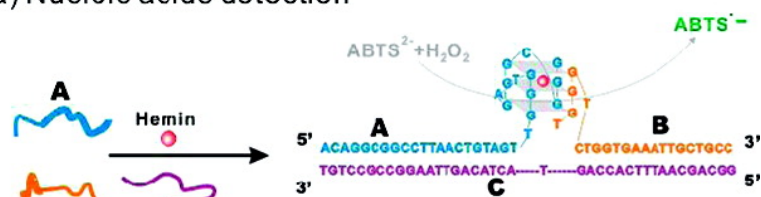Article

Highly Effective Colorimetric and Visual Detection of Nucleic Acids Using an Asymmetrically Split Peroxidase DNAzyme

Minggang Deng, Dan Zhang, Yangyang Zhou, and Xiang Zhou

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Highly Effective Colorimetric and Visual Detection of Nucleic Acids Using an Asymmetrically Split Peroxidase DNAzyme

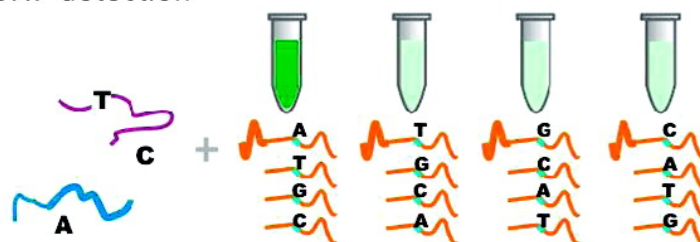Minggang Deng, Dan Zhang, Yangyang Zhou, and Xiang Zhou*

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Abstract: G-quadruplex containing peroxidase DNAzyme is a complex of hemin and a single-stranded guanine-rich DNA (hemin-binding DNA aptamer), which is used as an attractive catalytic label for biosensing recently. Therein, the hemin-binding DNA aptamer contains four *GGG* repeats and can fold into a G-quadruplex structure. In this paper, we have developed a new split mode to divide the hemin-binding DNA aptamer into two parts: one possesses three *GGG* repeats, and another part possesses one *GGG* repeat, namely, the 3:1 split mode. The combination of G-quadruplex and hemin binding could be used as a sensitive probe for the identification of single nucleotide polymorphisms by giving a color signal, visible to the naked eye at room temperature. The G-quadruplex containing peroxidase DNAzyme utilizes the 3:1 split mode and can be directly used for the identification of SNPs with a detection limit in the nM range when the matching length of the probe is short enough. When the matching length of the probe is relatively long, another method adding competition sequences to the probe could also operate effectively for the identification of SNPs. The results also suggested that we could detect the signal when the mutation sample was only 5% in the total target DNA with a competition strategy.

1. Introduction

HIV and all other retroviruses randomly generate mutations during their rapid multiplication process, which leads to resistance against antiviral drugs.[1] The development of sensitive and convenient methods to detect the occurrence of drug resistance is very important. Several methods have been reported, e.g., fluorescence[2] and electrochemistry,[3] but they require expensive reagents and equipment that potentially increase the cost of detection. The idea that a nucleotide change could be detected without the aid of such equipment and only with the naked eye is attractive and might be practical if it incorporates a general enzyme immunoassay. Horseradish peroxidase (HRP) can be used as a label for an enzyme-conjugated assay to detect DNA.[4] However, its modification is expensive: avidin-HRP must first be formed and then bonded to the biotinylated probe. Excess HRP must then be removed to minimize the background signal.
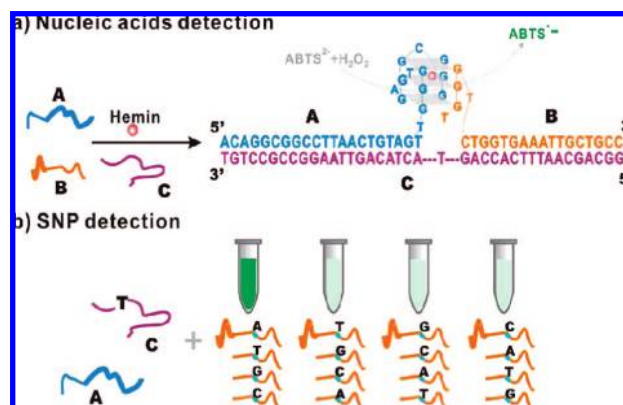

Figure 1. (a) Split DNA-A, -B can assemble on the target DNA-C in the presence of the complementary sequences in the two terminals of DNA-C, and the single-stranded overhanging DNA sequences of DNA-A and -B can form a G-quadruplex structure; when the aptamer binds to hemin, it can catalyze H_2O_2-mediated $ABTS^{2-}$ and obtain the oxidation product $ABTS^{•-}$ with color change. (b) The principle of the identification of single nucleotide polymorphisms using strategy (a) and adding competition sequences when the matching sequence was 17nt.

Sen[5] has designed and developed a DNAzyme, PS2.M, which possesses peroxidase-like activities and a special G-quadruplex structure. It contains a complex of hemin and a single-stranded guanine-rich nucleic acid, which can catalyze the oxidation of 2,2′-azino-bis(3-ethylbenzothiozoline-6-sulfonic acid) ($ABTS^{2-}$) mediated by H_2O_2 to produce the colored radical anion ($ABTS^{•-}$), therefore causing a detectable color change. Although

(1) Johnson, V. A.; Brun-Vézinet, F.; Clotet, B.; Günthard, H. F.; Kuritzkes, D. R.; Pillay, D.; Schapiro, J. M.; Richman, D. D. *Top HIV Med.* **2007**, *15*, 119–125.

(2) (a) Tyagi, S.; Kramer, F. R. *Nat. Biotechnol.* **1996**, *14*, 303–308. (b) Tan, W.; Wang, K.; Drake, T. J. *Curr. Opin. Chem. Biol.* **2004**, *8*, 547–553. (c) Tyagi, S.; Bratu, D. P.; Kramer, F. R. *Nat. Biotechnol.* **1998**, *16*, 49–53. (d) Cai, F.; Chen, H.; Hicks, C. B.; Bartlett, J. A.; Zhu, J.; Gao, F. *Nat. Methods.* **2007**, *4*, 123–125.

(3) (a) Liu, G.; Lin, Y. *J. Am. Chem. Soc.* **2007**, *129*, 10394–10401. (b) Xiao, Y.; Qu, X.; Plaxco, K. W.; Heeger, A. J. *J. Am. Chem. Soc.* **2007**, *129*, 11896–11897. (c) Thavarungkul, P.; Kanatharana, P.; Pretsch, E.; Wang, J.; Bakker, E. *J. Am. Chem. Soc.* **2008**, *129*, 410–411.

(4) Zhang, N.; Appella, D. H. *J. Am. Chem. Soc.* **2007**, *129*, 8424–8425.

(5) (a) Travascio, P.; Li, Y.; Sen, D. *Chem. Biol.* **1998**, 505–517. (b) Travascio, P.; Witting, P. K.; Mauk, A. G.; Sen, D. *J. Am. Chem. Soc.* **2001**, *123*, 1337–1348.

Table 1. Sequences of Oligomers Used in the Assembly of G-Quadruplex Containing Peroxidase DNAzyme

oligomer		sequence (from 5' to 3')	description
C	DNA-C	GGCAGCAATTTCACCAGTACTACAGTTAAGGCCGCCTGT	From nucleotides 4581-4619 of HIV-1 (NL4-3) RNA genome (the GenBank access number: HIVNL43)
	MutC-A	GGCAGCAAATTCACCAGTACTACAGTTAAGGCCGCCTGT	Mutant of DNA-C, whose mutant base is A
	MutC-G	GGCAGCAAGTTCACCAGTACTACAGTTAAGGCCGCCTGT	Mutant of DNA-C, whose mutant base is G
	MutC-C	GGCAGCAACTTCACCAGTACTACAGTTAAGGCCGCCTGT	Mutant of DNA-C, whose mutant base is C
	C-X-A	GGCAGCAATTTCACCAGAACTACAGTTAAGGCCGCCTGT	DNA-C contained a A between the two fragments complementary to the probe
	C-X-G	GGCAGCAATTTCACCAGGACTACAGTTAAGGCCGCCTGT	DNA-C contained a G between the two fragments complementary to the probe
	C-X-C	GGCAGCAATTTCACCAGCACTACAGTTAAGGCCGCCTGT	DNA-C contained a C between the two fragments complementary to the probe
	DNA-A	ACAGGCGGCCTTAACTGTAGTTGGGTAGGGCGGG	The first 21nt from 5'-terminal match the 3'-terminal of DNA-C, the left sequence can form three GGG repeats of the hemin binding apatmer with DNA-B
	IA	ACAGGCGGCCTTAACTGTAGT	Perfectly matched sequence between DNA-A and DNA-C
B	DNA-B (B-17-A)	TGGGTCTGGTGAAATTGCTGCC	The first 17nt from 3'-terminal match the 5'-terminal of DNA-C, the left sequence can form one GGG repeat of the hemin binding apatmer with DNA-A
	B-17-C	TGGGTCTGGTGAACTTGCTGCC	Mutant of DNA-B, whose mutant base is C
	B-17-T	TGGGTCTGGTGAATTTGCTGCC	Mutant of DNA-B, whose mutant base is T
	B-17-G	TGGGTCTGGTGAAGTTGCTGCC	Mutant of DNA-B, whose mutant base is G
	B-11-G	TGGGTCTGGTGAAATT	The first 11nt from 3'-terminal match the 5'-terminal of DNA-C, the left sequence can form one GGG repeat of the hemin binding apatmer with DNA-A
	B-11-C	TGGGTCTGGTCAAATT	Mutant of B-11-G, whose mutant base is C
	B-11-A	TGGGTCTGGTAAAATT	Mutant of B-11-G, whose mutant base is A
	B-11-T	TGGGTCTGGTTAAATT	Mutant of B-11-G, whose mutant base is T
IB-17-A		CTGGTGAAATTGCTGCC	Perfectly matched sequence between DNA-B and DNA-C
IB-17-T		CTGGTGAATTTGCTGCC	Mutant of IB-17-A, whose mutant base is T
IB-17-G		CTGGTGAAGTTGCTGCC	Mutant of IB-17-A, whose mutant base is G
IB-17-C		CTGGTGAACTTGCTGCC	Mutant of IB-17-A, whose mutant base is C
IW		GGGTAGGGCGGGTTGGG	Origin from the DNAzyme part of Catalytic Beacons[8]
PS2.M		GTGGGTAGGGCGGGTTGG	Hemin-binding aptamer reported by Sen[5a]

the catalytic activity of the DNAzyme was not better than that of horseradish peroxidase, the character of DNA is easily reproducible and the detection limit of target DNA was in the picomolar range.[6] The initial DNAzyme, PS2.M, possessed a unique quadruplex with unusual terminal guanines positioned in adjacent quartets,[5b,7] making this structure difficult to manipulate. However, Willner recomposed this sequence to include four GGG repeats that were assembled in a hairpin

structure to form catalytic beacons. They could be applied to detect target DNA and telomerase activity,[8] as well as small molecules and proteins when linked with other apatmers.[9] The unique structure of the four GGG repeats made it possible to split the full-length single strand into two parts which could connect with different DNA probes and assemble to form an aptamer in the presence of target DNA. When bound with

(6) (a) Weizmann, Y.; Beissenhirtz, M. K.; Cheglakov, Z.; Nowarski, R.; Kotler, M.; Willner, I. *Angew. Chem., Int. Ed.* **2006**, *45*, 7384–7388. (b) Tian, Y.; He, Y.; Mao, C. *ChemBioChem* **2006**, *7*, 1862–1864.
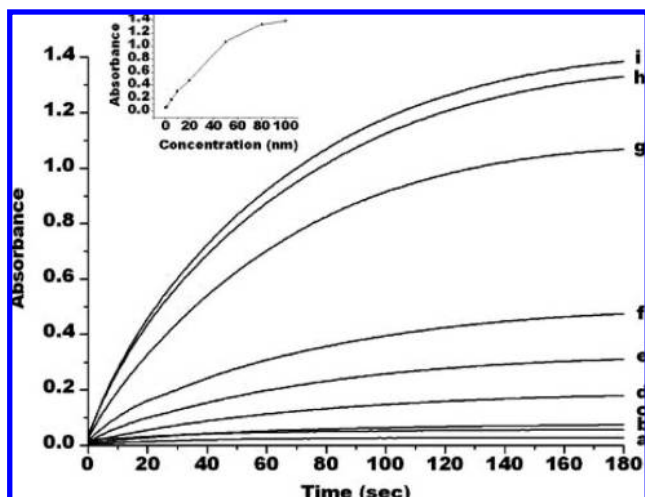
(7) Majhi, P. R.; Shafer, R. H. *Biopolymers* **2006**, *82*, 558–569.
(8) Xiao, Y.; Pavlov, V.; Niazov, T.; Dishon, A.; Kotler, M.; Willner, I. *J. Am. Chem. Soc.* **2004**, *126*, 7430–7431.
(9) Li, D.; Shlyahovsky, B.; Elbaz, J.; Willner, I. *J. Am. Chem. Soc.* **2007**, *129*, 5804–5805.

Figure 2. The activity of the assembly of G-quadruplex containing peroxidase DNAzyme is dependent on the existence of target DNA-C using an asymmetrically split mode. The absorbance changes within 180 s of the oxygenation product ABTS$^{\bullet-}$ at 414 nm in buffer which consisted of 25 mM HEPES-NH$_4$OH (pH 8.0), 20 mM KCl, 200 mM NaCl, 1% DMSO, and in the system of fixed concentration of split DNA-A, -B, hemin, H$_2$O$_2$ (2 mM), ABTS^{2-} (2 mM), and different concentration of target DNA-C. (a) Hemin alone (50 nM), (b–i) hemin (50 nM), split DNA-A (100 nM), -B (100 nM) and the concentration of target DNA-C were as follows: 0, 1, 5, 10, 20, 50, 80, 100 nM. Inset: Calibration curve corresponding to absorbance upon analyzing variable concentrations of target DNA-C (0, 1, 5, 10, 20, 50, 80, 100 nM) after a fixed time interval of 3 min.
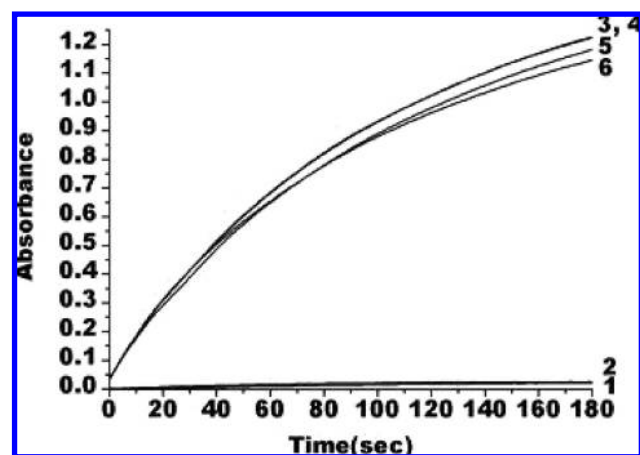

Figure 3. The activity of the assembly of G-quadruplex containing peroxidase DNAzyme is independent of the base which connected two complementary fragments in the middle of DNA-C. The absorbance changes within 180 s of the oxygenation product ABTS$^{\bullet-}$ in buffer which consisted of 25 mM HEPES-NH$_4$OH (pH 8.0), 20 mM KCl, 200 mM NaCl, 1% DMSO at 414 nm; the reaction system also includes a fixed concentration of split DNA-A, -B, hemin (50 nM), H$_2$O$_2$ (2 mM), ABTS^{2-} (2 mM), and different analytes (DNA-C, C-X-A, C-X-G, C-X-C). There was no DNA in curve 1, only the split DNA-A and -B (100 nM) in curve 2, and curves 3 to 6 represent the mixture of the split DNA-A, -B, and different analytes (100 nM respectively). The connecting base of target DNA-C which linked the two complementary fragments were T, A, G, C from 3 to 6, respectively (sequences were shown in Table 1).

hemin, the aptamer became an active DNAzyme and could detect target DNA through a colorimetric reaction. A similar strategy was applied by Kolpashchikov, who utilized a Malachite Green Aptamer for the fluorescent detection of DNA.[10] In fact, much work has been done on this split strategy, with a hemin-binding aptamer split into *two equal parts*, namely two guanine-rich block sequences.[11] However, their results also showed that

the two equally split parts could easily assemble to form an active aptamer in the absence of target DNA and then produce a background signal. In this paper, we report that we split the unabridged guanine-rich single-strand sequences reported by Willner[8] into 3:1 (namely, one part possesses three *GGG* repeats and another part possesses one *GGG* repeat) and found that we could easily assemble them. This allows for the G-rich segments to integrate into many patterns, therefore, reducing the possibility of forming an aptamer that is active in the absence of target DNA.

2. Results and Discussion

2.1. Kinetic Measurements of Peroxidase Reactions.

2.1.1. Assembly of a G-quadruplex and the Process of Oxidation are Dependent on the Presence of Target DNA-C in the 3:1 Split Mode. We applied the 3:1 split mode and detected target DNA with a detection limit in the nanomolar range. Furthermore, we could also distinguish a single mismatched nucleotide, which gave a colored signal visible to the naked eye. As shown in Figure 1a, we developed a peroxidase-like DNAzyme which was composed of the hemin-binding DNA aptamer.[5a] The DNA aptamer was assembled by an unequal split of DNA-A, -B and a target DNA-C (sequences are shown in Table 1). DNA-A possesses *three GGG repeats*, and the 5′-terminus matches the 3′-terminus of the target DNA; oppositely, DNA-B has *one GGG repeat*, and the 3′-terminus matched the 5′-terminus of the target DNA. Therefore, when adding the target DNA-C into the mixture of DNA-A and -B, there are two segments of double-stranded DNA formed at the two termini of DNA-C, allowing the overhanging single-stranded sequences of DNA-A and -B to form a G-quadruplex structure. When binding with hemin, it becomes a G-quadruplex containing DNAzyme which possesses peroxidase-like activities and can catalyze H$_2$O$_2$-mediated ABTS^{2-} to obtain the oxidation product ABTS$^{\bullet-}$ and a corresponding color change.

We utilized the 3:1 split mode to detect target DNA-C and found that it was ineffective at strengthening the catalytic activity of hemin in the absence of target DNA-C. Figure 2 indicates that the OD value of the oxygenation product ABTS$^{\bullet-}$ was 0.06 at the 3 min time point in the absence of target DNA-C (Figure 2b). However, when the concentration of target DNA-C was increased to 100 nM, which was equimolar with DNA-A and -B, the absorbance of the oxygenation product ABTS$^{\bullet-}$ increased to 1.38 (Figure 2i), more than 20 times the initial value. Therefore, we conclude that the process of oxidation is dependent on the concentration of target DNA-C in the 3:1 split mode. Furthermore, if T, which is connected two complementary fragments in the middle of DNA-C, was replaced by A, G, or C, we found that they still worked very well when using this approach under the same conditions (Figure 3).

2.1.2. The DNAzyme utilizing the 3:1 Split Mode can be Directly used for the Identification of Single Nucleotide Polymorphisms when the Matching Length is Short Enough. We next tried to determine whether the split mode could be used for the identification of single nucleotide polymorphisms (SNPs). Here, we expected to identify the mutated nucleotide in the target DNA-C through the color change caused by the H$_2$O$_2$-mediated

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(10) Kolpashchikov, D. M. *J. Am. Chem. Soc.* **2005**, *127*, 12442–12443.
(11) (a) Xiao, Y.; Pavlov, V.; Gill, R.; Bourenko, T.; Willner, I. *ChemBioChem* **2004**, *5*, 374–379. (b) Li, T.; Dong, S.; Wang, E. *Chem. Commun.* **2007**, 4209–4211. (c) Kolpashchikov, D. M. *J. Am. Chem. Soc.* **2008**, *130*, 2934–2935.
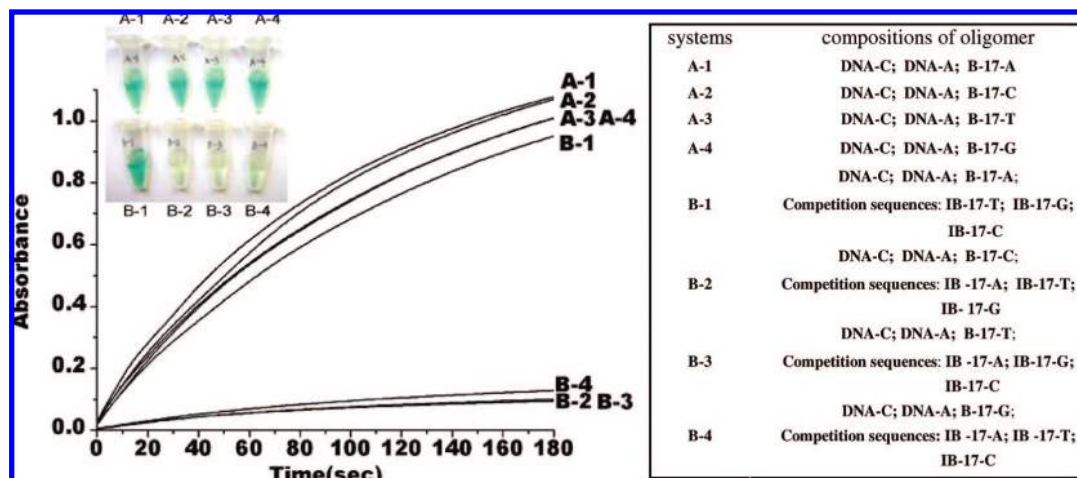
Figure 4. The identification of single nucleotide polymorphisms when the matching length of probe B is 17nt. The absorbance changes within 180 s of the oxygenation product ABTS$^{\bullet-}$ in buffer which consisted of 25 mM HEPES-NH$_4$OH (pH 8.0), 20 mM KCl, 200 mM NaCl, 1% DMSO at 414 nm. From A-1 to A-4, the parts of DNA-B were as follows: B-17-A, B-17-C, B-17-T, B-17-G, respectively. From B-1 to B-4, both the sequence of DNA-B and competition sequences (IB-17-A, IB-17-T, IB-17-G, IB-17-C) were different (sequences were shown in Table 1). The reaction system also includes 2 mM ABTS, 2 mM H$_2$O$_2$. Hemin 50 nM, DNA-C 50 nM, DNA-A 50 nM, both DNA-B and competition sequences 75 nM.
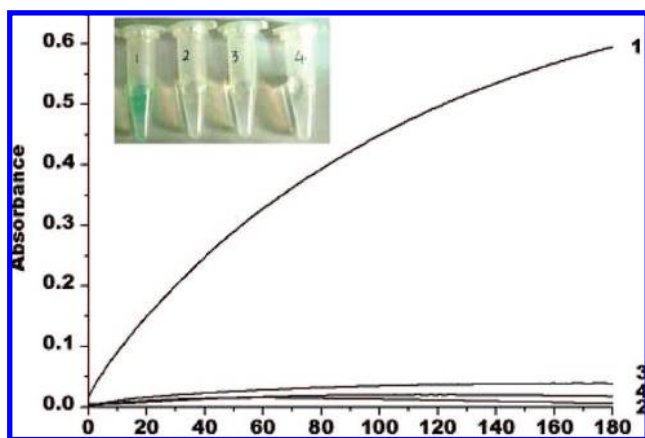

Figure 5. The identification of single nucleotide polymorphisms when the matching length of probe B is 11nt. The absorbance changes within 180 s of the oxygenation product ABTS$^{\bullet-}$ in buffer which consisted of 25 mM HEPES-NH$_4$OH (pH 8.0), 20 mM KCl, 200 mM NaCl, 1% DMSO at 414 nm. The reaction system also includes split DNA-A 50 nM, DNA-C 50 nM, hemin 50 nM, H$_2$O$_2$ (2 mM), ABTS^{2-} (2 mM), and a different sequence of split DNA-B 50 nM. The matching length between split DNA-B and target DNA-C was 11 base pairings. From sequence 1 to 4 the sequences of split DNA-B were as follows: B-11-G, B-11-C, B-11-A, B-11-T, respectively (sequences were shown in Table 1). Only B-11-G can match target DNA-C perfectly.

oxidation reaction. First, we replaced a base of DNA-B from A to C, T, or G, which was in the middle of the matching region between DNA-B and target DNA-C (shown in Figure 4 and sequences were shown in Table 1). Then we detected the corresponding blue oxygenation product, ABTS$^{\bullet-}$. The absorbance of ABTS$^{\bullet-}$ of the four systems are shown from A-1 to A-4 in Figure 4, which were close to each other, so it was proper to conclude that the mutated DNA-B could still assemble with DNA-A on the target DNA-C to form the G-quadruplex as long as the matching length (17nt) was long enough, even if one base was mismatched.

Since a shorter complementary region will be more sensitive in distinguishing mismatched from fully complementary nucleic acid duplexes,[10] we shortened the matching sequence from 17nt to 11 nt and also replaced the middle matching base of DNA-B from G to C, A, or T (sequences are shown in Table 1); the

results shown in Figure 5 indicated that only the perfectly complementary sequence B-11-G could assemble with DNA-A on the target DNA-C to form the G-quadruplex and catalyze the H$_2$O$_2$-mediated ABTS^{2-} to form the blue oxidation product ABTS$^{\bullet-}$.

2.1.3. The DNAzyme Utilizing the 3:1 Split Mode can Identify the SNPs of Relatively Long Matching Length when Adopting a Competition Strategy. When a long matching sequence was introduced, as shown in Figure 1b, another experiment adding competition sequences was utilized. We did four parallel experiments using different mutated sequences of DNA-B at the same time. In other words, there were three competition sequences mutated from DNA-B but without overhanging regions in each experiment, and the mutated nucleotides among them were all different from the unabridged DNA-B. These competition sequences could bind to the target DNA-C through Watson−Crick hydrogen bonds but without the G-quadruplex structure forming because they lacked overhanging regions. We could obtain a G-quadruplex containing peroxidase DNAzyme that catalyzed an oxidation reaction with a corresponding color change only when the overhanging DNA segment bound to its perfectly matched sequence. From the result shown in Figure 4 (from B-1 to B-4), we find that the absorbance of ABTS$^{\bullet-}$ in B-1 was much higher than that in the other three systems, B-2, B-3, and B-4. The split DNA-B in system B-1, B-17-A, not only matched the target DNA-C perfectly but also possessed the overhanging DNA segment which could assemble with DNA-A to form a G-quadruplex. Therefore, we conclude that the strategy adopting competition sequences operated effectively on the identification of single nucleotide polymorphisms when the matching length was not short enough. In fact an analogous strategy using competition sequences was applied with hairpin-structured DNA probes to identify single nucleotide polymorphisms.[12] An excess of blocking oligonucleotides was used to identify the SNPs by single-molecule fluorescence spectroscopy. However, in our experiment, the ratio of concentrations (competition sequences to DNA-B) was only 3 to 1. This strategy might be favorable

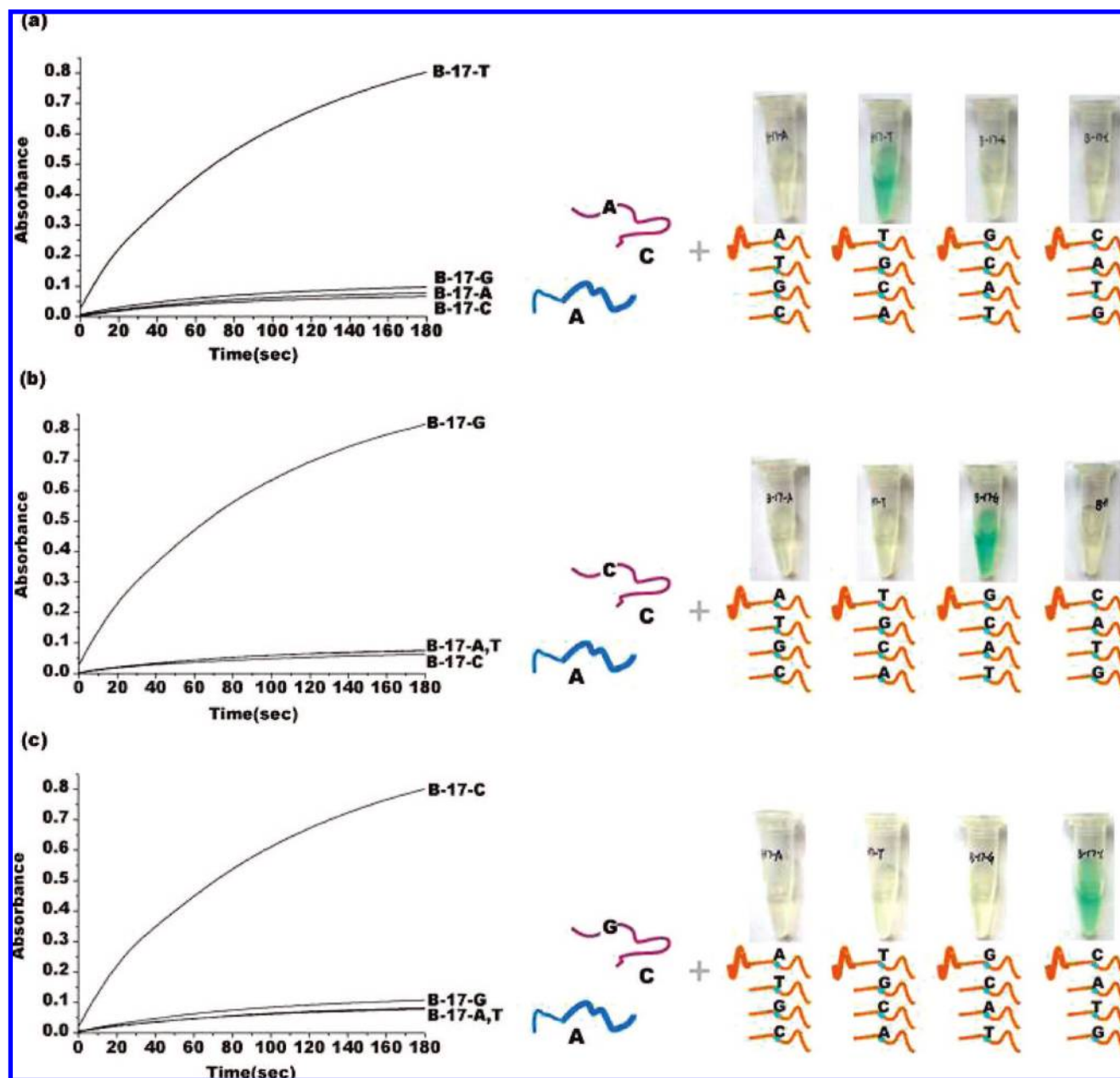(12) Friedrich, A.; Hoheisel, J. D.; Marmé, N.; Knemeyer, J. P. *FEBS Lett.* **2007**, *581*, 1644–1648.

Figure 6. Distinguishing three other variants at the hypothetical SNP site of DNA-C with competition strategy. The absorbance changes within 180 s of the oxygenation products ABTS$^{\bullet-}$ in buffer which consisted of 25 mM HEPES-NH$_4$OH (pH 8.0), 20 mM KCl, 200 mM NaCl, 1% DMSO at 414 nm, and the reaction system also includes 50 nM hemin, 2 mM ABTS, 2 mM H$_2$O$_2$, 50 nM DNA-A, 50 nM analytes (MutC-A, MutC-C, MutC-G), 75nM DNA-B (B-17-A, B-17-C, B-17-T, B-17-G), and 75 nM competition sequences (IB-17-A, IB-17-T, IB-17-G, IB-17-C). (a) The middle base of DNA-C (MutC-A) in the matching region between DNA-B and DNA-C was A. (b) The middle base of DNA-C (MutC-C) was C. (c) The middle base of DNA-C(MutC-G) was G (sequences were shown in Table 1).

for a system of linear probes when using competition sequences to distinguish single mismatched nucleotides.

We completed a further series of related experiments except that we changed the mutated base of DNA-C from T to A, C, or G, respectively, and detected the oxygenation product in the same system (Figure 6). Analogous results were obtained from the four parallel experiments, which clearly proved that the DNAzyme probe was sensitive enough to distinguish different mutated sequences.

To enhance detection sensitivity and application, we modified the competition sequences of the probe and attempted to detect the M184V mutation in the reverse transcriptase (RT) gene of HIV, which is associated with

resistance to some of nucleoside and nucleotide reverse transcriptase inhibitors.[1] Experimental data (Figure 7) indicated that we can reduce the mismatch probe signal and increase the difference gap of signal between the match and mismatch dramatically if the 5′ of competition sequence (Table 2, SIB-RT, MSIB-RT) contained the sequences (*CCGC*T) which could be partially complementary with the G-rich part of A-RT (TGGGTAGG*GCGG*G). The signal from the completely matching sequences (Figure 7, W1 system, M2 system) was 20−30-fold greater than that from the mismatched sequences (Figure 7, W2 system, M1 system). Based on these experiments, we used the probes (Figure 7, A-RT, B-RT, MutB-RT) and the modified competition
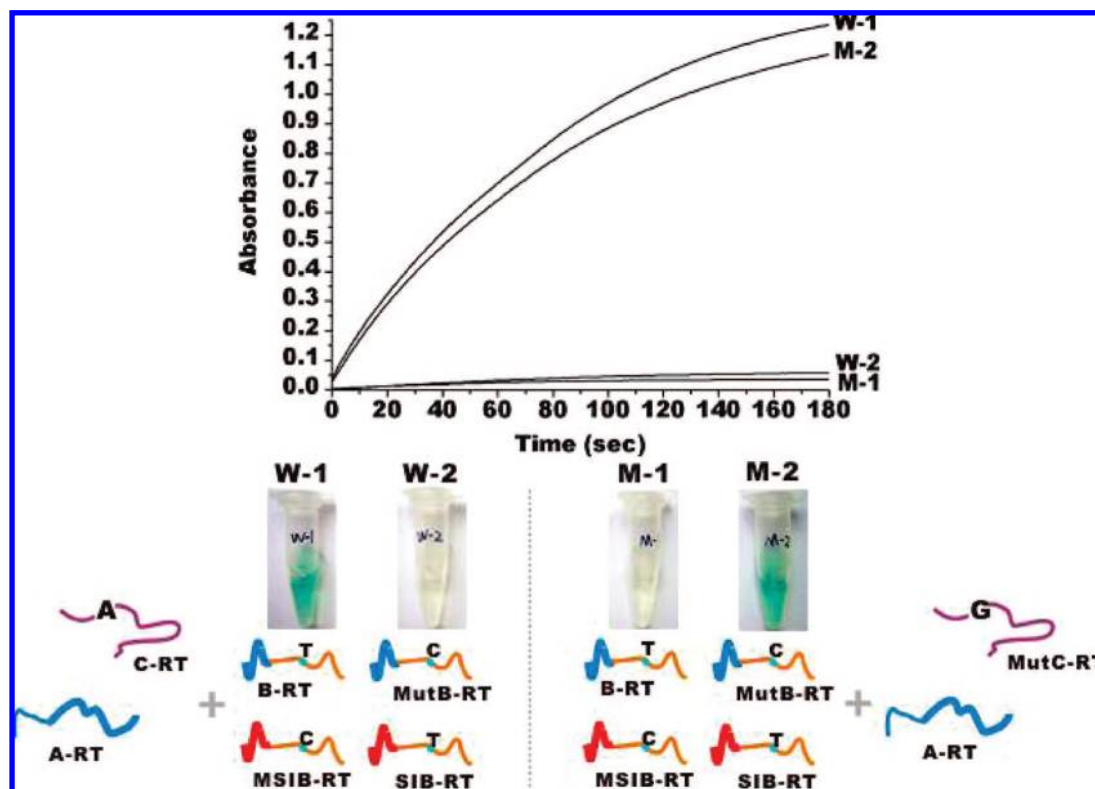
Figure 7. More distinguished detection of SNP through the modified competition sequence. The absorbance changes within 180 s of the oxygenation products ABTS$^{•-}$ at 414 nm in the systems of W-1, W-2, M-1, and M-2. The target DNA-C adopted in W-1 and W-2 was wide type, namely, C-RT; the concentrations of A-RT, B-RT, and C-RT were 100 nM, respectively, while the concentration of MSIB-RT which was the competition sequence of B-RT was 300 nM in the system of W-1. In the system of W-2, the concentrations of A-RT, MutB-RT, C-RT were 100 nM, respectively, while the concentration of SIB-RT was 300 nM, which was the competition sequence of MutB-RT. However, the target DNA-C adopted in the systems of M-1 and M-2 was MutC-RT, which was a mutated type compared to the wide type C-RT. Similarly, the concentrations of A-RT, B-RT, and MutC-RT were 100 nM, respectively, and the concentration of MSIB-RT was 300 nM in the system of M-1. In the system of M-2, the concentrations of A-RT, MutB-RT, MutC-RT, SIB-RT were 100, 100, 100, 300 nM, respectively (all sequences were shown in Table 2). These detections existed in 50 nM hemin, 2 mM ABTS, 2 mM H$_2$O$_2$, and the reaction buffer consisted of 25 mM HEPES-NH$_4$OH (pH 8.0), 20 mM KCl, 200 mM NaCl, 1% DMSO.

Table 2. Sequences of Oligomers Used in the Study of the More Distinguished Detection of SNPs

oligomer	sequence (from 5' to 3')	description
C-RT	TATCAATACATGGATGATTTGTATGTAGGATCTGACTTAGAAATA	From nucleotides 2636-2680 of HIV-1 (HXB2) RNA genome （the GenBank access number: K03455）
MutC-RT	TATCAATACGTGGATGATTTGTATGTAGGATCTGACTTAGAAATA	Mutant of C-RT, whose mutant base is G
A-RT	TATTTCTAAGTCAGATCCTACATACTGGGTAGGGCGGG	The first 25nt from 5'-terminal match the 3'-terminal of C-RT, the left sequence can form three GGG repeats of the hemin binding apatmer with DNA-B
B-RT	TGGGTAATCATCCATGTATTGATA	The first 19nt from 3'-terminal match the 5'-terminal of C-RT, the left sequence can form one GGG repeat of the hemin binding apatmer with DNA-A
MutB-RT	TGGGTAATCATCCACGTATTGATA	The first 19nt from 3'-terminal match the 5'-terminal of MutC-RT, the left sequence can form one GGG repeat of the hemin binding apatmer with DNA-A
SIB-RT	CCGCTAATCATCCATGTATTGATA	The first 19nt from 3'-terminal match the 5'-terminal of C-RT, the left sequence can partially match the G-rich part of A-RT
MSIB-RT	CCGCTAATCATCCACGTATTGATA	The first 19nt from 3'-terminal match the 5'-terminal of MutC-RT, the left sequence can partially match the G-rich part of A-RT

sequences (Table 2, SIB-RT, MSIB-RT) to distinguish between the wide type sequence C-RT and the mutant type sequence MutC-RT. After setup of this detecting system, we sought to discover whether we could detect minor mutations from mixed samples with this combination of A-RT, MutB-

RT, and SIB-RT (Figure 7). As shown in Figure 8, when the total target DNA concentration was 100 nM and the percentage of mutated target DNA was 5%, the signal showed an OD value of 0.123; if there was no mutated target DNA in the total target DNA, the OD value was only 0.035. This
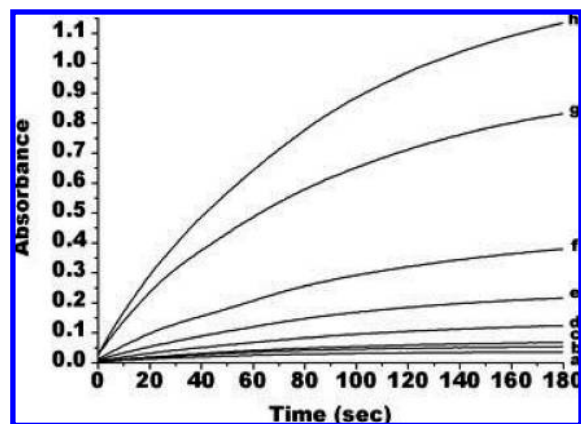
Figure 8. Detection of mutated gene in the mixed samples. The absorbance changes within 180 of the oxygenation products ABTS•– in buffer which consisted of 25 mM HEPES-NH₄OH (pH 8.0), 20 mM KCl, 200 mM NaCl, 1% DMSO at 414 nm, and the reaction system also includes 100 nM A-RT, 100 nM MutB-RT, 300 nM SIB-RT, 50 nM hemin, 2 mM ABTS, and 2 mM H₂O₂. The total concentration of C-RT and MutC-RT was 100 nM; from a to h, the percentage of MutC-RT in the 100 nM was 0, 1, 2, 5, 10, 20, 50, 100%, respectively.
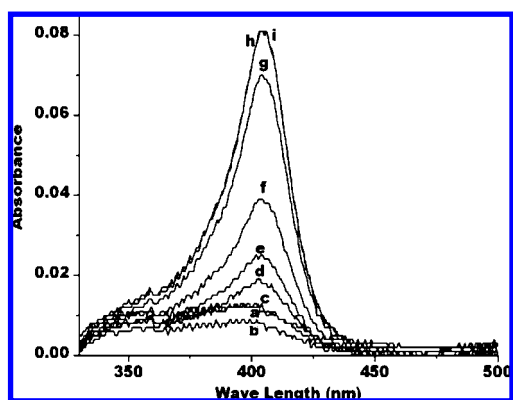

Figure 9. UV−visible spectra in the range 320−500 nm of hemin in buffer which consisted of 25 mM HEPES-NH₄OH (pH 8.0), 20 mM KCl, 200 mM NaCl, 1% DMSO. (a) 0.5 μM hemin alone. (b) 0.5 μM hemin and 1.0 μM target DNA-C. (c−i) The concentration of hemin (0.5 μM) and split DNA-A, -B (1.0 μM) were changeless; the concentration of target DNA-C was as follows: 0, 0.05, 0.1, 0.2, 0.5, 0.8, 1.0 μM.

result suggested that we could detect the signal when the mutation sample was only 5% in the total target DNA.

2.2. Formation of Assembled G-Quadruplex Structure When Utilizing the 3:1 Split Mode. We have demonstrated that the 3:1 split mode could distinguish a single mismatched nucleotide. We used UV titration to investigate whether hemin could be combined with the aptamer and participate in the catalytic reaction. The results, shown in Figure 9, indicate that the absorbance band of hemin increased dramatically when the concentration of target DNA-C was increased. The Soret band was red-shifted (from 397 to 404 nm) simultaneously, which indicated that the DNA aptamer, assembled by the split DNA-A, -B and target DNA-C, possessed a binding affinity for hemin.[5a]

To investigate and verify whether the assembled DNA aptamer had the ability to form a G-quartet structure, which is vital to the catalytic activity of the DNAzyme, we used CD spectra and the subtractive method to demonstrate that the overhanging single-stranded sequences of DNA-A and -B fold into a parallel G-quadruplex structure in the presence of 20 mM potassium ions and 200 mM sodium ions. Figure 10a shows
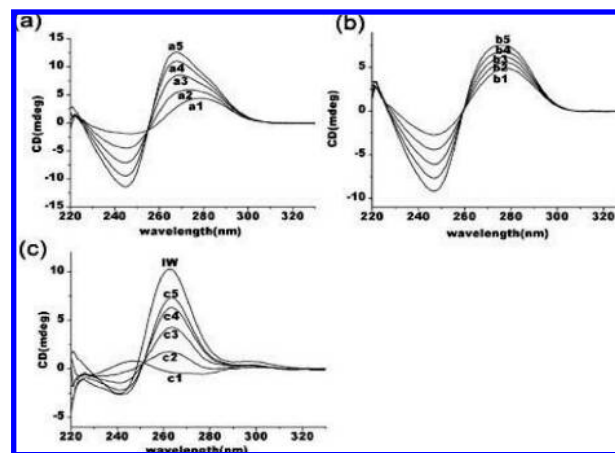

Figure 10. CD titration experiment in 25 mM HEPES-NH₄OH (pH 8.0) in the presence of 20 mM potassium ion and 200 mM sodium ion when increasing the concentration of target DNA-C. The concentration of target DNA-C was 0, 0.25, 0.5, 0.75, and 1.0 μM from 1−5. (a) Fixed the concentration of DNA-A and B at 1.0 μM. (b) Fixed the concentration of matching region of DNA-A and B at 1.0 μM. (c) c1−c5 subtracted (b) from corresponding (a); IW is the CD signal of DNAzyme part of Catalytic Beacons[8] as control.

the structure of the whole DNA aptamer, and Figure 10b can be interpreted as the baseline of Figure 10a, for it only shows the CD absorbance of the two parts of the double-stranded DNA region. The two experiments were carried out under the same conditions, so the structure formed by the overhanging single-stranded sequences can be determined by subtracting (b) from the corresponding spectrum in (a), which is presented in (c). It has been reported that CD spectra of a typical parallel G-quadruplex structure have a positive peak near 270 nm and negative band around 240 nm, whereas CD spectra of a typical antiparallel G-quadruplex structure have a positive peak at 295 nm and a negative peak close to 265 nm.[13] If there is a small positive peak at 265 nm along with a negative peak around 240 nm and a stronger positive peak at 295 nm, it may be characterized as a hybrid of parallel/antiparallel G-quadruplex structures.[13] From the resulting spectrum in Figure 10c, we find that an increasing concentration of target DNA-C (from c1 to c5) dramatically enhances the peak near 265 nm, while the band around 245 nm simultaneously decreases, suggesting that the DNA aptamer, assembled by the split DNA-A, -B and target DNA-C, forms a *parallel G-quadruplex structure*.[13]

3. Conclusion

In summary, we have developed a new and useful split mode of the G-quadruplex containing peroxidase DNAzyme and demonstrated that the assembled DNA aptamer can fold into a parallel G-quadruplex structure which possesses a binding capacity for hemin. This assay is simple and rapid, and there is no need to label DNA substrates. The results also suggested that we could detect the signal when the mutation sample was

(13) (a) Li, W.; Wu, P.; Ohmichi, T.; Sugimoto, N. *FEBS Lett.* **2002**, *526*, 77–81. (b) Ambrus, A.; Chen, D.; Dai, J. X.; Bialis, T.; Jones, R. A.; Yang, D. Z. *Nucleic Acids Res.* **2006**, *34*, 2723–2735. (c) Seenisamy, J.; Rezler, E. M.; Powell, T. J.; Tye, D.; Gokhale, V.; Joshi, C. S.; Siddiqui-Jain, A.; Hurley, L. H. *J. Am. Chem. Soc.* **2004**, *126*, 8702– 8709. (d) Balagurumoorthy, P.; Brahmachari, S. K. *J. Biol. Chem.* **1994**, *269*, 21858–21869. (e) Balagurumoorthy, P.; Brahmachari, S. K.; Mohanty, D.; Bansal, M.; Sasisekharan, V. *Nucleic Acids Res.* **1992**, *20*, 4061–4067. (f) Jin, R.; Gaffney, B. L.; Wang, C.; Jones, R. A.; Breslauer, K. J. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 8832–8836.

only 5% in the total target DNA. The most important characteristic of the assay is a sensitive probe for direct visualization of single nucleotide polymorphisms by the "naked-eye" at room temperature, which makes it more convenient than other methods that rely on instrumentation.

4. Experiments

4.1. Materials. Hemin was purchased from Acrose, and HEPES was purchased from Amresco. ABTS was purchased from Sigma, H_2O_2 was purchased from Sinopharm Chemical Reagent Co. Ltd., and the oligonucleotides were purchased from Invitrogen Technology (Shanghai, China).

4.2. Kinetic Measurements of Peroxidase Reactions. DNA-A and -B at 100 nM and different concentrations of DNA-C were heated at 95 °C for 5 min in a buffer containing 25 mM HEPES-NH_4OH (pH 8.0), 20 mM KCl, and 200 mM NaCl; samples were then incubated at room temperature for 1 h, and then hemin was added, making sure that the final concentration was 50 nM. Samples were incubated at room temperature for another 1 h, and then ABTS and H_2O_2 were added, the final concentrations of which were 2 mM. The detection was done in the Kinetics mode of a Shimadzu UV-2550 UV−vis spectrophotometer.

4.3. Absorbance Spectroscopy of DNA−Hemin Complexes. 1.0 μM DNA-A, -B and different concentrations of DNA-C were heated at 95 °C for 5 min in a buffer containing 25 mM HEPES-NH_4OH (pH 8.0), 20 mM KCl, and 200 mM NaCl. Samples were incubated at room temperature for 1 h, and then hemin was added, making sure the final concentration was at 0.5 μM. The detection was then done in the Spectrum mode of the Shimadzu UV-2550 UV−vis spectrophotometer.

4.4. Circular Dichroic Studies. CD experiments utilizing a Jasco-810 spectropolarimeter (Jasco, Easton, MD, USA) were measured at room temperature using a quartz cell with a 1 cm path length, CD spectra were collected from 220 to 330 nm and with a scanning speed of 200 nm/min. The bandwidth was 5 nm, and the response time was 2 s. All CD spectra were baseline-corrected for signal contributions due to the buffer and were the average of at least two runs. The process was as follows:

(a) Heating DNA-A, -B, 1 μM at 95 °C for 10 min in buffer containing 25 mM HEPES-NH_4OH (pH 8.0), 20 mM KCl and 200 mM NaCl and cooling to room temperature, then titrating in target DNA-C into the system, and collecting the CD signal ∼0.5 h after every titration, the final concentrations of DNA-C were as follows: 0, 0.25, 0.5, 0.75, and 1.0 μM.

(b) IA, IB were single-stranded sequences which could match the terminal ends of target DNA-C. DNA at 1.0 μM was heated to 95 °C for 10 min in buffer containing 25 mM HEPES-NH_4OH (pH 8.0), 20 mM KCl, and 200 mM NaCl and cooled to room temperature, and then DNA-C was titrated into the system. The CD signal was collected ∼0.5 h after every titration, and the final concentrations of DNA-C were as follows: 0, 0.25, 0.5, 0.75, and 1.0 μM.

IW was the sequence of the DNAzyme part of Catalytic Beacons.[8] DNA at 1.0 μM was heated to 95 °C for 10 min in buffer containing 25 mM HEPES-NH_4OH (pH 8.0), 20 mM KCl, and 200 mM NaCl and cooled to room temperature. Then, the CD signal was collected.