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Electrochemical Detection of VEGF165 Lung Cancer Marker Based on Au-Pd Alloy Assisted Aptasenor

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As we know that bimetallic nanomaterials containing Pd exhibit distinct properties as well as remarkable catalytic capacity. In this paper, a facile one-pot hydrothermal co-reduction method was employed to synthesize the alloy and core-shell Au-Pd nanomaterials. Then, the Au-Pd alloy/ionic liquid (AP/IL) was utilized to immobilize the aptamer on a glassy carbon electrode as the proper nanocomposite platform, which was used to detect lung cancer factor (VEGF165) in the ultra-trace level. The differential pulse voltammogram was recorded under the signal-off mode, exhibiting a linearly relationship with the concentration of VEGF165 in the sample solution increasing from 1 to 150 pM, where the limit of the detection is 0.5 pM. However, a positive linear relationship was observed in the signal-on mode between the resistance of charge transfer and the concentration of VEGF165 ranging from 5 to 200 pM, where the limit of the detection was 0.78 pM. Moreover, the proposed sensor has been successfully used for determination of VEGF165 in human serum sample. The result indicate our proposed method could be potentially used for clinic diagnosis of lung cancer.

Keywords: Au–Pd alloy; VEGF165; Hydrothermal coreduction; Core–shell; Aptasenor

1. INTRODUCTION

Vascular endothelial growth factor plays a key role in regulating both the physiologic and pathologic angiogenesis. Just like its name, VEGF can facilitate the growth, proliferation and survival of the vascular endothelial cell [1, 2]. Besides, VEGF, which is a signal protein, has been applied in numerous human diseases such as lung cancer [3-5], proliferating retinopathy [6], psoriasis [7] and rheumatoid arthritis [8] as the serum biomarker. For instance, the tumours require individual blood to

provide nutrients and oxygen for the further growth and metastasis, which can be satisfied through overexpressing VEGF so that a growing vascular network can be created and maintained [9]. Serum VEGF is often elevated in various histological types of disseminated cancer, including cancer of the lung, in comparison with healthy controls [10]. The importance of vascular endothelial growth factor (VEGF) in the angiogenic process of human malignancies is well established [11]. VEGF is a major angiogenic factor in non-small cell lung cancer (NSCLC), defining poor postoperative outcome [12]. High VEGF expression and/or high angiogenic activity is noted in about 30–40% of NSCLC [13, 14]. Hence, rapid and direct detection of VEGF with sensitivity and selectivity in the whole serum or blood of the patients is remarkably crucial for disease diagnosis and further therapy monitoring. So far, a variety of methods for the detection of VEGF have been developed, such as immunohistochemistry, enzyme-linked immunosorbent assays (ELISAs), radioimmunoassay and etc. Nevertheless, these methods are not suitable for the approximately real-time diagnostics in clinic, due to their time-consuming and labour-intensive disadvantage and requirement of complicated instruments.

Antibodies have been recognized as the biorecognition element of option in developing the biosensing assays in the past three decades. Hence, the long-term dominant status of the antibodies-based detection methods will be changed undoubtedly when a new kind of biorecognition elements appears. In 1990, the method to select aptamers recognized as the chemical antibodies was developed individually by two research groups to apply in both the therapy and biosensing [15, 16]. Systematic evolution of ligands by exponential enrichment (SELEX), which are recognized as the well-known *in vitro* selection approach, have been employed to develop aptamers composed of single-chain DNA, RNA or modified nucleic acids, where the special oligonucleotides are separated from the complicated class of the synthetic nucleic acids through binding the oligonucleotides to the target molecules repeatedly. Aptamers exhibit a high affinity and selectivity towards various targets, such as metal ions [15], small organic molecules [17], proteins [18] and especially the whole cells [19]. Besides, compared to antibodies, aptamers exhibit several advantages such as easy synthesis and labelling and outstanding stability. Hence, aptamers have been considered as the significantly promising biorecognition elements for the design of sensors recently. Thereafter, numerous sensors based on aptamer (aptasensor) have been developed, which almost cover all the biosensing approaches such as mass-sensitive, electrochemical and optical methods [20-22].

Since 2004, electrochemical aptasensors have received extensive attentions [23]. Especially for the folding-based aptasensors, they have attracted more interests among these sensors because of their sensing-platform relevant properties [24-27]. Besides, most of these sensors are basically sensitive, selective, recyclable, specific, rapid and reagentless, especially compatible with the platforms of the microfluidic-based detection [28]. So far, numerous folding-based aptasensors have been reported to detect the small molecules and proteins, whereas none of them are proper to directly detect VEGF, which is a significant serum biomarkers for diverse disease. Herein, a collapsible electrochemical aptasensor was developed to detect VEGF in a complicated medium which was clinic relevant. The first anti-VEGF DAN aptamer, which was reported in the previous literature, was used in this work [29-31]. Different from Pegaptanib (Macugen), the DNA aptamer employed in this work, which are well-defined and the most widespread anti-VEGF RNA aptamer, are more resistant to the degradation of nuclease. Additionally, in contrast to the other VEGF inhibitors which exhibit a stoichiometry of 2:1

(ligands:VEGF), a simple binding motive of 1:1 (aptamer:VEGF) is provided by the employed aptamer [32, 33]. In particular, the distinct fold of this DNA aptamer inspired us to develop a sensor which could directly transform the change of conformation induced by the target into the electrochemical signal change which could be measured. Thus, we immobilized the aptamers through Au-Pd (AP) on a glassy carbon electrode (GCE) and fabricated an electrochemical aptasensors without label to detect VEGF165 tumour marker through the "signal off" and "signal on" approaches which are based on differential pulse voltammetry and impedance. However, owing to the high specific surface area, low toxicity, facile functionalization and remarkable conductivity which are virtually different from their bulks, the proteins-stabilized nanoclusters have received an increasing attention in the electrochemical applications [34]. Besides, the gold nanoclusters exhibit a smaller size compared to the conventional nanoparticles [35]. Hence, based on these significant advantages, AP was employed combining ionic liquid (IL) to enhance the stability and sensitivity of the proposed aptasensor for the detection of VEGF165. Owing to the large surface area of AP as well as the massive active functional groups in the protein to attach the aptamers as the efficient stabilizing agents.

2. EXPERIMENTS

2.1. Materials

Tetrachloropalladate acid (H_2PdCl_4), hydrogen tetrachloroaurate(III) hydrate ($HAuCl_4$), cetyltrimethylammonium bromide (CTAB), ammonia (NH_3·H2O), sodium borohydride (NaBH4) and potassium hydroxide (KOH) and were commercially available (Shanghai Reagents Co., Shanghai, China). Hydrogen tetrachloroaurate(III) trihydrate ($HAuCl_4 \cdot {}_3H_2O$, ≥49.0% Au basis), methylene blue trihydrate (MB, 85%), glutaraldehyde solution (GA, 25% in water), polyvinylpyrrolidone (PVP, Mw = 360 000), bovine serum albumin (BSA, 98%), potassium hexacyanoferrate (II) ($K_4[Fe(CN)_6]$) (≥99.0%) and potassium hexacyanoferrate (III) ($K_3[Fe(CN)_6]$) were obtained from Sigma Aldrich. Besides, vascular endothelial growth factor (VEGF165) and anti-$VEGF_{165}$ DNA aptamer were purchased from Invitrogen™ (USA). NH2 group was introduced into the 5'-terminus to modify the aptamer, where the order was described as follows: 5′-NH_2-TTTCCCGTCTTCCAGACAAGAGTGCAGGG-3′. In this work, all the reagents were analytically pure and used directly without any further purification.

2.2. Preparation of Au–Pd Alloy Bimetallic Nanostructures

PVP (0.2 g) and CTAB (0.2 g) were added one by one into 10 mL mixed aqueous solution of $HAuCl_4$ (0.7 mM) and H_2PdCl_4 (0.9 mM) under vigorously stirring. Then, 5 mL $NH_3 \cdot H_2O$ (2.8%) was added to the as-formed solution and the solution was stirred for 10 min. Subsequently, the solution was transferred into the Teflon-lined stainless-steel autoclave with a volume of 25 mL and heated to 180 oC for 12 h. After that, the solution was cooled down naturally and the product was obtained through centrifugation and washed with deionized water and pure ethanol for three times.

2.3. Fabrication of electrochemical aptasensor

The end surface of GCE (i.d.=3.0 mm, Metrohm, Switzerland) was first polished through the synthetic cloths combining alumina slurry with a size of 0.3, 0.1 and 0.05 mm (Struers, Denmark) successively in prior to coating, where a mirror finish was obtained. Then, it was rinsed with the mixed solution of water and ethanol (1:1) under ultrasonication. After that, the electrode was placed into H_2SO_4 (0.1 M) and cycled voltammetrically under the potential ranging from -0.2 to 1.4 V until obtaining the typical CV. At last, the electrode was again rinsed with ultrapure water and dried through nitrogen. Then the aptamer was immobilized on the fresh electrode immediately.

To modify GCE, 0.2 g IL (EMIMPF6) was deposited on the surface of the electrode and heated to beyond the melting point of IL which was in the range of 58-62 ^{o}C through hairdryer so that the surface was covered with IL. Then, AP was covered on the surface of the electrode modified with IL through drop casting 10 μLon the surface and heating under ambient at 50 ^{o}C for 10 min [36]. It was demonstrated that the stability of AP was enhanced on the surface of the electrode through IL. Various researches reported that a considerably strong interaction present between IL and the surface of nanoparticles [37]. As a matter of fact, the imidazolium-based ILs-stabilized nanoclusters and nanoparticles have been recognized as the proper typical approach. Then, the AP/IL/GCE was dipped into the 2.5% GA in PBS for 1 h so that to immobilize the aminated aptamer onto the surface of the electrode [38]. Subsequently, the electrode was rinsed and 10 μL the aminated aptamer (0.6 □M) in PBS (0.1 M) with a pH of 7.4 was deposited on the surface of the electrode and cultivated at room temperature for 12 h. At last, the electrode was immersed into the 0.25% BSA solution for 30 min to reduce the non-specific binding.

2.4. VEGF165 detection

Owing to the outstanding redox and low-cost characteristics, MB was selected combining with $[Fe(CN)6]^{3-/4-}$for the “signal off” and “signal on” methods as the electrochemical probes, respectively. According to the previous reports, MB could be immobilized definitely through the guanine bases contained in ss-DNA [39]. In this work, the employed aptamer belonged to the class of ss-DNA which contains abundant guanine bases. To fabricate the MB/aptamer/AP/IL/GCE to determine VEGF165 with “signal off” approach, the end surface of the electrode was dipped into 0.1 M PBS with 25 □M MB for 15 min at room temperature under stirring. Then the electrode was rinsed by 0.1 M PBS and 20 μL VEGF165 with a certain concentration was deposited onto the MB/aptamer/IL/GCE surface for 40 min. Then, the reproducibility of the sensing phase was evaluated, where successive binding and washing process were performed. To determine VEGF165 through the “signal on” approach, 20 □L VEGF165 with a definite concentration was deposited on the surface of the aptamer/AP/IL/GCE and then cultivated for 40 min. Then, the electrode was rinsed through PBS for the removal of VEGF165. The EIS responses of the aptasensor were collected in 0.1 M PBS with a pH of 7.4 in the presence of 5 mM $[Fe(CN)6]^{3-/4-}$ couple (1:1).

3. RESULTS AND DISCUSSION

To monitor the process of fabricating the electrochemical aptasensor during each procedure, CV and EIS were employed. Figure 1 illustrated the CV characterization of the aptasensor, where the redox signal of MB was used. For the aptamer/AP/IL/GCE, no redox peaks were obtained. However, twain invertible redox peaks of MB were obtained, when accumulating MB on the surface of the electrode through the dipping the electrode into MB solution. Massive MB, which acted as the organic probe, was immobilized onto the aptamer through the particular interaction with the guanine bases contained in the ss-DNA. Nevertheless, the peak current of MB remarkably reduced after the exposure of the modified electrode into the solution of VEGF165 (1 pM). A rigid stem-loop structure was formed in the aptamer, resulting in the reduction of signal, where MB was desorbed from the surface of the electrode. Moreover, a target-induced folding approach was employed in the fabricated architecture of aptasensor, as the aptamer for anti-VEGF165 DNA could be folded via a specific three-dimensional construction through binding to the target.

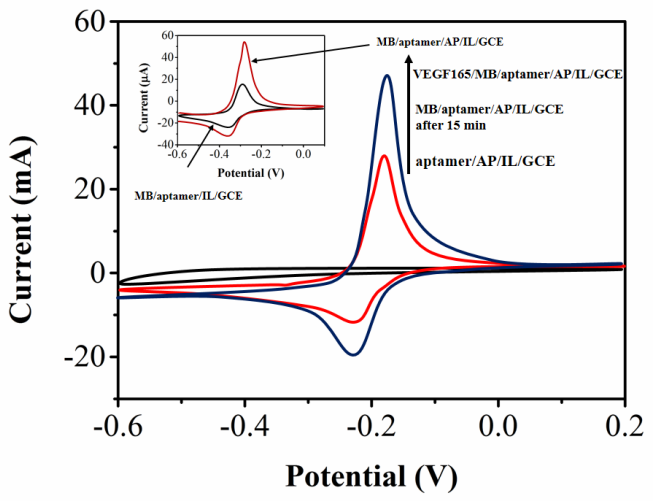

Figure 1. The CVs of aptamer/AP/IL/GCE and MB/aptamer/AP/IL/GCE after incubating for 15 min and VEGF165/MB/aptamer/AP/IL/GCE in 0.1 M PBS (pH 7.4), scan rate 100Mv/s. Inset is the CVs of MB/aptamer/AP/IL/GCE and MB/aptamer/IL/GCE.

EIS provide a direct insight into the mechanisms of the electrochemical processes was supplied by EIS, which could also obtained the information about the capacitance of the system [40]. Hence, the relevant Nyquist plots was collected in 0.1 M PBS with a pH of 7.4 in the presence of $[Fe(CN)6]^{3-/4-}$ with a concentration of 5 mM, which were illustrated in Figure 2A. A considerably small semi-circle domain was observed, where a remarkably low charge transfer resistance (R_{ct}) was measured to be 265 Ω. However, the R_{ct} value increased remarkably to 2855 Ω after the modification of the electrode surface with BSA-AP/IL. The generated layer would serve as a barrier to block the mass transfer as expected. Thus, it further insulated the conductive support as well as restricted the redox probe from accessing towards the surface of the electrode. The R_{ct} value remarkably increased to3677 Ω when binding the anti-VEGF aptamers to the AP, which might be owing to the negative-charged single-chain

nucleic acid on the phosphate backbone of the aptamer inducing the electrostatic repulsive force on the $[Fe(CN)6]^{3-/4-}$ probe. Nevertheless, MB with positive charge was accumulated on the aptamer/AP/IL/GCE surface, resulting in the reduce of the electrostatic repulsive force as well as the decrease of Rct to 2825 Ω. At last, MB was desorbed from the surface of the electrode through introducing VEGF165 with a concentration of 1 pM to the sensing platform, where R_{ct} increased significantly to 7841 Ω. In Figure 2B, a trend was observed in the changes of the relevant CVs of $[Fe(CN)6]^{3-/4-}$ as an electrochemical probe, which was in accordance with the results obtained through EIS measurements.

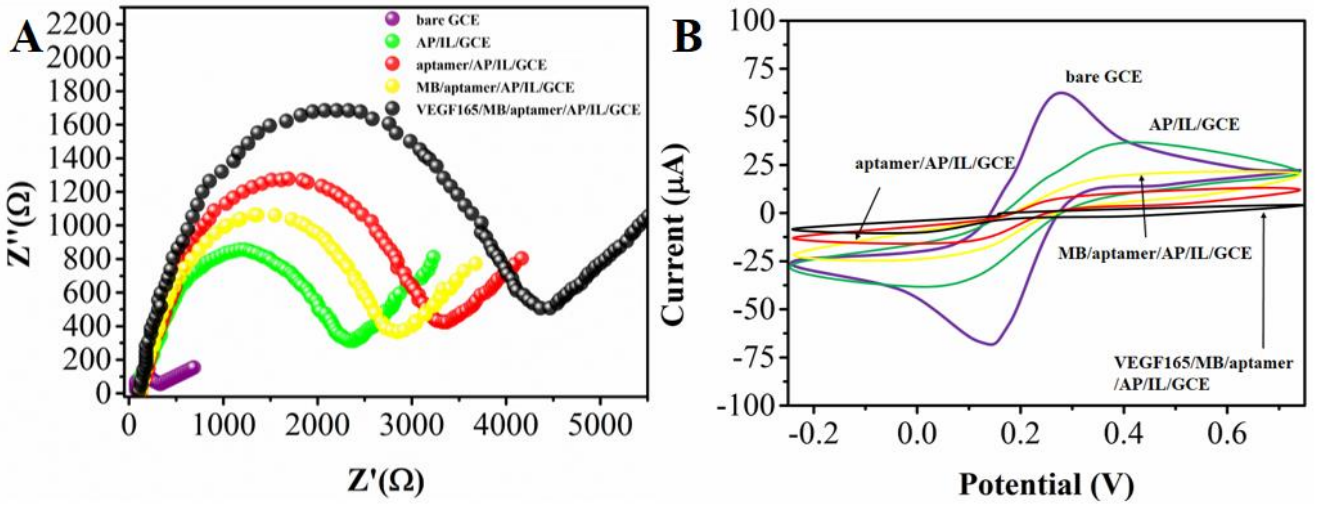

Figure 2. (A) Nyquist plots and (B) corresponding CVsof bare GCE, AP/IL/GCE, aptamer/AP/IL/GCE, MB/aptamer/AP/IL/GCE, VEGF165/MB/aptamer/AP/IL/GCE in 0.1 M PBS in the presence of 5 mM $[Fe(CN)_6]^{3-/4-}$ couple (1:1) (pH 7.4), scan rate 100 mV/s.

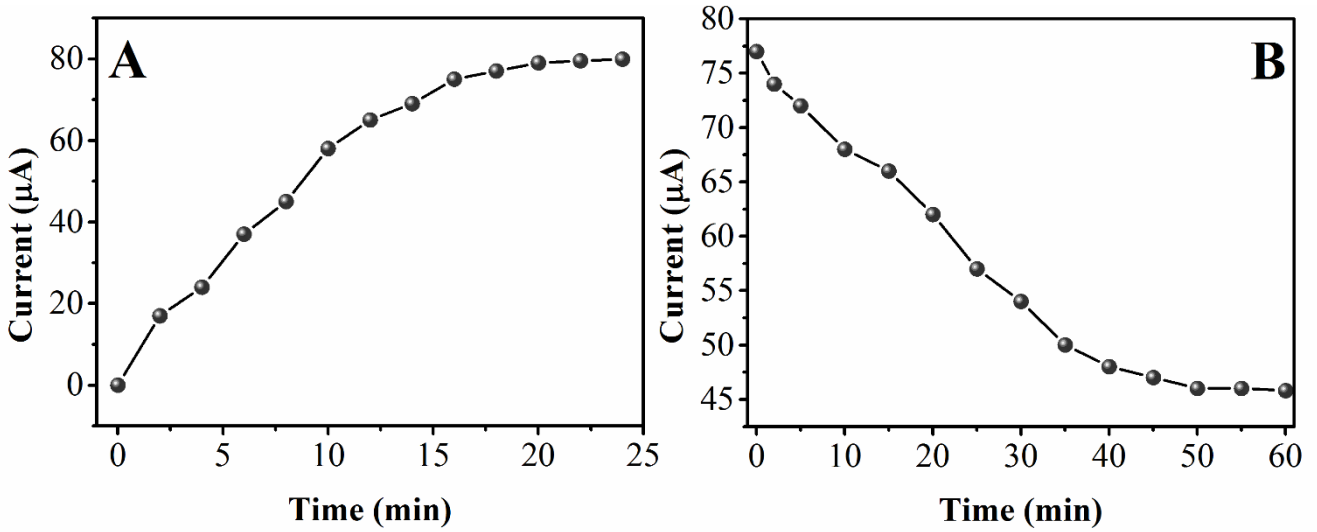

Figure 3. The influence of (A) the accumulation time of MB (25 μM) on the proposed aptasensor and (B) the incubation time of $VEGF_{165}$(100 pM) on the DPV responses of MB.

The influence of the accumulation time of MB with a concentration of 25 μM MB on the response of aptamer was studied. In Figure 3A, an increase was observed with the current of the immobilized MB when the accumulation time was increased to 25 min from 0. However, no change was observed with the redox signal current of MB when continuing to increase the accumulation time. Hence, the influence of the time of cultivatingVEGF165 with a concentration of 100 pM on the sensor response was also investigated, as the incubation time of the target was a crucial influence factor towards the response of the aptasensors [41]. In Figure 3B, it was obvious that the current response dramatically reduced when the incubation time increased to 50 min. However, it almost levelled off at much higher time, which indicated that the complex of VEGF165 and aptamer was generated on the electrode surface and reached to the saturated level.

The density of the surface probe played an essential role in the capacity of the aptasensor, as the results reported before [42]. The effect of the concentration of the aptamer employed to fabricate the sensor on the response properties of the obtained sensor was investigated, as the density of the probe was determined by the concentration of the aptamer, which was illustrated in Figure 4. It was obvious that the current of the absorbed MB increased when the concentration of the immobilized aptamer on the modified electrode surface increased up to 0.7 μM. Nevertheless, the signals of MB decreased when increasing the concentrations of the aptamer, as the electrode surface became saturated and induced the subsequent steric hindrance.

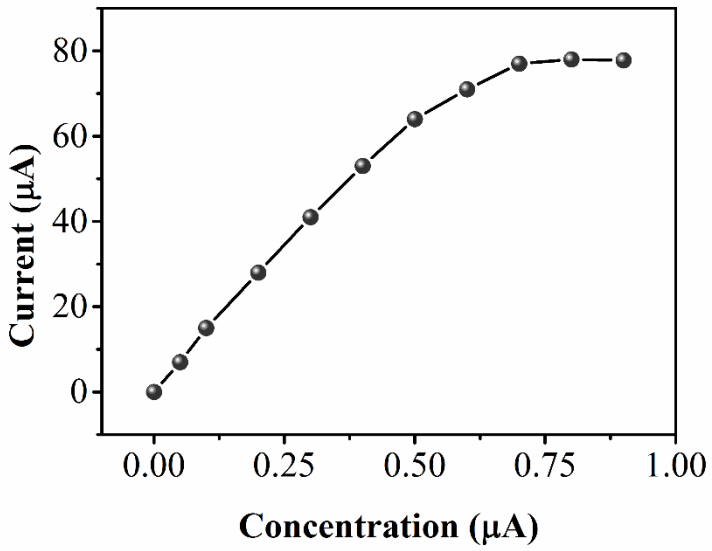

Figure 4. The influence of the surface of the modified electrode on the DPV responses of MB.

The fabricated aptasensor was used to determine VEGF 165 with two different techniques including DPV and EIS. The DPVs of the as-obtained aptasensor exposure to VEGF165 with various concentrations were illustrated in Figure 5A, which significantly exhibited a “signal off” tendency. MB could be absorbed by the aptamer and further dissolved into the solution when the hybridization took place between VEGF165 and the anti-VEGF165 aptamers, resulting in the decrease of the current

signal of MB. A linear change was observed between the current signal of MB and the concentration of VEGF165 ranging from 1 to 150 pM. The linear equation of the calibration curve was described as follow: I(μA)=-30.547 C (pM)+69.511, where the correlation coefficient was calculated to be 0.997. Besides, the limit of the fabricated aptasensor which was "signal off" was measured to be 0.5 pM (based as 3□). Furthermore, the determination of VEGF165 was also performed through the EIS method based on "signal on" response, where $[Fe(CN)6]^{3-/4-}$ was employed as the external probe. As shown in Figure 5B, VEGF165 was incubated through immobilizing anti-VEGF165 aptamers on the surface of the electrode, resulting in the more difficulty to diffuse $[Fe(CN)6]^{3-/4-}$ onto the surface of the electrode. Thus, an increase of the Rct value was observed when the number of the conjugates between anti-VEGF 165 aptamers and VEGF165 increased, as a barrier was generated during the electron transfer process of the [Fe(CN)]3-/4- on the modified electrode surface. According to the "signal on" tendency, a remarkable linear response was observed with the designed aptasensor to VEGF165 with a concentration in the range of 5 to 200 pM. The regression equation was described as follow: ΔR_{ct} (kΩ)=52.322 C (pM)+5411.2 (R^2=0.993), where the limit of the detection was measured to be 0.78 pM. Especially, the obtained limit of detection was higher compared with the clinical requirement for determining VFGF165, of which the cut-off value was 0.5 pM. The limit of the VEGF 165 determination with the MB/aptamer/AP/IL/GCE was lower than that with a multiplex sandwich aptamer microarray (3 pM) [43] and aptamer affinity probe in microchip capillary electrophoresis (4 μM) [44]. This analytical performance can be compared with those recently reported in the literature for Table 1. As compared with different determination methods, due to the high sensitivity of the proposed electrochemical performance, the MB/aptamer/AP/IL/GCE could be used for potentially detecting VEGF 165 at real samples.

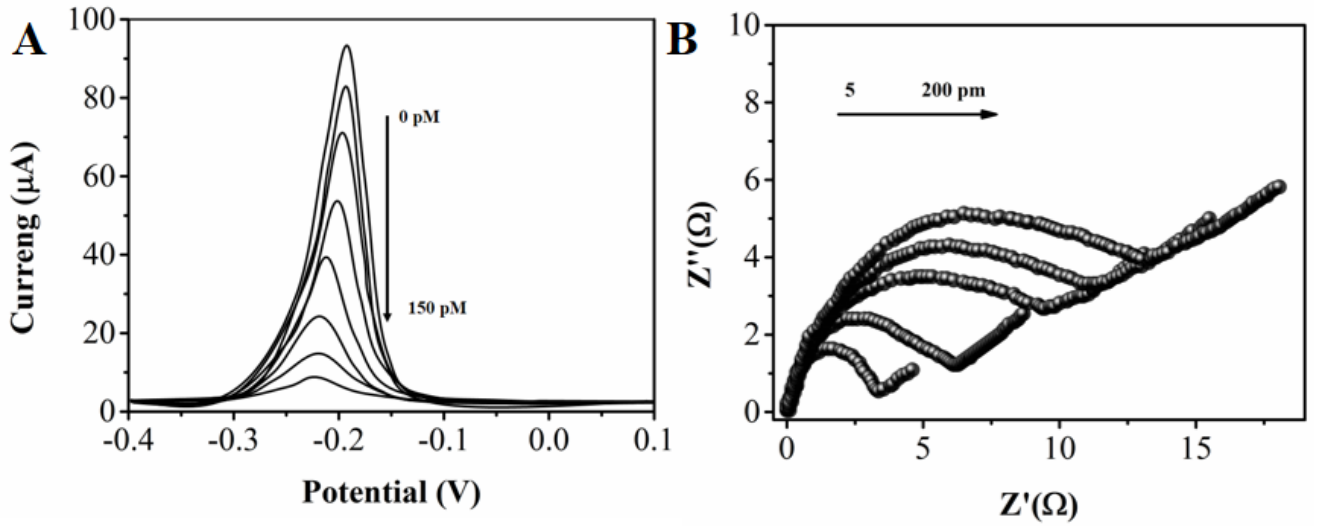

Figure 5. (A) The DPV responses of the MB/aptamer/AP/IL/GCE cultivated by VEGF 165 in 0.1 M PBS (pH 7.4) with various concentrations. (B) Nyquist plots of the aptamer/AP/IL/GCE incubated by VEGF 165 in 0.1 M PBS (pH 7.4) with various concentrations in the presence of 5 mM $[Fe(CN)_6]^{3-/4-}$ couple (1:1).

Table 1. Comparison of our proposed AIV H7 electrochemical sensor with other reports.

Electrode	Linear range (μM)	Limit of detection (μM)	Reference
Capillary electrophoresis	7-60 pM	4 pM	[44]
Multiplex sandwich aptamer microarray	5-100 pM	3 pM	[43]
MB/aptamer/AP/IL/GCE	1~150 pM	0.78 pM	This work

To evaluate the reproducibility of the proposed aptasensor, three modified electrodes, which were produced freshly, were employed to determine VEGF165 with a concentration of 1 pM under the optimized experimental conditions. The mean relative standard deviation (RSD) of the currents of the obtained DPV signals was calculated to be 3.4% based on these results. Besides, an RSD was measured to be 2.7%, according to the results of 10 continuous detection of VEGF165 with a concentration of 1 pM through a single electrode under the same conditions.

Furthermore, the stability of the aptasensor was evaluated, where the aptasensor was dipped into PBS with a pH of 7.4 for 14 days as well as placed at 4 ^{o}C under ambient for 14 days. Only 5.8% decrease was observed for the relevant DPV peak current, which indicated that the stability was acceptable. Nevertheless, the aptasensor exhibited a 10.1% loss of the activity when storing at room temperature for 14 days.

Since the VEGF plays an important role in lung tumour growth and metastasis. The potential analytical applicability of the fabricated sensor was evaluated through the detection of VEGF165 in three diverse serum samples, which was first diluted 2 folds with water in prior to the measurement. The precision of the aptasensor was accessed by comparing the obtained results with those measured through the standard Enzyme-Linked Immuno-sorbent Assay (ELISA) approach with a kit which is commercially obtained. The ELISA method is not suitable for the approximately real-time diagnostics in clinic, due to their time-consuming and labour-intensive disadvantage and requirement of complicated instruments. According to the results illustrated in Table 2, no obvious difference in statistics was observed between the results obtained via the aptasensor and those through the ELISA method, which indicated that the aptasensor was promising for determining VEGF165 in the pratical serum specimens as an approach with sensitivity and viability. Therefore, our proposed aptasensor has a large potential for clinic diagnosis of lung cancer.

Table 2. The determination of VEGF165 in the serum specimens.

Sample	Concentration (pg/mL)		
	Proposed method	ELISA	RSD
1	30.6	31.22	4.51
2	57.8	60.54	7.88%
3	102.56	100.25	6.21%

4. CONCLUSIONS

In conclusion, to detect the VEGF165 rapidly and sensitively, an electrochemical aptasensor without label was prepared with the Au-Pd alloys. The detection of VEGF165 was performed, where

the MB and $[Fe(CN6)]^{3-/4-}$ were utilized in the "signal off" (DPV) and "signal on" (EIS) approaches as the electrochemical probe, respectively. As a matter of fact, a large surface area could be supplied to immobilize VEGF165 by AP. For the "signal off" and "signal on" approaches, the developed aptasensor exhibited a linear range of 1-150 pM and 5-200 pM respectively, where the relevant limits of detection were 0.5 and 0.78 pM. Moreover, the developed aptasensor also exhibited a remarkable applicability for determining VEGF165 in the serum samples.

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