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Critical Analysis of Rate Constants and Turnover Frequency in Nucleic Acid-Templated Reactions: Reaching Terminal Velocity

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ABSTRACT: Nucleic acid-templated reactions have attracted significant attention for nucleic acid sensing and imaging. The level of signal amplification obtained from templated reactions is a function of the template turnover, wherein the template acts as the catalyst. Herein, we report the application of a pyridinium linker that immolates upon photocatalytic reduction with a ruthenium complex to yield the fastest nucleic acid templated reaction reported to date. We show that the templated reaction turnover is limited by the duplex dissociation kinetics beyond probes longer than a 6-mer and proceeded fastest for a 5-mer PNA probe. Using a beacon architecture that masks the catalytic template, we show that this methodology can be used for nucleic acid sensing extending the analyte recognition beyond a 5-mer. The system proceeds with a catalytic efficiency of 10^5 $M^{-1}s^{-1}$ and achieves turnover frequency of > 100 h^{-1}.

Nucleic acid templated chemical reactions are promoted by the high effective concentrations achieved upon hybridization of probes to a template.[1-2] While initial interest in nucleic acid templated reactions arose from its putative role in prebiotic chemistry,[3] this reaction format has more recently been harnessed to yield a fluorescent output as a means to sense and image nucleic acids,[4-6] uncage or synthesize bioactive molecules,[7-8] identify novel reaction by combinatorial reagent pairing[9-10] or more broadly to translate the instructions embedded into a DNA template into functional polymers and synthetic molecules.[11-12] These developments have been empowered by an expending scope of nucleic acid templated chemistry.[1-2,6] The first generation of reactions yielded a ligation between the probes resulting in a product with increased affinity to the template and in product inhibition.[13] Subsequently, several bioorthogonal reactions that do not yield a ligation were developed using DNA or PNA probes (Figure 1).[14-28] The ability to achieve high signal amplification is limited by the turnover frequency of the template. Collectively, the pseudo first order rate for the reported templated reactions range from k_{app} = 10^{-4} to 10^{-3} sec^{-1} (Figure 1) with the fastest reaction[25] reported to proceed at 11.6 x 10^{-3} sec^{-1}. We have recently developed a reaction that makes use of a $Ru(bpy)_3$ analog ($Ru(bpy)_2phen$) to photo-catalytically reduce an azide-based immolative linker.[26,28] The reaction was shown to work in live cells and vertebrates. The fact that the reaction is temporally controlled through light (irradiation at 455 nm) is appealing for in vivo applications and kinetic measurements, however, the rate of the reaction could not be directly assessed because of the slow linker immolation (k_{app} ≈ 10^{-3} sec^{-1}).[7] Herein, we report a novel linker for templated Ru-catalyzed photoreduction that achieved the fastest rate reported to date for nucleic acid templated reactions.

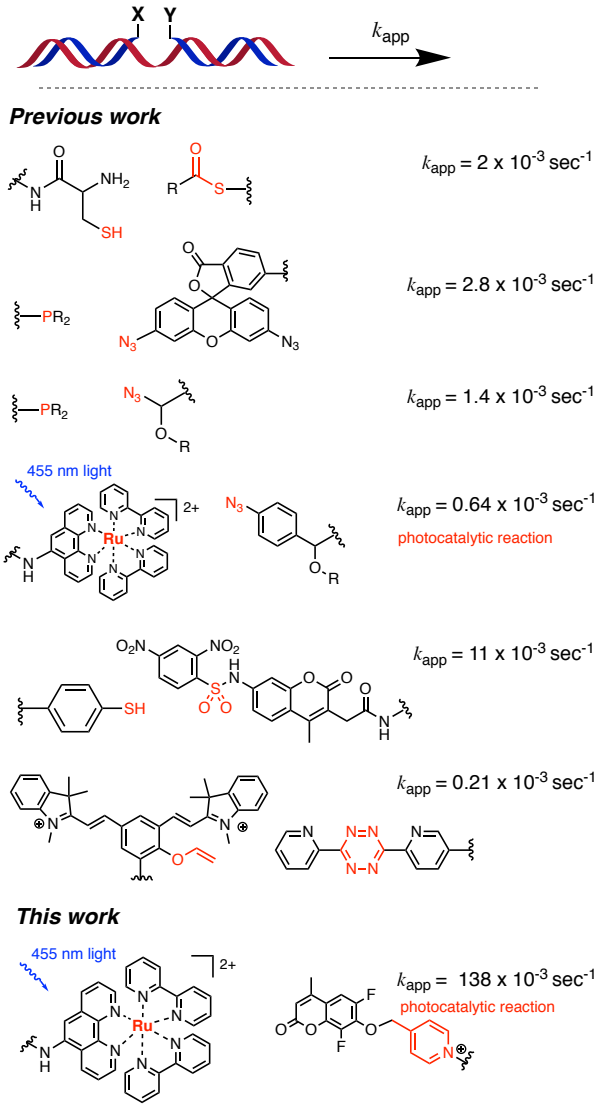


Figure 1. Schematic overview of templated reactions and apparent rate constant (k_{app}) calculated from pseudo first order reactions.

Based on the slow immolation of the 4-azidobenzyl moiety used in previous studies, we sought a linker with more favorable kinetics. Inspired by the report that N-methylpicolinium carbamates undergo C-O bond fragmentation upon photoreduction[29-30] with $Ru(bpy)_3$, we tested the viability of this linker for phenols (Figure 1, see figure S-1 for detailed chemical structures and proposed catalytic cycle of photoreduction). Preliminary experiments showed that phenolic linkages were as efficiently cleaved in the reductive fragmentation as carbamate. This linker can thus be used to unmask a broad variety of fluorophores such as coumarins (phenolic ether linkage) or rhodamines (carbamate linkage). To facilitate kinetic analysis, we opted to investigate a reaction between complementary probes with the reduction of a PNA-pyridinium-coumarin conjugate (Py) using a complimentary PNA-ruthenium conjugate (Ru) as shown in Figure 2. First, we calculated the rate of reaction using the half-life decomposition of a 1:1 Py : Ru mixture across a range of concentrations for different duplex lengths. The complimentary 4-mer did not yield significant signal at a concentration up to 400 nM suggesting that the half-life of the duplex is too short relatively to the reduction reaction. The 5-mer afforded a reaction with a rate constant k = 0.069 s^{-1} at saturation (concentration above 100 nM) and this rate further increased for the 6-mer and 7-mer (k = 0.115 and 0.138 s^{-1} respectively). However, no further gain was obtained by going to the 9-mer indicating that the half-life of the 7-mer duplex is sufficient for maximal reaction rate. Significantly, this reaction is 10 times faster that the fastest templated reaction reported to date (k_{app} = 0.0116 s^{-1}).[25] If the reaction is rate limiting in the templated reaction, the rate constant measured in these experiments should be equal to Vmax (0.069, 0.1115, 0.138 s^{-1} for the 5-, 6-, 7-mer respectively) while the concentration at half this rate should be equal to K_M (*ca.* 40 nM, 15 nM and < 10 nM for the 5-, 6-, 7-mer respectively).

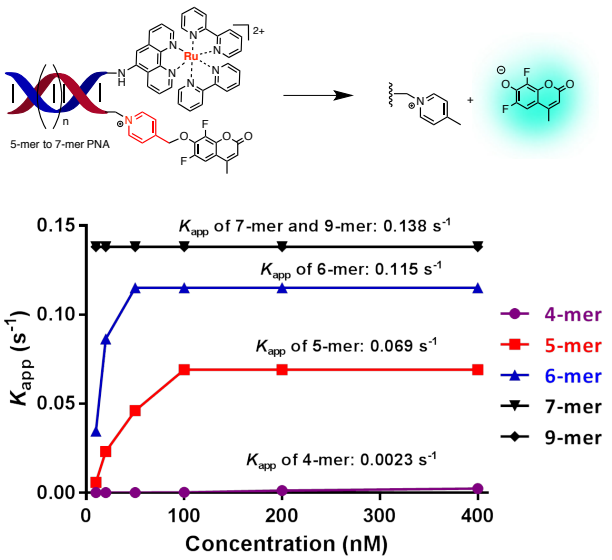


Figure 2. Calculation of k_{app} of templated reaction using 1:1 stoichiometry of Py and Ru probes at varying concentrations with 5 mM sodium ascorbate, LED 455 nm 1W (Buffer : PBS 0.1M, pH 7.4, 0.05% tween). k_{app} were calculated from the reaction half-life. See figure S-1 for detailed chemical structures.

We next investigated the reaction with catalytic amounts of the PNA-ruthenium conjugates (10 nM) with varying concentrations of PNA-pyridinium-coumarin conjugates (Py), measuring the initial rate of reaction and plotting the measured rate (k_{react}) against Py concentrations (Figure 3). Assuming Michaelis-Menten kinetics, the reaction with the 5-mer PNA nearly reached the expected *V*max (0.045 s^{-1} measured *vs.* 0.069 s^{-1}expected) and K_M (146 nM) whereas the 6- , 7- and 9-mer afforded a notably slower *V*max than anticipated based on the measured k_{app} (70 fold slower for the 7- and 9-mer). This discrepancy suggests that the rate limiting step in the reaction of probes longer than 5-mer is product dissociation. We measured the dissociation constant for the different PNA duplex by thermophoretic analysis,[31] wherein one strand is labeled with Cy3,

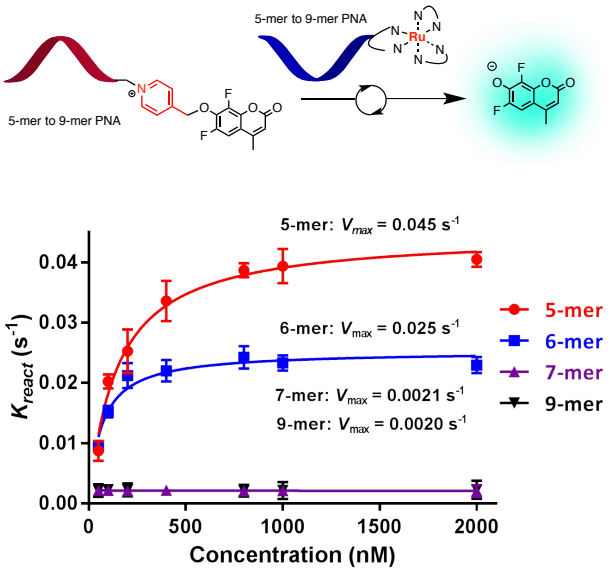
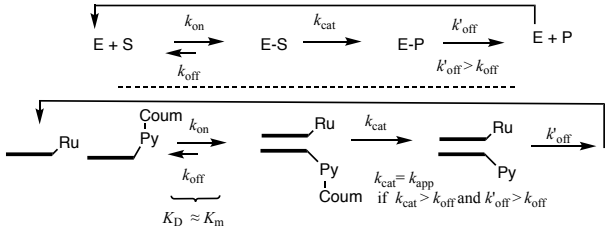


Figure 3. k_{react} of pyridinium reduction under different concentrations and different lengths of PNA at 25°C: 10 nM PNA-Ru, 5 mM sodium ascorbate, LED 455 nm 1 W, Buffer : PBS 0.1 M, pH 7.4, 0.05% tween (polyoxyethylene). See figure S-1 for detailed chemical structures.

finding K_D of 155 nM for the 5-mer, 22.4 nM for the 6-mer, 2.07 nM for the 7-mer (the 9-mer was below the detection threshold, Figure 4). We noted a fluorogenic effect of the Cy3 upon hybridization and used this fluorogenic effect to derive the rate of association of the 9-mer PNA from the half-life of association, finding a k_{on} of 1.28 x 10^6 $M^{-1}s^{-1}$. This value is

consistent with literature values for the rate of association (k_{on}) of DNA and LNA for short oligonucleotides.[32-33] Using the dissociation constant (K_D) and association kinetics (k_{on}), we calculated the dissociation kinetics (k_{off}, Figure 4). Based on the kinetic data measured for the k_{app}, the fact that the rate increased from the 5-mer to 7-mer and plateaued thereafter indicates that for PNAs longer than 7-mer $k_{app} >> k_{off}$. The fact that the 5-mer's k_{app} is half of the 7-mer's k_{app} suggest that the k_{app} of the 5-mer is close to its k_{off}. This is highly consistent with the value measured (Figure 4). Templated reactions for PNAs longer than 5-mer are limited by the duplex dissociation kinetics and the *V*max of reactions with the 6-, 7- and 9-mer are consistent with the duplex dissociation kinetics (Figure 4).



	K_D (nM)	K_M (nM)	k_{app} (s^{-1})	*V*max (s^{-1})	expected k_{off} (s^{-1})	calculated k_{off} (s^{-1})
5-mer	155	146	0.069	0.045	$k_{off} \approx k_{cat}$	0.198
6-mer	22.4	66	0.115	0.025	$k_{off} < k_{cat}$	0.029
7-mer	2.07	< 5 nM*	0.138	0.0021	$k_{off} \approx V$max $k_{off} << k_{cat}$	0.0026
9-mer	< 1 nM*	< 5 nM*	0.138	0.0020	$k_{off} << k_{cat}$	< 0.0013

Figure 4. Measured K_D, K_M k_{app}, *V*max and calculated k_{off} for the different PNAs (* value below the instrument detection threshold); k_{off} are calculated based on K_D and k_{on}. See figure S-1 for detailed chemical structures.

Based on the fact that the reaction of the 5-mer is not limited by strand exchange, it should be less sensitive to the presence of a competing hybridization than the 7-mer or 9-mer. To test this hypothesis, we performed the same kinetic experiment as in Figure 3 in the presence of 1 or 10 equivalents of an unconjugated PNA strand that can compete for duplex formation with the Ru strand (Figure 5). In the case of the 5-mer PNA, 1 equivalent of the competitor reduced the rate of reaction by 25%, and 10 equivalents of the competitor only slowed the rate by half. On the other hand the rate of reaction for 9-mer PNA was halved by 1 equivalent of competitor. The fact that the reaction of the 7-mer Py with 9-mer Ru and 1 equivalent of 9-mer still proceeded concurs the fact that the rate of reaction is faster than the rate of dissociation.

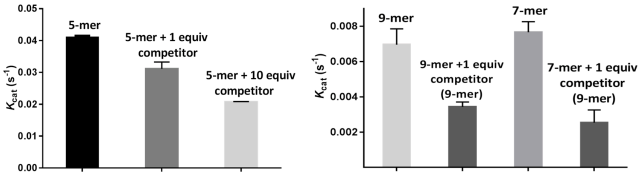


Figure 5. Kinetics of templated reactions in the presence of of probes competing for Ru-probe hybridization. Experiments carried out at the following concentrations: Ru-probe (10 nM), Py-probe (1 µM), competitor (0; 1 µM or 5 µM) with sodium ascorbate (5 mM), LED 455 nm 1 W, Buffer : PBS 0.1 M, pH 7.4, 0.05% tween (polyoxyethylene). See figure S-1 for detailed chemical structures.

Taken together, the data points to the fact that preforming templated reactions with short probes is desirable to maximize k_{cat} and alleviate product inhibition. However, a 5-mer probe is clearly too short for meaningful sequence discrimination of genomic nucleic acids. Nonetheless, we reasoned that this optimized reaction format could be used in a beacon architecture wherein recognition of a longer target sequence would open the beacon and reveal the template for catalysis (Figure 6). To bypass interference of the templating stretch or the Py-probe interacting with other genomic DNA, we made use of chiral γ-PNA wherein the *L* stereochemistry recognize DNA or RNA while the *D* does not.[34-35] Thus, the molecular beacon was built with an *L* stereochemistry 7-mer, followed by an achiral 8-mer for the recognition part, attached to a 5-mer with *D* stereochemistry complimentary to the achiral stretch for the template stretch. In the presence of the template (DNA sequence corresponding to MiR21), the beacon is opened and a templated reaction is observed strictly for the perfect match DNA and matching chirality of template and Py probe. However, a target DNA sequence that does not cover the nucleobase on the loop of the hairpin yielded a low K_M in the templated reaction (K_M = 700 µM), suggesting that the equilibrium towards the open beacon is unfavorable. Using the same DNA template with GG complementary to the CC nucleobase in the hairpin loop afforded a K_M < 200 nM, thus yielding a *V*max of 0.04 s^{-1} at 1 µM

of Py concentration. The fact that this rate is the same as the 5-mer Ru : 5-mer Py indicates that the beacon is fully open in the latter case. Applying this reaction format allowed the detection of a DNA probe with a TOF of 102 h^{-1} using 0.1% template loading.

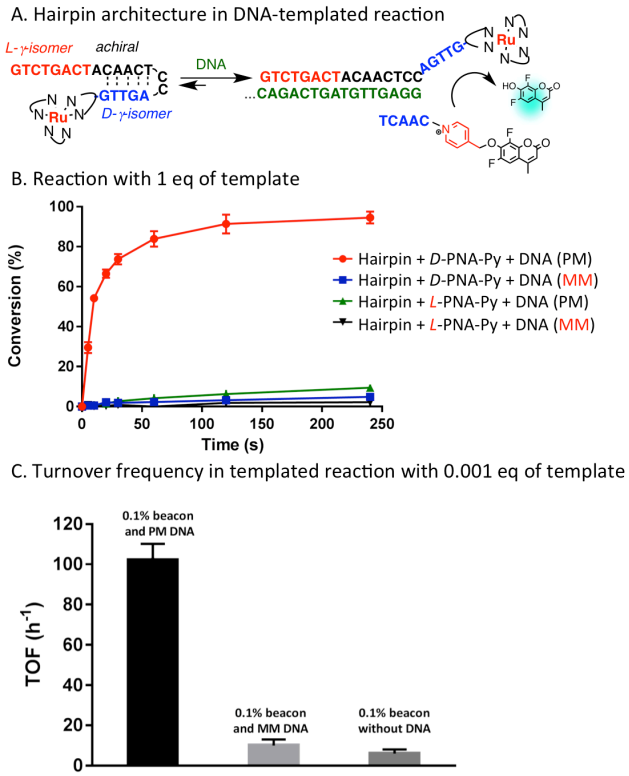


Figure 6. a) Beacon structure with masked catalytic sequence (Red denotes *L*-γ-PNA; Black denotes achiral PNA; Blue denotes *D*-γ-PNA). b) Kinetics of hairpin-Ru reaction at 25°C: 100 nM hairpin-Ru, 100 nM 5-mer-PNA-Coumarin, 100 nM miR21 DNA or 100 nM mismatched (MM) DNA, 5 mM sodium ascorbate, LED 455 nm 1 W, Buffer : PBS 0.1 M, pH 7.4, 0.05% tween (polyoxyethylene); c) Turnover frequency for reaction performed with DNA analyte (1 nM), hairpin beacon (1 nM), Py probe (1 µM) at 25°C under nitrogen atmosphere, Buffer: PBS 0.1 M, pH 7.4, 0.05% tween (polyoxyethylene).

In conclusion, we have developed a new templated reaction with a pyridinium linker that is photocatalytically reduced with a ruthenium complex. This reaction reaches the rate of notable enzymes (chymotrypsin: k_{cat} = 0.14 s^{-1}). The catalytic efficiency of the systems (k_{cat} / K_M) is 10^5 $M^{-1}s^{-1}$ (chymotrypsin: 9.3 $M^{-1}s^{-1}$; carbonic anhydrase: 10^7 $M^{-1}s^{-1}$). We provide a detailed kinetic analysis of the catalytic cycle and demonstrate that the reaction with PNA longer than 5-mer are limited by the exchange of reagents on the template, thus templated reactions cannot proceed faster, irrespective of the chemistry performed. However, this fast templated reaction can be applied to the sequence specific detection of longer target sequences by embedding the template into a beacon architecture which is opened in the presence of the analyte.

Supporting Information

Supplementary figures, experimental protocols, synthetic procedures and compound characterization.

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Notes

The authors declare no competing financial interest.

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