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Deoxyribozyme Cascade for Visual Detection of Bacterial RNA

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An ideal sensor should be user-friendly and inexpensive. It should report the presence of a specific analyte with high accuracy in a simple and straightforward assay.^[1] An example of a near-ideal sensor is the home pregnancy test, in which the exposure of an immunochromatographic strip to a urine sample containing a pregnancy marker, human chorionic gonadotropin, develops a colored test line. The important characteristics of the sensor include visual signal output that can be detected by the naked eye, and a simple protocol that does not require user expertise. Tests of this kind of facile format are yet to be developed for the detection of bacterial species. At the same time, straightforward, inexpensive and yet reliable bacteria-specific tests are urgently needed to expedite point-of-care (POC) disease diagnosis and monitor environmental or food contaminations. Here, we make use of recent advantages in functional DNA technology to design an assay that contributes to the development of an ‘ideal sensor’ against pathogenic bacteria.

The assay combines binary deoxyribozyme (Dz) technology^[2] and a peroxidase-like deoxyribozyme (PDz)^[3] in a cascade shown in Figure 1. The binary Dz probe consists of two DNA strands Dz_a and Dz_b (Figure 1A). Each strand contains a fragment complementary to the specific segment of bacterial RNA (analyte-binding arm) and a fragment complementary to the inhibited peroxidase-like deoxyribozyme (IPDz) substrate (IPDz-binding arms). IPDz is a stem-loop folded DNA with ribonucleotides embedded in the loop region (Figure 1A). A 5'-end terminal fragment of IPDz contains the G-rich sequence of PDz.^[3] Exposure of the two strands to a sample containing the target bacterial RNA results in the cascade of events as follows. Dz_a and Dz_b strands hybridize to the bacterial RNA and re-form a catalytic Dz core (Figure 1B). The RNA-cleaving Dz cleaves IPDz and releases the G-rich PDz fragment. PDz catalyzes oxidation of a colorless organic substrate, such as 3,3'-diaminobenzidine (DAB), into a colored product in the presence of hemin and hydrogen peroxide, thus changing the color of the solution (Figure 1C).

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In this proof-of-concept study, we used the sequence of a human pathogen *Mycobacterium tuberculosis* (*Mtb*)^[4] 16S rRNA as a practically significant analyte and demonstrated that the deoxyribozyme cascade is capable of differentiating the RNA from two closely related mycobacterial species. The sequence of 16S rRNA is 100% conserved among all members of the slow-growing *Mycobacterium Tuberculosis* Complex (MTC), which includes *Mtb* and the vaccine strain *M. bovis* BCG, among other species.^[4] In this study we used RNA purified from *M. bovis* BCG strain as a representative of MTC, called through the text MTC RNA. *M. smegmatis* is a non-pathogenic mycobacterium whose 16S rRNA is ~95% identical to that of members of the MTC.^[4] We designed two Dz cascade sensors specific to a 50-nt fragment (nts 181-231) of 16S rRNA from either MTC (Figure 2A) or *M. smegmatis* (Figure S1A).

Dz sensor reported by Mokany et al.^[2b] was adapted for the cleavage of IPDz (Figure S2). The design was uniform and straightforward for the two binary Dz probes specific to different analytes: IPDz-binding arms and the binary catalytic core of Dz_a and Dz_b were the same for the two sensors. Only the analyte-binding arms of Dz_a and Dz_b were designed complementary to their specific analytes (Figures 2A and S1A). The presence of multiple single nucleotide substitutions, insertions and deletions in the fragment of *M. smegmatis* RNA ensured high specificity of recognition of MTC RNA by the anti-MTC sensor. It was demonstrated earlier that structural adjustments of split probes allow differentiation of analytes that differ by a single nucleotide.^[2] Therefore, the Dz cascade is potentially applicable for the analysis of single nucleotide polymorphisms and point mutations, which opens a venue toward simple and inexpensive sensors for diagnosing human genetic disorders and drug-resistant bacteria.

The performance of the two sensors was initially optimized using synthetic DNA analytes corresponding to the targeted sequence of bacterial 16S rRNAs (Table 1). The LOD values quantified based on light absorbance at 500 nm using spectrophotometer (Figures S3, S4) were determined to be 0.4 nM and 0.6 nM for MTC- and *M. smegmatis*-specific sensors, respectively (Figures S3a and S4a). This LOD is at least 20 times lower than that reported for PDz-based sensor without the Dz cascade.^[1d,3a-d] Since an instrument-free detection format is preferable for the POC diagnostics, we explored TLC plate-based signal visualization. For this purpose, 3 μ L of the final samples were spotted on a TLC plate. The intensity of the spots correlated well with the absorbance values (Figure S3). We next performed the assay using total bacterial RNA isolated from either *M. smegmatis* or *M. bovis* BCG. The RNA preparations contained approximately equal amounts of 16S rRNA, as verified by electrophoretic analysis (Figure S5). For both MTC- and *M. smegmatis*-specific sensors, the absorbance of the samples increased as a function of the RNA amount added (Figure 2C and S6). The LOD was found to be 4.4 ng/ μ L and 4.8 ng/ μ L for MTC- and *M. smegmatis*-specific sensors, respectively. TLC-based visualization allowed reliable detection of as low as 12.5 ng of total bacterial RNA in a 3 μ L-spot (Figures 2B and S1B). Taking into account that a cell of slow-growing *Mtb* contains ~ 4,000 ribosomes and that rRNA comprises 83% of total bacterial RNA,^[7] we estimate that 12.5 ng of total RNA corresponds to ~10⁶ CFU. Our experimental correlation determined ~10⁷ CFU required for isolation of 12.5 ng of total RNA. Ten milliliters of a typical sputum sample of an *Mtb*-infected individual contains 10⁶-10⁷ CFU,^[8] which can be detected with the current detection limit. The detection limit can be further improved by employing multiple deoxyribozyme probes targeting all hyper-variable fragments of both 16S and 23S rRNAs. Next, we tested the ability of the sensors to differentiate between the RNA of closely related species. Both MTC and *M. smegmatis*-specific sensors produced significant color change only in the presence of their specific analytes (Figures 3 and S7). Moreover, the signal triggered by the non-specific RNA was only at the background level, thus demonstrating excellent selectivity of the assay.

Sensors based on cascades of Dzs for the detection of nucleic acids have been reported earlier.^[5,6] The distinguishing features of the assay proposed here include the following. (i) *Excellent selectivity*. Binary deoxyribozyme probes were found to have exceptional discrimination ability toward nucleotide substitutions in nucleic acids.^[1d,2] This is due to the possibility to design one of the two analyte-binding arms to be selective. In the present design, the analyte-binding arm of Dz_b strand was chosen to target a hyper-variable fragment of 16S rRNA (Figure 2A). (ii) *Signal can be detected by the naked eye*. Most of the deoxyribozyme cascades rely on fluorescence detection.^[5] Although more sensitive than visual assays, the fluorescent output requires a fluorometer for signal registration and, therefore, is less preferred for low-cost POC diagnostics. Visual assays based on RNA-cleaving deoxyribozymes have been reported earlier for the detection of metal ions^[9] and recently for the detection of a model DNA analyte.^[10] Together with the approach presented in this study, such assays may create a basis for a new generation of POC detection technologies. (iii) *The assay does not require protein enzymes*. A number of previously reported cascades rely on protein enzymes for signal production.^[6] However, proteins are significantly less stable than DNA under ambient conditions. Therefore, eliminating protein enzymes from a POC test is preferable for robust performance, reproducible results and economic shipment options. (iv) *Simplicity of the assay procedure*. Both RNA-cleavage and H₂O₂ decomposition reactions of the cascade operate in the same buffer, which eliminates the need of a buffer exchange between the reaction stages. The assay only requires incubation of a bacterial RNA with the reagents for 1 h at 50 °C and then for 30 min at room temperature followed by detection of a visual signal using TLC plates. The TLC plates can be scanned by a conventional scanner for quantification if needed. The hands-on time of the entire procedure is less than 10 min. (v) The assay was shown to work with real samples of a practically significant pathogen. To the best of our knowledge, there is no PCR-free assay that combines all the above properties essential for POC diagnostics. Our current study is focused on eliminating the RNA isolation step, which will further simplify the assay.

Experimental Section

RNA isolation

Total RNA was isolated from either *M. smegmatis* (strain MC2 155) or *M. bovis* (strain BCG) as previously published.^[1] The concentration of total RNA was calculated by measuring its absorption at 260 nm using a Nanodrop spectrophotometer and taking into account that 1 OD₂₆₀ corresponds to 40 µg/mL. The stock concentrations were 660 ng/µL and 1400 ng/µL for *M. bovis* and *M. smegmatis* RNA, respectively.

Gel electrophoresis

Total bacterial RNA isolated either from *M. smegmatis* (strain MC2 155) or from *M. bovis* (strain BCG) (1 µg in 5 µL) was mixed with 5 µL of a loading buffer×2 containing 2 mM EDTA, 0.01% bromophenol blue, 7 M urea, 13% Ficoll (w/v), 90 mM Tris-borate, pH 8.3. To serve as a size marker, ssRNA Ladder (New England BioLabs Inc, MA) (1 µg) was used. The samples were heated at 65 °C for 5 minutes, chilled on ice and loaded onto a 1% agarose gel. The gel was run at 100 V for 1 h, stained with GelRed (Biotium, CA), and the bands were visualized using a U:Genius gel documentation system (Syngene, MD).

Colorimetric assay

IPDz (1 µM), Dz_a (1 µM), Dz_b (30 nM) were mixed with either synthetic analyte or total bacterial RNA in the buffer containing 50 mM HEPES, pH 7.4, 50 mM MgCl₂, 20 mM KCl, 120 mM NaCl, 0.03% Triton X-100, 1% DMSO (reaction buffer) and incubated at 50 °C for 1 h. A typical reaction volume was 60 µL. In case of total bacterial RNA, the samples were heated at 95 °C for 5 min prior to incubation to ensure proper hybridization of the

deoxyribozyme strands to 16S rRNA. After incubation, the mixtures were cooled down to room temperature for 15 min to allow PDz to form a G-quadruplex structure. Then, hemin and DAB were added to the mixtures to the final concentration of 200 nM and 1 mM, respectively. Finally, H₂O₂ was added to the final concentration of 1 mM, and the mixture were incubated at room temperature for 30 min, then the absorbance of the final samples at 500 nm was measured using a Lambda 35 UV/Vis Spectrometer (Perkin Elmer, Inc., MA). The data was processed using Microsoft Excel 2010.

TLC plate-based visualization

To visualize an analyte-dependent color change, 3 μ L of the final samples were spotted on a silica gel TLC plate (EMD Chemicals Inc., Germany) and let dry on air. The plate was then scanned using a CanoScan LiDE210 scanner (Canon, NY), and the image was analyzed using a Gel-Pro Analyzer software. To calculate the relative intensity (RI) of the spots, the intensity of a sample was divided by the intensity of 100 nM Dz, which was used as a positive control for hemin-mediated DAB oxidation. The brightness and contrast of the image was adjusted using Corel PHOTO-Paint X6 software (Corel, Canada) to ensure proper visualization in a printed form.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. a) Rosi NL, Mirkin CA. *Chem Rev.* 2005; 105:1547–1562. [PubMed: 15826019] b) Kwok PY, Xiao M. *Cold Spring Harb Symp Quant Biol.* 2003; 68:65–67. [PubMed: 15338604] c) Zora A, Tribleris GP, Simserides C. *Recent Pat Nanotechnol.* 2011; 5:188–224. [PubMed: 21777179] d) Li D, Song S, Fan C. *Acc Chem Res.* 2010; 43:631–641. [PubMed: 20222738] e) Kolpashchikov DM. *Chem Rev.* 2010; 110:4709–4723. [PubMed: 20583806] f) Jenison RD, Bucala R, Maul D, Ward DC. *Expert Rev Mol Diagn.* 2006; 6:89–99. [PubMed: 16359270]
2. a) Kolpashchikov DM. *ChemBioChem.* 2007; 8:2039–2042. [PubMed: 17924377] b) Mokany E, Bone SM, Young PE, Doan TB, Todd AV. *J Am Chem Soc.* 2010; 132:1051–1059. [PubMed: 20038095] c) Gerasimova YV, Cornett E, Kolpashchikov DM. *ChemBioChem.* 2010; 11:811–817. [PubMed: 20301161] d) Gerasimova YV, Kolpashchikov DM. *Chem Biol.* 2010; 17:104–106. [PubMed: 20189100] e) Mokany E, Tan YL, Bone SM, Fuery CJ, Todd AV. *Clin Chem.* 2013; 59:419–426. [PubMed: 23232065]
3. a) Neo JL, Kamaladasan K, Uttamchandani M. *Curr Pharm Des.* 2012; 18:2048–2057. [PubMed: 22380516] b) Neo JL, Aw KD, Uttamchandani M. *Analyst.* 2011; 136:1569–1572. [PubMed: 21384012] c) Kolpashchikov DM. *J Am Chem Soc.* 2008; 130:2934–2935. [PubMed: 18281995] d) Deng MG, Zhang D, Zhou YY, Zhou X. *J Am Chem Soc.* 2008; 130:13095–132012. [PubMed: 18763776] e) Kosman J, Juskowiak B. *Anal Chim Acta.* 2011; 707:7–17. [PubMed: 22027115] f) McKeating KS, Graham D, Faulds K. *Chem Commun.* 2013; 49:3206–3208. g) Jiang C, Yan C, Jiang J, Yu R. *Anal Chim Acta.* 2013; 766:88–93. [PubMed: 23427805]
4. Rogall T, Wolters J, Flohr T, Böttger EC. *Int J Syst Bacteriol.* 1990; 40:323–330. [PubMed: 2275850]
5. a) Lu CH, Wang F, Willner I. *J Am Chem Soc.* 2012; 134:10651–10658. [PubMed: 22612395] b) Wang F, Elbaz J, Willner I. *J Am Chem Soc.* 2012; 134:5504–5507. [PubMed: 22404383] c) Lu LM, Zhang XB, Kong RM, Yang B, Tan W. *J Am Chem Soc.* 2011; 133:11686–11691. [PubMed:

- 21662240] d) Wang F, Elbaz J, Orbach R, Magen N, Willner I. *J Am Chem Soc.* 2011; 133:17149–17151. [PubMed: 21954996]
6. a) Wen Y, Xu Y, Mao X, Wei Y, Song H, Chen N, Huang Q, Fan C, Li D. *Anal Chem.* 2012; 84:7664–7669. [PubMed: 22928468] b) Tian T, Xiao H, Zhang Z, Long Y, Peng S, Wang S, Zhou X, Liu S, Zhou X. *Chemistry.* 2013; 19:92–95. [PubMed: 23225082] c) Li J, Yao QH, Fu HE, Zhang XL, Yang HH. *Talanta.* 2011; 85:91–96. [PubMed: 21645675]
7. Cox RA. *Microbiology.* 2003; 149:729–737. [PubMed: 12634341]
8. a) Joloba ML, Johnson JL, Namale A, Morrissey A, Assegghai AE, Mugerwa RD, Ellner JJ, Eisenach KD. *Int J Tuberc Lung Dis.* 2000; 4:528–536. [PubMed: 10864183] b) Palaci M, Dietze R, Hadad DJ, Ribeiro FK, Peres RL, Vinhas SA, Maciel EL, do Valle Dettoni V, Horter L, Boom WH, Johnson JL, Eisenach KD. *J Clin Microbiol.* 2007; 45:4064–4066. [PubMed: 17928422]
9. a) Chen J, Zhou X, Zeng L. *Chem Commun.* 2013; 49:984–866. b) Liu J, Lu Y. *J Am Chem Soc.* 2004; 126:12298–12305. [PubMed: 15453763] c) Zhang Q, Cai Y, Li H, Kong DM, Shen HX. *Biosens Bioelectron.* 2012; 38:331–336. [PubMed: 22784499] d) Lan T, Lu Y. *Met, Ions Life Sci.* 2012; 10:217–248. [PubMed: 22210341]
10. Zagorovsky K, Chan WC. *Angew Chem.* 2013; 125:3250–3253. *Angew Chem Int Ed Engl.* 2013; 52:3168–3171. [PubMed: 23401222]

Figure 1.

Principle scheme for deoxyribozyme cascade. A) Deoxyribozyme sensor consists of two strands of a split RNA-cleaving deoxyribozyme, Dz_a and Dz_b , and an inhibited peroxidase-like deoxyribozyme (IPDz). B) Binding to bacterial RNA re-forms the catalytic core of the RNA-cleaving deoxyribozyme, which cleaves IPDz and releases peroxidase-like DNA enzyme in solution. C) Peroxidase-like deoxyribozyme changes the color of the solution in the presence of hemin, H₂O₂ and an organic substrate.

Figure 2.

Deoxyribozyme cascade for the detection of MTC 16S rRNA. A) Design of MTC-specific deoxyribozyme cascade. Sequences of *M. smegmatis* and MTC 16S rRNA differ by several nucleotide substitutions. Insertions are indicated by a nucleotide above the sequence and the deletion is indicated by dashes (shown in orange). Analyte-binding arms of the split 10-23 deoxyribozyme^[2b] were complementary to MTC 16S rRNA sequence. Ribonucleotide inserts of IPDz are in red. B) TLC plate-based visualization of the signal for MTC-specific sensor in the presence of different amounts of total MTC RNA. C) Absorbance of the sensor at 500 nm in the presence of different Mtb analyte concentrations. The dashed line represents absorbance values correspondent to the limit of detection. The data is the average from three independent experiments, the error bars are given with standard deviations.

Figure 3. Selectivity of MTC- and *M. smegmatis*-specific deoxyribozyme sensors. TLC plate-based visualization of the samples containing no RNA or 100 ng total RNA from either *M. smegmatis* or BCG strain of *M. bovis*.

Table 1

Sequences of the oligonucleotides used in this study.

Mtb	5'- CAC GGG ATG CAT GTC TTG TGG TGG AAA GCG CTT TAG CGG TGT GGG ATG AG
M.smeg	5'- CCT GCT GGT CGC ATG GCC TGG TAG GGG AAA GCT TTT GCG GTG TGG GAT GG
Dz _a (MTC) [a], [b]	5'- CTC ATC CCA CAC CGC TAA AGC GCTT A CAA <i>CGA GAA CCC AAC C</i>
Dz _b (MTC)	5'- <u>GT TGC TCA TGG A</u> <i>GG CTA GCT</i> TCC ACC ACA AGA CAT GCA TCC CGT G
Dz _a (M.smeg)	5'- CCA TCC CAC ACC GCA AAA GCT TTCC A CAA <i>CGA GAACCC AAC C</i>
Dz _b (M.smeg)	5'- <u>GT TGC TCA TGG A</u> <i>GG CTA GCT</i> CCT ACC AGG CCA TGC GAC CAG CAG G
IPDz[c]	5'- GGG TAG GGC GGG TTG GGT TC rG rU CC ATG AGC AACTCG CCC
PDz	5'- GGG TAG GGC GGG TTG GGT TCG

[a] Nucleotides constituting the catalytic core of 10-23 deoxyribozyme are shown in italics.

[b] Nucleotides of split deoxyribozyme strands, Dz_a and Dz_b, that are complimentary to IPDz are underlined.

[c] Ribonucleotides are in bold.