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A label-free aptasensor for highly sensitive detection of homocysteine based on gold nanoparticles

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ABSTRACT

In the present study, an original electrode fabrication approach was devised to create a label free sensitive electrochemical aptasensor for the detection of Homocysteine (Hcy) (Homocysteine signal was used for detection). To bind certain targets, synthetic oligonucleotides used as aptamers (APs) were specifically selected. Aptamers are substitutes for antibodies for analytical devices because of their sensitivity and high affinity. In this study, Hcy-Binding-Aptamer (HBA) was grafted onto the surface of Au nanoparticles/Glassy Carbon Electrode (Au/GCE) in order to create an aptasensor. The effects of buffer concentration, buffer type, interaction time, and aptamer concentration were investigated and optimized. In addition, Differential Pulse Voltammetry (DPV) was implemented to identify homocysteine. Favorable performance was achieved at a detection limit of 0.01 μM (S/N = 3) and linear range 0.05–20.0 μM. Furthermore, the fabricated aptasensor displayed desirable stability and reproducibility. The developed electrochemical aptasensor was found to have reasonable selectivity for the detection of homocysteine in the presence of cysteine and methionine. Analysis of real samples showed good ability of the proposed homocysteine biosensor to provide sensitive, quick, easy, and cost effective measurement of homocysteine in human blood serum and urine samples.

1. Introduction

Homocysteine (Hcy), as a highly reactive sulfur-containing amino acid turns into an intermediate by-product during the metabolism of dietary methionine. Hyperhomocysteinemia, as a medical condition, is characterised by irregular elevation of plasma homocysteine concentration. Fasting values are used as reference to classify elevated levels of total homocysteine. These values are as follows: moderate (15–30 μmol/L), intermediate (31–100 μmol/L), and severe (>100 μmol/L). Homocysteine levels may increase as a result of a single or combination of the following factors: nutritional, pathological, physiological, genetic. Specifically, the administration of some drugs, chronic medical disorder, postmenopausal hormone status, lack of exercise, a methionine rich diet, older age, male sex, vitamin B deficiency, smoking and overconsumption of alcohol and coffee can be associated with high levels of homocysteine [1–5]. Hyperhomocysteinemia is also caused by folic acid, vitamin B_6, and B_{12} deficiency through effects on the methionine metabolic pathway. Kidney function is another factor playing a key role in the regulation of homocysteine levels. Hyperhomocysteinemia here may be due to impairment in glomerular filtration rate (creatinine clearance) especially in older adults. Plasma homocysteine concentrations may also be elevated in inherited genetic disorders. The Methylene Tetra Hydrofolate Reductase (MTHFR) C677T genetic variant is the most prevalent genetic defect associated with the hyperhomocysteinemia phenotype. The Cystathionine β-Synthase (CBS) 844ins68 variant is another disorder linked to premature vascular disorder and hyperhomocysteinemia [6–9].

Hyperhomocysteinemia is also caused by folic acid, vitamin B6, and B12 deficiency through Plasma homocysteine has been a focus of increased attention because to its significance in relation to population health. Diabetic vascular impairment, arterial and venous thromboembolism, cerebro and cardiovascular diseases are examples of vascular diseases with a possible association with hyperhomocysteinemia. Credible research has proposed that an increase of 5 μM in homocysteine level may cause a 32% increase in myocardial infraction risk and a 59% increase in stroke risk. Moreover, there is a significant causal relationship between development of

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cardiovascular disorders and elevation of homocysteine level [10–13]. Homocysteine as a redox molecule can be determined electrochemically. The overall oxidative reaction process is involves initial one -electron, one -proton oxidation of homocysteine to generate a free radical species (RS·), which undergoes rapid dimerization to form a disulfide-homocysteine species [14]:

$$2RSH \rightarrow 2RS\cdot + 2e^{-} + 2H^{+} \rightarrow RSSR \quad (1)$$

Aptamers are DNA or RNA single stranded nucleic acids able to selectively bind to target macromolecules such as proteins or to organic and inorganic species of low molecular weight. The stability constant of aptamers with their affinity target falls in the micromolar to nanomolar range and is of the order seen with antibodies with respect to target antigens [15]. Previous researches have shown that the characteristics of nano-material modified electrode surfaces considerably influence the analytical performance of electrochemical biosensors [16–29]. As selective binding agents, attention to aptamers stems from the simplicity of their separation *via* a transformative selection process, which does not require structural design of receptor sites. SELEX is an *in vitro* selection method designed to identify aptamers for specific targets. Aptamers offer considerable advantages in comparison with monoclonal antibodies as reagents for *in vitro*affinity assays. Notably, there is greater control over properties such as binding kinetics and selectivity [30–35].

Electrochemical -Aptamer Based sensors i.e., E-AB sensors are utilized extensively for rapid and sensitive detection of different targets such as proteins, e.g. thrombin and insulin, smaller molecules, eg cocaine and tobramycin, and even cells, due to their desirable properties of flexibility in design, easy labeling, lack of need for additional reagent and high, specific target affinity [36–49].

Based on the above background, the current study was conducted to develop and use an AP/nano-Au/GCE sensor for voltammetric determination of homocysteine. The fabricated device had the advantage of high performance and high detection sensitivity, which made it applicable for determination of homocysteine in serum and urine.

2. Experimental

2.1. Materials and reagents

The oligonucleotides sequences used were as follows: DNA, 5′SH-$(CH_2)_6$ ACCA GCAC ATTC GATT ATAC CAGC TTAT TCAA TTCA CAGC TATG TCCT ATAC CAGC TTAT TCAATT-3′ [47]. These oligonucleotides were obtained from Bio Basic Inc. (Canada). Ethanol, acetone, phosphoric acid, acetic acid, sodium hydroxide, and citric acid were purchased from Merck Company (Germany). DL-homocysteine and 6-mercapto 1-hexanol were obtained from Sigma-Aldrich Company (USA). All other chemicals were reagent grade.

2.2. Apparatus

An Autolab Potentiostat/Galvanostat (PGSTAT 302 N, Netherlands) was used to carry out all voltammetric measurements. A computer was used to control the process and to acquire data. A glassy carbon disk electrode (with a diameter of 2 mm) as working electrode (Azar Electrode, Urmia, Iran), Ag/AgCl/KCl (3.0 M) (Azar Electrode, Urmia, Iran) as reference and a platinum wire (Azar Electrode, Urmia, Iran) as the auxiliary electrode were used in a three -electrode system. To conduct pH measurements, a 713 pH meter Metrohm was utilized along with a glass-reference electrode combination. To conduct Differential Pulse Voltammetry (DPV) measurements, first, the working electrode was immersed in 5 mL homocysteine solution made up in 0.1 M Phosphate -Buffered Saline (PBS) (0.1 M Na_2HPO_4–NaH_2PO_4 in 0.1 M NaCl, pH = 7.0) stirred at room temperature. PBS was used for washing purposes, before the working electrode was transferred to the three -electrode system. 25 mL of 0.1 M PBS solution at a pH of 7.0 was used for DPV measurements at room temperature. Alumina powder (Sigma-Aldrich, Inc., USA with particle size of 45μ) was used to polish a glassy carbon electrode.

2.3. Preparation of modified glassy carbon electrode

Alumina powder (with a mean particle size of 45 μ, Sigma-Aldrich, Inc., USA) was used to polish glassy carbon electrodes rinsed entirely using double distilled water between every polishing step, before final sonication in 1:1 nitric acid:acetone. The electrodes were then left to dry at room temperature. Modification using gold nanoparticles was performed by immersion of an electrode in gold salt solution (6 mM $HAuCl_4$ and 0.1 M KNO_3) and then applying a potential of −400 mV (using constant potential mode) vs. Ag/AgCl/KCl (3.0 M) over 400 s (nanoAu/GCE). 5 μL of 4.0 μM aptamer solution was cast on a polished glassy carbon electrode surface. Then, the electrode was held upright in a humid chamber to complete the thiol aptamer self assembly. The modified glassy carbon electrode was then immersed in 1 mM 6-mercapto 1-hexanol solution for 1 h, with rinsing with 0.1 M PBS at pH 7.4, to restrict non-specific binding sites and to form the aptamer strands in linear chains for coordination. 25 mM of PBS solution at a pH 7.0 was used to wash the electrode at every step.

2.4. Detection of homocysteine

To detect homocysteine, 20 mL of homocysteine solution was used to immerse the modified glassy carbon electrode (nano-Au/GCE) at various concentrations in PBS (0.1 M PBS, 0.1 M NaCl, and pH = 7.0) for 1 h. Upon washing with buffer, bound homocysteine was measured electrochemically in a 25 -mL buffer (0.1 M PBS, 0.1 M NaCl, and pH = 7.0) using DPV.

3. Results and discussion

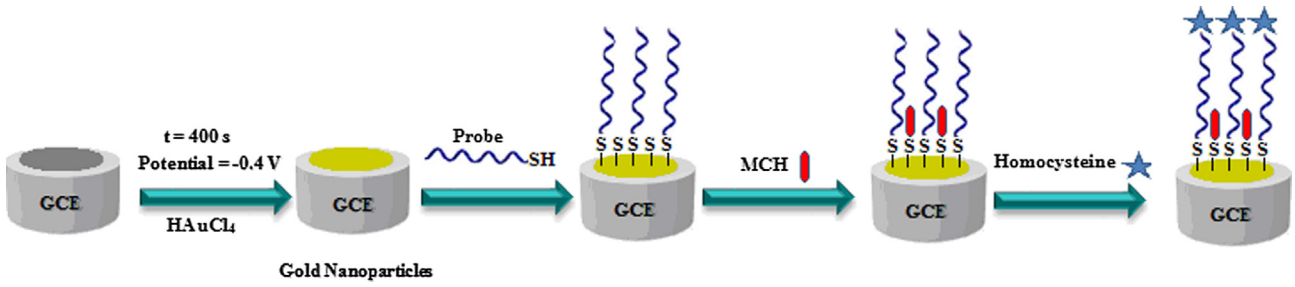
3.1. Fabrication of electrochemical aptasensor

To detect homocysteine, a label -free aptasensor was designed consisting of a thiolated aptamer as the capture probe. Scheme 1 presents the assay protocol for the biosensor. To investigate for gold nanoparticles on glassy carbon electrode surface, cyclic voltammetry of the $[Fe(CN)_6]^{4-/3-}$ redox couple was implemented as the indicator. As shown in Fig. 1 (curve b), the $[Fe(CN)_6]^{4-/3-}$ peak current at the bare glassy carbon electrode was lower than the electrode with gold particles (Fig. 1 curve a). Also, there was a reduction in potential difference between anodic and cathodic peak potentials. These changes were due to the gold nanoparticles at surface of the electrode resulting in an increased electrical conducting surface area.

3.2. Optimization of experimental conditions

To improve performance of the AP/nano-Au/GCE sensor, the effects of various factors such as aptamer concentration, time of interaction, type of buffer and concentration of buffer were investigated and optimized in a 0.1 μM homocysteine solution.

The effect of aptamer concentration in the range 0.5–6.0 μM on the detection of homocysteine was studied in solutions of 0.1 μM homocysteine in 0.1 M PBS (pH = 7.0) and the results are presented in Fig. 2. As shown in Fig. 2, the response of the electrode increased sharply at 4.0 μM of aptamer concentration.



Scheme 1. Schematic illustration of stepwise homocysteine aptasensor fabrication process.

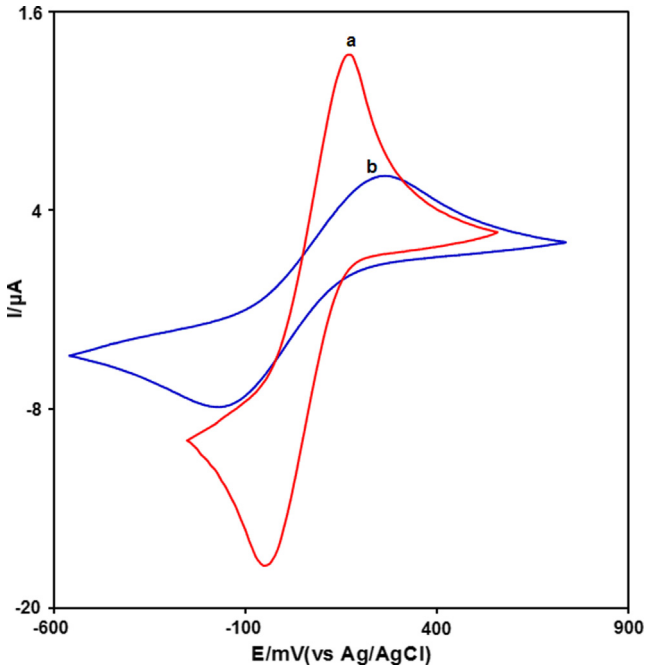


Fig. 1. Cyclic voltammograms of different modification steps for the GCE in presence of 5 μM $[Fe(CN)_6]^{4-/3-}$ redox couple and 0.1 M KCl. (a) Nano-Au/GCE and (b) bare GCE.

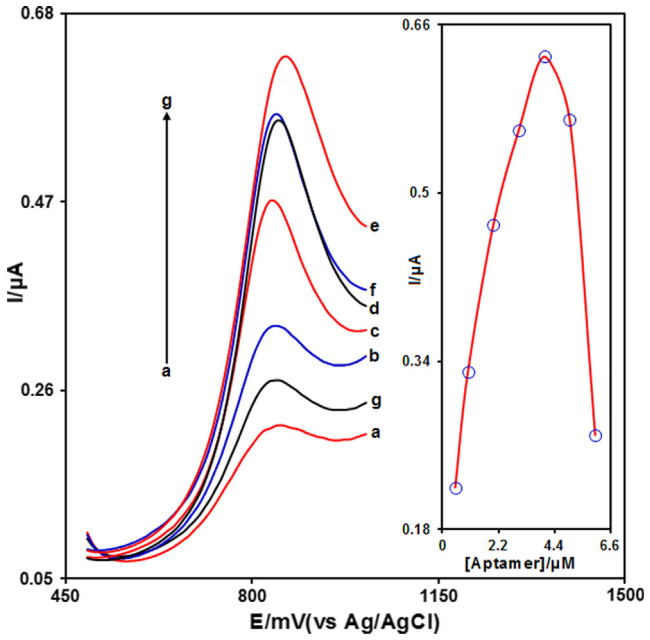


Fig. 2. DPV curves of nano-Au/GCE in solution with different concentrations of aptamer in 0.1 M PBS (pH = 7.0). a–g correspond to 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 μM of aptamer concentration, respectively. Inset: plot of the peak current as a function of aptamer concentration in the range of 0.5–6.0 μM.

The effect of the duration of incubation in the range 35–120 min on detection of homocysteine was studied in solutions of 0.1 μM homocysteine in 0.1 M PBS (pH = 7.0) and the results are presented in Fig. 3. These show that response increases sharply at 60 min of interaction time.

The effect of buffer type (citrate, acetate, and phosphate, all at pH 7.0) on the detection of homocysteine was studied in solutions of 0.1 μM homocysteine and the results are presented in Fig. 4. As can be seen from Fig. 4, the response of the electrode increases sharply with 0.1 M PBS (pH = 7.0). PBS possibly affords a more physiologically representative sample matrix.

The effect of concentration of PBS concentration in the range of 0.05–0.15 M on the detection of homocysteine was studied in solutions of 0.1 μM homocysteine and the results are presented in Fig. 5. Based on Fig. 5, the response of the electrode increased sharply at 0.1 M PBS.

Selected conditions used were accordingly accumulation time 1 h, aptamer concentration 4.0 μM and 0.1 M PBS.

3.3. Calibration plot and detection Limit

The homocysteine detection calibration curve was formed using the fabricated AP/nano-Au/GCE sensor under the optimized test conditions. The homocysteine oxidation peak at modified electrode surface was determined using DPV for various homocysteine concentrations, as presented in Fig. 6. Peak currents were proportional to homocysteine concentration giving a Linear Dynamic Concentration Range (LDCR) of 0.05–20.0 μM with a Limit of Detection (LOD) of 9.0 nM using the 3σ method ([Y(μA = 0.036X (μM) + 0.618, R^2 = 0.998]). Also, to make a comparison, a bare glassy carbon electrode was used for homocysteine determination in the solution in LDCR of 0.05–20.0 μM and LOD of 30.0 nM using 3σ method ([Y(μA = 0.019X (μM) + 0.306, R^2 = 0.998]).

3.4. Selectivity

Selectivity is a key factor for evaluating the usability of a sensor. To do so, control experiments were performed to investigate selectivity of the homocysteine aptasensor. To confirm binding selectivity for homocysteine, two amino acids with thiol group (cysteine and methionine) were tested. The calibration regression was Y(μA) = 0.0369X (μM) + 0.620, R^2 = 0.9992) in presence of 15.0 μM cysteine and Y(μA) = 0.0368X (μM) + 0.619, R^2 = 0.9984 in presence of 15.0 μM methionine. As can be seen, the slope of calibration curve did not change significantly. Results indicate that the electrochemical aptasensor has reasonable selectivity and can be used for the detection of homocysteine.

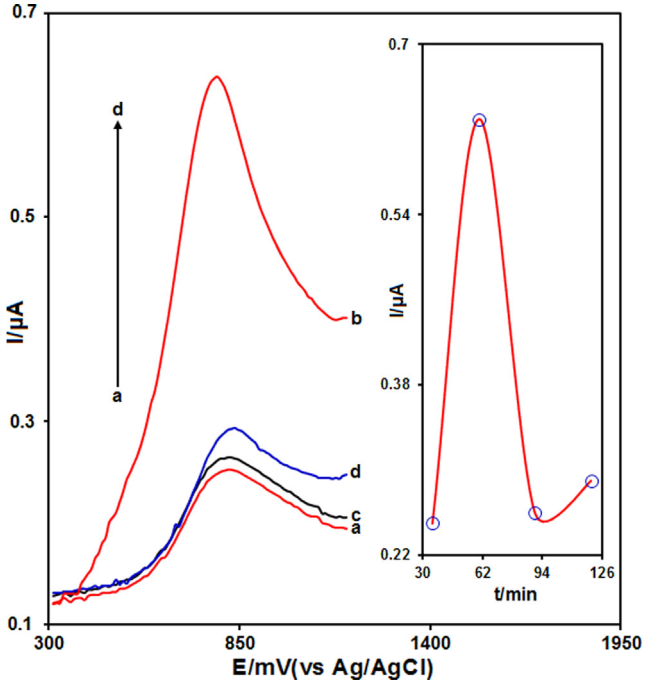


Fig. 3. DPV curves of AP/nano-Au/GCE sensor at 4.0 μM of aptamer concentration in 0.1 M PBS (pH = 7.0) at different interaction times of aptamer with homocysteine. a–d correspond to 35, 60, 90, and 120 min interaction times, respectively. Inset: plot of the peak current as a function of interaction times in the range of 35–120 min.

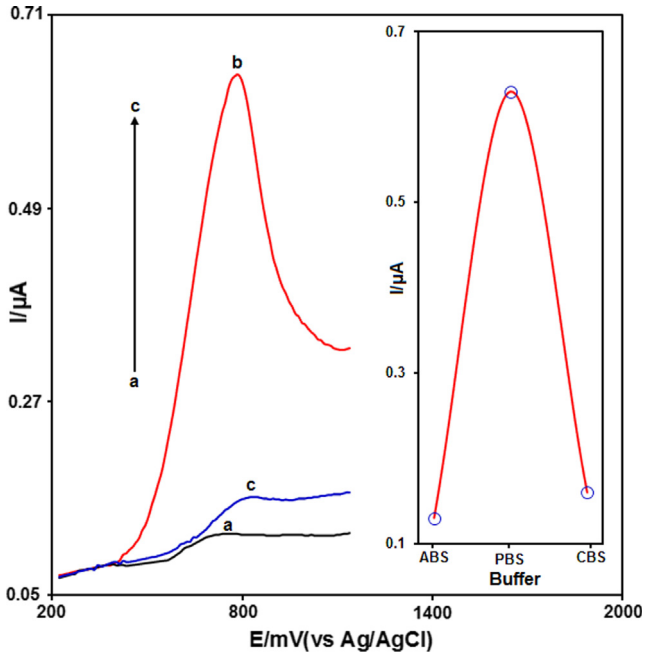


Fig. 4. DPV curves of AP/nano-Au/GCE sensor at 4.0 μM of aptamer concentration, at 60 min interaction time of aptamer with homocysteine and different types of buffer. a–c correspond to citrate, phosphate and acetate as types of buffer, respectively. Inset: plot of the peak current as a function of different types of buffer (pH = 7.0).

3.5. Analysis of real samples

Human serum and urine samples were analyzed using the standard addition method to study the possibility of applying a homocysteine aptasensor for analysis of real samples. To achieve accurate results, every sample must be promptly centrifuged and stored in a refrigerator at 4 °C. Hence, 1.0 mL of the human serum

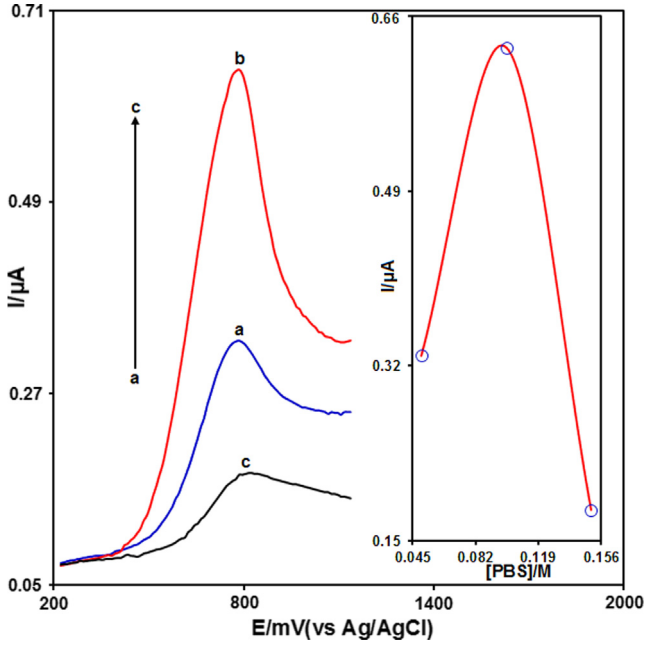


Fig. 5. DPV curves of AP/nano-Au/GCE sensor at 4.0 μM of aptamer concentration, at 60 min interaction time of aptamer with homocysteine in PBS (pH = 7.0) with different concentrations of phosphate buffer. a–c correspond to 0.05, 0.1, and 0.15 M buffer concentrations, respectively. Inset: plot of the peak current as a function of phosphate buffer concentrations.

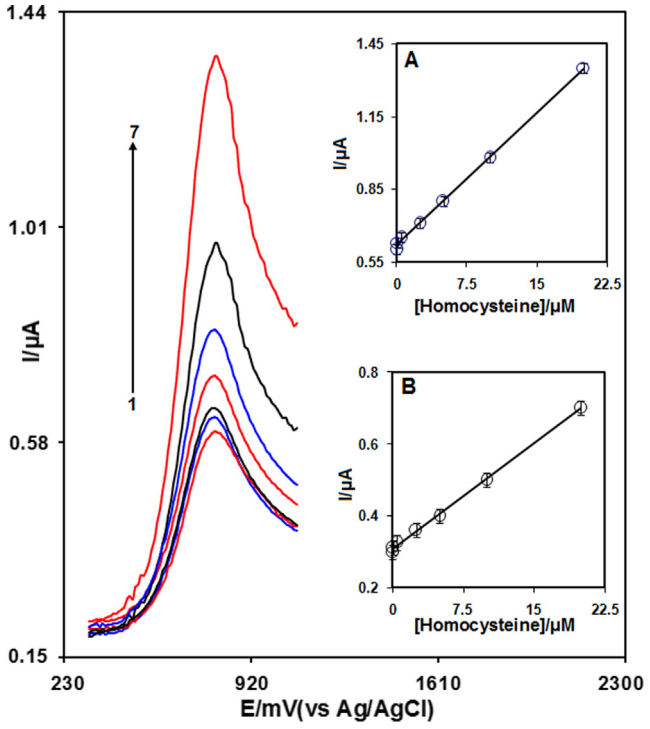


Fig. 6. DPV curves obtained at aptasensor for homocysteine at different concentrations in 0.1 M PBS (pH = 7.0). Numbers 1–7 correspond to 0.05, 0.1, 0.5, 2.5, 5.0, 10.0, and 20.0 μM of homocysteine concentration. Inset: (A) calibration curve for homocysteine, plot of peak current vs. homocysteine concentration using AP/nano-Au/GCE. (B) Calibration curve for homocysteine, plot of peak current vs. homocysteine concentration using bare GCE with the same concentrations used for AP/nano-Au/GCE. (Error bars were obtained from 5 replicates).

Table 1
Determination of homocysteine in human serum and urine samples (n = 5). All concentrations are in μM.

Sample	Spiked	Found	Recovery (%)	R.S.D. (%)
Urine	0	1.0	–	3.3
	4.0	4.9	98.0	2.5
	8.0	9.1	101.1	2.7
Human serum	0	4.1	–	2.8
	1.0	5.3	103.9	3.2
	5.0	9.0	98.9	2.1

Table 2
Comparison of homocysteine aptasensor with other reported electrochemical methods for determination of homocysteine (LOD: Limit of Detection and LDCR: Linear Dynamic Concentration Range).

Electrode	Method	LOD	LDCR	Ref.
Multiwall Carbon Nanotube Paste electrode	Square wave voltammetry	0.08 μM	0.1–210.0 μM	[4]
Gold electrode modified with homocysteine aptamer	Differential pulse voltammetry	0.89 μM	2.5–1000.0 μM	[47]
Glassy carbon electrode modified with carbon nanotube	Linear sweep voltammograms	3.3 μM	5.0–800.0 μM	[48]
Glassy carbon electrode modified with homocysteine aptamer	Differential pulse voltammetry	0.009 μM	0.05–20.0 μM	This work

was used upon separation of precipitated proteins and filtering using 0.45 μm millipore filter and the sample was then diluted with 1.0 mL of PBS (0.1 M PB, 0.1 M NaCl, pH = 7.0). For urine, 1.0 mL of sample was diluted with 1.0 mL of PBS. Table 1 presents the obtained results. As anticipated, after implementing the standard procedure, satisfactory recoveries of spiked homocysteine were obtained confirming that the aptasensor is suitable for use in biological samples.

3.6. Applying aptasensor for detection of homocysteine compared to other established electrochemical methods

Results obtained regarding the validation and assessment of the accuracy of the homocysteine aptasensor were compared with other electrochemical methods. The LOD is better and the linear ranges are commensurate to those ones reported (Table 2). Moreover, applying an aptamer to an electrode construction simplified determination of selectivity compared with previous methods as shown in Table 2.

4. Conclusions

In this research, an aptasensor was developed for label-free electrochemical detection of the homocysteine. A significant enhanced peak current indicated homocysteine pre-concentration at the surface of the modified electrode through the aptamer binding interaction. Upon optimization of experimental parameters, performance was assessed analytically. Thus, designed modified electrode is a useful and selective aptasensor for the electrochemical determination of the homocysteine in biological samples. Also, in future works the other morphology of Au nanostructures can be used for modification of the electrode surface. Also, other kinds of electrodes (carbon paste electrode, screen printed electrode or gold electrode) can be used or the researchers can use simultaneously two or more aptamers for modification of electrode surface to analysis two or more analytes simultaneously.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioelechem.2020.107497.

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