

Proximity Hybridization-Triggered Signal Switch for Homogeneous Chemiluminescent Bioanalysis

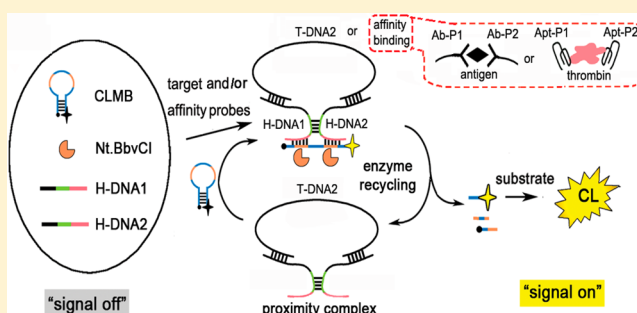
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S Supporting Information

ABSTRACT: A proximity hybridization-triggered signal switch was presented for convenient homogeneous chemiluminescent detection of a wide range of affinity target biomolecules, such as oligonucleotides, protein biomarkers, and aptamer-recognized targets. The presence of the target promoted the formation of a proximate complex via the proximity hybridization of two help DNA strands or the DNA strands labeled to affinity ligands, which subsequently unfolded the self-reporting molecular beacon to switch on the chemiluminescence signal. The response could be further amplified with an in situ enzymatic recycling strategy for highly sensitive chemiluminescence detection. By using an antibody as the affinity ligand, this simple protocol could sensitively detect protein biomarker in a concentration range of 6 orders of magnitude with a detection limit down to 80 fM. With the use of an aptamer as the affinity ligand, a method for homogeneous chemiluminescent detection of thrombin was proposed. The one-step and wash-free assay showed good selectivity and required only 1 μ L of sample. Its high sensitivity, acceptable accuracy, and satisfactory versatility of analytes led to various applications in bioanalysis.



The rapid advances in DNA nanotechnology offer great opportunities in various fields including molecular electronics, computation, imaging, and disease diagnostics.^{1,2} Through target-induced DNA assembly, some bioanalysis strategies have recently been designed for detection of proteins.^{3–6} This assembly induces the displacement of a dye-labeled DNA strand by a competing DNA^{4,5} or quencher-labeled DNA strand by the formed DNA three-way junction (TWJ)⁶ to separate the fluorescence dye from its quencher. Similar to the TWJ protocol, a DNA assembly strategy was earlier designed to develop an aptamer-based electrochemical proximity assay method for quantitation of proteins.⁷ These assemblies rely on the simultaneous recognition of target protein by two DNA-conjugated affinity ligands, which are generally called as affinity ligand- or antibody-based proximity assays. Among these DNA-assisted assays, the proximity ligation assay (PLA)^{8–11} is mostly used by converting the target event to amplifiable tag sequences for subsequent real-time PCR quantification^{9,12} or localized rolling-circle amplification to obtain high sensitivity.¹³

Generally, the DNA-assisted bioassays can be performed homogeneously without the requirement of time-consuming separation and washing steps, which can well overcome the limitations of the traditional protein assays. However, this protocol still face several challenges, including the simplification

of procedure for designing a miniaturizable and automatable detection system, the improvement of analytical performance, and more importantly, the extension of analyte ranges. This work designed a proximity hybridization-triggered signal switch by using a proximity complex to open a self-reporting molecular beacon (CLMB). This switch could be used for a one-step homogeneous chemiluminescent proximity assay (CLPA) of different kinds of target biomolecules with corresponding affinity ligands. A signal amplification strategy was further coupled with this switch by using nicking endonuclease for in situ recycling of the proximity complex. The CLPA protocol showed excellent detection versatility for sensitively detecting a wide range of targets such as oligonucleotides, proteins, or aptamer-recognized targets.

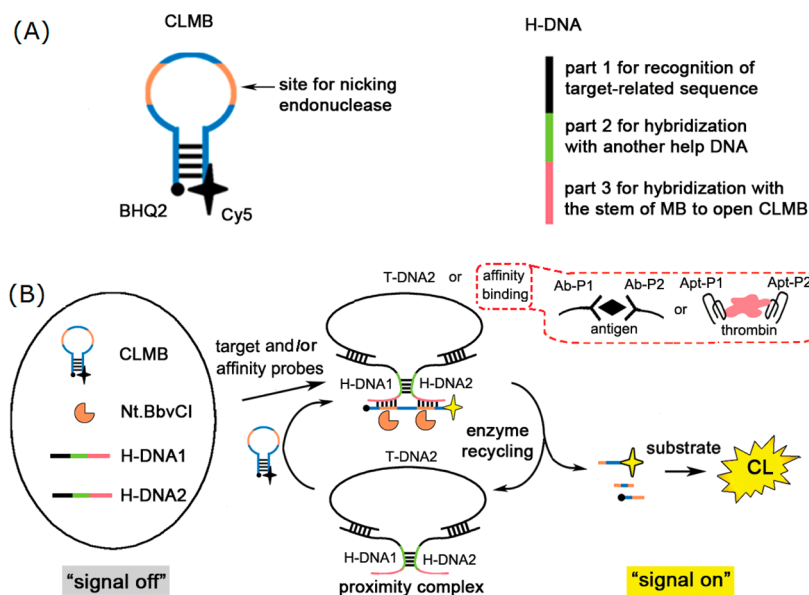
EXPERIMENTAL SECTION

Materials and Reagents. The oligonucleotides were synthesized and purified by Shanghai Sangon Biotechnology Co. Ltd. (China). Their sequences are listed in Table S1, Supporting Information. Carcinoembryonic antigen (CEA) and anti-CEA antibody (anti-CEA, mouse monoclonal antibodies,

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Scheme 1. General Principle of the Homogeneous Chemiluminescent Proximity Assay^a

^a(A) Structure of CLMB and H-DNA; (B) schematic diagram of the CLPA.

clone nos. Z-2011 and Z-2012) were purchased from Beijing Keybiotech Co. Ltd. (China). Human α thrombin was obtained from Tideradar Beijing Technology Co. Ltd. (China). Standard solutions of β -hCG and CA125 were from commercial β -hCG and CA125 CL ELISA kits supplied by Tigsun Diagnostics Co. Ltd. and Autobio Diagnostics Co. Ltd. (China), respectively. Sulfo succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) was supplied by Heowns Biochem LLC (China). Nt. BbvCI as well as 10X NEB buffer (pH 7.9 at 25 °C) was obtained from New England BioLabs Inc. (USA). Bis(2,4,6-trichlorophenyl) oxalate (TCPO) was obtained from Tokyo Chemical Industry Co. Ltd. (Japan), and dithiothreitol (DTT) was from Shanghai Sangon Biotechnology Co. Ltd. (China). All other reagents were of analytical grade and used without further purification. Ultrapure water obtained from a Millipore water purification system (≥ 18 M Ω , Milli-Q, Millipore) was used in all assays. TE buffer (10 mM, containing 1 mM EDTA and 0.3 M NaCl, pH 7.9) was used as the stock solution for oligonucleotides. PBS1 (55 mM, containing 150 mM NaCl and 20 mM EDTA, pH 7.2) and PBS2 (55 mM, containing 150 mM NaCl and 5 mM EDTA, pH 7.2) were used to prepare DNA-labeled antibodies. Tris-HCl buffer (20 mM, containing 8.5% glycerin, 5 mM K⁺, 100 mM Na⁺, 1 mM Ca²⁺, and 1 mM Mg²⁺, pH 7.4) was the stock solution for thrombin and its aptamer. NEB buffer (1X) was used for the CLPA. The clinical serum samples were from Jiangsu Cancer Hospital. The electrochemiluminescent (ECL) immunoassay reagent kits for reference detection were provided by Roche Diagnostics GmbH (Germany).

Apparatus. An IFFM-E luminescent analyzer (Remax, China) was used to collect the CL signal. A F97XP fluorospectrophotometer (Lengguang Tech., China) was used to record the FL signal. The gel electrophoresis was performed on the DYCP-31BN electrophoresis analyzer (Liuyi Instrument Company, China) and imaged on a Biorad ChemDoc XRS (Bio-Rad, USA).

Preparation of DNA-Labeled Antibody. The DNA-labeled antibody (Ab-P) was prepared by a modified coupling procedure.¹³ Anti-CEA (2 mg mL⁻¹) was first reacted with a

20-fold molar excess of SMCC in PBS1 for 2 h at room temperature. The obtained anti-CEA-SMCC was purified by ultrafiltration using a 100 KD millipore (10 000 r, 10 min). In parallel, 12 μ L of 100 μ M thiolated oligonucleotide (DNA1 or DNA2) was reduced with 16 μ L of 100 mM DTT in PBS1 at 37 °C for 1 h. The reduced oligonucleotide was purified by ultrafiltration using a 10 KD millipore (10 000 r, 10 min). Then, the obtained anti-CEA-SMCC and reduced oligonucleotide were mixed in PBS2 to incubate overnight at 4 °C, and the unreacted anti-CEA and DNA were removed by ultrafiltration using a 100 KD millipore (10 000 r, 10 min) for several times, and the obtained Ab-P was collected in 50 μ L of PBS2.

Experimental Confirmation of CL Signal Switch and PAGE Analysis. CL intensity was obtained at gain 2 and 800 V in 8.5 mM TCPO and 25 mM H₂O₂ as CL substrate, substrate and 1 μ M Cy5-DNA, substrate and 1 μ M Cy5-DNA and 1 μ M BHQ2-DNA, and substrate and 1 μ M CLMB, respectively. A 5% native polyacrylamide gel was prepared using 1X TBE buffer. The loading sample was the mixture of 14 μ L of DNA sample, 3 μ L of 6X loading buffer, and 3 μ L of UltraPower™ dye. Before injection into the polyacrylamide hydrogel, the loading sample was placed for 3 min. The gel electrophoresis was run at 75 V for 40 min. The resulting board was illuminated with UV light and photographed with a Molecular Imager Gel Doc XR.

Oligonucleotide-Based CLPA. The CLPA for DNA was performed by mixing 1 μ L of an 80 base DNA strand (T-DNA2) at different concentrations with 29 μ L of CL substrate solution containing 1 μ M CLMB, 1 μ M H-DNA1, 1 μ M H-DNA2, and 2 U of Nt. BbvCI and incubating the mixture at 37 °C for 30 min. Immediately, 20 μ L of CL substrate containing 8.5 mM TCPO and 25 mM H₂O₂ was added to record the CL intensity at gain 2 and 850 V.

Antibody-Based CLPA. Antibody-based CLPA was carried out by mixing 1 μ L of standard CEA solution or serum sample with 29 μ L of CLPA substrate solution, followed by a 30 min incubation at 37 °C. Immediately, 20 μ L of CL substrate containing 8.5 mM TCPO and 25 mM H₂O₂ was added to

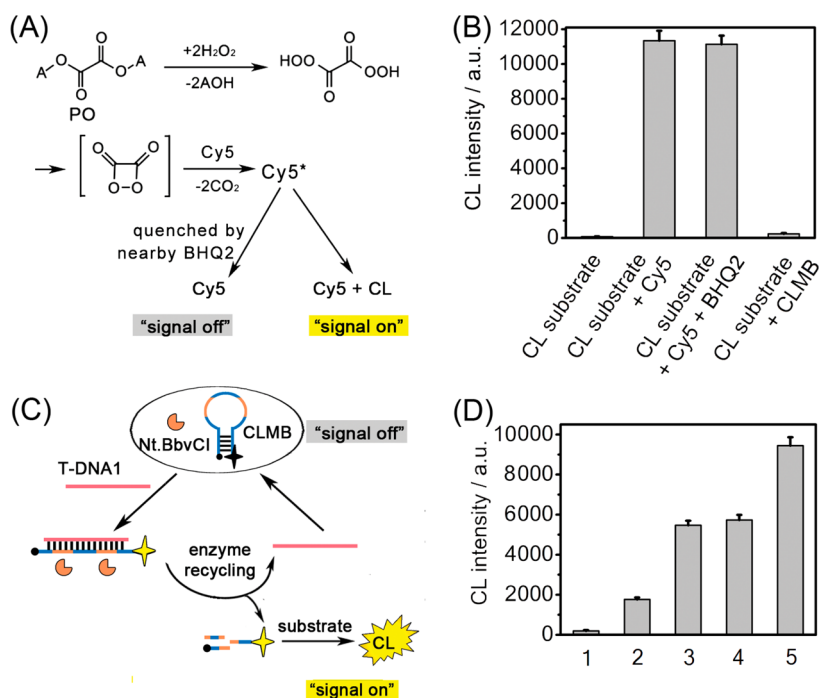


Figure 1. (A) Principle of PO-based CL reaction and CL quenching. (B) Experimental confirmation of CL emission and quenching. (C) Principle of hybridization-triggered signal switch. (D) CL intensity of (1) 1 μM CLMB, (2) (1) + 0.1 μM T-DNA1, (3) (2) + 2 U of Nt. BbvCI, (4) (1) + 0.5 μM T-DNA1, and (5) (4) + 2 U of Nt. BbvCI after incubation at 37 $^\circ\text{C}$ for 30 min in 1X NEB buffer.

record the CL intensity at gain 2 and 950 V. The CLPA substrate solution for protein detection was prepared by mixing 1 μL of equivalent volume mixture of as-prepared Ab-P1, Ab-P2, H-DNA1 (10 μM), and H-DNA2 (10 μM), 3 μL of CLMB (10 μM) with 0.2 μL of Nt. BbvCI (10000 U mL^{-1}) in 1X NEB buffer.

Aptamer-Based CLPA. Aptamer-based CLPA was performed by mixing 1 μL of thrombin at different concentrations with 29 μL of aptamer-based CLPA substrate solution containing 1 μM CLMB, 0.67 μM Apt-P1, 3.33 μM Apt-P2, 1 μM H-DNA1, 1 μM H-DNA2, and 2 U of Nt. BbvCI. After the mixture was incubated at 37 $^\circ\text{C}$ for 40 min, 20 μL of CL substrate containing 8.5 mM TCPO and 25 mM H_2O_2 was added to record the CL intensity at gain 2 and 900 V.

RESULTS AND DISCUSSION

Design of CL Signal Switch. The CLPA used two help DNA strands (H-DNA1 and H-DNA2), a CLMB (Table S1, Supporting Information), and a nicking enzyme Nt. BbvCI to perform the target recognition and signal amplification (Scheme 1). The CLMB probe was designed to contain 2 nicking endonuclease sites and 18 bases that were complementary to part 3 of two H-DNAs. The simultaneous recognition of part 1 in two H-DNAs to target oligonucleotide or target/affinity probes brought part 2 of two H-DNAs in sufficient proximity to undergo hybridization and form a proximity complex, which led to a sequence combining part 3 of two H-DNAs to subsequently unfold the CLMB and separate Cy5 as luminophore from its quencher BHQ2 for obtaining the detection signal. For further amplifying the signal, the unfolded CLMB was cleaved by Nt. BbvCI and dissociated from the proximity complex, which made the proximity complex unfold another CLMB and trigger the second cycle of cleavage. Eventually, each proximity complex could produce plentiful free Cy5 to amplify the CL signal.

This work used target oligonucleotide, P1-Ab/protein/Ab-P2, and P1-aptamer/thrombin/aptamer-P2 to form the proximity complex. In fact, other structures of the proximity complex can also open the CLMB to produce detection signal when they contain the sequence complementary to part of CLMB.

Feasibility of Hybridization-Triggered Signal Switch.

The luminescence signal of Cy5 was generated under the peroxalate (PO)-based CL reaction in which hydrogen peroxide (H_2O_2) oxidized PO to produce an electronically excited 1,2-dioxetanedione intermediate that subsequently transferred its energy to the Cy5 emitter and generate chemiluminescence (Figure 1A).¹⁴ Similar to fluorescent quenching phenomenon, the luminescence of Cy5 could be quenched greatly by a nearby black hole quencher, BHQ2, while its intensity was slightly changed in the presence of free BHQ2 (Figure 1B). The hybridization-triggered signal switch and in situ signal amplification were first demonstrated with a target oligonucleotide that was complementary to CLMB (T-DNA1). In the absence of T-DNA1, the hairpin structure of CLMB brought Cy5 nearby BHQ2 to quench the emission of Cy5, which was referred to as “signal off”; contrarily, the presence of T-DNA1 led to the dissociation of CLMB stem, which separated the Cy5 from BHQ2 to produce a “signal on” state (Figure 1C). The CL intensity increased with the increasing concentration of T-DNA1, suggesting that the CLMB along with the hybridization-triggered signal switch could be used for homogeneous CL bioassay. By introducing the Nt. BbvCI-assisted enzymatic recycling amplification, the CL intensity was greatly strengthened; moreover, the enhancement was more obvious at lower T-DNA1 concentration (Figure 1D), indicating the feasibility of the signal amplification strategy for sensitive homogeneous CL detection of targets, including the DNA sequence complementary to CLMB.

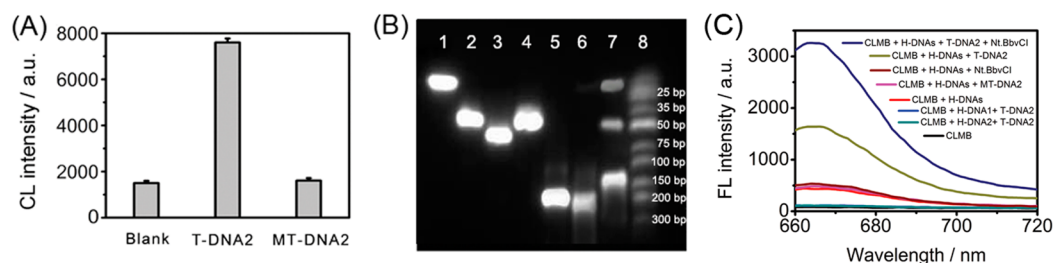


Figure 2. (A) CL intensity for the mixture of CLMB, H-DNA1, H-DNA2, and Nt. BbvCI as blank, blank + T-DNA2, and blank + MT-DNA2. (B) PAGE analysis: (1) unlabeled CLMB, (2) H-DNA2, (3) T-DNA2, (4) H-DNA1 + H-DNA2, (5) H-DNA1 + H-DNA2 + T-DNA2, (6) H-DNA1 + H-DNA2 + T-DNA2 + unlabeled CLMB, (7) H-DNA1 + H-DNA2 + MT-DNA2 + unlabeled CLMB, (8) DNA ladder marker. (C) Fluorescent spectra of different solutions containing the marked components.

To detect a wide concentration range of T-DNA, the concentration of CLMB as the signal indicator should be designed to be sufficient for complete “signal on”. This work chose 1.0 μM CLMB for whole experiments. In addition, because near-neutral pH was the best pH environment for DNA hybridization, immunoreaction and enzyme recycling process, this work used pH 7.4 at 37 $^{\circ}\text{C}$ for CLPA of different kinds of target biomolecules.

Characterization of Proximity Hybridization-Triggered Signal Switch. For opening the CLMB with the formed proximity complex, part 3 of the two H-DNAs contained nine bases complementary with CLMB, respectively. They could not unfold CLMB directly. After the proximity complex was formed in the presence of target, a sequence with 18 bases complementary with CLMB was produced, which could turn on the CL signal. To verify the hybridization processes, the following experiments were performed.

By using T-DNA2 as an example of target oligonucleotide to trigger the proximity hybridization of H-DNAs (Scheme 1B), the CLPA showed a strong CL signal, which went back to the blank signal after replacing T-DNA2 with a single-side mismatched T-DNA2 (MT-DNA2) (Figure 2A); therefore, the simultaneous recognition of two H-DNAs to the target was critical to CLPA. The proximity hybridization and subsequent unfolding of CLMB were verified by PAGE analysis (Figure 2B). Although two H-DNAs contained the complementary sequences, their mixture showed only a band at the same position as H-DNA2 (lanes 2 and 4), indicating no hybridization occurred between the two H-DNAs. When the mixture contained T-DNA2 (lane 3), a new band was observed at ~ 180 bp, demonstrating the formation of proximity complex H-DNA1/T-DNA2/H-DNA2 (lane 5). After adding CLMB in this mixture a new band occurred around 204 bp (lane 6), which could be attributed to the sum of CLMB (lane 1) and the proximity complex (lane 5) due to the formation of H-DNA1/T-DNA2/H-DNA2/CLMB. Contrarily, the presence of MT-DNA2 as the substitution of T-DNA2 displayed only the bands of H-DNA1/MT-DNA2 at ~ 130 bp and other original DNA sequences (lane 7). The unfolding of CLMB was further demonstrated by the fluorescence spectra (Figure 2C). The mixture of CLMB, two H-DNAs, and T-DNA2 showed strong fluorescence of Cy5, while much weaker fluorescence was observed for other mixtures. In the presence of Nt. BbvCI, the fluorescent intensity became doubly stronger, indicating the in situ recycling amplification. Moreover, the amplification process did not change the background, leading to a high efficiency of signal amplification.

Optimization of Detection Conditions. The signal-to-noise ratio depended on the number of complementary bases

between H-DNAs, the reaction time, and the concentration of H_2O_2 . As shown in Figure 3A, 10 complementary bases (10 bp)

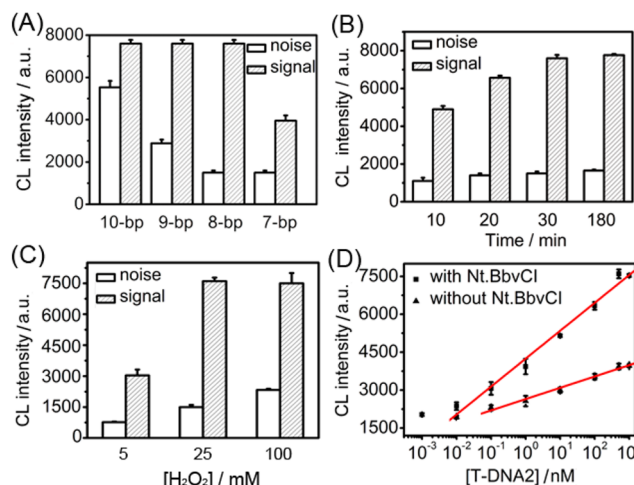


Figure 3. (A) Optimization of complementary base number between two H-DNAs. Effects of (B) incubation time and (C) H_2O_2 concentration on CL intensity for CLPA of 0.5 μM DNA. (D) Calibration curves for T-DNA2 detection in presence and absence of Nt. BbvCI.

produced a large noise due to the self-hybridization of two H-DNAs, which opened CLMB in the absence of proximate effect. The noise rather than signal decreased sharply with the decreasing number of complementary bases to 8 bp, at which the signal-to-noise ratio reached the maximum value. The signal decrease occurred at 7 bp, suggesting difficult hybridization between two H-DNAs. Thus, the H-DNAs with 8 pairs of complementary bases were chosen for the CLPA. At high H-DNA concentration, the CLPA showed large noise due to the self-hybridization of two H-DNAs, while the detectable concentration range of DNA was limited at low H-DNA concentration. Thus, 1.0 μM H-DNAs were chosen for DNA detection. The CL signal increased with the increasing assay time up to 30 min while the noise was slightly changed, indicating 30 min reaction was enough for the detection of oligonucleotide (Figure 3B). The effect of H_2O_2 concentration was also examined (Figure 3C), which led to an optimal concentration of 25 mM for obtaining the highest ratio of signal-to-noise.

CLPA of T-DNA2. Under the optimal conditions, a CLPA method for oligonucleotides was proposed. The plot of CL intensity versus the logarithm of T-DNA2 concentration from 0.01 to 500 nM showed a good linearity (Figure 3D). The limit

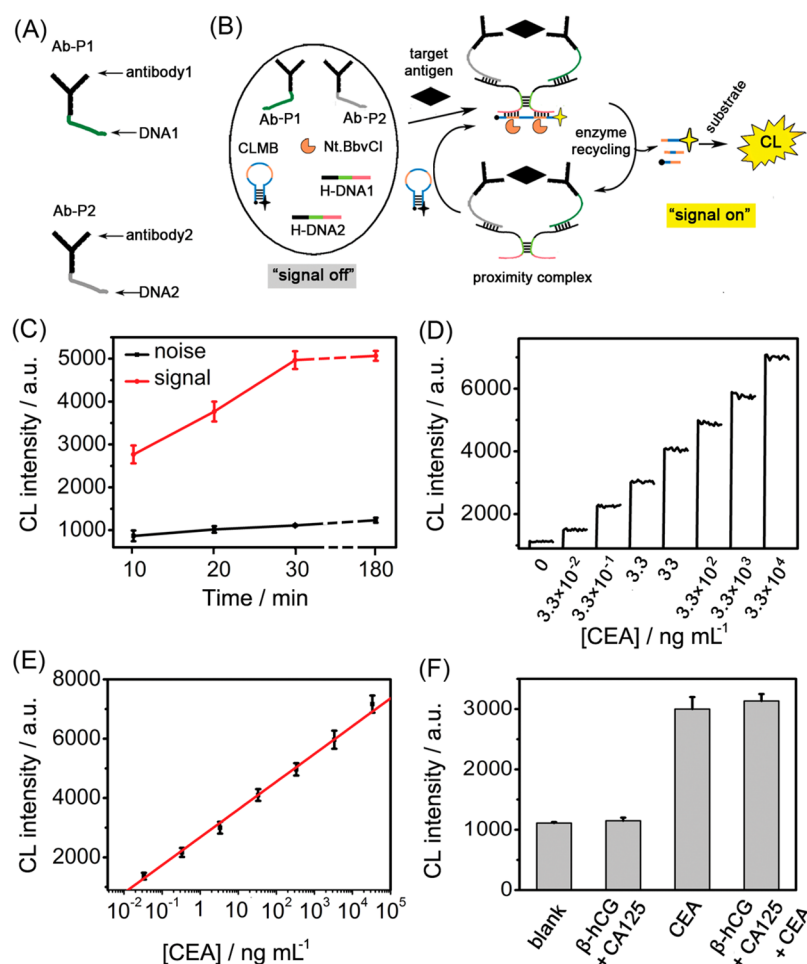


Figure 4. (A) Structure of DNA-labeled antibodies. (B) Principle of the antibody-based CLPA for protein. (C) Optimization of incubation time for CEA detection. (D) CL response to CEA at 0.033, 0.33, 3.3, 33, 330, 3.3×10^3 , and 3.3×10^4 ng mL⁻¹. (E) Calibration curve for CEA detection. (F) Selectivity evaluation of the CLPA using 3.3 ng mL⁻¹ CEA, 3.3 IU L⁻¹ β-hCG + 3.3 U mL⁻¹ CA125, and 3.3 ng mL⁻¹ CEA + 3.3 IU L⁻¹ β-hCG + 3.3 U mL⁻¹ CA125.

of detection corresponding to the signal of 3SD for T-DNA2 was 6.0 pM. Furthermore, the sensitivity (slope) was 2.4 times higher than that in the absence of Nt. BbvCI, in which the linear range was from 0.1 to 500 nM.

CLPA of Protein Biomarkers. Besides the oligonucleotides, this CLPA protocol could be conveniently used for the detection of protein biomarkers by using a pair of DNA-labeled antibodies (Ab-P1 and Ab-P2) as the affinity ligands (Figure 4A, B). The DNA strands labeled to antibodies contained 20 bases complementary with part 1 of H-DNA1 and H-DNA2, respectively. By using CEA as a model analyte, both the concentration of H-DNAs and the protein reaction time were optimized to be ~80 nM and 30 min (Figure 4C), respectively. The CL intensity increased linearly with the increasing logarithm of CEA concentration (Figure 4D), leading to a detectable range from 3.3×10^{-2} to 3.3×10^4 ng mL⁻¹ with a correlation coefficient of 0.9983 (Figure 4E). The limit of detection corresponding to the signal of 3SD for CEA was 24 pg mL⁻¹ (~80 fM), which was lower than those of other immunoassays with different amplification strategies,^{15,16} and comparable to other amplified proximity-depended methods^{5,7,9,11,17} (Table S2, Supporting Information). The CLPA method for protein showed excellent selectivity. The presence of β-hCG and/or CA125 did not affect the detection of target CEA (Figure 4F). The assay results of CEA in five clinical

serum samples were also in good agreement with the reference values (Table S3, Supporting Information). Overall, the favorable performance along with the additional benefits of one-step operation and less sample consumption provided a powerful protocol for point-of-care analysis.

CLPA of Thrombin. The CLPA could be easily extended to detect other targets by using corresponding DNA-labeled affinity ligands such as aptamers. Similar to the immunoassay, the simultaneous recognition of thrombin by two thrombin aptamer probes (Apt-P1 and Apt-P2) formed the proximity complex, which subsequently unfolded the CLMB to open the CL signal (Figure 5A). Here, the concentration of H-DNAs and the reaction time were optimized to be 1 μM and 40 min, respectively (Figure 5B). The linear range for thrombin detection was from 33 pM to 330 nM (Figure 5C), and the detection limit was 8.1 pM. The selectivity of the aptamer-based CLPA was evaluated by comparing the CL intensity toward the solutions containing thrombin or other antigen, for example, 10 μg mL⁻¹ CEA (Figure 5D). As expected, the CLPA showed obvious responses to the solutions containing target thrombin, while a negligible response was observed in the absence of target thrombin, indicating few nonspecific binding of interferences to the aptamer probes.

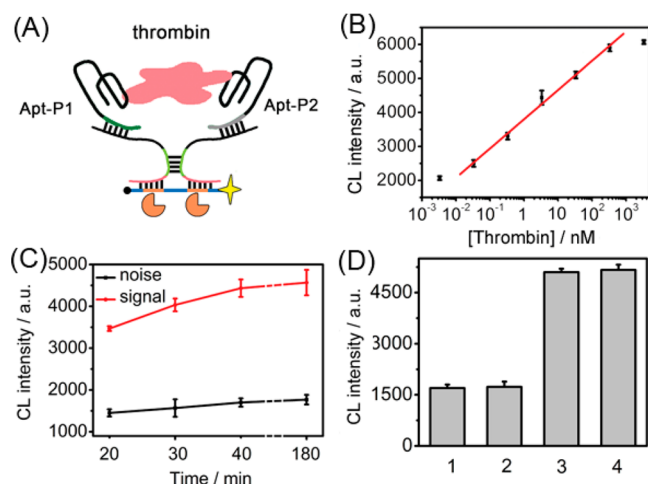


Figure 5. (A) Principle of the aptamer-based CLPA. (B) Effect of incubation time for the CLPA of thrombin at 3.3 nM on CL intensity. (C) Calibration curve for thrombin detection. (D) Selectivity evaluation of the CLPA for thrombin using (1) blank solution, (2) 10 $\mu\text{g mL}^{-1}$ CEA, (3) 33 nM thrombin, and (4) 33 nM thrombin + 10 $\mu\text{g mL}^{-1}$ CEA.

CONCLUSION

This work proposes a proximity hybridization-triggered signal switch and combines the switch with a designed in situ enzymatic recycling amplification to develop a highly sensitive and selective protocol for homogeneous chemiluminescent bioanalysis. This protocol has been successfully used for detection of oligonucleotide, protein biomarker, and thrombin. This methodology shows excellent analytical performance with satisfactory versatility of analytes. It can conveniently be extended to a wide range of analytes with available affinity ligands. Benefited from the one-step operation, the CLPA protocol possesses good practicability and can be used in a miniaturized system for automated bioanalysis. Due to the limitation of the PO-CL system such as a short lasting time of CL, this method can hardly be combined with high-throughput CL imaging technology. In the future, the proximity-dependent CL methods will use other CL systems to realize convenient multiplex and high-throughput CL detection.

ASSOCIATED CONTENT

Supporting Information

Sequences of all oligonucleotides used in this work; a performance comparison of this work with previous methods; and assay results of CEA in clinical serum samples using the proposed and reference methods. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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