Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 226 (2020) 117622

Contents lists available at ScienceDirect

Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

journal homepage: www.elsevier.com/locate/saa

# A highly sensitive colorimetric aptasensor for the detection of the vascular endothelial growth factor in human serum

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ARTICLE INFO

*Article history:*
Received 24 April 2019
Received in revised form
23 July 2019
Accepted 6 October 2019
Available online 7 October 2019

*Keywords:*
$VEGF_{165}$
Colorimetry
Aptamer
Biosensor

ABSTRACT

Early detection of cancer is of great significance for disease prevention and diagnosis. However, the levels of most cancer markers are quite low in the early stages of disease, so it is urgent to develop a highly sensitive detection method. In this study, a label-free and highly sensitive colorimetric strategy was developed for the detection of the vascular endothelial growth factor$_{165}$ ($VEGF_{165}$) in human serum. First, a convenient biosensor was constructed by immobilizing $VEGF_{165}$ on a microplate, where aptamers bound with $VEGF_{165}$ to form a complex. Then, streptavidin labeled-horseradish peroxidase (HRP-SA) combined with the complex via the interaction between streptavidin and biotin, thus catalyzing the 3,3′,5,5′-tetramethylbenzidine (TMB) and $H_2O_2$ system to produce colored products. In the presence of target, immobilized $VEGF_{165}$ and target competitively bound with the aptamers, resulting in a reduction of the colorimetric signal. Moreover, the optical density (OD) signal decreased with the increase of target concentration. The strategy showed a broad linear range (0.1−100 ng/mL) and a rather low detection limit of 10 pg/mL with good precision and selectivity. Further, the proposed method was successfully applied in detecting $VEGF_{165}$ in human serum. The detection results of serum samples showed that the proposed assay had a high correlation with CLEIA kits ($r = 0.971$, $P = 0.001$). It has potential for application in clinical research and diagnosis.



## 1. Introduction

Vascular endothelial growth factors (VEGF) are key factors in promoting growth of tumors [1]. They are the most potent and specific growth factors for inducing tumor angiogenesis [2]. In mammals, the VEGF family consists of five members, including VEGFA, VEGFB, VEGFC, VEGFD and the placenta growth factors [3,4]. The VEGFA group further includes four isoforms, consisting of $VEGF_{121}$,$VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$. Among them, $VEGF_{165}$ is the most potent pro-angiogenic isoform, which contains the heparin and the receptor binding domains [5]. The rapid growth and metastasis of tumor cells requires independent blood delivery for oxygen and nutrients, which results in overexpression of $VEGF_{165}$ [6−8]. As a signaling protein, $VEGF_{165}$ is associated with a variety of human tumor developments and metastasis. It has been regarded as a predominant biomarker for cancer monitoring and diagnosing [9,10].

To date, many strategies have been developed for the detection of $VEGF_{165}$ [11−14], for example, electrochemistry [15−18], fluorescence [19−22], colorimetric method [23,24], and surface plasmon resonance [25]. However, among these methods, electrochemistry and surface plasmon resonance often require complicated laborious pretreatment and sophisticated instruments. The fluorescence method needs an excitation light source, but the background interference of the light source is often unavoidable. The colorimetric method is the most commonly used method in immunoassays. Due to its unique advantages of simplicity, practicality and direct readout, it is especially suitable for the point-of-care test (POCT). The traditional colorimetric method for VEGF detection is an enzyme-linked immunosorbent assay (ELISA). However, the antibody-based colorimetric method is not conducive to the further development of the colorimetric

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https://doi.org/10.1016/j.saa.2019.117622
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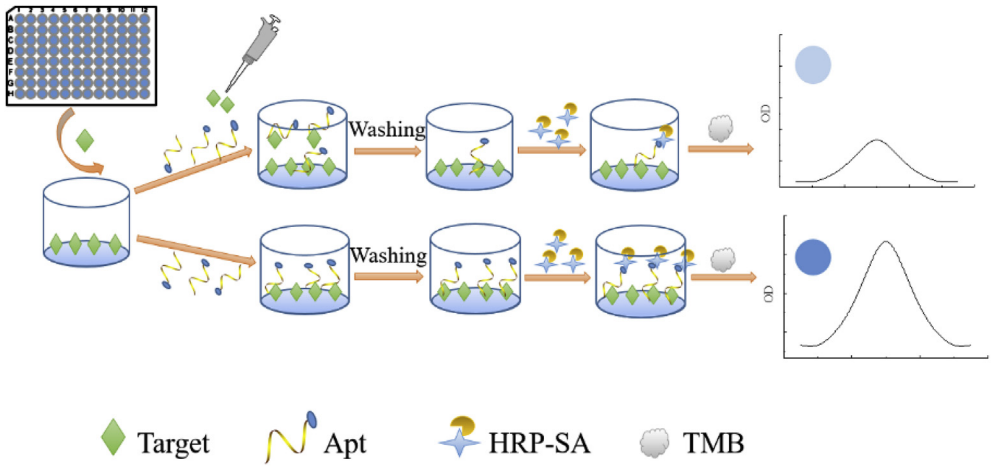


**Fig. 1.** Schematic illustration of colorimetric aptasensor for $VEGF_{165}$ detection.

method because the required antibodies are expensive, unstable and difficult to store.

In recent years, Aptamers (Apts) have been regarded as alternative reagents to antibodies due to their unique properties and advantages. Apts are single-stranded DNA or RNA oligonucleotides that are generated by systematic evolution of ligands by exponential enrichment (SELEX) [26–28]. They have a unique three-dimensional conformation, which is capable of bind with the functional groups of their targets with high affinity and selectivity including small molecules, nucleotides, proteins or cells [29,30]. Apts exhibit many advantages over natural antibodies such as low cost, stability, ease of synthesis, modification as well as reduced immunogenicity and toxicity [31]. Therefore, Apts have been widely applied in biological analysis and biosensor development [32–35].

Here, a highly sensitive colorimetric aptasensor for the detection of $VEGF_{165}$ in human serum is proposed. In this assay, Apts are applied to identify $VEGF_{165}$ instead of antibodies. In the presence of the target, immobilized $VEGF_{165}$ and target competitively bind with Apts to induce the change of the colorimetric signal, which in turn improves the sensitivity of the colorimetry. Instead of labeled-HRP at the end of Apts, streptavidin labeled-horseradish peroxidase (HPR-SA) was directly used to catalyze the 3,3′,5,5′-tetramethylbenzidine (TMB) and $H_2O_2$ system to produce a colored complex, which not only decreased cost, but also reduced the steric hindrance of the binding of the Apts with the target. This strategy has been successfully applied in the detection of $VEGF_{165}$ in human serum. The results show that it has potential to achieve rapid and highly sensitive detection of $VEGF_{165}$, in order to meet the needs of a POCT test in clinical research.

## 2. Experiment

### 2.1. Chemicals and materials

Recombinant Human $VEGF_{165}$ was purchased from *Peprotech Corporation* (USA). Streptavidin labeled-horseradish peroxidase (HRP-SA), Bovine serum albumin (BSA), Tween-20 and TMB Single Component Chromogenic Solution (contains $H_2O_2$) were acquired from Solarbio Life Sciences. 0.01 M PBS (pH = 7.4) and PBST (PBS with 0.05% tween-20) were used in the experiment. All reagents were of analytical grade. The water used in the experiments was deionized and ultrafiltered by Milli-Q system (Waters, Milford, MA). VEGF Apt: biotin-TTTTTTTTTTGTGGGGGTGGACGGGCCG GGTAGA were synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China).

### 2.2. Apparatus

Microplate Reader (Tecan Sunrise), High-binding 96-well microtiter plates used in the work were purchased from JET BIOFIL. The electrical thermostatic cultivation cabinet DHG-9146 A was manufactured by Shanghai Jing Hong Laboratory Instrument Co., Ltd., and the vortex mixing instrument by Tianyi Electronic Instrument Co., Ltd.

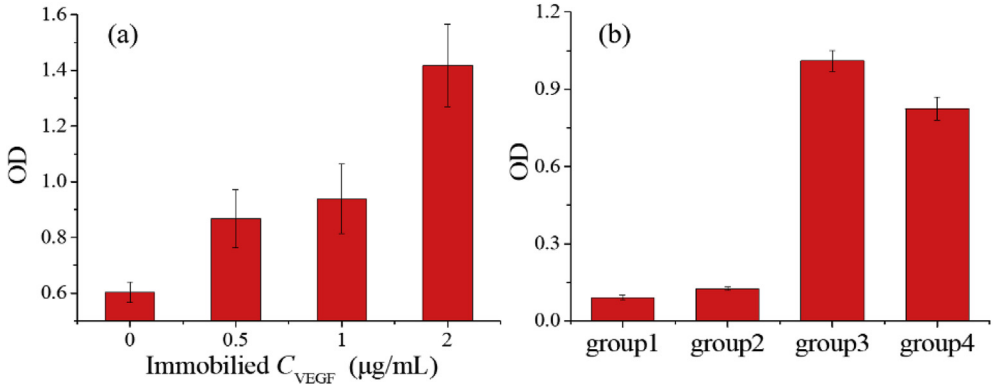


**Fig. 2.** (a) Validation of binding of Apts to $VEGF_{165}$, (b) OD signals of different immobilized $VEGF_{165}$ and target concentrations. Group 1 (no immobilized $VEGF_{165}$ + 50 ng/mL $VEGF_{165}$ + Apts), group 2 (no immobilized $VEGF_{165}$ + 100 ng/mL $VEGF_{165}$ + Apts), group 3 (immobilized $VEGF_{165}$ + 50 ng/mL $VEGF_{165}$ + Apt), group 4 (immobilized $VEGF_{165}$ + 100 ng/mL $VEGF_{165}$ + Apt).

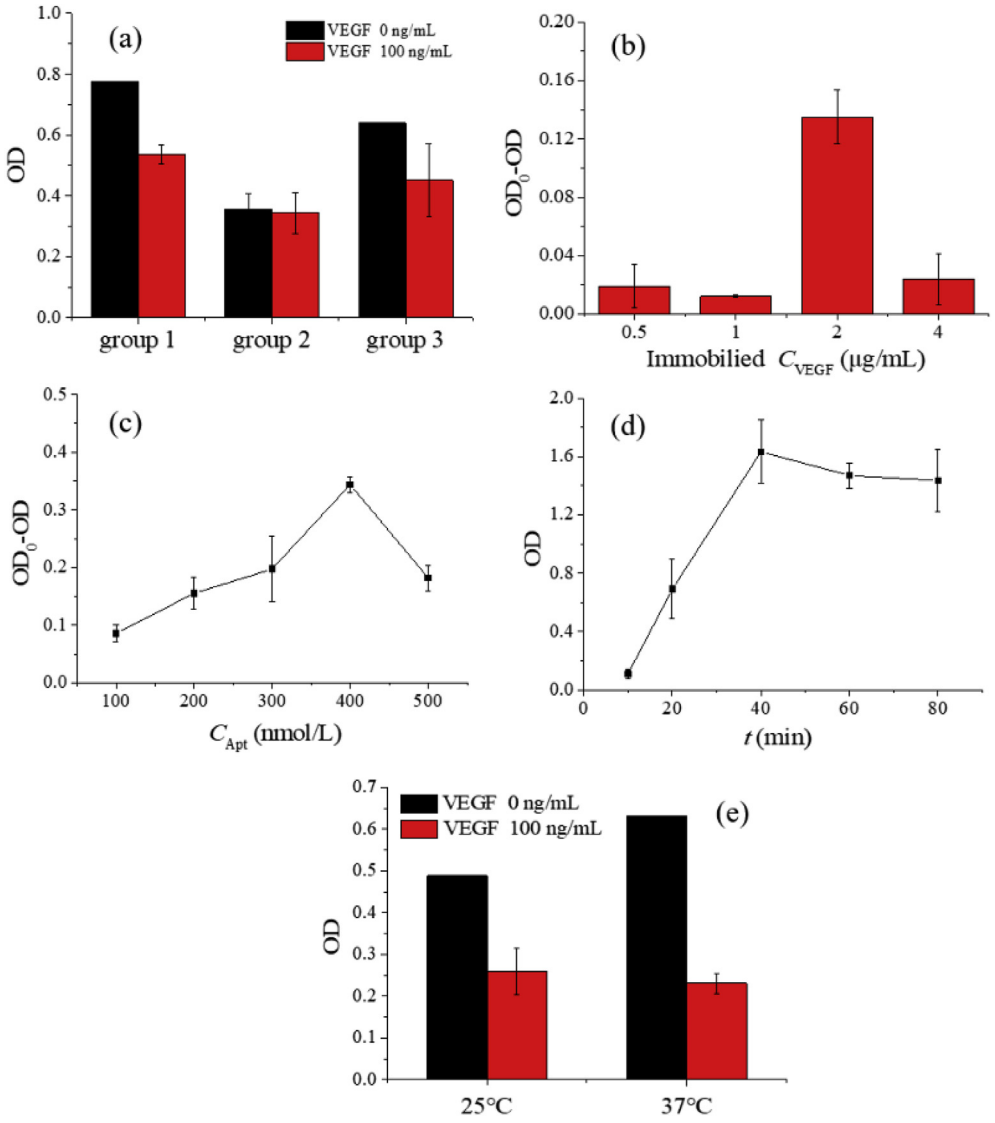


**Fig. 3.** Single factor method optimization of colorimetric assay reaction conditions (a) Effect of reagents addition sequence on OD signal. (b) Effect of the concentration of immobilized $VEGF_{165}$ on OD signal. (c) Effect of $VEGF_{165}$ Apts concentration on OD signal. (d) Effect of the reaction time of Apts and $VEGF_{165}$ on OD signal. (e) Effect of the reaction temperature of Apts and $VEGF_{165}$ on OD signal.

### 2.3. Detection principle of $VEGF_{165}$

First $VEGF_{165}$ was immobilized on the microplate as a biosensor. Apts combined with the immobilized $VEGF_{165}$ to form a complex. Then HRP-SA was added to bind with the complex via the interaction between streptavidin and biotin, thus catalyzing the TMB and $H_2O_2$ system to produce a colored complex. In the presence of target $VEGF_{165}$, target and immobilized $VEGF_{165}$ competitively

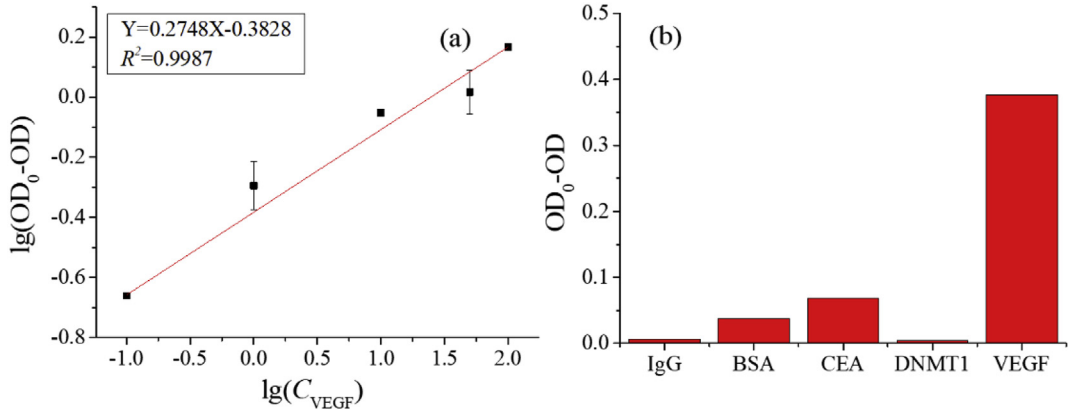


**Fig. 4.** (a) Calibration curve for the determination of $VEGF_{165}$. (b) Specificity evaluation of the developed method. $VEGF_{165}$, IgG, CEA, BSA and DNMT1 samples were all prepared in 100 ng/mL.

**Table 1**
Comparison of the designed method with alternate detection methods of $VEGF_{165}$.

| Method | Linear range (pg/mL) | Limit of detection (pg/mL) | Ref |
|---|---|---|---|
| Electrochemical | $50-1\times10^5$ | 50 | [15] |
| Fluorescence | 5–40 | 3.5 | [19] |
| Chemiluminescence | $1\times10^3-2\times10^4$ | $1\times10^3$ | [36] |
| Surface plasmon resonance | $100-1\times10^6$ | 100 | [25] |
| Fluorescence | $100-5\times10^5$ | 20 | [13] |
| Colorimetry | $100-1\times10^5$ | 10 | This work |

bound with the Apts inducing the change of color. Simultaneously, the OD signals decreased with the increase of target concentration. The schematics of this method was shown in Fig. 1.

### 2.4. Detection procedure of $VEGF_{165}$

First, 2 μg/mL of $VEGF_{165}$ standard solution were added to the microplate and incubated at 37 °C for 2 h. Then the well was washed twice with PBST. Then 100 μL 0.5% BSA was added and incubated for 1 h at 37 °C to reduce nonspecific adsorptions. Finally, excess 0.5% BSA solution was removed with PBST producing the desired biosensor with immobilized $VEGF_{165}$.

Different concentrations of $VEGF_{165}$ solution ranging from 0 to 100 ng/mL were mixed with Apts and vortexed for 20 min at 25 °C. Next, the mixture was added to a prepared biosensor and incubated at 37 °C for 40 min. The excess mixture was then removed and washed three times with PBST. Subsequently, HRP-SA was added and incubated for 20 min at 37 °C. After being washed with PBST, 100 μL TMB Solution (contains $H_2O_2$) was added and left to react for 20 min at 37 °C. Finally, the color reaction was terminated by the addition of 50 μL 2 mol/L $H_2SO_4$. Within 15 min the OD signal was read at 450 nm by microplate reader.

## 3. Results and discussion

### 3.1. Binding of Apts to $VEGF_{165}$

The specific binding between $VEGF_{165}$ and Apt was verified by the change of OD signal intensity. In this experiment, standard negative sample (0 ng/mL) and positive sample (0.5, 1 and 2 ng/mL) of $VEGF_{165}$ were incubated with Apt solution (200 nM), different OD signal intensities were found. Fig. 2(a) shows that the OD signal increased with increasing $VEGF_{165}$ concentration, indicating good affinity between Apts and $VEGF_{165}$.

### 3.2. Feasibility

In order to verify the feasibility of the developed method, four groups were tested and the results were displayed in Fig. 2(b). Even though the OD signals of group 1 and group 2 indicate certain nonspecific binding between Apts/HRP-SA and microtiter plates, this poses little problem as the OD signals of groups 3 and 4 were significantly larger than those of groups 1 and 2. The differences of OD signals were also detected between group 3 and group 4. The OD signal decreased significantly when target concentrations increased from 50 ng/mL to 100 ng/mL. There appears to be a competitive relationship between target and immobilized $VEGF_{165}$. The higher the concentration of the target, the lower the OD signal of the biosensor. These results suggest that the developed method was capable of detecting the different levels of $VEGF_{165}$.

### 3.3. Optimization of reaction conditions

#### 3.3.1. Addition sequence of reagents

Three different reagent addition sequences and their influence on signal intensity were evaluated. Sequence 1: First, the free $VEGF_{165}$ and Apts were mixed and left to react for 20 min at room temperature. Then, the mixture was added to the biosensor and incubated with the immobilized $VEGF_{165}$ for 40 min at 37 °C. Finally, HRP-SA was added to the system to bind with the Apts. Sequence 2: First, the Apts reacted with HRP-SA to form a HRP-SA@Apts complex based on the strong interaction between biotin and streptavidin, and then free $VEGF_{165}$ and HRP-SA@Apts were added to the biosensor. After competitive reaction, excess free $VEGF_{165}$ and HRP-SA@Apts complex were removed. Sequence 3: Free $VEGF_{165}$, Apts and HRP-SA were added to biosensor, then incubated together for 60 min at room temperature. The results of the three sequences show that the signal value of sequence 1 was greater than the other two (Fig. 3(a)). There was almost no difference in the signal values of sequence 2 between 0 ng/mL and 100 ng/mL $VEGF_{165}$ because Apts first combined with HRP-SA to form a large molecular weight complex producing a strong steric hindrance to the combination of Apts and $VEGF_{165}$. Based on the results of the three sequences, sequence 1 was chosen as the optimal reagent addition sequence.

#### 3.3.2. The concentration of immobilized $VEGF_{165}$

The concentration of immobilized $VEGF_{165}$ has great influence on the experimental results. Four control groups (different immobilized $C_{VEGF165}$: 0.5, 1, 2, 4 μg/mL) were set and the results compared in Fig. 3(b). When concentration of immobilized $VEGF_{165}$ was 2 μg/mL, the difference in OD signal value ($OD_0$-OD) was at its maximum. Therefore, the immobilized $VEGF_{165}$ concentration of 2 μg/mL was used in the subsequent experiment.

#### 3.3.3. Apts concentration

The concentration of Apts is a key factor; lower concentration may result in weaker signal intensity, and higher concentration may reduce the sensitivity of the method. Hence, five concentrations of Apts were evaluated. From Fig. 3(c), when the concentration of Apts increased, the signal intensity also increased from 100 nmol/L to 400 nmol/L. At a $VEGF_{165}$ concentration of 400 nmol/L, the OD signal reached its maximum, then began to decrease. Therefore, 400 nmol/L was selected as the optimal concentration.

#### 3.3.4. Reaction time and temperature of Apts and $VEGF_{165}$

To a certain extent, the reaction time and temperature affected the binding of $VEGF_{165}$ and Apts. Hence the reaction time and temperature of Apts and $VEGF_{165}$ in this assay were optimized. The effect of reaction time on the results is shown in Fig. 3(d). The signal value increased over the range from 10 to 40 min then started to decrease moderately, indicating that $VEGF_{165}$ and Apts initially bound rapidly, then reached saturation after which a small part of

**Table 2**
Precision of the proposed method for $VEGF_{165}$ detection (n = 3).

| $C_{VEGF165}$ (ng/mL) | Intra-assay | | | Inter-assay | | |
|---|---|---|---|---|---|---|
| | $\overline{X}$ | *SD* | *RSD* (%) | $\overline{X}$ | *SD* | *RSD* (%) |
| 1 | 1.25 | 0.062 | 5.0 | 1.13 | 0.187 | 16.6 |
| 10 | 8.06 | 0.498 | 6.2 | 9.01 | 1.359 | 15.1 |
| 50 | 43.66 | 2.402 | 5.5 | 48.69 | 7.113 | 14.6 |

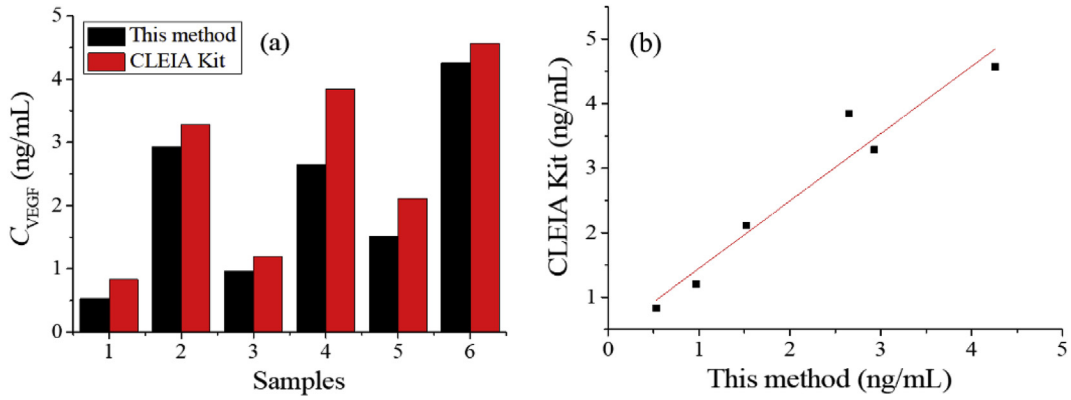


**Fig. 5.** Correlation analysis of serum samples results obtained by the developed method and CLEIA kit ($n = 6$).

the complex was dissociated and ultimately ended in a dynamic equilibrium. Fig. 3(e) shows the effect of temperature on the signal. The value of the signal at 37 °C was larger than at 25 °C, which is due to the higher activity of $VEGF_{165}$ at 37 °C and the greater likelihood of binding to the Apts. Therefore, 37 °C and 40 min were used as the reaction temperature and time.

### 3.4. Method evaluation

#### 3.4.1. Calibration curve

Under the optimum conditions, the developed method was applied to detect $VEGF_{165}$. A calibration curve was obtained by plotting lg($OD_0$-OD) against different concentrations of $VEGF_{165}$ solution ranging from 0.1 to 100 ng/mL. From Fig. 4(a), the increasing OD signal with increasing $VEGF_{165}$ concentration can be expressed by a linear regression equation ($Y = 0.2748X - 0.3824$, $R^2 = 0.9987$, Y and X denote the lg($OD_0$-OD) and lg($C_{VEGF165}$)).

Table 1 compares the analytical performance of the proposed strategy for the determination of $VEGF_{165}$ with other alternate methods reported in the literature. The results show that the proposed colorimetric strategy is superior to almost all listed methods in terms of linear range ($100-1\times10^5$ pg/mL) and detection limit (10 pg/mL).

#### 3.4.2. Selectivity

To evaluate the selectivity of the proposed method, several proteins including IgG, CEA, DNMT1, BSA were used as interference with concentrations of 100 ng/mL each. Fig. 4(b) shows that after the addition of equal concentrations of other kinds of proteins, low $OD_0$-OD signals were observed. The small difference in signal is due to the non-binding of non-target proteins with the Apts. The results further show that other proteins do not interfere with the signal, and that the developed strategy displays high selectivity.

#### 3.4.3. Precision

Precision is a key indicator in quantitative analysis, which is closely related to assay repeatability and accuracy. To evaluate the precision of the proposed method, three different concentrations (1, 10, 50 ng/mL) of $VEGF_{165}$ standard solutions were analyzed. Intra-assay precision was obtained by detecting each concentration three times in the same microplate. Similarly, the OD signal was determined in different microplates to evaluate the inter-assay precision. The precision of intra-assays and inter-assays ranged from 5.0% to 6.2% and 14.6%–16.6% (Table 2) showing that the precision of the proposed method is acceptable.

### 3.5. Sample analysis

To demonstrate the potential application of the assay in clinical research, six human serum samples from a hospital (the *First Affiliated Hospital of Zhengzhou University*, China) were tested by the proposed method and chemiluminescence enzyme immunoassay kit (CLEIA Kit) (Fig. 5). The paired sample *t*-test was used to analyze the experimental results. And the results indicated that there was a good correlation between this method and commercial CLEIA kits (correlation coefficient $r = 0.971$, $p = 0.001$). This method has great potential to be used in the detection of VEGF in human serum.

The recovery of $VEGF_{165}$ by the proposed method was also assessed. The different amounts of $VEGF_{165}$ (0.1, 1, 5 ng) were spiked into three human serum samples, and each sample was analyzed three times. Recoveries ranged from 81.4% to 116.4% (Table 3) and the veracity of the assay was satisfactory.

## 4. Conclusion

This study proposed and successfully tested a novel colorimetry strategy for the detection of $VEGF_{165}$. A simple biosensor was designed and used together with Apts to recognize $VEGF_{165}$. The high affinity and specific binding of Apts to $VEGF_{165}$ as well as the strong interaction between streptavidin and biotin ensured excellent selectivity and a rather low detection limit of 10 pg/mL. The method solves the problem of low sensitivity of colorimetry and meets the needs of clinical sample detection. Finally, the assay was successfully applied to detect $VEGF_{165}$ in human serum without any pretreatment and had a high correlation with commercial CLEIA kits. The reported method here certainly indicate great potential for future application in clinical analysis and diagnosis.

**Table 3**
Recoveries of $VEGF_{165}$ in pure human serum samples (n = 3).

| Samples | Background $VEGF_{165}$ (ng) | Add $VEGF_{165}$ (ng) | Measure $VEGF_{165}$ (ng) | Recovery (%) |
|---|---|---|---|---|
| 1 | 0.002 | 0.1 | $0.118 \pm 0.028$ | 116.4 |
| 2 | 0.113 | 1 | $0.906 \pm 0.130$ | 81.4 |
| 3 | 1.257 | 5 | $5.251 \pm 0.745$ | 83.9 |

## Acknowledgements

This work was supported by the National Natural Science Foundation of China (grant numbers. 81573203).

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