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Aptamer-based protein molecule detection via cyclic reverse transcription coupling with self-priming hairpin-triggered CRISPR-Cas12a system

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Abstract

Protein biomarkers (e.g. thrombin) are of great significance for the biological process of the organism, and its aberrant expression is closely associated with the development of diseases. With thrombin, a serine protease that plays a crucial role in maintaining homeostasis and promoting blood clotting, as detection target, this study introduces a novel approach for sensitive and accurate measurement of protein biomarker expression by utilization of cyclic reverse transcription (CRT) in combination with the self-priming hairpin-triggered CRISPR-Cas12a system. In this method, an elegantly designed sensing probe is utilized to specifically bind with the thrombin protein and convert the protein signals to nucleic acids signals, following by the CRT and CRISPR-Cas12a system-based signal amplification strategy. Taking the merit of the two-stage amplification, this assay has the capability to detect thrombin at the fM level. In addition, due to the aptamer sequence's strong selectivity to thrombin protein and the dual-check process in the signal amplification process (first in the CRT and second in the CRISPR system), the proposed test demonstrates exceptional specificity in detecting thrombin. By re-designing the sensing probe, the established method could be extended to various protein biomarker detection. Ultimately, this assay has successfully enabled the accurate evaluation of biomarker levels in constructed clinical samples, showing significant potential for application in the realm of clinical molecular diagnosis.

Keywords Thrombin, CRISPR-Cas12a, Cyclic reverse transcription (CRT), Self-priming

Introduction

Protein biomarkers refer to proteins present in an organism or in the external environment, including enzymes, hormones, antibodies, cytokines, etc. Its expression level is important for understanding the biological processes in the body and for the early diagnosis of diseases. Thromboembolic events (TEE) and spontaneous abortions are the most concerning consequences associated with antiphospholipid antibodies (aPL) (Abdel-Wahab et al. 2016; Grygiel-Gorniak and Mazurkiewicz 2023; Aron et al. 1995; Carolis et al. 2004). Primary prevention strategies necessitate the use of screening tests to detect patients with antibodies, as well as the implementation of safe and effective prophylactic anticoagulation for individuals at risk (Tufano et al. 2010). Thrombin is a serine protease that plays a crucial function in maintaining homeostasis and promoting blood clotting (Davie and Kulman 2006). Thrombin is produced from prothrombin, a plasma protein, in the event of arterial injury (Lundblad

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et al. 2004). Furthermore, thrombin is a highly effective agent for triggering platelet activation, resulting in the formation of blood clots to avoid hemorrhaging (Brass 2003). Thrombin monitoring is essential for the treatment and prevention of antiphospholipid syndrome. Efficient and accurate identification of thrombin is crucial in the field of biological analysis and clinical diagnosis.

Conventional thrombin detection method primarily includes the use of antibodies and enzymes (Chung et al. 2018; Kang et al. 2008; Chuang and Andrade 1985), which have certain drawbacks, including limited stability, high expenses, the need for enzyme labeling, lengthy reaction time, and the necessity of a large sample amount. As alternative, aptamer-based biosensors are commonly employed for thrombin detection because of the aptamer's unique identification capability (Pol et al. 2019; Bai et al. 2013). Aptamers are synthetic oligonucleotides selected using the SELEX (systematic evolution of ligands by exponential enrichment) technique, either DNA or RNA, that possess unique tertiary structures (Chen et al. 2020), resulting in a high affinity for certain target analytes (Gao et al. 2019; Eivazzadeh-Keihan et al. 2022). The aptamer-based thrombin detection biosensors integrate with various signaling modalities, including colorimetric (Song et al. 2018), fluorescence (Wen et al. 2017), and surface-enhanced Raman spectroscopy (SERS) (He et al. 2015). In the detection of thrombin, fluorescence assays have been utilized extensively due to their high sensitivity, accuracy, and repeatability. Li et al. (2018) proposed a simple fluorescent thrombin detection approach by using aptamer-based proximity recognition-dependent strand translocation. Furthermore, target circulation catalyzed by exonuclease (Exo) is also a prevalent technique employed for signal amplification. Based on this, Gao et al. (2017) implemented a technique for detecting thrombin using the aptamer-MoS2 sensor that was based on the strategy of fluorescence resonance energy transfer (FRET). For the detection of thrombin, these fluorescent methods have been extensively implemented by integrating them with diverse signal amplification strategies, including PCR and recombinase polymerase amplification. Nevertheless, these systems are frequently constrained by costly machinery, intricate operational procedures, and the need for extensively trained personnel. Therefore, a simple, specific, and sensitive analytical method for thrombin detection is urgently required.

The emergence of CRISPR-based techniques has provided a novel avenue for molecular diagnosis (Wang et al. 2020; Zhang et al. 2020; Zhao et al. 2020). The CRISPR-Cas12a system has gained significant interest because of its distinct collateral *trans*-cleavage capability. Activated Cas12a nucleases have the ability to cleave surrounding single-stranded non-targeted nucleic acids without discrimination, upon identification of DNA or RNA targets. This characteristic is utilized in the development of diverse assays for the identification of a broad spectrum of targets, which have been proven to be effective diagnostic tools.

In this work, we have developed a novel approach for biomarker analysis based on cyclic reverse transcription (CRT) coupling the self-priming hairpin-triggered CRISPR-Cas12a system. CRT is an effective isothermal signal amplification strategy without the requirement of enzymes. Upon thrombin recognition by the aptamer sequence, the primer was liberated, thereby instigating cyclic reverse transcription and triggering the self-priming process. Ultimately, the CRISPR-Cas12a system can be used to identify the product, which then initiates the *trans*-cleavage of "Reporter" probes, resulting in the release of a fluorescent signal. By detecting and analyzing the fluorescence intensities, we successfully detected thrombin with exceptional sensitivity. Furthermore, we confirmed the actual feasibility of the suggested approach by accurately identifying the desired objective in constructed clinical samples.

Experimental section

Reagents and apparatus

The DNA and RNA sequences used in this study were synthesized and purified by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China), and the synthesized nucleic acid sequences were dissolved in DEPC-treated distilled water. The details of the sequences are listed in Table S1 (Additional file 1). The enzyme and corresponding buffer solution, including the Engen Lba Cas12a, DNA polymerase, and the reverse transcriptase, Nb.BbvCI, Deoxynucleotide solution mix (dNTPs), and NEBuffer 2.1 buffer solution were provided New England Biolabs Inc. (Ipswich, MA, UK).

Construction of the sensing probe

10 μL aptamer sequence (5 μM) and 10 μL primer sequence (5 μM) were mixed in a tube containing 30 μL PBS buffer solution. The mixture was heated to 90 °C for 5 min and then slowly cooled to room temperature.

Thrombin detection by the system

Thrombin sensing: For the thrombin sensing, 10 μL sensing probe (5 μM) was mixed with 10 μL of the thrombin for 30 min at room temperature.

Cyclic reverse transcription: The reaction solution contains 5 μL of template, 5 U of ribozyme inhibitor, 0.5 mM dNTPs (1 μL), thrombin and sensing probe mixture, 1 μL of reverse transcriptase, and 8 μL buffer. The reaction mixture was incubated in a thermostatic metal bath for

45 min at 42 °C, followed by 5-min incubation at 85 °C to terminate the reaction.

Self-priming: 2 μL of DNA polymerase and 2 μL of endonuclease were added to the CRT reaction mixture. The mixture was incubated at room temperature for 30 min to produce the "d*" sequences. 2 μL of H probe and 4 μL of buffer solution were then added to the mixture, and the mixture was incubated for 30 min.

CRISPR-Cas12a-assisted signal generation: 30 μL of reaction product was mixed with 10 μL of digestion solution. The mixed digestion solution contains: 50 nM LbCas12a-crRNA, 1×NEBuffer 2.1, 1 μM "Reporter" probe. After incubating in a thermostatic metal bath and reacting at 37 °C for 1 h, the reaction was quenched by heating at 65 °C for 5 min.

Results and discussion

Overall procedure of the cyclic reverse transcription coupling with the self-priming hairpin-triggered CRISPR-Cas12a system

The working mechanism of the established approach is shown in Fig. 1. The proposed method involves the fabrication of a sensing probe that consists of a thrombin aptamer and a primer sequence. This design allows for the integration of thrombin identification and the commencement of a following cascade signal amplification process. When thrombin is present, the aptamer in the sensing probe binds to thrombin and subsequently releases the primer sequence. The linear hairpin variable RT template is designed to consist of parts "a*," "b," "c," and "d," in accordance with the previously published design philosophy of CRT. The component "a*" sequence is capable of identifying the primer sequence and serving as the loop in the resulting hairpin structure. Part "b" exhibits an identical sequence to the 5'-terminal regions of the primer. Part "c" is capable of transcribing the sites recognized by the endonuclease. In the presence of reverse transcriptase, the variable RT template undergoes elongation to form an incomplete hairpin structure (c–b–a*–b*) upon hybridization with the primer. This elongation results in the liberation of the primer for the CRT. The hairpin structure, initially incomplete (d–c–b–a*–b*), undergoes a process of self-replication, utilizing itself as a template, to extend and become a complete hairpin structure (d–c–b–a*–b*–c*–d*). The "c*" is recognized by the endonuclease, which constructs a nicking site.

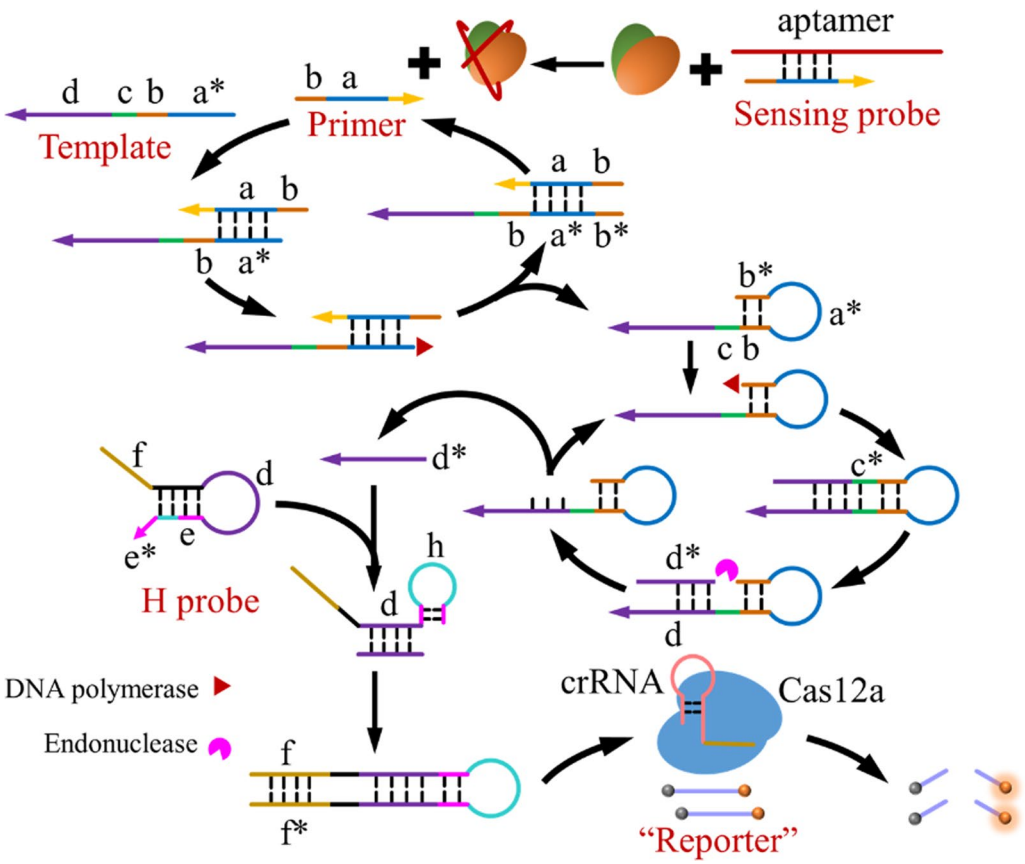

Fig. 1 The working mechanism of the approach for aptamer-based protein biomarker detection

Through the collaboration of DNA polymerase and endonuclease, a multitude of "d*" sequences are generated. The "d*" sequences form a binding interaction with the "d" region of the H probe, facilitating the self-priming of "e" and "e*." Using DNA polymerase, the component "e*" is extended, resulting in the production of "f*" section. The "f" section comprises the non-target strand (NTS) of LbCas12a, together with the 5′-TTTA-3′ protospacer adjacent motif (PAM). Subsequently, the recently formed hairpin (f–d–e–e*–d*–f*), which includes the PAM and the TS/NTS, has the ability to stimulate the LbCas12a/crRNA complex, resulting in the cleavage of the "Reporter," and ultimately the fluorescence signals reappeared.

Construction of the sensing probe and feasibility of the method

In order to confirm the construction of the sensing probe, the aptamer sequence and primer sequence were labeled with a fluorescent dye (FAM) and a quenched dye (BHQ-1), respectively. Upon hybridization of the aptamer and primer, there was a notable reduction in the fluorescence signal, suggesting effective hybridization of the two sequences (Fig. 2A). When the sensing probe was exposed to other interfering proteins, including CEA (carcinoembryonic antigen), CRP (C-reactive protein), and PCT (procalcitonin), there was no obvious alteration in the fluorescence signal. This indicates that the probe exhibits a strong preference for thrombin, as seen in Fig. 2B. In order to validate the CRT method, we cultivated equal amounts of primer sequences with FAM-tagged 3' ends together with template sequences labeled with BHQ at their 5' terminals. As the incubation time increased, the fluorescence signal of FAM progressively diminished, indicating that the primer sequences hybridized with the template sequence (Fig. 2C). When DNA polymerase is present, the fluorescence signal of FAM is restored, potentially because the template chain forms a hairpin structure to release primer. The results presented above demonstrate the practicality of CRT, as shown in Fig. 2D. The self-priming procedure was validated using synthesized "d*" sequences to demonstrate its practicality. SYBR-Green I is a fluorescent dye that selectively attaches to double-stranded DNA sequences. It is commonly employed in the analysis of DNA polymerase-assisted chain extension. As depicted in the diagram, the presence of "d*" and DNA polymerase leads to a substantial increase in the SYBR Green I signal. This indicates that "e*" undergoes elongation in a time-dependent manner (Fig. 2E).

Optimization of experimental parameter

Subsequently, we optimized the reaction parameters for both CRT and CRISPR-Cas12a, specifically aiming to enhance the amplification effectiveness of CRT and therefore increase the signal generating capacity of CRISPR-Cas12a. Figure 3A demonstrates that the fluorescent signal reached its peak when the concentration of the sensing probe was 100 nM. Subsequently, it was noted that Cas12a at a concentration of 250 nM exhibited the most prominent fluorescence signal, as depicted in Fig. 3B. As depicted in Fig. 3C, the highest level of fluorescence was achieved when the CRT reaction time was set to 60 min. Consequently, the fluorescence result (Fig. 3D) demonstrates that the fluorescent signal intensified as the reaction time progressed. After a duration of 30 min, the rate of increase in the fluorescent signal decelerated, indicating that the most favorable time for Cas12a to cleave the "Reporter" was 30 min.

Analytical performance of the approach

Next, we examined the analytical capabilities of the suggested cyclic reverse transcription combined with the self-priming hairpin-triggered CRISPR-Cas12a system. Figure 4A displays the analytical performance of the thrombin detection approach under the optimized experimental settings. With an increase in thrombin concentration, the fluorescence intensities resulting from the cleaved "Reporter" elevated. The fluorescence intensity at a wavelength of 520 nm exhibited a positive correlation with the concentration of thrombin within the range of 10 fM to 1 nM. The calibration curve is depicted in Fig. 4B, and the regression equation is expressed as $F = 1155 * \lg C - 172.8$. In this equation, C represents the quantity of thrombin, and F represents the measured fluorescence intensities. The linear curve had a coefficient (R^2) of 0.9929, and the detection limit for thrombin was determined to be 5.34 fM using the 3δ rule. This technique involves the conversion of each thrombin signal into numerous amplified "*f*"/"*f**" duplex outputs using

(See figure on next page.)

Fig. 2 Feasibility of the approach for thrombin detection. **A** Fluorescence spectrum of the aptamer sequence before and after being assembled to hairpin structure. **B** FAM signals of the sensing probe when thrombin and interfering protein molecules existed or not. 1, CEA (carcinoembryonic antigen); 2, thrombin; 3, CRP (C-reactive protein); 4, PCT (procalcitonin). **C** FAM signals of the primer/template duplex with different incubation durations. **D** Fluorescence spectrum of the primer/template duplex when DNA polymerase existed or not. **E** SYBR Green I signals during the self-priming process. 1, H probe; 2, H probe + "d*"; 3, H probe + DNA polymerase; 4, H probe + "d*" + DNA polymerase

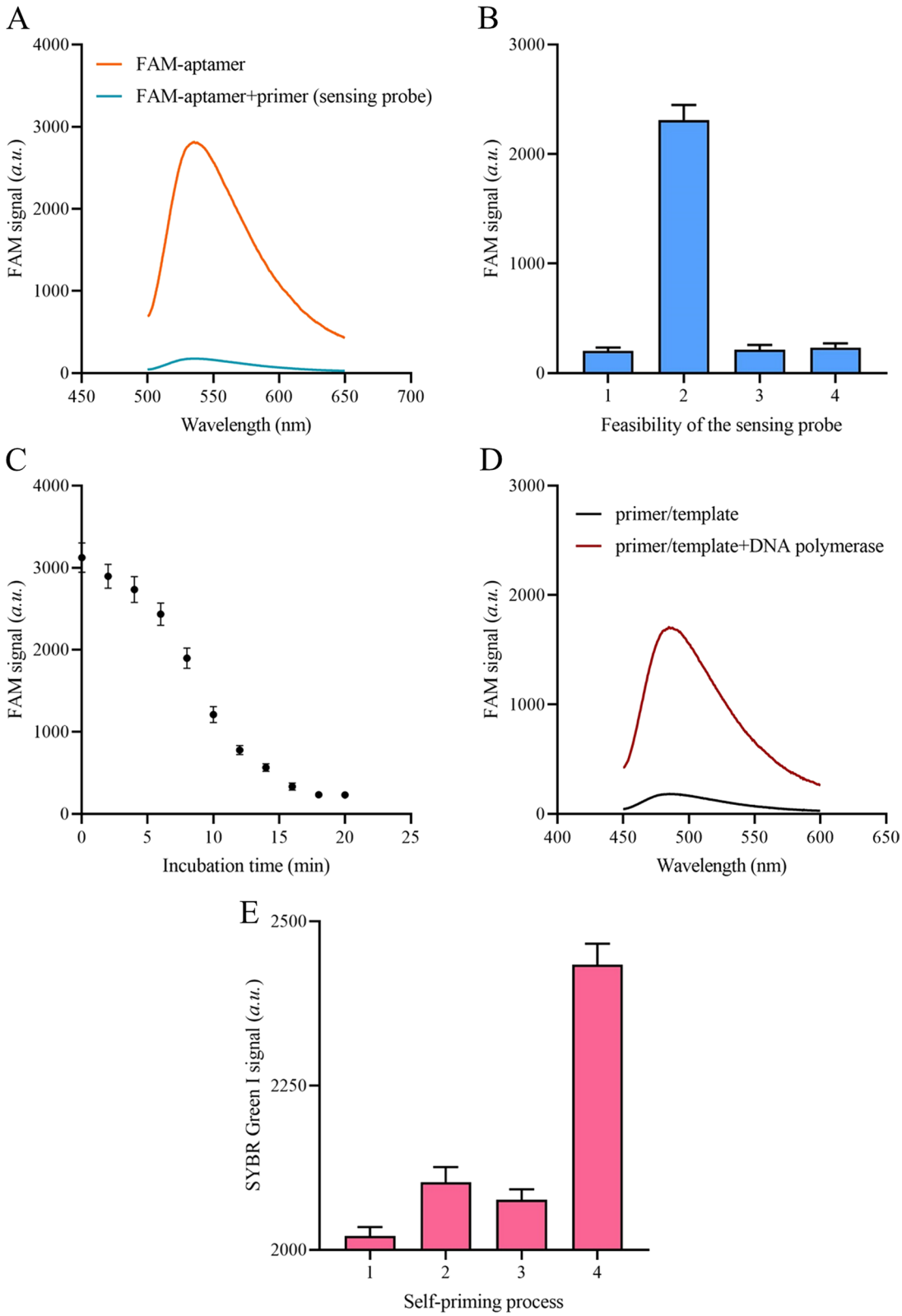

Fig. 2 (See legend on previous page.)

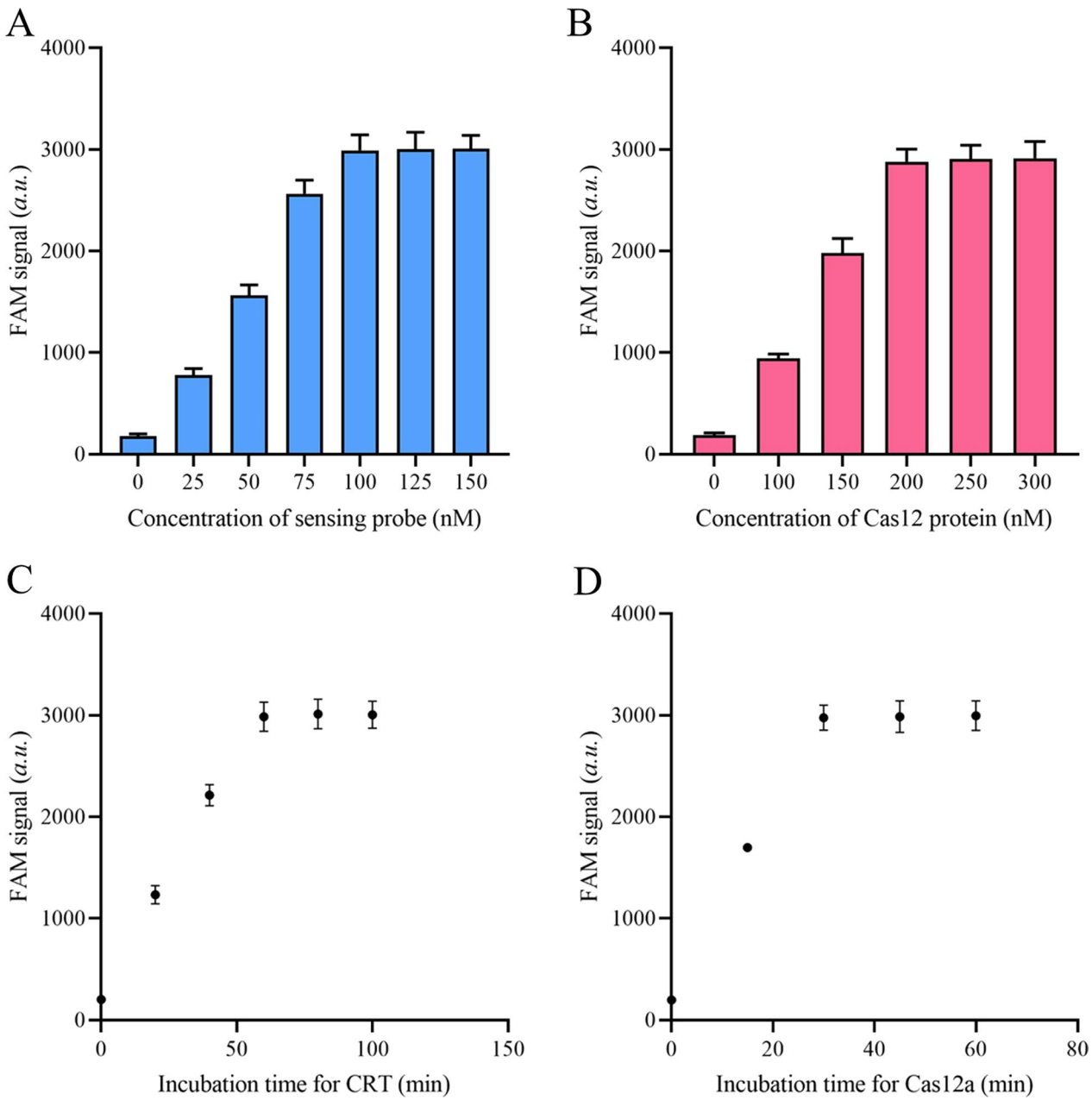

Fig. 3 Optimization of experimental parameters. FAM signals of the approach with **A** different concentrations of sensing probe, **B** different concentrations of Cas12a protein, **C** different incubation of CRT, and **D** different incubation for Cas12a protein

cyclic reverse transcription coupled with the self-priming process.

The approach's specificity was subsequently evaluated by testing the target thrombin and interfering protein molecules, including the carcinoembryonic antigen (CEA), C-reactive protein (CRP), and procalcitonin (PCT). When thrombin was present, a strong fluorescence was observed; however, there was very little fluorescence when non-target interfering protein molecules were present (Fig. 4C). The results demonstrated that the system exhibited a high level of specificity toward thrombin, hence verifying the exceptional specificity of the suggested method (Eivazzadeh-Keihan et al. 2022; He et al. 2015). The great specificity seen can be attributed to the CRISPR-Cas12a system (Wang et al. 2020; Zhao et al. 2020).

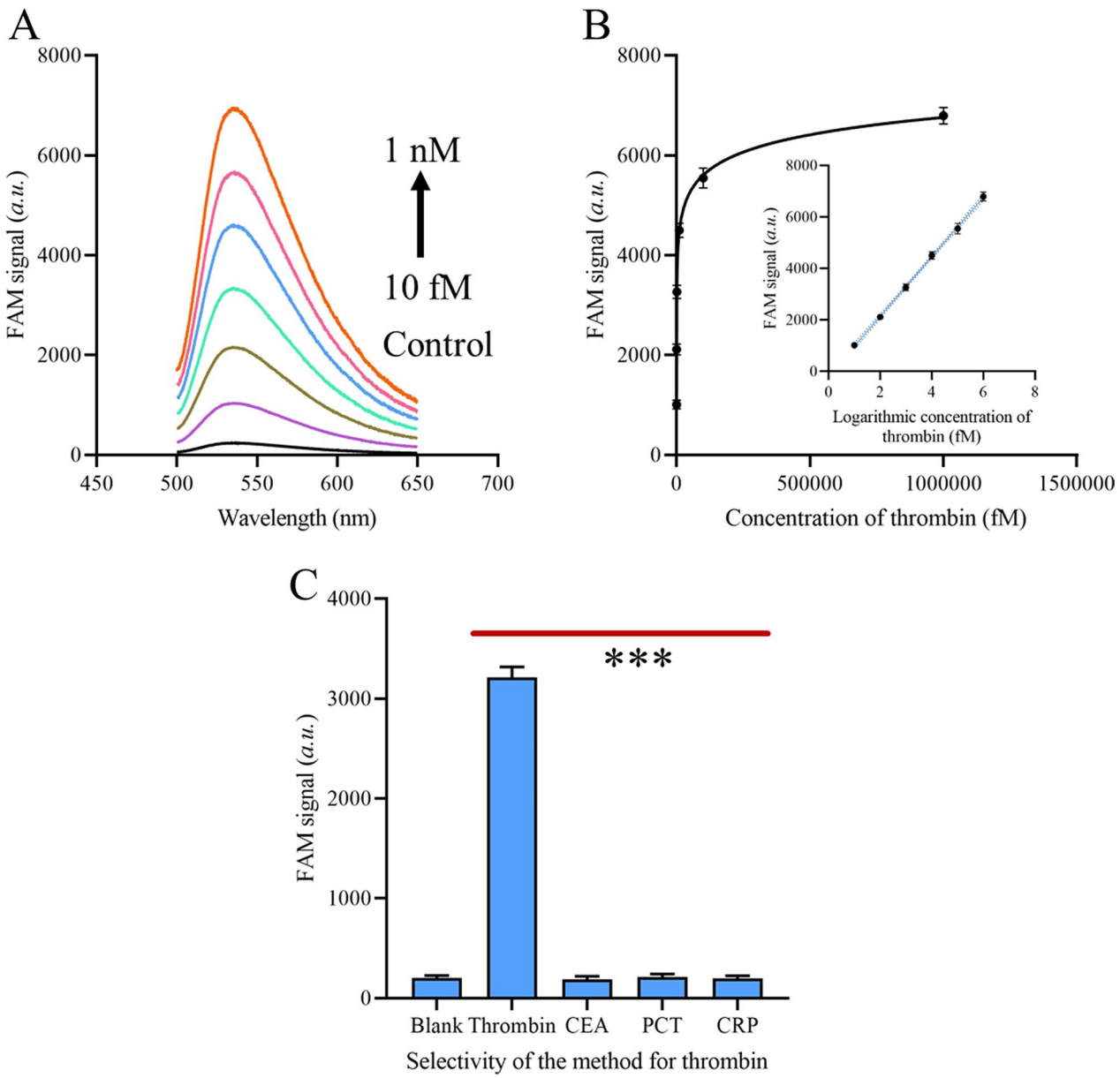

Fig. 4 Analytical performance of the approach. **A** Fluorescence spectrum of the approach when detecting different concentrations of thrombin. **B** Correlation between the FAM signals and the concentrations of thrombin. Inserted is the linear equation between the FAM signals and the logarithmic concentrations of thrombin. **C** FAM signals of the approach when detecting thrombin and interfering protein molecules. ***, $P < 0.05$

Practical applicability

The proposed strategy's practical applicability was proved by measuring thrombin in commercial human serum samples. Artificial clinical samples were created by adding different concentrations of thrombin to commercially available human serum. These samples were then subjected to the CRT coupling the self-priming hairpin-triggered CRISPR-Cas12a system. Figure 5 demonstrates a strong linear correlation ($R^2 = 0.99$) between the fluorescence intensities measured by the method and the concentration of thrombin in the clinical samples created. A recovery test was then conducted to evaluate the stability of the method through detecting thrombin from three samples. The concentrations of thrombin were accurately measured, with coefficients of variation (CV) below 5.14% and recovery rates ranging from 99.3 to 102.3%. These results demonstrate the high level of reproducibility and precision of the method used, as

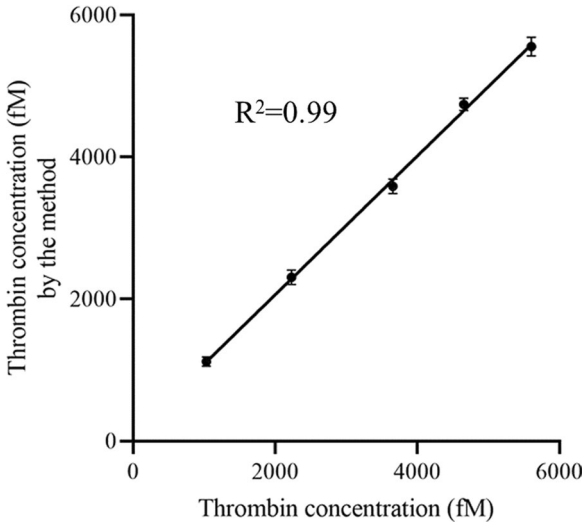

Fig. 5 Calculated thrombin concentration by the proposed method

Table 1 Recovery rate of the approach for thrombin detection

Samples	Added (fM)	Recovery (fM)	Recovery rate (%)
1	100	102.3	102.3
2	1000	993	99.3
3	5000	5102	102.04

shown in Table 1. These data confirm that the current approach has significant potential to be consistently applied to actual clinical samples.

Conclusion

We depict here a novel, simple, and highly sensitive protein biomarker detection method based on CRT coupling with the self-priming hairpin-triggered CRISPR-Cas12a system. The established method was ultimately employed for the detection of thrombin, exhibiting a remarkable sensitivity and an exceptionally low limit of detection of 5.34 fM. Based on the high selectivity and affinity of aptamer sequences to target and the dual-check process in the signal amplification process (first in the CRT and second in the CRISPR system), the proposed method demonstrated exceptional specificity in detecting thrombin. Furthermore, the high recovery rate of the method for thrombin detection from clinical samples demonstrated the clinical application potentials. In contrast to traditional isothermal amplification approaches, the proposed method possesses the advantages of simple probe design, high sensitivity, and stability for thrombin detection. In addition, the method could be extended to other protein molecules detection by simply re-designing the sensing probe. Taking the merit of the high sensitivity and stability of the method, it could be potentially applied for the disease diagnosis, clinical research, and point-of-care testing.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40543-024-00441-5.

Additional file 1: Table S1. Nucleic acid sequence used in the experiments.

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Author contributions
JG and XY designed the strategy, completed the preparation of the research, and wrote the manuscript; JG financed the research and reviewed the manuscript. All authors approved the submission of the manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article [and its Additional file 1: Supplementary information files].

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Study participants were written consent to publish.

Competing interests
The authors declare that they have no competing interests.

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