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Highly sensitive and multiple DNA biosensor based on isothermal strand-displacement polymerase reaction and functionalized magnetic microparticles

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ABSTRACT

A universal, highly sensitive and selective chemiluminescence (CL) imaging method has been developed for high throughput detection of DNA. After molecular beacon (MB) hybridized with the target DNA, the biotin-labeled primer was attached to a magnetic microparticle (MMP) surface by hybridization with the stem part of the MB to initiate a polymerization of DNA strand, which led to the release of the target and another polymerization cycle. Thus the polymerization produced the multiplication of biotin-labeled primer on the surface of MMPs. Sequentially, the horseradish peroxidase (HRP) was conjugated to MMPs surface through the biotin–streptavidin reaction. Then, the conjugated HRP was determined by the CL imaging method. This proposed method could detect the sequence-specific DNA as low as 0.4 pM and discriminate perfectly matched target DNA from the mismatch DNAs. All in all, this proposed method exhibited an efficient amplification performance, and would open new opportunities for sensitive and high throughput detection of DNA.

1. Introduction

The highly sensitive detection of DNA hybridization is of great demand in gene profiling, drug screening, clinical diagnostics and environmental analysis, food safety and a variety of biomedical studies (Fan et al., 2006; Turner et al., 2009; Luo et al., 2012a). Motivated by this demand, many signal amplification strategies have been employed to produce stronger signal in various DNA biosensors. To achieve strong signals, a series of functionalized nanoparticle probes, such as quantum dots (Chan and Nie, 1998; Chan et al., 2002), dye-doped nanoparticles (Zhao et al., 2003; Tan et al., 2004), and nanoparticles (Li et al., 2008; Song et al., 2010) whose surfaces were modified with many signal molecules, have been developed to enhance recognition events of targets and significantly lower the detection limit for DNA analysis. However, there are some limitations involved in using functionalized nanoparticles as signal probes, such as an extra functionalized process on the surface of these nanoparticles may affect the analytical accuracy and reproducibility. Furthermore, it is time-consuming to synthesize such functionalized nanoparticle probes, and the functionalized process is also tedious and complicated.

Meanwhile, signal amplification strategy based on nuclease, such as hybridization chain reaction (HCR) (Huang et al., 2011; Bi et al., 2013b), rolling circle amplification (RCA) (Cheng et al., 2010; Bi et al., 2010a), loop-mediated amplification (Hsieh et al., 2012) and the target DNA recycling amplification with endonuclease (Bi et al., 2010b; Chen et al., 2011; Jang et al., 2009) or exonuclease (Zuo et al., 2010; Luo et al., 2012b) have also been employed to improve the sensitivity of various DNA biosensors. Recently, isothermal strand-displacement polymerase reaction (ISDPS) has attracted considerable attention in target recycling amplification (Ye et al., 2003). This technique uses lengthening of a new strand to replace the target sequence, and thus releases the target to initiate a new polymerization reaction. Because ISDPS does not require a specific recognition site, it can be conveniently used for designing DNA biosensors. Wang's group reported a method for amplified detection of DNA based on the inherent signal-transduction mechanism of the hairpin fluorescence probe and strand-displacement property of polymerase (Guo et al., 2009). In addition, Ju's group developed several biosensors based on polymerase for the sensitive DNA detection (Dong et al., 2012; Gao et al., 2012, 2013). For example, they designed a novel dual signal amplification strategy which combined the ISDPS technique with nanoparticles-based signal amplification for electrochemical detection of the target DNA (Gao et al., 2012). Due to the extensive applications of DNA detection, novel sensitive DNA detection methods based on signal amplification strategies with high throughput are continually needed (Luo et al., 2012a,b; Xiang et al., 2013).

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Chemiluminescence (CL) imaging (Bi et al., 2013a; Dong et al., 2013; Yang et al., 2008, Zong et al., 2012), a powerful analytical technique, has been proved of particular utility in high throughput detection because it does not need external light source. The system of the horseradish peroxidase (HRP)-catalyzed luminol-*p*-iodophenol (PIP)-H_2O_2 reaction with PIP as the enhancer, allowing a higher sensitivity and steady-state light signal, is suitable for CL imaging detection. For example, our previous work developed a CL imaging biosensor for the DNA detection (Li and He, 2009). However, in order to expand the proposed method to detect various DNA sequences simultaneously, different capture DNA-modified MMPs were needed.

Herein, we reported a CL imaging method for amplified and high throughput detection of DNA based on functionalized magnetic microparticles (MMPs) and strand-displacement property of polymerase (Fig. 1). As shown in Fig. 1A, the molecular beacon (MB) acted as a template of polymerization reaction, while the target acted as a trigger of polymerization reaction. The activation of this DNA detection system was based on the conformational change of the MB induced by hybridization between the MB and the target DNA. In this method, the target DNA was displaced in the process of the polymerization reaction and then hybridized to another MB. Thus, in essence, this method allowed hybridization, polymerization reaction and displacement to occur cycle-after-cycle, producing, at the same time, an amplified CL signal sufficient to indicate the presence of trace amount of the target DNA. This sensing platform, however, needed to design specific functionalized MMPs for every target DNA. In order to resolve this apparent limitation, we used the short primer functionalized MMPs as a versatile probe (Fig. 1B). Only need to change MB, this biosensor could be expanded for the simultaneous detection of various DNA sequences in a microarray. In short, combined with the inherent advantages of CL imaging method, this new ISDPS-based biosensor could be widely applied to sensitive and high throughput DNA detection.

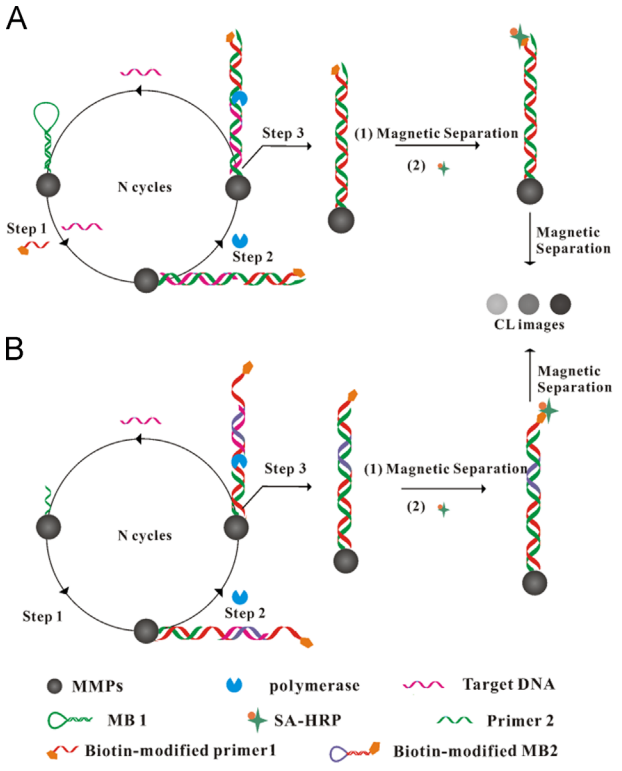

Fig. 1. Schematic representation of chemiluminescence (CL) imaging DNA detection relying on ISDPR and functionalized MMPs.

2. Materials and methods

2.1. Chemicals and reagents

The polymerase Klenow fragment exo- and the mixture of deoxyribonucleotides (dNTPs) were obtained from Fermentas Biotechnology Co. Ltd. (Canada). The carboxylated MMPs (1.02 μm, 10 mg mL^{-1}) were obtained from Invitrogen (Norway). Streptavidin-horseradish peroxidase (SA-HRP), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) were purchased from Sigma Chemical Co. Ltd. (USA). The other chemical reagents were all analytical reagent grades and purchased from Sigma Chemical Co. Ltd. (USA). The HRP substrate kit was purchased from Millipore. Polystyrene microplates (Costar) were used to perform CL imaging detection. DNA hybridization buffer was PBS (137 mM NaCl, 10 mM $MgCl_2 \cdot 12H_2O$, 10 mM Na_2HPO_4, and 2.0 mM KH_2PO_4, pH 7.4). The washing buffer was 10 mM PBS (0.1% (w/v) Tween-20, pH 7.4). All oligonucleotides with different sequences were synthesized and high-performance liquid chromatography (HPLC) purified by Sangon Biotechnology Co. Ltd. (Shanghai, China) and was stored in 10 mM Tris–HCl buffer (1 mM EDTA, pH=8.0). The sequences of the oligonucleotides were as follows:

The DNA sequences of Fig. 1A:

Molecular beacon (MB1): 5/-NH_2-GCT CGG CAT GAC CAC ATC ATC CAT ACA TGC CGA GC-3/
Target DNA for MB1 (HT1): 5/-TAT GGA TGA TGT GGT-3/
Primer DNA for MB1 (Primer1): 5/-biotin-GCT CGG C-3/

Mismatched target sequences for MB1:

Single-base mismatched (MT1): 5/-TAT GGA TAA TGT GGT-3/
Two-base mismatched (MT2): 5/-TAT GTA TGA TTT GGT-3/
Three-base mismatched (MT3): 5/-TAT TGA TAA TGC GGT-3/

The DNA sequences of Fig. 1B:

Molecular beacon (MB2): 5/-Biotin-GTG CTC GAG TCG AAT ACC ACA TCA TCC ATA TAC GAC TCG AGC AC-3/
Target DNA for MB2 (HT2): 5/-TAT ATG GAT GAT GTG GTA TT-3′
Primer DNA for MB2 (Primer2): 5/-NH_2-TTT TTT GTG CTC GAG-3/

Mismatched target sequences for MB2:

Single-base mismatched (MT1): 5/-TAT ATG GAT TAT GTG GTA TT-3/
Two-base mismatched (MT2): 5/-TAT ATG TAT GAT GCG GTA TT-3/
Three-base mismatched (MT3): 5/-TAT ACG GAT TAT GTA GTA TT-3′

The CL images were acquired with a ChemiDoc XRD system (Bio-Rad). A HZQ-F160 temperature oscillation incubator (Shanghai Anting Scientific Instrument Co. Ltd.) was used to control the temperature of the reaction. Ultrapure water was produced by a Millipore-Q Academic purification set (Millipore, Bedford, MA, USA). A pB-10 potentiometer (Sartorius) was used to measure pH of solutions.

2.2. Modification of MMPs

The conjugates between MMPs and DNA sequences (MB1 or Primer2) were prepared using a modified protocol suggested by the manufacturer (Invitrogen). Briefly, 0.5 mL of 10 mg mL^{-1} carboxyl-modified MMPs suspension was washed two times with 1 mL of the

binding buffer solution (10 mM PBS, 15 mM NaCl, pH 7.4) and resuspended in 300 μL the binding buffer solution. A 9 nanomole DNA sequences in 100 μL binding buffer solution were mixed with the MMPs suspension. The mixture of 50 μL EDC (0.1 M), 100 μL NHS (0.05 M) were then added into the above suspension and incubated at room temperature under stirring for over night. The modified MMPs were washed three times with 0.25 M Tris–HCl buffer (0.01% (w/v) Tween-20, pH=8.0) for 30 min to quench the unreacted activated carboxylic acid groups. Then, the modified MMPs were dispersed in 1 mL 10 mM Tris–HCl buffer (1 mM EDTA, 0.5% (w/v)% BSA, pH=8.0) and stored at 4 °C for latter usage.

2.3. Isothermal strand-displacement DNA polymerization reaction (ISDPR)

The polymerase-based ISDPR (Fig. 1A) was performed by mixing 10 μL of MB1 modified MMPs (5 mg mL^{-1}), 10 μL of primer1 (200 nM), 8 μL of polymerase Klenow fragment exo- (4 U), 2 μL dNTPs (1 mM for each component) and varying concentrations of the target DNA to a final volume of 100 μl in the PBS buffer (137 mM NaCl, 10 mM $MgCl_2 \cdot 12H_2O$, 10 mM Na_2HPO_4, and 2.0 mM KH_2PO_4, pH 7.4), followed by incubating at 37 °C for 150 min with gentle shaking. Finally, the MMPs were washed three times with 10 mM PBS buffer (0.1% (w/v) Tween-20, pH=7.4) to remove the excess DNA sequences. For Fig. 1B, the reaction was performed by mixing 6 μL of primer2 modified MMPs (5 mg mL^{-1}), 10 μL of MB2 (100 nM), 8 μL of polymerase Klenow fragment exo- (4 U), 2 μL dNTPs (1 mM for each component) and varying concentrations of the target DNA to a final volume of 100 μL in the PBS buffer (137 mM NaCl, 10 mM $MgCl_2 \cdot 12H_2O$, 10 mM Na_2HPO_4, and 2.0 mM KH_2PO_4, pH 7.4). The following process is the same as Fig. 1A.

2.4. CL imaging detection

After ISDPR, 100 μL of 200 ng mL^{-1} SA-HRP in10 mM PBS buffer (15 mM NaCl, pH=7.4) was added and the reaction proceeded for 30 min at 37 °C with gentle shaking. Afterwards, the MMPs were washed five times with 10 mM PBS buffer (0.1% (w/v) Tween-20, pH=7.4) to remove the excess SA-HRP. Then, the MMPs were transferred into the wells of a microplate and the washing buffer removed, then 50 μL of CL reaction solution were added into each well. After 2 min, the CL imaging detection was performed on the ChemiDoc XRD system and the images were dealt with the Quantity One analysis software. To perform this assay, the Chemi Hi Sensitivity application model was used. The CL signal of each well was individually analyzed and the intensity was recorded.

2.5. Single nucleotide polymorphism (SNP) analysis

A 10 μL of 0.2 nM (Fig. 1A) and 0.5 nM (Fig. 1B) the target DNA and the same amount of other mismatched strands were used to perform the polymerase-based ISDPR according to the procedure of detection of the target DNA mentioned above, and the CL signal of each well was individually analyzed and the intensity was recorded.

2.6. Gel electrophoresis

A 18% polyacrylamide gel electrophoresis (PAGE) analysis of the products via the ISDPR was carried out in 1 × Tris-borate-EDTA (pH 8.3) at 150 V constant voltage for about 2 h. After ethidium bromide (EB) staining, gel was scanned using a Molecular Imager Gel Doc XR (BIO-RAD, USA).

3. Results and discussions

3.1. Principle of ISDPR

The principle of this isothermal amplified detection DNA method was shown in Fig. 1. As shown in Fig. 1A, the DNA detection system consisted of a MB1-modified MMP, a short biotin-modified primer (primer1) and polymerase. The stem region of the MB1 probe was 10-nt sequences long, and the loop was complementary to the target DNA (HT1). The biotin-modified primer was a 7-nt sequence long, which was complementary to the stem region of the MB1 probe at 3′-end. In the absence of HT1, the stem-loop conformational probe was unable to anneal with the primer1 to induce a polymerization reaction. However, in the presence of HT1, the MB1 probe recognized and hybridized with it and undergone a conformational change, leading to stem separation (Step (1). Following this, the biotin-modified primer1 annealed with the open stem and triggered a polymerization reaction in the presence of dNTPs/polymerase (Step 2). Next, in the process of primer1 extension, the target DNA was displaced by the polymerase with strand-displacement activity, after which a complementary DNA (cDNA) was synthesized, forming a probe-cDNA complex (Step 3). Finally, to renew the cycle, the displaced target hybridized with another probe, which triggered yet another polymerization reaction, resulting in the multiplication of the biotin-modified primer1 on the surface of MMPs (Step 4). Sequentially, the SA-HRP afforded a signal trace for CL analysis of the target DNA (Step 5). Therefore, by monitoring the increase of CL intensities, we could detect the target with high sensitivity. This sensing platform, however, needed to design specific functionalized MMPs for every target DNA. In order to resolve this apparent limitation, we used the MMPs functionalized with the short primer (primer2) as a versatile probe for sensing different DNAs (Fig. 1B).

3.2. Verification of ISDPR products

First of all, it was essential to confirm the polymerization as expected for amplification of DNA detection signal. As shown in Fig. 2, in the presence of dNTPs and polymerase, the CL signal of the sensing system (column D) was significantly greater than that in the absence of dNTPs and polymerase (column C), showing obvious signal amplification. In the absence of the target DNA, the CL intensity for the mixture of polymerase and dNTPs was also very low (column B), which was a little higher than the blank (column A), indicating that polymerization reaction was triggered unobviously. Thus the DNA target could hybridize with the immobilized MB1 to open the cycle of MB1 and the opened stem part of MB1 could then bind the primer1 to the surface of MMPs to initiate the polymerization of DNA strand, which led to the release of the target DNA for rehybridization with another immobilized MB1. The ISDPR amplified the attachment of biotin-modified primer1 on surface of MMPs and greatly improved the sensitivity of detection.

The above results were further confirmed with 18% PAGE analysis (Fig. 2, inset). 1 μM MB1 exhibited only one band (lane a). After 0.1 μM HT1 and 1 μM primer1 were added to the solution, the sample also exhibited one band (lane b), which was similar to lane a. After polymerase and dNTPs were further added to the mixture, a new product band with a slow migration speed was observed (lane c). This result should be contributed to an intermediate product of MB1-target-polymerization DNA complex existing in the process of primer extension before the target was displaced, verifying the polymerization process.

3.3. Optimization of detection conditions

The parameters affecting ISDPR process and the CL detection were investigated. Fig. 3a depicted the influence of the amount of polymerase used in assay on the relative CL intensity. The relative CL intensity increased with increasing the amount of polymerase in the range from 1 to 4 U and then leveled off in the range from 4 to 5 U. Therefore, 4 U of polymerase was selected in this assay. The incubation time of the duplication process was examined from 0 to 3.0 h (Fig. 3b). After 2.5 h the relative CL intensity reached a plateau, so 2.5 h was chosen as the duplication time.

The amount of functionalized MMPs influenced the MBs amount on the surface of the MMPs and blocked the CL during measurement. The relative CL intensity increased with increasing the amount of functionalized MMPs in the range 25–50 μg and decreased quickly in the range 50–125 μg (Fig. 3c). The reason for this phenomenon is that the amount of functionalized MMPs influenced the MB amount on the surface of the MMPs and blocked the CL during measurement. Therefore, 50 μg of functionalized MMPs were used for the following experiments. The amount of primer1 also affected the relative CL intensity. The CL intensity increased with increasing primer1 amount until 20 nM and then decreased slowly (Fig. 3d). Thus, 20 nM of primer1 was selected for this study.

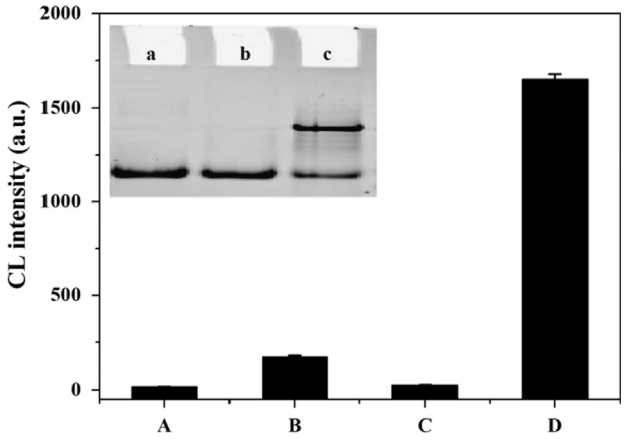

Fig. 2. The CL intensities of the sensing system in the presence of 50 μg MMPs (A), 50 μg MMPs, 4 U polymerase and 20 μM dNTPs (B), 50 μg MMPs and 20 pM HT1 (C), 50 μg MMPs, 20 pM HT1, 4 U polymerase and 20 μM dNTPs (D). Inset: PAGE analysis of the products via the ISDPR with 1 μM MB1 (a), 1 μM MB1, 1 μM primer1 and 0.1 M HT1 (b), 1 μM MB1, 1 μM primer1 and 0.1 μM HT1 in the presence of 4 U polymerase and 20 μM dNTPs (c).

3.4. Sensitivity for the target DNA detection

The system of the HRP-catalyzed luminol-PIP-H_2O_2 reaction with PIP as the enhancer, allowing a higher sensitivity and steady-state light signal, is suitable for CL imaging detection. Under optimal conditions, the DNA assay was performed with a series of target DNA (HT1) concentrations. As shown in Fig. 4 inset, with the increase of the concentration of HT1, the CL intensity increased gradually. The plot of the relative CL intensity versus HT1 concentration was also shown in Fig. 4. A good liner relationship could be obtained under the optimal conditions. The linear range was from 1 to 50 pM with linear equation $\Delta I = 65410.781 C_{HT1} + 49.325$, where ΔI is the relative CL intensity and C_{HT1} is the concentration of HT1 ($R^2 = 0.9979$). The limit of detection (LOD) for the target HT1 was calculated to be 0.4 pM, which corresponded to the detection of 40 amol of the target DNA in a 100 μL of sample solution.

3.5. Selectivity of the target DNA detection

In this study, we evaluated the specificity of this method. The assay was challenged with single-base mismatched target DNA (MT1), two-base mismatched target DNA (MT2) and three-mismatched target DNA (MT3). As shown in Fig. 5, the addition of the mismatched DNA targets only led to very slight CL intensity

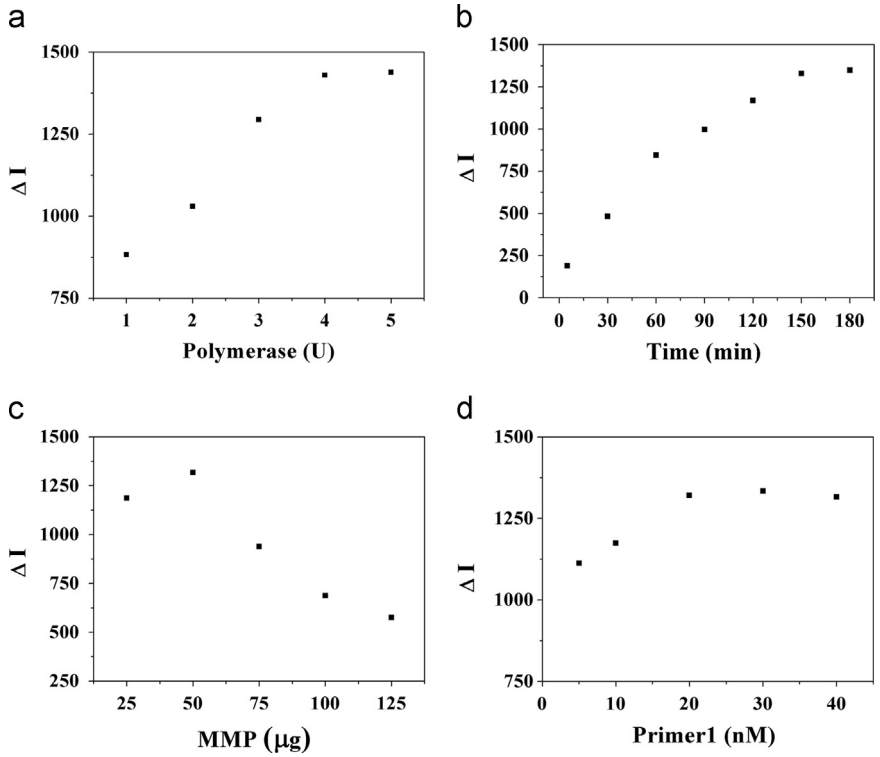

Fig. 3. Effects of (a) the amount of polymerase, (b) the duplication time of the ISDPR reaction, (c) the amount of MMPs and (d) the amount of primer on the relative CL intensity of the sensing system.

increase. This high specificity arose from the conformational constraint of the stem-loop structure of MB (Bonnet et al., 1999); that is, the presence of the stem made it thermodynamically unfavorable for the binding of the mismatched sequences to the loop. The proposed method can provide an excellent capability in differentiating between perfectly matched and mismatched DNA targets. Therefore, this proposed method has a high selectivity, and it holds a great opportunity to allow SNP analysis.

3.6. Multiplexed detection using ISDPR

It has been recognized that simultaneous detection of multiple targets held new promise in molecular diagnostics (Song et al., 2009; Xiang et al., 2011). The Fig. 1A, however, needed to design specific functionalized MMPs for every target DNA. In order to resolve this apparent limitation, the short primer2 functionalized MMPs were used as a versatile probe (Fig. 1B). Only need to change MB, the biosensor could be expanded for the high throughput DNA detection in a microarray. Under optimal conditions (Fig. S1), the DNA assay was performed with a series of target DNA concentrations. The linear range was from 5 to 100 pM with linear equation $\Delta I = 26156.300 C_{HT2} - 8.855$, where ΔI is the relative CL intensity and C_{HT2} is the concentration of HT2 ($R^2 = 0.9970$), with a detection limit of 1 pM. As shown in Fig S3, the method could also provide an excellent capability in differentiating between perfectly matched and mismatched DNA targets.

4. Conclusions

In conclusion, a highly sensitive and multiplexed DNA detection platform based on ISDPR and functionalized MMPs has been developed. The results indicated that this novel method could detect as low as 0.4 pM target DNA owning to the dual amplification of the assay. By switching the MBs, the proposed CL imaging method could be expanded for the high throughput DNA detection when primer-labeled MMPs were used as a probe in a microarray. Therefore, the method presented here will provide a universal platform to detect various DNA at an ultrasensitive level in biomedical and bioanalytical applications.

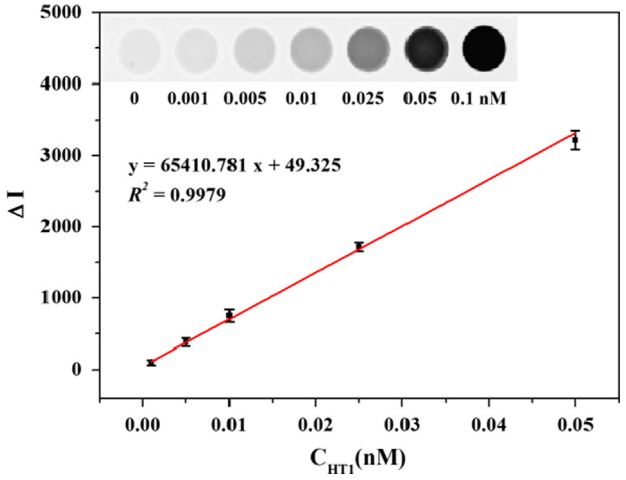

Fig. 4. The linear relationship between the relative CL intensity and the concentration of the target DNA ($\Delta I = I - I_0$, I_0 and I are CL intensities in the absence and the presence of HT1, respectively). Inset: CL images of the target DNA at different concentrations. Experimental conditions: 50 μg MMPs, 20 nM primer1, 4 U polymerase and 20 μM dNTPs.

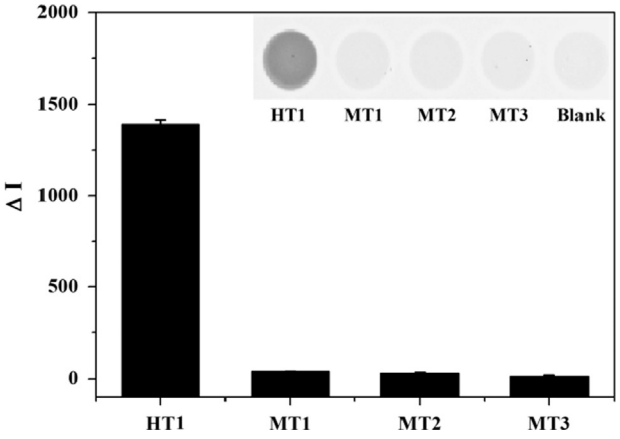

Fig. 5. The relative CL intensities histogram of the sensing system in the presence of 20 pM of the complementary target DNA (HT1), single-base mismatched target (MT1), two-base mismatched target (MT2) and three-base mismatched target (MT3). Inset: CL images of the sensing system in the absence (Blank) and the presence of 20 pM of the complementary target DNA (HT1), single-base mismatched target (MT1), two-base mismatched target (MT2) and three-base mismatched target (MT3). Experimental conditions: 50 μg MMPs, 20 nM primer1, 4 U polymerase and 20 μM dNTPs.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.11.066.

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