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DNA nanotechnology for nucleic acid analysis: multifunctional molecular DNA machine for RNA detection[†]

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Abstract

The Nobel prize in chemistry in 2016 was awarded for ‘the design and synthesis of molecular machines’. Here we designed and assembled a molecular machine for the detection of specific RNA molecules. An association of several DNA strands, named multifunctional DNA machine for RNA analysis (MDMR1), was designed to (i) unwind RNA with the help of RNA-binding arms, (ii) selectively recognize a targeted RNA fragment, (iii) attract a signal-producing substrate and (iv) amplify the fluorescent signal by catalysis. MDMR1 enabled detection of 16S rRNA at concentrations ~24 times lower than that by a traditional deoxyribozyme probe.

Hybridization probes have been used for the detection of specific DNA and RNA sequences for the last 55 years.^{1a} Common challenges of hybridization probes include insufficient sensitivity and selectivity.¹ Detection of specific RNA molecules is particularly challenging due to the stable secondary structures that inhibit or even prevent their interactions with hybridization probes.² For example, some molecular beacon probes, fluorophore- and quencher-labelled DNA stem-loop structures,³ were previously shown to fail in detecting folded RNA and DNA molecules.⁴ Probes based on nucleic acid analogues, *e.g.* peptide nucleic acids and locked nucleic acids, are required to tightly bind and unwind structured RNAs.⁵ Here we took advantage of recent developments of DNA nanotechnology⁶ to design a multifunctional platform that enables (i) unfolding of a specific RNA analyte, (ii) specific recognition of a targeted fragment, (iii) enhanced delivery of a signal-producing substrate to a target-activated catalytic sensor, and (iv) signal amplification by catalysis. We named the platform ‘multifunctional DNA machine’⁷ for RNA analysis of the 1st generation’ (MDMR1).

MDMR1 was designed based on binary deoxyribozyme (BiDZ) probe,⁸ in which two unmodified DNA strands, DZ_a and DZ_b, hybridize to the abutting positions of a complementary DNA or RNA analyte to form the catalytic core of an RNA-cleaving deoxyribozyme (Fig. 1A).⁹ The active core cleaves a fluorophore-and a quencher-labelled F_{sub} strand, which results in a production of a fluorescent signal. For BiDZ, as the signal

[†]Electronic supplementary information (ESI) available: Detailed experimental procedure and the structure of MDMR1; data used for calculations of LODs, as well as kinetics of MDRRA1 that lacks RNA-binding arm or Hook strand. See DOI: 10.1039/c6cc06889h

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increases over time, the limit of detection (LOD) decreases due to the catalytic turnover of F_{sub}. BiDZ can be considered as one of the most promising hybridization probes for homogeneous mix-and-read nucleic acid analysis, since it enables (i) high selectivity of point mutation analysis,⁸ (ii) reduced cost,[‡] and (iii) LOD down to 8–20 pM after 60 min of incubation,⁸ which is ~50 times lower than that of commercially available fluorescent probes.^{1,3b} However, the LOD of BiDZ sensors is compromised when long and highly folded RNA is to be analysed. In this case, the access of the BiDZ probe to the targeted analyte fragment is limited resulting in ~10–25 fold decrease in LOD in comparison to an unstructured analyte.^{8h,i} We therefore seek to equip the BiDZ probe with the RNA-unwinding function. We reasoned that the addition of an RNA-unwinding function to the BiDZ probe will help to facilitate its access to the targeted fragment and achieve lower LODs. Furthermore, deoxyribozyme 10–23 used in this study is ‘catalytically perfect’,¹⁰ which implies that its catalytic efficiency can only be increased by facilitated delivery of the substrate to the active site – a strategy used by some natural protein enzymes.¹¹ We recently reported the design and performance of BiDZ attached to an antenna DNA tile with a mechanism for enhanced delivery of a fluorogenic substrate and found improved sensitivity in comparison with the tile-free BiDZ probe.^{8j}

In this study, we combined the substrate delivering and RNA unwinding functions in a single molecular device, MDMR1, which consisted of 9 DNA strands, T1–T9, forming a DNA crossover tile.¹² Strand T5 contained the sequence of DZ_b strand at its 5′-terminal fragment (Fig. 1B). To ensure low background in the absence of target RNA, DZ_a strand remained free in solution in the absence of an analyte. The RNA-unwinding function was integrated into MDMR1 in the form of two additional RNA-binding arms (red lines in Fig. 1B), which bound to a specific RNA analyte and opened its secondary structure to facilitate hybridization of DZ_a and DZ_b to the targeted analyte sequence. DZ_a and DZ_b strands helped to further unwind the secondary structure and formed the catalytic core. MDMR1 was also equipped with the fluorogenic substrate-attracting function, which consisted of 14 identical Hook-binding sequences (cyan fragments in Fig. 1B). These fragments hybridized to the complex of Hook (orange) and F_{sub}-1 strands, thus increasing the local concentration of F_{sub}-1 near the analyte-activated BiDZ catalytic core.[§]

In this study, we chose to target 16S rRNA from *Mycobacterium smegmatis*, which is a fast-growing non-pathogenic model for studying pathogenic mycobacteria including a tuberculosis-causing pathogen *Mycobacterium tuberculosis*.¹³ Two analytes were initially tested: total RNA from *M. smegmatis* (R_{Msg}) and a synthetic DNA analyte corresponding to the fragment of 16S rRNA complementary to the analyte-binding arms of DZ_a and DZ_b (D_{Msg}, Table S1, ESI[†]). The latter was used as a control analyte with a relatively unstable secondary structure (Fig. S1A, ESI[†]). We compared the performance of BiDZ_Msg and MDMR1_Msg in terms of their abilities to detect R_{Msg} and D_{Msg}. We expected that

[‡]For BiDZ design (Fig. 1A), analysis of each new analyte requires syntheses of unmodified DNA stands DZ_a and DZ_b, while expensive double labelled F_{sub} is universal: it can be synthesised in bulk and used efficiently for the analysis of multiple target sequences.

[§]Note that simple increase in the concentration of the substrate elevates both the overall analyte-triggered fluorescence signal and the background cleavage reaction in the absence of analyte, thus leading to high LOD of BiDZ probe.

[†]Electronic supplementary information (ESI) available: Detailed experimental procedure and the structure of MDMR1; data used for calculations of LODs, as well as kinetics of MDRRA1 that lacks RNA-binding arm or Hook strand. See DOI: 10.1039/c6cc06889h

MDMR1 would detect lower analyte concentrations than BiDZ due to both the presence of the RNA-binding arms and the high local concentration of F_{sub}-1. The advantage of the new platform was expected to be more pronounced for R_{Msg} than for D_{Msg}, since the detection of short and relatively unstructured DNA analytes only marginally benefits from the RNA unwinding activity of MDMR1.

For both BiDZ and MDMR1 sensors, the presence of R_{Msg} or D_{Msg} triggered the increase in fluorescent signal over time (Fig. 2). Both sensors responded with a linear initial rate to D_{Msg}, which is consistent with a simple kinetic scheme for enzymatic catalysis. In contrast, fluorescence increase for the sensors in the presence of R_{Msg} had a time delay (Fig. 2, dashed lines), which was presumably caused by the weakened probe interaction with a folded RNA conformation. Interestingly, for BiDZ_{Msg}, this first ‘delayed’ phase had a similar slope to that of the ‘no analyte’ curve, reflecting the negligible analyte-dependent increase in the probe fluorescence within the initial incubation time period (Fig. 2A, compare dotted and dashed curves). For MDMR1, though, the slope of the initial phase in the presence of R_{Msg} is similar to that for D_{Msg}, which can be attributed to the unwinding of the RNA secondary structure by the RNA-binding arms of MDMR1 (Fig. 2B, compare dashed and solid lines). We also observed a higher slope for R_{Msg} than for D_{Msg} after longer incubation times. This is presumably due to the greater probe affinity to RNA than to DNA analyte, which is the result of the higher stability of DNA–RNA (for R_{Msg}) than DNA–DNA (for D_{Msg}) hybrids.¹⁴ Overall, the slopes of the fluorescence increase over time were higher for MDMR1 than for BiDZ in the case for either of the analytes used, which supports the hypothesis that addition of the RNA-unwinding and substrate delivery functions improves sensor performance. To quantify this improvement, we calculated the limits of detection (LOD) for both sensors in the presence of either analyte based on the data presented in Fig. S2 and S3 (ESI[†]). In a 20 min assay, BiDZ_{Msg} probe was found to detect 221 pM D_{Msg} and 4860 pM R_{Msg} (Table 1). This ~ 22-fold difference in LOD emphasizes the reduced affinity of the BiDZ probe to the folded RNA analyte in comparison with the relatively unstructured D_{Msg}. At the same time, MDMR1 demonstrated LOD of 35 and 204 pM for D_{Msg} and R_{Msg}, respectively, after 20 min, which is 6 and 24 times lower than that for the tile-free BiDZ_{Msg}. This result reflects the advantage of MDMR1 approach in the detection of folded RNA over unfolded short DNA analyte. In a 60 min assay, the advantage in LOD of MDMR1 for R_{Msg} was only about 20-fold, which correlates with the hypothesis that the efficiency of BiDZ_{Msg} binding to the RNA target increases during extended incubation time. This hypothesis is also supported by the kinetic data (Fig. 2A) – the rate of the signal production for BiDZ_{Msg} in the presence of R_{Msg} increases over time.

The MDMR1 used so far in this study employed two strategies for achieving low LOD: (i) RNA unwinding by RNA-binding arms and (ii) enhanced F_{sub}-1 delivery. In order to estimate the contribution from each individual strategy, we assembled RNA-binding arm-free MDMR1 by replacing strands T3 and T7 with the counterparts lacking the RNA-binding arms (Table S1, ESI[†]). This MDMR1 version eliminated the RNA unwinding effect, but preserved the substrate delivery function. We also used MDMR1 in the absence of Hook strands, which eliminated the F_{sub}-1 delivery function. It was found that the signal accumulation rate for MDMR1 in the presence of R_{Msg} decreases in the absence of either of the functions (Fig. S4, ESI[†]). Introduction of only the substrate-delivery functionality

(MDMR1_Msg, no RNA-binding arms) reduced LOD only about 2.6 times in comparison with that of the BiDZ_Msg probe after 20 min of incubation (Table 1), which also agrees with our previous findings.^{8f} At the same time, preserving only the RNA unwinding effect (MDMR1_Msg, no Hook strand) improved the LOD by about 7-fold in a 20 min assay (Table 1). We, therefore, concluded that the majority of the LOD improvement observed in the original MDMR1_Msg in a 20 min assay was due to the RNA unwinding activity inherent in the RNA-binding arms rather than substrate delivery function. The experiments with deletion of RNA-binding arms or substrate delivering function support our hypothesis of RNA unwinding and substrate delivering functions of the proposed system. However, more systematic study is required to collect accurate experimental evidence demonstrating each function of the MDMR1 approach.

Next, we studied the ability of MDMR1_Msg to detect the presence of a single base mismatch in the analyte. We designed MDMR1 with DZ_a strands, which contained a single base mispairing with the targeted fragment. It was found that the mismatched sensors were unable to produce signal above the background in the presence of either R_{Msg} or D_{Msg}, while they were able to report the presence of fully complementary synthetic DNA analytes (Fig. S6, ESI[†]). This result proves that MDMR format maintains the high selectivity of BiDZ probe reported earlier.⁸ To demonstrate the general applicability of the approach, we targeted an alternative RNA, 16S rRNA of *Mycobacterium tuberculosis* (*M.tb*) complex (MTC). BiDz_Mtb and MDMR1_Mtb sensors were designed to recognize a fragment of MTC 16S rRNA (Fig. S7A–C, ESI[†]), which was either represented by a synthetic DNA analyte, D_{Mtb}, or was a part of total RNA isolated from *M. bovis* BCG (R_{Mtb}), a mycobacterial strain with 100% identical rRNA sequences to *M.tb*. We found similar performance to that shown in Fig. 2: MDMR1_Mtb reported the presence of R_{Mtb} faster and with higher S/B response than BiDz_Mtb (Fig. S7 panels D and E, ESI[†]).

A number of isothermal techniques including loop-mediated (LAMP)^{15a} and rolling circle (RCA)^{15b–d} amplifications have been used for the detection of DNA and RNA. The deoxyribozyme-based technology amplifies the signal, not the target, and, therefore, senses directly RNA rather than DNA amplicons. Such approach can be used for the detection of RNA in its native environment, such as in living cells (please note that BiDZ sensor can operate at ambient temperatures).^{8a,d} Moreover, signal amplification approaches are less sensitive to contaminants.^{1d} Other advantages of BiDZ-based approaches include: (i) high selectivity towards SNP, which is not provided by amplification techniques directly, but requires additional probe-based assays; (ii) protein enzyme-free format, which potentially enables storage of the reagents at room temperature for a long time.

In conclusion, we introduced a multifunctional nucleic acid sensor platform that enables as much as 24-fold improvement of LOD for RNA analysis in comparison with a conventional binary deoxyribozyme probe.⁸ The platform is modular and can be easily adjusted to each new RNA target or transformed to accommodate additional functions including modifications to enable targeted sensor delivery to cancer cells, or drug load for catalytic drug release in the presence of specific RNA targets, the concepts developed earlier.¹⁶ We, therefore, hypothesize that complex multifunctional sensors like MDMR1 reported here will replace traditional probes in the analysis of nucleic acids.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Design of MDMR1. (A) Tile-free BiDZ probe.⁸ DNA strands DZ_a and DZ_b bind to the abutting fragments of a DNA or RNA analyte and form deoxyribozyme catalytic core, which cleaves a fluorophore (F)- and quencher (Q)-labelled substrate (F_{sub}) and produces fluorescent signal. (B) MDMR1 design: nine DNA strands associate to form a DNA tile. DZ_b sequence is connected to T5 strand, while DZ_a is free in solution in the absence of an RNA analyte. T3 and T7 strands are each equipped with an RNA-binding arm (red), which binds the RNA analyte and unwinds its secondary structure. This assists in positioning of DZ_a and DZ_b on the analyte and bringing the two strands together to activate the BiDZ reaction core for F_{sub-1} cleavage. Strands T1, T2, T3, T4, T6, T7, T8 and T9 contain 14 identical terminal fragments (cyan) complementary to Hook strand (orange, only one is shown), which serves as an adaptor between the tile and F_{sub-1} . The activated MDMR1 is, therefore, surrounded by up to 14 tile-associated F_{sub-1} molecules.

Fig. 2.

Increase of the fluorescent signal over time for the BiDZ probe (A) or MDMR1 (B) in the absence (dotted line) or presence of 100 pM D_{Msg} (solid line) or 100 pM R_{Msg} (dashed line). Samples were incubated at 55 °C in the reaction buffer (50 mM HEPES, pH 7.4, 50 mM $MgCl_2$, 20 mM KCl, 120 mM NaCl, 0.03% Triton X-100, 1% DMSO) with continuous fluorescent measurement using QuantStudio™ 6 Flex Real-Time PCR System (see ESI[†] for more details).

Table 1 Limits of detections (LOD) for BiDZ and MDMR1^a

Sensor	Analyte	LOD	
		Incubation time, min 20	60
BiDZ_Msg	D _{Msgb}	221 pM	68 pM
	R _{Msg}	4860 pM (9.32 ng μL^{-1}) ^c	974 pM (1.87 ng μL^{-1})
MDMR1_Msg, no RNA-binding arms	D _{Msg}	50 pM	8 pM
	R _{Msg}	1838 pM (3.53 ng μL^{-1})	196 pM (0.38 ng μL^{-1})
MDMR1_Msg, no Hook strands	D _{Msg}	119 pM	25 pM
	R _{Msg}	692 pM (1.33 ng μL^{-1})	40 pM (0.08 ng μL^{-1})
MDMR1_Msg	D _{Msg}	35 pM	11 pM
	R _{Msg}	204 pM (0.39 ng μL^{-1})	50 pM (0.10 ng μL^{-1})

^aLOD were calculated using the data presented in Fig. S2 and S3 according to the procedure described in ESI.^bTotal RNA isolated from *M. smegmatis* (MC²155 strain) was used.^cThe conversion of ng μL^{-1} total RNA into pM for 16S rRNA was performed as described in ESI.