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Turning a Kinase Deoxyribozyme into a Sensor

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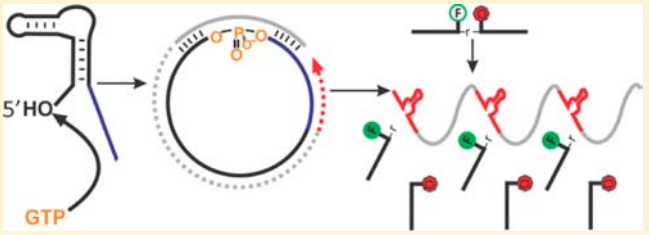
Supporting Information

ABSTRACT: The vast majority of deoxyribozyme-based sensors are designed using modified RNA-cleaving deoxyribozymes and detect analytes that act as allosteric regulators of their catalytic activity. These sensors are susceptible to background signals due to catalytic activity in the absence of target or contaminant molecules that cleave the RNA substrate, mimicking the deoxyribozyme reaction. In this manuscript, we introduce a novel system that avoids these problems by using the analyte as the substrate for a deoxyribozyme catalyzed self-phosphorylation reaction. This reaction creates a modified deoxyribozyme product that can be circularized and subjected to massive signal amplification by rolling circle amplification, leading to a sensor system with high sensitivity and low background, which can be coupled to numerous reporter systems. As an example of the potential of this system, we used the self-phosphorylating deoxyribozyme Dk2 to detect as little as 25 nM GTP even in the presence of 1 mM ATP, a potential contaminant. To demonstrate the adaptive properties of this system, we appended another DNA sequence to Dk2, which, once amplified by RCA, codes for a fluorescence generating deoxyribozyme. This two-deoxyribozyme system was able to report the presence of GTP from 4 μM to 1 mM, with specificity over other NTP molecules. Using this model system, we were able to show that small molecule modifying deoxyribozymes can be converted to analyte sensors by coupling their catalytic activity to signal amplification and reporting.



INTRODUCTION

Catalytic DNA, isolated through a process known as in vitro selection, has been utilized in many biosensing applications.[1] The first deoxyribozyme, isolated nearly 20 years ago, was shown to be able to cleave an RNA phosphoester bond embedded within a DNA substrate.[2] This deoxyribozyme as well as other RNA-cleaving deoxyribozymes with different cofactor requirements[3] have been developed into sensors for targets such as toxic metal ions (reviewed in ref 4) including lead(II)[5−7] and mercury(II)[8,9] and have been used to detect specific species of bacteria.[10] In most systems, deoxyribozyme-based sensors detect targets that act as cofactors or allosteric regulators of a few well-known deoxyribozymes. Upon target-binding, structural changes are induced in the deoxyribozyme, resulting in a change in activity that can be transduced into a detectable signal. Background and false positive signals can arise in these systems due to folding of the deoxyribozyme into its active structure in the absence of target. Other molecules in the sensing solution can also mimic the deoxyribozyme reaction, especially in the case of RNA-cleaving deoxyribozymes, which are susceptible to ribonuclease and chemical degradation. An alternate method of detection would be to use substrates from deoxyribozyme-catalyzed reactions as targets for new sensors. This would allow for reduced background, as contaminant molecules are not likely to induce substrate processing and substrate specificity can be tuned by in vitro selection. Apart from the aforementioned RNA-cleaving deoxyribozymes, many other deoxyribozymes are available that catalyze diverse reactions, such as RNA ligation[11] and nucleotide bond formation[12] or modify different substrates such as porphyrin.[13] Novel deoxyribozymes for other targets can further be isolated by in vitro selection. If the activity of these deoxyribozymes can be coupled to efficient reporting systems, they may serve as the foundation for the development of a plethora of new sensors.

Self-phosphorylating deoxyribozymes, or deoxyribozyme kinases, are catalytic DNA molecules that can transfer a γ-phosphate from an NTP substrate to their own 5′ ends. Through in vitro selection experiments, numerous self-phosphorylating deoxyribozymes with wide ranging properties have been isolated and characterized.[14−18] Such deoxyribozymes possess very specific substrate and cofactor requirements. These deoxyribozyme kinases, which can discriminate between a particular NTP and closely related analogues, appear to be ideal candidates for the development of sensors for NTP molecules. One such deoxyribozyme, Dk2, can transfer a phosphate from guanosine-5′-triphosphate (GTP) to its 5′ end at a rate of 0.2 min^{-1} in the presence of manganese(II).[15,16] It

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was found that this deoxyribozyme was highly specific for GTP as a substrate and could not process other purine-based nucleoside triphosphates, including several closely related GTP analogues.[17] As this deoxyribozyme can specifically process GTP, it is a prime candidate for conversion into a GTP sensor.

GTP plays many critical roles in the cell. In addition to being a building block for RNA, GTP is the substrate of numerous GTPases.[19] GTP hydrolysis by EF-Tu GTPase is critical for protein synthesis as it provides the energy for binding of aminoacyl tRNAs and the translocation of the ribosome.[20] Other GTPases are essential for protein trafficking,[21] microtubule depolymerisation,[22] and diverse regulatory roles.[23] Early assays for the detection of this important triphosphate molecule involved the chromographic separation of guanine nucleotides from the more abundant adenosine nucleotides followed by enzymatic conversion of GTP to ATP, which could be measured by luciferase-based detection.[24] In addition to enzymatic detection, GTP sensors have also been designed using artificial receptors. In these systems, binding of GTP to the receptor displaces or changes the environment of an associated fluorescent dye leading to a change in fluorescence. Receptors used in GTP sensors include guanidinium tripeptide receptors,[25] benzimidazolium receptors,[26,27] and cyclophane receptors.[28,29] These sensors can typically detect GTP in the micromolar to millimolar range. With regards to functional nucleic acids, aptamers that can bind to GTP have been isolated.[30,31] Ribonucleopeptide-based sensors have been developed for GTP, in which an RNA aptamer domain binds GTP, changing the environment of an associated fluorescently labeled peptide to generate a signal.[32,33] Covalent linking of the fluorescent peptide to the RNA aptamer portion of this sensor has allowed this system to be used in a multiplex format and simultaneously detect ATP and GTP.[34]

Currently, self-phosphorylating activity of Dk2 using GTP is indirectly detected through a ligation assay with 5′ phosphorylated Dk2 serving as a substrate for T4 DNA ligase and ligated to an acceptor DNA molecule, whereas inactive deoxyribozymes lacking the 5′ phosphate will not be ligated. The active sequences can then be electrophoretically separated from unreacted sequences based on their increased size. Using this system, it is possible to detect micromolar amounts of GTP (K_M = 550 μM).[15] To improve the sensitivity and thereby increase the detection limit, we have modified our ligation assay, rendering its product suitable for signal enhancement through rolling circle amplification (RCA). RCA is a process that involves continuous polymerization of a primer from a circular template, producing long single-stranded DNA molecules. Originally discovered in the replication of certain viruses, this technique has been adapted for the amplification of small single-stranded circular templates.[35] This system has been exploited to detect many DNA and RNA targets with the development of padlock probes (reviewed in ref 36). In this system, linear probes are designed such that their 3′ and 5′ ends will hybridize to a target sequence. In the presence of a ligase enzyme, these probes will ligate into a closed circle, which can be used as a template for RCA by a suitable polymerase such as ϕ29 DNA polymerase. A modified version of this padlock system has been used to detect ATP. In this case, a ligase deoxyribozyme allosterically controlled by ATP was used, and the linear probe would circularize following ATP binding.[37] Other analytes have also been detected using cleavage products, created after allosterically regulated deoxyribozyme-based RNA cleavage, as primers for RCA.[38] However, in these cases the use of RNA-cleaving deoxyribozymes presents the same background issues discussed earlier, with this background signal being amplified by RCA.

As RCA has been shown to be a powerful method for amplification, we examined the possibility of employing this technique to sense low levels of Dk2 self-phosphorylation and increase the detection limit for GTP. The scheme depicting our system is outlined in Figure 1. First, self-phosphorylation is

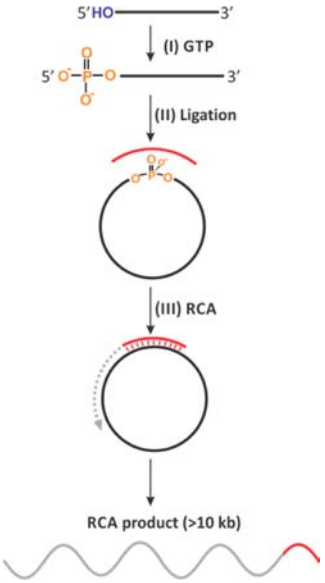

Figure 1. Amplifying self-phosphorylation signal through circular ligation and rolling circle amplification (RCA).

carried out in the presence of GTP and self-phosphorylation buffer (50 mM HEPES, 400 mM NaCl, 100 mM KCl, 10 mM $MnCl_2$, pH 7.0). This is followed by the addition of a splint DNA oligonucleotide that is complementary to the 5′ and 3′ ends of the deoxyribozyme. This will bring the phosphorylated 5′ end into proximity to the 3′ end of the same deoxyribozyme strand. Dilution of the sample to reduce the manganese(II) concentration and addition of T4 DNA ligase will lead to self-ligation of the deoxyribozyme into a single-stranded circle. This circularized DNA can then be used as a template for RCA upon the addition of ϕ29 DNA polymerase and 0.5 mM of each dNTP with the splint now acting as a primer for the RCA reaction. The resulting RCA reaction will produce long repetitive single-stranded products of more than 10 kilobases from phosphorylated deoxyribozyme templates of less than 100 nucleotides, thus greatly amplifying the initial self-phosphorylation.

EXPERIMENTAL SECTION

Oligonucleotides and Materials. DNA oligonucleotides were prepared using standard phosphoramidite chemistry (W. M. Keck Facility, Yale University, New Haven, CT, USA; and Integrated DNA Technologies, Coralville, IA, USA). Each oligonucleotide was purified by 10% denaturing (8 M urea) PAGE, and its concentration was determined spectroscopically. T4 DNA ligase and ϕ29 DNA polymerase were purchased from MBI Fermentas, Burlington, ON, Canada. [α-^{32}P]deoxy-GTP was acquired from Perkin-Elmer, Woodbridge, ON, Canada. All other chemicals were obtained from Sigma-Aldrich, Oakville, ON, Canada.

Self-Phosphorylation Reactions. Self-phosphorylation assays were carried out using 100 nM DNA in a typical volume of 10 μL. DNA was first heated to 90 °C for 1 min and cooled to room

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temperature. Self-phosphorylation buffer was then added to a final concentration of 50 mM HEPES (pH 7.0), 400 mM NaCl, 100 mM KCl, and 10 mM $MnCl_2$. Reactions were initiated by adding GTP to a final concentration of 100 μM. For the GTP sensitivity tests, final concentrations of GTP ranged from 1 nM to 2 μM. For the NTP specificity tests, GTP was replaced with 100 μM ATP, CTP. and UTP. For the divalent metal ion tests, $MnCl_2$ was replaced with 10 mM $CaCl_2$, $MgCl_2$, or $ZnCl_2$. Reactions were incubated at room temperature for 30 min and used immediately in the ligation reaction.

Circular Ligation Reactions. Self-phosphorylation reactions were diluted 3× for ligation reactions. Ligation reactions contained 200 nM circular ligation splint DNA (SPDK2 for Dk2, SPDKMgZ for Dk2/MgZ′, see the Supporting Information, Table 1), 1× T4 DNA ligase buffer (MBI Fermentas), and 0.01 U/μL T4 DNA ligase. DNA was heated to 90 °C and cooled to room temperature. Buffer and ligase were then added, and the reaction was incubated for 45 min at room temperature. The reaction was heated to 90 °C for 5 min to deactivate the ligase. These reactions were then immediately used for RCA.

RCA Assays. Ligation reactions were diluted 5× for RCA reactions. Reactions were performed in 1× ϕ29 DNA polymerase buffer (MBI Fermentas) with 500 μM dATP, dGTP, dCTP, and dTTP and 0.01 U/ μL ϕ29 DNA polymerase. For Figure 2a, [α-^{32}P]deoxy-GTP was

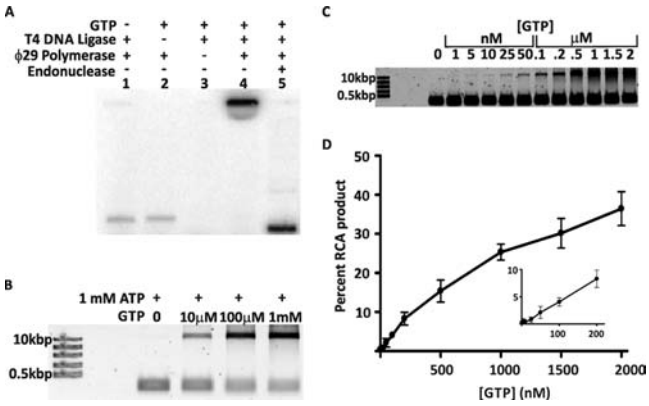

Figure 2. Rolling circle amplification (RCA) of a GTP responsive self-phosphorylating deoxyribozyme after circular ligation using the scheme shown in Figure 1. (A) The self-phosphorylating deoxyribozyme Dk2 was incubated sequentially with GTP, T4 DNA ligase and ϕ29 DNA polymerase (lane 4). The identity of the RCA product was confirmed by restriction enzyme digestion (lane 5). (B) RCA after self-phosphorylation and circular ligation with 1 mM ATP (contaminant) and increasing concentrations of GTP. (C) Sensitivity tests using decreasing concentrations of GTP for self-phosphorylation followed by circular ligation and RCA. (D) Quantification of sensitivity test shown in (C).

also added to allow for radioimaging. RCA reactions were incubated at 30 °C for 3 h. Reactions were then incubated at 70 °C for 20 min to deactivate the ϕ29 DNA polymerase.

Fluorescent Detection of RCA with MgZ and RS28. After heat inactivation of ϕ29 DNA polymerase, RCA product created from Dk2/MgZ′ circular DNA template was incubated with 2 μM RS28 substrate (see the Supporting Information, Table 1, for Dk2/MgZ′ and RS28 sequences) at room temperature. Fluorescence changes over time were monitored on a BioRad CFX96 Optical Reaction Module, as per the manufacturer's instructions. Fluorescence was normalized by subtracting the fluorescence signal generated by uncleaved RS28 substrate.

RESULTS AND DISCUSSION

We first tested whether an RCA product could be formed following self-phosphorylation and circular ligation of Dk2 using our experimental scheme. The sequence used for our study was a truncated 63-nucleotide construct previously shown to be the shortest variant of Dk2 capable of robust self-phosphorylating activity. Self-phosphorylation was carried out by incubating Dk2 with 100 μM GTP in self-phosphorylation buffer. Following phosphorylation, circular ligation was carried out using T4 DNA ligase and a splint oligonucleotide complementary to the 5′ and 3′ ends of Dk2. RCA was carried out in the presence of ϕ29 DNA polymerase, dNTPs, and [α-^{32}P]deoxy-GTP to allow for detection of the RCA product. The products were subsequently resolved on a 10% denaturing PAGE gel. The results of this assay are shown in Figure 2a. Carrying out these steps produced a large product, as seen in lane 4. In contrast, RCA product is not observed in the absence of GTP (lane 1) or if ligase is omitted (lane 2). In these cases, only a short faint product resulting from polymerization of linear Dk2 and the splint DNA is apparent. No product is seen when ϕ29 DNA polymerase is not added during the polymerization step (lane 3).

To confirm the identity of the product as a concatameric complementary copy of the circularized Dk2, the RCA product was subjected to restriction enzyme digestion. Addition of a nucleotide sequence identical to a portion of Dk2 created an MnlI restriction site in the RCA product. If the RCA product is a repetitive complement of Dk2, digestion with this restriction enzyme will cleave the RCA product into monomeric units of 63 nucleotides. As seen in lane 5 of Figure 2a, restriction digestion did indeed result in the loss of the large RCA product and the formation of a monomeric product.

To assess the sensitivity of our system at low concentrations of GTP, we tested our system over a range of GTP concentrations from 1 nM to 2 μM. As seen in Figure 2c, an RCA product larger than 10 kilobases is clearly visible at as low as 25 nM GTP, with a signal-to-noise ratio of 4.2. The signal increases in intensity as the GTP concentration is increased to 2 μM GTP, demonstrating that this is a sensitive sensor for GTP. The presence of RCA products is observable on the gel with as little as 1 nM GTP, although bands below 25 nM were difficult to quantify. Quantification of the RCA product bands showed a linear response for GTP concentrations up to 200 nM, as shown in the insert of Figure 2d. The short duplex produced by the template and noncircularized Dk2 is seen at the bottom of the gel. The system was also tested for its ability to sense GTP in the presence of the similar purine triphosphate ATP, which is likely to be a significant contaminant in many practical GTP sensing applications. As shown in Figure 2b, the presence of 1 mM ATP did not affect the ability of the sensor to detect different concentrations of GTP.

While the presence of RCA product on separation gels shows that RCA can significantly amplify the self-phosphorylation of Dk2 by GTP, the preparation and running of a polyacrylamide or agarose gel is labor intensive and reduces the ease and expediency of an otherwise facile series of reactions. To convert this RCA amplified self-phosphorylating deoxyribozyme into a practical sensor for GTP, it is necessary to couple it to a detectable readout in lieu of relying on gel electrophoresis. Several methods are available for detection of an RCA product. Fluorescence detection can be achieved with the use of molecular beacons,[39,40] whereas colorimetric detection has been made possible with deoxyribozyme-modulated peroxidase activity assays[41] and organic dyes.[38]

For this study, we chose to use a novel detection method incorporating a fluorogenic deoxyribozyme into the RCA product as shown in Figure 3. This method takes advantage of both the sensitivity of fluorescent detection and the processivity

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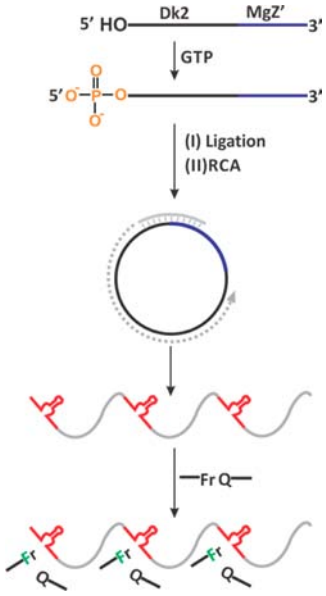

Figure 3. Fluorescent reporting of signal amplification using the MgZ fluorogenic deoxyribozyme. Scheme for generation of RCA products containing multiple MgZ RNA-cleaving fluorescence-generating deoxyribozymes, which can cleave the RS28 substrate to produce an increase in fluorescence.

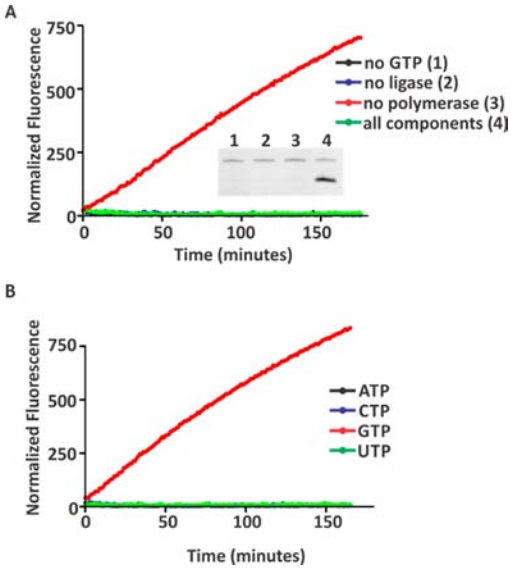

Figure 4. (A) Fluorescence generation upon incubation of RS28 substrate with Dk2/MgZ′ RCA product after self-phosphorylation, ligation, and RCA. A time-dependent fluorescence increase is seen when all components are added (red line), but not in the absence of GTP, ligase, or ϕ29 DNA polymerase. RS28 cleavage is shown in inserted gel. (B) NTP specificity of fluorescent sensor. Self-phosphorylation of Dk2/MgZ′ was performed in the presence of different NTPs followed by circular ligation, RCA amplification, and RS28 cleavage.

of a DNA catalyzed reaction. The deoxyribozyme we chose for fluorescence detection was MgZ, a fluorescence generating RNA-cleaving deoxyribozyme previously engineered by our group.[42] This deoxyribozyme cleaves a chimeric DNA/RNA substrate containing a fluorescein and DABCYL dye flanking an RNA residue that acts as the cleavage site. In the uncleaved state DABCYL quenches the fluorescence of fluorescein. Once cleaved, sequences containing fluorescein and DABCYL are separated and the quenching effect of DABCYL is lost, resulting in an increase in fluorescence. To allow for expression of MgZ in the RCA product, a Dk2 construct was made in which the antisense sequence of MgZ (MgZ′) was appended to the 3′ end of Dk2. The 3′ end was chosen for adding MgZ′ as 40 additional nucleotides were present at the 3′ end in the originally selected Dk2 sequence. Thus addition of this extra sequence should not effect the folding of Dk2 into its active structure. The MgZ deoxyribozyme is also compatible with ϕ29 reaction buffer, as both the polymerase and deoxyribozyme require magnesium(II) for activity.

Similar to the Dk2 construct used in the nonfluorogenic assay (Figure 2), this construct was able to produce an RCA product after incubation with GTP and ligation, as shown in the Supporting Information, Figure 1. This RCA product, which is a concatamer of the antisense of the Dk2/MgZ′, will contain multiple copies of the catalytic MgZ sequence. After RCA and heat inactivation, a substrate for MgZ can be added and will be cleaved by the multiple copies of MgZ in the RCA product producing an increase in fluorescence. As shown in Figure 4a, an increase in fluorescence is observed over time when GTP is added during the self-phosphorylation step (red line), while no fluorescence increase is observed when no GTP is provided or when no ligase or polymerase is added during the necessary reaction steps. Cleavage of the substrate is confirmed in the inserted gel of Figure 4a, as a fluorescent cleavage product is only seen when all reaction components are present (lane 4).

After establishing that RCA and fluorogenic deoxyribozyme activity allowed for the detection of GTP through self-phosphorylation, we assessed the selectivity of our fluorescent reporter system between the biologically relevant ribonucleoside triphosphates: ATP, CTP, GTP and UTP. We implemented the same reaction protocol described above. An RCA product was only observed when DK2/MgZ′ was incubated with GTP and not with any of the other three NTPs (Supporting Information, Figure 2a). When the product of the RCA reactions are incubated with RS28 substrate an increase in fluorescence over time is observed with GTP, while no increase in fluorescence is seen when Dk2/MgZ′ is incubated with ATP, CTP or UTP, as shown in Figure 4b. Gel analysis of the RS28 substrate after incubation showed this fluorescence increase to be the result of RS28 cleavage as shown in the Supporting Information, Figure 2b.

As this method of detection differs significantly from the gel-based separation method shown above, the response of the fluorescence system to different GTP concentrations was also tested, as the sensitivity of these two methods is likely to differ. As shown in Figure 5a, an increasing rate of fluorescence generation was seen from 10 μM to 1 mM GTP, showing that this fluorescent sensor is responsive to a range of GTP concentrations. The lower limit of fluorescent detection was investigated by incubating Dk2 with low micromolar amounts of GTP, as shown in Figure 5b. After the sequential reactions a time dependent fluorescent signal was observed with as little as 4 μM GTP, placing it as one of the most sensitive fluorescent sensors for GTP (as discussed below).

■ CONCLUSIONS

Our study has shown that it is possible to convert a deoxyribozyme substrate into a target for a novel sensor

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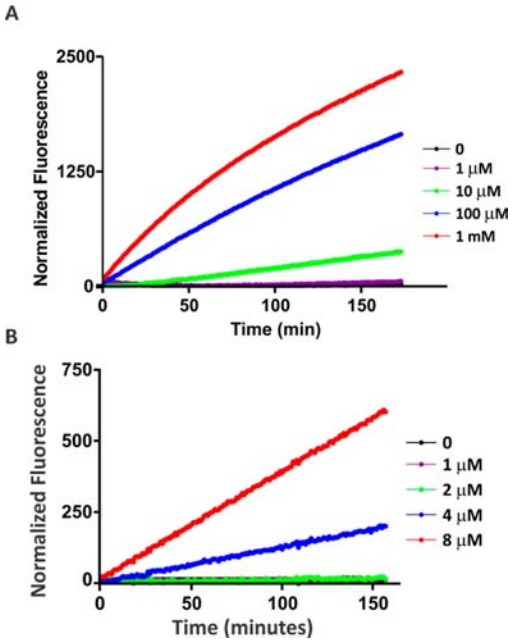

Figure 5. (A) Range of fluorescent GTP sensor. A time-dependent increase in fluorescence was seen when RCA product was incubated with RS28, after self-phosphorylation in the presence of 10 uM to 1 mM GTP, ligation and RCA. (B) Sensitivity of fluorescent sensor. Self-phosphorylation of Dk2/MgZ′ was performed in the presence of different micromolar concentrations of GTP followed by followed by ligation and RCA.

system. By utilizing properties unique to the reacted deoxyribozyme, we were able to perform two enzymatic reactions using the phosphorylated deoxyribozyme as a template, resulting in the production of large RCA products to specifically and sensitively report the presence of GTP. To couple this sensor to a spectroscopic readout, we appended nucleotides to Dk2 that resulted in the production of an RNA-cleaving deoxyribozyme that can cleave a fluorogenic RNA substrate. This modular design resulted in a sensor that could detect GTP at concentrations as low as 4 μM. Compared to the fluorescent GTP sensors mentioned in the introduction,[25−28,32] the detection limit of our assay is comparable to,[32] or lower than the other sensors tested, which were mostly designed to demonstrate specificity as opposed to sensitivity.

In terms of cellular applications, it has also been shown that fluorogenic DNAzyme sensors similar to our reporter can function in living cells.[43] Our sensor's ability to sense a range of GTP concentrations from 4 μM up to 1 mM (Figure 5) makes it suitable to cellular applications, as the physiological concentrations of GTP are typically 100 μM or higher[44,45] and fluctuations around this range can result in dramatic cellular changes. For example, moderate reduction of GTP concentration to 60 μM has been shown to induce apoptosis,[46] and this change in concentration is within the detection range of our sensor.

Our assays were performed without any purification between any of the steps from self-phosphorylation to fluorescence detection. Thus, this sensor system has the potential to be developed into a kit format with the entire procedure being carried out by repeated dilutions after each reaction using the components of the next reaction. To detect multiple targets simultaneously, the reporter portion can be easily modified with deoxyribozymes that cleave substrates containing fluorophores with different emission wavelengths.[47] With its ease of use, low detection limit, and ability to sense GTP in the presence of high concentrations of similar molecules, such as ATP, this sensor has the potential to be used in many practical applications. In addition to the demonstrated fluorescence signaling through coupling to a second deoxyriboyzme reaction, the production of an RCA product by our sensor system also gives our sensor the flexibility to be detected by many readouts. Many methods for reporting the polymerization of an RCA product have been developed and are continuously being improved[38−41](reviewed in ref 48). Our current system can be readily coupled to these and newly developed detection platforms, as the application dictates, in order to produce an efficient and facile sensor. If very high sensitivity is required over simplicity, the sensitivity of our sensor system could be further enhanced by addition of a step to separate the RCA product from the unreacted deoxyribozymes. This improvement of sensitivity by separation is demonstrated in the gel-based method in Figure 2, in which the separated product could be seen at as little as 25 nM GTP.

In addition to detecting GTP, the demonstrated system has the potential to be expanded to detect many more chemical analytes. In vitro selection could be used to isolate self-phosphorylating deoxyribozymes that can react with any substrate with a primary phosphate moiety. Other analytes could be detected as allosteric regulators or cofactors of self-phosphorylating activity. As an example, the sensor was shown to function in the presence of manganese(II) (for which the Dk2 deoxyribozyme requires for activity), but not other divalent metal ions, as shown in the Supporting Information, Figure 3. To detect other analytes, in vitro selection of de novo deoxyribozymes that only function in the presence of the analyte could be employed to prevent background due to analyte-independent catalysis seen with some allosteric RNA-cleaving systems, as discussed in the introduction. More generally, we have demonstrated that deoxyribozyme substrates can be converted into targets for sensors by amplification of the deoxyribozyme reaction and coupling to a detection system. As it is becoming more and more apparent that deoxyribozymes can catalyze any reaction for which a selection scheme can be developed,[49,50] the number of possible deoxyribozyme-based sensors is only dependent on the imagination of the researcher.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Oligonucleotide sequences and electrophoresis data for NTP and divalent metal ion specificity tests. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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