Fluorescence-based detection of single nucleotide permutation in DNA *via* catalytically templated reaction†

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Templated reduction of low fluorescence azidocoumarin–PNA conjugate to high fluorescence aminocoumarin was achieved using a catalytic amount of DNA with single nucleotide resolution.

Oligonucleotide-templated reactions have attracted tremendous interest for their implication in prebiotic chemistry and as a means to program and orchestrate designed reactions.[1] An important application of such reactions is the sequence specific detection of DNA and RNA which is of paramount importance in biomedical research.[2] For oligonucleotide detection, it is desirable to have a reaction where the template can turnover so to provide an amplification of the DNA or RNA signal with a single nucleotide resolution. Several strategies have already been reported notably based on autoligation,[3–6] hydrolysis,[7,8] the Staudinger reaction[9] and chemical ligations;[10–12] however, few methods were demonstrated to be catalytic in the template. Recently Seitz and Grossmann reported a system based on a transthioesterification to transfer a quencher group from one sequence to another with good turnover;[13] however, the impact of mutations was only reported for a pyrimidine to purine mutation.

Herein, we report the application of a system based on a masked 7-azidocoumarin–PNA conjugate which is unmasked *via* a Staudinger reaction by a triphenylphosphine–PNA conjugate (Fig. 1). The Staudinger reaction was seen as ideal based on its documented compatibility with cellular chemistry.[14,15] However, we found it important that the change in fluorescence be linked to the azide reduction rather than phosphine oxidation as we found it difficult to avoid partial phosphine oxidation in biologically relevant conditions.[9,16] For the oligonucleotide recognition, peptide nucleic acids (PNAs)[17,18] were deemed the optimal choice as they are more resistant to enzymatic or chemical degradation than natural oligonucleotides. Furthermore, several modifications have been reported to enhance their cellular permeability[19,20] and short PNAs do not activate an antisense response, suggesting that this approach may be compatible with cellular sensing of oligonucleotides.

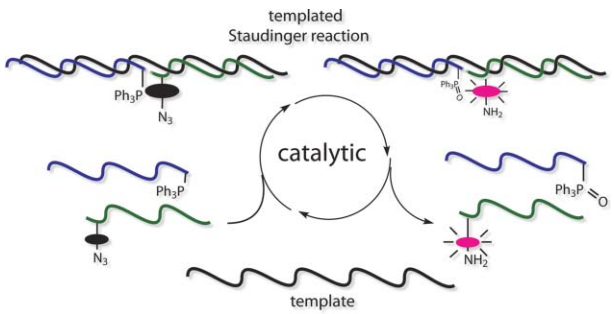

Fig. 1 The catalytic cycle of the templated Staudinger reaction yielding a fluorescent product.

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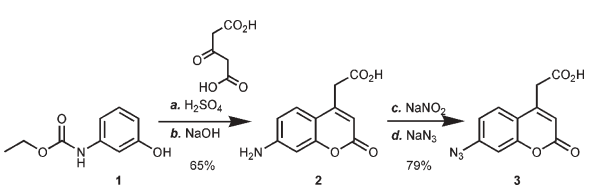
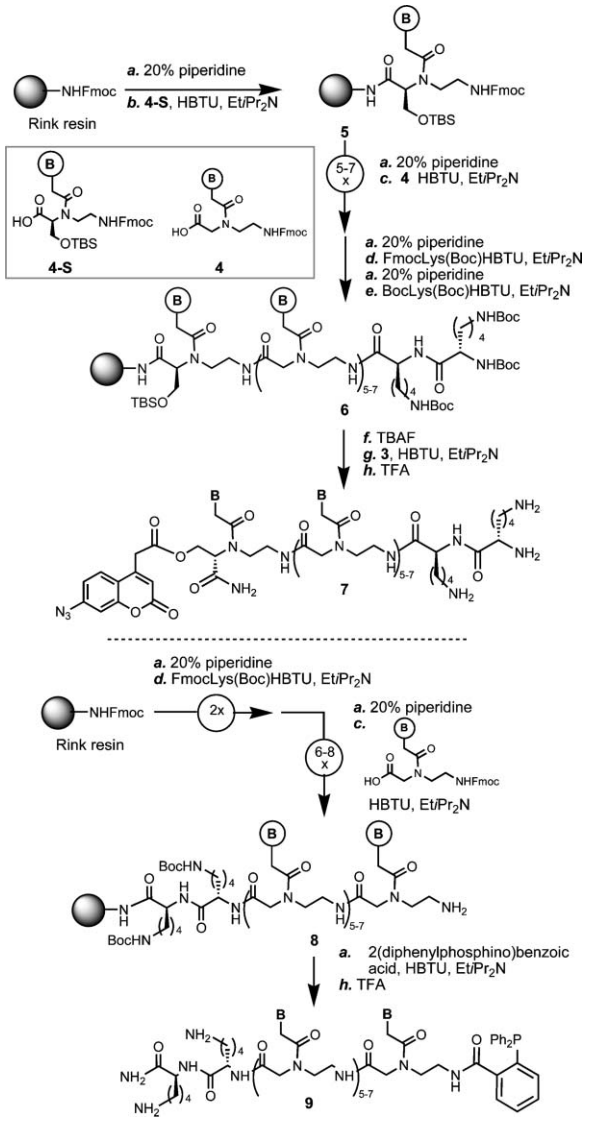
The suitably derivatized 7-azidocoumarin **3** was prepared according to modifications of known procedures[21,22] as summarized in Scheme 1. Thus, treatment of **1** with 1,3-acetonedicarboxylic acid in 70% sulfuric acid followed by hydrolysis of the ethyl carbamate afforded the 7-aminocoumarin **2**. Diazotization of **2** followed by sodium azide displacement afforded the required azidocoumarin **3** (51% yield for four steps).

In order to minimize the distance between the phosphine and azide upon hybridization, a PNA backbone modification was used to append the masked fluorophore at the C-terminus of the PNA. As shown in Scheme 2, monomer **4-S**, which has an *N*-(2-aminoethyl)serine backbone with a TBS-protected side chain, was loaded on Rink resin, affording resin **5** which was engaged in standard automated Fmoc synthesis thus leading to **6**. The silyl group was then removed using TBAF and the fluorophore was introduced *via* acylation. Cleavage of the final compounds with TFA : *meta*-cresol (4 : 1) afforded the labelled PNAs **7**. The phosphine was introduced at the N-terminus of the PNA oligomers **8** by coupling with 2-(diphenylphosphino)benzoic acid and the oligomers were released from the polymer using TFA : *meta*-cresol (4 : 1) thus affording phosphino–PNAs **9**. For all oligomers, two lysine residues were incorporated into the sequence to improve water solubility and disrupt potential aggregations.

We next investigated the change in fluorescence upon unmasking of the azidocoumarin **3** and its PNA conjugate **7** with excess phosphine (1 mM tris(carboxyethyl)phosphine). It is known that the fluorescence quantum yield of coumarins is strongly influenced by substituents in the 7-position as it alters the relative energies between the emissive state and the non-emissive state.[23] Electron



Scheme 1 Synthesis of 7-azidocoumarin-4-acetic acid **3**.



Scheme 2 Synthesis of coumarin–PNA conjugate **7** and phosphine–PNA conjugate **9**.

donor substituents at the 7-position and polar solvents lower the energy level of the emissive state relative to the non-emissive state thereby increasing the quantum yield of the fluorophore. Accordingly, a 22-fold increase in fluorescence was observed upon reduction of azidocoumarin **3** (for ex 360 nm, em 455 nm) while a 7-fold increase in fluorescence was observed for the reduction of azidocoumarin–PNA conjugate **7**.[24] The lower increase in fluorescence with the PNA conjugate was attributed to physico-chemical interactions between the nucleobases and the coumarin.[25] While this reaction was very fast with a phosphine at mM concentration, the reaction rate was slow between 10 μM and 1 μM concentration and negligible below 500 nM.

Next, we investigated the ability of DNA to template the reaction. For this purpose, we used an 18-mer single strand DNA sequence with overhangs on both sides (T1, Table 1) as well as sequences containing two to six residues between the hybridization sites or single mutations and a random template. The PNAs were incubated with the templates and the fluorescence emission level was monitored as a function of time (em λ = 455 nm; ex λ = 360 nm). For all experiments, a two to one ratio of phosphine **9** to azide **7** was used to compensate for partial phosphine oxidation which was observed.

Table 1 DNA template sequences

Symbol	DNA sequence (5′–3′)	
T1	5'-GGG TAT CCG GCA CGT CGG-3' KK-CC ATA GGC CGT GCA GC-KK Ph_3P N_3	Perfect match
T2	GGG TAT CCG **AA** GCA CGT CGG	Perfect match with two nucleotide separation
T2mG	GGG TAT ***G***CG **AA** GCA CGT CGG	C → G mutation
T2mA	GGG TAT ***A***CG **AA** GCA CGT CGG	C → A mutation
T2mT	GGG TAT ***T***CG **AA** GCA CGT CGG	C → T mutation

The templated process was first investigated in 1 mM PBS buffer (pH 7.0) using hexameric and octameric PNA probes at concentrations ranging from 100–500 nM of azidoPNA. In all cases, the reaction was very fast (nearly 30–35% conversion within 15 min) while the control reaction without DNA or using a random DNA sequence did not proceed at all. Furthermore, the reaction kinetic was slower for a single mismatch and the conversion exceeded template loading using catalytic amounts of template.[24]

We have previously noted that the fidelity and affinity of PNA/DNA hybridization can be enhanced using formamide solution in the context of microarray hybridizations involving PNAs.[26] The effect of formamide is due to a change in the relative contribution of hydrogen bonding and hydrophobic stacking of the nucleotides in the helices.[27] We thus turned our attention to the potential of the templated reaction involving PNA in a formamide solution. We found that the templated reaction was faster than in water (over 50% conversion within 15 min with 1 equivalent of template) and more importantly that the template turned over faster giving over 15% conversion within 15 min using just 1% of template (Fig. 2). Using an 8-mer PNA with one equivalent of template, the reaction rate was not particularly sensitive to the distance between the two reactive moieties,[24] consistent with the fact that the rate of dissociation of the PNA is slow relative to the rate of the Staudinger reaction under these conditions.[1,28] Next, we looked at the impact of single nucleotide mutation on the rate of reaction using catalytic amounts of template (5%). The reaction rates were significantly reduced for all mutations, with the C to A mutation having the lowest rate of reaction and the C to T mutation having

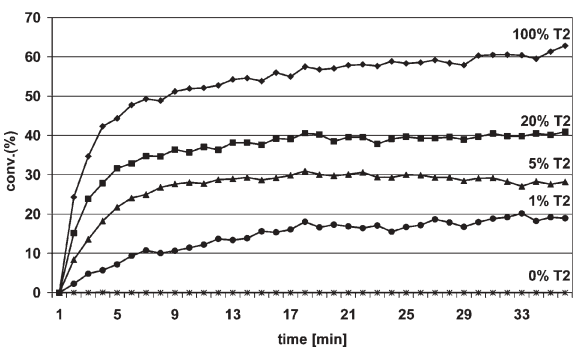


Fig. 2 Reactions with octameric PNA **7** (500 nM) and **9** in 1 : 1 formamide : 1 mM PBS (37 °C) using 0 to 100% of template.

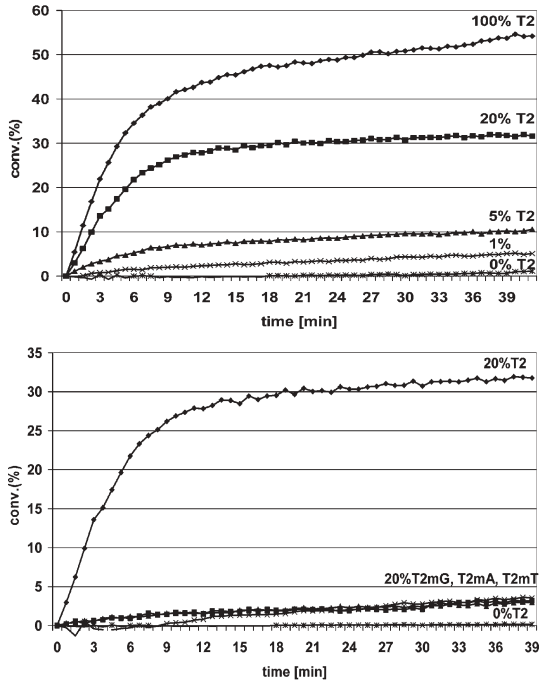


Fig. 3 Reactions with hexameric PNA **7** (500 nM) and **9** in 1 : 1 formamide : 1 mM PBS (37 °C) using 0 to 100% of template (top) and with templates containing a single nucleotide mismatch (bottom).

the highest rate. These results are consistent with the fact that a pyrimidine to purine mutation has a greater destabilization effect on the duplex due to the steric clash of the two purines from the mismatch strands. Importantly, the reaction was at least three times as fast for the perfect match template than for the template with a single nucleotide mutation. Similar results were obtained using just 1% template (1 nM).[24]

Using hexameric PNAs **7** and **9** rather than the aforementioned octamers under the same conditions still afforded a very fast templated reaction (over 50% conversion within 15 min); however, the distance between the two PNA fragments became significant, suggesting that the rate of the Staudinger reaction was no longer much faster than the rate of dissociation.[24] Under these conditions, a distance of two to four residues between the reacting PNAs was optimal. As with the 8-mer PNA, the reaction was found to still proceed with 1% template (Fig. 3 top). Most impressively, a 10-fold difference in fluorescence was observed for the perfect match template relative to single nucleotide mismatched templates within 15 min using catalytic amounts of template (Fig. 3 bottom).

In conclusion, we have developed a system based on a templated Staudinger reaction which can be used to detect single strand DNA down to 1 nM with single nucleotide resolution and signal amplification by virtue of the template turnover. The use of formamide to modulate the affinity of the PNA–DNA interaction was found to be important to achieve good selectivity and should be useful in DNA templated synthesis. The masked fluorogenic properties of 7-azidocoumarin which can be unveiled with a Staudinger reaction was demonstrated for DNA-templated reactions and should be of utility beyond the present work. The remarkable selectivity for single base pair mismatch and speed of analysis (10–15 min) should find applications in diagnostics.

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