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DNAzyme amplification of molecular beacon signal

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Abstract

This paper reports an improved catalytic molecular beacon. Addition of the target oligonucleotide activates a DNA enzyme (DNAzyme), which, in turn, activates multiple copies of molecular beacons (MB) and gives rise to a strong fluorescence signal. In a previous design, the activated DNAzyme could oligomerize, especially dimerize, and result in inactivation of the DNAzyme. The current design avoids this problem, upon activated by the target DNA, the DNAzyme will stay constantly active. With the improved method, a detection of 10 pM DNA has been demonstrated, which is 1000 times more sensitive than the method previously reported.

Keywords: DNA; DNAzyme; Molecular beacon (MB); Fluoroscence

1. Introduction

DNA/RNA detection is important in diagnosis, gene therapy and other biomedical studies. Sensitive yet inexpensive and reliable detection methods are essential for efficient use of genomic information. Significant efforts have been devoted to improve the sensitivity and accuracy of DNA detection [1–5]. Especially, nanobiotechnology is heavily involved, which integrates biotechnology and nanotechnology [6]. One of its branches is to apply nanotechnology into biomedical research. For example, nanoparticles and nanowires are extensively studied as biosensors [7]. Parallel to this, bimolecular self-assembly, such as DNA nanotechnology [8], is another active branch of nanobiotechnology. On one hand, self-assembly represents a promising route for preparation of nanopatterns; on the other, the nanostructures and strategies developed in self-assembly can also be used to design sensitive biosensors. For example, this paper will take advantage of strand displacement strategy, which was originally developed and has been widely used in DNA-based nanomachines [9].

In many cases, sample or signal amplification is an essential step in DNA detection. For example, DNA sample can be readily amplified by polymerase chain reaction (PCR) [10,11]. PCR is a powerful tool and widely used, however, it needs a special set-up (thermocyclers) and delicate chemicals (DNA polymerases). These drawbacks could potentially limit their applications in some circumstances. Is it possible to avoid these problems?

Real time detection of DNA or RNA are necessary for many applications and are normally carried out by in situ hybridization with oligonucleotide probes labeled with radioactive isotope, fluorescent dyes, quantum dots, metal or magnetic nanoparticles [7,12–15]. However, the detection and quantification of small amounts of nucleic acids under these conditions remain challenging, and often-delicate procedures are required in both preparation of those probes and in detection experiments. One major problem is the background arising from free probes. To overcome this problem, molecular beacons (MBs) have been developed [16–18]. A MB is a single DNA strand labeled with a fluorescent dye (fluorophore, F) and a fluorescent quenching molecule (quencher, Q) at its two ends, respectively. In a buffer solution, a MB adopts a stem-loop structure and the quencher is in close proximity to the fluorophore, and the fluorescence of the fluorophore is strongly quenched by the quencher. Upon binding to DNA of interest and forming a DNA duplex, the fluorophore and the quencher become well separated and a strong fluorescence appears. Due to the self-quenching of the free probes, this method has very low background. One target molecule, however, activates only one MB probe. It would

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be highly desirable to activate multiple MB probes with one target molecule.

To overcome the above-mentioned problems, a novel strategy, namely catalytic molecular beacons, has been reported [19]. It can detect DNA down to a concentration of 8 nM. In this paper, we have further improved this method to detect DNA at a concentration of 10 pM, which is almost 1000 times more sensitive than the previous reported method [19]. This strategy integrates RNA-cleaving DNA enzymes (DNAzyme) and molecular beacons. The DNAzyme is capable of cleaving RNA substrates at specific sites [20]. This method separates molecular recognition (activation of DNAzymes) and readout signal (activation of molecular beacons). In this design, upon binding with nucleic acid of interest, inactive DNAzymes become active and cleave molecular beacons, which, in turn, give strong fluorescence signal. Self-quenching of the molecular beacons generates very low background, and multiple turn over of the DNA enzymes results in signal amplification.

2. Experimental

2.1. Materials

All reagents were of A.R. grade. Boric acid, acetic acid and ethylenediamine-tetraacetate (EDTA) were from Mallinckrodt Chemicals. Magnesium acetate, tris(hydroxymethyl) aminomethane (Tris) base were from FisherBiotech (Fisher Scientific). Acrylamide was from Bio-Rad Laboratories. Ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) were from Amersham Pharmacia Biotech (Amersham Biosciences).

All oligonucleotides were purchased from Integrated DNA Technologies Inc., and purified by denaturing polyacrylamide gel electrophoresis. The DNA sequences are shown in Table 1. The molecular beacon is a DNA/RNA chimeric oligonucleotide labeled with a 5′-FAM and a 3′-black hole quencher. The two RNA residues are highlighted with bold letters. The cleavage site is between the two RNA residues. The DNAzyme is a 10–23 catalytic DNA, which has a 15 bases long catalytic domain (bold letters), two flanking substrate-recognition arms, and a 24 bases long inhibiting domain (italic letters) that harbors the complementary sequence of the target DNA. Enzyme domain and the inhibitory domain are linked by a T4 loop (letters with underlines).

2.2. Enzyme digestion

The target DNA (T, 0.1 μM), DNA enzyme (E, 0.1 μM) and DNA/RNA chimera molecular beacon analog (MB′, 7 μM) were incubated in TAE/Mg^{2+} buffer at 37 °C for various time periods and then analyzed by native polyacrylamide gel electrophoresis (PAGE). The TAE/Mg^{2+} buffer was at pH 8.0 and contained Tris base (40 mM), acetic acid (20 mM), EDTA (2 mM) and $(CH_3COO)_2Mg$ (150 mM).

2.3. Denaturing polyacrylamide gel electrophoresis

Gels contained 20% polyacrylamide (acrylamide/bisacrylamide, 19:1) and 8.3 M urea and were run at 55 °C. A TBE buffer was used as the separation buffer, which was at pH 8.0 and contained of Tris base (89 mM, pH 8.0), boric acid (89 mM) and EDTA (2 mM). Gels were run on a Hoefer SE 600 electrophoresis unit at 600 V (constant voltage).

2.4. Native polyacrylamide gel electrophoresis

Gels contained 15% polyacrylamide (acrylamide/bisacrylamide, 19:1) and TBE buffer. One microlitre tracking dye containing TAE/Mg^{2+}, 50% glycerol and 0.2% each of Bromophenol Blue and Xylene Cyanol FF was added to each sample (0.3 μg DNA in 10 μL of TBE). Gels were run on an FB VE-10-1 vertical electrophoresis system at 250 V (constant voltage) at room temperature.

2.5. Fluorescence spectroscopy

Target DNA was dissolved in TAE/Mg^{2+} buffer in two series of concentrations: (i) 0.2, 0.5 and 1 nM, when the concentrations of the DNAzyme and MB were 10 and 100 nM, respectively, and (ii) 0.01, 0.05, 0.10, 0.20 and 0.50 nM, when the concentrations of the DNAzyme and MB were 1 and 10 nM, respectively. Fluorescence emission spectra of each concentration sample were recorded on a Varian Cary Eclipse fluorescence spectrophotometer. All spectra were collected at 22 °C. The samples were excited at 470 nm and the emission data were collected from 500 to 650 nm. The maximal emission wavelength of FAM is 520 nm. The wavelength collection step was 1 nm. The sample cuvette (path length: 5 mm) was purchased from Starna Cells Inc. (#23-5.45-Q-5).

Table 1
DNA sequences

Strand name	Sequence
Target DNA (T)	5′-AAAA*GCACCCACTCTCTCTGAAGGACGC*-3′
Molecular beacon (MB)	5′-(FAM)-CCTGAGAGAG**rArU**GGGTGCAGG-(black hole quencher-1)-3′
Molecular beacon analog (MB′)	5′-CCTGAGAGAG**rArU**GGGTGCAGG-3′
DNAzyme (E)	5′-*GCGTCCTTCAGAGAGAGTGGGTGC*TTTTGCACCCA**GGCTAGCTACAACGA**CTCTCTC-3′

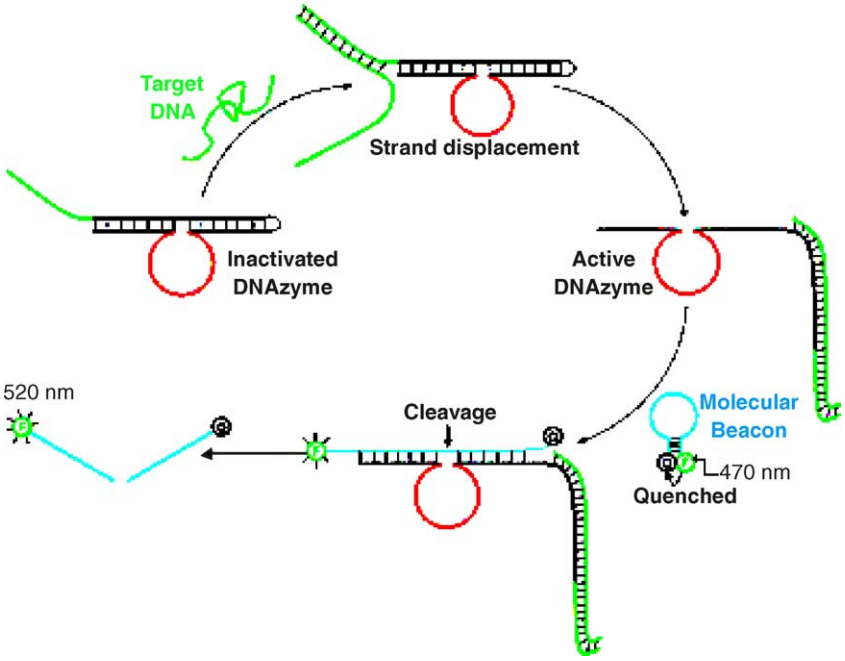

Fig. 1. Scheme of DNAzyme amplification of molecular beacon (MB) signals.

3. Results and discussion

3.1. Design of the catalytic molecular beacon and DNAzyme

In the current design, molecular beacons are not directly activated by conformational changes accompanying hybridization with target DNA. Instead, MBs' activation is mediated by 10–23 DNAzymes. The 10–23 DNAzyme has been resulted from an in vitro selection experiment. The DNAzyme is such named because it is the 23rd clone from the 10th round of selection [20]. The 10–23 DNAzyme contains three domains: one catalytic core (red) and two target recognition arms (Fig. 1). The 10–23 DNAzyme binds to an RNA substrate through Watson–Crick base-pairing and cleaves the RNA molecule into two fragments. Except the two residues flanking the cleavage site, all other RNA residues can be replaced by DNA residues. Here, we have covalently linked an inhibitory DNA strand to the 10–23 DNAzyme. The inhibitory DNA strand contains an inhibitory segment and a toehold segment. The inhibitory segment is a DNA analog of the RNA substrate, which can bind to the recognition arms of the 10–23 DNAzyme and prevent the 10–23 DNAzyme from binding to and cleaving its RNA substrate. Thus, the engineered DNAzyme is inactive.

The DNAzyme and the inhibitory strand are designed in such a way that the inhibitory strand is complementary to the DNA to-be-detected. When the target DNA is added to the solution, the target DNA will bind to the unpaired toehold segment first, and then gradually pulls the inhibitory DNA strand from the DNAzyme through strand displacement [9,21–23], and frees the DNAzyme. Thus, the DNAzyme becomes active. A molecular beacon has been designed to serve as the substrate of the activated DNAzyme. When the MB is cleaved by the DNAzyme into two fragments, the short fragments will dissociate from the enzyme. The two short fragments are complementary to each other only by four basepairs, which cannot form a stable complex in solutions to hold the fragments together. Thus, the fluorophore (F) and quencher (Q) will be physically separated from each other and F starts to fluoresce. One DNAzyme can cleave multiple copies of MBs and result in signal amplification. Overall, a very low concentration of target DNA can be detected by a normal fluorescence spectrophotometer.

Compared with the previous design [19], we have made two improvements. First, we used the 10–23 DNAzyme instead of other DNAzymes. Among all RNA-cleaving DNAzyme, the 10–23 DNAzyme is among the most efficient enzymes [24]. Second and most importantly, the current design avoids self-dimerization. In previous design, the activated DNAzyme has two unpaired segments that are complementary to each other. Though they could not hybridize within a molecule because of a conformational constrain, these segments are perfectly positioned to hybridize between the activated DNAzymes. As long as such hybridization happens, DNAzymes will lose their catalytic activities. In the current design, such self-complementarity has been eliminated. No unpaired segments in the activated DNAzyme are complementary to each other. After activation, the DNAzyme will be active constantly.

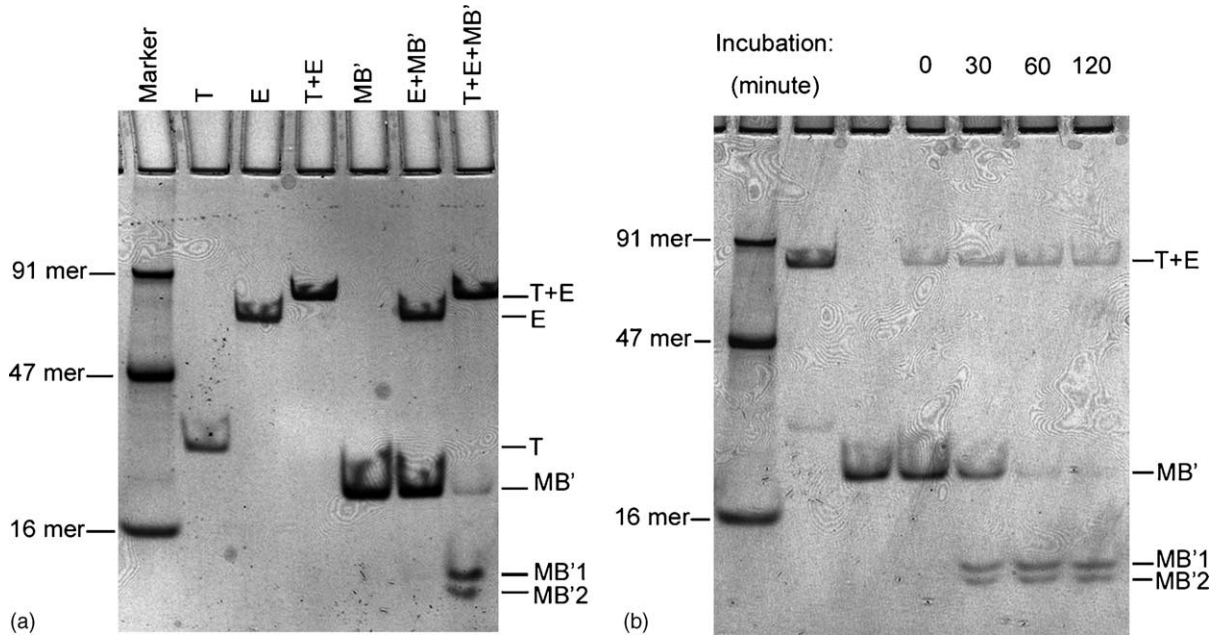

Fig. 2. Native polyacrylamide gel electrophoretic (PAGE) analysis of the MB′ digestion. (a) A demonstration of DNAzyme activation by target DNA and (b) a time course of MB′ digestion by activated DNAzyme (T, 0.1 μM; E, 0.1 μM; MB′, 7 μM). The components of each lane are indicated at the top of the gel images. T, target DNA; E, DNAzyme; MB′, molecular beacon analog; MB′1 and MB′2, cleaved MB′ fragments.

3.2. Enzymatic digestion of MB–polyacrylamide gel electrophoresis study

We examined the improved system initially with native PAGE analysis. Target DNA (T, 1 μM), DNAzyme (E, 1 μM) and molecular beacon analog (MB′, 20 μM) were incubated in different combinations at 37 °C for 2 h, and then analyzed by native PAGE (Fig. 2a). In this assay, the MB was replaced by its analog MB′. The MB′ had the same sequence as the MB but lacked the fluorophore and quencher. When equamolar amounts of target DNA and DNAzyme were combined together, one sharp clean band appeared in the gel, which indicated that the target DNA bound to the DNAzyme and formed a stable complex. When the inactivated DNAzyme was combined with the MB′, they did not interact with each other. The DNAzyme neither associated with nor cleaved the MB′. Consequently, two bands appeared, one corresponded to the DNAzyme and the other to the MB′. This observation clearly indicated that the DNAzyme was completely inhibited. In contrast, addition of the target DNA led to cleavage of the MB′ into two short fragments by the DNAzyme. The short fragments had low affinities to the DNAzyme and dissociated from the DNAzyme readily. Thus, multiple MB′ molecules could be cleaved by the DNAzyme. The cleavage demonstrated that the target DNA could activate the inhibited DNAzyme, and result in cleavage of multiple copies of MB′s, which was the signal amplification we pursuit.

A time course experiment was performed to show the kinetics of the digestion of the activated DNAzyme. DNA were mixed at a ratio of T:E:MB′ = 1:1:70 and incubated at 37 °C for different times, and then analyzed by native PAGE (Fig. 2b). The target-activated DNAzyme finished digestion of almost 70 copies of MB′s within 1 h. In other words, one target DNA molecule could result in activation of 70 copies of MB′s. It was clearly superior to traditional molecular beacon, where one target DNA molecule activated only one MB.

When MB was used in these two experiments, the digestion went much slower than with MB′ (data not shown). We speculate that the fluorophore and the quencher interfere with the DNAzyme activity. The mechanism is elusive at this stage and need more study.

3.3. Enzymatic amplification of MB signals–fluorescence study

We further characterized this system with fluorescence spectroscopy because fluorescence gives an easily detectable signal. Two different experimental sets were used. In the first set, we kept the concentration of DNAzyme and MB at 10 and 100 nM, respectively, but changed the target DNA concentration to estimate the detection limit (Fig. 3). The high MB concentration could produce high background and interfere with the detection of very low concentration of the target DNA. To test this hypothesis, we repeated the same experiment with 10 times diluted concentration of DNAzyme (1 nM) and MB (10 nM) (Fig. 4). In our current experiment, we can detect target DNA as low as 10 pM.

We incubated target DNA (0.2–1.0 nM), DNAzyme (10 nM) and MB (100 nM) at room temperature, and monitored the fluorescence signal at various time points. The fluorescent dye was irradiated at 470 nM and the emission was either monitored at 520 nm (the maximum emission wave-

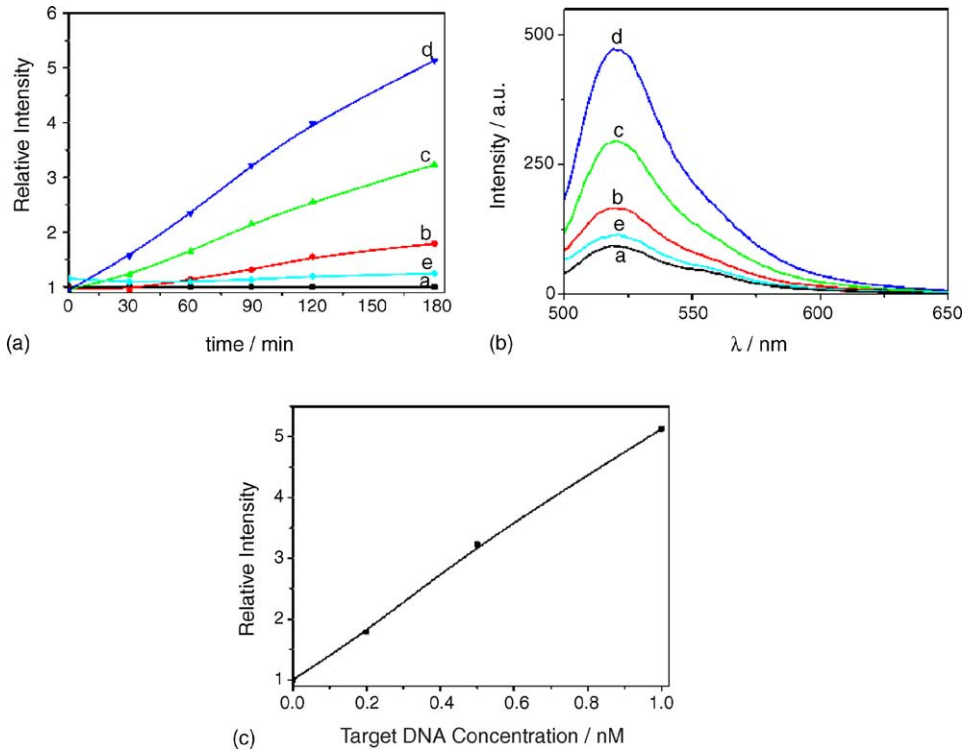

Fig. 3. Detection of target DNA by fluorescence with high concentrations of MB and DNAzyme. (a) Flourescence vs. incubation times at different target DNA concentrations: 0 nM (a), 0.2 nM (b), 0.5 nM (c), 1.0 nM (d) and 1.0 nM (e). In line e [MB] = 100 nM and [E] = 0 nM; in all other lines (a–d) [MB] = 100 nM and [E] = 10 nM. (b) Fluorescence spectrum after 3 h incubation. (c) Relative fluorescence intensity after 3 h incubation at different target DNA concentrations.

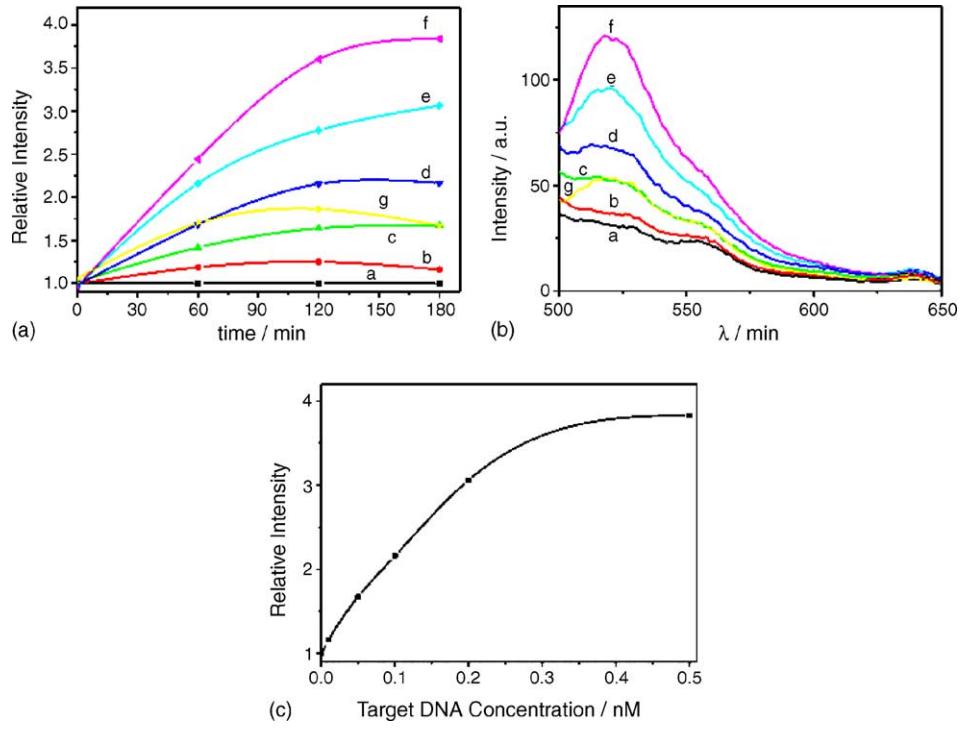

Fig. 4. Detection of target DNA by fluorescence with low concentration of MB and DNAzyme. (a) Flourescence vs. incubation times at different target DNA concentrations: 0 nM (a), 0.01 nM (b), 0.05 nM (c), 0.1 nM (d), 0.2 nM (e), 0.5 nM (f) and 1.0 nM (g). In line g [MB] = 10 nM and [E] = 0 nM; in all other lines (a–f) [MB] = 10 nM and [E] = 1 nM. (b) Fluorescence spectra after 3 h incubations. (c) Relative fluorescence intensity after 3 h incubation at different target DNA concentrations.

length) or scanned from 500 to 650 nm. Fig. 3a showed the time course data. Fluorescence intensities were normalized by the fluorescence intensity of a sample containing the same concentration of MB and DNAzyme but without target DNA at the same conditions. During incubation, the fluorescence intensity constantly increased, which indicated the progress of MB digestion. Fig. 3b showed the fluorescence spectra of the reactions mixture at 3 h. A control sample was prepared with hybridization of target DNA (1.0 nM) and MB (100 nM) but without DNAzyme (line e in Fig. 3). The fluorescence signal showed a low enhancement due to the low activation efficiency of the MB (at most only 1% of the MB was activated and exhibited fluorescence emission). In contrast, the addition of DNAzyme increased the fluorescence signal progressively when elongating the incubation time to 3 h. Fig. 3c showed the relationship between the fluorescence intensities and the target DNA concentration. The higher the target DNA concentration, the more MB became digested and the stronger fluorescence signal was resulted. There was almost a linear relationship between the target DNA concentration and the fluorescence signal in the tested concentration range of target DNA. At this experiment set, however, if the concentration of the target DNA was lower than 0.2 nM, the difference of fluorescence intensities between MB solutions with and without target DNA was not clear, so they could not be distinguished from each other. This phenomenon was presumably due to the background of the high concentration of MB.

To lower the background and increase the signal/noise ratio, we lowered the MB and DNAzyme concentration to 10 and 1 nM, respectively, and achieved a more sensitive detection (Fig. 4). At low concentration of the target DNA, the relative fluorescence intensity increased approximately linearly related to the target DNA concentration. However, as the target DNA concentration increased, the MB digestion became faster because more DNAzymes became activated, and reached completion before 3 h, so the intensity reached a plateau.

In this system, the MB concentration was very important. A low concentration of MB could detect target DNA at a low concentration but makes data collection difficult because of the limit of the instrumental sensitivity. Balancing between these two aspects, we achieved a sensitivity limit of this method as 0.01 nM.

4. Conclusion

We have improved the strategy of catalytic molecular beacon for DNA/RNA detection. Upon activation by target DNA, DNAzyme cannot oligomerize and will keep constantly active. This design is 1000 times more sensitive than the value previously reported [19]. The detection sensitivity is critically dependent on the concentrations of DNAzyme and molecular beacon.

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