Int. J. Electrochem. Sci., 12 **(2017)** 2865 – 2874, doi: 10.20964/2017.04.03

International Journal of
ELECTROCHEMICAL
SCIENCE
www.electrochemsci.org

Electrochemical Determination of Neopterin as Inflammatory Factor for Potential Clinic Vasculitis Diagnosis

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Received: 15 December 2016 / *Accepted:* 31 January 2017 / *Published:* 12 March 2017

In the present work, we develop a novel electrochemical strategy for immunoassay of neopterin by using screen-printed array as electrodes and Neopterin–alkaline phosphatase conjugation as label chemical. Electrochemical detection instead of traditional ELISA was employed, while the traditional plastic wells were replaced by screen-printed array electrodes. Various measurements were utilized for the detection of neopterin. Using the optimized electrochemical method, a limit. The obtained results show a possibility for the clinic diagnosis of vasculitis and a variety of other inflammatory diseases.

Keywords: Neopterin; Immunoassay; Screen-printed array; ELISA; Electrochemistry

1. INTRODUCTION

Neopterin (2-amino-4-hydroxy-6-(d-*erythro*-1′,2′,3′-trihydroxypropyl)pteridine), which can be derived from antigen-activated T lymphocytes, is generated from guanosine triphosphate when human monocytes and macrophages were stimulated by IFN-γ (interferon gamma) [1]. It will increase significantly in the human body fluids after the immune system was activated. Therefore, determination of neopterin is one of the effective method to confirm the activation state of cellular immune system during the subsequent stages of various diseases, for instance allograft rejection, cardiovascular disease [2], insulin resistance [3], neuropsychiatric abnormalities [4], rheumatoid arthritis (RA), tumors [5] and vasculitis. For the blood vessels system, it is comprised of arteries and veins. The former is for the delivering of oxygen-rich blood to the tissues of human body. And the latter is for the returning of oxygen-depleted blood from the tissues back to the lungs. Vasculitis is a general term for a group of uncommon diseases which is featured by a inflammation of blood vessels.

It cause a damage to the vessel walls. The definition of vasculitis diseases is based on involved blood vessels or organs. Thus, neopterin can act as an indicator for the clinic diagnosis of the vasculitis.

A raise of neopterin was also detected in viral infections [6-9] such as bacterial infections, cytomegalovirus, hepatitis-A, B and C, influenza, measles, and rubella [10]. Typically, in the case of sepsis caused by microbial invasion, neopterin concentration in plasma is much higher than that of healthy controls [11]. Up to now, various method have employed to analysis the concentration of neopterin in blood, for instance HPLC (high pressure liquid chromatography) [12] and ELISA (enzyme-linked immunosorbent assay) [13-17]. However, most of these methods have many drawbacks for example sophisticated instrumentation, high cost, long analysis time and so on. So, effective alternative methods for the determination of neopterin are urgently required.

Owing to its miniaturization, low cost, miniaturization, high sensitivity, and possibility of integration with multi-array tools, electrochemical biosensor is considered as a promising facility for point-of-care testing [18-22]. In a recent literature, functionalized monoclonal and polyclonal antibodies were developed. Those antibodies are with high specificity and affinity towards neopterin. It is contributed to enhance the detection sensitivity and to improve the performance and kinetic properties of the immunological reaction [23]. The detection capabilities of obtained hapten conjugates and antibodies were also verified by conventional ELISA formats with clinical samples. The limit of detection (LOD) of obtained monoclonal and polyclonal antibodies in direct ELISA format are 0.18 ng/mL and 0.05 ng/mL, that shows a comparatively higher sensitive of the polyclonal antibodies [23]. Recently, in the basis of fluorescence measurement in a binding inhibition assay, the same monoclonal antibody with a LOD of 0.45 ng/mL was reported [24, 25].

Conductive polymers (CPs), specially functionalized CPs, are useful for the modification of electrode surface in order to improve the high density of immobilized biomolecules, long-term stability of attached biomolecules, low non-specific binding, and specific interaction between electrode and proper biomoleculars. PABA (Poly o-aminobenzoic acid), a carboxyl functionalized polyaniline, is considered as one of the soluble conductive polymers[26]. Its carboxylic acid group can act as a matrix material to immobilize substrates forming covalent bonds with biomolecules like antibodies and proteins [27]. Herein, functionalized CPs are widely utilized as a platform for the immobilization of antibodies on the surface of electrode.

It is well known that MB (methylene blue) can accept two electrons and thus be reduced to LB (leucomethylene blue) on the surface of electrode. Hence, it can be used as an electrochemical indicator for the determination of target molecule. In the present study, this electrochemical indicator was employed to detect the interactions between target molecule-aptamers [28-30]. In addition, an novel aptamer-based electrochemical neopterin biosensor modified by functionalized conductive polymer on the surface of electrode was introduced.

2. EXPERIMENT DETAIL

All reagents were purchased as analytical grade and used as received. Carbodiimide hydrochloride, EDC (1-Ethyl-3-(3′-dimethylaminopropyl), NHS (N-hydroxysuccinimide), and o-ABA (o-Aminobenzoic acid) were purchased from Sigma-Aldrich. BSA (Bovine serum albumin),

ethanolamine, magnesium chloride, neopterin, potassium chloride, sodium chloride, and Tween 20 (poly-oxyethylene sorbitan monolaureate) were obtained from Sinopharm Group Co., Ltd. Disodium hydrogen phosphate, EDTA (ethylenediaminetetraacetic acid), MB (methylene blue), $K_4[Fe(CN)_6]$ (potassium ferrocyanide) $K_3[Fe(CN)_6]$ (potassium ferricyanide), sodium dihydrogen phosphate, sulfuric acid, and TRIS (Tris (hydroxymethyl)aminomethane) were purchased from Merck. Aptamer 5′-GCAGTTGATCCTTTGGATACCCTGGTTTTTTTTTTTTTT-3′ purified by HPLC was obtained from Xincheng Tech Co., Ltd., and it was dissolved in TRIS buffer (10 mM) containing EDTA (1 mM).

Autolab type II PGSTAT ((Metrohm, Italy, with a GPES 4.9 software package) was employed for electrochemical measurements. Screen-printed electrode, graphite electrode and silver pseudo electrode were used as working electrode, counterelectrode and reference electrode, respectively. All electrochemical experiments were measured by utilizing DPV (differential pulse voltammetry) at room temperature under the experimental conditions: potential range 0/-600 mV, interval time 0.1 s, step potential 5 mV, standby potential +100 mV, modulation amplitude 50 mV. And the conditions of electrochemical impedance measurements were as follows: 10 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (in PBS, NaCl 0.1 M, pH=7.4), frequency range 100 kHz–100 mHz, dc potential 0.13 V, alternative voltage 10 mV in amplitude (peak-to-peak).

The electropolymerization of o-ABA (o-aminobenzoic acid) at SPEs was accomplished under the following conditions: potential cycles from 0 to 1.0 V for 15 cycles, sweep rate 50 mV/s in a solution containing 0.1 M KCl, 1 M H_2SO_4, and 50 mM o-ABA. After polymerization, obtained electrode was washed with water and 1 M H_2SO_4 in order to remove the excess o-ABA monomer.

After electropolymerization, a buffer solution containing NHS and EDC in 0.1 M PBS (pH=5.0) were used to activate the terminal carboxylic groups which are contributed to the immobilization of the primary antibody. Briefly, a NHS/EDC mixture (0.2 M/0.4 M) was added and then was kept for 1 h to activate the carboxylic acid group to N-hydroxysuccinimide ester in poly-ABA. Subsequently, it was washed with PBS buffer (pH=7.4). 8 μL of Ab1 solution (40 ppm in 0.1 M PBS buffer, pH=7.4) was added soon afterwards, then the solution was incubated for 1 h and washed with PBS solution again. To eliminate the nonspecific binding, 10 mM EA (ethanolamine) solution was added and then solution was incubated for 15 min to deactivate the unreacted N-hydroxysuccinimide esters. Subsequently it was washed for three times with PBS solution. The poly-ABA modified electrodes were incubated in 8 μL TRIS buffer solution (pH=7.4, containing different amount of neopteri) for 90 min and then washed with TRIS buffer solution containing 0.005% Tween. Finally, 10 μM aptamer solution (mixture of 5 mM $MgCl_2$, 10 mM TRIS buffer, 100 mM KCl, and 100 mM NaCl) was added and then incubated overnight at 4 °C followed by washing with buffer. The aptamer solution was heated at 80 °C for 5 min, and cooled slowly to room temperature to fold into a proper secondary structure prior to experiments.

The electrochemical properties of MB were performed after treated with different amount of neopterin. After incubation in aptamer solution, the surface of electrode was washed carefully with TRIS buffer. MB accumulated on the aptamers by immersing in a 1 mM MB solution for 30 min. Unbound MB was removed by washing the electrode with buffer for several times. The electrochemical signals were collected by using DPV in PBS buffer (pH 7.4) at room temperature

condition. For the comparison of the results obtained by electrochemistry sensor, serum neopterin concentrations were assayed in the same samples, by an ELISA kit (DRG, Germany) according to the manufacturer's test procedure.

3. RESULT AND DISCUSSION

The cyclic voltammograms were recorded from a bare graphite screen printed electrode. The electro-polymerization was achieved on graphite SPE for 15 cycles in a solution containing 50 mM *o*-ABA, 0.1 M KCl and 1 M H_2SO_4, and the results of first and 15th cycles are shown in Fig. 1. From the cyclic voltammetry curves, it was clearly observed that PABA was formed on the surface of graphite SPEs during the electropolymerization process. This is agree with the previously reports [27, 31]. In addition, CV and EIS techniques were carried out to measure the properties of modified electrode in the presence of $[Fe(CN)_6]^{3-/4-}$ (10 mM in PBS containing 0.1 M NaCl, pH=7.4). The results show that the polymer modified electrode surface formed a biocompatible basis which can help to immobilize Ab1 onto the electrode. In comparison with bare graphite SPE, the PABA modified electrodes possesses significant differences in the impedance spectra [32]. As previously reported the current response obtained by CV obviously increases after modification with polymer film. All the electroconducting polymers used in this work have almost identical CV curves, indicating a good reproducibility of the PABA modified electrodes [33, 34].

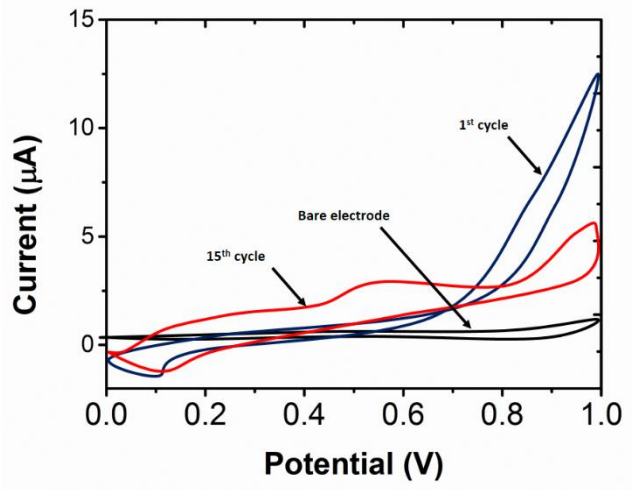

Figure 1. Cyclic voltammograms of bare graphite SPE, the first and 15th cycles of PABA/graphite SPEs (poly-anthranilic acid modified graphite screen-printed electrodes) in a solution containing 0.1 M KCl and 1 M H_2SO_4 with a scan rate of 50 mV/s.

The dilution of neopterin solution as well as the competition time was tuned to obtain the optimum conditions for the competitive experiments. In this work, the concentration of neopterin and the immobilization time were set to be 100 mg/mL and 20 min, respectively. Besides, the dilution ratio for the test was in the range of 1:500 to 1:100000. Figure 2 illustrated the obtained results, which exhibited a typical characteristic of the binding curve. The value of current increased when increasing

the concentration of tracer. Moreover, the current reached to a steady state when the dilution ratio was 1:500 or 1:1000, which indicated that all the target molecules were saturated. Hence, the dilution ratio of 1:1000 was selected to conduct the competition.

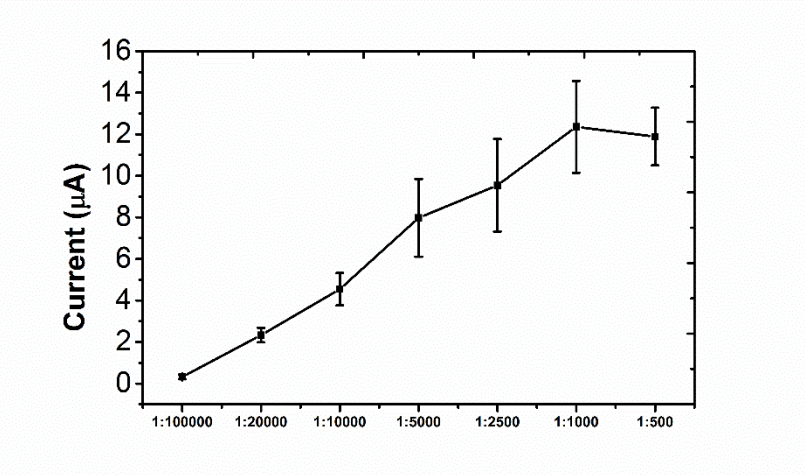

Figure 2. Choice of dilution ratio of neopterin employed for the competitive experiment as well as the immobilization time of the monoclonal antibody solution with a concentration of 100 mg/mL.

Furthermore, the competition time, which was the time needed to complete the reaction, was optimized, where these experiments were carried out via incubation at various times. In comparison, the same experiments were also performed without free neopterin (100% of signal). Figure 3 illustrated the comparison between the signals obtained with and without neopterin. 10 min incubation was selected as the competition time, where the optimum discrimination was obtained between the signals in the absence and presence of neopterin. This incubation period was agree with previous report [35].

The whole capacity of the biosensor based on aptamer was significantly determined by the experimental parameters including blocking agent, concentration and time. Hence, the concentrations of Ab1 and MB, the effect of the blocking agent as well as the incubation time were studied. The signal of the blank sample in sandwich assay in the absence of neopterin was measured to confirm that the effect of the variation of each employed parameter on the selectivity of the electrode was negligible. The choice process performed in this work was to find the maximum difference between the blank and antigen on the modified electrodes.

The concentration of Ab1 exhibited a remarkable influence on the response of biosensor. SPEs based on graphite, which was modified with PABA, were incubated in neopterin antibody (Ab1) of 20-50 ppm. In prior to perform DPV experiments described above, all the immunoassay procedures were conducted. A dramatic increase of the signal ratio of antigen to blank was observed when the concentration of primary antibody was 40 ppm. Hence, Ab1 solution with a concentration of 40 ppm was employed in the further experiments.

The electrochemical detection of MB, which was accumulated on the aptamers, was carried out to measure the concentration of MB, where the modified electrodes were dipped in PBS buffer (0.1 M) with a pH of 7.4 for 30 min in the presence of MB with a concentration of 0.1, 1.0 and 10 mM, respectively. The greatest response was observed when the concentration of MB was 1mM, which was

selected as the optimum concentration for the further experiments. BSA (0.02%) as well as EA (10 mM), which were both immobilized with Ab1, were used to study the effect of the blocking agent. The best results were obtained when the incubation time was 15 min and the concentration of EA was 10 mM. Besides, under the experimental conditions described above, the incubation time of Ab1, aptamer, MB and MUC1 protein were also optimized. For Ab1 and MUCI protein, aptamer and MB, the best results were obtained when the incubation times were 1h, overnight at 4 ^{o}C and 1 h, respectively.

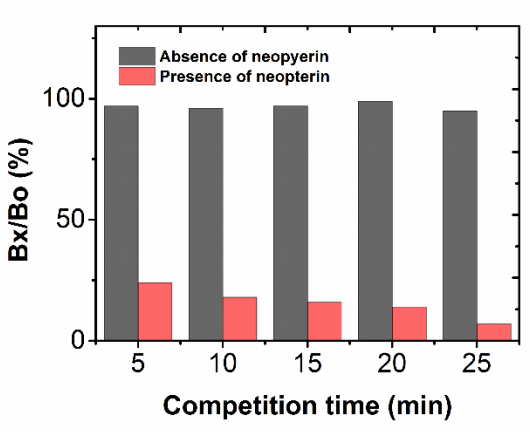

Figure 3. Optimization of the competition time. The points related to the average current ± S.D. calculated based on *n*=4 repetitions.

The relevant cyclic voltammograms as well as the impedance spectra were collected to monitor every immobilization and binding procedure, where $[Fe(CN)_6]^{3-/4-}$ anions were utilized as a redox probe. Figure 4 illustrated the Nyquist plots of the impedance spectra at various electrodes.

As shown in curve a, a small semicircle was observed with the PABA-modified graphite-based SPEs. In curve b, it was obvious that the diameter of semicircle increased when immobilizing Ab1 onto the PABA/SPEs. Moreover, in Figure 4, the Rct of the immunocomplex of the neopterin Ag and its antibody was increased by the attachment between neopterin and Ab1. At last, an insulating layer formed on the surface of the proposed biosensor when fabricating the aptamer-based biosensor with attachi, which slowed or even blocked the transfer of redox couple $[Fe\ (CN)_6]^{3-/4-}$ to the surface of PABA/SPEs. The Rct of the electrode after modification decreased, as the positive surface charge increased when the concentration of protein increased, which enhanced the electrostatic interaction with electroactive marker. These results obtained from EIS are in a good agreement with those obtained from the cyclic voltammograms previously reported [32, 36]. After each immobilization step, the registered current slightly decreases due to the decrease of the electron-transfer capability of the modified electrode. After overnight incubation with aptamer solution, a further decrease of the current and an increase of the peak-to peak separation can be seen in CVs. These results showed that the aptamers are adsorbed onto the electrode surface, although the change of CV signal is small and the sensing interface was successfully achieved.

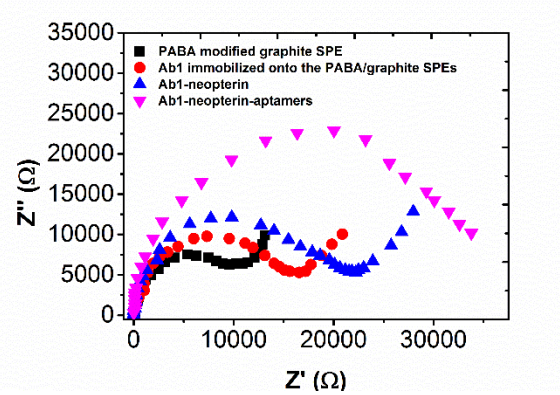

Figure 4. Nyquist plots of the impedance spectra of diverse electrodes including graphite SPE modified with PABA, PABA/graphite SPEs covered with Ab1, Ab1 and neopterin complex formation and aptamers attachment.

The performance of the designed biosensors was quantitatively evaluated under the optimized conditions. CV as well as DPV were utilized to detect the variation of peak current of MB reduction with the concentration of neopterin, where the obtained results were illustrated in Figure 5. The calibration curve was obtained through voltammetry with MB, where the potential ranged from 0.0 to -0.6 V with a scan rate of 50 mV/s. Noticeably, the charge of neopterin and most of its interferences under the measurement conditions (pH=10.0) was similar [37]. In Figure 4A, the accumulation of MB probes onto the surface of the anchored aptamer increased when the concentration of neopterin increased. Hence, the redox peaks of MB became larger. A linear relationship was observed between the concentration of neopterin and the peak current of MB reduction in the range of 2 to 10 ppb, where the limit of detection was 1.7 ppb.

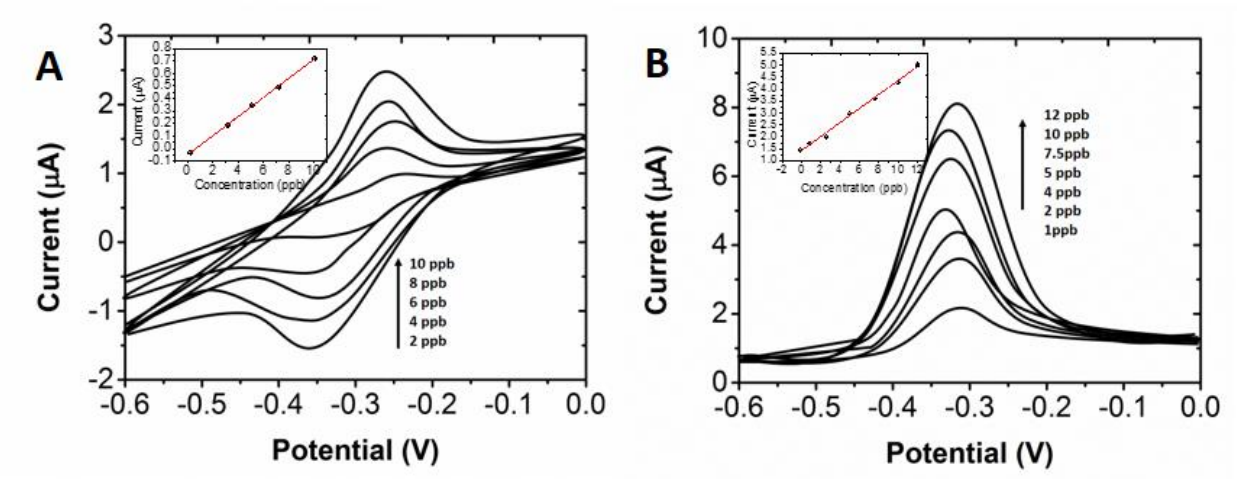

Figure 5. CVs (A) for aptamer in the absence of MUC1 (curve a) and in the presence of neopterin with a concentration of 2, 5, 7 and 10 ppb. (B) DPVs of aptamer in the presence of neopterin with a concentration of 0, 1, 2, 4, 5, 7.5, 10, and 12 ppb at the potential range for reducing MB. Insets illustrated the relationship plots of peak current with the concentration of neopterin.

DPV technique was employ to study the interaction between neopterin and the aptamer. Figure 4B clarified the recorded DPVs of aptamer in the absence of neopterin as well as in the presence of neopterin with a increasing concentration under the potential when the MB reduction could occur. In DPV curves, the maximum was observed when the potential was about -0.28 ± 0.01 V, which was corresponded to the reduction potential of MB. In this work, the neopterin with a concentration of 0, 1, 2.4, 5, 7.5, 10 and 12 ppb was used. It was obvious that the electrochemical of MB was significantly low without neopterin. This peak after removal of background signal was ascribed to the adsorption of MB and protein, which were at graphite-based SPE and immobilized on the electrode, respectively. The well-defined DPV signals were obtained when the concentration of neopterin increased in the range of 1 to 12 ppb. The limit of detection was measured to be 0.44 ppb when the ratio of signal to noise was 3. Compared with CV measurements, a remarkably low limit of detection of neopterin biomarker protein was obtained as expected with the DPV detection. The sensitivity of the proposed sensor was compared with that of other reported neopterin sensors and the results were presented in Table 1.

Table 1. Comparison of the present electrochemical sensor with other neopterin determination methods.

Method	Linear detection range	Detection limit	Reference
HPLC	0.5 to 7 ppb	0.21 ppb	[38]
ELISA	2 to 20 ppb	1.5 ppb	[39]
ELISA	1.5 to 20 ppb	0.5 ppb	[40]
Electrochemical immunoassay	1 to12 ppb	0.44 ppb	This work

The detection of a spiked serum specimen was a suitable approach to evaluate the system under the practical conditions with real sample. However, it was only a simulation. The measurement of the serum specimens collected from patients or healthy people should be taken in account to develop new approaches, although it was challenge. Hence, 10 serum specimens were utilized in the electrochemical immunoassay. A commercial ELISA test was employed as reference to determine the concentration of neopterin in all the specimens. Table 2 illustrated the comparison result of using proposed electrochemical method and commercial ELISA method. It can be seen that the result obtained from electrochemical senor with the clinical samples were remarkably in accordance with those obtained via the ELISA test, indicating that a remarkable correlation took place between the electrochemical assay and the commercial ELISA kit. Overall, the obtained results indicated that the diverse inflammatory diseases including vasculitis were possible to be diagnosed.

Table 2. Determination of neopterin in clinical serum samples.

Sample	Immunosensor (ppb)	ELISA (ppb)	RSD (%)
1	1.57	1.55	3.87
2	3.02	2.93	4.32
3	4.89	5.01	3.56
4	7.07	7.12	2.29
5	9.88	9.92	4.00

4. CONCLUSION

In conclusion, an electrochemical biosensor with high sensitivity towards the detection of protein was developed through employing neopterin and their corresponding antibody and aptamer. The biosensor based on aptamer was prepared through electro-polymerizing o-aminobenzoic acid (*o*-ABA) conductive polymer on the graphite-based SPEs surface. Subsequently, the MUC1 monoclonal mouse antibody was anchored on the surface of electrode via the covalent bond between the amino groups of antibody and the carboxyl groups of poly(*o*-ABA). After immobilizing aptamers, the immunoreaction between neopterin and antibody was performed, where MB was employed as the electrochemical indicator. As we know, MB would be reduced to a leucomethylene blue when accepting two electrons at the surface of electrode. In comparison with the commercial ELISA kit, the obtained results with a remarkable correlation indicated that the proposed biosensor was possible to differentiate ill or healthy subjects in the serum samples.

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