Published in final edited form as:

Chembiochem. 2010 October 18; 11(15): 2132–2137. doi:10.1002/cbic.201000329.

Templated Chemistry for Sequence-Specific Fluorogenic Detection of Duplex DNA

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Abstract

We describe the development of templated fluorogenic chemistry for detection of specific sequences of duplex DNA in solution. In this approach, two modified homopyrimidine oligodeoxynucleotide probes are designed to bind by triple helix formation at adjacent positions on a specific purine-rich target sequence of duplex DNA. One fluorescein-labeled probe contains an α -azidoether linker to a fluorescence quencher; the second (trigger) probe carries a triarylphosphine, designed to reduce the azide and cleave the linker. The data showed that at pH 5.6 these probes yielded a strong fluorescence signal within minutes on addition to a complementary homopurine duplex DNA target. The signal increased by a factor of ca. 60, and was completely dependent on the presence of the target DNA. Replacement of cytosine in the probes with pseudoisocytosine allowed the templated chemistry to proceed readily at pH 7. Single nucleotide mismatches in the target oligonucleotide slowed the templated reaction considerably, demonstrating high sequence selectivity. The use of templated fluorogenic chemistry for detection of duplex DNAs has not been previously reported and may allow detection of double stranded DNA, at least for homopurine-homopyrimidine target sites, under native, non-disturbing conditions.

Keywords

DNA recognition; triplex; fluorescent probes; templated chemistry

Introduction

The detection, identification and imaging of nucleic acids are of broad interest and utility in biology and medicine. Most existing methods of DNA detection use single-stranded DNA (ssDNA) as the analyte due to the requirement for hybridization of a probe to the analyte as a means of sequence recognition.^[1] However, double-stranded DNA (dsDNA) is the principal form of genomic information and the product of the polymerase chain reaction, making it a target of interest for analytical assays. Current examples of sequence-specific dsDNA detection methods include fluorophore-modified polyamides^[2] and oligonucleotides^[3] as well as protein complementation assays based on zinc-finger proteins.^[4] Because of the relative inaccessibility of dsDNA for sequence-specific binding, the development of detection assays for dsDNA has significantly lagged behind that for ssDNA. For example, template-mediated fluorescence activation has been established as a competent strategy to detect ssDNA but previous attempts to report dsDNA by this method

required denaturation of the duplex prior to detection.^[1] Such steps add to the complexity of the methods and instrumentation required, and preclude applications in living systems. Consequently, it is at present difficult to image or detect double-stranded DNA of specific sequences under native, non-heating and non-denaturing conditions.

A recent goal of several research groups has been the development of templated chemistry to detect nucleic acids.^[5] Template-mediated fluorescence activation reports on the presence of an analyte DNA or RNA sequence by the reaction of two chemically modified oligonucleotides upon hybridization to the target at adjacent positions, eliciting a fluorescence turn-on signal.^[6] This non-enzymatic approach can exhibit exceptional single-nucleotide specificity, provide isothermal signal amplification, and display a high tolerance for biological contaminants. However, double-stranded DNA has received very limited attention as a template for acceleration of chemical reactions. The few existing examples include triple-helix-forming oligonucleotides that bind via Hoogsteen base pairs^[7] or polyamides that associate in the minor groove.^[8] These reactions had limitations such as little or no fluorescence activation, short recognition sequences or dependence on acidic pH conditions. As such, efficient template-mediated fluorescence activation for the detection of dsDNA at physiological pH remains untested.

Here we explore the direct non-enzymatic detection of specific sequences of duplex DNA by fluorescence. In this approach, pairs of reactive DNA probes are designed to bind at adjacent sites on a DNA target by triple helix formation, triggering a templated fluorogenic reaction. To this end, we describe the design, synthesis and study of azidoether-containing quenched probes (Q-STAR probes), which are activated by reaction with adjacently hybridized triarylphosphine probes. Our results show that the templated chemistry can be carried out at room temperature and neutral pH to generate a robust fluorescence signal with high sequence specificity. Such a mode of fluorescence reporting may in the future allow detection, imaging and quantitative analysis without disturbing or destroying the targeted duplex DNA.

Results

Probe design and synthesis

The molecular strategy for templated chemistry employed in these experiments is illustrated in Figure 1. To ensure thermal stability of the DNA duplex, we designed a hairpin template (Table 1, entry **2**) with a 28 bp stem and a 4 nt loop (Figure 2); the stem sequence consists of a Watson-Crick base-paired polypurine tract (the target site). Two probes (14 nt each) were designed to bind side-by-side in a parallel manner (Figure 2a) on this target DNA by “pyrimidine motif” triple helix formation, in which thymine recognizes an A-T base pair and cytosine recognizes a G-C pair, forming Hoogsteen pairing interactions in the target DNA major groove (Figure 2b).^[9] Fully complementary pairing requires protonation of cytosine in the probe strand, which is promoted at acidic pH values.^[10]

To engender a fluorescence signal, we chose a reaction recently reported to proceed successfully on single-stranded DNA targets.^[11] In this approach, a fluorescein-labeled probe contains a dabsyl quencher conjugated to the DNA terminus via an α -azidoether

linker. A separate trigger probe carrying a phosphine group is designed to reduce the azide. Templated chemistry allows positioning of the two reacting groups in close proximity, resulting in azide reduction and rapid linker hydrolysis, thus releasing the quencher and activating the fluorophore. This quenched Staudinger-triggered α -azidoether release (Q-STAR) chemistry has been recently used to effectively detect single-stranded DNA and RNA targets. [11]

For the triplex-forming probes, we prepared 14-mer homopyrimidine oligodeoxynucleotides (Figure 2), one appended with a triphenylphosphine [12], and the second carrying fluorescein and conjugated with a 5'-terminal α -azidoether/quencher group. The quencher-linker was prepared as described previously, and the phosphine probe was synthesized by conjugating the *N*-hydroxysuccinimidyl 4-(diphenylphosphino) benzoate ester to amino-modified DNA. [5j] Successful probe conjugation was confirmed by MALDI-mass spectrometry (see Supporting Information (SI)).

Optimization of target binding

Our first experiments were directed at determining whether the duplex target could indeed template this fluorogenic reaction, and tested the effect of varied spacing between the probes. Since the triple helix is more crowded than a duplex as a result of a third strand occupying the major groove, it seemed possible that the reactive moieties and their linkers might require added space for optimal reaction. Thus, we prepared additional targets in which the designed probe binding sites were separated by 1-3 base pairs (Table 1, entry 3-5); these were compared to the duplex in which the probes were designed to bind without intervening base pairs. Probes were incubated at 200 nM (Q-STAR probe) and 600 nM (TPP probe) in the presence of 200 nM duplex target DNA in a pH 5.6 buffer containing 10 mM Mg^{2+} and 70 mM tris-acetate at 25 °C.

Initial tests of the Q-STAR and TPP probes with the target lacking an intervening space between probes (Table 1, Entry 2) showed the appearance of a distinct fluorogenic signal in the presence of the target (Figure 3). The reaction was significantly slower than a comparison reaction with a single-stranded DNA template (which reached completion in ca. 20 min), but still proceeded readily, approaching maximal conversion within ~3 h. A control experiment reaction lacking the target DNA showed insignificant increase of the signal over this time, demonstrating that the background signal in the absence of template is extremely low.

Comparisons of reaction profiles in the presence of the target DNAs containing 1-3 base pairs between the probe binding sites showed that while reactions proceeded in all cases (Figure 4 and SI), rates of fluorescence appearance substantially decreased with increasing number of nucleotides between the hybridization sites; the reaction with the target with three spacers was the slowest, with an initial rate ca. one-sixth that of duplex target without spacer. These results established that the optimum probe binding geometry for this reaction is the coaxial side-by-side arrangement.

Sequence selectivity at pH 5.6

Next we tested the effect of single mismatched base pairs in the duplex template on the rate of the reaction. Separate mismatched target DNAs containing noncomplementary G-C, T-A or C-G pairs (Table 1, entries 6-8) were synthesized and their effects on templated fluorescence activation were compared to the fully complementary DNA. Based on previous studies with single-stranded DNA targets, we placed the mismatch at a central position in the Q-STAR probe-binding site, where it is expected to have a maximal effect^[5a] on probe binding. The results for these reactions are shown in Figure 5. All three mismatched templates resulted in reactions substantially slower (by factors of ca. 7-17 in initial rates) than that of the fully complementary template (see SI), confirming single nucleotide selectivity. Among the three mismatched cases, the template corresponding to the T-CG mismatch generated the highest rate of fluorescence activation (about one-seventh the initial rate of the complementary template). This is consistent with previous studies that have shown the T-CG mismatch to be the least destabilizing of these three.^[13]

Pseudoisocytosine probes for dsDNA detection at neutral pH

The formation of standard pyrimidine-motif triple helices depends on protonation of cytosine, which is promoted at acidic pH.^[10] As anticipated, when we tested the above probes for their ability to perform the templated fluorogenic chemistry at pH 7.0, no reaction was observed under these conditions (see SI). To overcome the requirement for acidic reaction conditions, we designed and synthesized a pair of modified probes that allow triplex formation at physiological pH. As studied by Kan,^[14] 2'-pseudoisocytosine (Ψ C) is a neutral analogue of the protonated cytosine base, containing a hydrogen-bond donor at N³ (Figure 6a). This modification allows for Hoogsteen base pairing with guanine without the need for protonation (Figure 6b). We incorporated this commercially available nucleoside into our Q-STAR and TPP probes, replacing C at every position with Ψ C, without further modifications to the synthetic procedure.

The Ψ C Q-STAR and Ψ C TPP probes were then tested for their ability to carry out the templated chemistry at neutral conditions (pH 7.0) in a buffer otherwise the same as the previous experiments (10 mM MgCl₂, 70 mM tris-acetate, 25°C). Representative data are shown in Figure 7; the results show that the probes do indeed yield a template-dependent fluorogenic signal, showing a 25-fold fluorescence increase at 3 h. We further compared the new probes' ability to perform at the previous low-pH conditions (pH 5.6) (see SI), and found that the Ψ C probes were much less dependent on pH as compared to the probes containing cytosine, although we did observe faster reaction kinetics at more acidic pH.

We then tested the Ψ C probes' ability to discriminate between single base mismatches as we did previously with the cytosine-containing probes. Interestingly, the new Ψ C probes showed high selectivity against the mismatched targets (Figure 7), showing discrimination considerably greater than that seen for the cytosine-containing probes. The Ψ C probes yielded signals with the mismatched targets after 3.5 h that were 10- to 14-fold lower than for the correct target, and near that of the background reaction in which no target DNA was present. The initial rate of fluorescence for the Ψ C probes with matched template was also much higher than the mismatched templates (see SI).

Discussion

Our data establish that templated fluorogenic chemistry can be used to detect a specific sequence of double-stranded DNA with single nucleotide selectivity. We are unaware of prior reports of the use of templated chemistry in fluorogenic reporting of duplex DNA through triplex formation. However, there do exist a few prior reports of templated chemistry being performed in the context of DNA triple helices. Nearly two decades ago, Dervan demonstrated triplex-mediated chemical ligation of dsDNA, ^[7a] and we described the cyclization of DNAs by triplex formation on a single-stranded DNA template. ^[15] In related work, Nicolaou described the use of N-cyanoimidazole to form a phosphodiester bond in templated formation of triplex structures as part of a self-replication scheme. ^[7b] More recently, Mokhir demonstrated templated ester hydrolysis in triple helices. ^[7c]

The templated reaction rate we observe on a duplex target (involving a triple helical reactive complex) is slower than the analogous reaction on a single-stranded template, which was reported previously. ^[11] One possible explanation for the rate difference is that the reactions take place with different geometries. Since in both cases the reaction proceeds optimally with contiguous placement of the probes, this suggests that the reacting groups are not positioned between the probes, but rather are positioned elsewhere, either in the major/minor grooves or outside the helix altogether. In the duplex case (i.e., targeting single-stranded DNA to form a duplex) all of these may well be possible, but the fact that the triplex case is slower suggests that the preferred orientation is in the major groove. Since the triplex-forming probes occupy the major groove, this preferred orientation may be sterically hindered to some degree.

The present experiments combined with earlier results ^[11, 12b] demonstrate that Q-STAR probes are widely applicable DNA detection tools that can be used to detect both single and double-stranded DNA *in vitro*. The templated quencher release by Staudinger reduction proceeds with rapid reaction kinetics and exhibits high bioorthogonality. In the present triplex context, the Q-STAR templated chemistry is simple to perform in practice, requiring only addition of probes to the analyte (a duplex sample) followed by monitoring of fluorescence at room temperature. The fact that no denaturation or strand separation steps are required also contributes to the simplicity, as separate handling and thermocycling equipment are unnecessary. Moreover, our current experiments show that by replacing the cytosine on probes with 2'-pseudoisocytosine, the templated reaction can take place at physiologically relevant pH.

In a practical sense, the current probe design could potentially have utility in applications where small amounts of DNAs are present; for example, post-PCR sequence confirmation or after plasmid preparation. Of course, for the current approach there is a strict sequence limitation, requiring a polypurine target sequence to form the pyrimidine-purine-pyrimidine triple helix. In PCR such a sequence could be incorporated into a primer; a similar marker sequence could also be engineered into plasmids. Further studies will be needed to determine whether purine runs shorter than 28 bp, or containing a small number of interrupting pyrimidines, could also be used as targets for this templated chemistry.

Experimental Section

Material and methods

Anhydrous solvents were purchased from Fisher Scientific and used without further purification. Chemicals were purchased from either Sigma-Aldrich or Acros and used without further purification. Chemicals used for the solid-phase synthesis of oligonucleotides such as phosphoramidites, solid-supports, amino-modifiers, and synthesizer reagent-solutions were acquired from Glen Research. 2'-Deoxypseudoisocytidine CEP was purchased from Berry and Associates. Hairpin DNA templates used for fluorescent activation experiments were synthesized by the Stanford Protein and Nucleic Acid 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 using reverse phase C18 columns. Fluorescence measurements were performed either on the Fluorolog 3 Jobin Yvon fluorophotospectrometer equipped with an external temperature controller or the Flexstation II 384 microplate reader with built-in temperature control. Oligonucleotide masses were determined by the Stanford University Protein and Nucleic Acid Facility using a Perspective Voyager-DE RP Biospectrometry MALDI-TOF mass-spectrometry instrument using a 3-Hydroxypicolinic acid/di-ammonium hydrogen citrate matrix.

Synthesis of oligonucleotides

Unmodified oligonucleotides were synthesized on a 1 μ mol scale on 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 $^\circ\text{C}$ for 14 h unless otherwise specified. The oligonucleotides were purified using Poly-Pak II cartridges. Oligonucleotide concentrations were determined by UV-absorbance using extinction coefficients derived by the nearest neighbour approximation. The identity of the strands was confirmed by MALDI-TOF mass spectrometry.

Preparation of TPP-DNA conjugates

Oligonucleotides were synthesized on a 3'-PT-amino-modifier C3 CPG (Glen Research, cat. no. 20-2954-41) solid support and cleaved/deprotected as described for standard oligonucleotides, or incubated in concentrated aqueous NH_4OH solution at 25 $^\circ\text{C}$ for 24 hours and deprotected/purified using PolyPak II cartridges for oligonucleotides containing pseudoisocytosine. Amino-oligonucleotides (1 μ mol) were dissolved in 100mM sodium tetraborate (1.35 mL, pH 8.48) containing the sacrificial oxygen scavenger tris (2-carboxyethyl) phosphine (5.0 mg) and carboxy triphenylphosphine (TPP) N-hydroxysuccinimide ester (8.1 mg) in DMF (1.15 mL) was added to the solution. The suspension was placed under vacuum, backfilled with argon and incubated at 25 $^\circ\text{C}$ for 4 h with shaking. The TPP conjugated oligonucleotide was purified by ethanol precipitation, followed by reverse-phase HPLC. As the TPP-DNA probe is sensitive to oxidation by atmospheric oxygen, stock solutions were flushed with Argon, stored in small aliquots, and used within a month following synthesis.

Preparation of Q-STAR probes

Oligonucleotides were synthesized with a 5'-amino-modifier 5 (Glen Research, cat. no. 10-1905-90) appended to the 5'-terminus. Q-STAR probe containing deoxypseudoisocytines were synthesized on a 3'-phosphate CPG solid support as the first nucleotide to be incorporated was a Ψ C. The monomethoxy-trityl protecting group was removed on the synthesizer using alternating cycles of deprotection reagent (3 % trichloroacetic acid in DCM) and DCM washes. The solid support was added to a solution containing methyl 4-[(1-azido-3-dabsylsulfonamidopropoxy)methyl]benzoate (25mM), benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP, 25 mM), and diisopropylethylamine (50 mM) in DMF (500 μ L) and was shaken for 5 h protected from light. The DMF was decanted, the resin washed twice with MeCN, and dispersed in aqueous $\text{NH}_4\text{OH}/\text{MeNH}_2$ 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.

DNA sample preparation

Duplex DNA dissolved in water was annealed by heating to 90°C for 10 min and cooling down to room temperature.

Fluorescence time-course measurement

Q-STAR probes (200 nM) and the corresponding template (200 nM) were incubated at 25°C in tris-acetate buffer (70 mM, pH 5.6 or pH 7.0) containing MgCl_2 (10 mM). TPP probe (600 nM) was added and the fluorescence emission ($\lambda_{\text{ex}} = 494 \text{ nm}$; $\lambda_{\text{em}} = 521 \text{ nm}$ for Q-STAR) measured as function of time. Signal enhancement was calculated by taking the ratio of fluorescent reading at the indicated time to the initial emission upon TPP addition. Initial rate is calculated by taking slope of the emission curve when the steady-state is reached at the indicated time.

Acknowledgements

This work was supported by the U.S. National Institutes of Health (GM068122). HL acknowledges an A*STAR NSS scholarship, and RMF acknowledges a Stanford Graduate Fellowship.

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Figure 1. Strategy for detection of double-stranded DNA by template-mediated fluorescence activation of Q-STAR probes. Q-STAR and TPP-DNA probes bind to a complementary sequence of dsDNA through Hoogsteen base pairing. The proximity positions the triarylphosphine to cleave the α -azidoether linker on the Q-STAR probe, releasing the dabsyl quencher and yielding a fluorescence turn-on signal.

Figure 2.

Triplex formation through Hoogsteen base pairing of DNA probes to hairpin template. (A) Sequence of probes and hairpin target. (B) Structures of Hoogsteen pairing, involving T•AT and C⁺•GC triplet (note C⁺ = N³-protonated C).

Figure 3.

Fluorescence activation of Q-STAR probes on matched duplex DNA without intervening space between Q-STAR and TPP probes. Average traces ($n=3$) are shown for complementary single stranded template (ssDNA), hairpin template (dsDNA) and in the absence of DNA template upon TPP probe addition. Conditions: 200 nM Q-STAR probe, 200 nM DNA template, 600 nM TPP probe, 70 mM tris-acetate buffer (pH 5.6) containing 10mM MgCl₂, 25°C; $\lambda_{\text{ex}} = 494 \text{ nm}$, $\lambda_{\text{em}} = 521 \text{ nm}$.

Figure 4.

Effect of varying distance between the 3' triphenylphosphine moiety on TPP probe and the 5' azidoether linker on Q-STAR probe. Shown are initial reaction rates of kinetic traces ($n = 4$) of fluorescence activation after addition of TPP probe to hairpin templates containing no spacer, 1 spacer (C), 2 spacers (CG) and 3 spacers (CGC) (entries 3-5 in Table 1). Reaction conditions are as in Figure 3. Rate was determined by measuring the slope of the initial kinetics (2-5 min) after reaction reached steady-state.

Figure 5. Average kinetic traces ($n = 4$) of fluorescence activation for matched (T·AT) and single nucleotide mismatch containing (T·GC, T·TA, T·CG) templates using cytosine-containing probes. Reaction conditions are as in Figure 3.

Figure 6. Structure of 2'-deoxypseudoisocytidine as nucleoside (a) and as a member of the ΨC-GC triplet, ^[14] forming Hoogsteen hydrogen bonds with G in the Watson-Crick G-C basepair.

Figure 7.

Sequence specificity of C Q-STAR for detection of duplex DNA at neutral pH. Average time courses ($n = 3$) after C TPP probe addition to complementary and single base pair-mismatched templates are shown. No significant reaction is observed for the 3 mismatched templates; the curves for the T-TA and T-GC coincide with the curve where no DNA template is added. Conditions: 200 nM C Q-STAR probe, 200 nM DNA template, 600 nM C TPP probe, 70 mM tris-acetate buffer (pH 7.0) containing 10mM MgCl₂, 25°C; $\lambda_{\text{ex}} = 494$ nm, $\lambda_{\text{em}} = 521$ nm

Table 1

Sequence of DNA used as target strands for templated fluorescent probe activation. The 4 cytosines in the loop region of the hairpin are underlined. Entry 1 is the single-stranded DNA complementary to Q-STAR and TPP probes. Entry 2 is the complementary duplex template with no intervening space between the probes. Entries 3-5 are templates with 1, 2 and 3 nucleotide spacers (in bold) respectively. Entries 6-8 are templates containing no intervening nucleotide spacer and 1 single nucleotide mismatch at indicated position (bold, underlined).

Entry	Sequence of Hairpin Template
1	5' GAA AGA AAA AGA GAA AGA AAA AAA AGA A 3' 5' AAG AAA AAA AAG AAA GAG AAA AAG AAA GCC CCC TTT CTT
2	TTT CTC TTT CTT TTT TTT CTT 3' 5' AAG AAA AAA AAG AAC AGA GAA AAA GAA AGO CCC CTT TCT
3	TTT TCT CTG TTC TTT TTT TTC TT 3' 5' AAG AAA AAA AAG AAC GAG AGA AAA AGA AAG CCC CCT TTC
4	TTT TTC TCT GOT TCT TTT TTT TCT T 3' 5' AAG AAA AAA AAG AAC GCA GAG AAA AAG AAA GCC CCC TTT
5	CTT TTT CTC TCGTT CTT TTT TTT CTT 3' 5' AAG AAA AAA AAG AAA GAG AAG AAG AAA GCC CCC TTT CTT
5	CTT CTC TTT CTT TTT TTT CTT 3' 5' AAG AAA AAA AAG AAA GAG AAT AAG AAA GCC CCC TTT CTT
7	ATT CTC TTT CTT TTT TTT CTT 3' 5' AAG AAA AAA AAG AAA GAG AAC AAG AAA GCC CCC TTT CTT
8	GTT CTC TTT CTT TTT TTT 3'