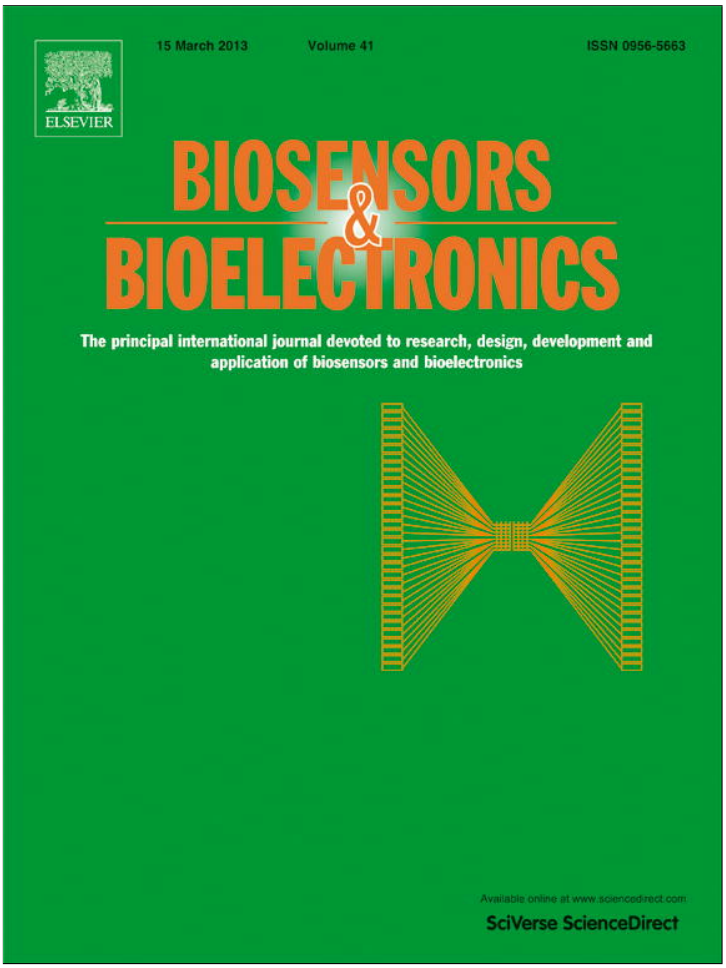

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Highly sensitive and selective chemiluminescent imaging for DNA detection by ligation-mediated rolling circle amplified synthesis of DNAzyme

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ABSTRACT

A highly sensitive DNA biosensing method down to sub-femtomolar level with excellent selectivity was proposed by designing an amplified synthesis of horseradish peroxidase mimicking DNAzyme and introducing the amplified DNAzyme to chemiluminescent (CL) imaging. The amplified synthesis was achieved by combining a target DNA related ligase reaction with rolling circle amplification (RCA), which produced thousands of repeated sequences to bind hemin and form a mass of horseradish peroxidase-mimicing DNAzyme units. The amplification strategy greatly enhanced the CL emission of the luminol–H_2O_2 system. The genotyping method displayed highly specific biochemistry in allele discrimination. The novel CL imaging strategy based on ligation-mediated RCA synthesis of DNAzyme showed high fidelity in discriminating single-base mismatch and efficiently facilitated signal amplification for sensitive target DNA detection. It could detect DNA ranging from 1×10^{-15} M to 1×10^{-11} M with a detection limit of 0.26 fM. The proposed approach provided a robust, cost-efficient, highly sensitive and specific platform for genetic target analysis in bioanalysis and clinic biomedical application.

1. Introduction

Single nucleotide polymorphisms (SNPs) related to many pathogenic and genetic diseases are the most abundant and stable form of genetic variations occurring in human genome (Sachidanandam et al., 2001; Syvanen, 2001; Michael and Andreas 2005). Genotyping SNP is a topic of intensive interest because the SNPs are useful targets for pharmacogenetic analyses and can always be regarded as markers of genetic disorders and disease predisposition. Genotyping SNP has been performed by combining some allele discrimination mechanisms, such as allele-specific hybridization (Xiao et al., 2007), cleavage (Chen et al., 2007) and oligonucleotide ligation (Steemers et al., 2006; Lizardi et al., 1998), with a specific detection strategy of allele-specific segment (Kim and Misra 2007). Some enzyme-based approaches, such as allele-specific nucleotide incorporation by DNA polymerases (Duan et al., 2007), cleavage by flap endonuclease (Lockett et al., 2007), and ligation by DNA ligases (Li et al., 2006), also display good practicability in single-base differentiation, which facilitates SNP genotyping due to the cost-efficiency, ease of operation and rapidness of implementation (Kirk et al., 2002).

Meanwhile, highly sensitive detection of DNA sequence has been attracting considerable interest due to the needs in biomedical molecular diagnostics, environment monitoring, anti-terrorism and forensic science (Dong et al., 2008; Sassolas et al., 2008; Bi et al., 2010a; Dong et al., 2010a). The widely used strategies to probe the low abundant DNA include amplifying the copy number of oligonucleotide with the polymerase-chain-reaction (PCR) technique (Ju et al., 2003; Ye et al., 2003), and the DNA hybridization event using enzymatic cycles (Munge et al., 2005; Liu et al., 2008), and nanoparticles to produce enhanced signal (Taton et al., 2000; Song et al., 2009). Recently rolling circle amplification (RCA), a simple but powerful isothermal amplification process that can yield long single-stranded DNA (ssDNA) with a repeating sequence unit (Daubendiek et al., 1995; Fire and Xu, 1995; Liu et al., 1996), has been recognized as a novel tool for amplified assay (Schweitzer et al., 2000; Zhao et al., 2008; Cheng et al., 2009). This technique can be employed for amplified synthesis of DNAzyme to catalyze the oxidation of dye to produce a colorimetric signal (Tian et al., 2006; Cheglakov et al., 2007) or stimulate the generation of chemiluminescence (CL) in the presence of H_2O_2 and luminal (Cheglakov et al., 2007), leading to several novel sensitive methods for DNA detection. The high catalytic and amplification efficiency of DNAzyme has greatly promoted the development of CL biosensing techniques for detection of DNA or protein (Li et al., 2008; Bi et al., 2010b; Wang et al., 2011; Luo, et al., 2012). Owing to the extensive applications of DNA

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detection, novel amplification strategies and sensitive detection methods with high throughput are continually needed (Wang et al., 2008; Dong et al., 2010b; Li et al., 2010b; Gao et al., 2011).

CL imaging by coupling CL reaction with a charge-coupled device (CCD) as detector can be used for high throughput detection because it does not need external light source. A CL imaging method has been designed for screening four pathogen bacteria in foodstuffs with a polystyrene 96×4 well microtiter plate (Magliulo et al., 2007). However, the CL signal from the typical horseradish peroxidase–H_2O_2–luminol system for single biorecognition event is relatively low, and difficult to be collected by a general CCD, which limits its application in the detection of low-abundance biomarkers in disease screening. In order to enhance the sensitivity of CL imaging analysis, different catalytic nanomaterials (Kong et al., 2011) and nanocarriers such as carbon nanotubes (Yang et al., 2008), and gold nanoparticles (Zong et al., 2012) have been introduced into CL sensor array. The nanocarriers can carry more enzyme molecules to catalyze the CL reaction of H_2O_2 and luminol for single biorecognition event, thus greatly improve the practicality of CL imaging in bioanalysis and detection of biomarkers. This work introduced the combination of a ligase reaction with RCA for amplifying the synthesis of DNAzyme into CL imaging and developed a highly selective and sensitive CL imaging method for DNA detection. The fidelity of DNA ligase reaction allowed highly specific typing of DNA sequences.

For genotyping of DNA, the capture probe 1 was firstly immobilized on the inner wall of wells at a 96-well plate via streptavidin–biotin interaction and annealed with DNA target, which then hybridized with discrimination probe 2 with 5′- phosphormonoester group to form a perfect sandwich complement with the target at the junction (Fig. 1). Probes 1 and 2 could further be covalently joined by *Escherichia coli (E. coli)* ligase. The 3′ end of probe 2 could further act as a primer to initiate an RCA reaction in the presence of Klenow exo- DNA polymerase and four nucleotides (dNTPs). After the RCA product bound hemin, thousands of HRP-mimicking DNAzyme units were generated. A DNAzyme catalyzed CL reaction in the presence of H_2O_2 and luminol was finally used to produce a strong detection signal for CL imaging. It is worthy to mention that, after "sandwiching" the target DNA between the capture probe 1 and the discrimination probe, the ligation reaction is carried out only when the discrimination probe perfectly anneals to the target. Therefore, the genotyping strategy involving ligase exhibited highly specific biochemistry in allele discrimination. Meanwhile, the coupled amplification steps of RCA and HRP-mimicking DNAzyme endowed the proposed biosensing method with high sensitivity due to the amplified hybridization event that was related to massive DNAzyme catalyzed reaction.

2. Materials and methods

2.1. Reagents

The dNTPs, *E. coli* ligase, T4 ligase and Klenow exo- DNA polymerase were obtained from Fermentas (Lithuania). The streptavidin-coated microplates were purchased from Greiner Bio-One (Germany). Hemin was purchased from Sigma (St. Louis, MO). Hemin stock solution (5 μM) was prepared in dimethyl sulfoxide (DMSO) and stored in the dark at 4 °C. Horseradish peroxidase-(HRP) substrate solution (luminol–p-iodophenol–H_2O_2) was obtained from Autobio Diagnostics Co., Ltd. (China). Tris–HCl (10 mM, pH 8.0) containing 1 mM ethylenediaminetetraacetic acid (EDTA) (10 mM Tris, 1 mM EDTA, 50 mM NaCl, 10 mM $MgCl_2$) was used as DNA hybridization buffer. 0.05% Tween-20 was added in Tris–HCl buffer (50 mM, pH 7.5) as rinsing buffer to minimize unspecific adsorption. DNA was stored in Tris–HCl (10 mM, pH 8.0). All other reagents were of analytical grade. All aqueous solutions were prepared using ultra-pure

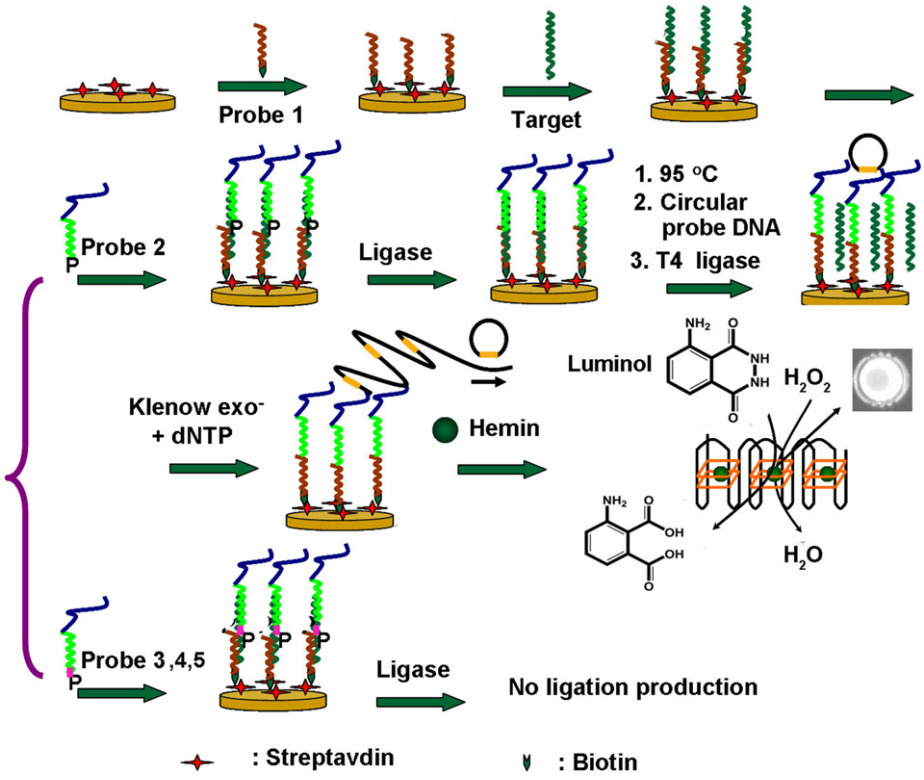

Fig. 1. Schematic presentation of DNA sequence biosensing by ligation-mediated RCA synthesis of DNAzyme for chemiluminescent imaging.

water (≥ 18 MΩ, Milli-Q, Millipore). The oligonucleotides were purchased from Sangon Biological Engineering Technology & Co. Ltd. Shanghai, China, and purified using high-performance liquid chromatography. Their sequences were:

probe 1: 5′-biotin-AAAAA AAAA CTT GTG GTA GTT GGA GCT GT-3';
discrimination probe 2: 5'–PO_4^{2-}-**T** GGC GTA GGC AAG AGT GCC C TTA GGATCG TGT GGT T-3';
discrimination probe 3: 5′–PO_4^{2-}-**A** GGC GTA GGC AAG AGT GCC C TTA GGA TCG TGT GGT T-3′;
discrimination probe 4: 5′–PO_4^{2-}-**G** GGC GTA GGC AAG AGT GCC C TTA GGA TCG TGT GGT T-3′;
discrimination probe 5: 5′–PO_4^{2-}-**C** GGC GTA GGC AAG AGT GCC C TTA GGA TCG TGT GGT T-3′;
target: 5′–GGGCACTCTTGCCTACGCC**A**ACAGCTCCAACTACCA-CAAGTTTT-3′;
circular probe: 5′–GATCCTAA CCC AAC CCG CCC TAC CCA AAA CCC AAC CCG CCC TAC CCA AAA CCC AAC CCG CCC TAC CC AACCACAC-3′;and
single DNAzyme probe: 5′–PO_4^{2-}-**T** GGC GTA GGC AAG AGT GCCC GGG TTG GGC GGG ATG GGT-3′.

2.2. DNA hybridization in microplate wells

Immobilization of biotinized probe 1 on the streptavidin-modified 96-well plate via streptavidin–biotin interaction was performed by dropping 100 μL probe 1 (10 pM) in each well, and allowing them to react at 37 °C for 0.5 h. The resulting microplate wells were washed thrice with 200 μL of rinsing buffer. The hybridization reaction was carried out by dropping 100 μL of the desirable target solution prepared with Tris–HCl solution (pH 8.0, 10 mM Tris, 1 mM EDTA, 50 mM NaCl, 10 mM $MgCl_2$) and incubating them at 37 °C for 0.5 h, washing thrice with 200 μL of rinsing buffer. Afterwards, 100 μL discrimination probe was added and incubated at 37 °C for 0.5 h, the resulting microplate wells were washed thrice with 200 μL of rinsing buffer before ligation reaction.

2.3. Ligation reaction

The ligation reaction was performed at 37 °C for 1 h in 100 μL of reaction buffer (50 mM NaCl, 10 mM KCl, 45 mM Tris–HCl, pH 8.0, 2 mM $MgSO_4$, 5 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.1% (w/v) Triton X-100, 1 mM NAD^+ and 0.5 mM dithiothreitol) containing 10 U *E. coli.* ligase. After ligation, *E. coli.* ligase was inactivated by heating the reaction mixture at 70 °C for 10 min. The mixture was then heated to 95 °C for 30 s and then rapidly cooled to 37 °C to dissociate the target from the ligated probe, followed by washing thrice with 200 μL of rinsing buffer. The resulting microplate was used for RCA process. Refrigerated heating bath K6-CC-NR (Hurber Germany) was employed in all the heat processes.

2.4. RCA process for CL imaging

After the hybridization and ligation reaction, the microplate wells were added with 100 μL ligation buffer (50 mM, pH 7.5 Tris–HCl buffer, 10 mM $MgCl_2$, 10 mM dithiothreitol, and 0.5 mM ATP) containing 10 pM of circular template oligonucleotide and incubated at 37 °C for 30 min. Then, 0.2 units of T4 DNA ligase was added and incubated at 22 °C for 1 h to form the circular DNA. Afterwards, T4 DNA ligase was inactivated by heating the reaction mixture at 65 °C for 10 min, and then incubating at 37 °C for further 30 min. The microplate wells were washed thrice with 200 μL of rinsing buffer, and then the RCA reaction was initiated by adding 0.2 units of Klenow exo$^-$ DNA polymerase in 100 μL of reaction buffer (50 mM, pH 7.5 Tris–HCl buffer, 10 mM magnesium acetate, 33 mM potassium acetate, 1 mM dithiothreitol, 10 mM dNTP, and 0.1% Tween 20) and continued for 1 h at 37 °C. After the microplate wells were carefully washed thrice with 200 μL of risning buffer, 100 μL of hemin (1 μM) was added into each well and incubated at 37 °C for 30 min. The microplates were carefully washed thrice with 200 μL of rinsing buffer to remove unspecific adsorption of hemin. 20 μL of CL substrate solutions (luminol–p-iodophenol–H_2O_2) were finally added to the wells to detect the CL signal at 10 min with a cooled low-light CCD camera with high resolution (BioImaging Systems Chemi HR 410 camera, UVP).

3. Results and discussion

3.1. Optimization of biosensing conditions

The amounts of probe 1 attached on the inner wall of wells and probe 2 acting as a primer to initiate an RCA reaction affected greatly the CL performance of the proposed imaging method. Therefore concentrations of both the probes, 1 and 2, were firstly optimized. The effect of probe 1 concentration used in the immobilization was tested at 10^{-14}, 10^{-13}, 10^{-12}, 10^{-11} and 10^{-10} M in triplicate. At the target DNA concentration of 10^{-13} M and probe 2 concentration of 10^{-11} M, with the increasing probe 1 concentration from 10^{-14} to 10^{-11} M, the signal increased, afterwards, the CL intensity decreased (Fig. 2A). Similarly, the effect of probe 2 level on the response was examined over the range 10^{-14}–10^{-10} M by immobilizing 10^{-11} M probe 1 on the wall to detect 10^{-13} M target DNA. As shown in Fig. 2B, the maximal signal was obtained at the concentration of 10^{-11} M. The signal decreased at higher concentrations resulted from the steric effect caused by probe 2 hindering the subsequent RCA. Therefore, a concentration of 10^{-11} M for both probes 1 and 2 was used in all subsequent work.

3.2. Reaction time of RCA

At the fixed concentration of probe 1, target and probe 2, the CL response of the luminol–H_2O_2 system catalyzed by the HRP mimicking DNAzyme depended on the time of RCA reaction (Fig. 3). The CL intensity increased rapidly with the increasing of RCA time from 0 to 30 min, and more slowly thereafter. This was explained on the basis that the longer the RCA process, the more DNAzyme units were generated, leading to an enhancement of the catalyzed CL reaction of luminol–H_2O_2 system. The reaction was almost completed at 60 min, in agreement with a previous report (Cheglakov et al., 2007). Therefore, a 60 min reaction time was selected as the RCA process.

3.3. Signal amplification by RCA

After the immobilized probe 1 hybridized in succession with the target and probe 2, probe 2 was covalently joined to probe 1 in the presence of *E. coli* ligase, and the target was then disassociated from the joined probes at 95 °C. The ligated probe 2 further hybridized with circular probe, which could form a DNA circle template in presence of T4 DNA ligase. A RCA reaction was then initiated in the presence of Klenow exo-DNA polymerase and dNTPs. The RCA products containing thousands of repeated sequences finally bound hemin to form HRP-mimicking DNAzyme units. In this case, each target hybridization event could trigger a RCA process to generate many copies of DNAzyme, therefore, the RCA made each target hybridization event relate to numerous

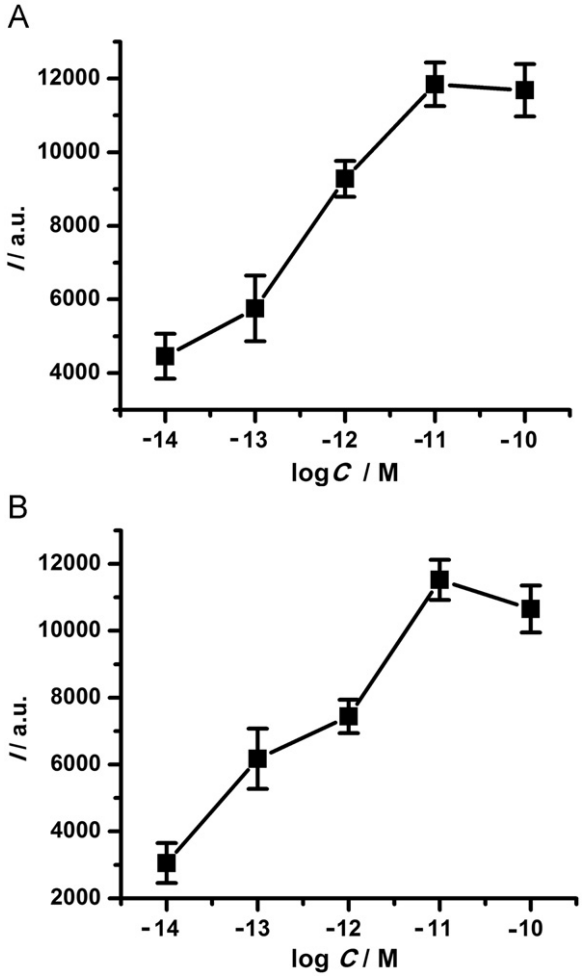

Fig. 2. Influences of probe 1 concentration at 10 pM probe 2 (A) and probe 2 concentration at 10 pM probe 1 (B) on CL response to 0.1 pM target DNA with 30 min incubation at each step (error bars: SD, $n=3$).

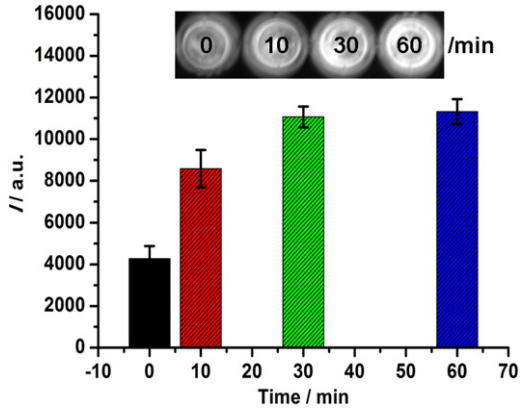

Fig. 3. CL response of proposed biosensor recorded at different times of RCA in the presence of 10 pM probe 1, 0.1 pM target and 10 pM probe 2. Inset: corresponding chemiluminescent images.

enzyme reactions and greatly amplify the detection signal. The significantly improved detection sensitivity could be demonstrated in Fig. 4. The CL signal originated from RCA process at 10 pM target was 4.3-fold larger than that from control experiment without RCA process just involving a single DNAzyme. Meanwhile, it displayed a CL signal of 13.4-fold larger than the

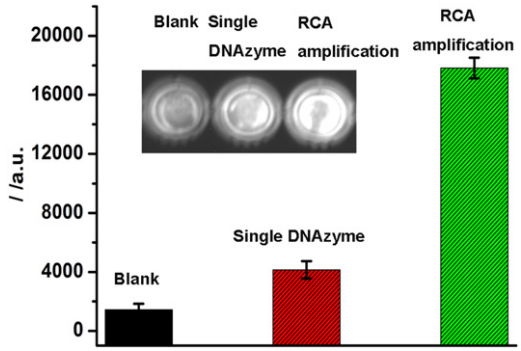

Fig. 4. CL responses of proposed biosensor in the absence (blank) and the presence of 10 pM target with single DNAzyme (without RCA) and RCA as tracer (error bars: SD, $n=3$). Inset: corresponding chemiluminescent images.

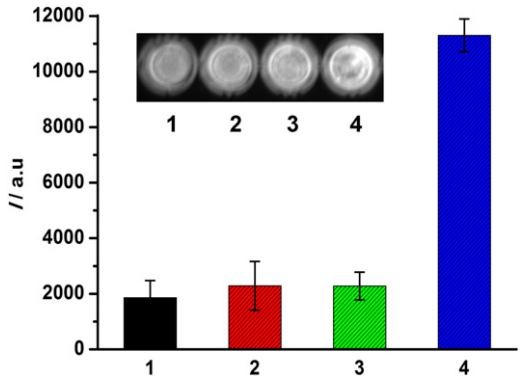

Fig. 5. CL responses by exposing to 10 pM mutant probes 3, 4, 5 (1–3) and complementary probe 2 (4) at target DNA concentrations of 0.1 pM ($n=3$). Inset: corresponding chemiluminescent images.

background, which was detected in the absence of target and came from the nonspecific adsorption of probe 2. Under this condition, the Z-factor and the coefficient of variation were calculated to be 0.8% and 3.9%, respectively, which indicated the proposed method had good robustness (Zhang et al., 1999).

3.4. Performance of specificity

The ligase-based allele discrimination capability was inspected by exposing it to three kinds of mutant DNA probes (probe 3, 4, and 5, 0.1 pM), and compared the CL intensities originated from the mutant DNA probes to the intensity produced by the complementary probe 2 (0.1 pM), which could produce a perfect complement with the target at the junction and form ligated product. The mutant DNA probes mismatched the target at the junction, thus could not produce ligation product. The CL intensity for probes 3, 4 and 5 displayed only 16%, 20% and 20% of that for probe 2, respectively (Fig. 5), indicating the method has high fidelity in discriminating single-base mismatch. The detectable differences among mutant probes might resulted from the dissimilarity of their hybridization efficiency and thermal stability (Gorodetsky et al., 2008). The proposed CL imaging method exhibited better performance to discriminate single-base mismatched than previous reports (Cai et al., 2002; Polsky et al., 2006; Mehrgardi and Daneshtalab, 2011). This desirable specificity in identification of SNP resulted from the ligase-based reaction and high signal-noise ratio (Abe and Kool 2004; Ogasawara and Fujimoto 2006; Li et al., 2009a; Wang et al., 2011).

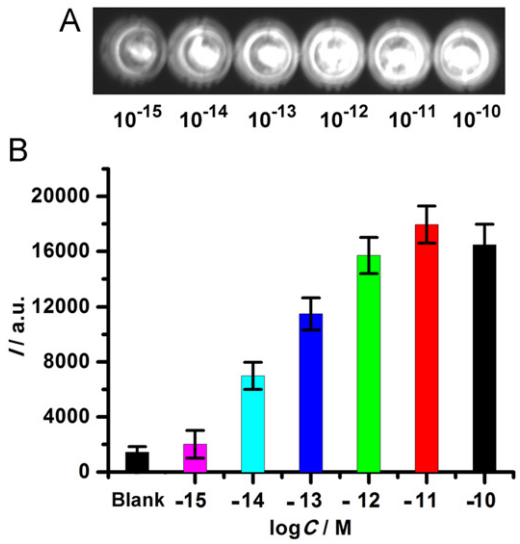

Fig. 6. (A) CL imaging at target DNA concentrations of 100 pM, 10 pM, 1 pM, 100 fM, 10 fM and 1 fM. (B) Linear relationship between CL intensity and logarithm of target DNA concentration ($n=3$).

3.5. Assay performance

Under optimal conditions, the CL intensity increased with the increasing concentration of target DNA (Fig. 6A). Furthermore, the CL intensity obtained in the absence of adjacent well emission did not show any difference from that obtained in presence of adjacent well emission, indicating negligible cross-talk effect. The plot of the response vs. the logarithm of target concentration displayed a linear relationship in the detected range from 1×10^{-15} M to 1×10^{-11} M (Fig. 6B). The limit of detection (LOD) was 0.26 fM calculated at the signal of three times standard deviation (SD) obtained in the absence of the standard. It was lower than the LOD of 1.0 pM obtained in the absence of RCA process, indicating that the RCA process sharply improved the sensitivity. Compared to the DNA detection employing single HRP-mimicking DNAzyme as catalytic tracer with a LOD of 1.0 nM, the proposed biosensor showed a much lower LOD (Pavlov et al., 2004). The LOD was also competitive with other sensitive detection involving RCA process for synthesizing HRP-mimiking DNAzyme as tracer with LOD of 10 fM (Cheglakov et al., 2007). The high sensitivity of the proposed biosensing method could be attributed to the low background and the circular DNA-mediated RCA reaction. To avoid the false results, the sample should be firstly diluted when its concentration was higher than 10 pM.

4. Conclusions

This work demonstrated a highly selective and sensitive DNA biosensing method by CL imaging based on a target related ligase reaction and RCA process to produce enormous HRP-mimicing DNAzyme. The genotyping strategy involving ligase could bring highly specific biochemistry in allele discrimination, and the RCA process generated thousands of HRP-mimicing DNAzyme units by associating the repeated sequences with hemin to enhance the CL emission from the catalyzed luminol–H_2O_2 system. This novel ligation-mediated RCA synthesis of DNAzyme greatly improved the sensitivity of CL imaging for detection of target DNA down to sub-femtomolar level, and the designed strategy can discriminate single-base mismatch with high capability. Due to the intrinsic advantage of CL imagining, the proposed approach possessed potential application in high-throughput screening of disease and provided a robust, cost-efficient, highly sensitive and specific platform for genetic target analysis in bioanalysis and clinic biomedical application.

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