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PAPER

Amplified electrochemiluminescence detection of nucleic acids by hairpin probe-based isothermal amplification

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Here, we present a straightforward method for isothermal amplified detection of nucleic acids. In this proof-of-concept study, a specific DNA sequence is amplified through hairpin probe-based isothermal strand-displacement polymerization reaction and then detected *via* a sensitive and commercially available ECL detection platform. Results show that the DNA sequence derived from the *Listeria monocytogenes hly* gene can be detected down to 10 pM in solution, together with correlation of the detected signal with the initial concentration of target DNA. Moreover, the designed stem–loop structured hairpin probe shows single-base variation differentiating ability. Considering the superior sensitivity and specificity, as well as the simple-to-implement features, the developed assay demonstrates a great potential of becoming a first-line tool for quantitative analysis of nucleic acids for biomedical research.

Introduction

Advances in diagnostic assays require innovative techniques that are sensitive, specific, and precise enough to quantify low levels of biological targets. Detection of specific sequences of DNA is vital for the detection of pathogenic infections, genetic diseases, forensic analysis, and the development of modern life sciences.[1–15] Analysis of DNA by amplification-based strategies is the most important concept since those strategies allow the highest analytical sensitivity. In order to enhance the sensitivity of a gene assay, one can either amplify the number of genes present in a sample or amplify the reporting signal produced by each of the target genes. PCR has become a mainstay of biological research and diagnostics for the rapid detection, isolation, and measurement of DNA sequences by their specific amplification. However, great care must be taken in the optimization of primer sequences to prevent amplification bias, and the need for a temperature cycling machine also imposes instrumentation constraints on the PCR method.[16,17] Therefore, its application in point of-care testing (POCT) is limited. Moreover, PCR is not suitable for applying to short-length DNA and RNA due to the difficulty in primer design.[18]

Thus, the need for a DNA assay with good selectivity, simplicity, and cost effectiveness in a wide range of applications has provided the driving force for the continuous development of new strategies and technologies. Recently, several isothermal nucleic acid amplification methods, such as strand displacement amplification (SDA),[19–21] helicase dependent amplification (HAD),[22] rolling circle amplification (RCA),[23–25] loop-mediated amplification (LAMP),[26] nucleic acid sequence-based amplification (NASBA),[27] and transcription-mediated amplification (TMA),[28] have been developed in order to overcome these limitations. Generally, these techniques can be classified into two main types according to whether they employ single primer for linear amplification and two primers or multi-primers for exponential amplification. For exponential amplification, the exponential nature of the reaction makes it difficult to obtain absolute quantitative data. Theoretically, linear amplification is quantitative but has limited sensitivity. The goal of the study is to develop an assay capable of achieving the ultra sensitive nucleic acid detection under isothermal linear amplification conditions with excellent specificity and quantitative ability. Motivated by recent advances in isothermal nucleic acid amplification, probe design, and detection tools,[29,30] we developed an amplified DNA assay using linear SDA and magnetic beads-based electrochemiluminescence (ECL) detection. In recent years, ECL, where light-emitting species are produced by reactions between electrogenerated intermediates, has been demonstrated to be a highly sensitive detection technique and a powerful analytical tool for quantifying amplified DNA.[31–37] ECL has some advantages, such as no radioisotopes are used, high sensitivity, wide dynamic range, the extremely stable labels, and the measurement is simple and rapid. High sensitivity is achieved by combining the amplified potential of SDA and the central merits of ECL detection, which is one of the most sensitive detection techniques. Good specificity is anticipated by the employment of a hairpin structural recognition probe. To demonstrate its potential in future application, the assays were performed on a commercial Roche Elecsys 2010 analyzer. As a proof-of-concept study, a DNA

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sequence derived from the *Listeria monocytogenes hly* gene is used as the target to validate the assay. The current method allowed the rapid detection of this target with a dynamic range from the picomolar to the nanomolar range. It has also been shown specifically to detect high homologous variants with single-base and two-base mismatches. These features should make current developed assay useful for various applications involving the detection of short DNAs, microRNAs, and the parallel analysis of single nucleotide polymorphisms of a large number of genes.

Experimental

Materials

The polymerase Klenow fragment exo- was purchased from New England Biolabs, Inc. Deoxynucleotide solution mixtures (dNTPs) were purchased from TaKaRa Bio Inc. (Dalian, China). Streptavidin microbeads (2.8 μm diameter) were products of Dynal Biotech (Lake Success, NY, USA). The chemicals to synthesize the $Ru(bpy)_3^{2+}$ *N*-hydroxysuccinimide ester (TBR-NHS ester) were purchased from Sigma (Louis, MO, USA). TPA buffer is purchased from Roche Diagnosis Corporation. 5′-Amino-modified hairpin probe, biotin-labeled primer, and target DNA were synthesized and HPLC purified by TaKaRa Bio Inc (Dalian, China). Their sequences were listed in the Table 1. The 5′-amino-modified hairpin probe was labeled with TBR-NHS ester according to our previous published paper.[1] Other chemicals employed were of analytical reagent grade and were used as received.

Hairpin probe labeling

TBR-NHS ester was synthesized according to the previous paper.[37] DNA hairpin-TBR labeling was accomplished by the following procedures. Briefly, 2 OD amino-modified DNA were dissolved in 50 μL 100 mM sodium bicarbonate buffer (pH 8.5), then 30 μL 10 mM TBR-NHS ester was added to the solution. This mixture was left to react in the dark and was gently shaken during 10 h. Labeled DNA was precipitated by the addition of cold absolute ethanol. The mixture was kept for 30 min at −20 °C and then centrifuged for 20 min at 12 000 rpm. The supernatant was removed and the pellet was rinsed twice with cold 80% ethanol. The pellet was allowed to dry under vacuum during 10 min, then was dissolved in pure water and stored at −20 °C until use.

Amplified detection of DNA target

Before the amplification reaction, TBR-labeled hairpin probes were heated to 80 °C, then the solutions were slowly cooled to room temperature for hairpin structure formation. Amplification experiments were performed in 50 μL solution consisting of 2.0×10^{-7} M TBR-labeled hairpin probe, 2.0×10^{-8} M biotin-labeled primer, 5U polymerase Klenow fragment exo-, 100 μM dNTPs, 1 mM DTT, 10 mM $MgCl_2$ and 50 mM NaCl in 10 mM Tris–HCl (pH 7.9) A series of targets at different concentrations were then added to every mixture solution and incubated at 37 °C for 1 h. The resulting products were detected using a Roche 2010 ECL detection platform. The process of the detection of DNA products was similar to that of immune assay. Firstly, products were added onto the sample cells, 10 μL samples was aspirated into an incubation reservoir using a robotic suction spindle. Secondly, binding buffer (10 mM Tris–HCl (pH 7.9), 300 mM NaCl) and streptavidin-coated magnetic beads were aspirated into the same incubation reservoir, and the reaction mixtures were incubated for 9 min at 37 °C. Finally, the liquids in the incubation reservoir were completely transferred into the flow ECL detection cell. The products were captured onto the surface of the working electrode, the unwanted probes were washed out, and resulting magnetic beads-captured products were detected by ECL. Photonics can be read out instantaneously after the measurements. Each run of two paralleled samples was analyzed by the employment of the calibration modes with the Roche 2010 instrument. The whole detection process can be completed automatically in 18 min.

Gel electrophoresis

Polymerization products were analyzed on a Biorad (Bio-Rad Laboratories, USA) slab electrophoresis system. 10 μL samples were loaded onto a 15% native polyacrylamide gel (29 : 1 acryl : bisacryl) in 0.5× Tris-borate-EDTA (TBE). Gels were run at room temperature for 45 min at 120 V. The gel was confirmed by SB green I staining and photographed by a Biorad imaging system.

Results and discussion

Assay design

The new nucleic acid detection protocol that combines isothermal SDA and an automatic ECL detection platform is shown in Fig. 1. A hairpin probe was designed for specific recognition of a DNA sequence derived from the *Listeria*

Table 1 DNA sequences and modifications

DNA	Sequence (5′–3′)
Biotin-primer	Biotin-AGACTCACAC
TBR-labeled hairpin probe	TBR-ACTCA*CACTGCATCTCCGTGGTATACTAA*CAGTGTGAGT[a]
Target (derived from *hly* gene)	TACAATAAAAACAATGTATTAGTATACCACGGAGATGCAGTG
Single-base-mismatched target	TACAATAAAAACAATGTATTAGTATAACACGGAGATGCAGTG
Two-base-mismatched target	TACAATAAAAACAATGTATTATTATACCACGTAGATGCAGTG
Random sequence	GCCTGCAAGTCCTAAGACGCCAATCGAAAAG

[a] Italic bases show the target binding region, and the underlined bases show the primer binding region.

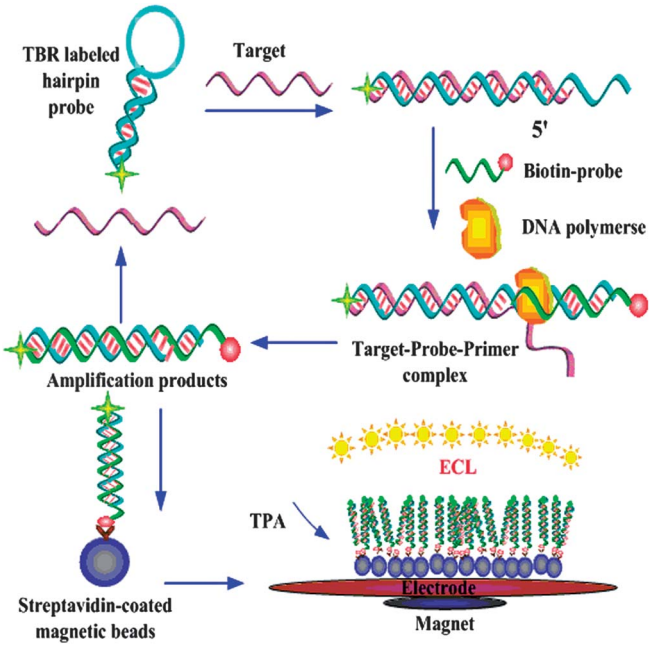

Fig. 1 The mechanism of the signal-amplified ECL DNA sensor based on isothermal strand-displacement polymerization.

monocytogenes hly gene, which is used as the target to validate the assay. The metastable state hairpin probes contain a loop-and-stem structure with a tris(bipyridine) ruthenium (TBR) linked to the 5′-end of the stem. It acts as a template of the polymerization reaction and as an ECL signal carrier. Hybridization between the hairpin probe and target DNA induced the activation of this DNA detection system which is based on the conformational change. The stem of the hairpin probe is a 10-nt long sequence, and the loop is complementary to the target. The primer (10-nt long sequences) is 8 bp complementary to the stem region of the probe at the 3′-end. In the absence of a target, it is hypothetical that the stem–loop conformational probe is in a metastable state and thus unable to anneal with the primer to induce a polymerization reaction. In the presence of the *hly* gene, which is functioned as the 'DNA trigger' and hybrid to the loop of the hairpin probe, this causes the hairpin probe to adopt the open form. As a result of activation, the biotinylated primer can anneal with the open stem and initiate the DNA polymerization reaction in the presence of dNTPs/polymerase. The target gene can be displaced by the polymerase with strand-displacement activity. Fully complementary double-strand DNA products were synthesized by biotinylated primer extension. After the extension reaction, the displaced target DNA is then hybridized to another hairpin probe to trigger cycle-after-cycle amplification. Double-strand DNA products carry the biotin label and the TBR label in its two ends. The resulting products can be easily detected using a magnetic ECL platform. After the amplificationt, the product detection process can be completed within 18 min without any manual operations.

Design of the hairpin probe

The success of the current assay is mainly dependent on the design of the hairpin probe. The stem of the current hairpin probe is designed to a 10-nt long sequence, and the loop is complementary to the target. The primer is a 10-nt long sequence, which is complementary to the stem region of the probe at the 3′-end. We first tested whether the designed hairpin probe works well. In fact, the hairpin probe has been reported to distinguish very similar sequences with high efficiency. In current experiments, due to the consideration of primer binding specificity, the stem of the hairpin sequence (10-nt) is designed to be longer than a classical molecule beacon-based probe, which usually contains a 5–6-nt long stem.[38] In our preliminary study, only the loop region of the hairpin probe is designed to be complementary to the target. However, we have found that this strategy did not allow the target nucleotide to bind to the loop and open the hairpin efficiently (data not shown). It may be due to the hybridization between target DNA and the loop being insufficient to disturb the metastable state structure protected by the long stem. In this case, it is unable to release the single-stranded stem sequence for primer binding. We next executed another strategy by the addition of a 5-nt stem sequence complementary to the target. The combination of hairpin probe and improved hybridization design appears to provide highly specific detection. In this proof-of-concept study, a DNA sequence derived from the *Listeria monocytogenes hly* gene is used as the target. As shown in Fig. 2, lines 1–4 show the non-denaturing PAGE analysis results of products from the amplification of 0 nM target DNA, 0.5 nM target DNA, 5 nM target DNA, and 5 nM random sequence. The reaction was visualized using gel electrophoresis which revealed the presence of the unreacted hairpin probe and the proposed duplex products. In the control experiments where duplex products do not appear, this indicates that the non-specific amplification reaction did not occur in the system. Addition of the target DNA into the system can lead to the formation of duplex products. The absence of

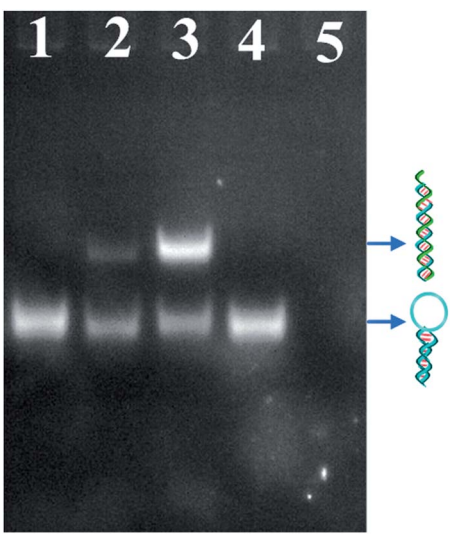

Fig. 2 Evaluation of the designed hairpin probe-based amplification assay by non-denaturing PAGE analysis: (1) hairpin probe + primer + polymerase mixtures, (2) 0.5 nM target DNA + hairpin probe + primer + polymerase mixtures, (3) 5 nM target DNA + hairpin probe + primer + polymerase mixtures, (4) random sequence + hairpin probe + primer + polymerase mixtures, and (5) primer only. It is noted that the primers were run on the gel.

duplex products in detecting random sequences also indicates that the system can only be initiated by the correct target sequence, as expected.

Optimization of amplification time

After the evaluation of the feasibility of the hairpin probe, there are also several factors which would affect the detection efficiency that need to be optimized. Amplification time is one of the core factors for amplification-based assay. In order to obtain the high assay sensitivity, the relationship between the amplification duration and ECL intensity was evaluated carefully. The concentration of target DNA was set at 5 nM for the optimized experiments. The amplification time was varied from 0 to 120 min with an interval of 30 min. As can be seen in Fig. 3, the ECL intensity maintained its increase with amplification time from 0 to 120 min, indicating that the continuous formation of double-strand DNA products and recycle amplification occurred. However, the signal exhibited no further significant increase when the reaction time went beyond 60 min. It may be attributed to the fact that the activity of the DNA polymerase had decreased and the dNTPs were used up. Therefore, 60 min was selected as the optimum amplification time in the following experiments.

Assay performance

To investigate the detection ability of the described amplification strategy, a series of different target DNA concentrations from 0 to 10 nM were measured under the 60 min amplification time. Fig. 4 shows that while the concentration of target DNA increases gradually, the intensity of the ECL signals rises accordingly. It also shows that the ECL signal of the blank control without target DNA is 646 ± 53 cps, thus the detection threshold value is set as 805 cps (mean of blank control plus three times SD). Note that the signal of 10 pM of target DNA can still be clearly identified from the threshold value, and thus in current system we estimate that the limit of DNA detection is 10 pM. It is noted that some recently developed hairpin probe-based amplification assays for DNA detection have achieved impressive

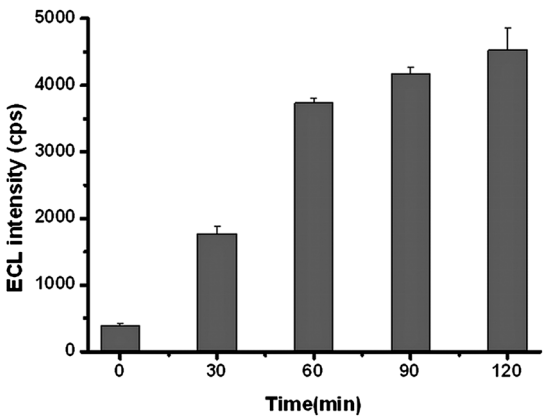

Fig. 3 ECL intensities plotted against time of hairpin probe-based amplification reaction. The assay exhibits a saturated signal within 90 min after the addition of 5 nM target DNA. Mean values and standard deviations are obtained from three independent experiments.

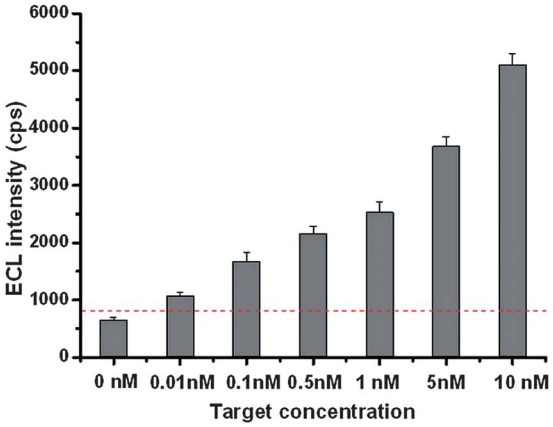

Fig. 4 Sensitivity of current assay. Hairpin probe-based amplification strategy and automatic ECL detection technique were combined for the analysis of 0 nM (control), 0.01 nM, 0.1 nM, 0.5 nM, 1 nM, 5 nM, 10 nM target DNA. Mean values and standard deviations are obtained from at least three independent experiments.

sensitivity: the reported fluorescence assay has a sensitivity of 6.4 fM,[19] an electrochemical assay with a sensitivity of 0.06 fM,[21] and a nanoparticle-assisted electrochemical method can detect as low as 1.5 fM of target DNA.[9] Although the sensitivity data of the current assay was incomparable to some previously developed hairpin probe-based amplification methods, however, we feel that the current assay has the potential utility that makes it worthwhile to optimize the amplification conditions. Moreover, it could be argued that some methods require more processing as compared with the current strategy. For example, electrochemical-based biosensors requiring pretreatment of the electrode, or the preparation of nanoparticle labeling. Based on the comparisons, it was evident that although each scheme has its particular advantages, each also demonstrates obvious disadvantages or limitations involved in aspects of sensitivity, simplicity or selectivity. However, our biosensor has a good balance between sensitivity and ease of operation.

It is worthy of note that the proposed method is compatible with a commercial ECL detection platform. In this platform, magnetic beads are introduced as the carrier, and thus the electrode material can be re-used rapidly and simply. As a result, the detection process can be completed rapidly and the detection cost is greatly reduced. This capability is indeed a desirable feature for future potential applications.

We then examined whether the hairpin probe-based amplification strategy is specific. The specificity of the sensor was determined by challenging it with single-base mismatched target and two-base mismatched target (see in Fig. 5). Control experiments were carried out in the absence of any target oligonucleotides. As expected, the ECL signals show a slight increase after treatment with single-base mismatched target compared to the control. Further signal enhancements were also investigated when two-base mismatched target was detected. However, a remarkable signal enhancement was obtained for measuring perfectly matched target. Accordingly, the designed strategy is highly selective that allows one to differentiate high homologous variants. We feel that the current developed gene detection assay is particularly suitable for detecting short-length DNA and

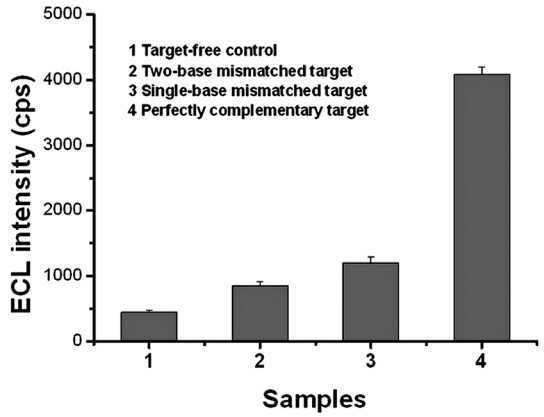

Fig. 5 Test of selectivity among closely related DNAs. Control (0 nM), 5 nM two-base mismatched target DNA, 5 nM single-base mismatched target, and 5 nM target DNA were determined. Mean values and standard deviations are obtained from at least three independent experiments.

RNA. For example, miRNAs, which contain only 19–23 nucleotides, with limited size and high sequence homology, are highly variable in their expression levels. These features place significant demands on microRNAs detection techniques. The current developed assay achieved a remarkable sensitivity (pM levels) and single-base variation differentiating ability that could obviously be an attractive tool for studying miRNA expression patterns.

Conclusions

Our experiments show that specific DNA targets can be successfully detected by combining a single-tube amplification experiment with a commercially ECL detection machine. The whole detection process can be completed within 18 min without manual operation. Moreover, the current developed hairpin probe-based amplification strategy achieved a remarkable sensitivity (pM levels) and single-base variation differentiating ability. The method reported here is advantageous over thermal cycling methods in that it is isothermal, and the amplification reaction can be carried out at one temperature and so only a simple heat block is required. Finally, this technique may not be confined to the detection of target DNA, and should be suitable for the detection of RNAs, especially miRNAs, which are the most important and challenging RNA targets for detection.

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