Analytica Chimica Acta xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Analytica Chimica Acta

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

A new method to assay hypoxia-inducible factor-1 based on small molecule binding DNA

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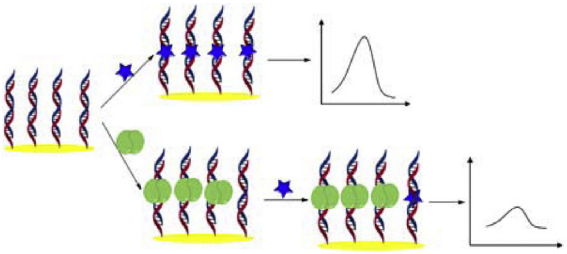
HIGHLIGHTS

- A novel electrochemical method has been established for hypoxia-inducible factor-1.
- The sensitivity of the method surpasses that of current immunoassays.
- The assay of placenta sample can be used to evaluate the severity of preeclampsia.

GRAPHICAL ABSTRACT

A novel method for HIF-1 detection is proposed by using electrochemical techniques based on small molecule binding DNA. The proposed method can be directly used to assay HIF-1 in placenta tissue, and the assay results can reliably reflect the severity of preeclampsia, a very dangerous condition during pregnancy.



ARTICLE INFO

Article history:
Received 4 September 2013
Received in revised form 25 April 2014
Accepted 22 May 2014
Available online xxx

Keywords:
Hypoxia-inducible factor-1
Echinomycin
Biosensor
Electroanalysis
Preeclampsia

ABSTRACT

Hypoxia-inducible factor-1 (HIF-1) is among the most important indicators of hypoxia in evaluating severity of many diseases. In this work, a novel method for HIF-1 detection is proposed by using electrochemical techniques based on small molecule binding DNA. In this method, since the designed DNA sequence can specifically bind with either an electroactive small molecule or HIF-1, the signal readout is inversely proportional to HIF-1 concentration, thus a simple and easily-operated method for HIF-1 detection can be developed. With the proposed method, HIF-1 can be determined in a linear range from 5 to 25 nM with a detection limit of 2.8 nM. Furthermore, the proposed method can be directly used to assay HIF-1 in placenta tissue, and the assay results can reliably reflect the severity of preeclampsia, a very dangerous condition during pregnancy. The proposed method also shows desirable sensitivity, high selectivity and excellent reproducibility, so this method can have potential applications in clinical practice.

1. Introduction

Hypoxia is a common condition found in a wide range of physiological and pathological states. Hypoxia promotes various biologically significant processes, such as cell glycolysis, angiogenesis, cell invasion, metastasis, by inducing hypoxia-inducible factor-1 (HIF-1) expression [1]. So, HIF-1 is the most important indicator identified to date of the cellular response to hypoxia [2,3]. Elevated HIF-1 level is frequently correlated with metastasis and

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http://dx.doi.org/10.1016/j.aca.2014.05.045
0003-2670/

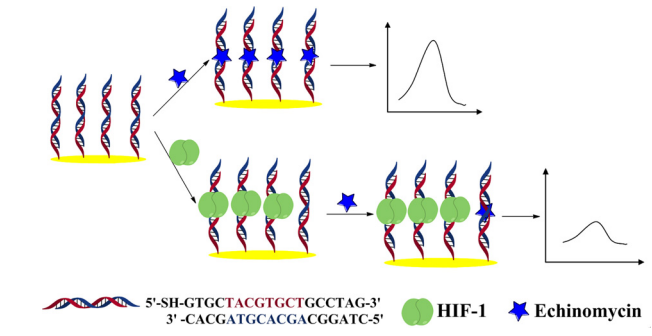
poor clinical outcomes in cancers of various origins [4,5]. HIF-1 overexpression can also imply the severity of hypoxia in other diseases such as preeclampsia. As a consequence, the assay of HIF-1 protein is critical for clinical prognosis and the monitoring of the curative effect of drug intervention [6].

In the present, only western blotting and ELISA have been proposed to assay HIF-1. Therefore, more methods should be developed on the one hand; on the other, the need of antibodies in the assays of western blotting and ELISA have posed limitations in their applications, because the highly complicated chemical structure of antibodies may greatly obstruct the use of novel sensing strategies.

In recent years, some low molecular weight equivalents to antibodies, such as structured DNA, peptides and small molecule drugs have been reported [7,8]. These molecules have far more simplified chemical structures, so they can be equipped with other function modules and can be orderly immobilized in the sensing layer without the loss of protein-binding affinity. Therefore, the development of chemically uncomplicated HIF-1 sensing reagent that enables highly sensitive, specific, and cost-efficient assays has aroused great interest in analytical chemistry as well as biomedical science.

In this work, sequence specific DNA is designed for both HIF-1 recognition and signal readout, so a simple and easily operated method with high sensitivity and selectivity is developed for HIF-1 assays. It has been known that some DNA sequences may have the characteristics of particular protein-binding motifs, such as the naturally occurring binding sites of transcriptional factors, and the artificially fabricated protein-binding surfaces on aptamers [9,10]. It has also been known that some DNA sequences can specifically bind with small molecules, such as organic metal complexes and various drug compounds via special sequence and conformation in the major groove. So, we propose that if the small molecules can be exploited as signal reporter, i.e., if a DNA sequence is designed to be the binding sites of both the target protein and a small molecule, then the readout signal from the small molecule will be inversely proportional to the concentration of the target protein, protein detection will be more easily and favorably performed. In this context, a DNA sequence featuring the hypoxia response element (HRE) is designed for the assay of HIF-1, based on the design that HRE will bind with either HIF-1 [11,12], or an electroactive small molecule, named Echinomycin (Echi) [13–16]. So, a new method for HIF-1 assay is established via the electrochemical signal readout of Echi inversely proportional to the amount of HIF-1. Meanwhile, since no bio-conjugation is required for the attachment of the electrochemical reporter Echi, performance of the HIF-1 assay is greatly simplified.

To demonstrate the feasibility of the proposed method, HIF-1 expression in placenta has also been determined to evaluate hypoxia during preeclampsia (PE). PE is a gestational idiopathic disease that complicates 5–8% of all pregnancies, resulting in



Scheme 1. Principle of the new method for HIF-1 assay. Not drawn to scale.

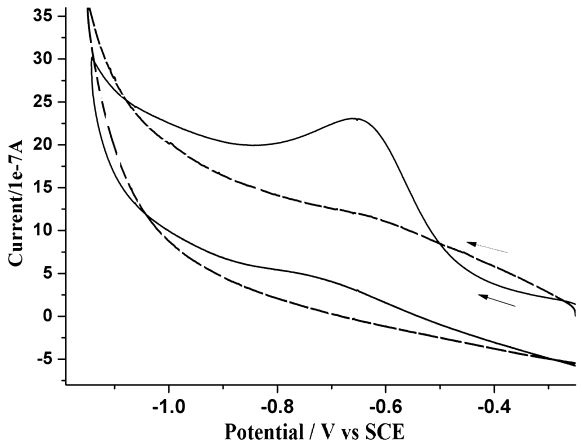


Fig. 1. Cyclic voltammograms obtained at the probe DNA (solid line) and the scrambled sequence (dotted line) modified electrode after incubation with 100 nM Echi at 37 °C for 1.5 h. Arrows mark the scan direction. Scan rate: 0.1 V/s.

significant prenatal morbidity and mortality [17]. Placental hypoxia is the principal clinical features of PE [18]. Our method enables efficient analysis of HIF-1 expression as a reliable indicator of the severity of PE.

2. Experimental

2.1. Chemicals and materials

Recombinant mammalian HIF-1α and HIF-1β were obtained from Abnova (America). Echi was ordered from Alexis (Switzerland). 6-mercapto-1-hexanol (MCH), tris(2-carboxyethy) phosphine hydrochloride (TCEP), bovine serum albumin (BSA), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-KB) from human plasma were purchased from Sigma–Aldrich. Nuclear extract kit was purchased from Active Motif. Ammonium formate was purchased from JK Scientific Ltd. (Beijing, China). Thiol-modified probe DNA (5′-GTGCTACGTGCTGCCTAG-SH-3′) and its complementary sequence (5′-CTAGGCAGCACGTAGCAC-3′), thiol-modified DNA with scrambled sequence (5′-GTGCTATC-TATCGCCTAG-SH-3′) and its complementary sequence (5′-CTAGGCGATAGATAGCAC-3′) (PAGE-purified) were synthesized from Takara Biotechnology Co., Ltd. (Dalian, China). The underlined sequence is the binding site of HIF-1 and Echi. The probe DNA and

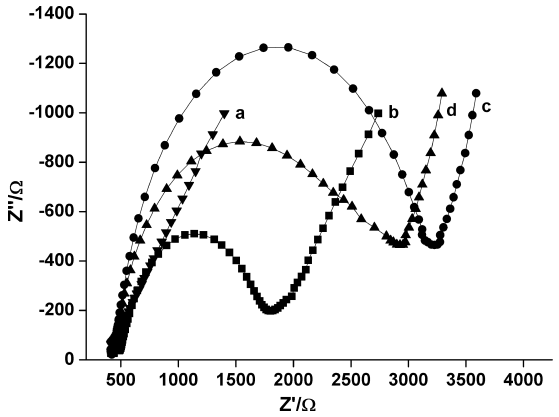


Fig. 2. Nyquist diagrams corresponding to (a) the bare gold electrode, (b) the probe DNA modified electrode, (c) the probe DNA modified electrode after being treated with 5 nM HIF-1, and (d) the probe DNA modified electrode after being treated firstly with 5 nM HIF-1, and then with 100 nM Echi. Electrochemical species: 5 mM $[Fe(CN)_6]^{3-/4-}$. Biasing potential: 0.224 V. Amplitude: 5 mV. Frequency range: 0.1 Hz–10 kHz.

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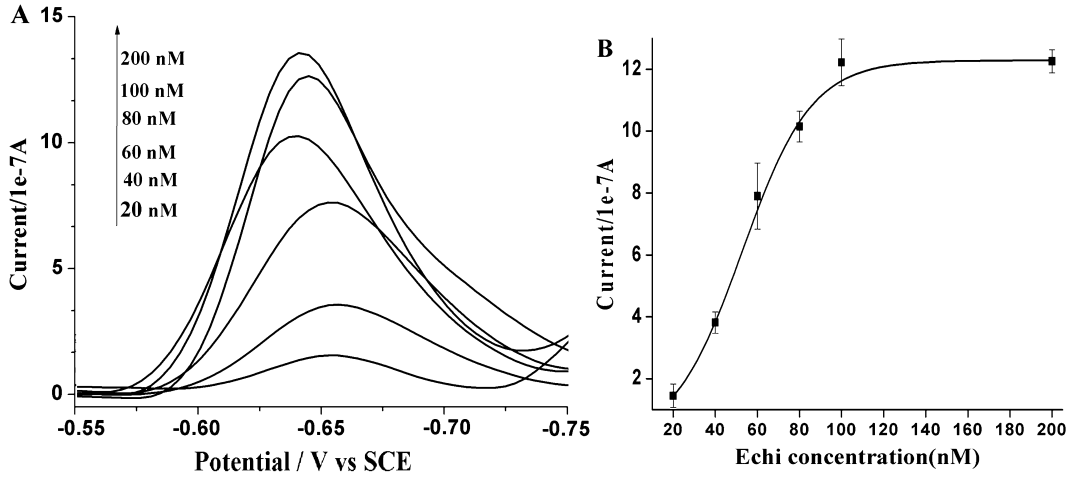

Fig. 3. (A) Differential pulse voltammograms obtained at the probe DNA modified electrode, which has been treated first with 5 nM HIF-1 and then with different concentration of Echi. (B) Relationship between the obtained peak current and the concentration of Echi. Error bars represent standard deviations of measurements ($n = 3$).

its complementary sequence, the scrambled DNA and its complementary sequence were heated at 95 °C for 5 min and annealed by slow cooling to room temperature to form duplex DNAs. Then they were modified onto the surface of the working electrode. Other reagents were of analytical reagent grade and used as received. Double distilled water that was purified with a Milli-Q purification system (Branstead, USA) was used for all solutions.

2.2. Preparation of the modified gold electrode

Pretreatment of the substrate gold electrode was described in detail in our previous paper [19]. Briefly, a gold electrode (3 mm diameter) was polished with alumina powder and sonicated in ethanol and water. After sonication, the electrode was immersed in piranha solution (Caution: Piranha solution reacts violently with organic solvents and should be handled with great care!) for 5 min. Finally, the electrode was electrochemically activated in 0.5 M H_2SO_4.

The above clean gold electrode was incubated with 0.2 μM thiol-terminated probe DNAs in DNA stock buffer solution (10 mM Tris–HCl buffer, pH 7.4, 10 mM TCEP, 0.1 M EDTA) at room temperature for 16 h, so dithiol group of the probe DNA could form Au—S covalent bond to form a self-assembled monolayer of DNAs on the electrode surface. Afterward, the electrode was reacted with 1 mM MCH for 1 h to block the nonspecific binding sites on the electrode surface, followed by being rinsed with double-distilled water.

2.3. Investigation of the binding activity of HIF-1 and DNA

HIF-1α was first incubated with HIF-1β in Tris buffer solution (20 mM Tris–HCl, 100 mM KCl, 50 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA) at 37 °C for 30 min to allow them to form heterodimer. Then, the above prepared electrode was treated with the heterodimers at 37 °C for 2 h, followed by thorough wash with washing buffer (20 mM Tris–HCl, 0.1 M NaCl, 5 mM MgCl, 1.0% Tween 20). After that, the electrode was kept in 100 nM Echi solution (20 mM Tris–HCl, 100 mM KCl, 50 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA) at 37 °C for 1.5 h. After subsequent repeated rinse with double distilled water, the electrode was ready to use.

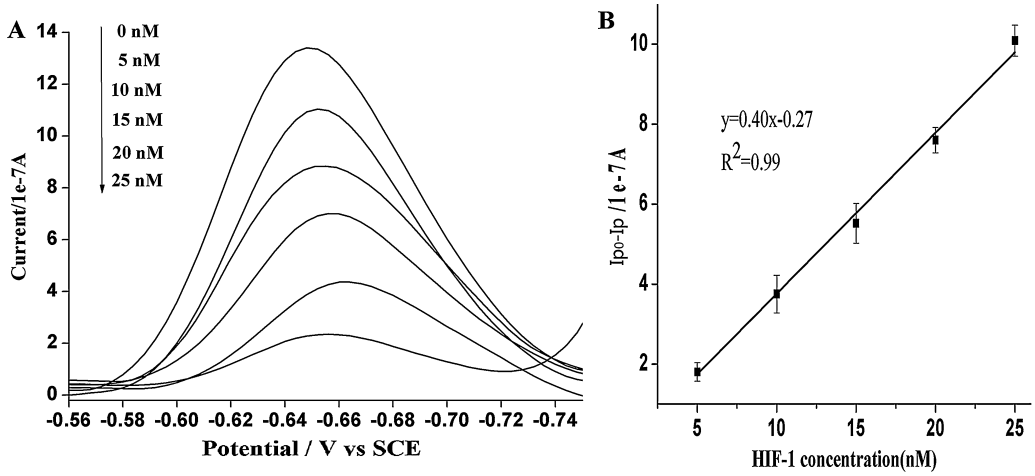

Fig. 4. (A) Differential pulse voltammograms obtained at the DNA probe modified electrode for the measurements of HIF-1 at different concentrations. (B) Linear relationship between ΔI_p and HIF-1 concentration. ΔI_p is the difference between the peak current obtained at 0 nM HIF-1 and those obtained at other HIF-1 concentrations. Error bars represent standard deviations of measurements ($n = 3$).

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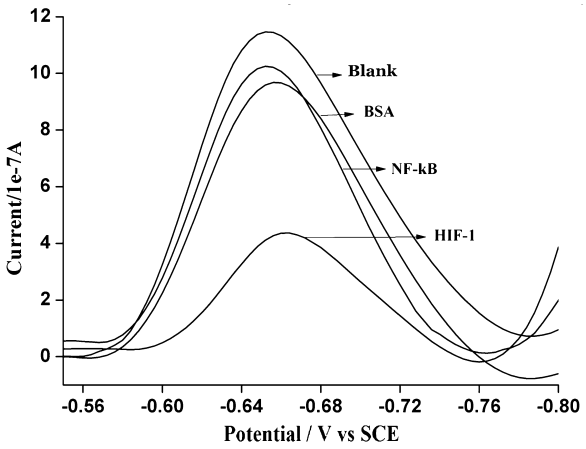

Fig. 5. Differential pulse voltammograms obtained at the probe DNA modified electrode upon analyzing different kinds of species. All targets are at 20 nM. Tris buffer solution is used as the blank control. Others same as in Fig. 3.

2.4. Preparation of placenta lysis

Human normotensive and preeclamptic placentas were obtained from the First Affiliated Hospital of Nanjing Medical University after elective cesarean, which were approved by the local hospital ethical committees. The nuclear fraction of placental tissue was extracted using nuclear extract kit, according to the manufacturers instructions. Freshly sampled placental tissue was diced into very small pieces and sonicated in ice-cold hypotonic buffer supplemented with 100 mM DTT (dithiothreitol) and the provided detergent on ice for 5 min. After incubation on ice for 15 min, the resultant suspensions were centrifuged at 850 × *g* for 10 min at 4 °C and the supernatant was discarded. Resuspend nuclear pellet in 50 μL complete lysis buffer containing 1 mM DTT and 0.1% protease inhibitor cocktail was vortexed for 10 s at highest setting, incubate for 30 min on ice on a rocking platform. After that, the suspensions were centrifuged at 14,000 × *g* for 10 min at 4 °C and the supernatants were collected. The extracted nuclear fraction was immediately brought to HIF-1 assay.

2.5. Electrochemical measurements

Electrochemical measurements were performed on 660D Electrochemical Analyzers (CH Instruments) with a conventional three-electrode cell at room temperature. The three electrode system was composed of a saturated calomel electrode (SCE) as the reference electrode, a platinum wire as the counter electrode, and the modified gold electrode as the working electrode. 300 mM ammonium formate (pH 6.9, 50 mM PBS) was used as the electrolyte solution for differential pulse voltammetry (DPV) experiments, while 5 mM $Fe(CN)_6^{3-/4-}$ with 1 M KNO_3 was used to obtain electrochemical impedance spectra (EIS). Before measurements, the electrolyte solution should be thoroughly purged with high purity nitrogen for about 30 min to avoid the interference from the reduction of oxygen. The data were obtained from at least three times of repetition of independent experiment.

3. Results and discussion

3.1. Validation of sensing mechanism

The principle of the new method for HIF-1 assay has been illustrated in Scheme 1. Firstly, the DNA probes are immobilized on the gold electrode surface through the Au—S bond to form a self-assembly monolayer. In the absence of HIF-1, Echi can completely bind with all the probes on the electrode surface, resulting in large electrochemical signal. However, when HIF-1 firstly binds with a fraction of the probes, only the remainder of the probes can bind with Echi, so the obtained electrochemical signal will be small. Furthermore, since higher concentration of HIF-1 will result in more surface-immobilized probes occupied by HIF-1, smaller electrochemical signals will be obtained. So, the readout signals are in reverse proportion to HIF-1 concentration, which forms the basis of the proposed new method to assay HIF-1.

Fig. 1 depicts the cyclic voltammograms of Echi bound with the probe DNA (solid line) and with the scrambled sequence (dotted line). As shown in Fig. 1, a peak at around −0.65 V can be observed by using the probe DNA, which contains the binding site of HIF-1, and the electrochemical response showing the redox peak of quinoxaline group of Echi is the same as the previous report [13]. However, in the absence of the binding site, as in the scrambled

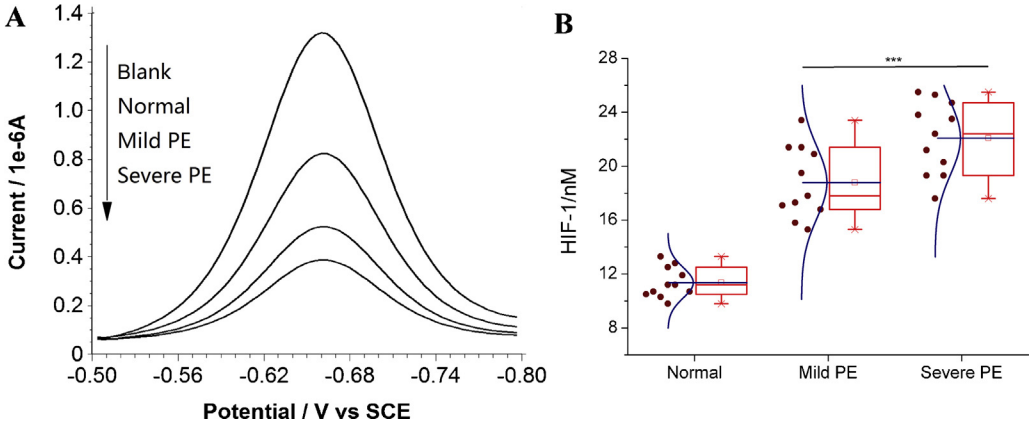

Fig. 6. (A) Differential pulse voltammograms obtained at the probe DNA modified electrode for the analysis of HIF-1 from the tissue lysis of placenta samples with normal pregnancy, mild PE and severe PE. (B) HIF-1 expression pattern of placenta samples.

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sequence, no peak is observed (Fig. 1). These experimental results confirm that Echi can bind with the probe DNA at the binding site which is also for the target protein binding in a sequence-specific fashion and give detectable electrochemical response.

In order to further validate the proposed method described in Scheme 1, EIS are recorded to manifest the stepwise modification and treatment of the electrode. As shown in Fig. 2(a), EIS of the bare electrode is similar to a straight line. Nevertheless, after the immobilization of the probe DNA, a semicircle can be observed (Fig. 2, curve b), indicating an increase in the interfacial electron transfer resistance, because the negatively charged DNA self-assembled on the electrode surface will electrostatically repel the also negative electroactive species $Fe(CN)_6^{3-/4-}$. Furthermore, when the electrode is further treated with HIF-1 protein, the diameter of the semicircle dramatically increases (Fig. 2, curve c), implying the HIF-1 binding with the probe DNA. Nevertheless, when the electrode is subsequently treated with Echi, the resistance evidently decreases (Fig. 2, curve d), due to the reason that the positively charged Echi may neutralize some negative charge of the probes, making the surface less repelling to $Fe(CN)_6^{3-/4-}$.

3.2. Optimization of experimental conditions

The concentration of Echi has been optimized in the presence of HIF-1 protein. As shown in Fig. 3(A), the peak current is positively related to Echi concentration, and reaches a plateau at 100 nM for 5 nM HIF-1 protein, indicating that the remainder of the probes after binding with HIF-1 protein have been completely bound with Echi. Therefore, 100 nM has been used in the subsequent experiments. The optimal temperature and pH for the electrode to bind with HIF-1 have also been investigated. As shown in Fig. S1 and Fig. S2, the interaction taken place at 37 °C in the buffer with a pH of 7.5 is the best, so these conditions are adopted in the following experiments. Moreover, the surface density of the DNA probe has also been optimized by varying the concentration of DNA employed for surface modification of the working electrode. As shown in Fig. S3, an intermediate concentration, namely 0.2 μM, can provide sufficiently high signal readout with no apparent steric hindrance for HIF-1/DNA interaction. Therefore, this concentration is adopted to modify the working electrode for HIF-1 detection.

3.3. Biosensing performance

Fig. 4(A) displays the differential pulse voltammograms obtained upon analyzing HIF-1 at different concentrations. It can be observed that the peak currents decrease along with the concentration of HIF-1. A linear response between ΔI_p and HIF-1 concentration can be obtained in the range of 5–25 nM (Fig. 4(B)). For each concentration of HIF-1, the electrochemical measurements have been repeated for at least three times independently. The average coefficient of variation is 3.8% and the detection limit has been calculated as 2.8 nM.

Fig. 5 depicts the DPV response obtained for a series of control experiments. As shown in Fig. 5, a well-defined DPV peak at around −0.65 V can be observed due to the oxidation of Echi; however, an apparently decreased signal can be observed due to the binding of HIF-1 with some of the DNA probes. Nevertheless, for the case to assay some other nonspecific proteins instead of HIF-1, decrease of the DPV peak is far less than that for the assay of HIF-1 since the DNA probes cannot bind to the other nonspecific proteins. So, these experimental results confirm that HIF-1 can bind with the DNA probe in a sequence-specific fashion, and the selectivity of our method is satisfactory.

In order to examine the feasibility of our new method for clinical applications, several placental tissue samples have been used for this study. Placental tissue samples from normal pregnancy ($n = 6$), mild PE ($n = 6$), severe PE ($n = 6$), are separately collected and fractionated for HIF-1 assays. As shown in Fig. 6(A), a high peak appears in the normal pregnancy, indicating that the expression of HIF-1 in normal pregnancy is in a small quantity, since the normal pregnant placenta is under physiological hypoxia status. However, compared with normal placentas, the peaks apparently decrease for the placentas with mild PE and severe PE, especially in severe PE, suggesting that overexpression of HIF-1 in PE placentas shows severe hypoxia. Moreover, the HIF-1 level may also reflect the severity of PE. Fig. 6(B) shows the comparison of the expression level of HIF-1 in normal pregnancy, mild PE and severe PE, which can clearly show the difference. Therefore, this new method proposed in this work can not only be of great value for the detection of HIF-1 protein in clinical applications, but our method can also evidently show the expression situation during normal pregnancy and pathological pregnancy. And in future, we will further employ this new method to determine the concentration of HIF-1 in peripheral blood because early detection is necessary to avoid the severity of preeclampsia during pregnancy.

4. Conclusion

In this paper, we report a novel method by using electrochemical technique for HIF-1 protein assay based on small molecule binding DNA. The DNA sequence contains the HRE which will bind with either the target protein HIF-1, or an electroactive small molecule Echi. Therefore, the electrochemical signals are obtained in reverse proportion to HIF-1 concentration, thus a new method for HIF-1 assay is developed with electrochemical technique. Experimental results have shown that the detection range is from 5 to 25 nM, and the detection limit is 2.8 nM, so the assay method is more sensitive than the current available methods. Moreover, the proposed method has also been used to determine the expression level of HIF-1 in placenta tissue. The assay results can reliably reflect the severity of preeclampsia. Therefore, the proposed method might have great potential applications in clinical prognosis and monitoring the curative effect of drug intervention in the future.

Acknowledgments

This work is supported by the National Science Fund for Distinguished Young Scholars (Grant No. 20925520), the National Natural Science Foundation of China (Grant Nos. 81270710, 21235003), and the Shanghai Science and Technology Committee (Grant No. 11DZ2272100).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2014.05.045.

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