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Graphene Oxide-Based Fluorescent "on/off" Switch for Visual Bioassay Using "Molecular Beacon"-Hosted Hoechst Dyes

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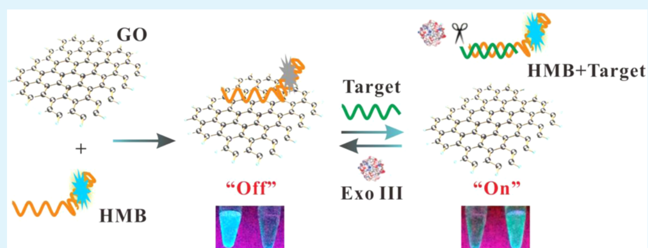
Supporting Information

ABSTRACT: In this study, the fluorescence of Hoechst dyes is significantly lit up by addition of our designed **MB** probe, forming a complex of "molecular beacon"-hosted Hoechst dyes (**HMB**). Combined with this property, a novel graphene oxide (GO)-based fluorescent "on/off" switch was developed to visually follow bioassay utilizing **HMB** as signal indicators and GO as an excellent energy acceptor to efficiently quench the fluorescence of **HMB** in a label-easy format. We have demonstrated its application for label-easy fluorescence "turn on" detection of sequence-specific DNA and "turn off" detection of exonuclease with sensitivity and selectivity in a single step in homogeneous solution. Compared to traditional molecular beacons, the proposed design is cost-effective and simple to prepare without fluorescence labeling or chemical modification.



KEYWORDS: *graphene, Hoechst, molecular beacon, biosensing, exonuclease*

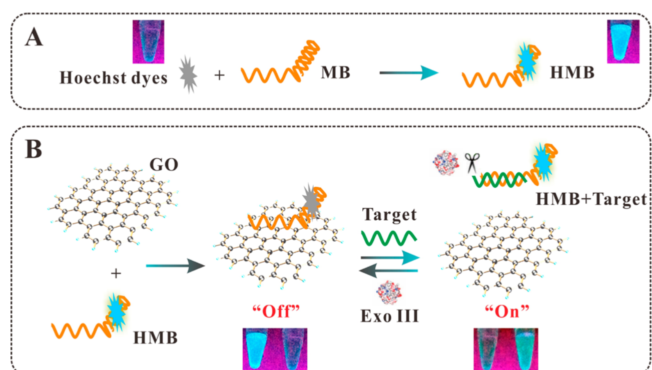
INTRODUCTION

Molecular beacons (MBs), first reported by Tyagi and Kramer, are dually labeled single-stranded DNA (ssDNA) that are internally quenched due to the proximity between a fluorophore and a quencher tagged at either end.[1] Traditional MBs have found marvelous applications in various fields, but always need dual labeling, which will unavoidably accompany with some limitations such as high-cost and time-consuming fluorescence labeling or chemical modification.[2−4] Therefore, it is highly desirable to exploit label-easy and simple MB-like probes.

Many research efforts have been devoted to the development of label-easy and convenient MB-like probes for fluorescent detection of varied target molecules.[5−13] These MB-like probes may render certain advantages over traditional MBs, because they are simple and inexpensive without covalent labeling of fluorophores/quenchers on the probes. Recently, our group developed a novel label-free MB-like probe based on the reversible directing fluorescence of Hoechst dyes for fluorescent detection of Hg^{2+} and biothiols.[14] Hoechst dyes are part of a family of blue fluorescent dyes used as cell permeable nucleic acid stains.[15] The dyes can bind to the minor groove of AT-rich double-stranded DNA (dsDNA) with a considerable fluorescence enhancement.[16]

Herein, we describe our ongoing effort to develop a facile and label-easy graphene oxide (GO)-based fluorescent switch, in which "molecular beacon"-hosted Hoechst dyes (**HMB**) act as signal indicators and GO as an excellent energy acceptor[4,17−21] to efficiently quench the fluorescence of **HMB** (Scheme 1). The **MB** probe used in this work containing

Scheme 1. (A) Fluorescence of Hoechst dyes is Significantly Lit up by Addition of MB Probe, Forming a Complex of "Molecular Beacon"-Hosted Hoechst Dyes (HMB); (B) Schematic Representation of the Label-Easy Graphene Oxide (GO)-Based Fluorescent Switch for Fluorescence "Turn on" Detection of Target DNA (Target) and "Turn off" Detection of Exonuclease (Exo III) Utilizing HMB as Signal Indicators[a]



[a]The photos were taken under a 365 nm UV lamp excitation using a digital camera.

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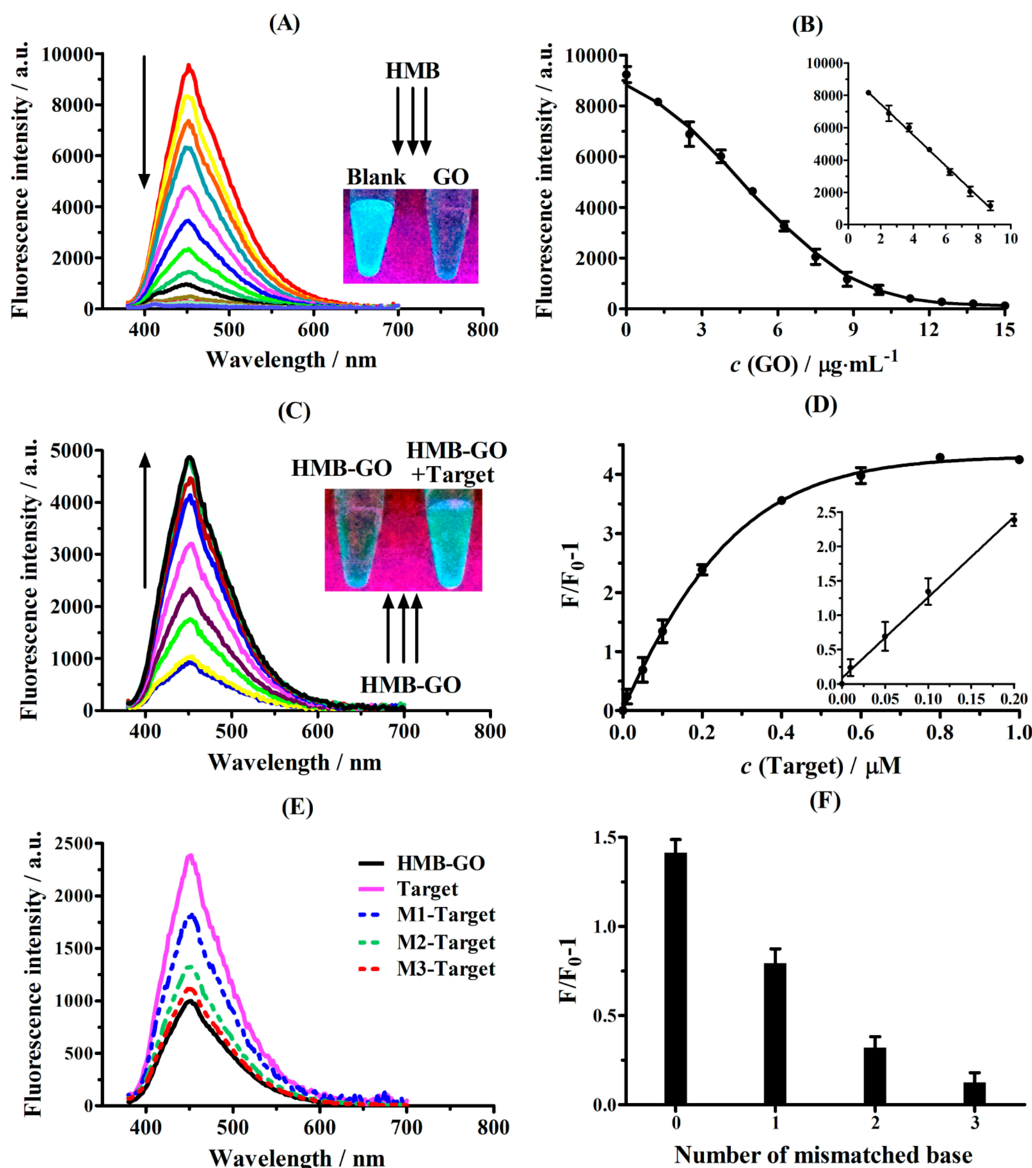

Figure 1. (A) Fluorescence emission spectra recorded during the process of titration of **HMB** in the PBS buffer (Hoechst dyes of 1 μg/mL and **MB** of 0.1 μM were used) with various concentrations of GO solution (in the range of 0−15 μg/mL). (B) The plot of the fluorescence intensities vs. the increasing concentrations of GO solution of the same data. (C) Fluorescence emission spectra (excitation at 360 nm, emission at 450 nm) of **HMB**-GO switch on adding increasing concentrations of **Target** (0, 0.01, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 μM); (D) Plot of (F/F_0-1) as function of the increasing concentrations of **Target**, where F_0 and F are the fluorescence intensity of **HMB** in the absence and the presence of different concentrations of **Target**. (E) Fluorescence response of **HMB**-GO switch in the presence of 100 nM different DNA inputs including perfect-matched **Target**, single-base-mismatched **M1-Target**, two-base-mismatched **M2-Target** and three-base-mismatched **M3-Target**. (F) Analysis of the loss of fluorescence restoration with the increased number of mutations on the **Target**. Each data point is an average of three replicate measurements and the error bars correspond to the standard deviation of each measurement.

two regions, the hairpin region with an AT-rich sequence as a sensitizer for Hoechst dyes, and the ssDNA region for the recognition of target DNA (**Target**) (see Figure S1 in the Supporting Information and Scheme 1). The fluorescence of Hoechst dyes is significantly lit up by addition of **MB** probe, forming a complex of "molecular beacon"-hosted Hoechst dyes (**HMB**) (Scheme 1A and Figure S2 in the Supporting Information, yellow line). The **HMB** can self-assemble on the GO surface via pi-stacking interactions between the ssDNA region of **MB** probe and the GO surface, effectively quenching the **HMB** and leading to the fluorescence "off" state (Scheme 1B and Figure S2 in the Supporting Information, green line). In the presence of **Target**, the duplex **HMB-Target** is liberated from the GO surface, resulting in fluorescence restoration of **HMB** and leading to the fluorescence "on" state (Scheme 1B and Figure S2 in the Supporting Information, purple line). It is reported that exonuclease III (Exo III) can catalyze the stepwise removal of mononucleotides from 3′-hydroxyl termini of duplex DNAs with blunt or recessed 3′ termini.[22−26] Thus, by introducing Exo III, the digestion of the 3′-end of **Target** strand in the duplex **HMB-Target** causes the release of **HMB** and it self-assembles anew on the GO surface, switching to the fluorescence "off" state again, which enable the detection of Exo III with high sensitivity and selectivity (Scheme 1B and Figure S2 in the Supporting Information, pink line). Exonucleases, a class of DNA-degrading enzymes, play vital roles in a wealth of

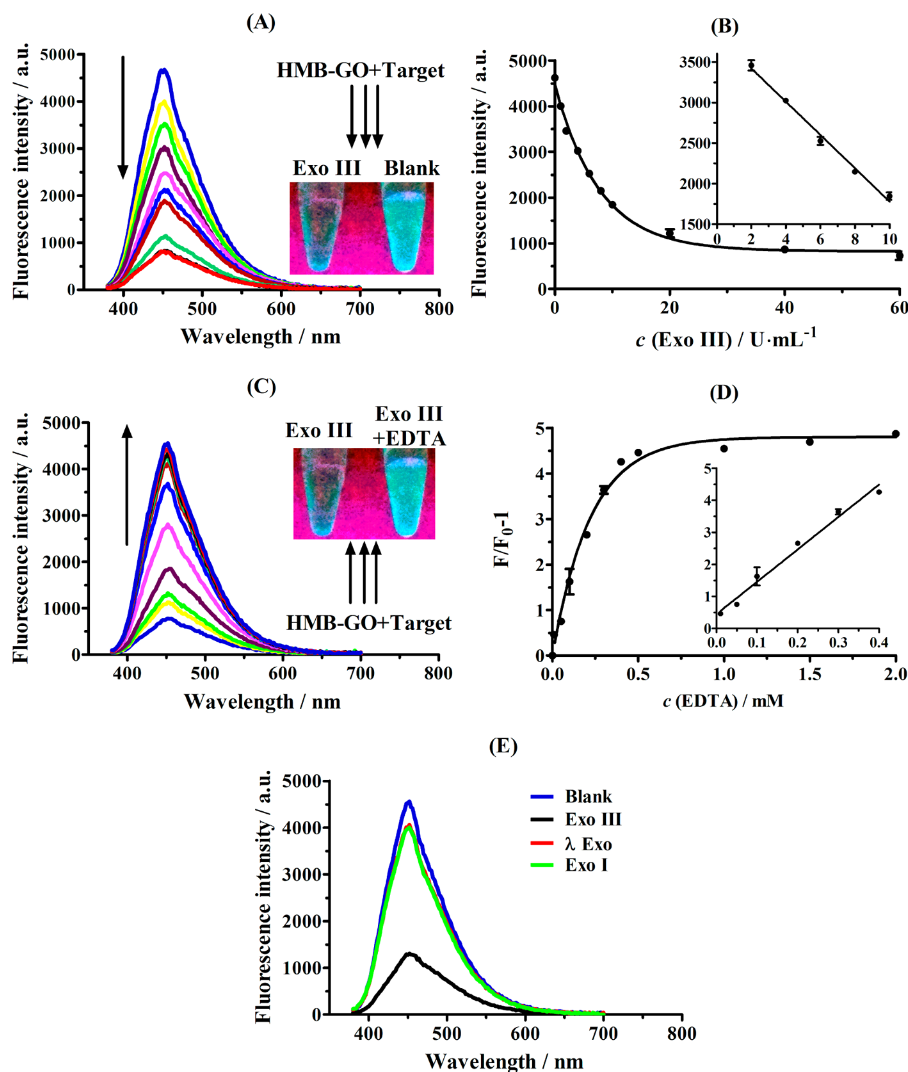

Figure 2. (A) Fluorescence response of **HMB**-GO switch in the open state (0.6 μM **Target** added) to Exo III. The fluorescence emission spectra are shown for various Exo III concentrations of 0, 1, 2, 4, 6, 8, 10, 20, 40, and 60 U/mL (excitation at 360 nm, emission at 450 nm). (B) Plot of the fluorescence intensities vs. the increasing concentrations of Exo III solution of the same data. (C) Fluorescence response of **HMB**-GO switch in the open state (0.6 μM **Target** added) for inhibition analysis of Exo III by EDTA (60 U/mL Exo III added). The fluorescence emission spectra are shown for various EDTA concentrations of 0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 1.5, and 2.0 mM. (D) Plot of $(F/F_0 - 1)$ as function of the increasing concentrations of EDTA, where F_0 and F are the fluorescence intensity of **HMB**-GO switch in the open state (0.6 μM **Target** added) to 60 U/mL Exo III in the absence and the presence of different concentrations of EDTA. (E) Selectivity analysis for Exo III. Fluorescence response of **HMB**-GO switch in the open state (0.6 μM **Target** added) to different enzyme inputs including 20 U/mL Exo III, 200 U/mL λ Exo, and 200 U/mL Exo I. The blank sample is indicated as **HMB**-GO switch in the open state (0.6 μM **Target** added). Each data point is an average of three replicate measurements and the error bars correspond to the standard deviation of each measurement.

physiological and cellular processes. Therefore, the studies of exonucleases and their inhibition are highly important and of general interest.

■ RESULTS AND DISCUSSION

To demonstrate the feasibility of our proposed approach, the experiments begin with the utilization of GO, as both a "nanoscaffold" and a "nanoquencher" for self-assembling **HMB**, to form the **HMB**-GO switch for **Target** sensing. After adding GO with increasing concentrations, a gradual fluorescence decrease in the **HMB** solution was observed (Figure 1A). When the GO concentration was higher than 11.25 μg/mL, the fluorescence intensity of **HMB** system (Hoechst dyes of 1 μg/mL and **MB** of 0.1 μM used) reached a nearly saturated signal, which indicated that the 11.25 μg/mL GO reached the adsorption equilibrium with the **HMB** system, leading to the forming **HMB**-GO switch "off" state (Figure 1B). However, in the presence of **Target**, the **HMB**-GO switch changed to the fluorescence "on" state as a result of the forming duplex **HMB-Target** detached from the GO surface (Figure 1C). The kinetic behaviors of fluorescence quenching of **HMB**-GO switch and fluorescence restoration via separation of **HMB**-GO switch in the presence of **Target** were monitored (see Figure S3 in the Supporting Information). The fluorescence intensity rapidly decreased to a minimum value within 5 min when adding GO to the **HMB** solution. The fluorescence restoration in the presence of **Target** was about 3.5 min.

The fluorescence restoration phenomenon is sensitive and specific to the **Target**. As the **Target** concentration increased, the fluorescence intensity of the resulting duplex **HMB-Target** gradually increased (Figure 1C). Figure 1D illustrates how the

addition of increasing concentrations of **Target** in the range of 0−1.0 μM results in the fluorescence intensity changes ($F/F_0 - 1$) of the duplex **HMB-Target**, where F_0 and F are the fluorescence intensity of **HMB** in the absence and the presence of **Target**, respectively. Figure 1D inset showed a good linear correlation between the ($F/F_0 - 1$) value and the concentration of **Target** over the range of 0−0.2 μM. A series of 4 repetitive measurements with 0.02, 0.04, 0.06, 0.08, 0.12, and 0.16 μM **Target** were used for investigating the precision of **HMB** responses, and obtained relative standard deviation (RSD) of 8.3, 3.5, 3.8, 2.4, 2.1, and 2.6%, respectively, demonstrating an excellent reproducibility of the assay. The limit of detection (LOD) of **Target** based on the three times of signal-to-noise level of the blank sample was estimated to be 2 nM.

We further investigated the sequence discrimination ability of the **HMB**-GO switch toward single-base-mismatched DNA (**M1-Target**), two-base-mismatched DNA (**M2-Target**) and three-base-mismatched DNA (**M3-Target**). The data of fluorescence restoration were shown in Figure 1E; the signal outputs for the **M1-Target**, **M2-Target**, and **M3-Target** in the same condition were weaker than the corresponding perfect-matched **Target** signal output and are appreciably discriminated with the number of mutation increased (Figure 1F). The results clearly illustrated that the **HMB**-GO switch can provide a powerful alternative to specifically detect target DNA and discriminate single-base mismatches with remarkably specific responses. The identification of single-base mismatch is important for the single nucleotide polymorphism (SNP) genotyping and routine screening of genetic mutations and diseases. The results demonstrated that our proposed probe provides great potential for the quantification of nucleic acid as well as the detection of point mutation.

On the other hand, detection of exonuclease was performed to evaluate the practicality of our proposed platform (Figure 2A, B). A **HMB**-GO solution containing 0.6 μM **Target** (**HMB**-GO+**Target**) was prepared. After adding Exo III with increasing concentrations, a gradual fluorescence decrease in the **HMB**-GO+**Target** was observed (Figure 2A). The results clearly verified that the digestion of the 3′-end of **Target** strand in the duplex **HMB-Target** release **HMB** to self-assemble anew on the GO surface. Figure 2B shows a dependence of fluorescent decrease on Exo III concentration. The detection limit was estimated to be 1 U/mL. The kinetic behaviors of fluorescence decrease of the **HMB**-GO+**Target** solution in the presence of Exo III were also monitored (see Figure S4 in the Supporting Information). The fluorescence intensity decreased to a minimum value within 10 min when adding Exo III to the **HMB**-GO+**Target** solution.

It is known that there are certain metal ions in the active center of enzymes. Ethylenedaminetetraacetic acid (EDTA), a chelating agent, can form complexes with metal ions to lower the concentration of metal ions in the active center of enzymes, resulting in the inactivation of enzymes.[27,28] In this respect, the inhibition effect of EDTA was investigated through our proposed platform. Figure 2C and 2D showed that fluorescence intensity of the **HMB**-GO+**Target** system upon addition of Exo III enhanced with an increasing concentration of EDTA presented, demonstrating that the digestion ability of Exo III was reduced by the presence of EDTA. These results, in turn, show that the method has a potential for the studies of exonuclease inhibition and the screening of exonuclease inhibitors.

Moreover, we investigated the specificity of the **HMB**-GO +**Target** system toward 20 U/mL Exo III, 200 U/ml lambda exonuclease (λ Exo) and 200 U/mL exonuclease I (Exo I) as controls. The data of fluorescence response were shown in Figure 2E, the signal decreases for the λ Exo and Exo I were significantly weaker than the Exo III signal decrease. The results clearly indicated that the **HMB**-GO+**Target** system can provide a powerful tool to specifically detect target exonuclease. As the results show, qualitative and quantitative detection of Exo III can be performed through the proposed system.

CONCLUSIONS

In summary, we have proposed a novel self-assembled graphene-ssDNA MB-like switch design using Hoechst dye as signal indicators and demonstrated its application for label-easy fluorescence "turn on" detection of sequence-specific DNA and "turn off" detection of exonuclease with sensitivity and selectivity. This novel "mix and measure" MB-based probe design provides many advantages, including simplicity of preparation and manipulation compared with other methods that employ specific strategies including tedious procedures,[29] and the need for labels,[30] etc.

EXPERIMENTAL SECTION

Reagents and Materials. The oligonucleotides used in this study were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China) with the following sequences: **MB**: 5′-TGGAAGGAGGCGTTATGAGGGGGTCCAATATTATTATAAAAATAATAATAT-3′; **Target**: 5′-TGGACCCCCTCATAACGCCTCCTTCCA-3′; **M1-Target**: 5′-TGGACCCCCTCATTACGCCTCCTTCCA-3′; **M2-Target**: 5′-TGGACCACCTCATTACGCCTCCTTCCA-3′; **M3-Target**: 5′-TGGACCACCTCATTACGCCTGCTTCCA-3′ (Note: the mutation base is indicated in underlined portion). Exonuclease III (Exo III), lambda exonuclease (λ Exo) and exonuclease I (Exo I) were purchased from New England Biolabs Inc. 5× PBS buffer (5 × 2.7 mM KCl, 5 × 2 mM KH_2PO_4, 5 × 136 mM NaCl, 5 × 10 mM Na_2HPO_4, pH 7.4) was prepared using metal-free reagents in distilled water purified by a Milli-Q water purification system (Millipore Corp., Bedford, MA) with an electrical resistance of 18.2 MΩ. All chemicals used in this work were of analytical reagent and obtained from commercial sources and directly used without additional purification.

Instrumentation. Fluorescence was measured in a fluorescence microplate reader (Bio-Tek Instrument, Winooski, USA) using a black 384 well microplate (Fluotrac 200, Greiner, Germany). Photographs were taken with a digital camera under a 365 nm UV lamp excitation.

Preparation of HMB-GO Switch for Fluorescent "On/off" Bioassay. Graphene oxide (GO) was synthesized from natural graphite powder by modified Hummers method.[31,32] Prior to the experiments, the GO powder was dissolved in Milli-Q water and then sonicated for 5 h to give a homogeneous brilliant yellow solution (see Figure S5 in the Supporting Information). The probe **HMB** (Hoechst dyes and **MB** were used) was first prepared and then mixed with GO to form **HMB**-GO switch using PBS buffer (2.7 mM KCl, 2 mM KH_2PO_4, 136 mM NaCl, 10 mM Na_2HPO_4, pH 7.4). Then the **HMB**-GO switch was incubated for 5 min at RT. For the fluorescent "on" detection of DNA target, an aliquot of the tested **Target** or Mill-Q water (as blank sample) was added to the **HMB**-GO switch. The final concentration of GO, Hoechst

dyes and **MB** was 11.25 μg/mL, 1 μg/mL, and 0.1 μM, respectively. The mixture was vortexed to mix all the reagents and then incubated for 10 min at RT and after that an aliquot of 0.1 mL mixture was placed in the black 384 well microplate to measure the fluorescence intensity. For the fluorescent "off" detection of Exo III, first, an aliquot of 0.6 μM **Target** was added to the **HMB**-GO switch and incubated at RT for 10 min. Then an aliquot of Exo III or Mill-Q water (as blank sample) or other enzyme inputs (as control samples) for the selectivity experiment was added to the above solution, and the mixture was allowed to incubate for another 30 min RT, while the emission spectra were recorded in the same way.

Data Analysis. The GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA) was employed to perform the data processing. Each sample was repeated in triplicate, and data were averaged. The DNA structure used in this study was predicted by the popular structure-prediction program Mfold (http://mfold.rna.albany.edu/?q=mfold/DNA-Folding-Form).

ASSOCIATED CONTENT

Supporting Information

The additional experimental data in this study. This material is available free of charge via the Internet at http://pubs.acs.org/.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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