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ORIGINAL PAPER

Highly sensitive fluorometric determination of thrombin by on-chip signal amplification initiated by terminal deoxynucleotidyl transferase

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Abstract

The article describes an on-chip amplification scheme initiated by a terminal deoxynucleotidyl transferase (TdT) for highly sensitive fluorometric determination of protein. Two thrombin-binding aptamers were designed to capture thrombin as they can form a sandwich structure for improved specificity. An amino-modified aptamer (TBA29) was first immobilized on a silicon chip. After capture of thrombin, a second aptamer (TBA15) was conjugated to the second binding site of thrombin. The 3'-terminal of aptamer TBA15 is exposed on the chip surface, and then fluorescein-labeled 12-dATP associates to the 3'-terminal with the help of TdT. This results in signal amplification, and eventually leads to highly sensitive detection. Under optimal conditions, fluorescence intensity is linearly related to the logarithm of thrombin concentration in the range of 100 fM - 0.1 μM, and the detection limit is as low as 2.0 fM. The assay is sensitive and selective even over potentially interfering proteins and in the presence of human serum.

Keywords Fluorescence microscopy · TdT · Aptamer · Protein · Biosensor · Sandwich · Fluorescein-12-dATP · Silicon chip

Introduction

The ultrasensitive detection of special proteins, such as osteopontin, interferon gamma, thrombin and immunoglobulin G, is essential for early disease diagnosis, tracking of diseases development, and medical research. Such as, thrombin, an effective protease of the coagulation cascade, can convert fibrin to fibrinogen catalytically by activating platelets, and plays a central role in the initiation and transmission of thrombotic diseases via promoting the stability of blood clots. Therefore, detection for thrombin performs an essential role in physiological and pathological conditions [1–4]. Many detection techniques have been used to fabricate protein biosensors, including electrochemical method, spectrophotometry, fluorescence, isotope, chemiluminescence method, quartz crystal microbalance and surface enhanced Raman scattering [2, 4, 5]. For thrombin detection, there have been some reports of aptasensor. Among them, fluorescence method shows a rapid response with high sensitivity due to its low background. However, low-abundance of proteins, complex sample preparation and multiple purification steps of existing techniques, remain an obstacle to ultralow level of proteins analysis. Take the enzyme-linked immunosorbent assay (ELISA) for example [6], it is heavily reliant on the quality of the antibodies; the preparation of antibody is expensive, laborious, and time-consuming. To overcome these drawbacks, new rapid, high specificity, more sensitive and wide availability analytical methods for trace proteins have drawn tremendous focuses of scientists [6–9].

Dongxiao Wen, Minhui He and Kefeng Ma contributed equally to this work.

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Special attentions have focused on signal amplification strategies, various amplification techniques based on nucleic acid and nanomaterial amplification assays have been developed to enhance the sensitivity of protein detection [10]. However, limitations do exist. Such as specific equipment and high assay cost of polymerase chain reaction (PCR) [11], the complexity of target sequence preparation of chain replacement amplification technology (SDA) [10, 12], multiple expensive enzymes of enzyme-linked immunosorbent assay (ELISA) [6, 13], the cumbersome, time-consuming and low sensitivity of Hybrid chain reaction [14],and so on. Aptamers, short single-strand DNA (ssDNA) or RNA oligonucleotides, as a kind of special nucleic acid, can replace antibodies to identified protein due to its unique 3D feature [1, 15], which has a specific ligand binding site. Compared to traditional recognition elements, nucleic acid based aptamer probes exhibit fascinating advantages. Such as high stability and resistant under harsh conditions for long-term storage, simple and inexpensive production, good compliance to chemical modifications in chip surfaces, easier to label and target diversity [2, 16]. These advantages enable them a high selectivity and affinity to target analytes with low detection limit for aptasensors. Much attention has been paid on aptamer-based recognition assays for peptides, proteins, drugs, cells and other small molecules [15–17].

Noteworthy, TdT, with its outstanding template-independent polymerase activity, has been used in DNA-templated biosensor field. Some studies had demonstrated the capacity of TdT mediated DNA elongation and this polymerization can be applied for multiple poly-labeled natural or unnatural nucleotides. Among them, deoxyadenosine triphosphate (dATP) is the most efficient nucleotide substrate [16, 18] and the mature nucleotide-like oligomers mostly are non-natural fluorophores for on-chip labeling and signal amplification [19–22]. Many detections of specific nucleic sequence have been designed by using of TdT. However, directly labeling of the target protein by the action of TdT is still a big challenge [23–25].

In order to fabricate a cheap, template-independent and sensitive protein sensor through a directly rapid “green” process, for the first time, we use fluorescein-12-dATP to label the thrombin-binding aptamers by catalytic polymerization of TdT. After polymerization, a long nucleic acid chain with multiply fluorescent labels is formed from the end of one ssDNA (aptamer). In addition, two different special thrombin-binding aptamers were designed to capture the thrombin, and form a sandwich structure for improved specificity. In this strategy, one end of the sandwich structure is connected to the surface of the silicon chip, the other 3’-OH end of the secondary aptamer is exposed on the silicon surface. These exposed tails can be used as the primers to make the fluorescein-12-dATP easier to connect with the 3' -OH end under the polymerase TdT, and make a multitude of fluorescein-dATP signal for thrombin detection labeled accurately. Eventually, a TdT-mediated signal amplification for ultrasensitive detection is realized. Modified chips were characterized to check the effect of modification and observe surface morphology by contact angle, X-ray photoelectron spectroscopy (XPS), atomic force microscope (AFM) and fluorescence microscope. The water contact angle was described to qualitative analysis of the modified chip surface. Furthermore, its analytical behavior was studied by fluorescence microscope under optimal condition.

Experimental

Materials

3-Aminopropyltriethoxysilane (APTES) was obtained from Aladdin Industrial Inc. (Shanghai, China, http://www.aladdin-e.com/zh_cn/). 1, 4-Phenylenediisothiocyanate (PDITC) was bought from Sigma-Aldrich (St. Louis, MO, http://www.sigmaaldrich.bioon.com.cn/). TdT enzyme and TdT buffer were acquired from Beyotime Institute of Biotechnology (Shanghai, China, http://beyotime.bioon.com.cn/). The fluorescein-12-dATP was obtained from Perkin Elmer (Shanghai, China, http://perkinelmer.bioon.com.cn/). Sinopharm Chemical Reagent Co., Ltd (Shanghai, China, http://u36494437.b2bname.com/) provided all other chemical reagents used in this study.

The primary thrombin-binding-aptamer DNA oligomer with a 3’-amino modification (TBA29: 5’-AGTCCGTG GTAGGGCAGGTTGGGGTGACT-(CH_2)$_6$-NH_2-3’), and the secondary thrombin-binding -aptamer (TBA15: 5’-GGTTGGTGTGGTTGGAAAAAAA-3’) DNA oligomer come from Invitrogen Biotechnology Co., Ltd (Shanghai, China, http://www.invitrogen.com/). Binding buffer: 20 mM Tris-HCl buffer (pH 7.4) are 140 mM NaCl, 5 mM KCl, 1mM $MgCl_2$, 1mM $CaCl_2$, and 1% BSA. Treatment solutions: (1) 98% H_2SO_4/ 99% H_2O_2 (7/3, v/v); (2) H_2O/ 23.5% $NH_4{\cdot}H_2O$/ 99% H_2O_2 (5/2/1, v/v); (3) H_2O/ 37.5% HCl/ 99% H_2O_2 (5/2/1, v/v). Ultrapure water used in this work was prepared with a Millipore Milli-Q water purification system (≥18.25 MΩ·cm).

Apparatus

Fluorescent measurements were carried out with a Zeiss Imager M2 Fluorescence microscope (X-Cite Series 120Q excitation light source, AXIOCam HRc CCD). Static water contact angle measurements data were obtained by the sessile drop technique using an Optical Tensionmeter (Theta Lite, Finland). An S-4800 (Hitachi Co., Ltd, Japan) and a PHI Quantera II (Japan) were used to perform the X-ray photoelectron spectroscopy (XPS). Atomic force microscope (AFM) images were acquired with Dimension Icon (Bruker Inc.)

Pretreatment of silicon chip

Prior to modification, silicon chip (5 × 5 mm^2.) was pretreated with piranha and so on. Then, it was prepared APTES and PDITC according to earlier works [26]. For details, please see the Electronic Supporting Material.

Formation of the sandwich structure

After pretreatment, the 3'-amino modified TBA29 (1 μM, 20 μL Tris-HCl buffer) reacted with isothiocyanate groups on the silicon chip for 2 h at room temperature [27, 28]. After washing with Tris-HCl buffer to remove the excess TBA29, the chip was immersed in ethanolamine (EA, 50 mM) to block the unreacted isothiocyanate group, and then 20 μL thrombin samples with different concentrations in Tris-HCl buffer (pH=7.4,20 mM Tris,5 mM KCl,140 mM NaCl,1mM $MgCl_2$,1 mM $CaCl_2$) were dropped onto the TBA29-modified chip surface and incubated for 1 h at 37 °C. The sensor chip was subsequently washed then with Tris-HCl buffer (0.05% tween, pH 7.4) to remove the unbound and superfluous thrombin. At last, by binding of a secondary dual-functional thrombin-binding aptamer (TBA15) to the captured thrombin, in 37 °C incubator, the chip interacted with a 1 μM solution of TBA15 (20 μL) for 1 h to form the "TBA29/thrombin/TBA15"sandwich structure.

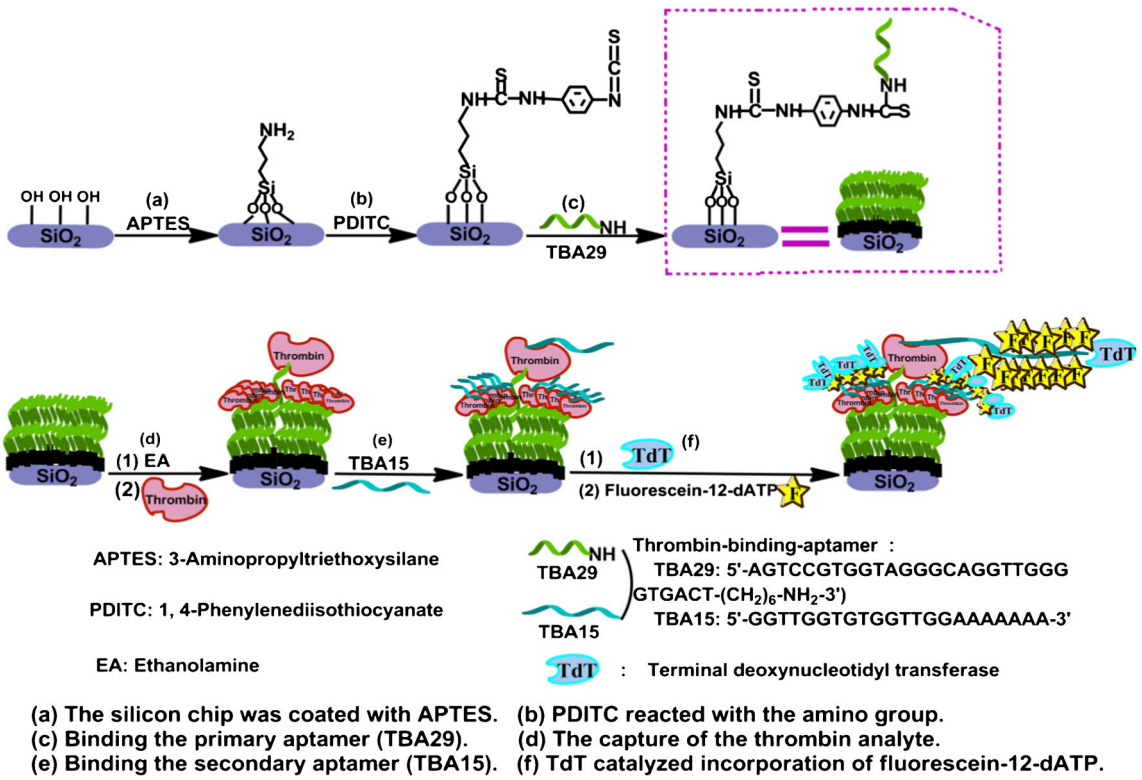
Fluorescence labeling by TdT-mediated for detection

The mixed solution, which included 1 U TdT, 100μM fluorescein-12-dATP and Tris-HCl reaction buffer (2:1:7, v/v/v) in a total volume of 20 μL, was dropped on the chip for 1 h at 37 °C. This results in a polymerization reaction and label thrombin with fluorecein. Afterwards, the chip was washed immediately to remove any unbound reactants, especially those superfluous fluorescein-12-dATP. The resulting chip was used for detecting by fluorescence microscopy.

Results and discussion

The strategy of the sandwich biosensor

As illustrates in the Scheme 1, the "aptamer/thrombin/aptamer" sandwich biosensor was fabricated under room temperature. It has been known that steric effects can effectively impact the binding of the receptor to its ligand [19]. PDITC has rigid planar structure and good water solubility. Thus, after the pretreatment of silicon chip (step a, b), the PDITC was selected to link amine-modified aptamer (TBA29) to APTES-modified silicon chip surface (step c). The 3'-NH_2 terminal of TBA29



Scheme 1 The strategy of the sandwich biosensor

was immobilized on the modified-chip by the covalent bond between its amine and the PDITC. Besides, the aptamer (TBA29)-functionalized chip was used to capture the thrombin based on the selective binding of the target (thrombin) (step d). Then a secondary thrombin binding aptamer (TBA15) was associated to the captured thrombin, and formed aptamer/thrombin/aptamer sandwich structure finally (step e). Although both aptamers captured the thrombin, their binding sites were different. The 29-mer DNA aptamer bound to the heparin-binding exosite of thrombin with a K_d of 0.5 nM [27] and another 15-mer DNA aptamer was bonded to the fibrinogen-recognition exosite of thrombin with a K_d around 100 nM [28]. They can fold to form a G-quadruplex conformation when the protein presents. These can be considered as a contribution to its relatively stable sandwich structure.

Recently, TdT has been reported to incubate with oligonucleotide self-assembled monolayers (SAMs) and catalyze the extension of this oligonucleotide chain from the 3'-end of a short oligonucleotide primer. Further, these DNA strands can be extended with different dNTPs. Based on their extension rate, dATP is the most preferred monomer [20]. There are certain kinds of scaffolds for getting together multiple fluorescent molecules, such as organic polymer backbones, inorganic framework and oligonucleotide [22]. In this sandwich biosensor strategy, the 3'-OH end of secondary aptamer (TBA15), exposed on the chip surface and acted as the primer and the scaffold of the polymerization reaction. The strands of TBA15 can be effectively extended with fluorescein-12-dATP under the action of TdT. With the extension, the fluorescence labels were introduced. A multitude of fluorescein-dATP molecule for thrombin detection were labeled accurately through TdT-mediated signal amplification initiated from the 3'-OH end of aptamer (TBA15), finally (step f). This selective amplification strategy eventually led to an obvious fluorescence signal and low detection limit at femtomole level.

Surface characterization

The modified chips were characterized by contact angle, XPS and AFM to check the effect of modification. The water contact angle (WCA) is described to qualitative analysis of surface group. In addition to the WCA of each modified step of the silicon chip, introduction of TBA29 and thrombin were also characterized in Fig. S1. For details, please see the Electronic Supporting Material. To further demonstrate that silicon chip was functionalized as expected. XPS experiment was performed. From the Fig. 1a, it can be seen that after cleaning, the XPS spectrum shows bare silicon chip only have silicon, oxygen and hydrogen element peaks. However, compared with the bare silicon chip, XPS analysis of APTES-modified surface appeared nitrogen element peak at around 400eV. Similarly, the spectra of XPS of the step by step modification appeared sulfur and phosphorus peaks on their surface in turn, evidences of introduction with PDITC and aptamer DNA respectively. Furthermore, widely energy XPS spectra show elements quantitatively by comparing the relative strength of the same elements to estimate its modification effect. As shown in Fig. 1a, the N (1s) peak of thrombin (5) was stronger than that of individual aptamer (4) which attribute to the abundant nitrogen content of protein. Compared to other prominent peaks, the S (2p) and P (2p) peaks were weak. This is mainly caused by their relative content. Comparison of XPS spectra indicates that this chip was modified well and thrombin was conjugated on the surface as anticipated.

The sandwich structure formed by thrombin and two kinds of aptamers was further examined by AFM. As described in literature [29, 30], lengths of TBA29 and TBA15 in G-quadruplex conformation are about 7 nm, and the diameter of thrombin always in 3-4 nm. In Fig. 1b, it is clear that there are some "islands" on the surface and the height of these "islands" roughly equals to the sum of length of TBA29, TBA15 in G-quadruplex and the diameter of thrombin, after modifying with aptamer/thrombin/aptamer on the chip surface. The surface morphology of the sandwich structure had

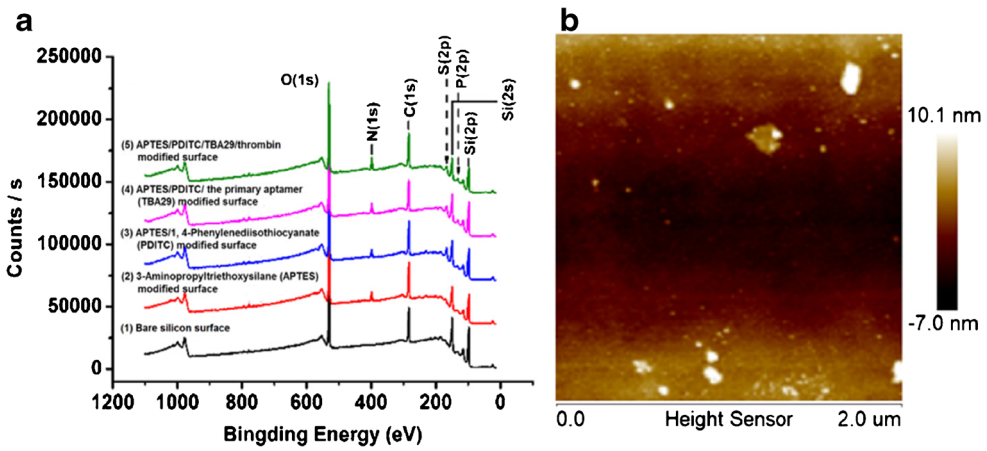


Fig. 1 **a** XPS spectra of silicon surfaces. **b** Atomic force microscope image of silicon chip after immobilization of "aptamer/thrombin/aptamer" sandwich structure

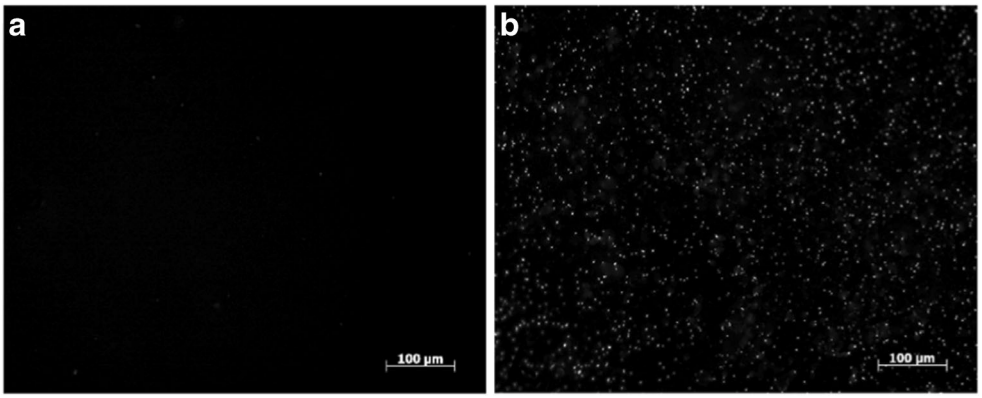


Fig. 2 Fluorescence images of the silicon surface toward 10 nM thrombin (**b**) and negative control group (**a**) (magnification: 10 × 20, exposure time: 400 ms)

no obvious fluctuate, which demonstrated that the sandwich structure was distributed uniformly on the surface.

Optimization of method

Several parameters were optimized: (a) conjunction time of aptamer with thrombin; (b) concentration of fluorescein-12-dATP; (c) reaction time of TdT; (d) temperature of fluorescence labeling by TdT. Respective data and Figures are given in the Electronic Supporting Material. Optimal conditions are: (a) conjunction time of aptamer with thrombin: 60 min; (b) concentration of fluorescein-12-dATP: 1 μM; (c) reaction time of TdT: 60 min; (d) temperature of fluorescence labeling by TdT, 37°C ".

Analytical performance of the sandwich biosensor

Under optimal conditions, 10 nM thrombin was used and the evenly distributed fluorescence spots were observed as shown in Fig. 2b. In view of the fluorescent signal is generated exclusively from the aptamer/thrombin/aptamer sandwich biosensor, the negative control group hardly showed fluorescence spots (Fig. 2a). Both TBA29 and TBA15 can fold into a G-quadruplex structure to bind thrombin. When the aptamer is absent, the sandwich structure cannot be formed on the chip and the protein cannot be labeled by fluorescence label. After labeling fluorescein-12-dATP at the 3' end of TBA15 under the initiation of TdT, forming the fluorescein polymerization, the protein detection can be realized. Therefore, the sandwich format system for protein detection is crucial.

Furthermore, signal changes were investigated with varying concentrations of thrombin. For its fluorescence measurement, an FITC filter (excitation band: 475±40 nm, emission band: 530 ±50 nm) was used. On-chip TdT labeling was determined by scanning the fluorescein-12-dATP at the excitation wavelength of 480 nm [25, 31, 32]. Each sample with different concentration was tested six times repeatedly using seven different modified chips to get the fluorescent signal. In the detection strategy, one thrombin molecule can form one aptamer/protein/aptamer

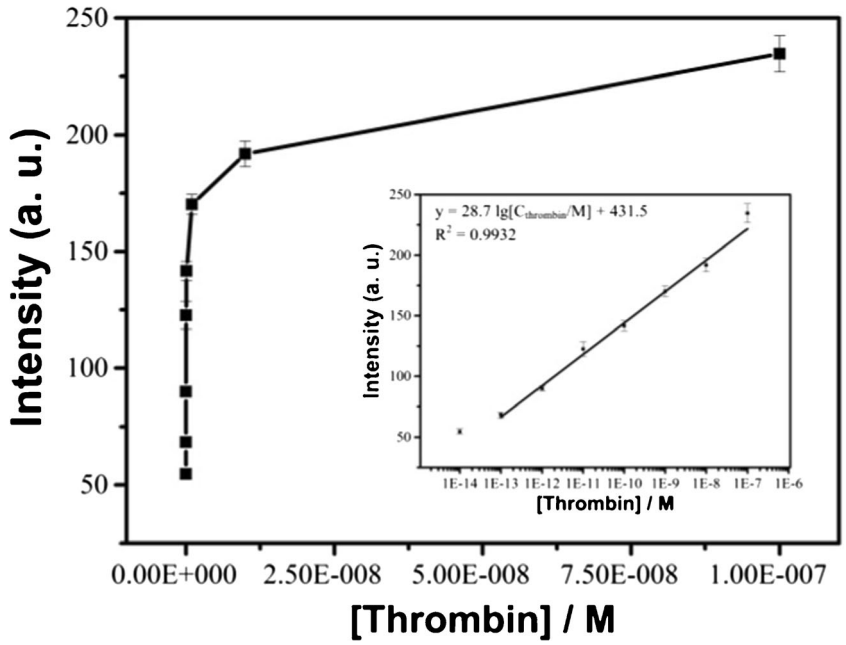


Fig. 3 Fluorescence intensity of the silicon chip toward varying concentrations of thrombin (FITC filter, Ex=480 nm)

Table 1 Comparison of recent methods for detection

Method	L. R. (M)	D.L.(M)	Reference
Fluorescent aptasensor	1.0×10^{-8}~4.0×10^{-7}	2.4×10^{-9}	[2]
Electrochemical	5×10^{-11}~$1.\times10^{-8}$	1.2×10^{-11}	[3]
Aptamer	1.0×10^{-8}~1.0×10^{-7}	7.3×10^{-12}	[7]
Aptamer	5.0×10^{-8}~2.0×10^{-7}	1.8×10^{-8}	[8]
Photoelectrochemical aptasensor	1.0×10^{-12}~1.0×10^{-11}	4.5×10^{-13}	[9]
Fluorescent aptasensor	1.0×10^{-10}~4.0×10^{-9}	5.0×10^{-11}	[10]
Colorimetric	1.0×10^{-12}~7.5×10^{-8}	1.4×10^{-13}	[33]
Fluorescent aptasensor	1.0×10^{-13}~1.0×10^{-7}	1.95×10^{-15}	This work

sandwich structure. This sandwich was labeled with a number of fluorescein-labeled 12-dATP molecules and result in one exponential signal amplification. Furthermore, because the complexity of surface chemistry, such as fluorescein-labeled 12-dATP's interactions on surface, as well as interactions between fluorescein-labeled 12-dATP and other molecules in this system. Therefore thrombin detectable range will be shorter, even the interaction mechanism is not very clear yet. As observed in Fig. 3, there was a good linear response between the fluorescence intensity and the logarithm of thrombin concentration over the range of 100 fM to 0.1 μM. The repeatability of the system was assayed by a relative standard deviation (R.S.D.) of intra- and inter-assays (3.2% and 3.4%, respectively). According to the regression equation: $y = 28.7 \lg [C_{\text{thrombin}}/\text{M}] + 431.5$, with a coefficient of determination (R^2) of 0.9932, the limit of detection (LOD) is calculated to be 2.0 fM (S/N = 3). Compared with the expensive or complicated readers in reports previously

Table 1 this fluorescent signal amplification strategy can achieve low limit detection. Besides, this "signal-on" positive feedback method is highly tolerant to the results of false-positive. After storage in moisture-saturated environment at 4 °C for four weeks, chips still can be reused. The improved sensitivity and the broad dynamic range of the sandwich biosensor also can be ascribed to the low background and the high density binding sites on the silicon surface. What's worth mentioned is its wide linear range, over 7 orders of magnitude, which is great importance to clinical trace analysis.

Specificity and application of serum samples

Several single non-target protein groups (as the negative control experiment), a blank group and a thrombin detection system (mixed five kinds of interference proteins) were studied under the same conditions to verify the selectivity of the sandwich biosensor. The chip showed remarkable fluorescence enhancement only when the detection objects is thrombin rather than any other protein present: alkaline phosphatase (ALP), human serum albumin (HSA), acid phosphatase (ACP), albumin from bovine serum (BSA) and trypsin (Try). As seen from Fig. 4a, the net fluorescence intensity(deducted the fluorescent background) of 0.1 μM thrombin was around 18 times when it compared to other five interfering species, whose concentrations were 10 μM. The special recognition between the aptamer and the corresponding protein achieved via the specific sequence of the aptamer, which can fold into G-quadruplex structure to stabilize the detection system [2, 4]. In this analytical system, the sandwich biosensor use two aptamers to specifically capture and immobilize the thrombin, and the "signal-on" fluorescence signal was amplified with the directly TdT-based rapid "green" process. Therefore, selectivity of the system and the accuracy of experiment results are greatly enhanced and guaranteed. Compared to the traditional design method, this assay improves selectivity and avoids the interference from other proteins.

Finally, to further evaluate the promising application of the sandwich biosensor for protein in clinical applications, the

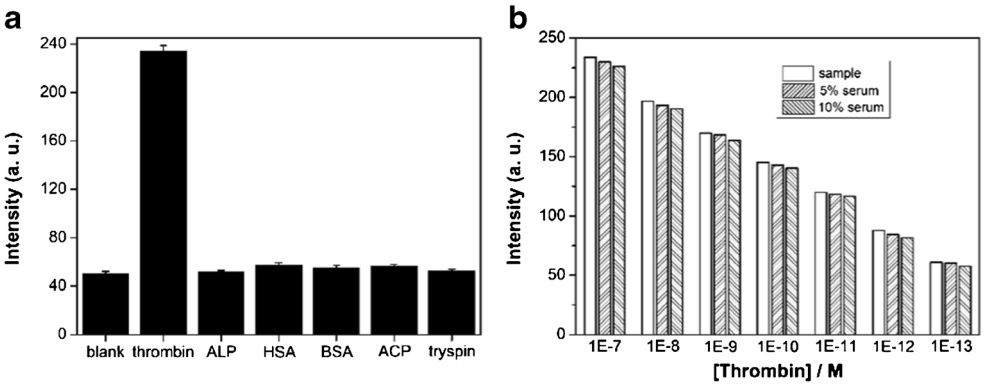


Fig. 4 **a** Selectivity of the aptasensor for thrombin detection. (The concentration of all interfering species was 10 μM and thrombin concentration was 0.1 μM.) **b** Application of the aptasensor for different concentration thrombin in the sample with 5% and 10% serum

analytical reliability of complex biological components with the interferences was investigated. As exhibited in Fig. 4b, the thrombin concentration used in all groups was 0.1 μM. Compared with TE buffer system, their average estimate absorptions in 5% and 10% serum samples were 96.65% and 95.31%, respectively. All these data highlighted that this sandwich fluorescent biosensor for protein detection revealed favorable analytical reliability and usefulness in complex biological environment, such as in real diluted human serum.

Conclusions

A highly selective and sensitive sandwich format is described for aptamer based determination of thrombin using a silicon chip. The method is based on TdT initiated fluorescence amplification and employs a fluorescein-12-dATP acting as the detection probe and two dual-functional aptamers as protein capture probes. Characterizations of different modified-silicon chip surface through WCA, XPS and AFM, confirmed the success of aptamer/ thrombin/aptamer sandwich fluorescence biosensor. Under optimized conditions, we obtained a linear response between the fluorescence intensity and logarithm of thrombin concentration, as well as a good low limit of detection. Furthermore, all reactions were carried out under isothermal conditions at room temperature to minimize its technological complexity and make it field portable for point-of-care analysis. The detection results in interferences and serum samples demonstrate that, sandwich structure ensured the probe accurately capture interested target protein; high density fluorescent signal by TdT enzyme initiated on-chip improved the accuracy and availability of the detection. Crucially, TdT catalyzed polymerization of fluorescein-12-dATP labeling for *in situ* fluorescence signal amplification greatly expand the detection range and decrease the limit of detection. This strategy has great potential for a wider range of the proteins with no customized detection probes or other signal amplifiers, by replacing the target proteins and their aptamers. There is no doubt that this template-independent is "green", highly selective and sensitive protein detection. Even so, there still are some disadvantages such as TdT enzyme used in this method is expensive and instable, which limited its potential for application. Future work will focus on the system of enzyme-free polymerization and application of various types of analytes in clinical serum samples detection.

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Compliance with ethical standards The author(s) declare that they have no competing interests.

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