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Label-free Molecular Beacons for Biomolecular Detection

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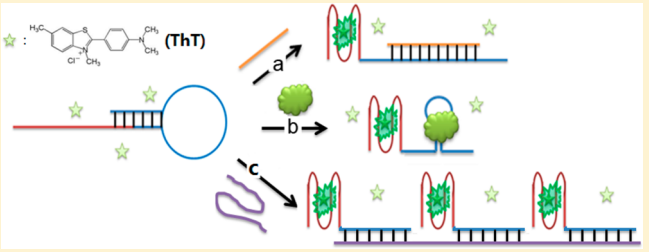
ABSTRACT: Biomolecular detection and imaging methods provide quantitative measurements essential for biological research. In this context, molecular beacon based sensors have emerged as powerful, no-wash imaging agents, providing target-specific fluorescent activation for nucleic acids, proteins, and small molecules. Conventional molecular beacons require double-labeled DNA sequences, which are costly and time-consuming to prepare. To address this issue, we developed DNA based label-free molecular beacons consisting of two regions: a signal-generating region based on human telomeric G-quadruplex sequence that activates Thioflavin T fluorescence and a target recognition sequence designed to interact in a molecular beacon format. We demonstrated the utility of these probes for the selective detection of DNA, RNA, and protein. Multiple probes were applied against a single target to achieve improved brightness in fluorescence detection of nucleic acid targets. This label-free strategy provides a straightforward, cost-effective alternative to fluorescently labeled oligonucleotides in biomolecular detection and imaging.



Molecular beacons (MBs) are fluorogenic DNA based detection agents that were first reported in 1996.[1] Typically, MBs are dual-labeled with fluorophore and quencher groups at the 5′ and 3′ ends, respectively, and fold into a stem−loop, i.e., hairpin, secondary structure.[1−6] Binding of the target, e.g., a nucleic acid sequence complementary to the loop region, induces a conformational change that opens the stem, separating the fluorophore and quencher moieties and reactivating the fluorescence. Because of this property, MBs have been applied in numerous in vitro hybridization assays.[4,7,8] In addition, MBs have been widely used in many other areas, such as the detection of PCR products, mutational analysis, and medical diagnostics.[5,6,9−18]

MBs have also been used for detection of non-nucleic acid targets such as small molecules and proteins utilizing aptamer-substrate recognition. Aptamers are single-stranded nucleic acids generated by SELEX (systematic evolution of ligands by exponential enrichment).[19,20] An aptamer-MB, also called an aptamer switch probe[21] or activatable aptamer probe,[22] is typically composed of three elements (in addition to the fluorophore and quencher on the ends): an aptamer for target recognition, a short DNA sequence complementary to part of the aptamer (to form the stem), and a connecting linker, e.g., PEG. To bind to the target, an aptamer-MB undergoes spontaneous structural reorganization, which opens the stem, leading to fluorescence recovery. Aptamer-MBs can specifically recognize various target molecules, such as ATP, proteins, or even cells.[21−24]

Although conventional MBs have been widely used in bioanalysis and biomedical research, preparation of these dual-labeled oligonucleotides is relatively expensive and time-consuming. The bulky fluorophore or quencher group may also influence the selectivity or binding affinity of the functional oligonucleotide.[25] To avoid the need for dual-labeling, some "label-free" nucleic acid probes were generated; however, they still rely on chemical modifications on DNAs.[26−28] On the other hand, completely label-free approaches were developed to construct DNA,[29−31] RNA,[32−35] or DNA-RNA hybrid[35,36] based probes. RNA based probes suffer intrinsic problems such as low biostability and high cost because of the RNA backbone. Although DNA based label-free probes can overcome these limitations, the previous DNA probes were constructed in the form of DNA duplexes: separation of the duplexes released one strand for recognition and the other one for signal generation.[29,30] Due to the requirement of a separate oligonucleotide competitor in these probes, they cannot be used in some applications, such as in situ detection due to problems with localization of the released signaling strand. Therefore, it is highly desirable to develop a single DNA based label-free approach for detection of various biomolecules.

Recently, Mohanty et al. reported that thioflavin T (ThT) can specifically bind to a DNA quadruplex derived from the human telomeric repeat sequence (HTG), generating a 2100-fold fluorescence enhancement,[37] which Mergny and co-workers subsequently extended to several other quadruplexes.[38] We envisioned using this interaction to generate a label-free

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Scheme 1. Schematic Drawing of ThT based Molecular Beacon for Detection of (a) Nucleic Acids and (b) Other Biomolecules such as Proteins

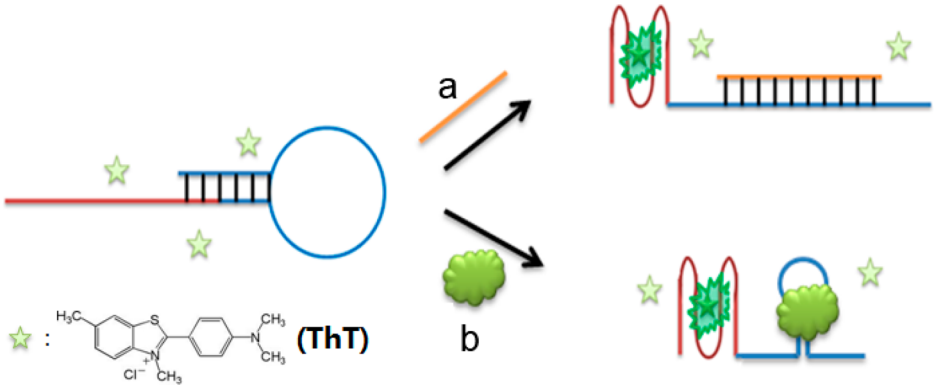

Table 1. Design of TMBs Targeting the DNA S-1[a]

name	DNA sequence	stem length	F/F_0
S-1	CTTGAGAAAGGGCTGCCA		
probe 1	*AGGGTTAGGGTTAGGGTTAGGG*TGGCAGCCCTTTCTCAAG	4	1.12 ± 0.07
probe 2	*AGGGTTAGGGTTAGGGTTAGGG*CTTGAGTGGCAGCCCTTTCTCAAGCCC	9	4.64 ± 0.20
probe 3	*AGGGTTAGGGTTAGGGTTAGGG*CTTGAGTGGCAGCCCTTTCTCAAGCCCTA	11	6.52 ± 0.05
probe 4	*AGGGTTAGGGTTAGGGTTAGGG*CTTGAGTGGCAGCCCTTTCTCAAGCCCTAA	12	8.03 ± 0.33
probe 5 (TMB-S-1)	*AGGGTTAGGGTTAGGGTTAGGG*CTTGAGTGGCAGCCCTTTCTCAAGCCCTAAC	13	10.49 ± 0.29
probe 6	*AGGGTTAGGGTTAGGGTTAGGG*CTTGAGTGGCAGCCCTTTCTCAAGCCCTAACCC	15	5.06 ± 0.18

[a]Italicized bases are the human telomeric G-quadruplex sequence (HTG). Bold bases are the cDNA sequence of S-1. Underlined bases denote the stem of TMBs. F_0 was the fluorescence intensity when TMB was incubated with ThT only and F was the fluorescence intensity when TMB was incubated with ThT in the presence of S-1.

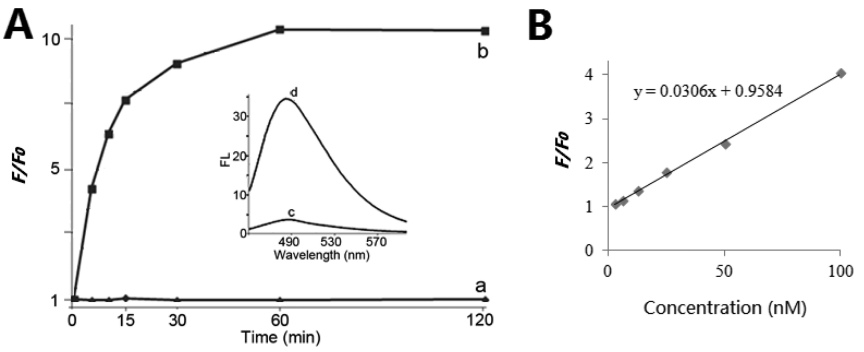

Figure 1. (A) Fluorescence signal of TMB-S-1/ThT in the presence of DNA S-1 as a function of time. TMB-S-1 (100 nM) was incubated with ThT (3 μM) in the absence (a) or presence (b) of DNA S-1 (500 nM). The inset displays fluorescence emission spectra after 1 h of incubation at RT in the absence (c) or presence (d) of DNA S-1. (B) Linear relationship between the fluorescence change and the concentration of DNA S-1. [ThT] = 3 μM, [TMB] = 400 nM, [S-1] = 0–100 nM. Excitation: 425 nm. Emission: 492 nm.

molecular beacon with the HTG/ThT complex providing the fluorogenic signal. By extending the HTG sequence to promote hairpin formation, folding of HTG into a quadruplex and binding of ThT should be suppressed, while disruption of the stem by target binding liberates the HTG and allows fluorogenic association with ThT in solution (Scheme 1). This ThT based molecular beacon (TMB) is demonstrated here as a label-free sensor for detection of DNA, RNA, and protein. The key to the design shown in Scheme 1 is the weak interaction between ThT and double-stranded DNA (dsDNA) or single-stranded DNA (ssDNA),[37] which implies that the background fluorescence of the hairpin structure will be low. Binding of an analyte to the recognition domain (blue line) provides energy needed to unfold the hairpin, allowing the HTG domain (red line) to refold into a G-quadruplex, which then binds and activates the fluorescence of ThT. Thus, covalently attached fluorophores and quenchers are unnecessary.

To demonstrate the utility of this approach, we constructed TMB sequences with varying stem lengths complementary to DNA target S-1, as shown in Table 1. The HTG signaling sequence is located in the 5′-portion of the TMB, whereas the 3′-portion is complementary to the DNA S-1 target with extra bases to allow base pairing with part of HTG, disfavoring G-quadruplex formation (see Table 1, probes 1–6). Complementary DNA detection was carried out at room temperature (RT = 23 °C) under optimized conditions (100 nM TMB, 3 μM ThT in 20 mM Tris containing 75 mM KCl, pH 8.3). F_0 was the background fluorescence intensity of the individual TMB with ThT in the analysis buffer, but no complementary

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DNA. In the presence of target DNA S-1, the hairpin structure opens, and the HTG G-quadruplex forms and binds to ThT, generating increased fluorescence intensity (F). As shown in Table 1, the length of the beacon stem increases from 4 to 15 base pairs for the series of six different TMBs. The longer stems initially lead to progressive increases in F/F_0 due to reduced background signal, i.e., more stable folding of the TMB. However, if we extended the self-complementarity to form a 15 base pair stem (probe 6), the F/F_0 value decreased, indicating that the stem of probe 6 is too strong to be opened by the target DNA. Thus, probe 5, renamed TMB-S-1, gave the highest F/F_0 value and was used for further experiments. The assay kinetics were monitored using the TMB-S-1 probe with S-1 target at RT. As shown in Figure 1, in the presence of S-1, the fluorescence of TMB-S-1/ThT reached the maximum value after 1 h. The hybridization rate of TMB is slower than that of conventional MBs.[39] Possible reasons include: (1) the stem portion of conventional MBs is usually much shorter than in the TMBs, hence the TMBs require a longer time for opening by the analyte; (2) for TMB, the signal generation requires stem disruption, G-quadruplex formation, and dye binding; however, for conventional MB, the signal generation only requires stem disruption, leading to a relatively faster one-step signal generation in conventional MBs. The enhancement (F/F_0) ~11 is comparable to the conventional dual labeled MBs.[1,40−43] In addition, the hybridization rate of TMB is concentration-dependent, i.e., the activation is slower when the target concentration is reduced (data not shown). The limit of detection (LOD) of DNA S-1, based on 3 times the signal-to-noise level, was about 6.5 nM (Figure 1B).

To examine the specificity of TMB-S-1, several mismatch-containing DNA targets were tested. As shown in Figure 2,

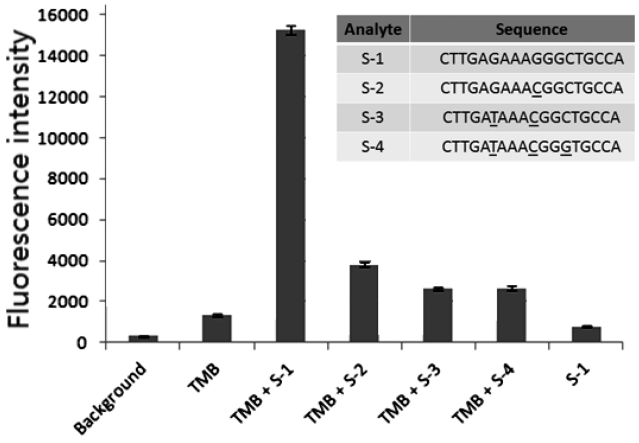

Figure 2. Fluorescence intensity of ThT under different incubation conditions. The inset displays the DNA sequences of target S-1 and its various mismatches.

compared with S-1, which gives an 11-fold signal increase, the single mismatch S-2 displayed only a 2.8-fold signal enhancement, whereas double (S-3) or triple mismatch (S-4) sequences produced only a 2.0-fold enhancement. TMB-S-1 or S-1 alone generated very low signals, presumably due to the low affinity of ThT for the individual DNAs.

Hybridization to a beacon can also be used for RNA detection and quantitation.[44,45] A complementary RNA (RNA S-1) was evaluated for activation of TMB-S-1. As shown in Figure 3, strong fluorescence enhancement was observed when

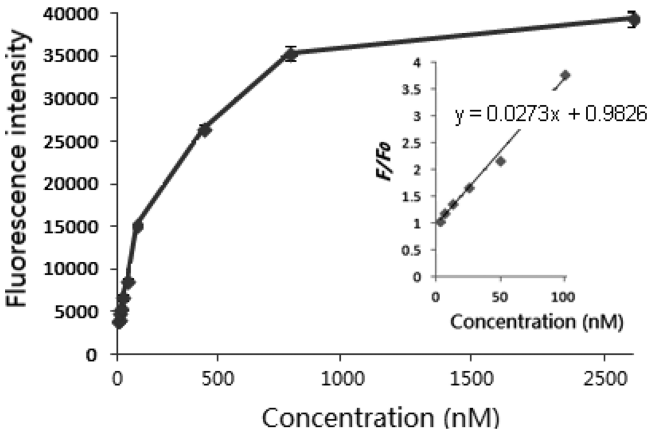

Figure 3. Fluorescence intensity of TMB-S-1/ThT in the presence of various concentrations of target RNA S-1. Inset: linear relationship between the fluorescence change and the concentration of RNA S-1.

TMB-S-1 was incubated with saturating RNA S-1, showing a similar overall fold enhancement to that seen with DNA targets. The fluorescence signal increased linearly when the RNA was added in the assay (Figure 3, inset). The limit of detection (LOD) of RNA S-1, based on 3 times the signal-to-noise level, was about 5.7 nM.

To extend the use of TMBs to protein detection, the DNA/RNA recognition domain used above was replaced with a DNA aptamer that binds to the protein PDGF-BB. Following the same strategy used to design TMB-S-1, we constructed TMB-PDGF (*AGGGTTAGGGTTAGGGTTAGGG*CAGGA**CAGGCTACGGCACGTAGAGCATCACCATGATCCTG**CCC), which includes the anti-PDGF aptamer (bold sequence)[46] in the loop. TMB-PDGF should fold into a hairpin containing an 8 bp stem. As shown in Figure 4A, the fluorescence of TMB-PDGF significantly increased in the presence of PDGF-BB, but not in the presence of nontarget proteins such as BSA, thrombin, and lysozyme, indicating that TMB-PDGF specifically recognizes PDGF, and that recognition generates a fluorescent response. Different concentrations of PDGF-BB were incubated with TMB-PDGF/ThT, generating a linear concentration-dependent titration (Figure 4B) with a LOD of about 13 nM (~300 pg/μL).

The low cost of our unmodified DNA probes and thioflavin T allows the use of multiple TMBs, rather than a single TMB, to quantitatively image or detect a single nucleic acid target. The binding of multiple TMBs to a long nucleic acid target should produce enhanced fluorescent signals compared with single TMB probe (as shown in Scheme 2).

As a proof-of-concept study, TMB probes were designed to hybridize to adjacent complementary sequences on a long bead-anchored DNA target (see the Experimental Section for probe sequence details). As shown in Figure 5A, S-5 was bead-anchored through avidin/biotin interaction, and different TMB probes were incubated with these beads before ThT staining. As shown in Figure 5B, three noncomplementary control TMB probes cannot bind S-5 and ThT staining generated only weak background signals on the beads. A single complementary TMB probe a specifically binds one segment of S-5 and generates significant fluorescence enhancement (Figure 5C). Next, when two TMBs, probes a and c, which bind to two segments of S-5 were applied, higher fluorescent signals were observed (Figure 5D). Fluorescence signals were also clearly observed when three TMBs (probes a, b, and c) were employed simulta-

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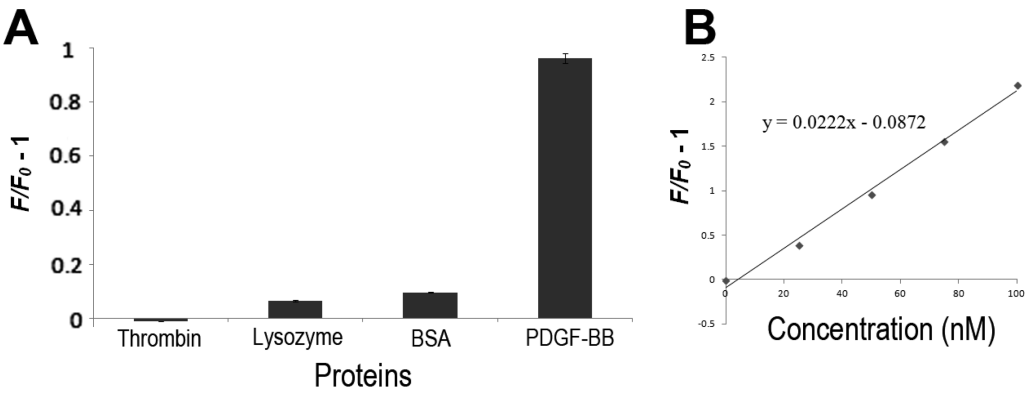
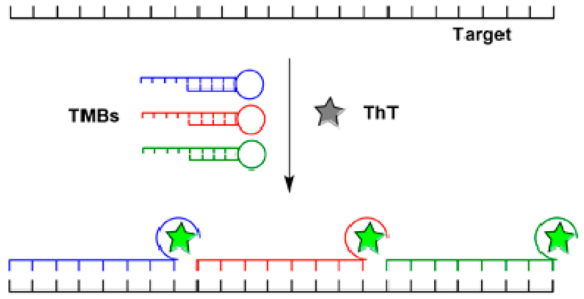


Figure 4. (A) Fluorescence intensity changes $(F/F_0 - 1)$ of TMB-PDGF/ThT toward different proteins. F_0 and F are fluorescence intensities in the absence or presence of detected proteins, respectively. (B) Linear relation between F/F_0 and the concentration of PDGF-BB.

Scheme 2. Schematic Drawing of Using Multiple TMBs for Detection of a Nucleic Acid Target



neously, which can bind to the whole three segments of S-5 (Figure 5E).

To prove that multiple TMB probes can generate corresponding multiple-fold fluorescence enhancement compared with the single TMB probe, we quantitatively measured fluorescent signals on beads treated by different probes. As shown in Figure 5F, the normalized fluorescence intensity generated by probes a and c was 2-fold more than that generated by probe a alone, and this enhancement increased to even 4-fold when probes a, b, and c were used, which may be possibly caused by (1) the cooperativity in binding among three TMBs, i.e., neighboring TMBs will facilitate each other to bind the target, therefore three beacons can generate a more than 3-fold increase than one beacon; (2) the higher local concentration of quadruplexes leading to binding of more ThT at equilibrium. In a short summary, these data indicate that multiple TMBs can generate corresponding enhanced fluorescence signals relative to a single TMB probe.

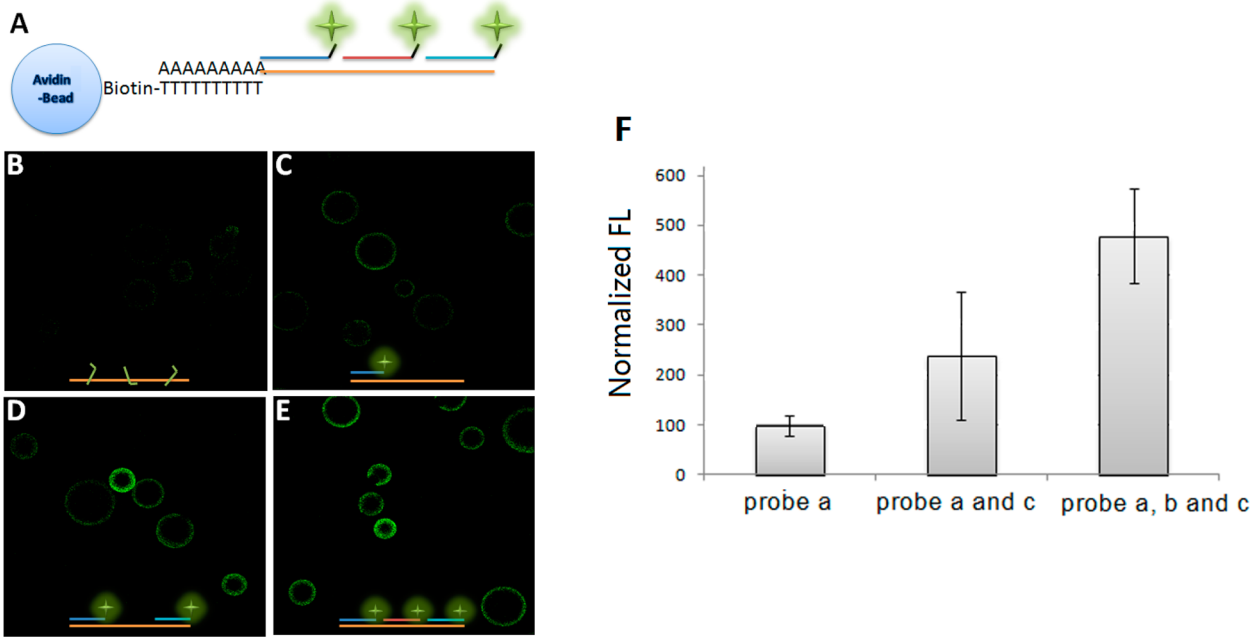


Figure 5. Application of multiple TMBs for imaging or quantitative detection the target DNA on beads. (A) schematic illustrating how to apply multiple TMB probes (probes a, b, and c) for imaging target DNA by fluorescent signals generated from TMB/ThT interaction. The confocal microscope images were recorded after ThT staining of beads that were incubated with (B) three noncomplementary control probes; (C) probe a; (D) probes a and c; (E) probes a, b, and c; (F) the fluorescence intensities on beads were recorded by a fluorescence plate reader after ThT staining of beads that were incubated with probe a, probes a and c, or probes a, b, and c.

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In summary, the human telomeric G-quadruplex can be used as a robust fluorogenic transducer in a molecular beacon design to activate thioflavin T in an analyte-dependent manner. This approach has allowed generation of label-free TMBs that function for specific detection of DNA, RNA, or protein. This new concept for molecular beacons offers an inexpensive and simple approach for biomolecule detection, e.g., the cost of DNA synthesis can be reduced by more than 95% relative to traditional fluorophore-quencher labeled MBs. Exploiting the low cost allows development of bioimaging applications using multiple TMBs to target bead-captured nucleic acids. Ongoing work is directed toward further improving the brightness of TMB probes, which is currently limited by the relatively weak K_d (1.5 μM) between HTG and ThT (Figure 6). New designs in which higher affinity recognition between the fluorogenic reporter and the refolded MB should lead to higher fluorescence enhancements and improved sensitivity.

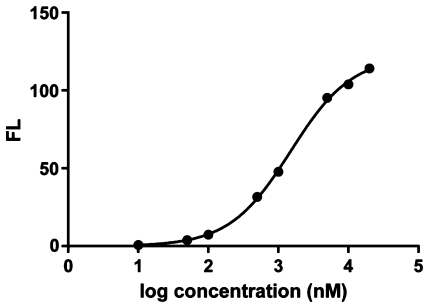

Figure 6. Measurement of K_d value of ThT/HTG interaction (reaction condition: 100 nM of HTG sequence was incubated with different concentrations of ThT at RT for 30 min). The K_d value was determined to be 1500 nM using a single-site hyperbolic binding curve.

EXPERIMENTAL SECTION

Materials and Methods. DNA molecules were ordered from Integrated DNA Technologies (Coralville, IA, USA) with standard desalting. Thioflavin T was purchased from Abcam (Cambridge, MA, USA). PDGF-BB was obtained from Shenandoah Biotechnology (Warwick, PA, USA). Monomeric avidin-coated agarose beads were purchased from Pierce and streptavidin-coated polystyrene beads were purchased from spherotech. All other reagents were of analytical grade. Fluorescence intensity was measured in a 384-well Nunc microplate on a multimode fluorescence plate reader (M1000-Tecan, Männedorf, Switzerland) and fluorescence emission spectra were collected on a Varian Cary Eclipse Fluorescence Spectrophotometer (Agilent, Santa Clara, CA, USA).

Detection of Nucleic Acid Targets by TMBs. The DNA detection was carried out at RT, including 100 nM of TMBs, 500 nM of DNA target S-1 and 3 μM of ThT in TMB buffer (20 mM Tris containing 75 mM KCl, pH 8.3). The RNA detection solution contained 400 nM of TMBs, 3.13−100 nM of RNA target S-1 and 3 μM of ThT in TMB buffer. Fluorescence intensity was measured at a fluorescence plate reader with an excitation at 425 nm and an emission at 492 nm. Data was obtained from the average of three parallel experiments.

Nucleic acid sequences:

DNA S-1: CTTGAGAAAGGGCTGCCA

RNA S-1: CUUGAGAAAGGGCUGCCA

Probe 1: AGGGTTAGGGTTAGGGTTAGGGTGGCAGCCCTTTCTCAAG

Probe 2: AGGGTTAGGGTTAGGGTTAGGGCTTGAGTGGCAGCCCTTTCTCAAGCCC

Probe 3: AGGGTTAGGGTTAGGGTTAGGGCTTGAGTGGCAGCCCTTTCTCAAGCCCTA

Probe 4: AGGGTTAGGGTTAGGGTTAGGGCTTGAGTGGCAGCCCTTTCTCAAGCCCTAA

Probe5 (TMB-S-1): AGGGTTAGGGTTAGGGTTAGGGCTTGAGTGGCAGCCCTTTCTCAAGCCCTAAC

Probe 6: AGGGTTAGGGTTAGGGTTAGGGCTTGAGTGGCAGCCCTTTCTCAAGCCCTAACCC

Detection of Proteins by TMB. The protein detection assay was carried out at RT for 1 h, including 100 nM TMB, 50 nM proteins and 3 μM ThT in TMB buffer (20 mM Tris containing 75 mM KCl, pH 8.3). For measureing the detection limitation of PDGF-BB by TMB, the reaction solution contained 100 nM of TMB, 20−100 nM of PDGF and 3 μM of ThT in TMB buffer. Fluorescence intensity was measured on an M1000 Tecan instrument with an excitation at 425 nm and an emission at 492 nm. Data was obtained from the average of three parallel experiments.

On-Beads Assay. For imaging assay, avidin-coated agarose beads (50 μL) were reacted with 5 μM biotin-T_{20} in PBS for 1 h. After washing twice by PBS, beads were incubated with 5 μM target DNA S-5 in PBS for 1 h and were washed twice by PBS to remove unbound DNA. Then beads were incubated in 2 x SSC solution (0.15 M sodium chloride and 15 mM trisodium citrate, adjusted to pH 7.0 with HCl) with TMB probe cocktail. The final concentration of DNA probes for all conditions was 3 μM. After 3 h incubation at RT, beads were washed by 2 x SSC and TMB buffer (20 mM Tris containing 75 mM KCl, pH 8.3). Thereafter, beads were stained by 5 μM ThT in TMB buffer for 2 min and replaced by 200 nM ThT in TMB buffer. Fluorescence imaging was taken using a Carl Zeiss LSM-510_META_UV_DuoScan inverted spectral confocal microscope with excitation at 458 nm and emission at 475−525 nm.

For fluorescence quantification, streptavidin-coated polystyrene beads were treated by the same procedures as in the imaging assay and the final fluorescence was recorded by a fluorescence plate reader with excitation at 425 nm and emission at 492 nm. All final fluorescence intensities were obtained after background (beads alone) subtraction.

DNA sequences:

Probe a: AGGGTTAGGGTTAGGGTTAGGGAAATCTCCTCCGGTTGCGGCATTTCCC

Probe b: AGGGTTAGGGTTAGGGTTAGGGAGCTTGGAGTGTATCAGTCAGCTCCC

Probe c: AGGGTTAGGGTTAGGGTTAGGGGAGATCTTCTAGTTGGTCTGTCTCCCC

Control probe a: AGGGTTAGGGTTAGGGTTAGGGCAGGTCTTCACAATGCCAGCCCTGCCC

Control probe b: AGGGTTAGGGTTAGGGTTAGGGATGATGCGCTCGGCTTCCTGTCATCCC

Control probe c: AGGGTTAGGGTTAGGGTTAGGGACTACCTTACCCCTCCTGCCAGTCCC

Biotin T20: Biotin-TTTTTTTTTTTTTTTTTTTT

DNA S-5: ACTGGCAGGAGGGGTAAGGTAAAATGACAGGAAGCCGAGCGCAAAACAGGGCTGGCATTGTGAAG AAAAAAAAAAAAAAA

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Notes

The authors declare no competing financial interest.

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