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DNA Antenna Tile-Associated Deoxyribozyme Sensor with Improved Sensitivity

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Abstract

Some natural enzymes increase the rate of diffusion-limited reactions by facilitating substrate flow to their active sites. Inspired by this natural phenomenon, we developed a strategy for efficient substrate delivery to a deoxyribozyme (DZ) catalytic sensor. This resulted in 3- to 4-fold increase in its sensitivity and up to 9-fold improvement of the detection limit. The reported strategy can be used to enhance catalytic efficiency of diffusion-limited enzymes and to improve sensitivity of enzyme-based biosensors.

Graphical Abstract

Deoxyribozyme (DZ) sensors for fluorescent detection of nucleic acids demonstrate improved sensitivities and limits of detection when attached to a DNA tile, which attracts and increases local concentration of the fluorogenic reporter substrate.

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Keywords

Deoxyribozymes; split hybridization probes; DNA crossover tile; mycobacterium tuberculosis; diffusion limited reactions

Deoxyribozymes (DZ) are non-natural catalytic DNA oligomers derived by *in vitro* selection.^[1] The most abundant classes of DZ are those that cleave RNA phosphodiester bonds.^[2] DZ-based biosensors are currently employed for sensing metal ions.^[3] They have also been proposed for analysis of nucleic acids^[4] and other biological molecules.^[5] The advantageous characteristics of DZ sensors include biocompatibility, affordable cost, versatility in design, and chemical stability (in comparison with protein and RNA sensors).^[1,2,6] We and others have been developing binary (split) DZ sensors (BiDZ) for nucleic acid analysis (Figure 1A).^[7] In this approach, two DNA strands called DZ_a and DZ_b, hybridize to a specific DNA or RNA analyte and form a DZ catalytic core that can then cleave an RNA phosphodiester bond in a fluorophore(F)- and quencher(Q)-labeled fluorogenic substrate (F_{sub} in Figure 1A). When the fluorophore is separated from the quencher, it emits high fluorescence, which produces a detectable signal. An important advantage of BiDZ sensors over other hybridization probes (e.g. molecular beacon,^[8] strand displacement,^[9] adjacent hybridization^[10] probes) are their ability to amplify the detection signal through catalysis: one activated BiDZ can cleave many substrate molecules, which increases the signal over time. This signal amplification feature allows for low limit of detection (LOD), which is an important characteristic of any biosensor and significant for PCR-free detection of RNA and bacteria.^[7g,h,11] The most sensitive BiDZ was designed by Mokany *et al.*^[7c] from 10–23 DZ.^[12] The catalytic activity of 10–23 DZ is limited by the rate of substrate delivery to the active site, while the cleavage efficiency is close to ‘perfect’ under special *in vitro* conditions.^[12b] Mokany *et al.* achieved a LOD of 5 pM for 10–23 BiDZ in a 3 hr assay using elevated (55°C)^[7c] temperatures that facilitated substrate diffusion. Is it possible to further increase the catalytic efficiency of diffusion-limited 10–23 and by this means achieve lower LOD for 10–23 DZ sensors?

We were inspired by the strategy used by some natural enzymes to overcome diffusion limitation by speeding up the flow of substrates to their active sites.^[13] Indeed, according to the Chou model,^[13] the surface of some proteins can act as a “promoter”, accelerating the flow of substrates into their active sites, which can increase the rate of diffusion-controlled reactions by one order of magnitude.^[13a] We theorized that increased flow of F_{sub} to the analyte-activated BiDZ through facilitated delivery with the concomitant increase of DZ catalytic efficiency would make it possible to lower LOD of DZ-based biosensors.

We used self-assembling DNA tiles^[14] as a platform for both the analyte-dependent formation of DZ catalytic core and F_{sub} delivery (Figure 1B). As target analytes, we chose fragments of DNA encoding 16S rRNA of either *Mycobacterium tuberculosis* (Mtb) (named here **D_{Mtb}**) or a non-tuberculosis *Mycobacterium smegmatis* (Msg) (**D_{Msg}**).^[15] We designed tile-free BiDZ sensors **BiDZ_{Mtb}** and **BiDZ_{Msg}**, which were specific to the correspondent analytes (Table S1). We then designed two tile-associated BiDZ (Tile-BiDZ) sensors, **Tile-BiDZ_{Mtb}** and **Tile-BiDZ_{Msg}** with specificity to one of the two analytes. In

our proof-of-concept design, a Tile-BiDZ sensor consisted of 9 DNA strands, one of which was linked to one of the two BiDZ strands (**DZ_b**, purple strand in Figure 1B). Eight other tile-forming strands were equipped with a total of 16 identical capture sequences (both at 5'- and 3'-ends) complementary to the substrate-delivering **Hook_D** strand, which was partially complementary to **F_{sub-1}**. **Hook_D** was expected to bind to the DNA tile and attract **F_{sub-1}** from solution, which would increase its local concentration around the analyte-activated BiDZ sensor (see Figure S1 for more detailed design). **Hook_D** hybridized to **F_{sub-1}** via a separate hilt sequence (dashed black line in Figure 1B) to minimize the competition for **F_{sub-1}** binding between **Hook_D** and the substrate-binding arms of the activated BiDZ. The complete formation of **Tile-BiDZ_Mtb** was revealed by gel electrophoresis (Figure S2). We measured the dependence of the fluorescent response of the tile-associated DZ on the analyte concentration and compared the slope of the graph (sensitivity)^[16] and the LOD^[17] with that of the free BiDZ sensor in solution (standard design, Figure 1A). It is important to note that the reaction conditions for the tile-free BiDZ sensor were optimized earlier.^[7c] For example, a higher concentration of **F_{sub}** would result in a higher background fluorescence due to the increased spontaneous cleavage of the substrate in the absence of analytes, which would negatively impact LOD. Furthermore, substrate-binding arms of BiDZ sensors were optimized for operation at 55°C,^[7c] which enabled greater catalytic activity than at lower assay temperatures.^[12]

Figure 2 demonstrates that for both Mtb and Msg-specific sensors, sensitivities of **Tile-BiDZ** was greater than that of the tile-free BiDZ. This is reflected by the difference in the slopes of the correspondent concentration-dependence linear trendlines. For Mtb-specific sensor, this difference was about 2.7 (compare slopes (a) and (c) in Figure 2A). For the Msg-specific sensor, this difference was about 3.7 (compare slopes (a) and (c) in Figure 2B). At the same time, the slope of the trendlines in the absence of **Hook_D** was about the same as for the tile-free BiDZ (compare slopes (b) and (a) in Figure 2A and B). This implies that the presence of **Hook_D** is essential for the improved sensitivity of **Tile-BiDZ** sensors. To further quantify the effect of the tile on sensor performance, we compared the LODs for the tile-free and tile-associated BiDZ sensors. The tile-associated BiDZ sensors were able to detect lower concentrations of the analytes than tile-free BiDZ in all studied cases (Table S2). For example, in a 1 hr assay, **Tile-BiDZ_Mtb** detected as low as 6 pM **D_{Mtb}**, which was about 6.5 times lower than for the tile-free sensor (39 pM). LOD of 5 and 8 pM after 3 hrs for tile-free BiDZ was comparable with that reported in other studies (3–10 pM).^{7c,h} Similar LOD was achieved by the tile-associated BiDZ just in 1 hr assay, which is ~6–8.5 times lower than that for tile-free BiDZ (Table S2, compare LODs after 60 min). However after 3 hr of incubation the difference in LOD of the two approaches was not as impressive (only ~1.3–2.5 times). Therefore, the effect of alleviated substrate delivery is more pronounced during shorter incubation times, which in practice can lead to shortening the assay time.

To prove that the observed effect of the tile is preserved when more complex samples are used, we compared the performance of the tile-free and tile-associated BiDZ sensors using total RNA isolated from Mtb surrogate organism – *M. bovis* BCG strain (**R_{Mtb}**) for Mtb-specific sensors and from *M. smegmatis* (**R_{Msg}**), for Msg-specific sensors. The rRNA

sequence of BCG strain is 100% identical to that of Mtb. Panels C and D in Figure 2 demonstrate similar trends for sensitivity and LOD as described for panels A and B. Comparison of the two approaches revealed about 3.3- and 4.3-fold improvement in sensitivity for Tile-BiDZ sensors in the presence of **R_{Mtb}** and **R_{Msg}** analytes, respectively (compare slopes (a) and (c) in Figure 2C and D). The LOD for **Tile-BiDZ_{Mtb}** was measured to be about 9.6 ng/μL or approximately 5 nM,^[18] which was ~ 9 times lower than that of the tile-free sensor (~87 ng/μL or ~ 45 nM) after 1 hr of incubation (Table S2). The LOD of **Tile-BiDZ_{Msg}** (0.4 ng/μL or ~196 pM) was ~5 times lower than that of the free sensor (1.9 ng/μL or 975 pM) after 1 hr of incubation (Table S2). Note that LODs determined for the RNA samples were higher than those determined for the synthetic DNA analytes. This is most likely due to the stable RNA secondary structure, which inhibits hybridization of the BiDZ sensor to its target RNA.

In this study, we followed the natural path for overcoming the efficiency limitation for the ‘catalytically perfect’ enzymes^[13] and applied it to a practically significant scenario: detection of nucleic acids by BiDZ sensors. We found that association of BiDZ with a DNA tile that attracts the reporter substrate and thereby increases its local concentration demonstrated improved sensitivity and detection limits. The success of the design reported here may be attributed to the following features. (i) Melting temperatures of **Hook_D** and **F_{sub-1}** strands were chosen to be close to the reaction temperature (55 °C), which enabled exchange of DNA tile-associated **F_{sub-1}** and **Hook_D** with that in solution. (ii) **DZ_b** and **Hook_D** were equipped with flexible TTT or triethylene glycol linkers (see table S1 for sequences). Both features (i) and (ii) impart certain flexibility to the system which enabled **F_{sub-1}** to reach the DZ catalytic core. This might be important considering that the sequences of both **F_{sub-1}** and **Hook_D**, as well as the attachment of the Hook strands to the DNA tile or their orientations toward DZ core, were not thoroughly optimized. (iii) In the absence of the analyte, DZ core is not exposed to the locally elevated concentrations of **F_{sub-1}**, since **DZ_a** remained free in solution. This last feature helped to maintain low background in the absence of a specific analyte, which would be impossible if an alternative structure-switching DZ sensor format (e.g. ‘catalytic molecular beacons’)^[4a,b] was implemented. (iv) The tile associated BiDZ should inherit its high selectivity common to all binary (split) sensors.^[7,10] Indeed, both **Tile-BiDZ_{Mtb}** and **Tile-BiDZ_{Msg}** were able to specifically recognize cognate analytes producing no signal above the background in the presence of non-specific analytes (Figure S7). Further optimization experiments can include variation of the number and the orientation of the Hook strands attached to the DNA tile. It would also be interesting to investigate the performance of the system at ambient temperatures, considering the significance of room-temperature assays for point-of-care diagnostics.

In conclusion, the advantage of the tile-associated DZ sensor over its tile-free counterpart was demonstrated for the detection of DNA and RNA molecules. Assembling of the tile requires simple thermal annealing of DNA strands, which are inexpensive commercial products. One can envision application of similar technology for the detection of metal ions^[3] and other biological molecules.^[5] On the other hand, tile-associated deoxyribozymes and ribozymes could possibly serve as simple models for the study of mechanisms of

enzymatic catalysis, e.g. strategies for overcoming the diffusion limitations or the substrate channelling along the active sites of several enzymes.^[14c,19]

Experimental Section

Concentration Dependence

Experiments with tile-free BiDz were completed as described previously^[7g] (see SI for detailed information). Tile-DZ_b associates were prepared by annealing 9 tile-forming strands (each 100 nM) (see Table S1 for sequences) in the reaction buffer (50 mM HEPES, pH 7.4, 50 mM MgCl₂, 20 mM KCl, 120 mM NaCl, 0.03% Triton X-100, 1% DMSO) and slow cooling of the samples overnight. **F_{sub-1}** (200 nM), tile-associated DZ_b (10 nM), DZ_a (2 nM) strands were incubated in the absence (negative control) or presence of different concentrations of complementary analytes in the reaction buffer at 55°C. The fluorescent intensities after 1 hr, and 3 hrs were measured at 517 nm (excitation at 485 nm).

RNA isolation

Total RNA was isolated from either *M. smegmatis* (strain MC2 155) or *M. bovis* (strain BCG) as previously described.^[15]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Design of the DNA antenna tile-associated binary deoxyribozyme (Tile-BiDZ). A) Tile-free BiDZ sensor reported earlier.^{7b} DNA strands DZ_a and DZ_b bind to the abutting fragments of a DNA or RNA analyte to form a DZ catalytic core, which cleaves the fluorophore (F)- and quencher (Q)-labelled fluorogenic substrate (F_{sub}) and produces a fluorescent signal. The analyte-binding arm (dashed lines) of DZ_b is short enough to sense a single base mis-pairing, thus allowing recognition of single nucleotide substitutions (SNS).^[7] B) Tile-BiDZ sensor. Nine DNA strands associate to form a DNA antenna tile. The central strand of DNA antenna tile is equipped with a linker connected to DZ_b strand (purple). In the presence of a specific analyte, the DZ_a strand (dissolved in solution) associates with the tile to form activated Tile-BiDz. Each of the 8 green strands contains two identical terminal fragments complementary to **Hook_D** strand (orange, only one is shown), which serves as an adaptor between the tile and F_{sub-1} . The activated Tile-BiDZ is, therefore, surrounded by up to 16 tile-associated F_{sub-1} molecules.

Figure 2.

Fluorescent response of tile-free and tile-associated BiDZ sensors to the presence of different concentrations of D_{Mtb} (panel A), D_{Msg} (panel B), R_{Mtb} (panel C) and R_{Msg} (panel D); (a) Tile-free BiDZ as shown in Figure 1A; (b) Tile-associated BiDZ, no **Hook_D** control; (c) Tile-associated BiDZ in the presence of **Hook_D** as shown in Figure 1B. Reaction mixtures containing BiDZ sensors (see Experimental Section for concentrations of DNA strands) were incubated with different concentrations of correspondent analytes at 55°C. Fluorescent intensities were measured at 517 nm ($\lambda_{ex} = 485$ nm) after 3 hrs of incubation. The data (average values of 3 independent experiments) were used to calculate the LODs listed in Table S2. Linear equations that correspond to each trendline are shown.