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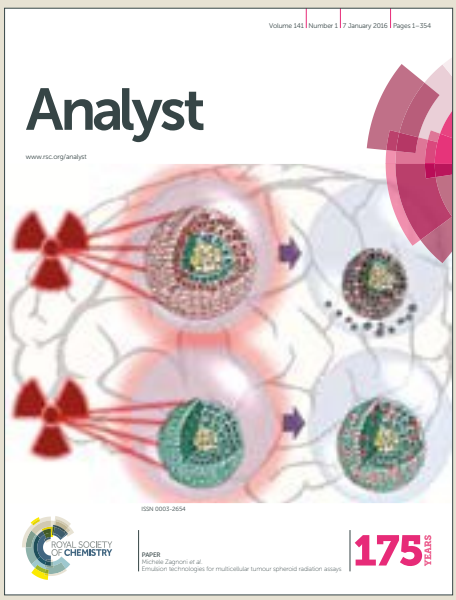

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ARTICLE

A label-free colorimetric biosensor for sensitive detection of vascular endothelial growth factor-165

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Huige Zhang,[a,b] Liang Peng,[c] Maoxing Li,[d] Ji Ma,[d] Shengda Qi,[a,b] Hongli Chen,[a,b] Lei Zhou[a,b] and Xingguo Chen[a,b,*]

Sensitive detection of low abundant protein is essential to biomedical research and clinical diagnostics. Herein, we develop a label-free colorimetric biosensor for sensitive detection of recombinant human vascular endothelial growth factor-165 ($VEGF_{165}$). This biosensor consists of an aptamer-based hairpin probe, an assistant DNA-trigger duplex and a linear template. In the presence of $VEGF_{165}$, the specific binding of $VEGF_{165}$ with the aptamer-based hairpin probe results in the opening of a hairpin probe and the opened hairpin probe subsequently hybridizes with the single-stranded region of assistant DNA-trigger duplex to initiate the strand displacement amplification (SDA) to yield abundant triggers. The released triggers can further function as the primers to anneal with the hairpin probe and lead to the opening of hairpin structure, which subsequently hybridizes with the assistant DNA-trigger duplex to initiate the next round of SDA reaction and generates more triggers. Large amounts of triggers could be generated by synergistic operation of dual SDA reaction, and the obtained triggers can initiate a new round of SDA reaction to yield numerous G-quadruplex DNAzymes, which subsequently catalyze the conversion of $ABTS^{2-}$ to $ABTS^{\bullet-}$ by H_2O_2 to yield a color change with the assistance of cofactor hemin. In contrast, in the absence of target $VEGF_{165}$, the hairpin probe, the assistant DNA-trigger duplex and the linear template can stably coexist in solution, and thus no color change is observed because no trigger can initiate SDA to generate G-quadruplex DNAzyme. This biosensor has a low detection limit of 1.70 pM and a dynamic range over 3 orders of magnitude from 24.00 pM to 11.25 nM. Moreover, the biosensor shows excellent specificity toward the target $VEGF_{165}$ and the entire reaction can be carried out in an isothermal manner without the involvement of high precision thermal cycler, making current assay extremely cost effective.

Introduction

Recombinant human vascular endothelial growth factor-165 ($VEGF_{165}$) plays central roles in the regulation of angiogenesis and vascular permeabilization.[1] Notably, $VEGF_{165}$ has significant implication as biomarker for early disease diagnosis and subsequent monitoring of treatment.[2] To date, a variety of $VEGF_{165}$ detection methods have been reported such as immunohistochemistry (IHC),[3] enzyme-linked immunosorbent assays (ELISAs),[4] surface plasmon resonance (SPR),[5] surface-enhanced raman scattering (SERS),[6] fluorescence,[2] fluorescence polarization,[7] chemiluminescence[8] and colorimetry.[9,10] Among these methods, colorimetry is especially promising because of its low cost, visualization and simplicity.[11,12] For example, hemin/G-quadruplex horseradish peroxidase (HRP)-mimicking DNAzyme was introduced to develop optical biosensor for detection of the hepatitis B gene,[13] Hg^{2+}[14], thrombin,[15] coralyne[16] and uracil DNA glycosylase (UDG) activity[17]. Although a few attempts have been exploited to improve the sensitivity,[18,19] colorimetric detection of trace amounts of the target represents another challenge due to the extremely low concentration of target in complicated sample solution.

To improve the sensitivity of G-quadruplex DNAzyme-based colorimetric biosensor, appropriate G-quadruplex DNAzyme amplification strategy should be explored. Because G-

[a] National Key Laboratory of Applied Ogranic Chemistry, Lanzhou University, Lanzhou 730000, China E-mail: chenxg@lzu.edu.cn; Tel: 86-931-8912763; Fax: 86-931-8912582
[b] Department of Chemistry, Lanzhou University, Lanzhou 730000, China.
[c] Facility center of Life Science Research, School of Life Sciences, Lanzhou University, Lanzhou 730000, China
[d] Lanzhou General Hospital of PLA, Lanzhou 730050,China

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quadruplex DNAzymes are nucleic acid molecules with catalytic characteristics, current nucleic acid amplification techniques might be easily introduced in G-quadruplex DNAzyme-based biosensors, such as polymerase chain reaction (PCR),[20] rolling circle amplification (RCA)[21] and strand displacement amplification (SDA).[22] Notably, SDA has been attracting much attention due to its unique characteristics such as mild reaction conditions and ease of operation.[23] Moreover, SDA does not require precise thermal cycling compared with PCR[24] and becomes a good choice for sensitive sensor design. Weizmann's group introduced a template DNA with one nicking site to initiate SDA to generate G-quadruplex DNAzymes with a detection limit of 1×10^{-14} M for the detection of single-stranded DNA.[25] Ye's group reported that a template with two nicking sites could initiate exponential amplification reaction (EXPAR) to generate large amounts of G-quadruplex DNAzymes with a detection limit of 1×10^{-15} M for microRNAs detection.[26] A template with more than one nicking site number could facilitate the exponential amplification reaction to generate numerous short oligonucleotides. However, the template could become long with increase of nicking site number and the long template might increase the complexity of DNA secondary configuration and the risk of producing undesirable by-products.[27]

Here, we develop a label-free colorimetric biosensor for sensitive detection of recombinant human $VEGF_{165}$. The specific binding of $VEGF_{165}$ with hairpin probe results in initiating the SDA to yield abundant triggers**.** The released triggers can further function as the primers to anneal with the hairpin probe and lead to the opening of hairpin structure, which subsequently initiates the next round of SDA reaction and generates more triggers. Large amounts of triggers could be generated by synergistic operation of dual SDA reaction, and the obtained triggers could initiate a new round SDA reaction to yield numerous G-quadruplex DNAzymes, which subsequently catalyze the conversion of a colorless $ABTS^{2-}$ to a green $ABTS^{\bullet-}$ in the presence of H_2O_2 and hemin. The detection limit of current biosensor is as low as 1.70 pM and a large dynamic range over 3 orders of magnitude from 24.00 pM to 11.25 nM. The biosensor exhibits high sensitivity and specificity toward $VEGF_{165}$ versus other nonspecific proteins. Furthermore, the suitability of this biosensor for the quantification of $VEGF_{165}$ in diluted human serum sample has also been demonstrated. Thus, this biosensor has potential for the development of an effective detection method for $VEGF_{165}$.

Experimental

Reagents

Oligonucleotides used in this work were synthesized and purified by Sangon Biotechnology Co. Ltd. (Shanghai, China), and their sequences were listed in Table 1. They were diluted in 20 mM Tris-HCl buffer solution (containing 100 mM NaCl, 20 mM KCl, and 4.68 mM $MgCl_2$, pH 7.7). Before use, the hairpin probes were diluted to 1.2 μM in the Tris-HCl buffer. The hairpin probes were incubated at 95 °C for 10 min and then slowly cooled to room temperature over 2 h to make the probe perfectly fold into hairpin structure. $VEGF_{165}$ was purchased from Abcam (Beijing, China); Cytochrome C, transferrin and human serum albumin (HSA) were purchased from Sangon Biotechnology Co. Ltd. (Shanghai, China); Albumin egg and interferon γ (IFN-γ) were received from SinoBio Biotech Ltd. (Shanghai, China). HEPES (4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All reagents were prepared and handled according to the supplier's specifications. Klenow fragment polymerase (3→5 exo-, KF polymerase), the nicking endonuclease Nt.BbvCI and 10 × NEB buffer 2 (500 mM NaCl, 100 mM Tris-HCl, 100 mM $MgCl_2$, and 10 mM dithiothreitol, pH 7.9) were purchased from New England Biolabs Ltd.

Polyacrylamide gel electrophoresis

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The product solution was analyzed by 15% nondenaturating polyacrylamide gel electrophoresis (PAGE) in Tris-borate-EDTA (TBE) buffer (9 mM Tris-HCl, pH 8.3, 9 mM boric acid, and 0.2 mM EDTA) buffer at a 100 V constant voltage for 150 min at room temperature. After ethidium bromide staining, the gels were scanned using the ChemiDoc XRS+ Gel imaging system (BIO-RAD, USA)

Table 1 DNA sequences used to construct the biosensor

name	Sequences description
Hairpin probe	5'-ACCCGTCTTCCAGACAAGAGTGCAGGGTTT TTTTTTTGAAGACGGGT-3'
Assistant DNA	5'-ACCCGTCTTCCAGACAAGCTGA↓GGTTACCC GTCT-3'
Trigger	5'-CTTGTCTGGAAGACGGGT-3'
Linear template	5'-CCCAACCCGCCCTACCCAGGCTGA↓GGTTAC CCGTCTTCCAGACAAG-3'

The italic letters of hairpin probe are the sequence of $VEGF_{165}$ aptamer. The underlined letters of hairpin probe are the stem and the arrow indicates the nicking position.

Detection procedures

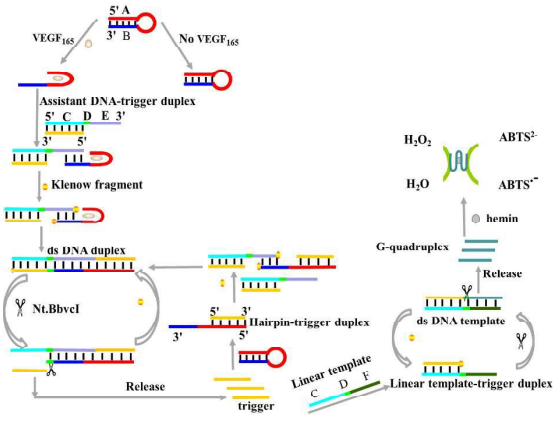
The detailed procedure of the detection was as follows. First, to prepare the assistant DNA-trigger duplex, two relevant single-stranded oligonucleotides were mixed at an equal molar ratio in the above Tris-HCl buffer. Then, the mixture were annealed by heating to 95 °C for 10 min and slowly cooled down to room temperature over 2 h. Second, the amplification reaction was performed in a volume of 20 μL solution containing the hairpin probe (5 μL, 1.2 μM), the assistant DNA-trigger duplex (5 μL, 1.8 μM), linear template (2.5 μL, 10 μM), Nt.BbvCI (0.5μL, 10 U/μL), KF polymerase (0.8 μL, 5 U/μL), 0.5 μL of dNTPs (10 mM), and a series of $VEGF_{165}$ at different concentrations. The reaction was performed at 37 °C for 90 min. Next, 3 μL hemin (10 μM) and 44 μL HEPES buffer solution (pH 8.0, 25 mM HEPES, 0.05% (w/v) Triton X-100, 1% (v/v) DMSO, 20 mM KCl, 200 mM NaCl) were added, and the solution was incubated for 1 h at room temperature. Finally, the $ABTS^{2-}$ and H_2O_2 were added to the mixture to reach final concentrations of 2 mM and 2 mM, respectively, at room temperature. The resulting sample was diluted with deionized water to 180 μL and tested with a TU-1901 UV-vis spectrometer (Beijing Purkinje General Instrument Co.).

Preparation of Human Serum Sample

The normal human plasma sample collected in the citrate anticoagulated tube was provided by the healthy volunteer in our laboratory. Separated serum sample (12000 rpm for 10 min) was stored at -80 °C until used. The human serum sample was diluted with 1 × NEB buffer 2 and different concentration of $VEGF_{165}$ was spiked to the diluted human serum sample to evaluate the potential application.

Live subject statement

The authors state that all experiments were performed in compliance with the relevant laws and institutional guidelines. The institutional committee of the Lanzhou University approved the experiments. The authors also state that informed consent was obtained for any experimentation with human subjects and Lanzhou University is committed to the protection and safety of human subjects involved in the research.



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Scheme 1 Schematic illustration of $VEGF_{165}$ detection based colorimetric biosensor.

Results and discussion

Assay Principle

The principle of the label-free colorimetric biosensor is illustrated in Scheme 1. The biosensor consists of an aptamer-based hairpin probe, an assistant DNA-trigger duplex and a linear template. The hairpin probe consists of two regions termed as A and B, and region A is the aptamer region for $VEGF_{165}$. The assistant DNA includes three regions: the trigger recognition region (C), a recognition region (D) for Nt.BbvCI and a special 3' protruding end (E) as the binding region for region B from the opened hairpin structure. The trigger is partly complementary to region A. The assistant DNA-trigger binding leads to a part assistant DNA-trigger duplex at region C. The linear template is designed to have the same recognition regions (C and D) as the assistant DNA and a recognition region (F) for G-quadruplex sequence (Table 1).

In the presence of $VEGF_{165}$, the specific $VEGF_{165}$-hairpin probe binding at region A leads to the opening of a hairpin structure and exposure of the region B, which hybridizes with region E of the assistant DNA-trigger duplex to form a part double stranded DNA (dsDNA) duplex, making the DNA be extended along template to form a complete dsDNA duplex in the presence of Klenow fragment DNA polymerase and dNTPs. Subsequently, the nicking enzyme recognizes the dsDNA duplex site at region D and cleaves the lower DNA strand, generating a new replication site for polymerase. The SDA cycle includes polymerization, nicking, and subsequent release of the oligonucleotides. The released triggers function as the primers to anneal with region A of the hairpin probe and lead to the opening of hairpin structure and exposure of the region B, which subsequently hybridizes with region E of the assistant DNA-trigger duplex to initiate the next round of SDA reaction and generates more triggers. The obtained triggers can hybridize with the linear template to form a part duplex at region C, making the trigger be extended along the linear template to form a complete dsDNA template in the presence of Klenow fragment DNA polymerase and dNTPs. Subsequently, the nicking enzyme recognizes the dsDNA template site at region D and cleaves the upper DNA strand, generating a new replication site for polymerase. As a result of strand displacement activity of KF polymerase, a large number of G-quadruplex DNAzymes are generated through the repeated extension, cleavage, and the release of short oligonucleotides. The obtained G-quadruplex DNAzymes subsequently catalyze the conversion of $ABTS^{2-}$ to $ABTS^{\bullet-}$ by H_2O_2 to yield a color change with the assistance of cofactor hemin. In contrast, in the absence of target $VEGF_{165}$, the hairpin probe, the assistant DNA-trigger duplex and linear template can stably coexist in solution, and thus no color change is observed because no trigger can hybridize with linear template to initiate SDA to generate G-quadruplex DNAzyme. The scheme described here has significant advantages, large amounts of triggers can be generated by synergistic operation of dual SDA reaction, and the obtained triggers can hybridize with linear template to initiate SDA to generate numerous G-quadruplex DNAzymes.

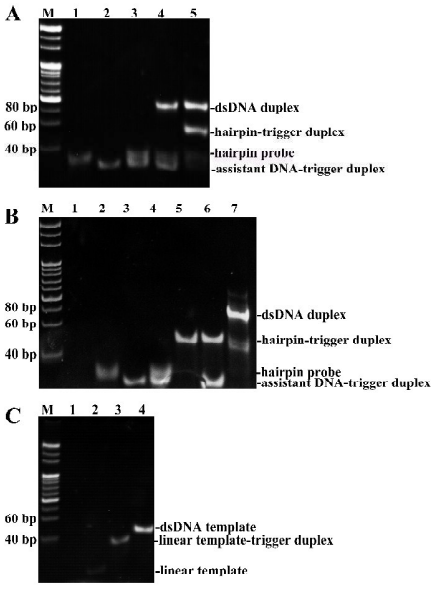

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Fig.1 Results of polyacrylamide gel electrophoresis analysis. (A) (M) DNA marker; (1) hairpin; (2) assistant DNA-trigger duplex; (3) hairpin + assistant DNA-trigger duplex + KF polymerase; (4) hairpin + assistant DNA-trigger duplex + $VEGF_{165}$ + KF polymerase; (5) hairpin+ assistant DNA- trigger duplex + $VEGF_{165}$ + KF polymerase + Nt.BbvcI. (B) (1) trigger; (2) hairpin; (3) assistant DNA-trigger duplex; (4) hairpin + assistant DNA-trigger duplex; (5) hairpin + trigger; (6) hairpin + trigger + assistant DNA-trigger duplex; (7) hairpin + trigger + assistant DNA-trigger duplex + KF polymerase. (C) (1) trigger; (2) linear template; (3) trigger + linear template; (4) trigger + linear template + KF polymerase.

The amplification reactions are further verified by using polyacrylamide gel electrophoresis (PAGE). As can be seen in Fig.1A, in the absence of $VEGF_{165}$, the hairpin probe and the assistant DNA-trigger duplex maintain their structure even in the presence of KF polymerase and dNTPs, the bands (lane 3) locate at a comparable distance from the notch (lanes 1 and 2), indicating that no polymerization reaction occurs. However, in the presence of $VEGF_{165}$, $VEGF_{165}$ could initiate polymerization reaction to produce a complete dsDNA duplex, and the band of dsDNA duplex is observed distinctly (lane 4). Moreover, when Nt.BbvCI is added to the polymerization solution, a small amount of $VEGF_{165}$ can be converted to large numbers of triggers through SDA reaction and the triggers can also hybridize with hairpin probe to form a part hairpin-trigger duplex. The bands of dsDNA duplex and hairpin-trigger duplex are observed distinctly (lane 5). As can be seen in Fig.1B, no obvious band is observed in lane 1 due to the short single-stranded structure of trigger. The two bands in lane 4 locate at almost the same migration position as that in lanes 2 and 3, indicating that no interaction between the hairpin probe and the assistant DNA-trigger duplex occurs. When hairpin probe mixes with trigger, a part hairpin-trigger duplex is generated due to the hybridization, and the band of hairpin-trigger duplex in lane 5 is observed. Two bands in lane 6 locate at almost the same migration position as that in lanes 5 and 4, indicating that no interaction between the hairpin-trigger duplex and the assistant-trigger DNA duplex occurs. However, when KF polymerase and dNTPs are added to the mixture of the hairpin-trigger duplex and the assistant-trigger DNA duplex, the polymerization reaction could proceed and generate dsDNA duplex, and the dsDNA duplex band is observed (lane 7). As can be seen in Fig.1C, when linear template mixes with trigger, the linear template-trigger duplex can be generated due to hybridization, and the linear template-trigger duplex band is observed (lane 3). In the presence of KF polymerase and dNTPs, the trigger could initiate polymerization reaction to form a complete dsDNA template, and the band of dsDNA template is observed distinctly (lane 4). These PAGE results indicate the successful proceeding of the amplification reactions.

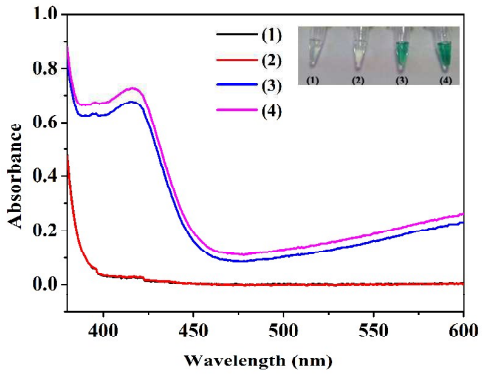

Fig.2 Absorption spectra of sample solutions under different conditions: (1) hairpin + assistant DNA + linear template; (2) hairpin + assistant DNA-trigger duplex + linear template; (3) hairpin + assistant DNA + linear template + $VEGF_{165}$; (4) hairpin + assistant DNA-trigger duplex + linear template + $VEGF_{165}$. The inset shows the corresponding color change.

Feasibility Study

To demonstrate the feasibility of the biosensor for $VEGF_{165}$ detection, we perform the optical measurement. As shown in Fig. 2, the low absorption signals are observed (curves 1 and 2), indicating that few G-quadruplex DNAzymes is produced without the addition of $VEGF_{165}$. However, the addition of $VEGF_{165}$ induces a strong absorption signal at 418 nm (curve 3), indicating

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that $VEGF_{165}$ initiates the SDA reaction to produce large amount of triggers, which subsequently initiate a new round of SDA to produce abundant G-quadruplex DNAzymes. The obtained G-quadruplex DNAzymes subsequently catalyze the conversion of $ABTS^{2-}$ to $ABTS^{\bullet-}$ by H_2O_2 to yield a color change with the assistance of cofactor hemin. Moreover, a stronger absorption signal at 418 nm is observed after the addition of assistant DNA-trigger duplex instead of the assistant DNA (curve 4), indicating that $VEGF_{165}$ initiates the SDA reaction and the displacement of trigger during the polymerization process to produce more triggers, which hybridize with linear template and subsequently initiate SDA to produce more G-quadruplex DNAzymes. These results are also confirmed by comparing the color difference with and without the addition of $VEGF_{165}$ (inset Fig. 2). No distinct green color is visible without the addition of $VEGF_{165}$ (inset Fig. 2, 1 and 2), while a green color of the solution is visible with the addition of $VEGF_{165}$ (inset of Fig. 2, 3). And the green color of the solution is intensified when the assistant DNA-trigger duplex is used instead (inset Fig. 2, 4 vs 3).

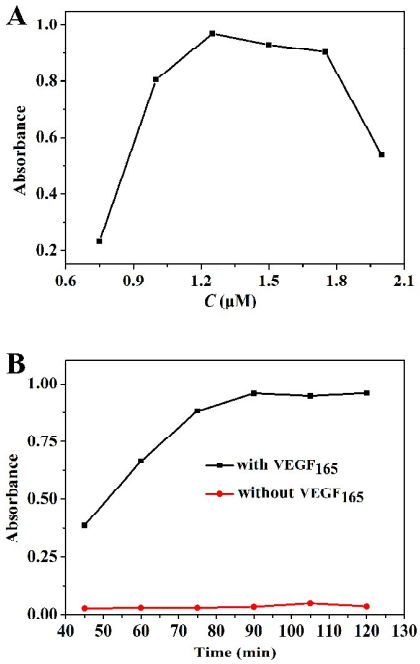

Fig.3 (A) Absorption intensity monitoring at the different concentration of the linear template. (B) Time-dependent absorption intensity obtained from SDA reaction in response to 11.25 nM $VEGF_{165}$ (black line) and the control group without $VEGF_{165}$ (red line).

Optimization of the experimental conditions. Different experimental parameters, such as the concentration of linear template and the reaction time could influence the performance of the detection system. To achieve optimal sensing performance, the effects of these factors are investigated.

Effect of the linear template concentration. The concentration of linear template has a crucial effect on the efficiency of amplification. On one hand, a high concentration of linear template might lead to a high amplification efficiency and the generation of large amounts of G-quadruplex sequences, but the released free G-quadruplex sequences could subsequently hybridize with the excess linear templates at the F region (Scheme 1), resulting in the decrease of free G-quadruplex sequences.[28] On the other hand, the low concentration linear template might adversely affect detection sensitivity because the amplification efficiency of SDA reaction depends on the ability of triggers to hybridize with the linear templates.[29] Therefore, the concentration of linear template should be optimized carefully. As shown in Fig. 3A, the absorption intensity increases gradually with the linear template concentration from 0.75 to 1.25 μM. However, the absorption intensity decreases gradually when the concentration was above 1.25 μM. Therefore, the linear template concentration of 1.25 μM is selected in the following assay.

Optimization of the reaction time. The optimization of the reaction time on absorption intensity is investigated with and without the addition of $VEGF_{165}$, and the experimental results are shown in Fig. 3B. No intensified absorption intensity is detected in the solution without $VEGF_{165}$ (Fig. 3B, red line), indicating that there is

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few G-quadruplex sequences produced by SDA reaction in the absence of $VEGF_{165}$. In contrast, the addition of $VEGF_{165}$ induces an increasing absorption intensity in the initial 90 min, followed by reaching a plateau (Fig. 3B, black line). Thus, the reaction time of 90 min is selected for subsequent experiments.

Detection specificity

The specificity of current method primarily depends on the specific binding of $VEGF_{165}$ with the aptamer-based on hairpin probe. To investigate the selectivity of the proposed method, we measure some irrelevant proteins, such as HSA, albumin egg, transferrin, cytochrome C and IFN-γ, which cannot specifically bind to hairpin probes. In the presence of these nonspecific proteins, the SDA reaction could not be initiated to generate G-quadruplex DNAzymes due to no free triggers. As expected, a significant absorbance enhancement is observed only in the presence of $VEGF_{165}$ (Fig. 4A). And the specific $VEGF_{165}$ lead to an increase in the absorption intensity at 418 nm, while no distinct absorption intensity is observed in the presence of nonspecific proteins including HSA, albumin egg, transferrin, cytochrome C and IFN-γ even at high concentration of 25 nM (Fig. 4B). Such high specificity can be related to the highly specific binding capability of the aptamer sequence to $VEGF_{165}$.

Sensitivity of $VEGF_{165}$ assay

We further measure $VEGF_{165}$ at different concentrations using the proposed colorimetric biosensor. Under optimal conditions, the absorption signal at 418 nm increases with the concentration of $VEGF_{165}$ from 24.00 pM to 11.25 nM (Fig.5A), and the absorption intensity increases with the $VEGF_{165}$ concentration (Fig.5B). In logarithmic scales, the absorption intensity exhibits a linear correlation with $VEGF_{165}$ concentration over a range of 3 orders of magnitude from 24.00 pM to 11.25 nM (inset of Fig. 5B). The regression equation was $A=0.373\log_{10}C-0.472$ with a correlation coefficient of 0.9884, where A and C represents the absorption intensity and the $VEGF_{165}$ concentration, respectively. The detection limit is estimated to be 1.70 pM from three times the standard deviation corresponding to the blank without the addition of $VEGF_{165}$. It is worth noting that the sensitivity of current method has improved in comparison with those of the previous reported colorimetric assay (185 pM and 0.1 nM),[9,10] and even comparable with the fluorescence method (1 pM).[30] The improved sensitivity of this biosensor can be attributed to that the enrichment of triggers by synergistic operation of dual SDA reaction will increase the amplification efficiency of G-quadruplex DNAzyme dramatically.

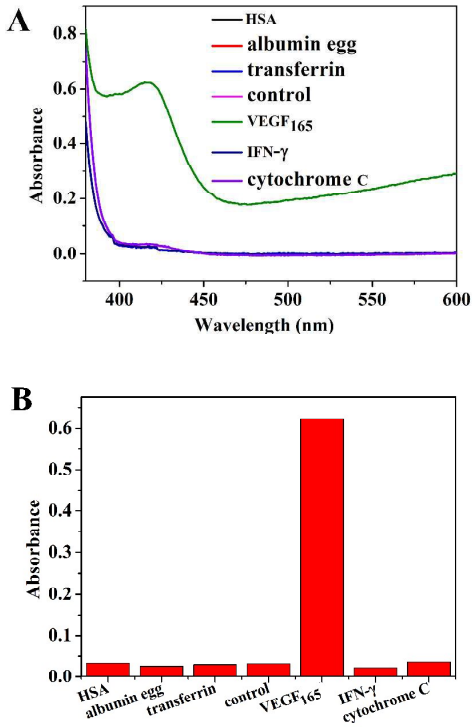

Fig.4 (A) Absorption spectra induced by 25.0 nM HSA, 25.0 nM albumin egg, 25.0 nM transferrin, 0.45 nM $VEGF_{165}$, 25.0 nM IFN-γ, 25.0 nM cytochrome C and a control without any proteins. (B) Absorption intensity of the system toward different proteins.

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Detection of $VEGF_{165}$ in Human Serum

To be useful in the bioassays, the developed detection method should be able to tolerate the interference from the biological samples. Therefore, we prepare a series of samples by adding different concentration of $VEGF_{165}$ to 12.5% diluted human serum from healthy volunteer. The experimental results are summarized in Table 2, which shows that recovery is in the range of 91.72-101.86% with the RSDs between 3.53% and 6.89%. These results indicate that the developed colorimetric biosensor has potential to be applied for $VEGF_{165}$ detection in real physiological media.

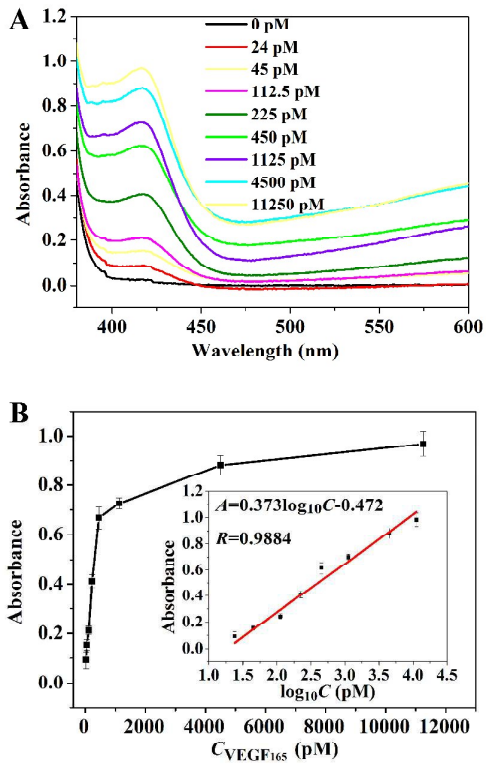

Fig.5 (A) Absorption spectra of the system toward different concentrations of $VEGF_{165}$. (B) Variance of absorption intensity as a function of the concentration of $VEGF_{165}$. Inset: the absorption intensity is a log-linear correlation with the concentration of $VEGF_{165}$ in the range from 24.00 pM to 11.25 nM. Error bars show the standard deviation of three experiments.

Table 2 Results of the recovery tests of $VEGF_{165}$ in 12.5% diluted human serum.

Sample	Added $VEGF_{165}$ concentration	Measured $VEGF_{165}$ concentration	Recovery (%)	RSD (%, n= 3)
1	225 pM	227 pM	100.99	3.53
2	450 pM	458 pM	101.86	6.89
3	1125 pM	1032 pM	91.72	3.87

Conclusions

In summary, we have developed a label-free colorimetric biosensor for sensitive detection of $VEGF_{165}$. The specific binding of $VEGF_{165}$ with the hairpin probe can not only ensure the detection specificity, but also induce the SDA reaction to produce triggers, which can hybridize with the hairpin probe to initiate the next round of SDA reaction to generate more triggers. Large amounts of triggers could be generated by synergistic operation of dual SDA reaction, and the obtained triggers can hybridize with linear template and subsequently initiate a new round of SDA reaction to yield numerous G-quadruplex DNAzymes for further colorimetry. The detection limit of current method can reach as low as 1.70 pM, which is sensitive than those of previously reported colorimetric methods[9,10] and even comparable with the fluorescence method (1 pM).[30] Notably, the colorimetric biosensor used in this research require neither any modification nor long template with more than one nicking site number, and the entire reaction can be carried out in an isothermal manner without the involvement of high precision thermal cycler, making current assay extremely cost effective.

Acknowledgments

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Notes and references

1 D.W. Leung, G. Cachianes, W.J. Kuang, D.V. Goeddel and N. Ferrara, *Science*, 1989, **246**, 1306-1309.
2 J.L. Li, K.X. Sun, Z.P. Chen, J.F. Shi, D.D. Zhou and G.M. Xie, *Biosens. Bioelectron.*, doi.org/10.1016/j.bios.2016.09.078
3 S.M. Anderson, T.T. Chen, M.L. Iruela-Arispe and T. Segura, *Biomaterials*, 2009, **30**, 4618-4628.
4 M.-Y. Hsu, S.-J. Chen, K.-H. Chen, Y.-C. Hung, H.-Y. Tsai and C.-M. Cheng, *Lab Chip*, 2015, **15**, 2357-2363.
5 C. Liu, T. Lei, K. Ino, T. Matsue, N. Tao and C.-Z. Li, *Chem. Commun*., 2012, **48**, 10389-10391.
6 S. Zhao, W. Ma, L.G. Xu, X.L. Wu, H. Kuang, L.B.Wang and C.L. Xu, *Biosens. Bioelectron*., 2015, **68**, 593-597.
7 Y. Huang, X.Q. Liu, H.K. Huang, J. Qin, L.L. Zhang, S.L. Zhao, Z.-F. Chen and H. Liang, *Anal. Chem*., 2015, **87**, 8107-8114.
8 W. Li, Q. Zhang, H. Zhou, J. Chen, Y. Li, C. Zhang and C. Yu, *Anal. Chem.*, 2015, **87**, 8336-8341.
9 C.-C. Chang, C.-Y. Chen, T.-L. Chuang, T.-H. Wu, S.-C. Wei, H.E. Liao and C.-W. Lin, *Biosens. Bioelectron.*, 2016, **78**, 200-205.
10 D. Wu, T. Gao, L. Lei, D.W. Yang, X.X. Mao and G.X. Li, *Anal. Chim. Acta*., 2016, **942**, 68-73.
11 J. Li, H.-E. Fu, L.-J. Wu, A.-X. Zheng, G.-N. Chen and H.-H. Yang, *Anal. Chem*., 2012, **84**, 5309-5315.
12 Y. Zhang, J. Hu and C.-Y. Zhang, *Anal. Chem*., 2012, **84**, 9544-9549.
13 Y. Weizmann, Z. Cheglakov and I. Willner, *J. Am. Chem. Soc.*, 2008, **130**, 17224-17225.
14 T. Li, S.J. Dong and E.K. Wang, *Anal. Chem*., 2009, **81**, 2144-2149.
15 L.B. Zhang, J.B. Zhu, T. Li and E.K. Wang, *Anal. Chem*., 2011, **83**, 8871-8876.
16 T. Hou, X.Z. Wang, X.J. Liu, S.F. Liu, Z.F. Du, F. Li, *Analyst*, 2013, **138**, 4728-4731.
17 X.J. Liu, M.Q. Chen, T. Hou, X.Z. Wang, S.F. Liu, F. Li, *Biosens. Bioelectron*., 2014, **54**, 598-602.
18 D. Li, B. Shlyahovsky, J.Elbaz and I. Willner, *J. Am. Chem. Soc.*, 2007, **129**, 5804-5805.
19 .J. Zhou, X. Gong, Y. Xiang, R. Yuan, and Y.Q. Chai, *Anal. Chem*. 2014, **86**, 953-958.
20 N. Wang, D.-M. Kong and H.-X. Shen, *Chem. Commun*., 2011, **47**, 1728-1730.
21 Y. Chen, Y. Song, F. Wu, W. Liu, B. Fu, B. Feng and X. Zhou, *Chem. Commun*., 2015, **51**, 6980-6983.
22 W. Li, Z. Liu, H. Lin, Z. Nie, J. Chen, X. Xu and S. Yao, *Anal. Chem*., 2010, **82**, 1935-1941.
23 Z.-Z. Zhang and C.-Y. Zhang, *Anal. Chem.*, 2012, **84**, 1623-1629.
24 S. Fredriksson, M. Gullberg, J. Jarvius, C.Olsson, K. Pietras, S. M. Gustafsdottir, A. Ostman and U. Landegren, *Nat. Biotechnol.*, 2002, **20**, 473-477.
25 Y. Weizmann, M. K. Beissenhirtz, Z. Cheglakov, R. Nowarski, M. Kotler and I. Willner, *Angew.Chem*., 2006, **118**, 7544-7548.
26 X.-P. Wang, B.-C. Yin, P. Wang and B.-C. Ye, *Biosens. Bioelectron*., 2013, **42**, 131-135.
27 Y.-C. Du, H.-X. Jiang, Y.-F. Huo, G.-M. Han and D.-M. Kong, *Biosens. Bioelectron*., 2016, **77**, 971-977.
28 L.-R. Zhang, G.C. Zhu and C.-Y. Zhang, *Anal. Chem.*, 2014, **86**, 6703-6709.
29 H. Jia, Z. Li, C. Liu and Y. Cheng, *Angew. Chem. Int. Ed.*, 2010, **49**, 5498-5501.
30 X. Li, X.L. Ding and J. Fan, *Analyst*, 2015, **140**, 7918-7925.