Nucleic Acid Detection Hot Paper

International Edition: DOI: 10.1002/anie.201509131
German Edition: DOI: 10.1002/ange.201509131

ATP-Releasing Nucleotides: Linking DNA Synthesis to Luciferase Signaling

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Abstract: *A new strategy is reported for the production of luminescence signals from DNA synthesis through the use of chimeric nucleoside tetraphosphate dimers in which ATP, rather than pyrophosphate, is the leaving group. ATP-releasing nucleotides (ARNs) were synthesized as derivatives of the four canonical nucleotides. All four derivatives are good substrates for DNA polymerase, with K_m values averaging 13-fold higher than those of natural dNTPs, and k_{cat} values within 1.5-fold of those of native nucleotides. Importantly, ARNs were found to yield very little background signal with luciferase. DNA synthesis experiments show that the ATP byproduct can be harnessed to elicit a chemiluminescence signal in the presence of luciferase. When using a polymerase together with the chimeric nucleotides, target DNAs/RNAs trigger the release of stoichiometrically large quantities of ATP, thereby allowing sensitive isothermal luminescence detection of nucleic acids as diverse as phage DNAs and short miRNAs.*

Methods for the detection of DNA synthesis are broadly useful in biology and medicine. For example, common next-generation sequencing methods use fluorescence signals associated with sequencing-by-synthesis.[1,2] Luminescence emission signals are also important for measuring amplification in real-time PCR,[3–5] including the use of DNA-binding dyes or fluorogenic probes. Detection of DNA synthesis in cellular specimens is also useful; this is commonly carried out by polymerase incorporation of BrdU with subsequent antibody detection,[6] or by incorporation of bioorthogonally reactive functional groups into DNA.[7]

The use of luciferase signaling can also provide sensitive reporting of DNA synthesis. This enzyme is employed in the "pyrosequencing" methodology developed for high-throughput DNA sequencing.[8] In this technology, four enzymes are employed, two of them to recycle the pyrophosphate product of the DNA polymerase reaction to generate modified ATP. This method is sensitive, but is also relatively complex, and as a result, the method is not used broadly beyond its application in pyrosequencing instruments.

Further improvements in methods for the general reporting of polymerase synthesis may be useful for the amplified detection of native nucleic acids, reporting on isothermal amplification methods such as rolling-circle amplification (RCA),[9–12] and future-generation approaches to DNA sequencing. To this end, it would be desirable to take advantage of the high sensitivity and specificity of luciferase in the detection of DNA synthesis.

Herein, we describe the design and application of ATP-releasing nucleotides (ARNs, Figure 1) as reporters of DNA synthesis. These tetraphosphate-bridged chimeric RNA–DNA dinucleotides are employed sequentially as substrates for DNA polymerases and luciferase. In this design, DNA polymerase uses the ARNs to copy a target strand, thereby releasing one equivalent of ATP for every deoxynucleotide incorporated. In a subsequent reaction, luciferase processes the ATP products to generate light signals in the presence of luciferin. In principle, the longer the target nucleic acid molecule, the more signal is generated, thus giving the possibility of high sensitivity.

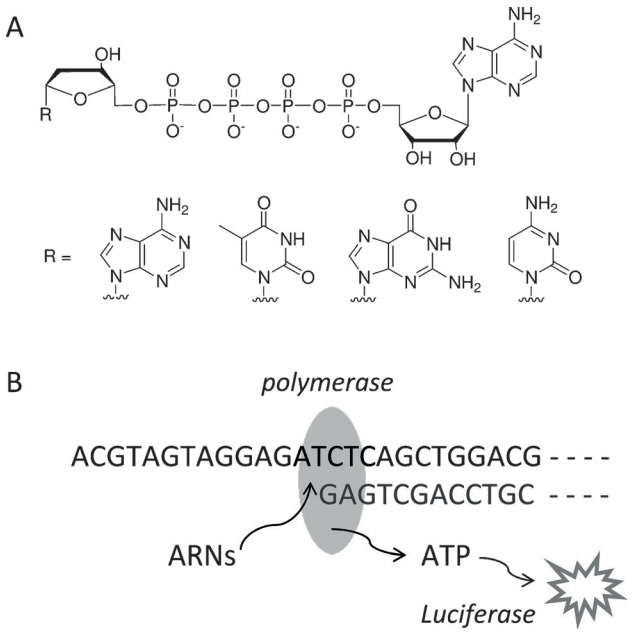

Figure 1. Structures and strategy in this study. A) The four chimeric ATP-releasing deoxynucleotides (dApppA, dTpppA, dGpppA, and dCpppA). B) Scheme showing how DNA polymerase activity incorporates the deoxynucleotide portion of an ARNs while copying a template, releasing ATP in the process, which then activates luciferase luminescence signaling.

Although dimeric polyphosphate-linked nucleotides are known,[13–15] ATP-releasing chimeric nucleotides have not been studied previously. Tetraphosphate-bridged deoxy–deoxy dinucleotides have been the subject of a report testing them as substrates for DNA polymerases.[13] Tetraphosphate-

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201509131.

linked ribo–ribo dimers have been studied more widely as enzyme inhibitors.[14,15] Despite these precedents, we know of no reported studies of chimeric ribo–deoxy tetraphosphate dinucleotides. One patent describes the theoretical use of such compounds for DNA sequencing,[16] but no experimental data are given on their synthesis or on any enzymatic properties. Thus we undertook the current study. It was not known at the outset 1) whether DNA polymerases would accept the dinucleotides without interference from the chemically similar ATP group at the opposite end; 2) whether luciferase might accept the dinucleotides as substrates, thus bypassing the polymerase and short-circuiting this concept; 3) what enzymes and conditions would yield optimal signals; or 4) what sensitivity the approach might have in reporting on nucleic acid targets.

We report that these chimeric dinucleotides are in fact efficient substrates for DNA polymerase, but they are inefficient with luciferase, thereby minimizing background signal. These properties enable their use in the luminescence reporting of DNA polymerase activity, including the sensitive detection of DNA and RNA targets.

A full set of four chimeric ATP-releasing nucleotides (dTppppA, dAppppA, dCppppA, and dGppppA) was synthesized by activating deoxynucleoside monophosphates (dNMPs) with carbonyldiimidazole and then reacting them with 5′-ATP to produce the desired chimeric dimers in 42–60% yields (see the Supporting Information). To test whether these modified nucleotides can be substrates for a DNA polymerase, we carried out primer extension on short synthetic duplexes (Figure 2; 1 μM) in the presence of a 3′-exonuclease deficient variant of the Klenow fragment of DNA polymerase I (Kf exo$^-$). We supplied one ARN at a time (20 μM) to its complementary template; if synthesis were successful, it should generate up to 20 μM ATP as a byproduct of the reaction. We removed a small aliquot of the polymerase reaction and measured luminescence from the ATP with a commercial luciferase + luciferin reaction buffer (Figure 2B). Signals were clearly generated for each of the four DNA templates, resulting in about equal intensities except for the G_{20} template sequence, which generated a moderately smaller signal, possibly due to G-quadruplex structures that may inhibit the polymerase.[18] In all four cases, signals were considerably (13–33-fold) higher than background when no primer/template was used.

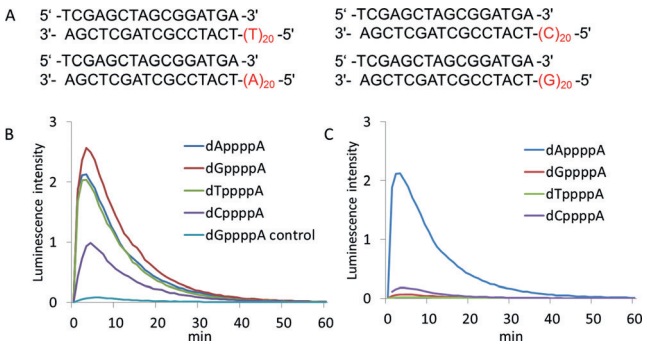

Figure 2. Initial primer extension studies of chimeric nucleotides with Kf exo$^-$ polymerase. A) Primer–template duplexes with $(N)_{20}$ ends used in this study. B) Luminescence signals resulting from the incorporation of ATP-releasing nucleotides by Kf exo$^-$ polymerase. The Kf (exo$^-$) polymerase reaction was carried out with 20 μM chimeric nucleotides and 1 μM corresponding primer–template at 37°C for 1 h. 5 μL of polymerase reaction solution were used for the luciferase reaction. The bioluminescence signal was recorded in 1 min intervals for 1 h. In the dGppppA control, no primer was added. C) Kf exo$^-$ polymerase selectivity with chimeric nucleotides. The reaction was carried out with the $(T)_{20}$ template and each of the four ARNs under the same reaction conditions as in Figure 2B.

Next, we tested the sequence selectivity of the chimeric nucleotides, evaluating sixteen combinations of ARNs with the four DNA sequences (Figure 2C and Figure S1 in the Supporting Information). In all cases, the correct nucleotide/target sequence combinations yielded much higher signals than incorrect combinations, thus showing clear nucleotide/template base selectivity. Importantly, the adenosine ribonucleotide moiety of these chimeras was not noticeably misincorporated by the Kf polymerase (Kf pol), as evidenced by the lack of enhanced signal on the T_{20} template sequence with dTppppA, dCppppA, or dGppppA. The main background signal appeared from experiments containing dCppppA; subsequent experiments revealed that this arises primarily from a very small contamination of the nucleotide with ATP (Figure S3).

If ARNs could directly act as efficient luciferase substrates, one would observe strong signals whether or not a DNA polymerase or a template DNA were present, thus nullifying their utility in reporting on DNA synthesis. Therefore, we compared luciferase signals in the absence of DNA or polymerase, supplying each of the ARNs separately. The results show that the ARNs are poor substrates for luciferase, yielding from 50-fold to more than 300-fold lower signals than ATP (Figure S2). Background signals from these chimeric ARNs are therefore quite low.

Next, we performed experiments to quantify the efficiency of ARNs as DNA polymerase substrates, measuring steady-state kinetics of the four nucleotides in experiments with Kf exo$^-$. The experiments reveal that the ARNs are substrates with efficiencies moderately less than those of native dNTPs (Table 1 and Figure S6). K_m values average 2.5 μM, which is higher than those of natural nucleotides, for which the values average 0.2 μM. Values for k_{cat}, on the other hand, are very similar for the chimeric nucleotides (7.7 min^{-1}) and native dNTPs (11.7 min^{-1}). Thus, although somewhat higher concentrations may be required to achieve near-

Table 1: Steady-state DNA polymerase (Kf exo$^-$) efficiency with chimeric ATP-releasing nucleotides.

dNTP	k_{cat} [min^{-1}]	K_m [μM]	k_{cat}/K_m [μM^{-1} min^{-1}]
dGTP	15.4 ± 0.5	0.11 ± 0.01	140
dGppppA	7.1 ± 0.5	3.5 ± 0.4	2.0
dCTP	14.0 ± 0.4	0.07 ± 0.01	200
dCppppA	12.9 ± 0.1	3.0 ± 0.2	4.3
dATP	8.6 ± 0.3	0.35 ± 0.05	25
dAppppA	7.1 ± 0.2	1.3 ± 0.4	5.5
dTTP	8.7 ± 0.3	0.24 ± 0.06	36
dTppppA	3.7 ± 0.1	2.2 ± 0.6	1.7

maximum reaction velocities for ARNs, the maximal rates for polymerase incorporation are expected to be nearly the same as those of native nucleotides. The most efficient ARN (compared to its native congener) is dAppppA, which exhibits a k_{cat}/K_m value only 5-fold less than that of dATP, while the least efficient is dGppppA, which is less efficient than dGTP by a larger factor of 70 (with most of this factor in the K_m term).

We then explored the question of whether other DNA polymerases can accept ARNs as substrates by testing a range of DNA polymerases and reverse transcriptases (Figure S4A,B). The data show that several polymerases successfully extend primers exclusively by using these chimeric nucleotides. Interestingly, the strongest signals were seen with Kf pol with exonuclease activity (Figure S4B), thus suggesting that proofreading activity may enhance signals. This may occur through the enzyme incorporating a nucleotide, then removing it, and then incorporating it again, thus generating multiple equivalents of ATP for each nucleotide ultimately incorporated.

The preliminary data suggested a use for ARNs in reporting on varied classes of DNA or RNA targets. Although in principle, one might use all four ARNs for detecting a target, we considered whether one might enhance the signal-to-background ratio by using a smaller subset of ARNs in combination with native dNTPs. We tested combinations of ARNs in primer extension experiments with single-stranded phage M13 DNA. We found that all four ARNs could indeed be used simultaneously, generating a robust signal (Figure S7, lane 1). However, replacement of dCppppA with dCTP yielded an approximately 10% higher signal, rather than 25% lower as expected from the stoichiometry. Similarly, replacing both dCppppA and dTppppA yielded an even higher signal (lane 3). Measuring the background for these combinations (with no DNA target) showed that omission of two of the nucleotides also lowered the background signal by several-fold (compare lane 3c with lane 1c).

The above experiments establish that chimeric ATP-releasing nucleotides can be used to generate luminescence signals through luciferase when a DNA polymerase has been active on a nucleic acid template. In principle, one might use this signaling to detect a genetic target. A longer template is expected to yield more signal than a short one, since there are stoichiometrically more nucleotides consumed (and ATP generated) per molecule. This suggests that long or circular biological nucleic acids might be detected quite sensitively. Short genetic targets would yield only small signals when used as templates, but might generate larger signals if employed instead as primers on long or circular templates. We explored these issues in subsequent experiments with two classes of genetic targets: bacteriophage DNA and miRNA.

Bacteriophage M13mp18 DNA is a single-stranded circular DNA 7249 nt in length. We envisioned the use of a phage-specific primer (see above) for the detection of this target. In initial tests with with Kf polymerase, testing three different primers (Figure 3A) showed that two DNA primers complementary at distinct sites in the phage each yielded identical amounts of signal with 1 nM phage DNA, while a noncomplementary primer (sense rather than antisense in complementarity) yielded little signal, the same as the control lacking DNA. Similarly, a primer mismatched at the three 3′-terminal nucleotides also yielded approximately background levels of signal, which is consistent with the need for 3′ end priming to initiate the reaction (Figure 3A). Experiments with 10 h versus 24 h polymerase reactions (Kf pol) showed significant signal enhancement between these times (data not shown), thus confirming that the Kf DNA polymerase remained active for a long period, as expected on this circular target. Experiments at shorter times (50 fmol target) confirmed that there was significant signal over background at times shorter than one hour (Figure S9). Next we evaluated the limit of detection, employing 20 μM dAppppA, dGppppA, dTTP and dCTP, and diluting the DNA (Figure 3B). The data show that 5 attomoles (5×10^{-18} moles) of phage M13 DNA could be reproducibly detected over background.

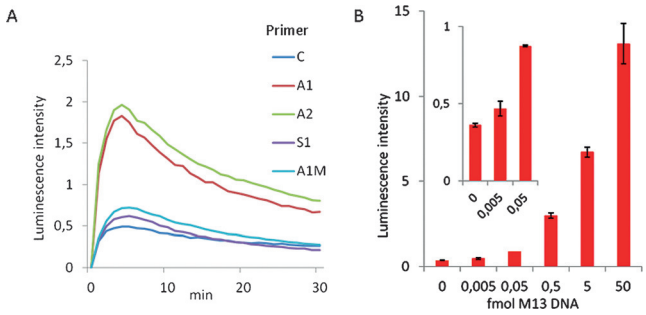

Figure 3. Detection of circular M13 DNA by using chimeric nucleotides and luciferase. A) Signals with varied primers on M13 DNA. Luminescence signal from 5 μL of polymerase reaction with 1 nM primer and 1 nM M13 DNA at 37°C for 5 h. A1 and A2 are antisense M13 DNA primers; A1M is the A1 primer mismatched at the three 3′-terminal nucleotides; S1 is a noncomplementary sense M13 primer; and C is a control with primer A1 but lacking DNA. B) Testing the limit of detection for M13 DNA. Polymerase reactions were carried out with 0.005 to 50 fmol of primer A1/phage DNA at 37°C for 24 h. Luciferase signals are shown as the 5 minute values; error bars represent standard deviations from three replicates. Inset: the lower-concentration samples shown on a magnified scale.

Next, we turned our attention to the detection of miRNAs, since detecting these small single-stranded RNAs has been an active research goal.[19–26] The let-7 family of miRNAs in particular has been shown to play significant roles in ovarian, prostate, liver, and pancreatic cancer.[27–30] Since miRNAs are short, polymerase chain reaction (PCR) cannot be carried out on the unmodified target. Additional steps (such as ligation) are needed to modify miRNAs for PCR-based detection,[19] and so simpler approaches merit investigation. We took the approach of employing them as primers, using a small circular DNA template complementary to the target let-7a miRNA. In this strategy, RCA is carried out, primed by the miRNA on the circular DNA.[21–26] This can in principle be extended yet further with hyperbranched RCA.[11] Isothermal detection of miRNAs through the use of rolling circle templates has been reported previously with templated fluorogenic chemistry[10,31] or DNA-binding fluorescent dyes[22–25] to report on the products.

Experiments in the presence of ARNs showed that the 22mer let-7a RNA could indeed prime DNA synthesis by the

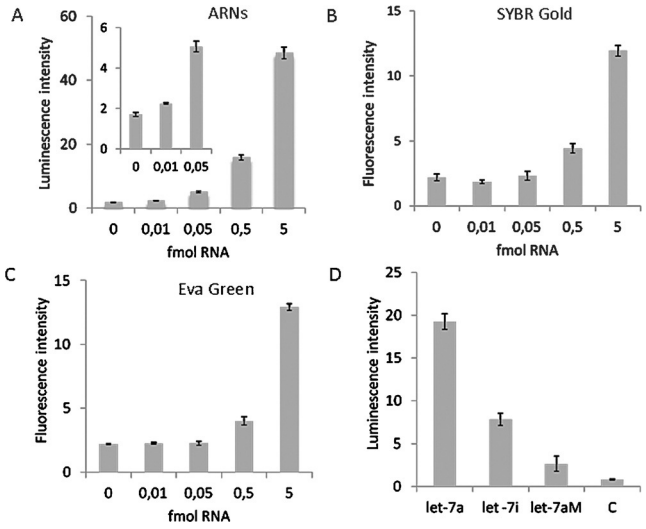

Figure 4. Detection of miRNA with chimeric nucleotides. A) The limit of detection for miRNA let-7a when using chimeric nucleotides. The branched RCA reactions were carried out simultaneously with varied amounts of miRNA let-7a at 30 °C for 24 h. Then, 5 μL polymerase reaction and 95 μL luciferase reaction mixtures were combined and the luminescence signals at 5 min were recorded. Error bars represent the standard deviation from three trials. B) The the limit of detection for let-7a RNA when using SYBR Gold Dye (emission at 538 nm). Inset: the lower-concentrations shown on a magnified scale. C) The limit of detection of miRNA when using EvaGreen Dye (emission at 525 nm). D) Test of selectivity among related let-7 RNA family members and a mismatched version (let-7aM; 20 h polymerase reaction). Luminescence signals were measured at 5 min.

highly processive Φ29 DNA polymerase when using a 50 nt circular DNA complementary to the miRNA as template. Signal appeared above background for reactions as short as 1 h (Figure S10), and longer polymerase reactions produced yet greater signals. To measure sensitivity, reactions were carried out with 10 nM circular DNA template and 50 μM dAppppA, dGppppA, dTTP, and dCTP. Luminescence detection showed signals above background for as little as 10 attomoles of target RNA (Figure 4A). For comparison we tested the use of DNA-binding fluorescent dyes for detecting product in otherwise identical reactions (Figure 4B,C), and sensitivity was approximately 1–2 orders of magnitude less than detection with ARNs and luciferase. Controls with varied sequence (DNA or RNA targets) confirmed selective signaling for the let-7a target; a 3′-terminally mismatched target showed diminished signal (Figure 4D, let-7aM), as did a naturally occurring variant with a mismatch 4 nt from the 3′ end (let-7i). Targets mismatched near the center, however, showed lower selectivity, as expected since the target 3′ end remains complementary to the circular DNA (Figure S11). Nevertheless, a single nucleotide mismatch (let-7e sequence) did produce a measurable reduction in signal.

Taken together, our experiments have shown that ATP-releasing deoxynucleotides act as good polymerase substrates and yield little background reaction with the luciferase enzyme. These facts enable these chimeric nucleotides to be employed in the sensitive detection of nucleic acids. The method is isothermal and simple, requiring only one DNA probe and a standard DNA polymerase. No labelling is required. The strategy is versatile, detecting DNA or RNA, and short or long targets can be sensitively detected with judicious design of the primer or circular template. The separation of the luciferase reaction from the polymerase reaction[32] allows one to measure signals at a convenient time after multiple polymerase reactions.

The sensitivity of the ARN/luciferase method compares well to reported methods for the isothermal detection of nucleic acids, such as RCA detection of miRNAs.[25,26] Compared to PCR-based approaches to miRNA detection,[19] the current method is simpler, requiring fewer primers and enzymes, fewer steps, and no thermal cycling equipment. One possible limitation of the current approach is that it is difficult to detect single-nucleotide variants in a miRNA target if the polymorphism occurs near the center or 5′ end, since only a complementary 3′ terminus is needed to prime synthesis. Further design modifications and experiments will be needed to address this.

Future studies will explore new applications of the ARNs for reporting on biomolecules. Since ATP acts as an energy source in multiple biological processes, ATP-releasing nucleotides could potentially find use in the polymerase-mediated activation of other enzymatic activities beyond luciferase.

Acknowledgements

We thank the U.S. National Institutes of Health (GM068122, GM110050) for support. E.M.H. acknowledges support from an NSF graduate fellowship.

Keywords: ATP · DNA polymerase · luciferase assay · modified nucleotides · nucleic acids

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 2087–2091
Angew. Chem. **2016**, *128*, 2127–2131

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Received: October 21, 2015
Revised: November 20, 2015
Published online: January 6, 2016

