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Sequence-Specific Detection of Short-Length DNA via Template-Dependent Surface-Hybridization Events

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Short-length DNA and RNA, such as mature small RNA, which contains only 17−25 nucleotides, are always a problem in hybridization-based detection assays. In this paper, we report a proof-of-concept for a new short-length DNA detection technology which encompasses a design strategy whereby capture and reporter probes that do not hybridize to each other at 20 °C can be made to anneal to each other in the presence of a template via the formation of a stable three-component complex. The thermodynamics of this magnetic bead-based DNA biosensor was then investigated in detail by monitoring chemiluminescence (CL) changes in the absence and presence of targets over a temperature profile. The data show that this new biosensor offers the possibility of highly selective and sensitive detection of the short-length target DNA. In view of these advantages, this template-dependent surface-hybridization assay, as a new CL strategy, might create a universal technology for developing simple biosensors in sensitive and selective detection of short-length DNA and RNA.

The availability of fast and reliable sequence-specific DNA and RNA detection assays has grown tremendously in the past few years, fueled by the significant advantages that they provide in various fields such as the diagnosis of infectious and genetic diseases and environmental and forensic applications. Many alternative DNA and RNA detection assays have been developed, for example, using optical (fluorescent labels,[1−11] including molecular beacons[4–8] and polymers,[9–11] chemiluminescent (CL) labels,[12−15] colorimetric nanoparticles,[16] etc.) or electrochemical (small redox molecules,[17] redox polymers,[18,19] redox enzymes,[20,21] silicon nanowires,[22] liposomes,[23−25] etc.) techniques. Among these techniques, the three-component strategy, which involves a pair of DNA probes (capture and labeled reporter probes) that flank the target DNA sequence, has been widely employed. Such dual hybridization processes significantly improve the signal-to-noise ratio; however, this technique is mostly limited to the detection of target sequences longer than 24 bases, otherwise the binding efficiency may be low and double-stranded structures may not withstand the harsh wash conditions that are applied to remove nonspecific bindings.

Short-length DNA and RNA, such as mature small RNA,[26,27] which contains only 17−25 nucleotides, are always a cause for concern in hybridization-based detection assays. These short-length entities present great challenges in studies using conventional three-component strategies, because their inherent small size provides only short sequences for appending labels or for designing probes; probe ligation is sometimes used to stabilize the binding of the target short-length RNA to the capture

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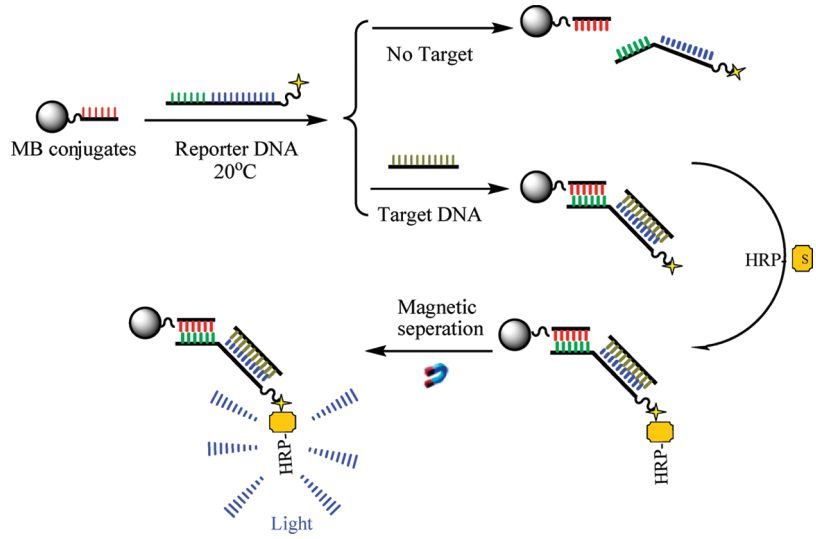
Scheme 1. Schematic Representation of Sequence-Specific Detection of Short-Length DNA via Template-Dependent Surface-Hybridization Events



probe.[28,29] Here, we report a proof-of-concept for a new short-length DNA detection technology, which encompasses a design strategy whereby capture and reporter probes that do not hybridize to each other at 20 °C can be made to anneal to each other in the presence of a template via the formation of a ternary complex. Specifically, the reporter probe has a 3′-biotin-labeled eight-nucleotide tail sequence that is complementary to a surface-tethered capture DNA with a predesigned low melting temperature (usually <20 °C) and an additional tethered nucleic acid domain complementary to the target DNA. In the absence of target DNA, the reporter DNA does not associate with the surface-tethered capture strands because the complementary fragment is too short to promote effective annealing. When target DNA binds to the reporter DNA, allowing the reporter DNA to hybridize with the surface-tethered capture DNA, the biotin label then attaches to the surface, reacts with streptavidin−horseradish peroxidase (HRP), and is detected by an enhanced CL HRP substrate. Because magnetic beads are easy to handle with a magnet, they are employed as the solid surface for our assay, thus allowing relatively easy optimization of operational and separation procedures.

EXPERIMENTAL SECTION

Apparatus. CL measurements were carried out using a PC-controlled Fluoroskan Ascent FL (Thermo Electron Corporation, Waltham, MA). Absorbances were determined using a Hitachi U-2900 spectrophotometer (Hitachi, Tokyo, Japan).

Reagents. All chemicals were of analytical reagent grade and were used as received. Water was purified using a Millipore Milli-XQ system (Millipore, Bedford, MA). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was purchased from Sigma (St. Louis, MO). The carboxyl-terminated magnetic beads (1.5 μm, 20 mg/mL) were purchased from Polysciences, Inc. (Warrington, PA), and streptavidin−HRP was bought from Sigma-Aldrich. The HRP substrate kit was purchased from Millipore. Oligonucleotides were acquired from Invitrogen Biotechnology Co., Ltd. (Shanghai, China), and all the capture probes contained a 10 nucleotide adenine spacer at the 3′ end, which provides optimal immobilization and hybridization efficiency.

Preparation of Magnetic Bead−Capture Probe Conjugates. First, 24 μL of the carboxyl-terminated magnetic beads were transferred to a 1.5 mL centrifuge tube, washed three times with 150 μL of 0.1 M imidazole buffer (pH 7.0), and then activated in 300 μL of 0.1 M imidazole buffer containing 0.03 M EDC for 20 min with gentle shaking. Second, 1200 pmol of capture probes were added to the tube and incubated for 1 h at 37 °C with continuous shaking. The resultant magnetic bead−capture probe conjugates were then magnetically separated, washed three times with 300 μL of wash buffer (7 mM Tris−HCl, pH 8.0, 0.17 M NaCl, 0.05% Tween 20), and resuspended in 240 μL of buffer A (20 mM Tri−HCl, pH 8.0, 0.5 M NaCl).

Assay Procedures on Magnetic Beads. In a typical experiment, 20 μL of the magnetic bead−capture probe conjugates were first mixed with the desired amount of the target DNA, or mismatched DNA, and reporter DNA in 100 μL of buffer A. The hybridization reaction was allowed to proceed for 1 h at 20 °C. The hybridized bead conjugates were then washed three times with wash buffer; 100 μL of 66.7 pg/mL of streptavidin−HRP were added, and the mixture was incubated for 1 h at 37 °C. The resultant magnetic bead−DNA−HRP conjugates were washed three times with 150 μL of wash buffer, and CL signals on the surface of the magnetic beads were detected directly with 100 μL of commercial CL HRP substrate.

RESULTS AND DISCUSSION

This magnetic bead-based DNA technique demonstrated a turn-on architecture in response to a target DNA (Scheme 1). In the absence of a target A, only an insignificant CL signal was observed (Figure 1), showing that the hybridization efficiency of the biotin-labeled reporter probes with immobilized capture DNA was extremely low. After reaction with 100 fmol of target A, a strong CL emission, with a 16-fold signal gain compared with the signal for the blank, appeared (Figure 1).

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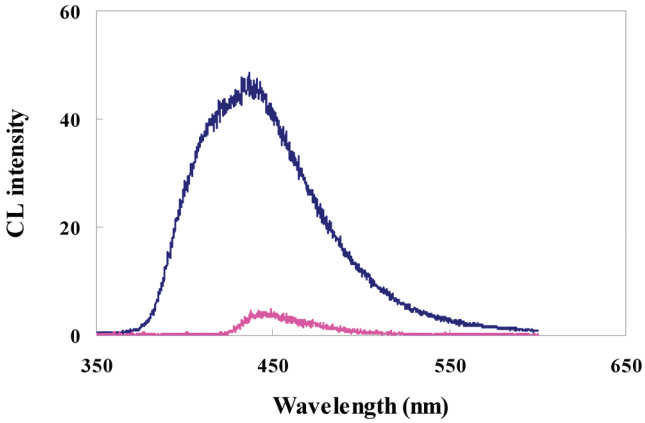

Figure 1. CL spectra before (red line) and after (blue line) reaction with 100 fmol target A. Experimental conditions: magnetic beads, 40 μg; capture A and reporter A, 100 pmol and 2 pmol, respectively; streptavidin−HRP, 6.67 pg; reaction temperature, 20 °C. The detection procedure was carried out as described in the Experimental Section.

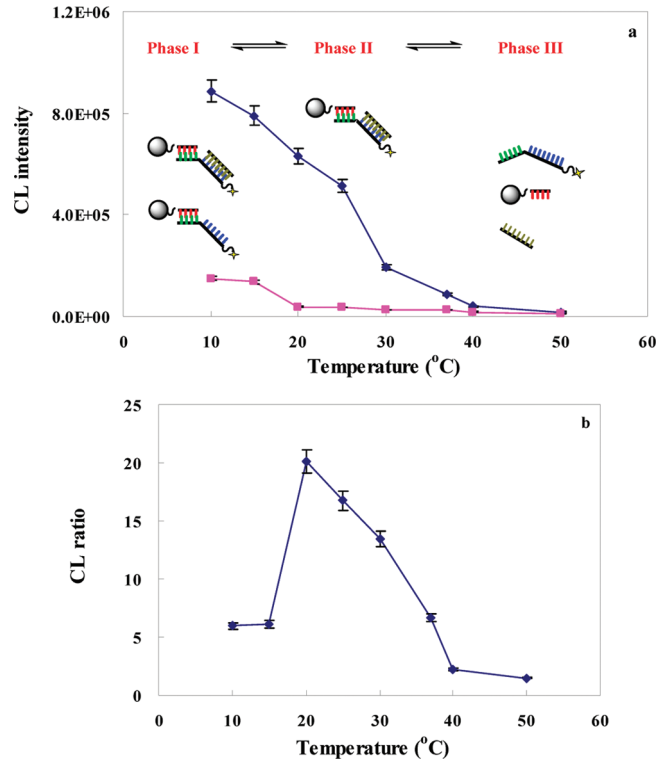

Figure 2. Thermodynamic studies of CL biosensor. (a) Variation of the CL intensity of the biosensor in the absence (red line) and presence (blue line) of target A over a temperature profile. The structures shown demonstrate the conformational changes of surface-attached probes during the phase transition. (b) CL signal-to-noise ratio vs the reaction temperature. Experimental conditions: magnetic beads, 40 μg; capture A and reporter A, 100 pmol and 2 pmol, respectively; and streptavidin−HRP, 6.67 pg. The detection procedure was carried out as described in the Experimental Section.

While duplex stability is affected by several factors, such as sequence composition, secondary structure and strand length, ionic strength, and temperature,[30] two essential parameters contribute the major forces for DNA duplex stability: base pairing between complementary strands and stacking between adjacent bases.[31] Base stacking is the dipole-induced dipole−dipole interaction between the planar aromatic bases in two contiguous nucleotides. When two contiguous tandem sequences (here capture and target DNA) are annealed on a longer strand (here reporter DNA), coaxial stacking at the nick brings additional stability and efficiency to the hybridization.[32,33] The melting temperature of a capture DNA and the template reporter DNA is, thus, increased by several degrees in the presence of an adjacent target DNA, leading to an increase in the stability of the duplex formed. Experiments performed by Khrapko et al.[34] have proved that continuous stacking hybridization of two duplexes results in their mutual stabilization and that the dissociation temperature of the duplex is increased.

The thermodynamics of this magnetic bead-based DNA biosensor was then investigated by monitoring CL changes in the absence and presence of targets over a temperature profile, as shown in Figure 2a. In the absence of targets, the biosensor is partly held in the "hybridized" state with a slightly high CL at low temperatures such as 10 °C (Phase I). The CL intensity gradually decreases with increasing temperature, and then, at temperatures above 20 °C, thermal destabilization of the duplex structure occurs, with a very low CL emission, which means that the capture and reporter DNA are almost completely separated. In the presence of target DNA, the biosensor exhibits similar behavior, but coaxial stacking greatly improves the thermal stability of the DNA double helix. Initially, the biosensor has a very high CL intensity at low temperatures as result of the formation of a stable three-component complex (Phase I). The CL intensity slowly decreases with increasing temperature, and a maximum CL ratio is reached at 20 °C (Phase II, Figure 2b). At temperatures above 40 °C, the biosensor is in the "open" state, in which the three-component complex is also completely dehybridized, which leads to a low CL intensity (Phase III).

Overall, in the absence of target DNA, the strand length is only eight base pairings; as a result, the DNA double helix is not stable and, thus, the CL intensity is very low, as shown in Figure 1. When target DNA binds to complementary reporter DNA, coaxial stacking at the nick brings additional stability and efficiency to the hybridization as a result of increased association and decreased dissociation.[35,36] Thus, the cumulative base pairing and stacking between adjacent bases stabilize the formation of a three-component complex, leading to a strong CL emission.

A comparison between another two capture probes, containing four and 12 base pairings, was also carried out in parallel (Figure 3). The data show that, even in the presence of target DNA, the cumulative base pairing and stacking still could not stabilize hybridization between reporter B and capture B, leading to a very low CL intensity. In contrast, capture C can stably hybridize with the reporter C with a high CL intensity, even in the absence of

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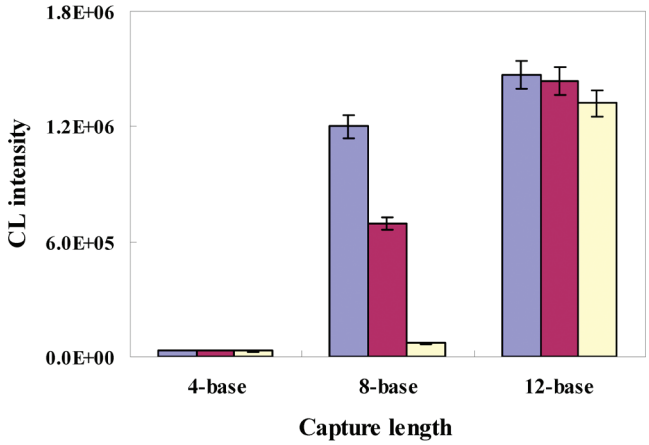

Figure 3. CL intensity vs different capture DNA and reporter DNA. Experimental conditions: magnetic beads, 40 μg; capture probe and biotinylated reporter DNA, 100 pmol and 2 pmol (4-base means capture B/reporter B, 8-base means capture A/reporter A, 12-base means capture C/reporter C), respectively; 500 fmol (blue), 100 fmol (red), and 0 fmol (yellow) target A; streptavidin−HRP, 6.67 pg; reaction temperature, 20 °C. The detection procedure was carried out as described in the Experimental Section.

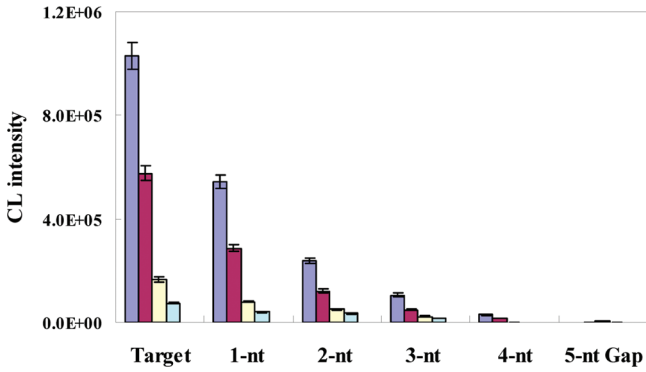

Figure 4. CL intensity vs different target DNAs. Experimental conditions: magnetic beads, 40 μg; capture A and reporter A, 100 pmol and 2 pmol, respectively; 500 fmol (blue), 100 fmol (red), 20 fmol (yellow), and 2 fmol (green) target; streptavidin−HRP, 6.67 pg; and reaction temperature, 20 °C. The detection procedure was carried out as described in the Experimental Section.

target DNA, and thus, coaxial stacking in the presence of target DNA only causes a slight increase in CL intensity. This suggests that the length of the capture probe also plays a key role in this new sequence-specific detection of short-length DNA via template-dependent surface-hybridization events.

We also tested the detection when the gap between the formed helixes was one, two, three, four, and five nucleotides; the results are shown in Figure 4. The CL intensity was found to decrease gradually with increasing number of gap nucleotides. The hybridization was destabilized because a 1, 2, 3, 4, or 5 nucleotide gap was generated between the capture and target DNA. This interruption in the coaxial stacking results in destabilization of the three-component hybridization, thus decreasing the CL signal. These gap experiments further confirm that the origin of the enhancement of the thermodynamic stability of adjacent helixes in the presence of target DNA could result from coaxial stacking.[37]

It was, therefore, reasoned that recognition of target DNA by the reporter probe was a prerequisite for efficiently facilitating

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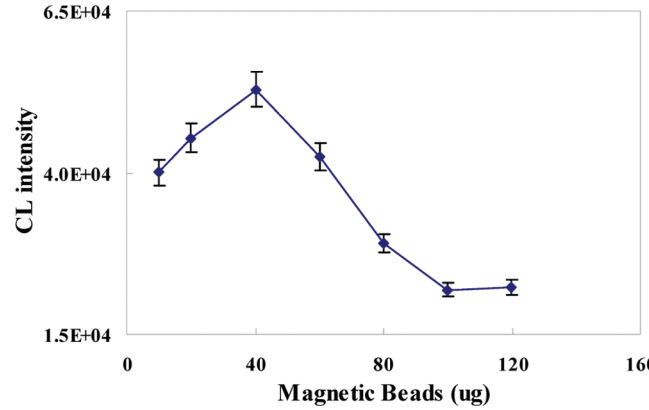

Figure 5. CL intensity vs the amount of magnetic beads. Experimental conditions: capture A, target A, and reporter A, 20 pmol, 0.1 pmol, and 4 pmol, respectively; streptavidin−HRP, 20 pg; and reaction temperature, 20 °C. The detection procedure was carried out as described in the Experimental Section.

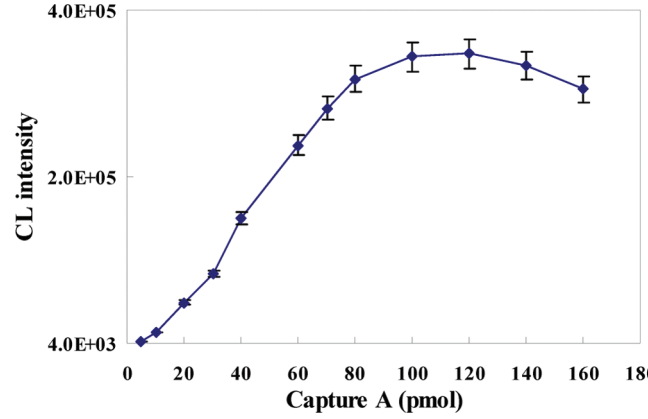

Figure 6. CL intensity vs the amount of capture A. Experimental conditions: magnetic beads, 40 μg; target A and reporter A, 0.1 pmol and 4 pmol, respectively; streptavidin−HRP, 20 pg; and reaction temperature, 20 °C. The detection procedure was carried out as described in the Experimental Section.

hybridization of the reporter probe with the magnetic bead-attached capture strand. It was also observed that there was no appreciable CL signal in the control experiments with magnetic bead-attached noncomplementary capture X, suggesting that surface hybridization was essential in signaling target DNA. Actually, the recognition of target DNA by the reporter probe and hybridization of the reporter probe with immobilized strands represent two crucial steps in the template-dependent surface-hybridization assay, offering the possibility of highly selective detection of the short-length target DNA.

Optimization of Reaction Parameters. Several parameters, including the amounts of magnetic beads, capture probe, biotinylated reporter sequence, and streptavidin−HRP, were investigated systematically to establish optimal conditions for CL detection of the HBV DNA.

As shown in Figure 5, the CL intensity increased with increasing amounts of magnetic beads, reached a maximum at 40 μg, and then decreased, possibly due to CL quenching by an inner filter effect from an excess of black magnetic beads. Hence, 40 μg of magnetic beads were used in subsequent work.

The effects of different amounts of capture A on CL intensity are shown in Figure 6. CL intensity was observed to increase over the range 5−100 pmol of capture A and then remain almost

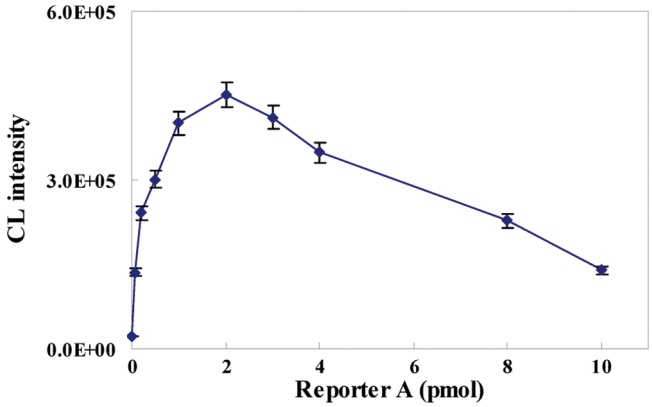

Figure 7. CL intensity vs the amount of reporter A. Experimental conditions: magnetic beads, 40 μg; capture A and target A, 100 pmol and 0.1 pmol, respectively; streptavidin−HRP, 20 pg; and reaction temperature, 20 °C. The detection procedure was carried out as described in the Experimental Section.

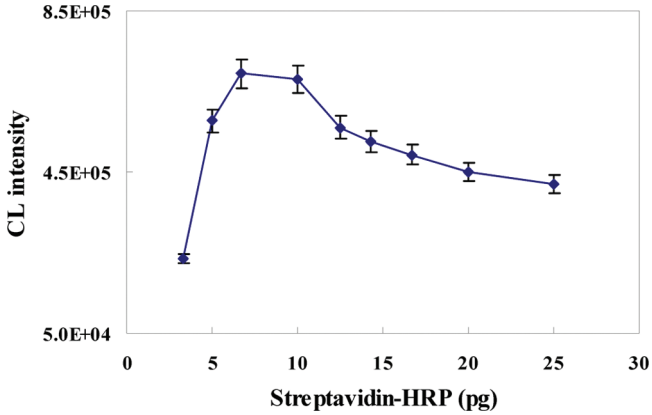

Figure 8. CL intensity vs the amount of streptavidin−HRP. Experimental conditions: magnetic beads, 40 μg; capture A, target A, and reporter A, 100 pmol, 0.1 pmol, and 2 pmol, respectively; and reaction temperature, 20 °C. The detection procedure was carried out as described in the Experimental Section.

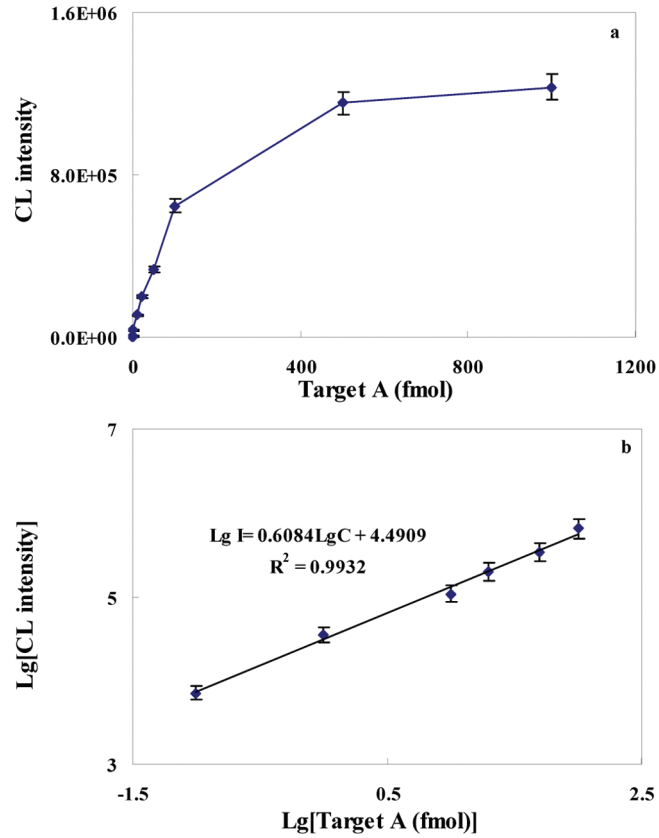

Figure 9. Calibration plot (a) and log−log calibration data (b) for target A. Experimental conditions: magnetic beads, 40 μg; capture A and reporter A, 100 pmol and 2 pmol, respectively; streptavidin−HRP, 6.67 pg; and reaction temperature, 20 °C. The detection procedure was carried out as described in the Experimental Section.

constant, possibly because the binding sites on the magnetic beads were progressively saturated, and thus, no more three-component complex was formed on the magnetic beads. Thus, 100 pmol of capture A was selected as the amount used in subsequent experiments.

The effects of the amounts of streptavidin−HRP and biotinylated reporter sequence were subsequently examined and opti-

Table 1. DNA Sequences Used

name	abbreviation	sequence
capture DNA with 8 base pairings	capture A	5′-CACACAGAAAAAAAAAAA-NH_2-3′
capture DNA with 4 base pairings	capture B	5′-CACAAAAAAAAAAA-NH_2-3′
capture DNA with 12 base pairings	capture C	5′-CACACAGAGAGAAAAAAAAAAA-NH_2-3′
reporter DNA	reporter A	5′-TCTGTGTGCCCCAACTCCTCCCAAAAAA-biotin-3′
reporter DNA with 4 base pairings	reporter B	5′-TGTGCCCCAACTCCTCCCAAAAAA-biotin-3′
reporter DNA with 12 base pairings	reporter C	5′-TCTCTCTGTGTGCCCCAACTCCTCCCAAAAAA-biotin-3′
target DNA A	target A	5′-TGGGAGGAGTTGGGG-3′
target DNA A with a 1-nt gap	1-nt Gap	5′-TGGGAGGAGTTGGG-3′
target DNA A with a 2-nt gap	2-nt Gap	5′-TGGGAGGAGTTGG-3′
target DNA A with a 3-nt gap	3-nt Gap	5′-TGGGAGGAGTTG-3′
target DNA A with a 4-nt gap	4-nt Gap	5′-TGGGAGGAGTT-3′
target DNA A with a 5-nt gap	5-nt Gap	5′-TGGGAGGAGT-3′
C−C mismatch	C−C	5′-TGGGAGGAGTTGGCG-3′
C−T mismatch	C−T	5′-TGGGAGGAGTTGGTG-3′
C−A mismatch	C−A	5′-TGGGAGGAGTTGGAG-3′
two-base mismatch	two base	5′-TGGGAGGAGTTGAAG-3′
noncomplementary strands	non	5′-ACCTGGGTGGGAAGTAATTTGGAAGACCCAGCATC-3′
capture DNA X	capture X	5′-TTGATCAAAAAAAAAA-NH_2-3′
target DNA D	target D	5′-TGGGAGGAGTTGGGGGAGGA-3′
target DNA E	target E	5′-GATTAGGTTAAAGGT-3′
reporter DNA E	reporter E	5′-TCTGTGTGACCTTTAACCTAATCAAAAA-biotin-3′

Table 2. Comparison of Sensitivity for Different DNA Assay Methods

analytical method	label	no. of target bases	detection limit
electrochemical detection	HRP	38	20 pM[40]
electrochemical detection	polypyrrole	25	10 pM[18]
electrochemical detection	liposome	27	1.2 pM[23]
fluorescence imaging	Cy5	30	1 pM[41]
fluorescence imaging	counting silica NPs	27	0.8 fM[42]
fiber-optic sensor	FL dyes	21	100 pM[43]
biocatalytic fluorescence	ecarin-biotin and dyes	27	3 pM[44]
colorimetric detection	Au nanoparticles (NPs)	30	50 pM[45]
colorimetric detection	Au NPs	24−80	10 pM[46]
colorimetric detection	Au NPs and fluorous	27	10 pM[47]
raman	Au NPs and dyes	30	20 fM[48]
light scattering	Au NPs	30	0.1 pM[49]
SPR	label free	21	0.677 pM[50]
SPR	Au NPs	30	1 pM[51]
ICPMS	Au NPs	40	0.2 pM[52]
QCM	Au NPs	24	32 pM[53]
CL detection	Au NPs	30	1 pM[54]
CL detection	DNAzyme	36	1 nM[55]
CL detection	Pt NPs	27	10 pM[56]
CL biosensor (this work)	HRP	15−20	0.1 pM

mized. The CL signal intensity increased with increasing amounts of reporter A in the range 0.01−2 pmol and then slowly decreased in the range 3−10 pmol (Figure 7). We attribute the decrease in the number of duplexes formed on the magnetic beads to an excess of free reporter sequences in the solution competitively capturing target DNA; the amount of target DNA captured on the surface of the magnetic beads, therefore, decreased as a result. The CL intensity increased in the range 3.3−6.67 pg of streptavidin−HRP and then decreased (Figure 8). Thus, 6.67 pg of streptavidin−HRP and 2 pmol of reporter A were selected as the amounts for use in further studies.

Quantification of the Target DNA. The quantitative behavior of the method under the optimized experimental conditions was assessed by monitoring the dependence of the CL intensity on the target DNA amount. A calibration graph in the range 0.1−100 fmol showed an approximately linear correlation ($R^2 = 0.9932$, Figure 9b) between the amount of target A and the CL intensity (represented by $\log I = 0.6084 \log C + 4.4909$, where I is the CL intensity and C is the amount of target A). At amounts higher than 400 fmol, the CL intensity leveled off and deviated from the calibration curve (Figure 9a). A readily achieved detection limit was found to be 0.01 fmol, which is equal to or better than most previous methods for short DNA sequences (Table 2).

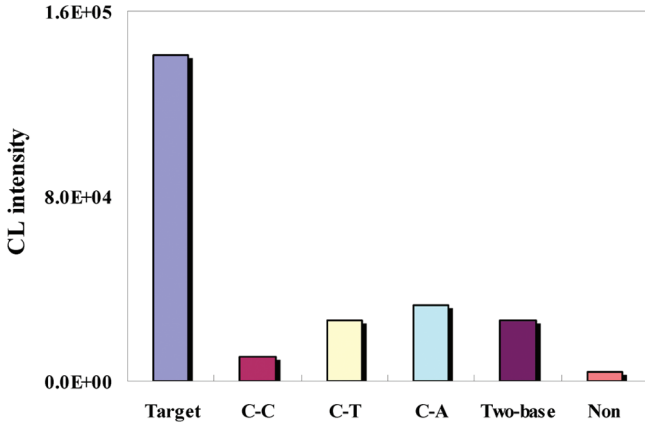

Figure 10. CL intensity vs target A/mismatch DNA. Experimental conditions: magnetic beads, 40 μg; capture A and reporter A, 100 pmol and 2 pmol, respectively; streptavidin−HRP, 6.67 pg; and reaction temperature, 20 °C. The detection procedure was carried out as described in the Experimental Section.

Discrimination of cDNA Sequences from Mismatched Sequences. To validate the sequence specificity of the detection system, the proposed assay was examined by detecting the CL response of perfectly complementary targets, noncomplementary strands, C−C, C−T, and C−A one-base mismatched strands, and two-base mismatched strands (Table 1), all at an amount of 100 fmol. The ratio of the CL intensities of the six sequences was 100: 3:7.5:18.5:23.1:18.4, respectively (Figure 10). When mismatch is placed in the center of target, mismatch discrimination is less distinguished. As reported by Allawi et al.,[38,39] the efficiency of mismatch recognition depends on the type of mismatch and its nearest-neighbor base pairs, and C−A mismatches are more stable than C−C and C−T mismatches. Note also that the CL signal was greatly reduced when the target lost several nucleotides (Figure 4). Hence, good selectivity can be obtained using this new CL DNA biosensor.

In addition to detection sensitivity and sequence specificity, this magnetic bead-based DNA approach also shows good generality for target DNA with varied base numbers. As shown in Figures 11 and 12, by further increasing the target length from 15-mer to 20-mer or changing the G−C content in the target, a calibration graph in the range 0.1−100 fmol was also obtained.

CONCLUSION

We developed a magnetic bead-based sensing platform for simple detection of short-length DNA, on the basis of a template-

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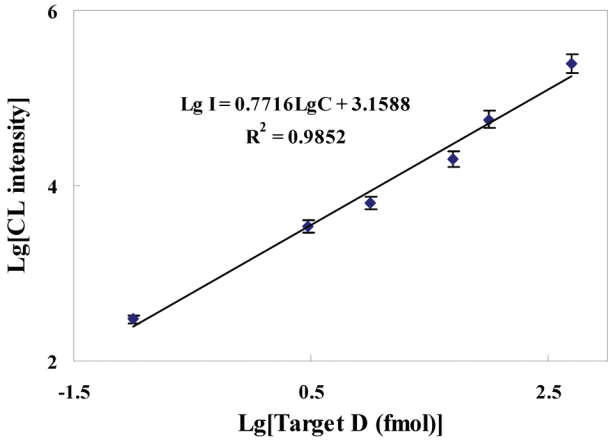

Figure 11. Log−log calibration data for the 20-mer target D. Experimental conditions: magnetic beads, 40 μg; capture A and reporter A, 100 pmol and 2 pmol, respectively; streptavidin−HRP, 6.67 pg; and reaction temperature, 20 °C. The detection procedure was carried out as described in the Experimental Section.

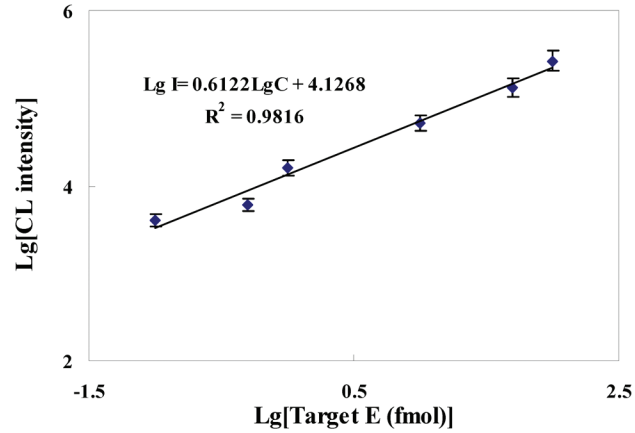

Figure 12. Log−log calibration data for the 15-mer target E. Experimental conditions: magnetic beads, 40 μg; capture A and reporter E, 100 pmol and 2 pmol, respectively; streptavidin−HRP, 6.67 pg; and reaction temperature, 20 °C. The detection procedure was carried out as described in the Experimental Section.

dependent surface-hybridization assay. The method required the recognition of the target by a reporter probe to promote their efficient annealing with magnetic bead-attached capture DNA. Moreover, with different reporter DNAs with different labels, such as biotin and FITC, each specifically designed for an individual target gene, the strategy could be implemented for multiplex detection of multiple genes in a single sample. In view of these advantages, the template-dependent surface-hybridization assay, as a new CL strategy, could create a universal technology for developing simple biosensors for sensitive and selective detection of short-length DNA and RNA.

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