



Aptamer-functionalized magnetic and fluorescent nanospheres for one-step sensitive detection of thrombin

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Abstract

A one-step sandwich method is described for detecting proteins with magnetic nanospheres (MNs) and fluorescent nanospheres (FNs). Thrombin is selected as a model analyte to validate the method. Two DNA aptamers (Apt 29 and Apt 15 targeting two different exosites of thrombin) are chosen as recognition elements to modify MNs and FN. The superparamagnetic MN-Apt 29 conjugate is used to separate and concentrate thrombin. The FN-Apt 15 conjugate encapsulates hundreds of fluorescent quantum dots and is used as reporter to provide a stable signal. Magnetic capture and fluorescence identification are performed simultaneously to form a sandwich complex (MN-Apt 29-thrombin-FN-Apt 15) for fluorescence determination (at excitation/emission wavelengths of 380/622 nm). The method is convenient, time saving, and gives a strong signal (compared to the two-step method where capture and identification are performed in two steps). The one-step method presented here is completed within 30 min and has a $3.5 \text{ ng}\cdot\text{mL}^{-1}$ (97 pM) detection limit. The method is reproducible, has an intra-assay variability of 1.5%, and an inter-assay variability of 4.9%. Other serum proteins (HSA, CEA, PSA, and AFP) do not interfere. The method was also applied to analyze serum samples. Almost the same fluorescence intensity was measured when analyzing 1% serum samples (compared to buffer samples).

Keywords Magnetic nanospheres · Fluorescent nanospheres · Quantum dots · Magnetic nanoparticles · Biomarker · One-step detection · Aptamer

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Introduction

Protein biomarkers are one of most important kinds of biomarkers. Their detection is of great significance to clinical application [1]. For instance, thrombin is a central enzyme involved in platelet aggregation and blood coagulation. It plays important roles in hemostasis, inflammation, thrombosis, angiogenesis, tumor growth and metastasis. It has become an important therapeutic and biomarker for diseases associated with coagulation abnormalities [2, 3]. So far, in practical applications, chromatography/mass spectrometry-based methods and antibody-based immunoassays (e.g., enzyme-linked immunosorbent assay, ELISA) are still the most commonly used diagnostic methods for protein detection [4–6]. These methods have good accuracy. But they suffer from the disadvantages of time consuming, laborious and complex manipulation, high cost, or requiring sophisticated instruments and skilled technical staffs. Hence, sensitive, convenient, and reliable method for detecting protein biomarkers has always been pursued.

With the rapid progress of nanoscience and nanotechnology, numerous nanomaterials have been applied to biological detection and medical diagnosis. Quantum dots (QDs) and magnetic nanoparticles have shown great superiority and potential [7, 8]. QDs are newly developed versatile fluorescent nanoparticles. They have unique optical properties, such as good photochemical stability, high photoluminescence quantum yields, size/composition-dependent and narrow emission spectra with broad absorption, etc. QDs have become excellent report probes for improving detection sensitivity and multiplexed analysis [9, 10]. Magnetic nanoparticles exhibit superparamagnetic properties at room temperature. They behave just like a giant paramagnetic atom, and they have fast magnetic response and negligible remanence. These properties avoid magnetic nanoparticles agglomerating and allow them to be manipulated easily just by a magnet [11, 12]. Magnetic nanoparticles-based separation techniques have been used for efficient enrichment and purification in complex sample analysis. They greatly reduce interference and enhance signal sensitivity [13, 14].

Nowadays, QDs and magnetic nanoparticles have been widely applied to protein detections. However, despite their promising potential, some challenges still exist in practical operation. For instance, their optical or magnetic properties are usually affected during the modification or biofunctionalization process. They suffer from the problems of aggregation, nonspecific adsorption, or colloidal instability in complex matrices [7, 9, 15]. In our previous work, by incorporating QDs and magnetic nanoparticles with polymer nanospheres, we constructed fluorescent and magnetic nanospheres (FNs and MNs) [16, 17]. The FN and MN inherit the unique fluorescence and magnetic properties of QDs and magnetic nanoparticles. Moreover, they also show superiority: high stability, amplified signal, great versatility, and convenient manipulation. Accordingly, the FN and MN may be expected as promising tools for protein biomarker detection.

In the present work, a one-step convenient and sensitive method for detecting protein biomarkers is developed utilizing our prepared MNs and FNs. Thrombin is used as a model protein. Aptamers are used as the recognition elements. The aptamers exhibit the superiority over antibodies. They have good affinity and specificity, convenient generation, low cost, easy modification, and high resistance against denaturation [18]. In the method, MNs and FNs are respectively modified with Apt 29 (29-base DNA aptamer targeting the heparin-binding exosite of thrombin) and Apt 15 (15-base DNA aptamer targeting the fibrinogen-recognition exosite of thrombin) [19, 20]. Then, the MN-Apt 29 and FN-Apt 15 are used to specifically capture and recognize thrombin simultaneously.

This method is completed within 30 min, and has a detection limit of $3.5 \text{ ng}\cdot\text{mL}^{-1}$ (97 pM). It is more time-saving and can get higher detection signal than the two-step method. (Capture and identification are performed separately in the two step method.) Besides, this method exhibits good reproducibility, high specificity, and can be applied in serum samples. Thus, this method has potential application in practice.

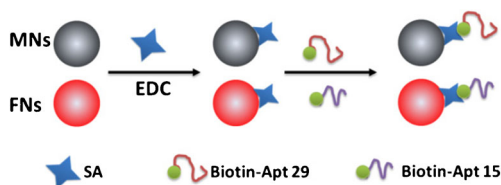
Experimental

Materials and reagents

Carboxyl-terminated poly(styrene/acrylamide) polymer nanospheres (Pst-AAm-COOH) were prepared by emulsifier-free polymerization method [21]. Hydrophobic oleic acid-capped magnetic nanoparticles were synthesized by high-temperature pyrolysis [17]. Hydrophobic trioctylphosphine oxide (TOPO)-capped CdSe/ZnS QDs (emission wavelength: 620 nm) were purchased from Wuhan Jiayuan QDs Co., Ltd. (<http://www.qds.net.cn/>). Branched poly(ethylene imine) (PEI), bovine serum albumin (BSA), tetraethyl orthosilicate (TEOS), (3-aminopropyl)triethoxysilane (APTES), polyvinylpyrrolidone (PVP), human serum albumin (HSA), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), and *N*-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich. (<https://www.sigmaaldrich.com/china-mainland.html>). Human α -thrombin was bought from Haematologic Technologies, Inc. (<https://www.haemtech.com/>). Streptavidin (SA) was purchased from Amresco. (<http://www.amresco-inc.com/>). Difco skim milk was got from Becton, Dickinson and Company. (<http://www.bd.com/>). Carcinoembryonic antigen (CEA), Human alpha fetoprotein (AFP), and prostate specific antigen (PSA) were purchased from Nanjing sanchen biotechnology Co., Ltd. (<http://njsc2015.bioon.com.cn/>). Fetal bovine serum (FBS) was bought from Gibco. (<https://www.thermofisher.com/cn/zh/home/brands/gibco.html>). The biotinylated 15-base DNA aptamer (Apt 15) and 29-base DNA aptamer (Apt 29) against Human α -thrombin were purchased from Invitrogen Corp. (<https://www.thermofisher.com/cn/zh/home/brands/invitrogen.html>). The aptamers have the following sequences, and an oligodeoxythymidine (oligo(dT)) spacer (12 T) was added to reduce the binding steric hindrance:

Apt 15: Biotin-5'-TTT TTT TTT TTT GGT TGG TGT GGT TGG-3'

Apt 29: Biotin-5'-TTT TTT TTT TTT AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3'



Scheme 1 Schematic diagram for construction of thrombin-targeting MNs and FNs

Apparatus

Transmission electron microscopy (TEM) images were got by an electron microscope (FEI Tecnai G² 20 TWIN). Fluorescence images were acquired by an inverted fluorescence microscope (Ti-U, Nikon, Japan) mounted with a CCD camera (Nikon digital sight DS-U3). Magnetic hysteresis loops were determined by a Lake Shore 7410 vibrating sample magnetometer. Magnetic separation was performed with a commercial magnetic scaffold (Invitrogen, 12320D, the field strength on the surface of the magnetic scaffold is 325 ± 25 mT). Fluorescence emission spectra were recorded with a fluorescence spectrometer (Hitachi, F-7000).

Construction of thrombin-targeting MNs and FNs

The MNs and FNs were respectively prepared with embedding method and assembly method according to our previous work. The detail procedures are described in the Electronic Supporting Material (S.1). The MNs and FNs were first modified with SA by carbodiimide chemistry, and then coupled with the biotinylated DNA aptamer via biotin-streptavidin conjugation (Scheme 1). Typically, 5 mg of MNs or FNs were dispersed in 1 mL of phosphate buffer (0.01 M pH 7.2) containing 50 mM EDC to react with 0.1 mg of SA. They were shaken gently at room temperature. After 4 h reaction, the MNs were separated by magnetic attraction and the FNs were separated by centrifugation. They were washed five times by phosphate buffer to remove any unreacted SA. Then, the SA

conjugated MNs and FNs were respectively incubated with 10 μ L of biotin-Apt 29 (100 mM) and 10 μ L of biotin-Apt 15 (100 mM) for 2 h at room temperature. Afterwards, the resultants were washed with phosphate buffer to remove any unreacted aptamer. The thrombin-targeting MNs and FNs (MN-Apt 29 and FN-Apt 15) were obtained. Finally, they were blocked with 1% BSA- phosphate buffer and stored at 4 °C for use.

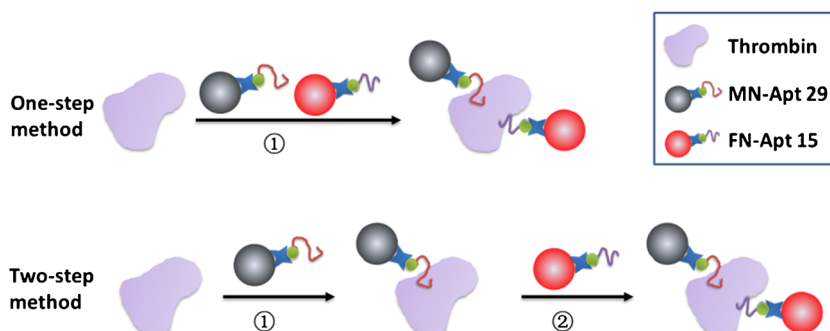
Thrombin detection protocols

Two kinds of strategies (one-step method and two-step method) were investigated for detecting thrombin, as illustrated in Scheme 2. In the one-step method, 0.10 mg of MN-Apt 29 and 0.90 mg of FN-Apt 15 were simultaneously added to 1 mL of sample (thrombin in buffer containing 50 mM Tris, pH 7.4, 140 mM NaCl, 1 mM MgCl₂, 5 mM KCl, and 0.1% skim milk). After a gentle shaking for 30 min, the sandwich complexes were separated with a magnetic scaffold. They were washed four times by the buffer and once by the buffer without skim milk. Finally, they were resuspended in 400 μ L of skim milk-free buffer for fluorescence spectrum measurements (Excitation wavelength: 380 nm; Emission wavelength: 622 nm). In the two-step method, 0.10 mg of MN-Apt 29 was first added to the sample solution for incubation (30 min) to capture thrombin. Then, the MN-Apt 29-thrombin complexes were separated with a magnetic scaffold and resuspended in the buffer. Then, 0.90 mg of FN-Apt 15 was added. After incubation for 30 min, the complexes were washed similarly with the one-step strategy and detected by a fluorescence spectrometer.

Detection of thrombin in serum samples

Thrombin (0.15 μ g) was spiked into 1 mL of diluted FBS (10, 100, and 1000 times dilution of FBS with the above buffer) as synthetic samples. MN-Apt 29 and FN-Apt 15 were added to the serum samples following the above one-step detection procedure. Control experiments were performed under the same condition except no thrombin was added.

Scheme 2 Schematic diagram of one-step strategy and two-step strategy for detecting thrombin



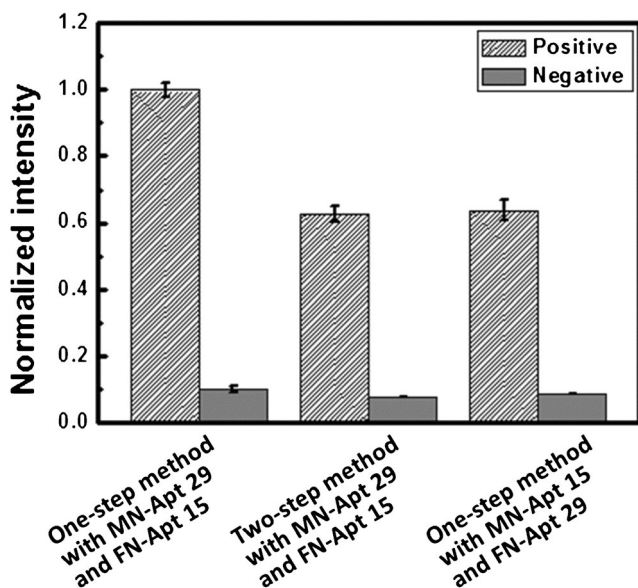


Fig. 1 Histogram for fluorescence intensities of positive and negative samples with different detection protocols. Error bars = \pm SD ($n = 3$). (Excitation wavelength: 380 nm; Emission wavelength: 622 nm)

Results and discussion

Choice of materials

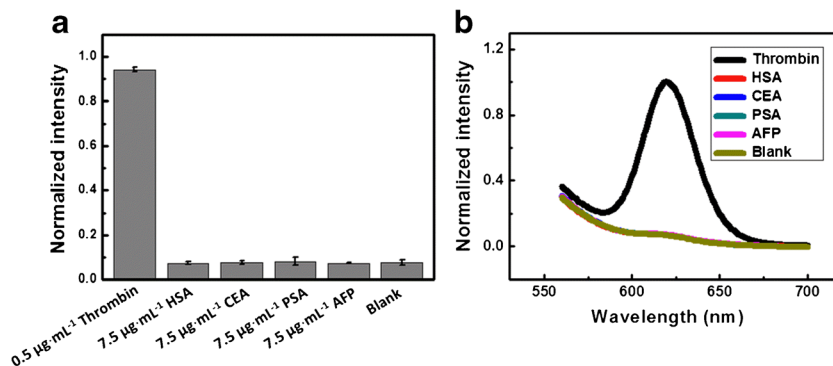
Protein biomarkers usually exist in complex body fluid with low concentrations. This makes their detection suffer from the problems of interference and weak signal. Hence, on the one hand, our prepared MNs with excellent superparamagnetic properties, high stability, and efficient recognition capacity are used to capture target proteins from complex matrices [17]. This is expected to remove the contaminants, and achieve purification and enrichment of target proteins. On the other hand, our prepared FNs encapsulating hundreds of QDs are used as reporters [16]. This is expected to give highly amplified signal. The characterizations of the FNs and MNs are shown in the Electronic Supporting Material (S.2) to

confirm their good fluorescence and magnetic properties. Based on these considerations, we infer that the combination of MNs and FNs can improve the sensitivity of protein detection.

Selection of the favourable detection protocol

DNA aptamers against thrombin have been extensively investigated. Two specific aptamers (donated as Apt 29 and Apt 15) have been used most widely. Apt 29 contains 29 bases targeting the heparin-binding exosite of thrombin. Apt 15 contains 15 bases mainly interacting with the fibrinogen-recognition exosite of thrombin [19, 20]. In this method, Apt 29 and Apt 15 are chosen for the sandwich assay: Apt 29 modified MNs are used to capture thrombin, and Apt 15 modified FNs are used to identify thrombin. It is noteworthy that during the modification, an oligo(dT) spacer (12T) rather than a commonly used dodecyl is introduced between the nanospheres and the aptamers. That's because a negatively charged, flexible oligo(dT) spacer can induce much more effective binding than a dodecyl spacer [22]. For thrombin detection, one-step method and two-step method were both investigated (Scheme 2). The detection results are shown in Fig. 1. It can be seen that the one-step strategy achieve higher signal-to-noise ratio (the ratio of the fluorescence intensity detected from positive sample to that from blank sample, S/N) compared with the two-step strategy. As mentioned above, Apt 29 and Apt 15 mainly recognize different sites of the thrombin. Thus, there is little competition over the binding sites between them. However, in the two-step method, MN-Apt 29 is first added to the samples and preferentially binds with thrombin. The formed MN-Apt 29-thrombin complexes may need overcoming larger steric hindrance to bind with FN-Apt 15. This probably induces less FN-Apt 15 binding and lower fluorescence signal compared with the one-step strategy where free thrombin interacting with FN-Apt 15. Apart from the enhanced signal and higher sensitivity, the one-step method also

Fig. 2 a Histogram for fluorescence intensities of positive samples ($0.5 \mu\text{g}\cdot\text{mL}^{-1}$ thrombin), negative samples ($7.5 \mu\text{g}\cdot\text{mL}^{-1}$ HSA, CEA, PSA, and AFP), and blank samples. Error bars = \pm SD ($n = 3$). **b** Corresponding typical fluorescence spectra. (Excitation wavelength: 380 nm; Emission wavelength: 622 nm)



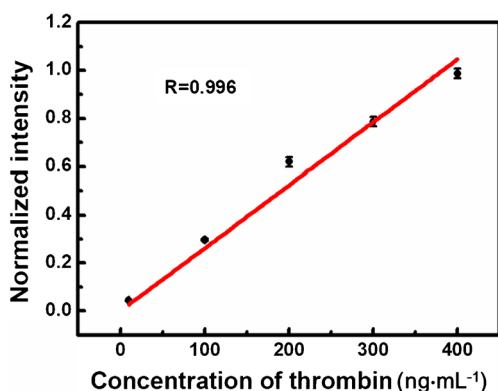


Fig. 3 Calibration curve of the fluorescence intensity versus thrombin concentration from 10 to 400 ng·mL⁻¹ (That's from 0.3 to 11.1 nM). Error bars = \pm SD (n = 3). (Excitation wavelength: 380 nm; Emission wavelength: 622 nm)

exhibits more convenient and more time-saving manipulation. Thus, one-step strategy is chosen for thrombin detection rather than two-step strategy. In addition, we investigated the effect of exchanging capture aptamer and identification aptamer on the detection signal. MN-Apt 15 and FN-Apt 29 were used for one-step detecting thrombin. Compared with the initial design (one-step method with MN-Apt 29 and FN-Apt 15), the latter shows lower S/N (Fig. 1). The MNs have larger size (350 nm) compared with the FNs (200 nm), as shown in Fig. S2 in the Electronic Supporting Material. Larger size makes the MNs have larger steric hindrance and lower flexibility in bio-recognition. As reported, Apt 29 binds to thrombin with 20 to 50-fold higher affinity than Apt 15 [19, 20]. Thus, we presume that using MN-Apt 29 for capture can compensate for the disadvantages of MNs in recognizing

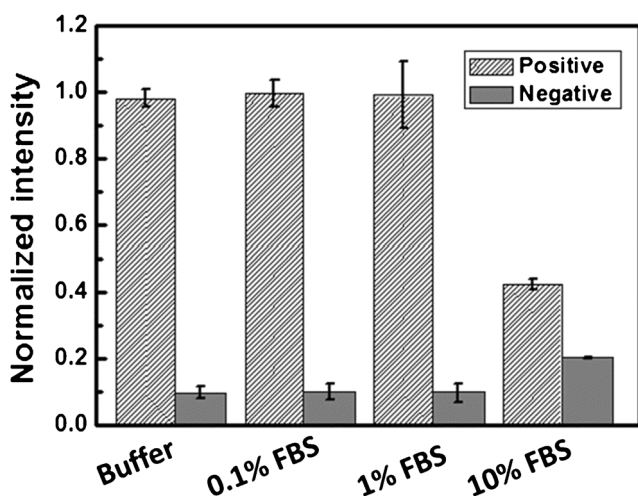


Fig. 4 Histogram for fluorescence intensities of positive and negative samples in presence of different concentrations of FBS. Error bars = \pm SD (n = 3). (Excitation wavelength: 380 nm; Emission wavelength: 622 nm)

thrombin, thereby achieving higher detection signal than using MN-Apt 15. Based on the above results, the one-step method with MN-Apt 29 and FN-Apt 15 is selected as the favourable protocol for detecting thrombin.

Optimization of the method

The following parameters were optimized: (a) Blocking condition; (b) Incubation temperature; (c) MN-Apt 29 dosage; (d) FN-Apt 15 dosage; and (e) Incubation time. Respective data and Figures (S.3) are given in the Electronic Supporting Material. The following experimental conditions are chosen: (a) a blocking condition of 0.1% skim milk; (b) an incubation temperature of 30 °C; (c) a MN-Apt 29 dosage of 0.10 mg; (d) a FN-Apt 15 dosage of 0.90 mg; and (e) an incubation time of 30 min.

Reliability and specificity

To investigate the specificity of this method, this method was used to detect 0.5 $\mu\text{g}\cdot\text{mL}^{-1}$ thrombin as positive samples, several types of serum proteins (HSA, CEA, PSA, and AFP, 7.5 $\mu\text{g}\cdot\text{mL}^{-1}$) as negative controls, and blank buffer samples. Fluorescence intensities collected from the six samples are shown in Fig. 2a, and typical fluorescence spectra are in Fig. 2b. The positive samples exhibit strong FN fluorescence. No obvious FN peak is found in the negative samples and their fluorescence intensity is almost as low as that of the blank samples. These results suggest that those nonspecific proteins with concentrations even more than 10 times higher than that of the thrombin do not produce detectable fluorescence signal. These confirm that the aptamer-based sandwich method has good specificity and selectivity. The intra-assay variability (variation coefficient of the results from the parallel samples detected with the same batch of thrombin-targeting nanospheres) and inter-assay variability (variation coefficient of the results from the parallel samples detected with different batches of thrombin-targeting nanospheres) are calculated as 1.5 and 4.9%. These indicate this method has good reproducibility.

Quantitative analysis and detection limit

Quantitative analysis is achieved by measuring the fluorescence intensity of the sandwich complexes. The fluorescence intensities of samples at different thrombin concentrations were recorded by a fluorescence spectrometer. As shown in Fig. 3, a linear relationship is exhibited at the concentrations from 10 to 400 ng·mL⁻¹. Furthermore, by calculating the ratio of 3 times the standard deviation of the blanks to the slope of the linear equation of the calibration curve, the limit of detection (LOD) is calculated as 3.5 ng·mL⁻¹ (LOD = 3SD/slope, n = 11). Based on the reported molecular weight of the

Table 1 An overview on recently reported aptamer-functionalized nanoparticle-based fluorescence methods for the detection of thrombin

Nanoparticles used	Linear range (nM)	Detection limit (nM)	Detection time	References
Fluorescent copper nanoparticles	10–400	2.4	> 6 h	[24]
Metal-organic framework platform	0.05–100	0.015	> 3 h	[25]
Ag@SiO ₂ nanoparticles and graphene oxide	0.1–4	0.050	30 min	[26]
CdTe quantum dots and Pt nanoparticles	0.00005–10	0.000015	> 24 h	[27]
Silica nanoparticles	0.07–2	0.020	ca. 1 h	[28]
CuInS ₂ quantum dots	0.034–102	0.012	ca. 1 h	[29]
Ag nanoclusters	50–900	8.4	>3 h	[30]
Bio-dots and gold nanoparticles	0–35	0.59	ca. 1 h	[31]
Magnetic nanoparticles	1–60	0.5	40 min	[32]
MnO ₂ nanosheets	0–100	11	15 min	[33]
Gold nanoparticles	0.5–110	0.5	1 h	[34]
Magnetic/Fluorescent nanospheres	0.3–11.1	0.097	30 min	This work

thrombin (36 kDa) [23], the one-step method quantitatively detects thrombin with the concentrations ranging from 0.3 to 11.1 nM with a LOD of 97 pM.

Detection of thrombin in serum samples

Thrombin is a plasma protein, and thus it is essential that this new method can be applied in serum. To assess the potential application of this one-step sandwich method in practice, FBS was used to mimic real samples for detection. As shown in Fig. 4, the fluorescence intensities measured from the 1000-fold and 100-fold diluted serum samples (0.1% FBS and 1% FBS) are very close to those obtained with plain buffer samples. The fluorescence signals from the 10-fold diluted serum samples (10% FBS) obviously decrease. The negative 0.1 and 1% FBS samples and the buffer samples exhibit similar low background signals. The negative 10% FBS samples generate slightly higher signals. These results suggest that serum diluted 100 times and higher dose not significantly interfere with the detection signal. But lower dilutions of serum reduce the detection signal attributing to the complex matrices in the serum. Therefore, to ensure detection accuracy, serum samples should be diluted 100 times for the one-step sandwich detection. This is acceptable given the high sensitivity of this method.

Conclusions

In summary, we report a one-step sandwich method for detecting thrombin. MN-Apt 29 and FN-Apt 15 are used to

capture and identify thrombin simultaneously. This method can be completed within 30 min, and has high sensitivity with picomolar detection limit. Compared with most of the recently reported methods for thrombin detection (Table 1), our method has lower or comparable detection limit, and consumes much less time. (The method based on CdTe quantum dots and Pt nanoparticles achieves much higher sensitivity, yet it takes too much time; The method with MnO₂ nanosheets just needs 15 min, yet it has much lower sensitivity.) Besides, our method has good reproducibility, high specificity, and can be manipulated conveniently with low cost. It does not require sophisticated instruments, complex pretreatment or highly trained personnel. Finally, this method is successfully applied to 100-fold diluted serum samples, indicating its potential application in practice. Furthermore, the concept of this method can be expanded to other biomarker detection and can be designed for simultaneously detecting multiple biomarkers with multicolor reporters. While it should be noted that this sandwich strategy has a major limitation. The target protein should have at least two binding sites for respectively combining MNs and FNs to form the sandwich structure. Nonetheless, this method is still expected for sensitive, reliable and rapid detection of thrombin and facilitating research on diseases associated with coagulation abnormalities.

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Compliance with ethical standards The authors declare that they have no competing interests.

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