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Ligation-Based High-Performance Mimetic Enzyme Sensing Platform for Nucleic Acid Detection

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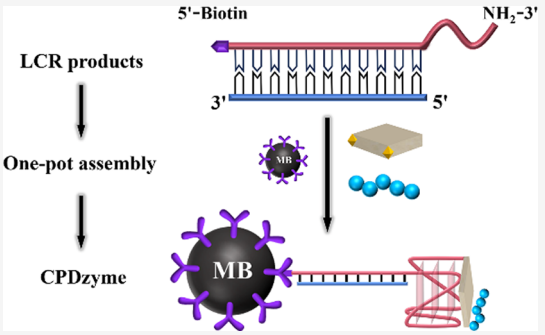
ABSTRACT: G-quadruplex (G4)/hemin DNAzyme is a promising candidate to substitute horseradish peroxidase in biosensing systems, especially for the detection of nucleic acids. However, the relatively suboptimal catalytic capacity limits its potential applications. This makes it imperative to develop an ideal signal for the construction of highly sensitive biosensing platforms. Herein, we integrated a novel chimeric peptide-DNAzyme (CPDzyme) with the ligase chain reaction (LCR) for the cost-efficient and highly sensitive detection of nucleic acids. By employing microRNA (miRNA) and single-nucleotide polymorphism detection as the model, we designed a G4-forming sequence on the LCR probe with a terminally labeled amino group. Subsequently, asymmetric hemin with carboxylic arms allowed assembly with the LCR products and peptide to form CPDzyme, followed by the magnetic separation of the extraneous components and chemiluminescence detection. Compared with the conventional G4/hemin signaling-based method, the LCR-CPDzyme system demonstrated 3 orders of magnitude improved sensitivity, with accurate quantification of as low as 25 aM miRNA and differentiation of 0.1% of mutant DNA from the pool containing a large amount of wild-type DNA. The proposed LCR-CPDzyme strategy is a potentially powerful method for in vitro diagnostics and serves as a reference for the development of other ligation- or hybridization-based nucleic acid amplification assays.



Nucleic acids, including deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), are among the four major types of macromolecules essential to all known lifeforms; these macromolecules store, transmit, and express genetic information.[1−3] The regulation of gene expression is intricately associated with disease development and aberrant expression of key genes may lead to the onset and progression of a variety of diseases.[4,5] Therefore, nucleic acids are considered to be important biomarkers for biological research and medical diagnosis. In vitro nucleic acid amplification (NAA) is a powerful tool and has been widely used over the past 40 years.[6,7] Although several techniques have been developed for rapidly detecting nucleic acids using synthetic biomolecular components,[8−11] highly sensitive and cost-effective NAA assays are still required for various applications; for instance, to be used for detecting clinical biomarkers and pathogens.[12,13] Polymerization-based amplification assays, such as polymerase chain reaction (PCR),[14] exponential amplification reaction,[15,16] loop-mediated isothermal amplification,[17] and recombinase polymerase amplification,[18,19] can be easily implemented and monitored by designing specific DNA probes or using nucleic acid-specific dyes. Nevertheless, some ligation or hybridization-based NAA techniques, such as catalytic hairpin assembly, hybridization chain reaction, and ligase chain reaction (LCR), are very challenging for the commonly used monitoring methods.[20−22]

LCR employs thermostable ligases for allele recognition, which is one of the recently developed amplification techniques for detecting point mutations and microbial pathogens.[23−26] However, the detection of LCR products is challenging and usually requires the use of electrophoresis for the separation.[27] Although homogeneous detection methods based on FRET and CRISPR/Cas12a have been developed in recent years,[28,29] they are not favored because of high costs and complex optics. Thus, developing a simple and universal biosensing platform that can be used for ligation- or hybridization-based amplification is desirable.

G-quadruplex (G4)/hemin is a class of DNAzymes with peroxidase-mimicking activity; these enzymes are typically formed by G-rich DNA in the presence of metal ions and cofactor hemin.[30,31] Since G4/hemin can be edited and easily integrated with NAA technologies, it is considered an ideal signal element for the analysis of nucleic acids, proteins, and small molecules.[32−34] Furthermore, G4/hemin can act on

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different substrates and produce colorimetric, chemiluminescent, fluorescent, and electrochemical signals. However, The unsatisfactory catalytic performance of G4/hemin severely limits its application in different biosensing platforms.[35] To address this issue, researchers have improved the catalytic activity of G4/hemin by adding exogenous enzyme enhancers (such as ATP),[36] optimizing DNA sequences,[37,38] and chemically conjugating hemin to G4.[39,40] However, these efforts have not significantly improved the catalytic performance of G4/hemin and are often accompanied by undesirable background signals.

Recently, our group has reported a novel chimeric peptide-DNAzyme (CPDzyme) based on the covalent assembly of DNA, cofactor hemin, and peptide in a single scaffold.[41] The catalytic activity of CPDzyme is approximately 2000 times higher than that of G4/hemin. This unique performance is based on a series of incremental improvements that ensure efficient and stable stacking of hemin on the G4. In addition, the peptide provides a catalytic microenvironment for hemin similar to that of horseradish peroxidase (HRP), fulfilling the role of an iron-assisted activator. The high catalytic activity and editability of CPDzyme make it a promising candidate for catalysis and biosensing. Chemiluminescence (CL), the generation of light produced by chemical reactions, has become an attractive tool for biochemical research.[42] By eliminating the need for external optical excitation, CL effectively avoids background interference and provides a high signal-to-noise ratio and sensitivity for biosensing and bioimaging.[43,44]

In this study, the LCR was chosen as the instance to take full advantage of CPDzyme's high performance. To achieve that purpose, one pair of LCR probes was designed and specially labeled to ensure the formation of the CPDzyme and allow separation by using the streptavidin-modified magnetic beads (SA-MBs). The LCR-CPDzyme assay was then used to detect microRNA (miRNA) and single-nucleotide polymorphism (SNP) for potential clinical applications. Here, we demonstrated that this novel low-cost, high-sensitivity signal element can be seamlessly integrated with the LCR. The sensitivity of LCR-CPDzyme was 3 orders of magnitude higher compared to that of G4/hemin-based assays. LCR-CPDzyme opens up new avenues for the ultrasensitive and cost-effective detection of nucleic acids and establishes a favorable signaling bridge for the development of novel biosensors.

EXPERIMENTAL SECTION

Materials and Reagents. Thermostable ampligase and 10× reaction buffer were purchased from LGC Biosearch Technologies (CA, USA). SplintR ligase and 10× reaction buffer were obtained from New England Biolabs (MA, USA). All DNA oligonucleotides used in this study were synthesized by Sangon Biotechnology (Shanghai, China). Reverse transcriptase M-MLV (RNase H−), dNTP mixture (10 mM), and synthesized miRNAs were obtained from Takara Biotechnology Co., Ltd. (Dalian, China). SA-MB was purchased from Thermo Fisher Scientific Co., Ltd. (MA, USA). NHS (*N*-hydroxysuccinimide)-hemin-NHS was purchased from Xi'an Rui Xi Biotechnology Co., Ltd. (Xi'an, China), and the peptide was synthesized by GenScript Biotech Co., Ltd. (Nanjing, China). All other reagents used in this experiment were analytical grade and used without further purification.

Apparatus. The miRNA-dependent ligation reaction and LCR were performed using the Mastercycler nexus gradient thermal cycler (Eppendorf, Germany). The quantification of miRNA samples (extracted from cells) using a StepOnePlus real-time fluorescence PCR system (Applied Biosystems, USA). CL detection was performed by injecting H_2O_2 into the MPI-A system (Remex, China) through an MSP60-3A flow syringe pump (HUIYUWEIYE, China).

LCR for miRNA Detection. Probes A_2 and B_2 were specifically ligated using miR-221 as a template; this reaction (10 μL) was catalyzed by splintR ligase and included 20 nM probes A_2 and B_2, 1× reaction buffer, 8 U of recombinant RNase inhibitor, and an appropriate amount of miRNA solution. The reaction mixture was heated to 80 °C for 3 min and then incubated at 25 °C for 20 min. Subsequently, 5 U splintR ligase was added to the mixture, and the ligation reaction was carried out at 25 °C for 40 min. Finally, the reaction was terminated by heating at 80 °C for 10 min. LCR (20 μL) contained 1× reaction buffer, 100 nM probes A_2, B_2, A_2', B_2', and 2 μL ligation products. The mixture was predenatured at 95 °C for 3 min, and subsequently, 2 U of ampligase was added after cooling to 75 °C, followed by 45 thermal cycles at 95 °C for 30 s and 58 °C for 30 s.

LCR for SNP Detection. The LCR for SNP detection was designed without target pretreatment and was performed in a 20 μL system containing 1× reaction buffer, 200 nM probes A_1, B_1, A_1', B_1', and 2 μL of mutant or wild-type DNA. The mixture was first predenatured at 95 °C for 3 min, cooled to 75 °C with the addition of 2 U of ampligase, followed by 45 thermal cycles of 95 °C for 30 s and 66 °C for 30 s.

Specificity for Allele Frequency Detection. Mutant and wild-type DNA were premixed at various ratios (from 0 to 100%) to mimic samples with different mutation frequencies. The total concentration of the target DNA was 12.5 fM. LCR amplification and CL detection were performed as described earlier.

One-Pot Synthesis of CPDzyme and CL Detection. One-pot preparation of CPDzyme was carried out in a 40 μL system. This system contained 15 μL of LCR products, 0.1 mg/mL 4-dimethylaminopyridine, 5 μM of the peptide (KHRRH), 10 mM HEPES (pH, 7.0), and 5 μM NHS-hemin-NHS. This reaction mixture was oscillated at 1500 rpm for 1 h at 4 °C. Subsequently, 10 μL of 2 M KCl was added to the mixture, and the denaturation was conducted at 95 °C for 5 min, followed by slowly cooling to room temperature to form the assembled products. SA-MB (2 μL of 10 mg/mL) was resuspended in 48 μL of 2× BW (10 mM Tris−HCl, 1 mM EDTA, 2 M KCl, 0.2% Tween-20, and 2% DMSO) and mixed with the products of the above reaction in equal volumes. Finally, after shaking at 1500 rpm for 2 h at room temperature, the beads were washed three times with 1× BW buffer using magnetic separation and then resuspended to a volume of 30 μL.

CL intensity was recorded by injecting 200 μL of 300 mM H_2O_2 into a mixture that included 10 μL of resuspended products, 140 μL of TK buffer (10 mM Tris−HCl and 100 mM KCl, pH 7.0), and 50 μL of 1 mM luminol (dissolved in 0.1 M NaOH).

Cell Culture and Total Small RNA Extraction. Human umbilical vein endothelial cells (HUVECs), cervical cancer cells (HeLa), and human breast cancer cells (MCF-7) were cultured in Dulbecco's Modified Eagle's Medium which contained 10% fetal bovine serum, 100 μg/mL streptomycin, and 100 U/mL penicillin. All cells were cultured in a humidified incubator at 37 °C and maintained with 5% CO_2

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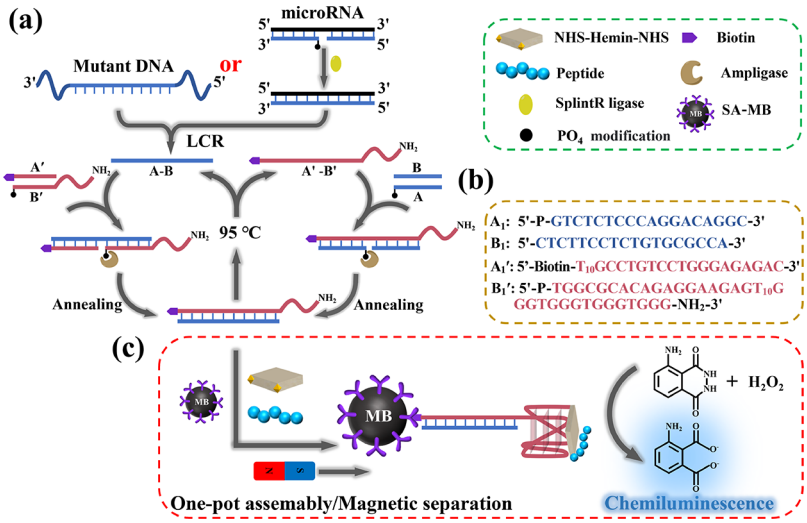

Figure 1. Schematic illustration of the LCR-CPDzyme system for SNP or miRNA detection. (a) LCR for SNP or miRNA detection. (b) Sequences of the LCR probes employed for SNP detection. (c) One-pot assembly of CPDzyme and CL detection.

and 95% atmosphere. Total small RNA was extracted according to the manufacturer's instruction of the RNAiso.

Quantification of miRNA in Cells Using RT-qPCR. A specific stem-loop primer for miR-221 reverse transcription was designed and used in the reverse transcription reaction (10 μL) that contained 1× M-MLV (H−) reaction buffer, 500 μM dNTPs, 50 nM stem-loop primer, 10 U Recombinant RNase inhibitor, 100 U M-MLV (H−), and an appropriate amount of RNA sample. The mixture was first incubated at 16 °C for 30 min, followed by incubation at 42 °C for 1 h. The reaction was terminated by heating to 75 °C for 15 min. Next, 1 μL of transcription products were transferred to a 20 μL qPCR system containing 200 nM forward and 200 nM reverse primers and 1× ChamQ SYBR qPCR Master Mix (Vazyme biotech, Nanjing). The mixture was predenatured at 95 °C for 3 min, followed by 50 thermal cycles (each cycle consisted of 95 °C for 30 s and 66 °C for 30 s) in the StepOnePlus real-time PCR system.

RESULTS AND DISCUSSION

Mechanistic Study of the LCR-CPDzyme Assay. Figure 1 shows the principles of the proposed strategy. In this approach, SNP and miRNA serve as detection models individually. To detect miRNA, one pair of probes was initially ligated using splintR ligase, and the ligated product was used as the template for initiating the LCR. The SNP site was found to be located in exon 8 of the P53 gene, with C/G and T/A in wild-type and mutant DNA, respectively. Table S1 describes the probe pairs practically used. As shown in Figure 1a, LCR involved two pairs of adjacent probes; after triggering by the template, the products of each ligation could be used as the template for the next cycle, followed by thermal cycling to realize exponential amplification. In addition, the inherent specificity of the ligase allowed discrimination of a single base mismatch in each ligation, enabling favorable specificity for the LCR. Besides the typical phosphate modification at the 5′ end of the ligation sites, the 3′ end of probe B′ incorporated a G4-forming sequence and was terminally labeled with an amino group (Figure 1b).

Subsequently, the hemin with asymmetric carboxylate arms could covalently assemble with both the amino group found in the LCR products and the peptide (Lys-His-Arg-Arg-His, KHRRH) to form the CPDzyme via amide bonds (Figure 1c). Furthermore, the 5′ end of probe A′ was labeled with biotin to achieve separation using SA-MB, whereas extra probes were washed off. In this proposed method, the main ligation products (AB/A′B′) were isolated after the formation of CPDzyme, which was employed to catalyze the luminol-H_2O_2 CL system for secondary amplification. As a result, the LCR-CPDzyme system enables the detection of nucleic acids in an ultrasensitive, cost-effective, and low-background manner.

LCR-CPDzyme System for miRNA Analysis. To verify the feasibility of the proposed strategy, the LCR products of miR-221 were tested by using 9% natural polyacrylamide gel electrophoresis (PAGE). Figure 2a shows the component

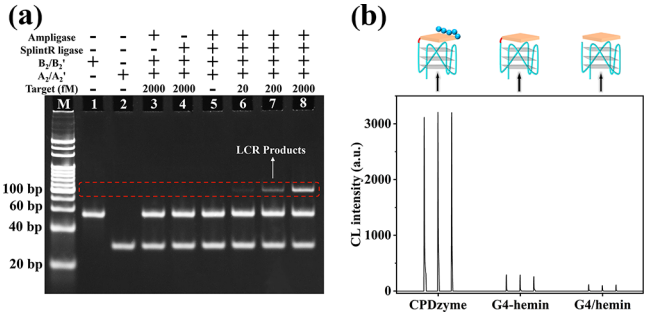

Figure 2. Validation of the feasibility of the proposed strategy. (a) Component deletion assays in the LCR, where the missing component is labeled at the top of Figure 2a, and the target used in the LCR was miR-221. (b) Comparison of the CL intensity of different types of peroxidase mimics using standard DNA.

deletion assay, and probes B_2/B_2' and A_2/A_2' were distributed with different migration rates. Moreover, no bands were observed in the absence of splintR ligase, ampligase, or target. The LCR products were observed only when all components were present, and the intensity of the bands was enhanced as the target concentration increased. We also prepared different types of peroxidase mimics using standard DNA (Table S1) as the virtual LCR products and compared their catalytic activities. As shown in Figure 2b, the difference between G4/hemin and G4-hemin is that NHS-hemin was used in the

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preparation of G4-hemin, so that hemin was covalently linked to G4 through the amide bond, whereas unmodified hemin was used in the preparation of conventional G4/hemin. All other experimental conditions were consistent with the synthesis of CPDzyme. The activity of CPDzyme was significantly higher than that of G4-hemin and G4/hemin, which is consistent with previous studies and fully proved the rationality of this design.[41]

The high performance of CPDzyme is attributed to the multiple selection and alignment of the components, enabling comparable performance to that of HRP.[41] Furthermore, after systematic and comprehensive optimization, we established the LCR-CPDzyme and LCR-G4/hemin systems under optimal conditions (Figures S1−S6) and compared their sensitivities. As shown in Figure 3, the LCR-CPDzyme system has

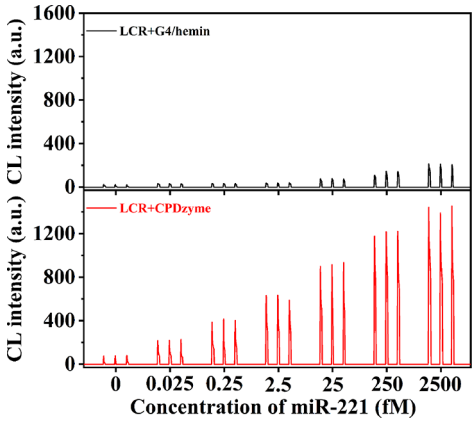

Figure 3. Comparison of the sensitivity of the assay by constructing G4/hemin and CPDzyme with LCR products (gradient concentrations of miR-221 were used).

significantly improved the detection sensitivity by at least 3 orders of magnitude compared with the LCR-G4/hemin system. This should be considered a breakthrough for miRNA detection. The editable property of CPDzyme will also allow this system to be fully integrated with other amplification techniques, further enhancing the sensitivity.

Subsequently, we determined the linear range of the LCR-CPDzyme system for the detection of miR-221 by using different concentrations of miR-221. The intensity of CL was enhanced gradually with the increased concentration of miR-221 (Figure 4a), and even a target concentration of 0.025 fM was significantly distinguished from the background. Meanwhile, as demonstrated in Figure 4b, a good linear correlation between the logarithm of miR-221 concentration and the intensity of CL was exhibited, and the limit of detection (3σ, n = 11) was estimated to be 18.6 aM, with a linear range of 25.0 aM−25.0 fM. The linear equation was $\Delta CL = 225.23 \lg(C/\text{aM}) - 149.43$, with Pearson's r = 0.9975. The relative standard deviation was calculated to be 5.2% by measuring 2.5 fM miR-221 seven times in parallel, demonstrating good reproducibility (Figure S7). Taken together, the above-mentioned results demonstrate that LCR-CPDzyme was able to accurately detect miRNA-221 of 3 orders of magnitude with high sensitivity and accuracy.

To evaluate the specificity of the LCR-CPDzyme system for miRNA detection, we confirmed that let-7a, miR-141, miR-155, miR-21, and miR-222, which were 10-fold in concentration than miR-221, also showed no significant differences

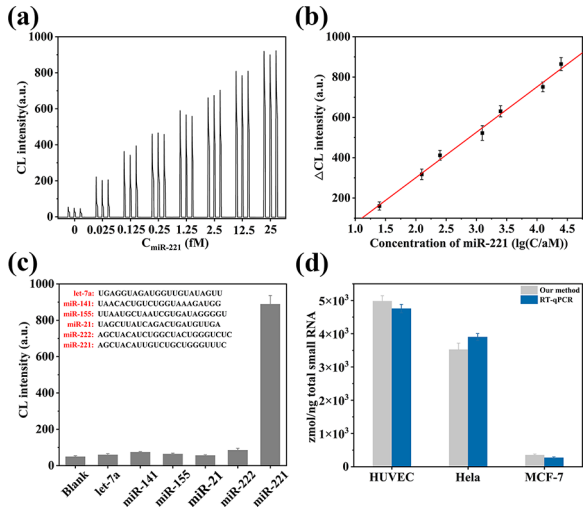

Figure 4. LCR-CPDzyme system used for miRNA detection. (a) CL intensity mediated by different miR-221 concentrations. (b) Relationship between relative CL intensity (background has been deducted) and the logarithm of miR-221 concentrations. (c) Evaluation of the specificity of the LCR-CPDzyme system for miRNA detection. The concentration of interfering substrates was 250 fM, and all experiment steps were performed in parallel. (d) Expression levels of miR-221 in HUVEC, Hela, and MCF-7 cells were determined by the LCR-CPDzyme system and compared with the results of the RT-qPCR. Error bars indicated the standard deviation of three parallel measurements.

compared with the blank control (Figure 4c). The intensity generated by miR-221 was significantly higher than that of other miRNAs, demonstrating the higher specific recognition ability of the LCR-CPDzyme system. This was attributed to the high-specific ligation of splintR ligase and ampligase.

Satisfied with the potential of LCR-CPDzyme as a tool for miRNA analysis, we shifted our attention toward more complicated biological issues, such as the quantification of miRNAs in different cells. As a proof of concept, the expression levels of miR-221 in HUVEC, Hela, and MCF-7 cells (2 ng of total small RNA) were determined, and the results revealed that the contents of miR-221 in HUVEC, Hela, and MCF-7 cells were 4980.1, 3527.5, and 354.6 zmol/ng, respectively. We also compared the results of the proposed method with that of RT-qPCR, which is considered the gold standard for miRNA detection; the results of both methods were consistent (Figures 4d, S8, and S9). As shown in Table S2, the recoveries of miR-221 ranged from 92.0 to 108.6%, demonstrating the application potential of the proposed method for the accurate quantification of miRNAs in real samples.

LCR-CPDzyme System for SNP Analysis. Next, we attempted to demonstrate the versatility of the LCR-CPDzyme system for SNP detection. SNP is one of the most common types of human genetic variation and is widely observed in the human genome. As demonstrated in Figure 5a, the CL intensity floated consistently with the concentration of mutant DNA, and even 2.5 aM of mutant DNA was clearly distinguished from the blank control. Meanwhile, the relative CL intensity exhibited a favorable linear correlation with the logarithm of the concentration of mutant DNA, and the limit of detection (3σ, n = 11) was estimated to be 1.8 aM, with a linear range of 2.5 aM−2.5 fM. The linear equation was $\Delta CL = 239.45 \lg(C/\text{aM}) + 92.34$ with Pearson's r = 0.9956 (Figure 5b). The improved sensitivity for mutant DNA detection

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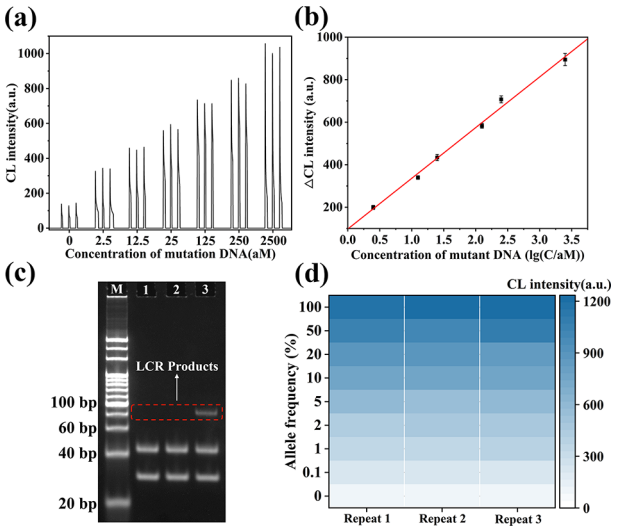

Figure 5. LCR-CPDzyme for the detection of SNP. (a) Intensity of CL is mediated by different concentrations of mutant DNA. (b) Relationship between the logarithm of mutant DNA concentration and relative CL intensity (background has been deducted). (c) LCR products were characterized by 9% natural PAGE. Lane M is the DNA ladder, lane 1 is the blank control, and lanes 2 and 3 are the products after thermal cycling of wild DNA and mutant DNA, respectively. (d) Blue depth of the heat map represents the CL intensity generated by the mixture of mutant DNA and wild DNA in different ratios, with a total concentration of 12.5 fM. Error bars indicated the standard deviation of three repetitions.

compared with miRNA is mainly explained by insufficient miRNA-dependent ligation. In the early diagnosis of a few diseases, the concentration of wild-type DNA was much higher than that of mutant DNA. To this end, the specificity of the LCR was confirmed by 9% natural PAGE. As revealed in Figure 5c, only mutant DNA triggered the amplification. Following this, we mixed the mutant and wild-type DNA in different ratios, and we can see from Figure 5d that the blue depth of the heatmap gradually enhanced as the allele frequency increased from 0 to 100%. To demonstrate the practicality of the proposed method and its performance in complex matrices, recovery assays were performed in 10-fold dilutions of human serum with the addition of different mutant DNA, and the recoveries were in the range of 94.0−108.0% (Table S3). These results demonstrated the applicability of the LCR-CPDzyme system for SNP detection. In comparison with other nucleic acid detection assays, as revealed in Table S4, the LCR-CPDzyme system exhibits the advantages of being cost-effective and having high sensitivity. In particular, the sensitivity of this study is much higher than that of other biosensing platforms by using G4/hemin.[45−47]

CONCLUSIONS

In conclusion, a novel LCR-CPDzyme system was developed for miRNA and SNP detection in this study. LCR could perfectly integrate high amplification efficiency and excellent base discrimination, ensuring highly sensitive and specific nucleic acid detection. Furthermore, the peroxidase-mimicking activity of CPDzyme improved significantly compared with that of other DNAzymes, and the inherent properties of CPDzyme provide great potential for the application of other ligation- or hybridization-based NAA approaches. In addition, the CL assay eliminates the need for sophisticated apparatus or trained personnel, enabling highly sensitive, cost-effective analysis. Finally, magnetic separation perfectly removed the unwanted components to provide a sufficiently low background. Taking the above merits together, the limit of detection of the proposed method is 18.6 and 1.8 aM for miRNA and mutant DNA, respectively. Even 0.1% of mutant DNA could be detected in the presence of a large proportion of wild-type DNA. The LCR-CPDzyme system as a powerful signal amplification strategy is promising to be applied in the highly sensitive detection of clinical biomarkers. Furthermore, CPDzyme can be integrated with DNA nanotechnology and novel functional nanomaterials in the future, which can significantly facilitate the development of biosensors and their application in biomedical analysis.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.3c04417.

The sequence of oligonucleotides used in the experiment, recovery assay in total small RNA and 10% human serum, comparison of the LCR-CPDzyme system with other nucleic acid detection assays, optimization of the experimental conditions, reproducibility of the miRNA assay, quantification of miR-221 in different cells by RT-qPCR (PDF)

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Author Contributions

All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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