

A general protein aptasensing strategy based on untemplated nucleic acid elongation and the use of fluorescent copper nanoparticles: Application to the detection of thrombin and the vascular endothelial growth factor

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Abstract The authors describe an aptamer-based detection scheme that is based on untemplated nucleic acid elongation and the use of copper nanoparticles (CuNPs) as a fluorescent probe. An aptamer without any other auxiliary sequence and label is required only which makes the method rather convenient. Under the catalysis of terminal deoxynucleotidyl transferase (TdT), the single-stranded aptamer is elongated without template. By using dTTPs as the substrate, long linear poly T can be produced, and these can act as templates for the synthesis of CuNPs which display red (617 nm) fluorescence under 349 nm photoexcitation. In the presence of the analyte, the TdT-catalyzed production of poly T is blocked, and this results in suppressed fluorescence. The strategy was successfully applied to the determination of the proteins thrombin and vascular endothelial growth factor 165. Only three steps are involved in the whole assay. This aptamer-based assay is believed to have a wide scope in that it may be applied to the analysis of many other proteins if the corresponding aptamers are available.

Keywords Aptamer · Terminal deoxynucleotidyl transferase · Poly T · DNA templated copper nanoparticles · Fluorescence · Protein detection

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Introduction

Aptamers are single-stranded oligonucleotides isolated from random-sequence DNA or RNA libraries by an in vitro selection process termed the systematic evolution of ligands by exponential enrichment (SELEX) [1–3]. Aptamers have been greatly developed in the field of bioassays as a new branch of aptamer-based assays since their first discovery in 1990s [2]. Compared with antibodies, aptamers can bind to a wide range of target molecules with high affinity and specificity including small molecules [4–6], proteins [7–11] and even entire cells [12–14]. And they can be selected by SELEX process and produced by chemical synthesis with low cost, simple production, design flexibility, low immunogenicity and good stability. Many techniques have been utilized in aptamer-based assays, such as electrochemistry [15–19], fluorescence [5, 6, 20–22], colorimetry [8, 23, 24], electrochemiluminescence [25, 26] and surface plasmon resonance [10, 27], *etc.* Among these methods, fluorescence assays have attracted much attention due to its simplicity, sensitivity and rapidness. However, most fluorescence aptamer-based assays require labelled chromophores and quenchers either at the ends or selective sites of the aptamers [6, 28]. The labeled fluorescence aptamer-based assays have certain drawbacks, such as relatively expensive labeled oligonucleotides and high background signal between fluorophores and quenchers. In addition, the fluorescent labelling may affect the binding affinity of aptamers with their targets. Therefore, it is necessary to develop a sensitive, low cost, and label-free fluorescence method for aptamer-based assays [29]. On the other side, sequence specified nucleic acid amplification is often used to elevate the sensitivity of the aptamer-based assays. These techniques include hybridization chain reaction, rolling circle amplification, enzyme-assisted recycling amplification and so on [23, 30]. The problem of these kinds of strategy is the

dependence on the sequence of the aptamer, which makes the system lack of universality. To resolve this problem, we would like to look to the lately emerging findings.

Nowadays, double-stranded DNA (dsDNA) templated copper nanoparticles (CuNPs) have many applications in DNA assay, protein assay and ion detection due to the good photochemical properties and rapid synthesis [31, 32]. Morkhir et al. reported that dsDNA acted as the template for the formation of CuNPs at a low concentration of CuSO₄, whereas single-stranded DNA (ssDNA) did not support CuNPs formation [33]. Later, Wang et al. reported that poly(thymine) (poly T) can be used as ssDNA template for the formation of fluorescent CuNPs, while poly A, poly C, poly G failed to serve as the template for CuNPs formation under the same conditions [34]. Compared to other fluorescent metal nanoparticles, such as silver nanoclusters and gold nanoclusters, DNA templated CuNPs are more environment-friendly and have better photo-stability besides the advantages of low cost and rapid synthesis, and thus have great potential as signaling reporters in fluorescent assays. Currently, a nuclease-assisted aptamer-based assay for detecting ATP is developed by fluorescent CuNPs [35]. However, the exploration of poly T templated CuNPs for application in aptamer-based assays is still at an early stage, which may be because the fluorescence intensity of CuNPs is length-dependent but the design of most reported assay strategies is confined to ready-made short DNA sequences that contain only 25–40 continuous T bases, resulting in limited detection sensitivity.

In this work, terminal deoxynucleotidyl transferase (TdT) is selected as the unique enzyme which aids the untemplated nucleic acid elongation and produces the product of long linear poly T. In comparison to double chain DNA or poly T with fixed length, the long linear poly T yielded by TdT may provide a much longer template for fluorescent CuNPs synthesis and therefore may enable superior sensitivity for analytical applications. By ingeniously combining the TdT catalyzed nucleic acid elongation and the DNA templated formation of CuNPs, we here report a novel aptamer-based method to detect proteins. In this design, besides being a probe of the target protein, aptamer with a free 3'-OH terminus also act as the primer of TdT catalyzed elongation which endows the strategy with the universality. In the presence of the target protein, it will bind with the aptamer and block the free 3'-OH terminus of the aptamer, preventing the formation of long linear poly T and fluorescent CuNPs, resulting in suppressed fluorescence. In the absence of the target protein, the fluorescent CuNPs may be synthesized using long linear poly T as the template and serve as the label-free fluorescent reporters which make the assay very sensitive. Taking use of the strategy, thrombin and vascular endothelial growth factor 165 (VEGF165) are analyzed respectively. It is believed that the method is versatile in analyzing other proteins by applying their corresponding aptamers as the probes and primers.

Experimental

Materials and Reagents

Aptamer DNAs were synthesized and purified by Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China; <http://www.sangon.com>). The sequences of the aptamers for thrombin and VEGF165 respectively are as follows: 5'-GGT TGG TGT GGT TGG-3' [21] and 5'-TGT GGG GGT GGA CGG GCC GGG-3' [10]. Terminal deoxynucleotidyl transferase (TdT) was purchased from New England Biolabs (Beijing, China; <http://www.neb-china.com>). Recombinant murine VEGF165 and 100 mM dTTP solution were obtained from Bio Basic Inc. (Shanghai, China; <http://www.sangon.com>) Thrombin (Tb), ascorbic acid, ovalbumin (OVA), human serum albumin (HSA), immunoglobulin G (IgG) and hemoglobin (Hb) were purchased from Sigma-Aldrich Co. (<http://www.sigmaaldrich.com>). Copper sulfate (CuSO₄·5H₂O), potassium acetate (KAc), Tris-acetate (Tris-Ac), magnesium acetate (Mg(Ac)₂), cobalt chloride (CoCl₂) and all other chemical reagents were of analytical grade, obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China; <http://en.reagent.com.cn>) and used without further purification. For all experiments, Milli-Q water (>18.0 MΩ) was purified by a Milli-Q ultrapure water system (Millipore purification pack).

Apparatus

Fluorescence spectra were measured on an F-7000 fluorescence spectrometer (Hitachi, Japan), with both excitation and emission slits set at 5.0 nm, the PMT voltage set at 900 V. The emission spectra were exhibited in the range of 500–675 nm at room temperature with the excitation wavelength of 349 nm. Results of fluorescent emission images were recorded by the Gel Doc XR gel imaging system (BioRad). All fluorescent photographs were recorded by a nikon camera DL-7000.

TdT catalyzed nucleic acid elongation and poly T templated CuNPs formation

TdT catalyzes the repetitive addition of dNTPs to the free 3'-OH terminus of DNA primer with three or more nucleotides. With only dTTP as the substrate, TdT catalyzes the repetitive addition of dTTPs and poly T is consequently produced. In the implement of this work, the aptamer DNA with the free 3'-OH terminus was used as the primer. The specific elongation was realized by first preparing 25 μL of polymerization mixture which consisted of 20 nM aptamer DNA, 2 mM dTTP, 10 U TdT, 1 × TdT reaction buffer (50 mM KAc, 20 mM Tris-Ac, 10 mM Mg(Ac)₂, pH 7.9) and 0.25 mM CoCl₂ in the reaction system. Then the mixture was allowed to react at 37 °C for 4 h

in the PCR tube. Finally, the mixture was heated to 70 °C and kept for 10 min to stop the polymerization. Thus the TdT catalyzed nucleic acid elongation was finished and a long poly T tail was obtained following the 3' terminus of the aptamer DNA. The synthesis of CuNPs was accomplished with the poly T as the template. The procedure was as follows. Ascorbic acid (2.5 µL, 20 mM) and CuSO₄ (22.5 µL, 200 µM) were added to the mixture. After the whole solution being kept in dark at room temperature for 10 min, CuNPs with high fluorescence were formed. The fluorescence emission spectrum over 500 to 675 nm of the mixture was measured by F-7000 fluorescence spectrometer immediately under 900 V of PMT voltage and 5 nm of slit width. The excitation and emission wavelength was 349 nm and 617 nm respectively.

Preparation of aptamer-protein complex and fluorescence detection of proteins

The thrombin aptamer DNA (TBA, 100 µM) and the VEGF165 aptamer DNA (100 µM) were dissolved in Tris-HCl buffer (10 mM, pH 7.4). The solution was heated to 95 °C and kept for 5 min and then slowly cooled down to room temperature, permitting the aptamer to fold into its specific secondary structure. Thrombin was dissolved in the thrombin buffer (10 mM Tris-HCl, 50 mM NaCl, 50 mM KCl, 0.1% BSA, pH 7.5).

The TBA-thrombin complex was prepared by mixing 2.5 µL 200 nM TBA with 2.5 µL different concentrations of thrombin, followed by being incubated for 2 h at room temperature in order that the antiparallel G-quadruplexes of TBA would bind with thrombin to form the TBA-thrombin complex. For VEGF165, the VEGF165 aptamer (2.5 µL, 200 nM) was allowed to incubate with different concentrations of VEGF165 (2.5 µL) in Tris-HCl buffer (10 mM, pH 7.4) for 2 h at room temperature to fold.

A typical experiment for the detection of thrombin or VEGF165 was conducted as follows. Firstly, 5 µL of freshly prepared aptamer-protein complex was mixed with 5 µL of 10 mM dTTP, 1 µL of 250 U TdT, 2.5 µL of 10 × TdT reaction buffer, 2.5 µL of 2.5 mM CoCl₂ and 9 µL of Milli-Q water. The mixture was then maintained at 37 °C to perform TdT catalyzed nucleic acid elongation. After 4 h, the elongation was terminated by a 70 °C treatment for 10 min. Afterward, the above mixture was brought to a volume of 50 µL by adding 2.5 µL of 20 mM ascorbic acid and 22.5 µL of 200 µM CuSO₄. After being kept in dark at room temperature for 10 min, the fluorescence spectrum over 500 to 675 nm of the resulting mixture was collected with the excitation wavelength at 349 nm.

Gel electrophoresis analysis

Polyacrylamide gel electrophoresis was performed for the characterization of the products of TdT catalyzed nucleic acid

elongation. Briefly, 10 µL of the products together with 1 µL of 6 × loading buffer was loaded onto a 20% non-denaturing polyacrylamide gel. The electrophoresis experiments were carried out in 1 × Tris-boric acid-EDTA (TBE) at 120 V. After being separated for 90 min, the resulting gel was silver stained and photographed with a Bio-Rad GelDoc XR Imaging System.

Results and discussion

The design principle of the method

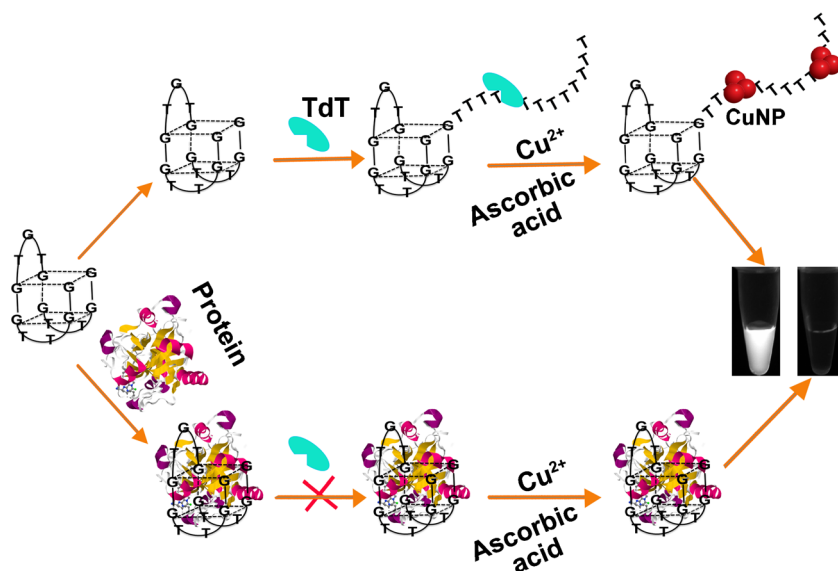
As Scheme 1 illustrated, the aptamer-based assay method designed in this work is sensitive upon the presence of the target protein. If the target protein is absent in the system, high fluorescence may be observed. When the target protein is introduced into the system, fluorescence intensity will decrease. The sensitivity is derived from TdT catalyzed poly T generation and poly T templated CuNPs formation which are highly fluorescent, while the difference between the presence and absence of the target protein is originated from the block of the 3'-OH terminus of the aptamer or not. Specifically, in the absence of the target protein, the aptamer of the target protein is served as the primer for TdT catalyzed nucleic acid elongation, which results in the formation of poly T. Next, when copper ion and ascorbate are added to the mixture, poly T is used as the template for CuNPs formation, which is highly fluorescent and may be accurately measured as the detection signal. Things are rather different when the target protein has been introduced into the system. As the protein will bind with the aptamer and form aptamer-protein complex, the 3'-OH terminus of the aptamer would be blocked or hard for TdT to access for the reason of steric hindrance. The aptamer-protein complex cannot be acted as the effective primer. Thus the aptamer cannot be elongated. Neither can CuNPs be formed. The fluorescence intensity is supposed to be very low in the present of the target protein.

Optimization of the experimental conditions for CuNPs formation

As the fluorescent properties of the CuNPs are directly related to the detection sensitivity, several experimental conditions that may influence the formation and the fluorescence intensity of CuNPs were intensively studied and optimized.

Different nucleic acid templates including poly T, poly A, poly C and poly G were examined to find out the best template for the formation of high fluorescent CuNPs. It is worth noting that poly N, where N denotes any one of the four bases, is synthesized by TdT catalysis using the aptamer as the primer and only one of the dNTPs as the substrate. The results are shown in Fig. 1. It is clear to observe that CuNPs synthesized

Scheme 1 Schematic illustration of the principle of the method for thrombin detection based on TdT catalyzed nucleic acid elongation and poly T templated CuNPs formation



using poly T as the template give the strongest fluorescence while solutions with other three dNTPs as the substrates nearly show no fluorescence response in the detected wavelength range (Fig. 1A). The reason might be that Cu^{2+} has stronger specific interaction with thymidine than the other three bases and therefore CuNPs may be formed with poly T as the template and ascorbic acid as the reducing reagent [35]. Different proportions of dTTP / dATP were also tested to synthesize different templates for CuNPs formation. Results show that 100% T is the best for high fluorescent CuNPs formation (Fig. 1B). Therefore, only dTTP was used as the substrate for the primer elongation and thereafter CuNPs formation in the following experiments.

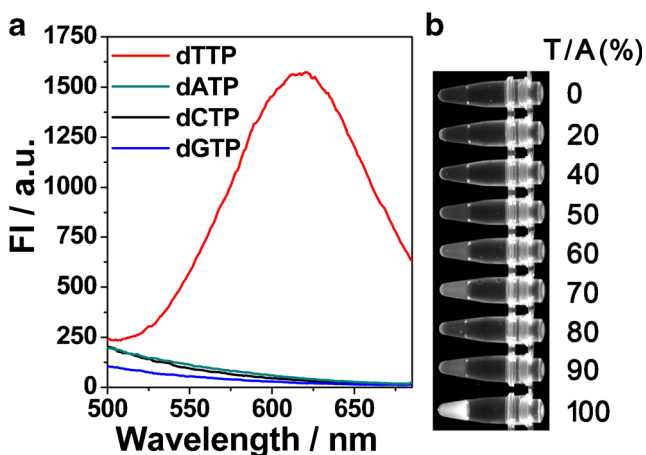


Fig. 1 (A) Fluorescence spectra of the solution under 349 nm photoexcitation after TdT catalyzed polymerization with dTTP, dATP, dCTP, dGTP as the substrate respectively and the formation of CuNPs. (B) Fluorescence images of the solution after TdT catalyzed polymerization with different proportions of dTTP / dATP as the substrate and the formation of CuNPs

Optimization of the experimental conditions for TdT catalyzed poly T formation

As poly T is the template for CuNPs formation and therefore deeply affects the fluorescence properties of CuNPs, the factors that influence the synthesis of poly T were extensively studied. It is noteworthy that the majority of the results of poly T synthesis are displayed as the fluorescence intensities of thereafter formed CuNPs since they are more accurate and convenient. Specifically, the following parameters were optimized: (a) concentration of TdT and (b) reaction time of TdT catalysis. Respective data and figures are given in the [Electronic supporting information](#). The following experimental conditions were found to give best results: (a) a TdT concentration of 10 U; (b) a reaction time of 4 h for TdT catalysis.

Feasibility investigation of the strategy and optimization of the binding time of thrombin and TBA

In order to verify our proposal, thrombin was employed as a model target for detection of protein. Fig. 2A depicts the fluorescence spectra under different conditions. In the absence of thrombin, poly T is synthesized by repeatedly dTTP adding to the 3'-OH terminal of TBA catalysed by TdT and CuNPs are formed with poly T as template. Specific fluorescence appears in curve a, Fig. 2A. In contrast, when thrombin is added to the solution, the TBA-thrombin complex cannot be extended by TdT and no fluorescence signal was detected (curve d, Fig. 2A). Besides, in the absence of TBA or TdT, the signals are also off (curve b and c, Fig. 2A). The results show that strategy is successful for the analysis of thrombin.

To obtain the maximum contrast before and after thrombin is added, the binding time of thrombin and TBA was explored. As is shown in Fig. 2B, the fluorescence intensity decreases

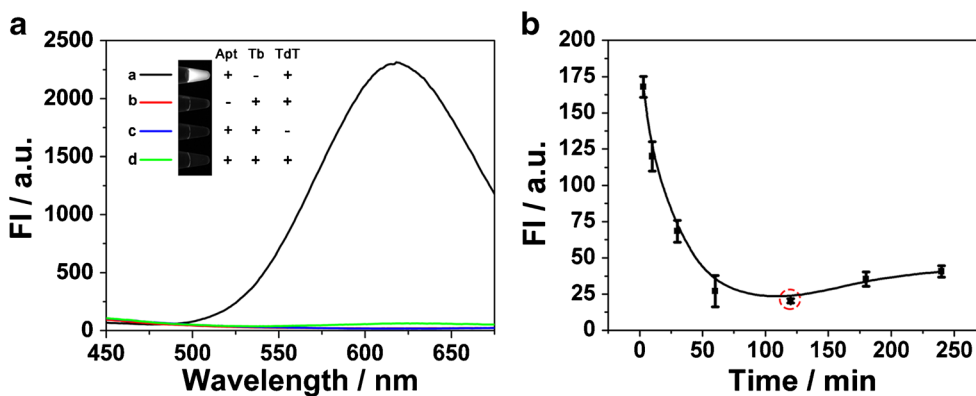


Fig. 2 (A) Fluorescence spectra of the solutions under 349 nm photoexcitation in the absence of thrombin (a), aptamer (b) or TdT (c), and in case that thrombin, aptamer and TdT were added (d). Inset is the photograph of different solutions. (B) Relationship between the

fluorescence intensity measured at 617 nm and the binding time of thrombin and TBA. The red spot shows the time for the minimum fluorescence intensity. The error bars were obtained by three separated measurements ($n = 3$)

sharply with the increase of the binding time and reaches the lowest signal in about 2 h. In order to obtain the best response, 2 h of binding time was used in further experiments.

Implementation of the strategy to the detection of thrombin

Under optimal conditions, different concentrations of thrombin were added to the detection system. Results are shown in Fig. 3. It can be seen from Fig. 3A that the fluorescence intensity measured at 617 nm decreases with the increase of the concentration of thrombin from 0 nM to 1 μ M. The specific relationship between the fluorescence intensity and the

concentration of thrombin is depicted in Fig. 3B. Obviously, the fluorescence intensity decreases linearly when the concentration of thrombin increases from 10 nM to 400 nM. The detection limit is calculated to be 2.4 nM at a signal to noise ratio of 3.

The specificity of the detection of thrombin

Other proteins were tested to inspect the selectivity of the assay. Human serum albumin (HSA), ovalbumin (OVA), hemoglobin (Hb), human IgG was used here. As is shown in Fig. 3C, only in the presence of thrombin, the fluorescence intensity measured at 617 nm decreases sharply. While other

Fig. 3 (A) Fluorescence spectra of the system under 349 nm photoexcitation after different concentrations of thrombin (0, 0.01, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 μ M, respectively) are added. (B) The fluorescence intensity measure at 617 nm is plotted with increased concentration of thrombin. Inset is the photograph of the system with increased concentration of thrombin and the linear relationship between the fluorescence intensity and the thrombin concentration. The error bars were obtained by three separated measurements ($n = 3$). (C) The fluorescence intensity measure at 617 nm of the system in the presence of no protein, thrombin, HSA, OVA, Hb and IgG. The concentration of each protein is 1 μ M. The error bars were obtained by three separated measurements ($n = 3$)

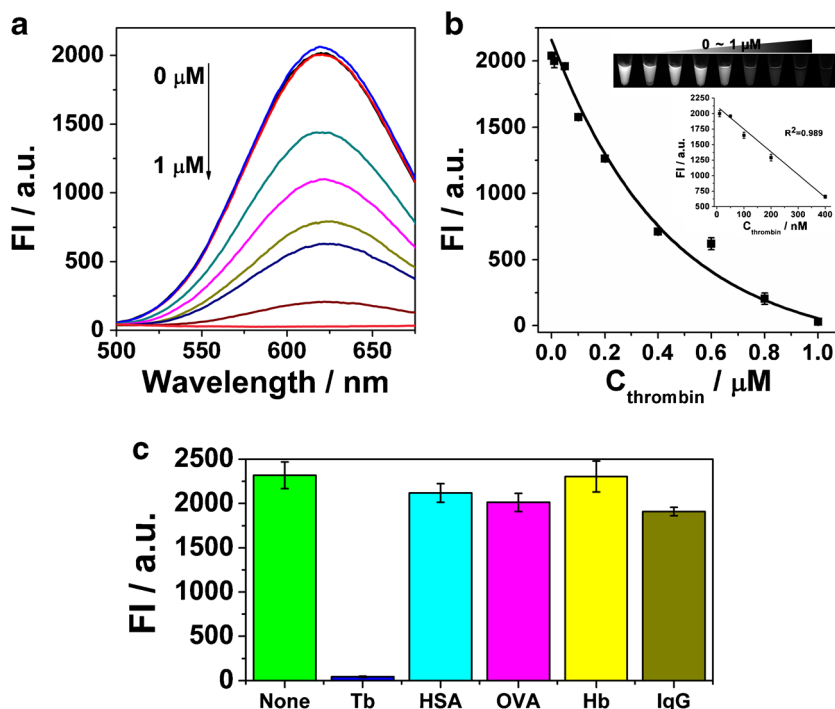
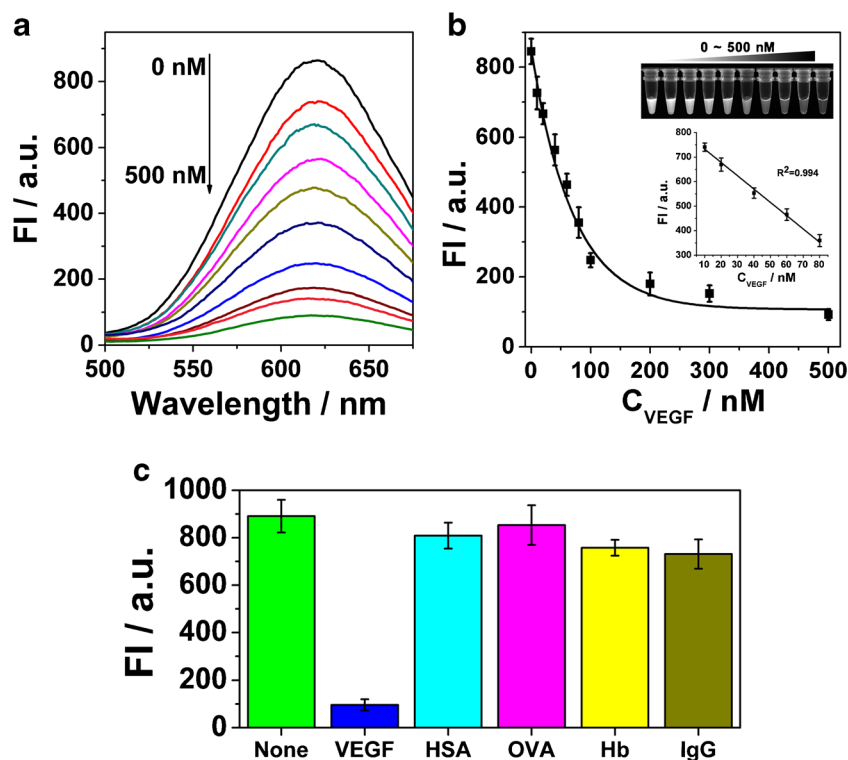


Fig. 4 (A) Fluorescence spectra of the system under 349 nm photoexcitation with different concentrations of VEGF165 (0, 10, 20, 30, 40, 50, 80, 100, 200 and 500 nM, respectively). (B) The fluorescence intensity measure at 617 nm is plotted with increased concentration of VEGF165. Inset is the photograph of the system with increased concentration of VEGF165 and the linear relationship between the fluorescence intensity and the VEGF165 concentration. The error bars were obtained by three separated measurements ($n = 3$). (C) The fluorescence intensity of the system measure at 617 nm in the presence of no protein, VEGF165, HSA, OVA, Hb and IgG. The concentration of each protein is 200 nM. The error bars were obtained by three separated measurements ($n = 3$)



proteins bring about signals as high as blank does, demonstrating that the method has great potential for the target protein detection with good selectivity.

Implementation of the strategy to the detection of VEGF165

To investigate the universality of the strategy, VEGF165 was further detected using our strategy. And the aptamer in the system was changed to be the aptamer of VEGF165. Similarly, following the increase of VEGF165, the fluorescence intensity of the system measured at 617 nm decreases (Fig. 4A). It can be seen from Fig. 4B that the fluorescence

intensity decreases gradually and finally reaches a plateau when the concentration of VEGF165 is 500 nM. A linear relationship dependent on the concentration of VEGF165 over the range 10 nM to 80 nM can be observed in the inset of Fig. 4B. The detection limit is calculated to be 1.3 nM at a signal to noise ratio of 3. We then compared the analytical performance of the present work with recently reported nanoparticle-based fluorescence methods for the detection of thrombin and VEGF165. As shown in Table 1, although the detection limit of the present work is only comparable or even higher than those of recently reported methods, this method provides a wide linear range for protein detection and avoids expensive aptamer labeling process [20, 21, 36–39]. The

Table 1 An overview on recently reported nanoparticle-based fluorescence methods for the detection of thrombin and VEGF165

Targets	Nanoparticles used	Label with aptamer	Linear range	Detection limit	References
Thrombin	Ag@SiO ₂ nanoparticles	Cy5	0.1–4 nM	0.05 nM	20
	DNA/Ag nanoclusters	/	50–900 nM	8.4 nM	21
	Gold nanoparticles and TBA-dots	/	0–35 nM	0.59 nM	36
	Quantum dots	/	Not provided	5 nM	37
	Poly T templated CuNPs	/	10–400 nM	2.4 nM	This work
VEGF 165	Mn-doped ZnS quantum dots and silver nanoparticles	Biotin	0.1–16 nM	0.08 nM	38
	CdSe/ZnS quantum dots	Cy5	Not provided	12 nM	39
	Poly T templated CuNPs	/	10–80 nM	1.3 nM	This work

selectivity of the VEGF165 detection platform was also tested. As Fig. 4C manifests, the results are similar with those done in the specificity test of thrombin, showing that the strategy also has good selectivity in the detection of VEGF165.

Conclusions

In summary, we developed a versatile method for protein detection based on TdT catalyzed poly T generation and poly T templated CuNPs formation. The design has been successfully used for the detection of thrombin and VEGF165, indicating the effectiveness of our methods. More importantly, the method can generally be applied to the detection of other proteins by simply changing the used aptamer as the method have no demand for the length and structure of the aptamers. Certainly, there still exist some limitations for the method. For example, this method may be unsuitable for the detection of proteins of small molecular weight because the binding of these proteins with their aptamers cannot bring about significant steric hindrance and block the free 3'-OH terminus of the aptamers. Nevertheless, this work is expected to have other applications in biological areas, such as the interaction of small molecules and proteins, DNA and protein analysis and environmental monitoring.

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Compliance with Ethical Standards The author(s) declare that they have no competing interests.

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