence specificity was calculated by dividing the fluorescence intensity for the reaction mediated by the matched template SE-DNA by the fluorescence intensity for the reaction mediated by the mismatched template EC-DNA. Error bars represent the standard deviation of the mismatch sensitivity at the time point of maximal specificity. Conditions: c(2-STAR) = 100 nM, c(TPP–DNA) = 600 nM, c(template) = 100 nM; tris-borate buffer (70 mM, pH 7.55) containing $MgCl_2$ (10 mM), T = 37 °C.

EC-DNA (100 nM). Fluorescence monitoring revealed that the template efficiently mediated the reaction between 2-STAR EC and 9m TPP–DNA, generating a strong increase in fluorescein emission (λ_{ex} = 494 nm; λ_{em} = 521 nm) (Figure 1a). The fluorescence activation trace exhibited a brief delay time at the beginning of the experiment, consistent with the requirement for two consecutive quencher release events to occur and buildup of non-fluorescent mono-dabsyl intermediates. Nevertheless, the overall rate of 2-STAR activation was rapid and reached 75% fluorescence activation in less than 40 min.

To evaluate the role of probe exchange, we investigated the activation of 2-STAR EC probes by the longer 15-mer (15m) TPP probe (Figure 1a). The rate of fluorescence activation of 2-STAR EC by 15m TPP–DNA was considerably lower than that for 9m TPP–DNA. This finding confirms that slow exchange of TPP–DNA probes impedes two successive binding/reaction events and presumably causes substantial accumulation of non-fluorescent mono-quencher intermediates. This outcome provides strong evidence that probe exchange is required for efficient fluorescence turn-on of 2-STAR probes by single TPP–DNAs (Scheme 1a).

Having established that short mono-TPP–DNAs can activate the fluorescence of 2-STAR probes, we examined whether the double quencher design enhances sequence selectivity and reduces background fluorescence relative to probes with a single quencher. To assess the sequence specificity, we monitored the fluorescence activation of 2-STAR EC as a function of time by using SE-DNA as a single-mismatch-containing template (Figure 1a and Figure S1 in the Supporting Information). The effect of the single T–C mismatch on the rate of fluorescence activation was dramatic. After 115 min, the 2-STAR emission intensity generated by 9m TPP–DNA and the mismatched template reached only (0.9 ± 0.2)% of the value for the complementary EC-DNA template. By comparison, the corresponding value for the control single-quencher probes was (11.0 ± 1.8)% for the same mismatched target.[10] Therefore, double displacement probes improve the single-mismatch specificity for this set of probes by a factor of more than ten. Moreover, the fluorescence generated without the template was as low as (or slightly lower than) that with the singly mismatched template (Figure 1a and Figure S1 in the Supporting Information), demonstrating that double quencher release probes minimize the background fluorescence associated with off-template reactions of the probes. Furthermore, the fluorescence turn-on value of 2-STAR probes (emission of 2-STAR probes after complete activation divided by emission prior to addition of TPP–DNA) was remarkably high ((370 ± 20)-fold), substantially exceeding the values reported for other quencher displacement probes including the control single-quencher probes (61-fold).[10] The high fluorescence turn-on value can be attributed to the presence of two dabsyl groups,[13] which quench the fluorophore of 2-STAR probes with > 99.7% efficiency. Interestingly, this quenching efficiency is higher than that reported for molecular beacon probes containing two dabsyl groups (98.75%),[13] possibly because of the different arrangement of the quenchers/fluorophore in these probes.

These results establish that double quencher release probes enhance the turn-on value of templated fluorescence activation schemes, whereas greatly suppressing off-template reaction signals. Moreover, the experiments with the T–C mismatch-containing SE-DNA template established that probes, relying on two quencher release events provide greatly enhanced sequence discrimination by reducing the signal from the mismatched template. We hypothesize that the enhancement of sequence specificity for double quencher release probes is a stochastic effect; the requirement of two binding/reaction events to occur for fluorescence activation magnifies the difference in total signal-generating rates between the matched and mismatched templates.

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To provide an even more stringent test of selectivity, we further assessed the potential of dual-quencher 2-STAR probes to enhance the sequence-specificity for a weakly destabilizing A–G mismatch in comparison to single-quencher Q-STAR probes. We prepared a second 2-STAR probe (2-STAR SE) complementary to the same target but specific to the sequence of *S. enterica* and tested it in combination with 9m TPP–DNA to discriminate between the complementary target (SE-DNA) and the corresponding sequence of *E. coli* (EC-DNA), which contains the mismatch (Table 1). The minor difference in thermal stability between the matched (A–T) and the mismatched (A–G) renders the sequence selectivity particularly challenging. Incubation of the control mono-quencher SE probe (100 nM) with 9m TPP–DNA (600 nM) and either the matched SE-DNA or the mismatched EC-DNA template (100 nM) provided only moderate selectivity for this challenging mismatch; the fluorescence signal generated after 115 min in the presence of the mismatched target EC-DNA reached (23±7)% of that of the matched target (see the Supporting Information, Figure S3). In contrast, the EC-DNA template-mediated reaction of 2-STAR SE was visibly decelerated relative to the control reaction of Q-STAR SE, providing only (12±4)% background signal relative to the matched target (see the Supporting Information, Figure S2). To analyze this difference quantitatively over time, we plotted the sequence specificity of 2-STAR SE and of mono-quencher SE (fluorescence emission of the reaction with SE-DNA divided by that with EC-DNA) as a function of time (Figure 1b). With this challenging mismatch, the 2-STAR probes reached a maximal sequence selectivity of (16±4)-fold after 29.5 min. In comparison, the sequence selectivity for the mono-quencher case peaked earlier (17.5 min) and maximized at a value of only (7.8±1.3)-fold. Therefore, 2-STAR probes can enhance single-mismatch selectivity even for challenging targets by more than a factor of two.

Fluorescence activation of 2-STAR probes with dual-TPP–DNAs: In principle, probes that carry two TPP groups (dual-TPP–DNAs) could trigger the release of both quenchers and obviate probe exchange (Scheme 1b). Therefore, we evaluated the ability of dual-TPP–DNA probes (9d or 15d, Table 1) to activate 2-STAR probes in a template-dependent configuration. Under the same conditions as for the templated reactions with mono-TPP–DNAs, incubation of 2-STAR EC (100 nM) with 9d TPP–DNA (300 nM) and the EC-DNA template (100 nM) provided a strong fluorescence turn-on signal (Figure 2). Fluorescence activation was slightly more rapid for 9d TPP–DNA than for 9m TPP–DNA, and no lag phase was observable for 9d/15d TPP–DNAs. The experiment with longer the 15d TPP–DNA probe generated similar kinetic traces relative to 9d TPP–DNA, which indicates that fluorescence activation was independent of strand exchange. This result is in strong contrast to the mono-TPP activator probes, which clearly required multiple binding events for 2-STAR activation.

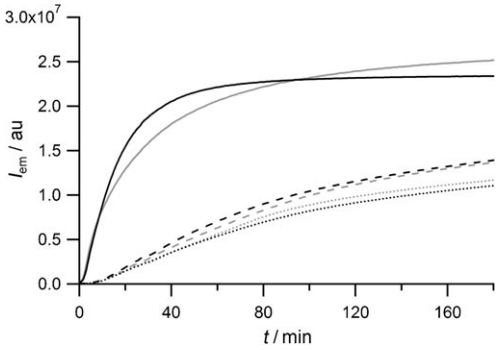

Figure 2. Kinetic analysis of template-mediated fluorescence activation of 2-STAR EC by DNA probes with two TPP modifications. Two dual-TPP–DNA probes of different length were investigated: 9-mer (9d) TPP–DNA (black traces) and 15-mer (15d) TPP–DNA (grey traces). Template dependence was assessed by incubating the probes with the matched template EC-DNA (——), the mismatch-containing template SE-DNA (-----) and without template (•••••). Conditions: *c*(2-STAR EC) = 100 nM, *c*(TPP–DNA) = 300 nM, *c*(template) = 100 nM; tris-borate buffer (70 mM, pH 7.55) containing $MgCl_2$ (10 mM), T = 37 °C.

In contrast to the experiments with mono-TPP–DNAs, which yielded extremely low background signal without template (Figure 1a), dual-TPP–DNA probes generated a considerable off-template background signal and lower selectivity against a singly mismatched template (SE-DNA). The off-template signal for dual-TPP–DNAs was 40-fold higher than with mono-TPP–DNA probes after 115 min (Figures 1a and 2); the mismatch specificity at the same time point was only (2.3±0.7)-fold for 2-STAR EC and 9d TPP–DNA. Decreased mismatch specificity and higher off-template reaction for dual-TPP–DNA probes were anticipated because both quenchers of the 2-STAR probe can be released in a single binding event. However, this background reaction significantly exceeded the level observed previously for single-quencher Q-STAR probes with single TPP–DNA.[10] Therefore, dual-TPP–DNA probes generate increased background reaction by an additional, unknown mechanism. One possible explanation for the high background signal for dual-TPP–DNAs is that the covalent iminophosphorane ylide intermediate for the first Staudinger reduction persists long enough to initiate the second reduction prior to dissociation. Alternatively, the two probes may noncovalently associate because of the increased hydrophobicity of the dual-TPP modification.

2-STAR probes provide amplified detection signal with reduced background reaction: In a previous study, mono-quencher (Q-STAR) probes yielded signal amplification of >75-fold by an isothermal catalytic cycle of target binding, reaction, and dissociation.[6,10] In principle, signal amplification should also be possible for 2-STAR probes. However, the requirement for two sequential quencher release reactions may decrease the rate of fluorescence activation and amplification. Consequently, longer reaction times might be necessary to engender amplified fluorescence activation for 2-STAR probes.

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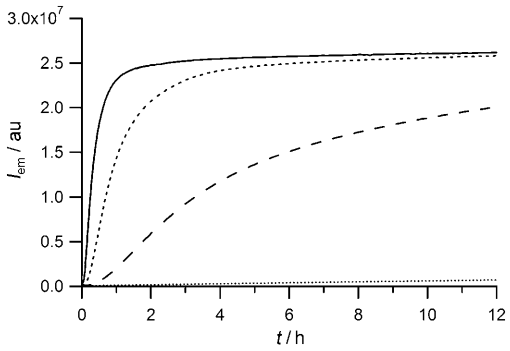

Figure 3. Signal amplification for 2-STAR EC and 9m TPP–DNA with substoichiometric concentrations of the target EC-DNA (c(EC-DNA=100 (——), 20 (-----), 4.0 nM (– – –), and no template (•••••), other conditions are the same as described in Figure 1).

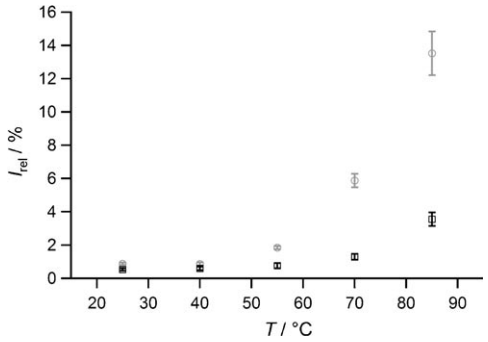

Figure 4. Comparison of the thermal stability of 2-STAR SE (□) and mono-quencher SE (○) probes. The quenched probes were incubated in buffer for 90 min at different temperatures. Fluorescence emission (y axis) indicates the fluorescence intensity of the samples as a percentage of fluorescence intensity of completely activated 2-STAR probes. Conditions: c(Q-STAR) = 100 nM, c(template) = 100 nM; tris-borate buffer (70 mM, pH 7.55) containing $MgCl_2$ (10 mM).

To test the performance of 2-STAR probes under turnover conditions, we incubated 2-STAR EC (100 nM) and 9m TPP–DNA (600 nM) with the EC-DNA template (100, 20, and 4 nM) and measured the intensity of fluorescence as a function of time (Figure 3). Within few hours, the fluorescence emission for the substoichiometric concentrations of EC-DNA approached the level of complete probe activation, which establishes that 2-STAR probes are able to provide an amplified signal corresponding to at least fifteen turnovers. Not surprisingly, the generation of an amplified signal was slower for 2-STAR probes than for mono-quencher probes, and this unfavorable tendency intensified as the concentration of EC-DNA declined. However, this effect was compensated by an overproportional decrease of the signal associated with the off-template reaction between 2-STAR EC and 9m TPP–DNA relative to the mono-quencher EC. After 12 h, the fluorescence of 2-STAR EC in the absence of template reached only $(3.0 \pm 1.0)\%$ of the value measured for complete conversion, compared to $(17.0 \pm 1.6)\%$ for mono-quencher probes.[10] Thus, the data show that 2-STAR probes provide an amplified reporter signal with a higher signal-to-background ratio than corresponding single quencher probes.

Thermal stability of 2-STAR probes: Prolonged incubation in aqueous solution potentially generates background fluorescence for mono-quencher probes because of hydrolysis of the α-azidoether groups.[14] Although the stability of such probes is sufficient at physiological conditions, during this study we observed that these probes release quenchers non-specifically at elevated temperatures. Thermal instability could preclude the application of previous mono-quencher probes in detection assays that require elevated temperatures or thermal cycling. To investigate whether double release probes can provide enhanced thermal stability, we compared the fluorescence levels of 2-STAR SE and mono-quencher SE probes measured after incubation in buffer for 90 min at various temperatures (Figure 4); the fluorescence intensities were normalized to the fluorescence levels corresponding to complete fluorescence activation. At ambient temperature, neither probe showed a significant increase in background fluorescence over 90 min. In contrast, with higher temperatures ($T > 50\,°C$) the fluorescence level increased substantially. For example, mono-quencher SE incubated for 90 min at 85 °C reached a fluorescence level corresponding to $(13.5 \pm 1.3)\%$ probe activation, a degree that could be problematic for certain analytical assays. The fluorescence was significantly lower for 2-STAR SE, reaching only $(3.6 \pm 0.4)\%$ conversion under the same conditions. Thus, the data show that 2-STAR probes have reduced background fluorescence at elevated temperatures and are suitable for applications that require prolonged heating.

Conclusion

We have described a novel strategy to increase both the signal-to-background ratio and the sequence specificity of fluorescence DNA/RNA detection schemes that employ templated chemistry. The design of the presented 2-STAR probes requires that two successive quencher release reactions with TPP–DNA activators occur before fluorescence emerges, minimizing background signal. For example, an unintended reaction with a single TPP group releases one quencher, but leaves a second quencher to maintain the dark state of the probe.

Kinetic analysis revealed that activator DNA probes with a single TPP molecule efficiently induced the fluorescence of 2-STAR probes in a two-step mechanism in which two mono-TPP–DNAs consecutively bind a target adjacent to the 2-STAR probe and successively release both quenchers. In general, the activation kinetics of 2-STAR was rapid and enabled the generation of an amplified detection signal. In contrast to mono-TPP–DNAs, dual-TPP–DNA probes rapidly activated the fluorescence of 2-STAR probes without probe exchange. However, the dual-TPP activator probes yielded a significant template-independent signal and poor sequence selectivity.

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The 2-STAR probes greatly reduced the fluorescence background resulting from unwanted side reactions relative to single quencher probes. This effect was evident for multiple sources of background, including reactions mediated by mismatched templates, off-template reactions between the probes, and thermal hydrolysis of the α-azidoether linker. Furthermore, two dabsyl molecules quench fluorescein more efficiently than a single one[13] and reduce the inherent background fluorescence of the quenched probes considerably. Together, these results clearly illustrate the potential of double release probes to minimize background signal and strongly enhance mismatch selectivity for fluorescence-based genetic identification. The beneficial strategy of carrying out two reactions on a template may be generalizable to multiple classes of templated fluorogenic reactions.

Experimental Section

General: Anhydrous solvents were purchased from Fisher Scientific and used without further purification. Chemical reagents were purchased from either Sigma–Aldrich or Acros and used without further purification. Reagents used for the solid-phase synthesis of oligonucleotides such as phosphoramidites, solid supports, amino modifiers, and reagent solutions for the synthesizer were acquired from Glen Research (Sterling, VA, USA). ^{1}H and ^{13}C NMR spectra were recorded on either a Varian Innova 500 MHz or a Varian Mercury 400 MHz NMR spectrometer. High-resolution mass spectrometry analyses were performed by the UC Riverside Mass Spectrometry Facility. Analytical and semi-preparative high performance liquid chromatography was performed on a LC-CAD Shimadzu liquid chromatograph, equipped with a SPD-M10A VD diode array detector and a SCL 10A VP system controller and by using reverse-phase C18 columns. Fluorescence measurements were performed on a Fluorolog 3 Jobin–Yvon fluorophotospectrometer equipped with an external temperature controller. Oligonucleotide masses were determined by the Stanford University Protein and Nucleic Acid Facility by using a Perspective Voyager-DE RP Biospectrometry MALDI-TOF mass-spectrometry instrument with a 3-hydroxypicolinic acid/diammonium hydrogen citrate matrix. Thermal stability experiments were performed on a Flexstation II 384 microplate reader with a 96-well quartz plate. Oligonucleotide concentrations were determined by UV absorbance at 260 nm for denatured oligonucleotides ($T=90$ °C) by using linear combinations of the nucleobases extinction coefficients.

***N,N′*-bis-[3-(4-Monomethoxytrityl)aminopropionyl]-1,3-diamino-2-propanol (1)**: *N,N′*-dicyclohexylcarbodiimide (0.82 g, 4.0 mmol), 1,3-diamino-2-propanol (0.16 g, 1.8 mmol), and a catalytic amount of 4-(dimethylamino)pyridine (50 mg) were added to a solution of *N*-(4-monomethoxytrityl)-β-alanine[12] (1.45 g, 4.0 mmol) in anhydrous CH_2Cl_2 (10 mL). The mixture was stirred for 14 h at room temperature. The formed precipitate was eliminated by filtration and the product was purified by silica column chromatography (hexane/EtOAc 2:3 + 0→5% MeOH + 2% triethylamine (TEA)) to provide **1** as a white foam (1.2 g, 86%). ^{1}H NMR (500 MHz, $CDCl_3$): $\delta=2.05$ (br s, 2H; NH), 2.36 (t, $^3J(H,H)=6.0$ Hz, 4H; CH_2), 2.46 (t, $^3J(H,H)=6.0$ Hz, 4H; CH_2), 3.24–3.30 (m, 2H; CH_2), 3.39–3.45 (m, 2H; CH_2), 3.76 (s, 6H; CH_3), 4.05–4.18 (m, 1H; CH), 6.80 (d, $^3J(H,H)=9.0$ Hz, 4H; Ar-H), 6.96 (t, $^3J(H,H)=6.0$ Hz, 2H; NH), 7.17 (t, $^3J(H,H)=7.5$ Hz, 4H; Ar-H), 7.26 (t, $^3J(H,H)=7.5$ Hz, 8H; Ar-H), 7.33 (d, $^3J(H,H)=9.0$ Hz, 4H; Ar-H), 7.42 ppm (d, $^3J(H,H)=8.0$ Hz, 8H; Ar-H); ^{13}C NMR (500 MHz, $CDCl_3$): $\delta=37.13$, 39.93, 42.86, 55.23, 70.52, 113.21, 126.38, 127.92, 128.53, 129.84, 137.85, 146.01, 157.94, 174.12 ppm; HRMS [+ scan]: *m/z*: calcd for $C_{49}H_{53}N_4O_5$: 777.4011; found: 777.4014.

***N,N′*-bis-[3-(4-Monomethoxytrityl)aminopropionyl]-1,3-diamino-2-propanol 2-cyanoethyl diisopropylphosphoramidite (2)**: 2-Cyanoethyl-*N,N*-diisopropyl chlorophosphoramidite (183 mg, 0.77 mmol) was added to a solution of *N,N′*-bis-[3-(4-monomethoxytrityl)aminopropionyl]-1,3-diamino-2-propanol (300 mg, 0.39 mmol) and diisopropylethylamine (168 μL, 0.97 mmol) in anhydrous MeCN (3 mL). The solution was stirred for 90 min at room temperature under an argon atmosphere and concentrated under vacuum. The residue was purified by silica column chromatography (hexane/EtOAc/MeCN 7:3:1 + 2% TEA) to provide **2** as a white foam (368 mg, 97%). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=1.16$–1.21 (m, 12H; CH_3), 2.35–2.55 (m, 10H; CH_2), 2.92–3.00 (m, 1H; CH), 3.08–3.19 (m, 1H; CH), 3.55–3.66 (m, 2H; CH_2), 3.70–3.82(m, 10H; CH_3 and CH_2), 3.88–3.98 (m, 1H; CH), 6.71–6.81 (m, 6H; Ar-H and NH), 7.13–7.18 (m, 4H; Ar-H), 7.22–7.28 (m, 8H; Ar-H), 7.32–7.36 (m, 4H; Ar-H), 7.43–7.46 ppm (m, 8H; Ar-H); ^{13}C NMR (400 MHz, $CDCl_3$): $\delta=20.45$ (20.38), 24.72 (24.57), 37.30 (37.24), 39.96, 40.56, 43.16 (43.03), 55.12, 58.41 (58.20), 70.37, 113.06, 118.25, 126.17, 127.78, 128.49, 129.78, 138.04 (138.02), 146.19, 157.71, 172.74, 173.14 ppm (the values in parentheses refer to the signals that are different for the second diastereomer); HRMS [+ scan]: *m/z*: calcd for $C_{58}H_{70}N_6O_6P$: 977.5089; found: 977.5106.

Unmodified oligonucleotides: Oligonucleotides were synthesized on a 1 μmol scale by an ABI model 392 synthesizer using standard β-cyanoethylphosphoramidite coupling chemistry. Removal of the protecting groups and cleavage from the CPG support were carried out by incubation in concentrated aqueous NH_4OH solution at 55 °C for 14 h. The oligonucleotides were purified by using Poly-Pak II cartridges. The identity and purity of the probes was confirmed by MALDI-TOF mass spectrometry (see the Supporting Information, Table S1).

Preparation of 2-STAR probes: Oligonucleotides were synthesized with the 5′-bis-amino-modifier **2** appended to the 5′ terminus. The MMt protecting groups were removed on the DNA synthesizer by using alternating cycles of deprotection reagent (3% trichloroacetic acid in CH_2Cl_2) and CH_2Cl_2 washes. The solid support was added to a solution containing compound **2** (25 mM), benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP, 25 mM), and diisopropylethylamine (50 mM) in DMF (250 μL) and gently shaken at room temperature for 5 h protected from light. The DMF was decanted, the resin washed twice with DMF and three times with MeCN, and dispersed in aqueous NH_3/$MeNH_2$ deprotection/cleavage solution (1 mL) and incubated for 1 h at 55 °C. Beads were removed by filtration and the oligonucleotide probes were purified by reverse phase HPLC. The purity of the Q-STAR probes was assessed by analytical HPLC and found to be >95% with a minor impurity that contains two dabsyl and one fluorescein molecules. Mono-quencher Q-STAR probes were prepared as described previously.[10]

Dual-TPP–DNA conjugates: Oligonucleotides were prepared by reverse (5′→3′) synthesis by using the 5′-bis-amino-modifier **2** as the terminal phosphoramidite for modification of the 3′ terminus. The MMt protecting groups were removed on the synthesizer by using alternating cycles of deprotection reagent (3% trichloroacetic acid in CH_2Cl_2) and CH_2Cl_2 washes. The solid support was added to a solution containing 4-(diphenylphosphino)benzoic acid (0.1 M), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (0.1 M), and diisopropylethylamine (0.2 M) in DMF (500 μL). Air trapped by the solid support was removed by briefly vacuumizing the mixture followed by backfilling with argon. The reaction mixture was gently shaken for 2.5 h at room temperature. DMF was decanted, the resin washed twice with DMF and three times with MeCN, dispersed in aqueous NH_4OH/$MeNH_2$ deprotection/cleavage solution (1 mL) containing the oxygen scavenger tris-(2-carboxyethyl)phosphine (4 mg) and incubated for 1 h at 55 °C. The solid support was removed by filtration, the solution concentrated (60 min) on the Speed-Vac to remove the volatile amines. Dual-TPP–DNAs were purified by semi-preparative reverse-phase HPLC concentrated on the Speed-Vac, divided in aliquots, flushed with argon and stored at −78 °C. Samples were used within one month after preparation to ensure maximal reactivity of the probes. Mono-TPP–DNAs were prepared as described previously.[10]

Kinetic analysis of 2-STAR fluorescence activation: 2-STAR probes (100 nM) and the corresponding template (100 nM, unless stated differently) were incubated at 37 °C in tris-borate buffer (70 mM, pH 7.55) containing $MgCl_2$ (10 mM). TPP–DNA (600 nM for mono TPP–DNA and

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300 nM for dual-TPP–DNA) was added and the fluorescence emission (λ_{ex} = 494 nm, λ_{em} = 521 nm) was measured as a function of time.

Analysis of thermal stability of 2-STAR probes: A solution containing either the mono-dabsyl or 2-STAR probe (100 nM) and the template strand SE-DNA (100 nM) was incubated for 90 min without TPP–DNA in tris-borate buffer (70 mM, pH 7.55) containing $MgCl_2$ (10 mM) at the specified temperature. The solutions were cooled for 10 min on ice. Fluorescence signals were measured by using a 96-well microplate reader. As a reference for complete conversion, the Q-STAR or 2-STAR probe (100 nM) was incubated for 5.5 h at 25 °C with SE-DNA (100 nM) and 9m TPP–DNA (600 nM).

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