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RESEARCH ARTICLE

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Dual signal amplification coupling with DNA-templated silver nanoclusters for sensitive and label-free detection of thrombin

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Abstract

Sensitive and reliable determination of thrombin is relevant in the realms of medical and biological research as it serves as an essential biomarker of a number of blood-related illnesses. Herein, we integrate allosteric probe-based specific identification of thrombin and dual signal amplification to present an unique fluorescent technique for label-free and sensitive thrombin detection. Based on DNA polymerase and endonuclease-assisted signal amplification, the method exhibits a high sensitivity with a low limit of detection of 2.3 pM, while maintaining an excellent selectivity and stability. More importantly, the approach is successfully applied in analyzing the effect of nalbuphine on coagulation function of mice. Overall, this approach possesses the advantages of high specificity and sensitivity in label-free detection of thrombin, which is promising in the diagnosis of blood-related diseases.

Keywords Allosteric probe, Thrombin, Nalbuphine, DNA template, Silver nanoclusters

Introduction

Thrombin, as a key serine protease that plays crucial roles in converting soluble fibrinogen to insoluble fibrin and catalyzing the activation of platelet, is involved in a variety of physiological diseases, such as thrombosis (Jordan et al. 2021; Shlobin et al. 2021). In light of that aberrant level of thrombin may lead to blood coagulation dysfunction and cause uncontrollable bleeding in surgery, quantifying the level of thrombin is essential in preoperative examination (Al-Amer 2022; Cardenas 2021). As a main criterion for coagulation disorders, accurate and quantitative determination of thrombin levels assists the diagnosis of many diseases and developing treatment plans.

The most traditional technique for thrombin detection is immunoassay, which is frequently used in clinical settings. Despite that the immunoassay can accurately identify thrombin and provide a convincing results, the weak response to the trace amount of thrombin and low sensitivity to changes of thrombin concentration confine its wider application (Yousef et al. 2022; Salmasi et al. 2022; Kintigh et al. 2018). In recent years, several types of techniques based on different transducers have been employed for detection of thrombin, such as the electrochemical assay (Chen et al. 2020; Lin et al. 2020, 2017), fluorometric assay (Shen et al. 2017; Cui et al. 2021; Yun et al. 2019), and colorimetric detection assay (Yin et al. 2020; Shen et al. 2021). As an electrode-dependent sensor to record signal of interactions between biomolecules and targets on an electrode surface, electrochemical

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assay has made significant advancements in thrombin detection, such as the high sensitivity (pM level) and quick response. However, the low repeatability and complicated preparation compromise its widespread use. has currently attracted abundant attention due to its superior features in easy production, high stability, low cost, and affectability. For example, *Zhen Zhang* proposed a sensitive thrombin detection approach based on cationic conjugated polymer-assisted signal recycles (Zhang et al. 2021). The device eventually exhibited a wide detection range of 3–54 nM with the limit of detection of 11 pM. Even though fluorometric assays have made remarkable progresses to the immunoassay and electrochemical assay, they still have several drawbacks, including (1) the sensitivity cannot meet the high requirement of detecting trace amount of thrombin; and (2) labeling of fluorescence moiety and quenching moiety (e.g., FAM and BHQ) on probes is easily affected by reaction conditions and thus inevitably increasing the experimental complexity, cost, and risk of false positive results (Li et al. 2020, 2017; Zhu et al. 2019; Xu et al. 2017). Therefore, it is great demand to develop label-free and fluorescent thrombin detection assay with improved sensitivity.

Silver nanoclusters (AgNCs) have emerged as a novel fluorophore in recent years and have attracted considerable attention in chemical/biomolecular detection and cellular imaging due to its unique features of lower toxicity, better brightness, and high photostability (Kim et al. 2022; Kim and Park 2021; Lee et al. 2021). In addition, the fluorescent response of the DNA-templated AgNCs, in which DNA template works as a scaffold, relies on the surrounding microenvironment and the variation of the lengths of DNA template sequence. DNA-templated AgNCs have been widely used in establishing label-free and fluorescent approaches in recent years (Eivazzadeh-Keihan et al. 2022; Maleki et al. 2016). For example, *Guimin Ma* developed a sensitive miRNA detection based on turn-on fluorescence of DNA-templated silver nanoclusters which inspired us to utilize AgNCs for fluorescent signal generation (Ma et al. 2021).

Herein, a label-free and sensitive thrombin detection approach is constructed based on allosteric probe-assisted dual signal amplification (Fig. 1). In this approach, an allosteric probe is designed with hairpin structure. There are five functional sections in the designed allosteric probe (***a***, ***a′***, ***b***, ***c***, ***d***). Among them, the ***a*** section is thrombin aptamer that can specifically bind with thrombin in samples; ***a′*** section is complementary with ***a*** sequence; ***c*** is the nicking site of endonuclease. In the existence of thrombin, it can specifically bind with ***a*** section in allosteric probe and consequently lead to the allosterism of the probe from hairpin structure to linear, exposing the ***a′*** and ***d*** sections. The released sequences composing of ***a′*** and ***d*** sections gradually unfold the H probe through hybridizing with the ***e*** and ***d′*** section in H probe. Upon the addition of DNA polymerase, chains are extended with ***d*** and ***e*** as primers. As a result, a double-strand DNA (dsDNA) product is formed and thrombin

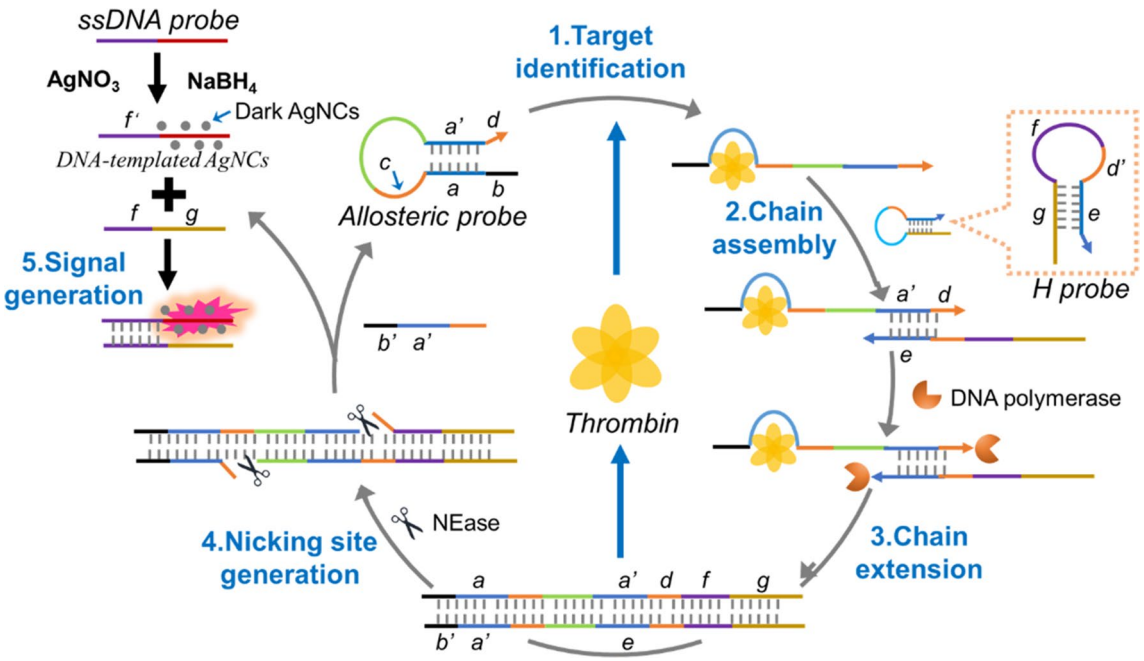

Fig. 1 The working mechanism of the approach

is disassociated with ***a*** section. The released thrombin can attend a next recycle through binding with allosteric probe, forming the first signal amplification. The formed dsDNA product contains two endonuclease recognizing sites, which can be specifically cut by endonuclease to generate nicking sites. Under the assistance of DNA polymerase, two signal-strand DNA (ssDNA) are produced. The first ssDNA sequence which contains ***b′*** and ***a′*** section can hybridize with ***a′*** section and ***b*** section in allosteric probe. As a result, allosteric probe is unfolded to linear state and can induce a second signal recycle. The second chain which is composed of ***f*** and ***g*** (G-rich) sections binds with the dark AgNCs, narrowing the distance between the dark AgNCs and the G-rich sequence and stimulating AgNCs to emit strong red fluorescence. Notably, the mechanism of emission of red fluorescence of DNA-AgNCs remains unknown.

Experimental conditions

Reagents and materials

The details of DNA probes are listed in Additional file 1: Table S1. The oligonucleotides sequences were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China). Thrombin, ATP (Adenosine triphosphate), CEA (Carcinoma embryonic antigen), and CRP (C-reactive protein), and immunoglobulin G (IgG) were purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China). Enzymes used for chain extension including Klenow fragment (KF) polymerase ($3'\rightarrow 5'$ exo-) and Nt.AIWI endonuclease were brought from New England Biolabs (Beijing, China). The other regents were in analytical grade and were purchased from Beijing Chemical Co. (Beijing, China) without further purification. Preparation of the dark silver nanoclusters is shown in Additional file 1.

Preparation of the dark silver nanoclusters (AgNCs)

The preparation of the dark AgNCs follows the reaction of $NaBH_4$ based on the reduction of $AgNO_3$. In brief, 4 μL of ssDNA probe (100 μmol/L), 4 μL of $AgNO_3$ (1.25 mmol/L), 5 μL 10 × PB (pH 6.6) and 33 μL of distilled water were mixed in the tube, and the mixture was incubated at room temperature for 30 min. Afterward, 6 μL of the $NaBH_4$ (1.25 mmol/L) was added in the mixture, and the mixture was incubated at room temperature for 120 min to reduce free Ag ions to Ag atom (more details in Additional file 1).

Characterization of allosteric probe and DNA-templated AgNCs

Assembly of the allosteric probe the obtained allosteric probe was firstly diluted to 100 nM by DEPC water. 10 μL allosteric probe (100 nM) was heated to 90 °C for 10 min and gradually cooled to room temperature.

Fluorescence assay to characterize allosteric probe the fluorescence signals of 10 μL linear allosteric probe and 10 μL assembled allosteric probe were recorded by Hitachi fluorospectro photometer (Tokyo, Japan). To test the identification of thrombin, 10 μL assembled allosteric probe was mixed with 10 μL thrombin (10 nM) and incubated at 37 °C for 20 min.

Detection sensitivity of the approach

2 μL allosteric probe (100 nM), 2 μL thrombin (100 nM), and 2 μL H probe were mixed in a tube and incubated for 10 min. Afterward, 1 μL KF polymerase (0.5 U μL^{-1}), 1 μL Nt.AIWI endonuclease (0.2 U μL^{-1}), and 1 × PBS buffer. When the mixture was incubated at 37 °C for 40 min, 1 μL DNA-templated AgNCs were added in the mixture and incubated for attached 10 min. The fluorescence signals were recorded by Hitachi fluorospectro photometer (Tokyo, Japan) with the excitation wavelength of 565 nm and the emission wavelength of 630 nm. In addition, the slit width is 6 nm. Lastly, the correlation between fluorescence signals and concentrations of thrombin was calculated.

Statistical analysis

The data in this research were collected and calculated by GraphPad Prism 8.0 and Microsoft Excel 2016. The data were shown as mean ± standard deviations. Differences between two groups were determined by using the two-tailed Student's *t* test. $P < 0.01$ was the threshold for significance.

Results and discussion

Characterization of allosteric probe and DNA-templated AgNCs

The assembly of allosteric probe determined the accurate identification of thrombin and initiation of subsequent dual signal amplification. Therefore, we firstly investigated the assembly of allosteric probe and tested its capability to identity thrombin through a fluorescence assay. In the fluorescence assay, the two terminals of the allosteric probe were labeled with FAM and BHQ. The FAM signal observed when the probe was in linear state would be quenched when it was assembled to hairpin structure (Additional file 1: Fig. S1). The result in Fig. 2a showed a greatly decreased fluorescence intensity when linear allosteric probe was heated to 90 °C for 10 min and cooled to room temperature, indicating the successful assembly of allosteric probe to hairpin structure. Upon the addition of thrombin, the signal recovered to 2655 *a.u.*, implying that thrombin could unfold assembled allosteric probe. In addition, no significant signal

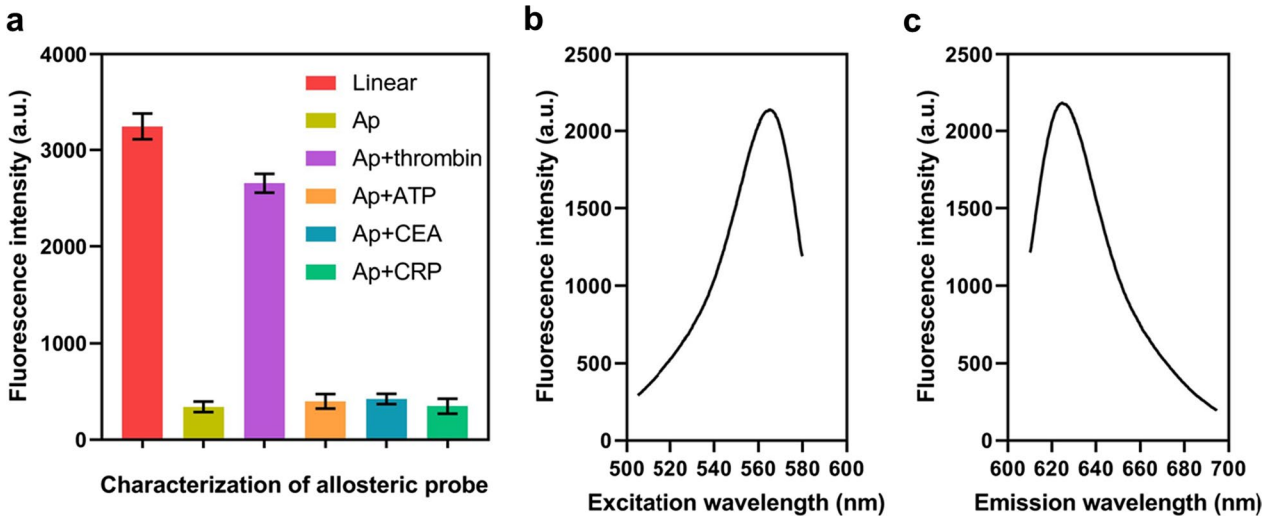

Fig. 2 Characterization of allosteric probe and DNA polymerase and endonuclease-assisted chain extension. **a** Fluorescence intensities of the linear allosteric probe and hairpin structure allosteric probe (Ap); **b** excitation spectra of the DNA-templated AgNCs. **c** Emission spectra of the DNA-templated AgNCs

increasements were obtained when the assembled allosteric probe was incubated with ATP (Adenosine triphosphate), CEA (Carcinoma embryonic antigen), and CRP (C-reactive protein), suggesting a high specificity of the designed allosteric probe.

Optical properties of DNA-templated AgNCs are crucial to the signal generation. To test the optimal excitation wavelength and emission wavelength, we recorded the excitation and emission spectra of DNA-templated AgNCs. As shown in Fig. 2b, the excitation spectra showed a most significant fluorescence respond when DNA-templated AgNCs were excited by light at 565 nm. Meanwhile, the emission spectra showed the optimal emission wavelength was 630 nm (Fig. 2c).

PAGE analysis was applied to study the feasibility of the dual signal recycles. From the result in Additional file 1: Fig. S2, a new band appeared in Lane 3 when allosteric probe was incubated with the H probe. When DNA polymerase and endonuclease were added in the system, two band appeared (marked with the red and purple boxes, respectively), indicating the successful generation of two chains.

Optimization of experimental conditions

In the established DNA-templated AgNCs-based label-free and sensitive thrombin detection approach, the parameters that affect detection performance have been optimized. To test the effect of amounts of DNA-templated AgNCs on the thrombin detection, different concentrations of DNA-templated AgNCs were prepared from 20 to 80 pM to detect 5 nM of thrombin samples. The result in Fig. 3a showed a gradually increased fluorescence signal with the increase of DNA-templated AgNCs from 20 to 40 pM, while no remarkable increment was observed in more than 40 pM DNA-templated AgNCs. Therefore, the optimized concentration of DNA-templated AgNCs was 40 pM. The influence of the amount of DNA polymerase on the thrombin detection was tested. Accompanying the elevation of DNA polymerase dosage from 0.1 to 0.5 U/L, enhanced fluorescence signals were observed (Fig. 3b). When the system was incubated with more DNA polymerase, no more fluorescence signal increasements were observed. Thus, the concentration of DNA polymerase was 0.5 U/L. Meanwhile, the optimized concentration of endonuclease was 0.2 U/L (Additional file 1: Fig. S3) and the incubation time was determined 60 min (Fig. 3c).

Analytical performance of the approach

Under the optimized experimental conditions, we have investigated the detection performance of the approach. The sensitivity of the approach for thrombin detection was studied through detecting different concentrations of the thrombin. The recorded fluorescence intensity at 630 nm in Fig. 4a elevated with the concentration of thrombin range from 10 pM to 100 nM (excitation wavelength = 565 nm). The fluorescence intensities showed a good linear relationship with the concentration of thrombin. The result in Fig. 4b showed the correlation between the concentration of thrombin and the recorded fluorescence signals. The linear regression equation can be expressed with $Y = 799.7^{*}\lg C + 4438$ ($R^2 = 0.9813$), where y was the obtained fluorescence intensities and C represented

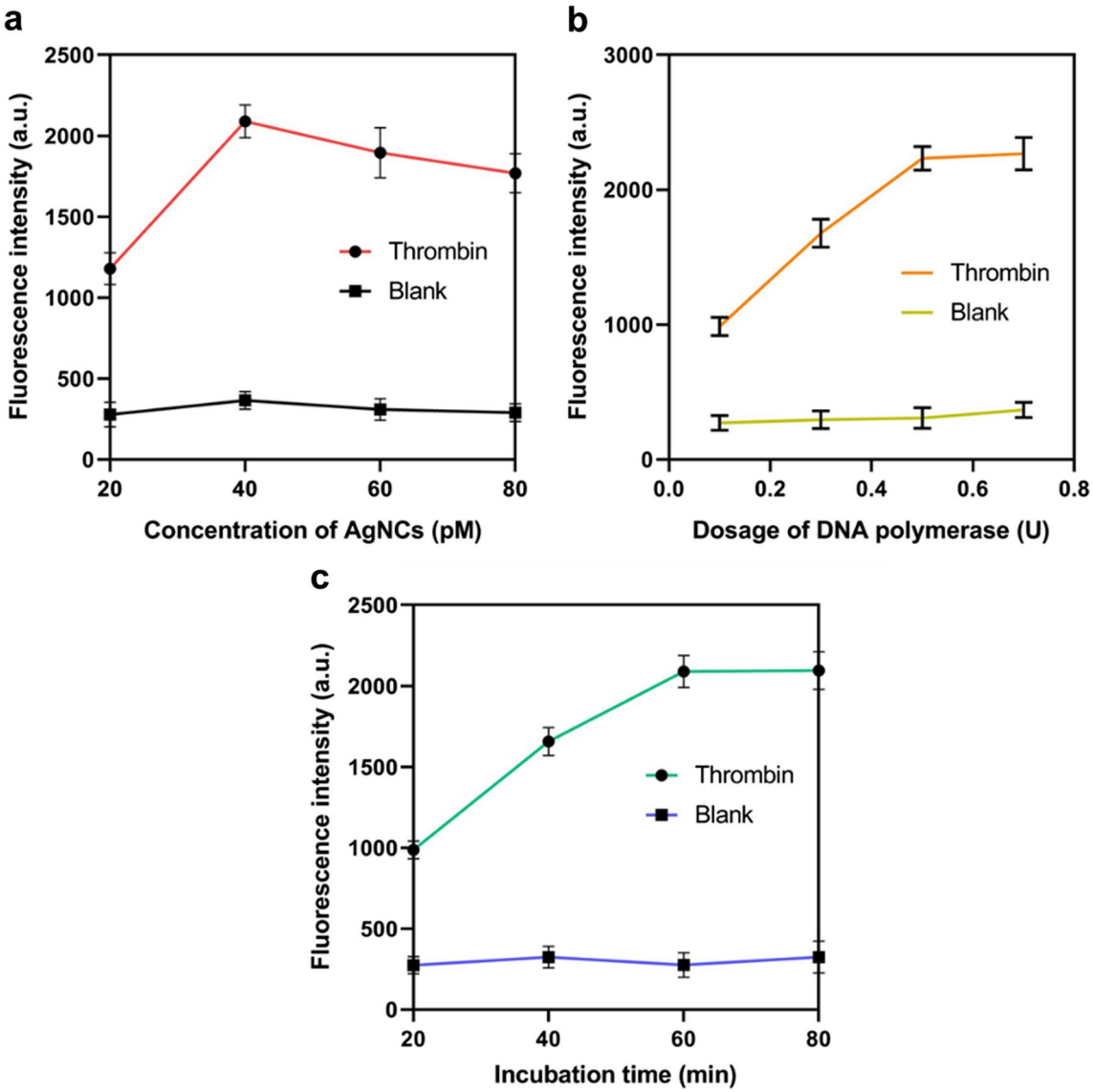

Fig. 3 Optimizations of experimental conditions. Fluorescence intensities of the approach with different concentrations of **a** DNA-templated AgNCs, **b** DNA polymerase, and **c** incubation time

the thrombin concentration. The LOD was estimated to be 2.3 pM based on the 3σ standard rule (σ, standard deviation). In addition, the selectivity of the approach was explored through detecting thrombin and several interferents (IgG, CEA, CRP, and lecithin). Notably, the concentrations of thrombin and the interferents were 500 pM. As shown in Fig. 4c, the approach exhibited a remarkable elevation of fluorescence signal when detecting thrombin, while no significant fluorescence signal increasements were obtained when detecting the interferents, indicating a favorable selectivity of the approach. Compared with former established thrombin detection approaches, the method exhibited a low LOD and possessed the capability to output signals in a label-free manner (Additional file 1: Table S2).

Clinical application in detecting thrombin

The established approach was then applied to evaluate the level of thrombin in nalbuphine-treated serum samples and untreated samples to study the effect of nalbuphine on coagulation function. The former research has demonstrated that the thrombin binding aptamer (HD22) is the only aptamer that has the capability to detect thrombin in plasma and is capable of identifying thrombin in murine plasma. Therefore, the method could possibly utilized in mice sample

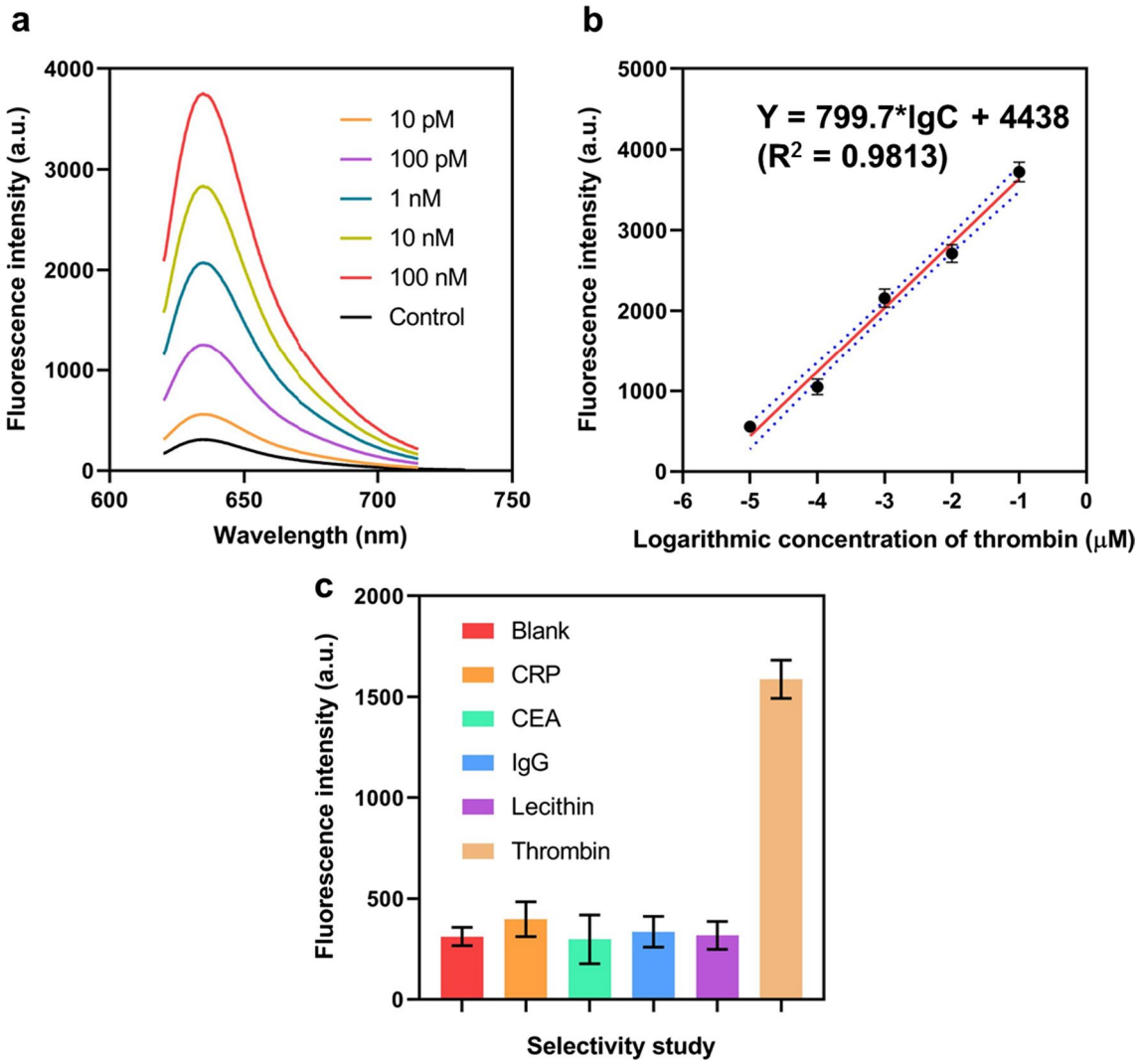

Fig. 4 Analytical performance of the established approach. **a** Fluorescence spectra of the approach when detecting different concentrations of thrombin. **b** Linear regression equation between fluorescence intensities and concentration of thrombin. **c** Fluorescence intensity of the approach when detecting thrombin and other interferents (IgG, CEA, CRP, and lecithin)

detection. For the preparation of serum samples, the mice were divided into two groups, one group was treated with nalbuphine and the other was treated with normal saline. Serum samples were collected 5 min after the nalbuphine, and normal saline was injected. The established approach and immunoassay were utilized to detect the amount of thrombin in the collected serum samples. With thrombin concentration of one sample in nalbuphine group calculated by immunoassay as control, relative expression level was calculated by comparing calculated thrombin concentration with the control. The result in Fig. 5 showed that thrombin was not up-regulated in nalbuphine-treated group ($P > 0.01$), which was consistent with former references.

Conclusion

We developed in this research a DNA-templated AgNCs-based label-free and sensitive thrombin detection approach through ingeniously integrating allosteric probe based specifically identification of thrombin and dual signal recycles. The hairpin structure of allosteric probe endowed the probe the capability to specifically bind with thrombin and convert thrombin signal to nucleic acids signals. Based on the DNA polymerase and endonuclease-assisted signal amplification, the approach exhibited a wide detection range from 10 pM to 100 nM with a low LOD of 2.3 pM. Furthermore, the practical applicability of the approach was demonstrated in real biological samples. Therefore, we

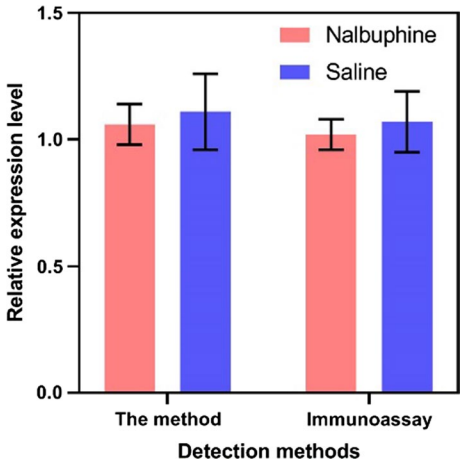

Fig. 5 Relative thrombin expression level in nalbuphine-treated group and saline-treated group

believe that the approach could be a powerful tool for thrombin detection and could be potentially expanded in the clinical diagnosis of various diseases (i.e., thrombosis and hemophilia) in which thrombin levels should be considered. Besides the thrombin detection, the proposed approach could be also utilized in other protein biomarker analysis. In future, we will extend the approach to other biomarkers detection and develop a universal platform for disease diagnosis.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40543-023-00372-7.

Additional file 1. **Preparation of the dark silver nanoclusters (AgNCs)**. **Table S1**. The details of the probes used in this research. **Fig. S1**. The illustration of the fluorescence assay to study the assembly of allosteric probe. **Fig. S2**. PAGE analysis of DNA polymerase and endonuclease assisted chain extension. **Fig. S3**. Fluorescence intensities of the approach when incubated with different concentrations of endonuclease. **Table S2**. Comparisons of the detection performance of the established approach with former ones.

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Author contributions
YY is the supervisor of the team in all research steps including designing, data analysis and manuscript writing. BZ, as the first author, has the main role for experimental data collection, data gathering, preparation of results, and data analysis. WM, JG, QZ, CZ, SZ, and HX assist the data analysis. All authors read and approved the final manuscript.

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Availability of data and materials
Almost all details of experimental data are presented in the article or additional file.

Declarations

Ethics approval and consent to participate
The manuscript does not contain clinical or trial studies on patients, humans, or animals.

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

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