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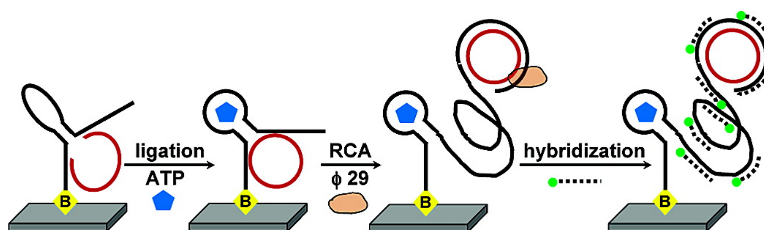

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Using a Deoxyribozyme Ligase and Rolling Circle Amplification To Detect a Non-nucleic Acid Analyte, ATP

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In vitro selection can yield nucleic acid binding species (aptamers) against a wide variety of targets, from small organics to proteins, peptides, and cells.[1] By coupling known aptamer binding domains with ribozyme catalytic domains, it has proven possible to generate allosteric ribozymes or "aptazymes" whose catalytic activities are modulated by effector molecules.[2,3] Aptazymes that can sense small analytes such as ATP, FMN, and theophylline have been both designed and selected. A variety of detection schemes have been developed on the basis of the ability of aptazymes to transduce molecular recognition into either covalent cleavage or ligation, including several aptazyme-based chip arrays.[4,5] However, since aptazymes can uniquely convert the recognition of analytes into the production of amplicons, the most novel and robust detection schemes for aptazymes have involved sequence amplification.[3]

While PCR is typically used for ligase-mediated sequence amplification including proximity DNA ligation,[6] rolling circle amplification (RCA) methods have also been shown to detect nucleic acids with great sensitivity[7] and to be adaptable to a wide variety of analytical schemes and devices.[8,9] In RCA assays, small linear DNA probes hybridize to target sequences and are circularized; the circularized templates (padlock probes) are then copied into linear concatamers by DNA or RNA polymerases under isothermal conditions.[10] The concatamers are frequently thousands of nucleotides in length, leading to a large amplification of the initial circular "signal". RCA has been an especially useful method for on-chip signal amplification because RCA-amplified concatamers can be localized to a given microarray spot.[9,11]

We now meld the unique abilities of aptazymes to transduce molecular recognition to sequence information with the capabilities of RCA to amplify nucleic acid sequences. To this end, we use a DNA aptazyme that was previously generated by appending an anti-ATP DNA aptamer[12] to a selected deoxyribozyme ligase.[13] In the resultant deoxyribozyme the autoligation[14] of a 3′-phosphorothioate on a 5′-iodine residue was activated by up to 460-fold by the cognate analyte, ATP. We hypothesized that if this aptazyme could catalyze the ligation and circularization of a single-stranded DNA substrate upon ATP recognition, this would generate a probe that could be directly amplified via RCA and thereby generate a visible signal on a chip-based array.

For this proof-of-principle demonstration, we engineered an ATP-sensing DNA aptazyme that could ligate its substrate in trans, as shown in Figure 1A. The substrate was a 63 mer oligonucleotide that contained iodo and phosphorothioate moieties at its 5′ and 3′ ends, respectively, was complementary to the aptazyme, and could be circularized upon ligation. Initially, we examined ATP-dependent ligation followed by RCA in solution. The aptazyme was mixed with its substrate in either the presence or absence of 10 mM ATP at room temperature. Aliquots of the ligation mixture were taken after 1 h, ligated products were directly amplified without further purification by RCA using the ϕ29 DNA polymerase at 37 °C, and

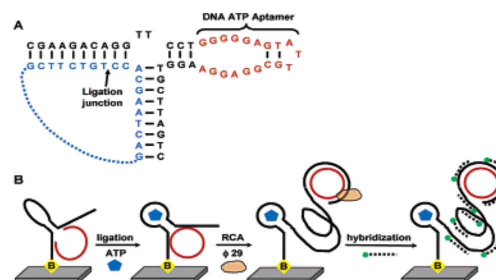

Figure 1. (A) Deoxyribozyme ligase and substrate. The ligase is shown in black, the allosteric, ATP-binding domain is in red, and the padlock probe is in blue. An arrow indicates the ligation junction. (B) Schematic of deoxyribozyme-mediated RCA. The aptazyme labeled with biotin at its 5′ end is immobilized on a streptavidin-coated glass slide. The aptazyme is activated by ATP and ligates a padlock probe. RCA is initiated from the 3′ end of the aptazyme, and the elongated aptazyme is visualized using fluorescent oligonucleotide probes labeled with Cy3 (green ●).

the amplified products were separated by gel electrophoresis. The amount of product that accumulated was determined by staining with a fluorescent dye and scanning on a FluoroImager (Molecular Dynamics, Sunnyvale, CA).

The signal due to RCA increased approximately 1500-fold after 10 min of amplification. The signal in the presence of ATP was about 19-fold higher than in the absence of ATP. The apparent activation by ATP is not as high as previously reported,[12] likely because the amplification provided by RCA overrepresented the background ligation reaction. Even without optimization of the ligation and RCA reactions, the positive signal was clearly detectable when compared to the negative control. Additionally these results suggested the potential for analyte quantitation in a multiplexed format.

This novel assay strategy could be readily adapted to a chip array format (Figure 1B), and all further optimizations were carried out in the context of the chip. A biotin-modified aptazyme was immobilized on streptavidin-coated glass slides. We and others[4,15] have previously reported that the immobilization of RNA aptamers and aptazymes onto solid substrates altered their target-binding and kinetic characteristics; therefore, it was possible this would occur for the deoxyribozyme ligase as well.

To address this issue, we generated biotinylated aptazymes with linkers (dT and T_{10}) at their 5′ end that would extend them away from the surface. Initially, we examined ligation activation in solution in the presence and absence of 10 mM ATP. Monitoring ligated products for 2 h, we found that biotinylated aptazymes showed a slightly higher extent of ligation (40−42%) compared with unmodified aptazymes (34%) in the presence of ATP, but they also showed a significant increase in background ligation in the absence of ATP (14−24% of ligation for biotin-labeled aptazymes vs 6% ligation for unmodified aptazyme). As a result, the ligation activation (ratio of percent ligation in the presence of ATP vs percent ligation in the absence of ATP) for the biotin-labeled

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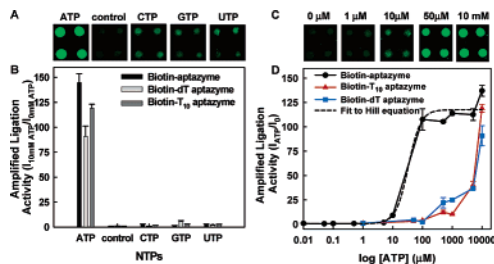

Figure 2. ATP-sensing performance on a chip array. (A) Fluorescence images of biotin-labeled aptazyme (without linker) spots on a chip array showing the specificities over various nucleotides. (B) Amplified ligation activity measured in the presence of NTPs (10 mM ATP, UTP, GTP and CTP) and in the absence of NTPs (denoted as control) for biotin-modified aptazymes with various linkers: no linker, dT, and T_{10}. $I_{10mM-ATP}$ represents fluorescence intensity at 10 mM ATP, while I_0 represents fluorescent intensity at 0 mM of ATP. (C) Fluorescence images of biotin-labeled aptazyme (without linker) spots on a chip array at the ATP concentration of 0, 1, 10, and 50 μM and 10 mM. (D) Amplified ligation activity as a function of ATP concentration for biotin-modified aptazymes with various linkers: no linker, dT, and T_{10}. The dotted line represents the best fit to the Hill equation (SigmaPlot, SPSS, Chicago, IL).

aptazymes was between 1.7 and 4.7, whereas the ligation activation for the unmodified aptazyme was 7.0. Interestingly, the catalytic activity of biotin-labeled aptazymes decreased by about 32−64% by adding linkers such as dT and T_{10}.

Next, we prepared chip arrays by printing biotin-modified aptazymes with and without linkers. Printed aptazymes were incubated at room temperature for 1 h under high humidity (60−80%) and then exposed to either 10 mM ATP, UTP, CTP, or GTP. After 1 h ligation, slides were washed with ligation buffer (50 mM Tris-Cl buffer, pH 7.4, and 50 mM $MgCl_2$) containing 0.05% Tween 20. Ligated products were amplified by RCA for 5 min at 37 °C, and the slides were again washed two times with SSC buffer containing 0.05% Tween 20. Finally, amplified products were detected by hybridization with 0.5 μM of a complementary, fluorescent oligonucleotide probe for 30 min at 37 °C. Figure 2A shows the fluorescence images of biotin-modified aptazyme spots when they were subjected to various nucleotides. Even though the images were taken at relatively low excitation power (PMT power of 400V at λ_{ex} 532 nm using Axon microarray scanner), bright signals due to signal amplification using RCA (∼13-fold) could readily be seen. Figure 2B summarizes the amplified ligation activities for three biotin-modified aptazymes with various linkers. In general, the aptazyme without a linker showed higher activation than aptazymes containing linkers, as was the case in solution. However, all aptazymes exhibited more than 100-fold activation in the presence of ATP and showed no or very weak activation (<5%) above background by other NTPs. The improved responsivity on the surface relative to that in solution could be attributed in part to the fact that unreacted, linear substrate could be washed away prior to initiating RCA and was therefore unable to react further during the amplification step. These results demonstrate that aptazyme-catalyzed effector-dependent ligation coupled with RCA can differentiate between structurally similar molecules such as UTP, CTP, and GTP on a sensor platform with a preparation time of an hour and a development time of only a few minutes.

Finally, to determine if a combination of aptazyme-catalyzed ligation and RCA could be used to quantitate analytes in the chip array format, response curves were obtained by measuring the fluorescence intensity of RCA products as a function of ATP concentration. Responses were measured for all three ATP-responsive deoxyribozyme ligase aptazymes with different linker sequences. Figure 2C shows the scanned images of fluorescence labeled aptazyme-catalyzed circularization and RCA reactions as a function of ATP concentration. The biotinylated aptazyme without any linker again yielded the best responsivity (Figure 2D). As previously reported, the anti-ATP aptamer has two ATP binding sites, which seem to function cooperatively.[12,16] Therefore, the sharpness of the sigmodial response curve is not surprising. The calculated Hill coefficient for the biotin-labeled aptazyme was 2.1 $\pm$ 0.56. While as little as 1 μM ATP could be reproducibly detected by the deoxryibozyme ligase, a large response was produced over a relatively narrow concentration range (from 10 to 100 μM). The deoxyribozyme essentially serves as a "switch" that activates amplification only at a threshold concentration of ATP. Selected aptazymes that we and others have reported have lower background rates of ligation and thus might give better limits of detection. In addition, the fact that the assay takes places directly on a chip surface in the presence of a very small amount of aptazyme (ca. 300 amol) may make it possible to prevent the dilution of cellular metabolites into large volumes, as is the case for many conventional, enzyme-based assays.

In summary, we have described a novel analytical approach in which analytes are transduced into amplicons, in this case circular probes, that can be readily amplified by rolling circle amplification. The utility of this strategy has been demonstrated for the sensitive detection of ATP. Aptazyme-coupled RCA was adapted to a sensor platform that could be easily read and multiplexed, and reproducible signals were acquired within a few minutes.

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Supporting Information Available: Oligonucleotide sequences and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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