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Original Research Article

A rapid nucleic acid detection platform based on phosphorothioate-DNA and sulfur binding domain

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ABSTRACT

Nucleic acid detection plays a key role in diverse diagnosis and disease control. Currently available nucleic acid detection techniques are challenged by trade-offs among speed, simplicity, precision and cost. Here, we described a novel method, designated SENSOR (Sulfur DNA mediated nucleic acid sensing platform), for rapid nucleic acid detection. SENSOR was developed from phosphorothioate (PT)-DNA and sulfur binding domain (SBD) which specifically binds double-stranded PT-modified DNA. SENSOR utilizes PT-DNA oligo and SBD as targeting module, which is linked with split luciferase reporter to generate luminescence signal within 10 min. We tested detection on synthesized nucleic acid and COVID-19 pseudovirus, achieving attomolar sensitivity combined with an amplification procedure. Single nucleotide polymorphisms (SNP) could also be discriminated. Indicating SENSOR a new promising nucleic acid detection technique.

1. Introduction

Nucleic acid detection plays a key role in clinical and analytical applications. The prevalent polymerase chain reaction (PCR) method requires expensive equipment and lacks point-of-care detection for samples [1]. Isothermal amplification methods such as recombinase polymerase amplification (RPA), rolling circle amplification (RCA), are usually not highly effective or sensitive [2]. Recently, new rapid and efficient nucleic acid detection systems have been developed based on novel enzymes like CRISPR-Cas and Agonaute [3–11]. In the CRISPR-based systems, the *trans*-cleavage activity of Cas proteins could be activated by recognition of target sequence and then the reporter oligo modified with fluorescent dye and quencher is cleaved to generate signal [3–7]. And the nickase activity [9] and binding activity [8] of Cas proteins could also be utilized for amplification of nucleic acid and for complementation of split reporter. In the Agonaute-based systems, the Agonaute proteins also cleave the reporter oligo in presence of guide DNA [10,11]. Since the recognition sequence of Cas and Agonaute proteins is programmable, these techniques could theoretically detect any specific sequence of samples. Despite remarkable progress, there are stills some limitations in these systems. The CRISPR-Cas proteins were restricted due to their reliance on the protospacer-adjacent motif (PAM), which significantly limited its application [12]. The CRISPR-based systems require handling of RNA and the Agonaute-based systems need expensive phosphate modified guide DNA and fluorescent dye and quencher modified reporter oligo. New techniques are required for trade-offs among speed, simplicity, precision and cost.

Recently, a new type of phosphorothioate (PT) modification, in which a non-bridging oxygen is replaced by a sulfur atom in DNA backbone [13], has been discovered in prokaryotes [14]. And the PT-dependent restriction endonucleases (REases) which specifically cleave PT-modified DNA were also discovered in bacteria [13–17]. PT-modification and PT-dependent REases comprising the PT-based bacterial defense system that plays a role in restriction on foreign DNA [18]. PT-dependent REases rely on a sulfur binding domain (SBD) to specifically recognize PT-DNA and make double strand break 5–30 bases away from the PT-modification site [16,17,19,20]. A typical SBD is monomeric, composed of ~160 amino acids, and contains a

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hydrophobic surface cavity. The SBD recognizes PT-DNA by embedding sulfur into its hydrophobic cavity, hydrogen bonds and electrostatic interactions between the SBD and the DNA also significantly contribute to binding [20,21]. SBD proteins have strong binding affinity on PT-DNA, especially for binding the PT-double-strand (ds)DNA. This allows SBD to be utilized as a targeting tool, in which synthetic PT-modified oligos were used as probes to anneal with target single strand DNA, and form a hemi PT-modified double strand to be recognized by SBD. Based on this, SBD has potential to be developed as a new tool in nucleic acid detection.

In this work, we developed SENSOR (Sulfur DNA mediated nucleic acid sensing platform), a novel *in vitro* detection platform based on PT-probe-guided SBD coupled with bioluminescent reporter, which allowed for rapid detecting nucleic acids with single-base specificity, and achieved attomolar (aM) sensitivity by introducing amplification procedure. It is suitable for infectious diseases detection and single nucleotide polymorphisms (SNP) identification. In addition, SENSOR uses stable, programmable, simple-design and inexpensive PT-modified probe. Considering these features, SENSOR holds great potential to provide an alternative solution for nucleic acid detection.

2. Materials and methods

2.1. Reagents and materials

Oligonucleotides used in this study were synthesized by GENEWIZ co. (Suzhou, China), with detailed sequences listed in Tables S3–S7. Plasmids and strains used in this study are listed in Table S8. Bright-Lumi™ Firefly Luciferase Reporter Gene Assay Kit and Bradford Protein Assay Kit were purchased from Beyotime (Shanghai, China). RNA Isothermal Rapid Amplification Kit (Fluorescent) was purchased from AMPLIFICATION FUTURE (Weifang, China). COVID-19-pseudovirus Kit was purchased from Yeasen (Shanghai, China). iTaq™ universal SYBR® Green supermix was purchased from Bio-Rad (Shanghai, China). SYBR™ Gold Nucleic Acid Gel Stain was purchased from Thermo Fisher Scientific Inc. (China). Taq polymerase was purchased from Takara Bio Inc. (Beijing, China). Ni-NTA column, PD-10 Desalting column and AKTA FPLC system were purchased from Cytiva. EZmax one step seamless cloning kit was purchased from ToloBio-technology (Shanghai, China). The codon-optimized DNA sequences of firefly luciferase (pET28a-luciferase) were ordered from GenScript (Nanjing, China) with sequence listed in supplementary note 1. All chemicals and reagents used were analytical grade and prepared with deionized water (18.2 MΩ cm) from Milli-Q Water Purification System (Millipore, USA).

2.2. Plasmid construction and protein purification

The SBD_{Mmo}-encoding gene and split firefly luciferase-encoding genes (Firefly luciferase-N, Fluc-N; Firefly luciferase-C, Fluc-C) were amplified (Primers are listed in Table S3) and cloned into pMAL-C2 and pET28a plasmids, respectively, with a 2*(GGGGS) in between, resulting in expression vectors for SBD_{Mmo} + FlucN (abbreviated as M + N) and FlucC + SBD_{Mmo} (abbreviated as C + M). For protein expression, pGro7/*Escherichia coli* BL21 (DE3) strains harboring plasmid encoding target protein were incubated overnight at 37 °C in Luria-Bertani (LB) medium containing corresponding antibiotics. The overnight culture was diluted 1:100 into 1000 mL of the same medium and grown at 37 °C until OD_{600} reached 0.5. The culture was then induced with 0.2 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and incubated for 16–18 h with shaking at 16 °C. Protein purification was performed at 4 °C. Cells were collected by centrifugation and resuspended in Ni-NTA column-binding buffer (25 mM Tris-HCl, pH 7.2, 300 mM NaCl). The cell pellets were lysed by homogenizer (JN-Mini pro), followed by centrifugation at 10,000 g for 60 min. The supernatant was collected and applied to 2 mL Ni-NTA column. The proteins were then eluted with 5 mL elution buffer (25 mM Tris-HCl, pH 7.2, 300 mM NaCl, 500 mM imidazole) after washing with wash buffer (25 mM Tris-HCl, pH7.2, 300 mM NaCl, 20 mM imidazole). The MBP-tagged protein products were cleaved by TEV protease (Skipping this step if proteins not containing the MBP-tag), followed by desalting with a PD-10 Desalting column, using desalting buffer (25 mM Tris-HCl, pH7.2, 50 mM NaCl). The proteins products were then purified with a HiTrap Heparin HP affinity chromatography column, equilibrated with desalting buffer using an AKTA FPLC system, and eluted with a NaCl linear gradient (50-1000 mM). Purified proteins were checked by 10% SDS–PAGE (Fig. S2), and proteins concentration were determined using Bradford Protein Assay Kit.

2.3. The electrophoretic mobility shift assays (EMSA) for SBD binding PT-modified DNA

For EMSA reaction of SBD binding PT-DNA, 10 bp annealed DNAs were used as substrates (GGCC-10 F/10 R, GGCC-NC10F, Table S3). The 10 μL reaction mixture consisted of 240 pmol SBD_{Mmo}, 15 pmol DNA substrates, 400 mM NaCl, 25 mM Tris-HCl (pH 8.8), and 5% glycerol. After incubation on ice for 30 min, the mixtures were loaded onto 12% non-denaturing polyacrylamide gels (acrylamide: bisacrylamide 79:1, w/w) and electrophoresed in 0.5 × Tris-borate-EDTA (TBE) buffer at 150 V for 60 min. Gels were stained by SYBR Gold and analyzed with the ChemiDoc XRS Gel Imaging System (Bio-Rad, USA).

2.4. Feasibility test of SENSOR

For one typical SENSOR reaction, 20 μL mixture was prepared in a Nunc 384 plate at room temperature as follow: 6 pmol M + N, 5 pmol C + M, 1 pmol probes (Probe-1PT, Table S4), 0.5/0.05 pmol target ssDNA (TMP-70, Table S4), 400 mM NaCl, 25 mM Tris-HCl (pH 8.0), with distilled water added up to 10 μL, then 10 μL luciferin (from Bright-Lumi™ Firefly Luciferase Reporter Gene Assay Kit) was added last. Luminescence was detected with a BMG CLARIOstar microplate reader (BMG Labtech, UK) at 30 °C for 10 min immediately without incubation.

2.5. Optimization of SENSOR parameters

Several key reaction parameters of SENSOR were investigated. We first determined the influence of the enzyme ratio. Seven molar ratios of 1:0, 1:4, 1:2, 1:1, 2:1 4:1, 0:1 and the control group 0:0 for M + N: C + M were tested. The reaction (20 μL) consisted of 1 pmol probes (Probe-1PT), 0.5 pmol target ssDNA (TMP-70), 400 mM NaCl, 25 mM Tris-HCl (pH 8.0), 10 μL luciferin, M + N and C + M with above tested ratios. Next, we compared the efficiencies of tandem PT-modification on probes (0 PT, 1 PT, 3 PT, 5 PT, and 7 PT) and tested the variations between single and dual probes (utilizing TMP-70 as target ssDNA, corresponding probes are show in Table S4). We also determined the length of nucleotides spacing between the PT-modifications in two adjacent probes, spacer varied from 4 to 34 bases were compared (probes and complementary ssDNA sequence are listed in Table S5). The reaction consisted of 7.5 pmol M + N, 7.5 pmol C + M, 1 pmol probes, 0.5 pmol target ssDNA, 400 mM NaCl, 25 mM Tris-HCl (pH 8.0), and 10 μL luciferin with a total volume of a 20 μL. Lastly, to explore the optimal amounts of probes in reaction, six amount gradients of 0.1 pmol, 0.25 pmol, 0.5 pmol, 1 pmol, 1.5 pmol, and 2 pmol were tested. The reaction (20 μL) consisted of 7.5 pmol M + N, 7.5 pmol C + M, 0.5 pmol target ssDNA (TMP-70), 400 mM NaCl, 25 mM Tris-HCl (pH 8.0), 10 μL luciferin and probes with above tested amounts (fs_DuPrb-7PT & bs_DuPrb-7PT, Table S4). All the experiment operations were followed the above feasibility test of SENSOR.

2.6. Specificity and sensitivity investigation of SENSOR

The optimum reaction conditions were as follow: 7.5 pmol M + N, 7.5 pmol C + M, 0.5 pmol probes, 0.5 pmol target ssDNA, 400 mM NaCl, 25 mM Tris-HCl (pH 8.0), and 10 μL luciferin with a total volume of a 20

μL. We designed two pairs of probe-substrate combinations (fPrb_spacer_56 & bPrb_spacer_67 with Tmp_spacer_6, fPrb_spacer_156 & bPrb_spacer_167 with Tmp_spacer_16, Table S5) to investigate the specificity of SENSOR. In addition, a series ssDNA sample concentration of 0.5 μM, 0.25 μM, 0.1 μM, 50 nM, 25 nM, and 10 nM were prepared (TMP-70), with the probes (fs_DuPrb-7PT & bs_DuPrb-7PT) to investigate the sensitivity of SENSOR. All the experiment operations were followed the above feasibility test of SENSOR.

2.7. Sensitivity optimization by combing with nucleic-acid amplification procedures

We explored RPA and PCR amplification procedures to improve the sensitivity of SENSOR. For RPA amplification procedure: 1) utilizing the asymmetric RPA to obtain the single-strand DNA (ssDNA) amplification products by using the RNA Isothermal Rapid Amplification Kit (Fluorescent); 2) the asymmetric RPA operation followed the kit Instructions, with corresponding primers and template (Target-F: Target-R with the molar ratio of 4:1 in reaction, TMP-221, Table S6) to amplify the target ssDNA; 3) the RPA reactions were run with 2 μL of input for 40 min at 42 °C. For PCR amplification procedure: 1) we amplified the nucleic acids sample by asymmetric PCR using rTaq 2 × mix, with corresponding primers and template (Target-F: Target-R with the molar ratio of 4:1 in reaction, TMP-221, Table S6); 2) the PCR reactions were carried out using the hot start of 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 20 s, finally an extension at 72 °C for 5 min. The amplification products were then detected by SENSOR under the optimum reaction conditions (utilizing the dual probes fs_DuPrb-7PT & bs_DuPrb-7PT). The experiment operations were followed the above feasibility test of SENSOR.

2.8. COVID-19 pseudoviruses RNA and SNP detection by SENSOR

We prepared a series dilution of COVID-19 pseudovirus RNA samples (from COVID-19-pseudovirus Kit) with different concentrations. Next, we rapidly released RNA by incubation at 70 °C for 10 min and then detected by SENSOR with asymmetric RT-RPA, operations followed the above RPA amplification procedure. For SNP detection, we examine the SNP locus of L452R and E484Q in COVID-19 genome by chemical synthesis the target mutant sequence (DNA sequences are listed in Table S7), and using the optimum reaction conditions to detect by SENSOR.

3. Results

3.1. Design and validation of SENSOR with synthetic DNA substrate

In previous researches, SBDs were found binding with PT-modified dsDNA. The crystal structure of SBD complexed with PT-DNA revealed that efficient binding requires the interactions between SBD and dsDNA [20], therefore, SBD may have different binding efficiency between PT-dsDNA and PT-ssDNA, so we came to the idea of harnessing single-stranded PT-probe to anneal with the target ssDNA. In presence of target ssDNA, PT-probe and target ssDNA will form the double strand PT-DNA, which could be specifically bound by SBD. If no target ssDNA, SBD will not bind the PT-probe.

Based on this, we designed a new nucleic acid detection method, designated SENSOR. SENSOR could detect DNA which extraction from various samples and combine with amplification methods to increase sensitivity (Fig. 1a). It is suitable for diagnosis of bacteria, virus, cancer, *etc.* (Fig. 1b).

To test the discrimination of SBD on PT-ssDNA and PT-dsDNA, we performed binding assay using DNA substrates and SBD_{Mmo} ($_{Mmo}$ denotes the SBD of Type IV REase MmoMcrA from *Morganella morganii*) which was characterized with enhanced binding affinities in our previous study (unpublished data). EMSA revealed that SBD_{Mmo} binds only the PT-dsDNA. Neither PT-ssDNA, nor unmodified DNA could be bound by SBD_{Mmo} (Fig. 2a and Fig. S1). This allows SBD to be utilized as a targeting tool, in which PT-probes anneal with target nucleic acid and form a hemi PT-modified double strand to be recognized by SBD. To achieve rapid and robust signal production, we utilized the split firefly luciferase reporter which was previously screened for studying protein−protein interactions, also applied in nucleic acids detection [8, 22–25]. The working principle of SENSOR is illustrated in Fig. 2b. Two PT-probes which are complementary with adjacent sites in target ssDNA, and SBDs fusing with split firefly luciferase reporters (Fluc-N and Fluc-C) are used. In presence of target ssDNA, the PT-probes anneals with target ssDNA, resulting in double-stranded PT-DNA; then SBDs

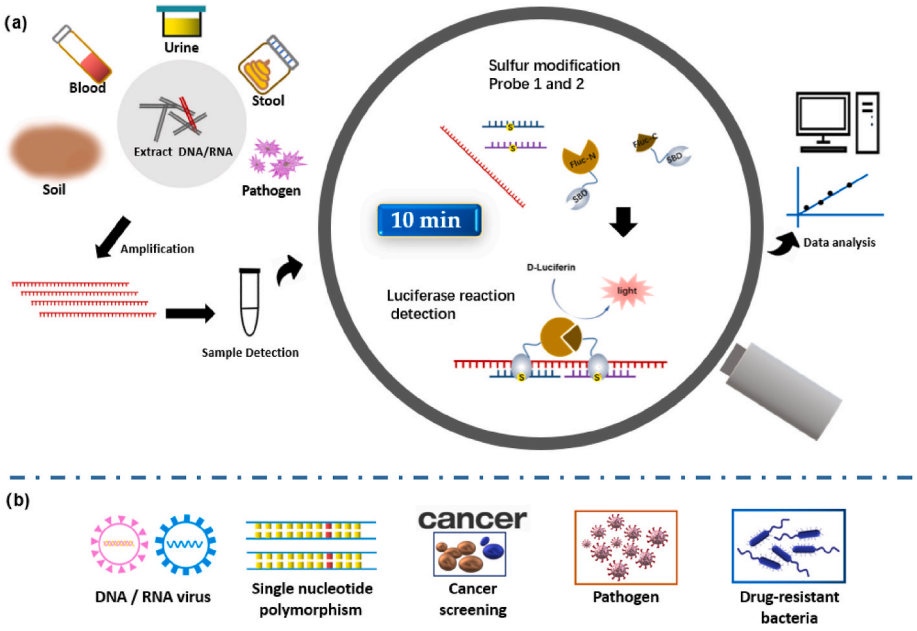

Fig. 1. Schematic outlining SENSOR detection of DNA/RNA extraction from different environmental samples. (a) The scheme of SENSOR detection, with the key design marked in the magnifier. (b) Detectable samples of SENSOR.

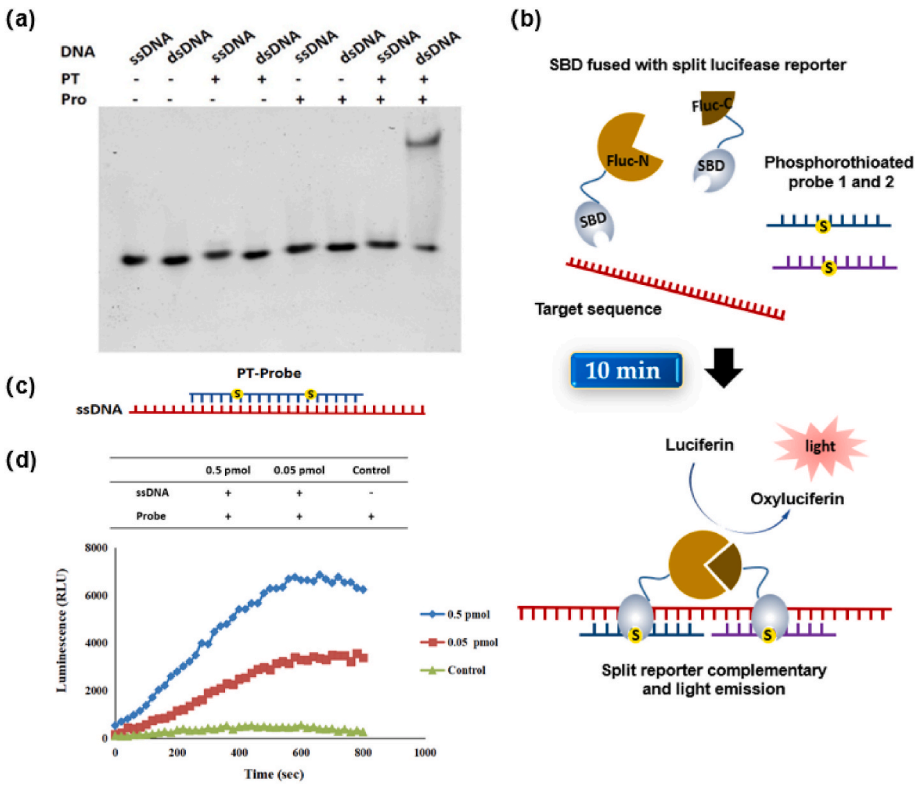

Fig. 2. Establishing SENSOR detection platform. (a) PAGE analysis of the SBD_{Mmo} bound double-strand and single-strand PT-DNA. Reaction products were analyzed by 12% non-denaturing PAGE and stained by SYBR Gold. (b) The working principle of SENSOR. SENSOR contains SBDs fusing with split firefly luciferase reporter (Fluc-N and Fluc-C) and two PT-probes adjacent to each other. The probes would anneal with target sequence by base pairing followed by cascade reactions of SBD binding, split reporter complementary, and light emission. No luminescence signal detected without target sequence. (c) Schematic of the feasibility verification for SENSOR by using a PT-probe to target the ssDNA. (d) Feasibility investigation of SENSOR. Luminescence intensity curves for different concentrations of ssDNA samples (utilizing water as control). RLU, relative luminescent units.

fusing the split reporter will bind the PT-DNA, helping complementation of the split reporter for signal production.

We first examined the feasibility of SENSOR with synthetic oligo. A 30 nucleotide (nt) oligo was designed as probe that contained two PT-modifications and corresponding ssDNA substrate (Fig. 2c). As show in Fig. 2d, reaction achieved a stable luminescence output within 10 min, and generated 12-fold and 6-fold signal-to-noise ratio (SNR) for 0.5 pmol and 0.05 pmol target ssDNA, respectively. The results confirmed

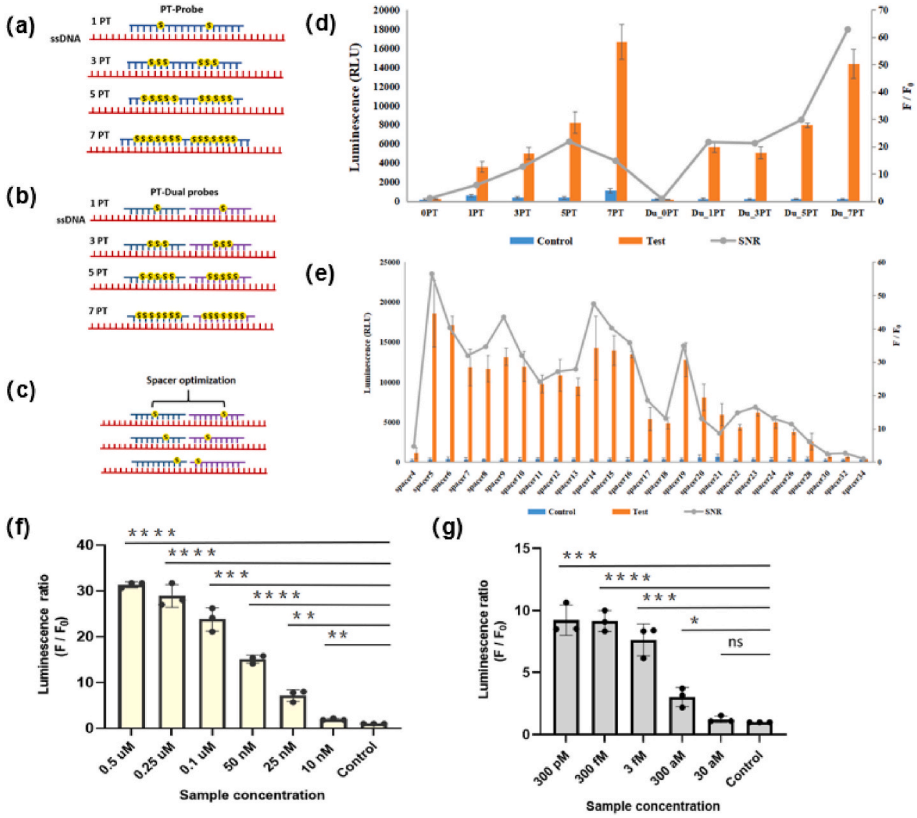

Fig. 3. Parameters and sensitivity optimization of SENSOR. (a, b, c) Schematic of SENSOR optimization about tandem PT-modifications, dual probes, and spacer. (d) Probe optimization of SENSOR by tandem PT-modifications and dual probes. (e) The influence of spacer on the performance of SENSOR. The spacing between two PT-modifications on probe varied from 4 to 34 bases were compared. Test group represent the reaction containing the substrate DNA, while control group without it. F and F_0 are the luminescence at 10 min in test and control group, respectively. SNR: signal to noise ratio. (f, g) Sensitivity of SENSOR identified by direct detection or coupled with RPA (utilizing water as control). F and F_0 are the luminescence at 10 min in sample and control group, respectively. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 from the two-tailed student's *t*-test, error bars indicate the standard deviation of three replicates. ns: no significance. RLU, relative luminescent units.

the feasibility of SENSOR.

3.2. Optimization of SENSOR

For optimization of SENSOR, we designed tandem-PT-probes, tested distance between PT sites of two probes and adjusted the reaction conditions. We first tested the single-probe (Fig. 3a) and dual-probe (Fig. 3b) designs, and found that they generated almost equal signal strength, but the SNR is much higher for the dual-probe design (Fig. 3d). We also designed probes with 1, 3, 5 and 7 consecutive PT-modifications (Fig. 3a) according to our previous study that tandem PT-modification in substrate enhanced binding affinities of SBD (unpublished data). Luminescence increased from 3000 (1-PT-Probe) to 16,000 (7-PT-Probe) with the increased number of PT-modification in the probe (Fig. 3d, left panel), demonstrating tandem PT-modifications enhanced the performance of SENSOR by improving the binding affinity of SBD. However, the SNR in single 7-PT-probe was obvious decreased owing to the high background noise (Fig. 3d, left panel). We speculated that the 30 nt probe may form secondary structure or dimer in absence of substrates, and the consecutive 7-PT modification site enabled binding of two SBDs, which leaded to generation of high background luminescence signal. To reduce the background noise, dual PT-probes were proposed to replace the single PT-probe (Fig. 3b). We observed that SNR in dual 7-PT-probes was 63, 4.2-fold higher than single 7-PT-probe, and the SNR in dual 5-PT, 3-PT and 1-PT-probes all increased compared to the corresponding single PT-probe (Fig. 3d, right panel), proving that dual probe design is better than single probe design in SNR. And the tandem PT-modification design is better than single PT-modification design.

Moreover, we also detected the length of nucleotides spacing between the PT-modifications in two adjacent probes (Fig. 3c). Probes with same PT-modification site but different spacer length varied from 4 to 34 bases were measured. Luminescence significantly decreased with spacer length less than 4 bp. We speculate that too short spacer is not enough for binding of two molecules of SBD, since in the structural of SBD complexed with PT-DNA, SBD domain consist of about 160 amino acids with a diameter that required at least 8 bp DNA to binding [20]. We got high signal with spacer 5–16, revealing this distance is optimal for binding of two molecules of SBD and complementation for the split reporter. For spacer longer than 17 bases, the signal decreased due to poor complementation of the split reporter caused by far distance (Fig. 3e).

In addition, SENSOR reaction parameters including the amounts of probes and the enzyme ratio in reaction were also investigated (Figs. S3–S4). We found that the optimum reaction conditions of SENSOR were as follows: the enzyme ratio was 1:1 (mol/mol); using dual probes that each contained 7 PT-modifications with spacing 12 bases; each probe added in reaction was 0.5 pmol. Simply mixing the above reagents in a total volume of 20 μL reaction at room temperature, followed by luminescence readout without incubation, and could obtain a stable signal within 10 min.

3.3. Detection of pseudovirus and SNP determination by SENSOR

We then validated the specificity of SENSOR under the optimum reaction conditions. We designed two pairs of probe-templates (Fig. S5a) and tested in different combinations. The results showed that high luminescence only generated from the matched pairs, 18-fold of the mismatched pairs (Fig. S5b). Subsequently, serial dilutions of ssDNA sample were prepared and tested to investigate the sensitivity of SENSOR. As show in Fig. 3f, SENSOR yielded a detection sensitivity of 25 nM (sample concentration). To improve the sensitivity, we explored to combine SENSOR with different amplification techniques. The sensitivity of 300 aM and 10 aM sample concentration was achieved when SENSOR was combined with RPA and PCR procedures, respectively (Fig. 3g and Fig. S6). Demonstrating excellent sensitivity of SENSOR.

Finally, we examined whether SENSOR would be effective in infectious disease applications. Utilizing the coronavirus disease 2019 (COVID-19) pseudovirus as samples, we rapidly released virus RNA by incubation at 70 °C for 10 min, then amplified by reverse transcription (RT)-RPA and detected by SENSOR. It showed that SENSOR could detect pseudovirus sample as low as 2.5 fM (Fig. 4c). Moreover, the ability to detect single nucleotide variations opens the opportunity for using SENSOR to identify new viral mutant strains. We chose two mutant loci that spanning of COVID-19 genome related SNPs. As show in Fig. 4a, we designed three probes to identify the mutant by luminescence analysis of the ratio of probe 1 & 2/probe 2 & 3. PT-modification was designed near the SNP in probe 1, Probe 2 & 3 used to detect the target sequence, Probe 1 & 2 used to detect the mutant base in target sequence, and the binding efficiency of probe 1 would decrease if the bases are mismatched, resulting in a decrease in luminescence readout. The results showed about 2-fold ratio difference between mutant and wild type (WT) (Fig. 4b), indicating that SENSOR allowing for precise discrimination for the single nucleotide variations.

4. Discussion

In this study, we developed SENSOR platform based on PT-probe-guided SBD linked to split luciferase reporter, allowing for rapid detection of nucleic acids with single-base specificity and attomolar sensitivity. To the best of our knowledge, this is the first application of SBD that derived from PT-dependent bacterial defence system as tool for molecular diagnostic. SENSOR differs from previously reported nucleic acid detection methods in several important ways. First, SENSOR is rapid and easy-operating, in one typical detecting reaction, the operation is only preparing the mixture of enzymes, probes, luminescence substrate and the sample, followed by luminescence readout, achieving fast detection within 10 min, with no incubation needed. Second, in contrast to CRISPR-Cas based systems [26,27], SBD does not require specific sequence motif like the PAM site, PT-probe can be designed to direct SBD to target any sequence. Third, SENSOR utilizes DNA as a probe, no requirement for operating RNA, and the probe bearing PT bonds could be commercially synthesized [28], which is simple and cheaper than fluorophore quencher-labeled probes that utilized in most CRISPR-based methods. The cost of the SENSOR can be as low as $0.4 per reaction (Tables S1 and S2). Fourth, in comparison with traditional PCR and isothermal amplification methods [1,29,30], for these methods sensitivity comes at the cost of specificity, SENSOR utilized two adjacent PT-probes achieved specificity and precise sequence targeting from amplification products by base paring, reducing the probability of false positive results due to non-specific amplification. Luminescence reporter was utilized in this method, whereas visual readout detection might be more desirable for point-of-care diagnostic field, such as electrochemical signals and ELISA colorimetric readout. There are some imperfections in SENSOR system, such as the two complementary parts of the split reporter have 50% possibility to form a functional hetero pair (Fluc-N and Fluc-C), another 50% forming a non-functional homo pair (Fluc-N and Fluc-N, or Fluc-C and Fluc-C), reducing the detection sensitivity. This could be further improved by using multiple probes, which not only increases the probability of functional hetero pair, but also provides opportunity to form more than one functional enzyme for single substrate ssDNA. Additional advances are still required in signal output that SENSOR could further be promoted by combined with visual split reporter.

Our study indicates that SENSOR is a fast, easy-operating, and inexpensive method that could detect DNA or RNA with single-base specificity and attomolar sensitivity. This platform is promising in further applications, which could be integrated into current diagnostic platforms, including the PCR machine for relative or absolute quantitation, and the microfluidic platform for multiple targets. We are confident that SENSOR will become an excellent candidate for nucleic acid detection.

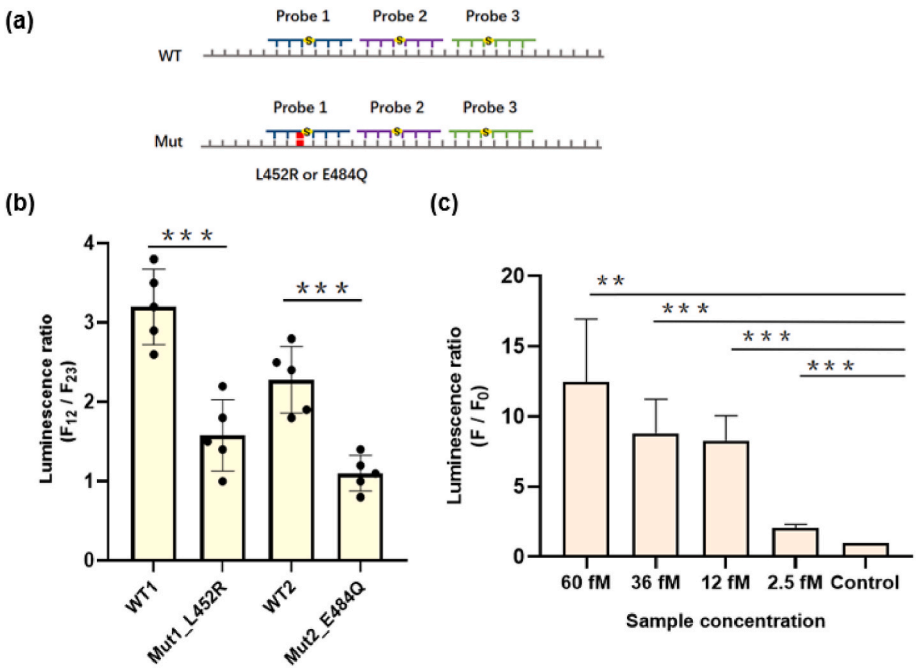

Fig. 4. SNP and COVID-19 pseudovirus RNA samples detection by SENSOR. (a) Schematic of probe design for COVID-19 mutant (L452R and E484Q) detection by SENSOR. PT-modification was near the mutant site in probe 1. Probe 2 & 3 used to detect the target sequence and Probe 1 & 2 used to detect the mutant base in the target sequence. (b) SNP detection for COVID-19 mutant by SENSOR. F_{12} and F_{23} are the background subtracted luminescence at 10 min for Probe 1 & 2 and Probe 2 & 3, respectively. (c) Detection of COVID-19 pseudovirus RNA samples by SENSOR. Extracting RNA after serially diluted the samples, then amplified and detected by SENSOR with RT-RPA (using water as control). F and F_0 are the luminescence at 5 min in sample and control group, respectively. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 from the two-tailed student's *t*-test. Error bars indicate the standard deviation of at least four replicates.

CRediT authorship contribution statement

Yuting Shuai: performed the experiments. **Yi Ju:** performed the experiments. **Yuanhang Li:** performed the experiments. **Dini Ma:** performed the experiments. **Lan Jiang:** processed the data and plotted the figures. **Jingyu Zhang:** processed the data and plotted the figures. **Gao-Yi Tan:** processed the data and plotted the figures. **Xueting Liu:** processed the data and plotted the figures. **Shenlin Wang:** designed the project, Writing – original draft. **Lixin Zhang:** designed the project, Writing – original draft. **Guang Liu:** designed the project, Writing – original draft.

Declaration of competing interest

The authors indicate that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2023.02.002.

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