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Aptamer-based Biosensor for Detection of Phenylalanine at Physiological pH

Eskandar Omidinia · Nasrin Shadjou · Mohammad Hasanzadeh

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Abstract A simple, sensitive aptamer-based biosensor for the detection of phenylalanine is developed using the electrochemical transduction method. For this proposed aptasensor, a 5-thiol-terminated aptamer is covalently attached onto a gold electrode. At the first time, the electrode was evaluated as an electrochemical aptasensor for determination of phenylalanine in aqueous solutions. This sensor was tested in a Tris–HCl buffer with physiological pH=7.4 by cyclic voltammetry and differential pulse voltammetry. The detection limit and sensitivity of the modified electrode toward phenylalanine were estimated to be 1 nM (S/N=3) and 0.367 μA nM^{-1}, respectively. The linear range of the signal was observed between 1 and 10 nM of phenylalanine with 0.9914 correlation factor. The herein-described approach is expected to promote the exploitation of aptamer-based biosensors for protein assays in biochemical and biomedical studies.

Keywords Aptamer · Electrochemical biosensor · Phenylalanine · Covalently attachment

Introduction

The oxidation and adsorption behaviors of amino acids on electrode surfaces are relevant to the interfacial behaviors of these biological molecules and also to the medical and industrial problems associated with the adsorption of the amino acids on the surfaces [1–4]. One of important amino acids is phenylalanine (Pha). Capillary electrophoresis, chromatography, mass spectrometry, ion exchange column chromatography, spectrophotometry and fluorometry are the most widely used methods for detection of Pha [5–7]. These techniques may not be well-suited for routine clinical screening of Pha, as they are often time consuming and require some tedious preparation and expensive equipment.

E. Omidinia (✉) · N. Shadjou
Biochemistry Department, Genetic and Metabolism Group, Pasteur Institute of Iran, P.O. Box 13164, Tehran, Iran
e-mail: skandar@pasteur.ac.ir

M. Hasanzadeh (✉)
Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz 51664, Iran
e-mail: mhmmd_hasanzadeh@yahoo.com

Since the pioneering studies on aptamers [8], substantial research efforts have been directed in order to apply the aptamers as the bioreceptor in different detection schemes such as micro gravimetric [9], and electrochemical aptasensors [10–12]. Aptamers are artificial nucleic acid ligands showing specific binding affinity for amino acids, drugs, proteins and other molecules, which can be screened through the systematic evolution of ligands by exponential enrichment (SELEX) process from random RNA or DNA libraries [8]. Compared with antibodies, aptamers possess some outstanding features, including high specificity of binding affinity, nice stabilization, easy synthesis and modification with electrochemical active markers, optical dyes, enzymes and other desired substances. Furthermore, aptamers can reversibly capture and release their target molecule. It is more facile for the aptamer to transduce the recognition events in to detectable signals [13].

The fabrication of aptamer-based electrochemical biosensors as an emerging technology has made the detection of small and macromolecular analytes easier, faster, and more suited for the ongoing transition from fundamental analytical science to the early detection of protein biomarkers. In the fabrication of aptamer-based electrochemical biosensors, one of the most popularly used electrode materials is gold, upon which thiolated DNA/RNA strands can be immobilized via strong Au–S linkages [14, 15]. The modification of DNA or RNA with a terminal thiol is straightforward: simple phosphoramidite chemistry on the DNA/RNA synthesizer suffices. The modification of Au surfaces with the thiolated oligonucleotide strands is readily accomplished by dipping a cleaned Au slide into the deposition solution to form an aptamer layer. Although the initial adsorption of thiolated DNA strands onto gold surfaces occurs within minutes, it is necessary to allow the reaction to continue for up to 24 h to ensure a homogenous surface. The resulting surface is usually subjected to a passivation step (i.e., treated with 1-mercapto-6-hexanol solution) to minimize non-specifically bound oligonucleotide strands.

In the present study, at continuation of our studies on detection of lower concentration of amino acids using electrochemical sensors [5–7, 21–23] we investigated a biosensor based on aptamer immobilized on the surface of gold electrode for simple, sensitive, rapid and cost-effective determination of Phe. To the best our knowledge, this device is the first selective aptasensor for Phe detection which has not been previously reported. This report is the first example of Phe aptamer-based electrochemical sensor.

Experimental

Reagent and Chemicals

5′ Thiolated L-Pha aptamer modification was custom-synthesized by Takapu Zist Co. (Iran) and its base sequence is as follows: 5-SH-GCGCGAGAAACGGUCACUAGAAUAGUGGGC CGUCAUGCUAACGCCUCUUUCGGUGUUGGGGGAAUAUUGGCCAAUCGAGU-3.

2-Mercaptoethanol was purchased from Sigma Aldrich (USA). All chemicals used in this work were of analytical reagent grade, from Merck (Germany). All solutions were prepared with bidistilled water.

Apparatus

Electrochemical measurements were carried out in a conventional three-electrode cell (from Metrohm) powered by an electrochemical system comprising of AUTOLAB system with PGSTAT 302N (Eco Chemie, Utrecht, The Netherlands). The system was run on a PC using

NOVA 1.7 software. The ac voltage amplitude used was 10 mV and the equilibrium time was 5 s. An Ag/AgCl-sat'd KCl (from Metrohm) and a platinum wire were used as reference and counter electrodes, respectively. The working electrode was a gold (Au) electrode (from Azar Electrode Co., Urumia, Iran). For differential pulse voltammetry (DPV) measurements, a pulse width of 25 mV, a pulse time of 50 ms, and a scan rate of 100 mV s^{-1} were employed. All the electroanalytical tests were carried out at 25 °C in 10 mM Tris–HCl buffer (pH=7.4) containing 5.0 mM $[Ru(NH_3)_6]^{3+}$.

Preparation of Aptasensor

The gold electrode was polished with 1.0, 0.3, 0.05 μm alumina powders, respectively, and rinsed with bidistilled water and ethanol. The electrode was then sonicated in bidistilled water for 5 min to remove bound particles, then rinsed thoroughly with bidistilled water and dried in nitrogen stream. Primary aptamer (1 μM) was diluted in 50 mM Tris–HCl buffer (140 mM NaCl, 1 mM $MgCl_2$, pH 7.4). The Au electrode was incubated with primary aptamer overnight at room temperature. Then, the blocking reagent mercapthoethanol was added to the surface of the gold electrode to reduce non-specific adsorption for 1 h.

Characterization of Electrode Surface Modification

Cyclic voltammetry (CV) has been proven as one of the common tool for probing the features of surface modified electrodes [16, 17]. In this work, CV was carried out to study the modification of the electrode surface. CV curves obtained in 10 mM Tris–HCl containing 5 mM $K_3[Fe(CN)_6]$, 5 mM $K_4[Fe(CN)_6]$, 100 mM KCl. As shown in Fig. 1b, a couple of well-defined redox peaks could be observed at the modified electrode. In the presence of Pha, it was observed that the anodic peak current decreased drastically. This implies that most of the electrode surface is covered by aptamer, with the result of blocking the electron-transfer efficiency of $[Fe(CN)_6]^{3-/4-}$ at interface due to this fact that the electrostatic repulsion between anionic $[Fe(CN)_6]^{3-/4-}$ and the surface bound aptamer with negative charges retards the interfacial electron-transfer kinetics. This observation demonstrates that the covalent immobilization of aptamer has been achieved successfully. The results indicate that a sensing interface is effectively constructed and successfully applied for the detection of Pha.

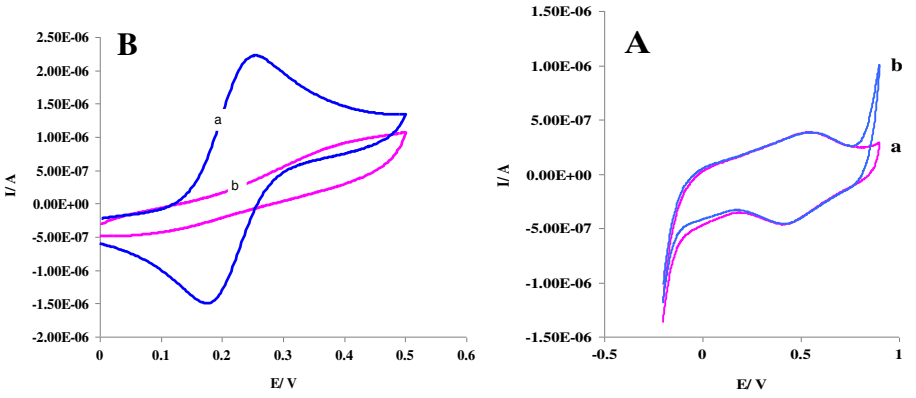


Fig. 1 **a**, **b** CVs of bare Au electrode in 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) with 100 mM KCl in the absence (*a*), and presence of Pha (*b*). **b** CVs of aptamer/Au electrode in 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) with 100 mM KCl in the absence (*a*), and presence of Pha (*b*)

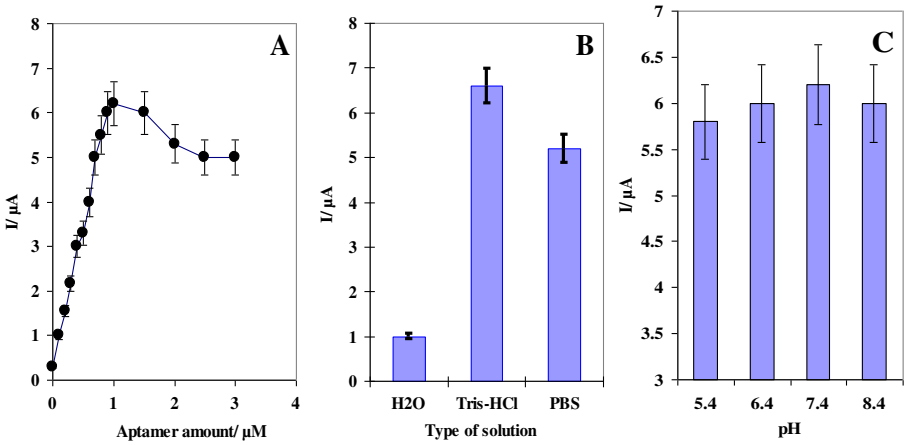


Fig. 2 **a** Effect of aptamer amount on the peak current. **b** Effect of type of buffer solution. **c** Effect of pH on the peak current (SD=3.0 %, n=3)

Optimization of Fabrication Conditions

Effect of Modifier (Aptamer) Amount on Sensing Activity

As shown in Fig. 2a, the first step of aptamer modification had a saturation value for the amount of aptamer on the surface of the gold electrode. The peak current increased with increasing amounts of aptamer (0–3 μM), from nearly zero to 5 μA. Higher concentration of aptamer showed a decrease in peak current. This is presumably due to reduction of conductivity of the aptasensor due to saturation of surface. Consequently, 1 μM of aptamer was chosen as the optimal concentration.

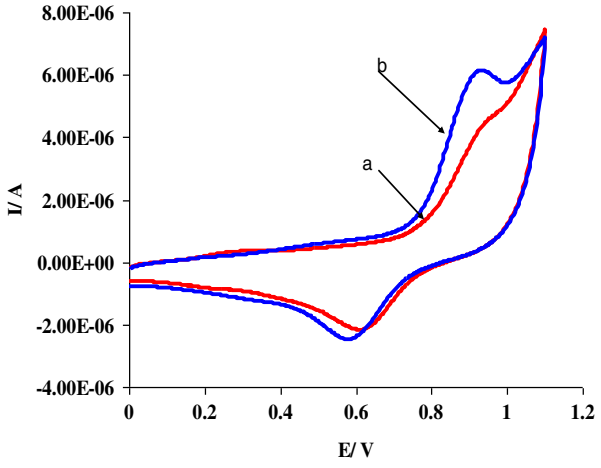


Fig. 3 CVs of 10 mM Tris–HCl buffer (pH=7.4) containing 5.0 mM $[Ru(NH_3)_6]^{3+}$ using aptamer/Au electrode in the absence (curve *a*) and presence (curve *b*) of 10 nM Pha. Potential sweep rate was 10 mVs^{-1}

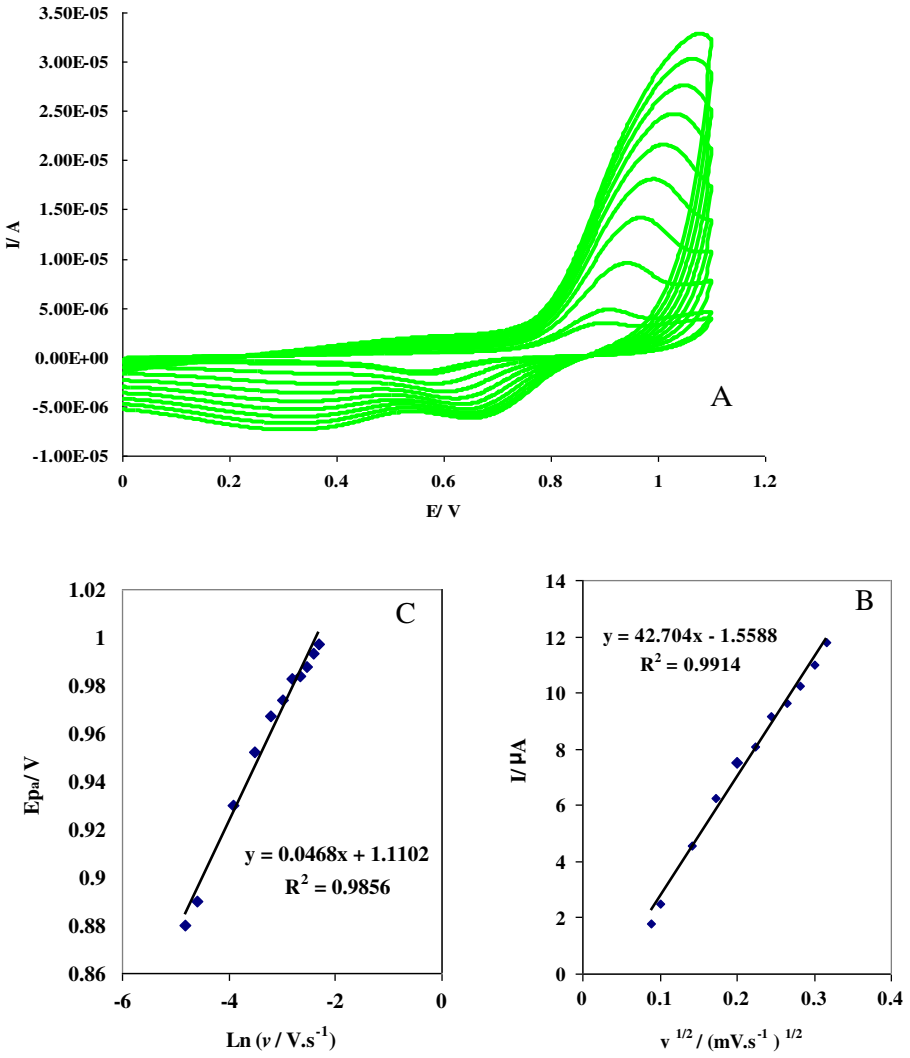


Fig. 4 **a** CVs of aptamer/Au electrode in 10 mM Tris–HCl buffer (pH=7.4) containing 5.0 mM $[Ru(NH_3)_6]^{3+}$ at different scan rates (from inner to outer): 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 mVs^{-1} respectively. **b** Peak current vs. square root of scan rate. **c** Peak potential vs. logarithm of scan rate (SD=3.0 %, n=3)

Effect of Buffer Solution

Selection of solution in fabrication procedures depends is the most important point in the electrochemical detections. Type of solution helps to increases the contact electrode surface with the sample solution. In order to study the type of disperser solution on fabrication, three solutions, including phosphate-buffered saline (PBS), Tris–HCl and H_2O were used in the detection system. PBS and Tris–HCl were better than pure H_2O as a support medium for sensing the target molecule, and Tris–HCl had a more effective signal transport capability, and was therefore adopted as the detection medium

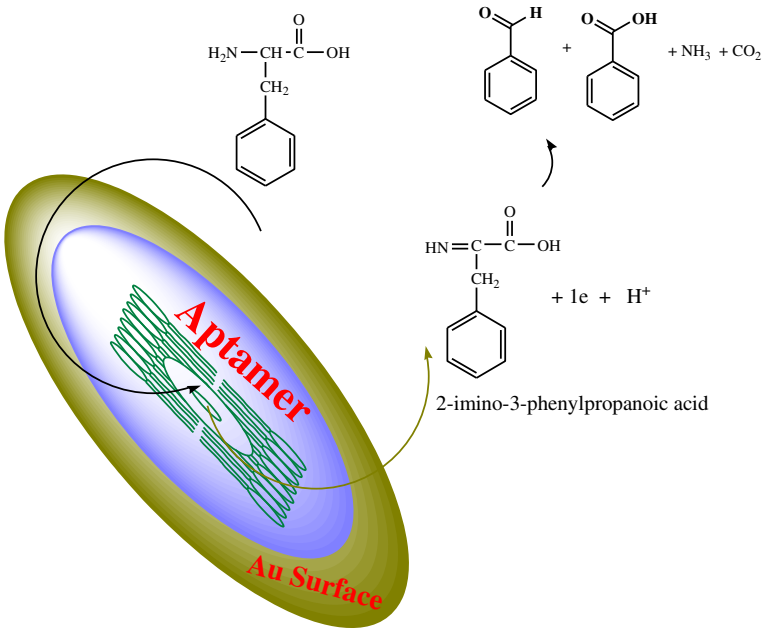
Fig. 2b. The possible reason for this is that Tris–HCl be more suitable for the detection of Pha than that of PBS.

Effect of pH

Another important factor that could influence the final sensing of the target molecules was pH. Based on physiological conditions, pH values from 5.4 to 8.4 were tested with no substantial differences in sensing peak currents under different pH conditions. Therefore, pH 7.4 was adopted for sensing Fig. 2c.

Results and Discussion

The cyclic voltammetric behavior of the sensing interface in the absence and presence of Pha were investigated, and the results were shown in Fig. 3. The cyclic voltammogram of the aptasensor in 10 mM Tris–HCl solutions was shown as curve a. A pair of well-defined peaks with a mid-peak potential of 0.6 and 0.87 V appears in the voltammograms, and the peak-to-peak potential separation (at the potential sweep rate of 10 mV s^{-1}) is 0.28 V. The peak-to-peak potential separation deviates from the theoretical value of zero and increases at higher potential sweep rates. This result indicates a limitation in the charge-transfer kinetics, which is due to chemical interactions between the electrolyte ions and aptamer [1, 18]. In the presence of the Pha, it was observed that the anodic current and the associated anodic charge increased drastically. Also, curve b shows that in the presence of Phe the oxidation current was greatly increased due to catalytic oxidation of Pha. The decreased of overvoltage and increased peak current of Phe oxidation confirms that aptamer/Au electrode has suitable ability for Pha



Scheme 1 Oxidation mechanism of Pha

oxidation. Therefore, aptamer/Au electrode is suitable as mediator to shuttle electron between Phe and working electrode and facilitate electrochemical regeneration following electron exchange with Pha.

To investigate the kinetic parameters of the modified electrode, CVs of aptasensor at different scan rates were recorded and the results were shown in Fig. 4. As shown in Fig. 4, the oxidation peak current of Pha was linearly related to the scan rate in the range of 10–100 mVs^{-1} (R^2=0.9914). This indicates that the electrochemical oxidation of Pha at aptamer/Au surface was controlled by diffusion. Meanwhile, the effect of scan rate on E_{pa} was also investigated. A linear relationship between the E_{pa} and lnν was obtained using the equation: E_{pa}=0.468lnν+2.12 (R^2=0.9856). For a diffusion-controlled and totally irreversible electrode process, the relationship between E_{pa} and lnν is expressed through the Laviron equation [19]:

$$E_p = \left(\frac{RT}{2\alpha_n F}\right)\ln\nu + \text{constant} \quad (1)$$

where α_n, and ν are the electron-transfer coefficient, and potential sweep rate, respectively. Other symbols have their usual significance. The value of α_n can be easily calculated from the slope of E_{pa} vs. lnν. In this study, α_n was calculated to be 0.55. Usually, α is assumed as 0.5 in a totally irreversible electrode process. The value obtained for n is 1.09≈1. Therefore, one electron is involved in the electrochemical oxidation of Pha.

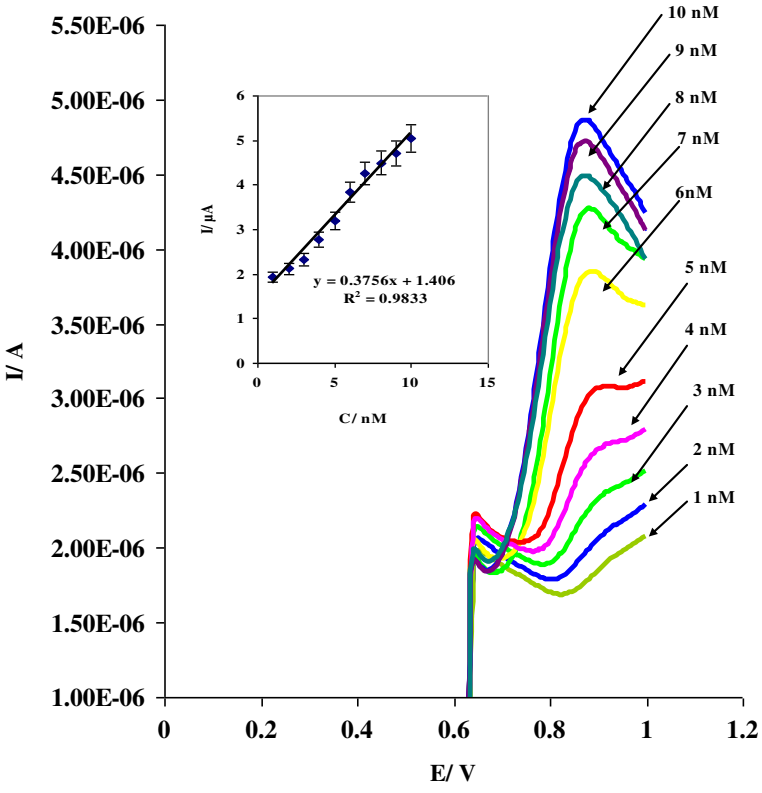


Fig. 5 DPVs for 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 nM of Phe in 10 mM Tris–HCl buffer (pH=7.4) containing 5.0 mM $[Ru(NH_3)_6]^{3+}$. *Inset* Plot of peak current vs. Pha concentration (SD=3.0 %, n=3)

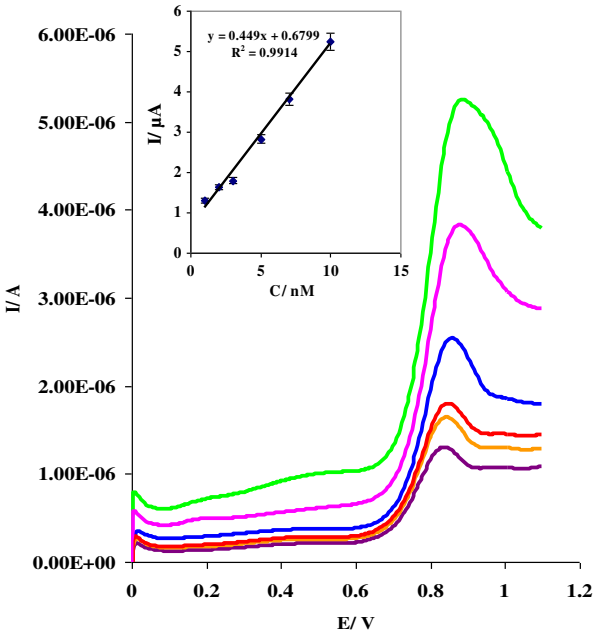


Fig. 6 SQWVs for 1, 2, 3, 5, 7, and 10 nM of Pha in 10 mM Tris–HCl buffer (pH=7.4) containing 5.0 mM $[Ru(NH_3)_6]^{3+}$. *Inset* Plot of peak current vs. Pha concentration (SD=3.0 %, n=3)

Also, the reaction mechanism was exhibited in Scheme 1. As shown in Scheme 1, at first 2-imino-3-phenylpropanoic acid as an intermediate was generated by oxidation of Phe via one electron and one proton. Subsequently, elimination of NH_3 and CO_2 occurred and the benzaldehyde and benzoic acid were formed as main products.

According to results and also above mechanisms, it can be considered that desorption step of the intermediated (according to proposed mechanism-produced from firs electron transfer step) and formation of product becomes more probable as the rate-determination step. Such behavior for the electrochemical oxidation of Pha was confirmed by the proposed mechanism.

The next attempt was taken to detect Pha at the modified electrode by differential pulse voltammetry (DPV) because of its higher current sensitivity and better resolution than CV. To

Table 1 Analytical parameters for detection of Phe at previously reported methods

Sensor	Linear range	LOD[a]	Ref.
Cobalt hydroxide/GCE	30–2000 nM	13.70 nM	[5]
Fe (III)-Schiff base CN/GCE	9–5000 nM	1.10 nM	[23]
MCM-41-NH_2/GCE	98–500 nM	13.0 nM	[6]
Biofunctionalization of dextran/enzyme/GCE	0.5–6 mM	0.5 mM	[22]
Polymer/enzyme/GCE	–	0.5 mM	[21]
MCM-41-Fe_2O_3/GCE	320–510 nM	126 nM	[7]
Aptamer/Au	1–10 nM	1 nM	This work

[a] Limit of detection

Table 2 Determination of Pha in human serum by aptamer/Au electrode

Sample	Added (μM)	Found (μM)	Recovery (%)
Human serum	0	Not detected	–
	10	9.7	97.2
	20	19.8	99.0
	30	32.0	106.0
	50	45.5	91.0
	60	57.9	96.5
	70	67.0	95.7

verify the linear relationship between DPV peak current and Pha concentrations, calibration curves were constructed under optimum conditions in 10 mM Tris–HCl of pH 7.4. Figure 5 shows DPVs obtained at aptamer/Au in various concentrations of phenylalanine. The peak current is linearly related to Phe concentration, over 1–10 nM (Fig. 5, inset), with correlation coefficients of 0.9923. The detection limit was estimated to be 1 nM (S/N=3). Using this device, we accessed a detection limit of 1 nM target concentration (RSD=4.3 %), which was a few orders of magnitude lower than the previous methods for Phe detection (1.10–126 nM) [20–25]. As shown in Fig. 5 (inset), the corresponding calibration plot of peak currents versus of Phe concentration was linear over the range from 1 to 10 nM, which was suitable for quantitative analysis of low-level Pha.

In addition, square-wave voltammetry (SQWV), as well as DPV, has been employed for the investigation of linear relationship between oxidation peak current and concentrations. Figure 6 shows SQWVs obtained at aptamer/Au surface in various concentrations of Pha. It can be seen that the aptamer/Au offered reasonable linear range for Pha detection. The analytical results were summarized in Table 1 along with the comparison of aptamer/Au with other modified electrodes for Phe determination. These results indicated that aptamer/Au is an appropriate platform for the determination of Pha.

Applicability of the aptasensor was examined for the determination of Phe in human serum. The high sensitivity of the aptasensor allows the determination of Pha in spiked human serum samples. The recovery of the analyte was measured by spiked Pha into diluted serum samples. The DPVs were recorded after the serum was spiked with various amounts of the Phe within the working concentration rang. Recoveries were found to lie in the range of 91.0 to 106.0 %. The results are listed in Table 2.

The reproducibility of the aptasensor is a very important feature, and thus the parameter was estimated. Six repetitive measurements of 10 nM of Pha solution with the proposed biosensor was used for estimating the precision of the aptasensor. The results indicated acceptable and reproducible signals with RSD of 3.87 % were obtained. Furthermore, in order to study the reproducibility of the aptasensor fabrication strategy, five aptamer/Au electrodes prepared independently. The RSD of the aptasensors responses toward 10 nM of Pha was 4.9 %, which indicated that the detection results based on the proposed strategy are reproducible and the aptasensor is of good reproducibility.

Conclusion

In summary, a sensitive aptamer-based biosensor was developed for the detection of Pha in aqueous solution. At the first time, an Au electrode was modified with abrasive immobilization

of Pha aptamer. The detection limit was obtained as low as 1 nM. The proposed aptasensor has been demonstrated to offer a sensitive method for detecting Pha. This biosensor can be used as a voltammetric detector for routine analysis of Pha in flow systems when coupled to chromatographic and electrophoretic separation systems.

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