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A new electrochemical aptasensor for the analysis of the Vascular Endothelial Growth Factor

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Abstract

The vascular endothelial growth factor (VEGF) is a mitogen and important regulator of angiogenesis by activation the VEGF-receptor tyrosine kinases in endothelial cells. VEGF finds growing interest as a therapeutic target, and its analytical detection has important implications as biomarker for di□erent clinical disorders. Herein, we propose a new strategy to fabricate elegant electrochemical aptasensors, and this strategy has been employed to develop a biosensor for the assay of the concentration of VEGF. In our strategy, an electroactive probe, hemin, is not covalently modified at the end of the oligonucleotide. The method was simple and economical, allowing monitoring of VEGF to low concentrations of 1 nM.

KEYWORDS: vascular endothelial growth factor (VEGF); electrochemical; aptasensor; Hemin; Protein detection

INTRODUCTION

The vascular endothelial growth factor (VEGF) is a mitogen and important regulator of angiogenesis by activation the VEGF-receptor tyrosine kinases in endothelial cells. [1] VEGF is important in the pathophysiology of preeclampsia for women. Abnormal levels of VEGF were suggested to promote neuron degeneration by limiting neural tissue perfusion, and low levels of VEGF are associated with neurological disorders, brain injuries, and Parkinson's disease. [2] VEGF finds growing interest as a therapeutic target, and its analytical detection has important implications as biomarker for di□erent clinical disorders. On account of the significance of the protein, assays of VEGF have received more and more interest, and various techniques have been employed including ELISA assay, [3] fluorescence resonance energy transfer (FRET)-based assay[1], etc. However, each method has its own pros and cons in respect to the limits of the strategy and the apparatus. Therefore, it is highly required to develop more methods for the assay of VEGF.

On the other hand, aptamer-based sensors (aptasensors) attract substantial research efforts due to the availability of aptamers,[4, 5] and the ease to select and identify aptamers for small molecules or macromolecules.[6] Numerous electrochemical, optical, and other sensors have been developed.[7]

Electrochemical aptasensors, integrating the advantage of the functionalization of chemically modified surface and the sensitivity of electrochemical technologies, are senior for the detection of a variety of targets. [8] These technologies always require a labelled with an electroactive probe which will inevitably result in some complicated or expensive operations. Since target-induced conformational change of the aptamer will make the probe close to the surface of the electrode, electrochemical signal is thus achieved. [9] In the meantime, despite a large extent of success in such labeled strategy, attempts to adopt label-free strategy have been also made, aiming to further increase the simplicity and cost-efficiency, although most of these proposed methods suffer from the limitations as "signal-off" architectures. Such "signal-off" strategies might compromise selectivity since other substances or environmental stimulus might also lead to signal decrease and give "false positive" results.

Here, attempting to integrate the advantages of labeled architectures and label-free architectures, we propose a new strategy to fabricate elegant electrochemical aptasensors, and this strategy has been employed to develop a biosensor for the assay of the concentration of VEGF. In our strategy, an electroactive probe, hemin, is not covalently modified at the end of the oligonucleotide. This novel strategy also allows our architecture to be not only "signal-on" but also cost-effective.

EXPERIMENTAL SECTION

Chemicals

VEGF was purchased from Diazyme (USA) without further purification. Hemin and 6-mercapto-1-hexanol (MCH), were obtained from Sigma. Oligonucleotides (Guaranteed Oligos, HPLC-purified) were purchased from Sangon Biotechnology Co., Ltd (Shanghai, China). TCEP (Tris (2-carboxyethyl) phosphine) was obtained from J&K Scientific (China). Other chemicals were all of analytical grade. All solutions were prepared with double-distilled water, which was purified with a Milli-Q purification system (Branstead, USA) to a specific resistance of >18 MΩ cm.

Two strands of oligonucleotides were adopted in our experiments. The sequences are as follows:

DNA1: 5'-GGG TAG GGC GGG TTG GG**A GTC TAC GGC CGG GTA GA**-3'

DNA2: 5'- **TGT GGG GGT GGA CGG ACT** TTT TTT TTT T-$(CH_2)_6$-SH-3'

The sequence shown in bold is a split aptamer of VEGF as previous report; [1] the underlined sequence is an aptamer of hemin; the italic is a linker complementary to the italic sequence of DNA2. The stock solutions of these two oligonucleotides were prepared with a final concentration of 10 μM in Tris buffer (pH 8.0, 10 mM Tris, 10 mM TCEP, 1 mM EDTA). The stock solution of hemin (2.5 mM) was prepared in NaOH (0.01 M), and diluted to desirable concentration with 40KT buffer.

Preparation Of Working Electrode

A gold electrode (2 mm in diameter) was firstly cleaned with freshly prepared piranha solution (1:3 mixture of 30% H_2O_2 and concentrated H_2SO_4) for 5 min and rinsed thoroughly with double-distilled water. Then it was polished carefully with rough and fine sand papers and subsequently alumina slurries (1.0, 0.3, and 0.05 mm), followed by sonication in absolute alcohol and double-distilled water for 5 min, respectively. Finally, the electrode was electrochemically cleaned in 0.5 M H_2SO_4 until a stable cyclic voltammogram was obtained, and dried with purified nitrogen.

After the pretreatment, the freshly cleaned gold electrode was immediately immersed in the stock solution of DNA2 at 4 °C for about 14 h to form a self-assembled monolayer of DNA2 on the surface of the electrode. Afterward, the electrode was reacted with 1 mM MCH in an aqueous solution for 1 h to fill the pinholes in the monolayer with MCH.

VEGF and DNA1 was then grafted to the surface of the electrode. In detail, the DNA2 modified gold electrode was reacted with aqueous DNA1 and VEGF in a buffer solution (50 mM Tris-HCl buffer, pH 7.4, 10 mM KCl, 5 mM $MgCl_2$ and 0.1 M NaCl) for 30 min to make the two subunits VEGF aptamer region reacting with VEGF completely.

VEGF Concentration Assay

The above prepared working electrode was firstly thorough washed with double-distilled water. Then the electrode was kept in a 40KT buffer (Tris-HCl buffer containing 40 mM

KCl, pH 6.2, 100 mM Tris, 50 mM MES, 40 mM KCl, 0.05% Triton X-100, 1% (v/v) DMSO) for 30 min to allow proper unfolding of DNA1. After that, the electrode was immersed in a solution containing 100 μM hemin for 30 min, so hemin, the electrochemical probe, might well interact with the hemin aptamer. [7] Thus, electrochemical signal, which may assay the concentration of VEGF, was achieved.

Electrochemical Measurements

Electrochemical measurements were carried out with a CHI660C Potentiostat (CH Instruments). The commonly used three-electrode configuration was employed for the electrochemical measurements, while a saturated calomel electrode (SCE) served as the reference and a platinum wire as the counter electrode. A 20 mM HEPES buffer (pH 8) containing 20 mM KCl was adopted as the electrolyte. Before the measurement, the buffer solution should be firstly bubbled thoroughly with high purity nitrogen for 30 min. Then a stream of nitrogen was blown gently across the surface of the solution in order to maintain the solution anaerobic throughout the experiment.

Real Sample Analysis

To apply the method, VEGF in 5-fold diluted human serum was detected. The serum sample was voluntarily provided by healthy people and stored at 4 °C. Before detection, the 5-fold diluted human serum were spiked with 0, 10, 20, and 50 nM VEGF.

RESULTS AND DISCUSSION

Scheme 1 may illustrate the mechanism of the strategy in this work to fabricate the aptasensor. The aptamer against VEGF was split into two subunits. First, the DNA2 is immobilized on the gold electrode. Then the modified gold electrode was treated with different concentrations of VEGF and DNA1. In the absence of VEGF, the two subunits lack the affinity to form a stable complex. However, in the presence of VEGF, the respective aptamer subunits-VEGF complex is stabilized. Consequently, the hemin aptamer may cooperate to accept hemin, the electroactive label, giving electrochemical response.

Therefore, we have employed the aptasensor to detect VEGF concentration. Figure 1 shows the differential pulse voltammetric (DPV) waves of the VEGF/DNA1/DNA2/gold electrode. Obviously, the peak currents increase along with the increasing concentration of VEGF. The result is certainly reasonable, because a high concentration of VEGF will bind with large quantity of DNA1, and more hemin will bind with the hemin apatamer and to further give electrochemical signals. Figure 2 presents the relationship between the peak currents and the quantity of VEGF. The insert of Figure 2 reveals that the current is linearly dependent on the VEGF concentration in a range from 0 nM to 80 nM ($R^2 = 0.994$). And a detection limit of 1 nM can be obtained, which is lower than other aptasensor's detection limit (2.6 nM, 12 nM and 18 nM). [1] Thus, the results of the assay of the enzymatic activity by this aptamer-based electrochemical biosensor are demonstrated to work well. Although

the detection limit is higher than other reports: 104 pM, [10] 5 pM, [11] and 0.4 pM, [12] and cannot meet the clinical requirement of VEGF cutoff value (6.7 pM), [13] this strategy may provide an idea for future label-free and signal-on protein assay. By using the elaborate architecture, we are able to endow the hardly detectable protein concentration with an electroactive label, whose signals are in correspondence with the protein concentration.

The selectivity of this assay was also investigated by examining whether other common proteins would interfere with the assay for VEGF. Three proteins, bovine serum albumin (BSA), thrombin and carcinoembryonic antigen (CEA),) were chosen to be tested under the same experimental conditions as those for VEGF (Figure 3). The DPV waves remained low for these proteins even though their concentrations were 1 mM. These results suggest good selectivity of the assay for VEGF.

We next challenged the assay by analyzing samples of complicated matrix to evaluate the applicability of the assay to real samples. Three concentrations of VEGF were spiked into 5-fold diluted human serum. The recovery values were determined and were acceptable (Table 1). Therefore, this turn-on assay could potentially be applied to realistic biological samples, such as human serum. Compared with Zhang's strategy, [7] our strategy can be used in complicated matrix with a low limit of detection (1 nM).

The selectivity of this strategy in 5-fold diluted human serum was also investigated. Three proteins, BSA), thrombin CEA were also chosen to be tested in 5-fold diluted human serum which spiked with 50 nM VEGF (Table 2). The datum show that the concentration are the same as the data obtained from the 5-fold diluted human serum.

CONCLUSIONS

We have developed a novel aptasensor for the assay of the activity of VEGF. The elaborate architecture is designed on the surface of a gold electrode. The substrate of VEGF and the electroactive label, hemin, equal for the DNA1 on the surface, allowing the detection of VEGF by measuring the electrochemical signal of hemin. Experimental results show that the electrochemical response is in favorable accordance with the VEGF concentration, with a detection limit of 1 nM. From the view of the field of enzyme assay, the method we propose here may suggest a new way for the assay of protein concentration. Meanwhile, from the view of the field of electrochemical aptasensor, we have proposed a new strategy, integrating the advantages of labeled and label-free technique. Furthermore, based on this work, more architecture can be designed for the detection of other protein which has two subunit aptamers.

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Figure captions

Table 1. Results of the recovery test of VEGF in 20% human serum.

Samples	VEGF Added (nM)	Found (nM)	Recovery (%)
1	0	2.5	
2	10	11.8	93
3	20	20.3	89
4	50	50.5	96

Table 2. The selectivity test of this strategy in 5-fold diluted human serum.

Interfering Material	Concentrations (mM)	VEGF Found (nM)
	0	50.5
BSA	1	48.9
Thrombin	1	47.5
CEA	1	49.8

Scheme 1. Schematic illustration of the strategy to fabricate an electrochemical aptasensor for the assay of VEGF concentration. In the presence of VEGF, the VEGF subunits aptamers will form supramolecular structure once binding with VEGF. Consequently, the G-quadruplex may corporate to accept hemin, the electroactive label, giving an electrochemical response.

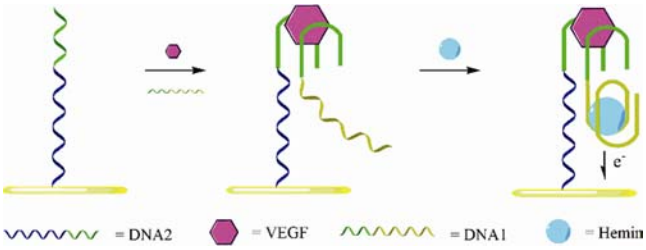

Figure 1. DPV response of the sensor for the assay of VEGF concentration.

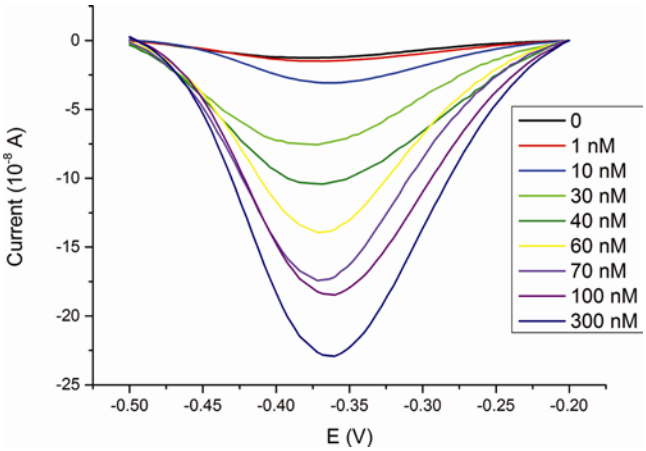

Figure 2. The relationship between the DPV peak and the concentrations of VEGF. The inset is shown with the linear range from 0 to 80 nM. All the data are taken from independent experiments with repetition for at least three times, and the presented data are the results of averaging.

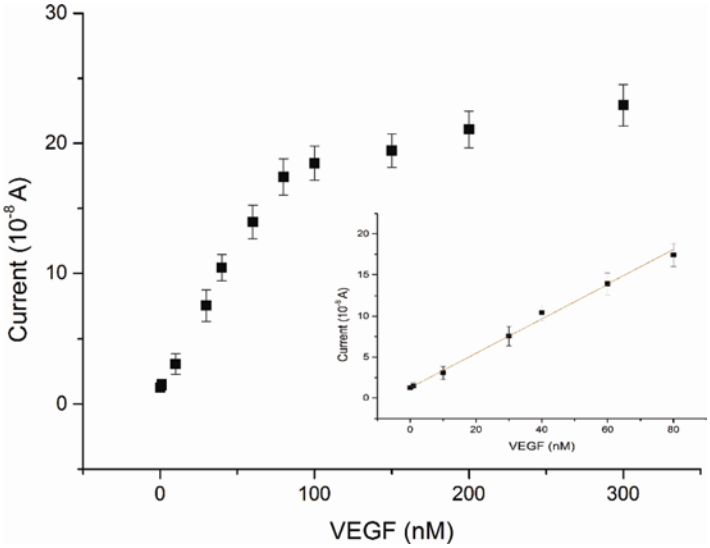

Figure 3. Selectivity analysis for VEGF detection. Bars represent the DPV wave of the sensor for VEGF (80 nM), BSA (1 mM), Thrombin (1 mM), and CEA (1 mM), respectively.