Regular Article

ANALYTICAL AND BIOANALYTICAL CHEMISTRY RESEARCH

Published by the Iranian Chemical Society

Anal. Bioanal. Chem. Res., Vol. 4, No. 2, 341-352, December 2017.

Fabrication of an Electrochemical Immunosensor for Determination of Human Chorionic Gonadotropin Based on PtNPs/Cysteamine/AgNPs as an Efficient Interface

Akram Valipour and Mahmoud Roushani*

Department of Chemistry, Ilam University, Ilam, Iran

(Received 10 April 2017, Accepted 22 July 2017)

An ultrasensitive electrochemical immunosensor for the detection of tumor marker human chorionic gonadotropin (hCG) was developed with a limit of detection as low as 2 pg ml^{-1} in phosphate buffer. The platinum nanoparticles (PtNPs) were electrodeposited to modify the gold surface and to increase enlarging the electrochemically active sites, resulting in the facilitation of electron exchange. Cysteamine (Cys) self-assembled monolayer was chemisorbed spontaneously on Pt substrates *via* the Pt-S bond and the amine groups array exposed on the electrode surface was used to anchor AgNPs through electrostatic interaction. AgNPs increased the immobilized amount of antibodies on the electrode to enhance the sensitivity of the sensor. Under optimal experimental parameters, differential pulse voltammetry (DPV) signal changes of the $[Fe(CN)_6]^{3-/4-}$ are used to detect hCG with two broad linear ranges: 0.007-1.11 and 1.11-68 ng ml^{-1}. The reported strategy has provided a promising platform for highly sensitive and selective detection of hCG. Finally, the proposed immunosensor is successfully used in detecting hCG in human serum samples.

Keywords: Electrochemical immunosensor, Cysteamine, Electrodeposition of PtNPs, Human chorionic gonadotropin, AgNPs

INTRODUCTION

Human chorionic gonadotropin (hCG) can be used as a tumor marker, especially in gestational trophoblastic disease. The early detection of hCG produced by the placenta is very important in preventing the spread of pregnancy complications [1]. Therefore, developing a simple, rapid, and non-invasive method with low cost for hCG detection is a challenge. Up to now, as shown in Table 1, several methods have been introduced for hCG detection. Despite their accuracy and reliability, some of these approaches have certain practical disadvantages such as complexity. Also, the sensitivity of these methods can be improved. Therefore, it is necessary to develop a simple approach for ultrasensitive and selective measurement of hCG.

Immunoassays with high selectivity and affinity of antibody molecules to their corresponding antigens have been widely used in clinical diagnosis [2]. The interactions between an antibody and an antigen are known to be very specific chemical reactions. Such a specific molecular recognition of antigens by antibodies has been exploited in immunoassays to develop highly selective detection methods in many clinical analyses and medical diagnostics as well as for environmental monitoring [3].

Electrochemical immunosensors are gaining a growing attention due to their low cost and relatively fast response times [4]. The main applications of electrochemical immunosensors are in health care. In recent decades, a wide range of immunosensors based on electrochemical transducers have been developed, such as potentiometric [5, 6], conductimetri [7], amperometric [8], impedimetric [9], and piezoelectric immunosensors [10]. Among these, amperometric immunosensors are of great interest owing to a relatively low detection limit and high sensitivity. Electrochemical immunosensors are usually prepared on the

*Corresponding author. E-mail: m.roushani@ilam.ac.ir

Table 1. Comparison of Different Immunosensors for Detection of hCG

Modified Electrode	Method	LR (ng ml^{-1})[a]	LOD (pg ml^{-1})[b]	Ref.
anti-hCG/NP-Pd/ MWCNTs[c]-$BMIMPF_6$/GCE	CV	0.05-50	3.2	[1]
Pd-Al alloy/HRP[d]-Ab_2[e]/hCG/Ab_1/ GNps[f] /PB/GNps/GCE	CV	0.5-200	9.3	[23]
anti hCG/NPG[g]-Gs[h]/GCE	AMP[k]	0.5-40.00	34	[12]
anti-hCG/Pd@SBA-15/TH/HSO_3-GS/GCE	AMP	0.01-16.00	8.60	[15]
Pt@MSN[i]/HRP/Ab_2/hCG Antigen/Ab_1/ TH[j]/Graphene/GCE	AMP	0.01-12	7.50	[13]
anti-hCG/nano-Au/MB[l]/GCE				
anti-hCG/nano-gold and CS hybrid film/GCE	CV	1-1000	0.3	[24]
anti-hCG/Pt–Au alloy nanotube array/GCE	CA[m]	0.2-1000	0.1	[25]
anti-hCG/gold nanotubesarray/GCE				
anti-hCG/Au@SiC–CS/GCE	CA	25-400	12	[26]
anti-hCG/GNPs[n]/pPA[o]/MWCNTs/GCE				
anti-hCG/AgNPs/Cys/PtNPs/GE	CA	0.1-100	O.08	[27]
	DPV	0.1-5,5-1000	0.042	[28]
	CV	1-10,10-160	0.3	[29]
	DPV	0.007-1.11,1.11-68	2	This work

[a]Linear rang. [b]Limit of Detection. [c]Multiwalled carbon nanotubes. [d]Horseradish peroxidase. [e]Anti body. [f]Gold nanoparticles. [g]Nanoporous gold. [h]Graphene nanosheets. [i]Mesoporous silica nanoparticles. [j]Thionine. [k]Amperometry. [l]Methyleneblue. [m]Chronoamperometry. [n]Gold nanoparticles. [o]Poly-(2,6-pyridinediamine).

basis of their accuracy in recognition of antigens and antibodies. In the process of the design and fabrication of highly sensitive electrochemical immunosensors, signal amplification, antibody immobilization and noise reduction are the crucial steps. Among the various immobilization methods, the traditional method was to form covalent couplings, which provided stable and strong bindings between the desired biomolecule and the electrode substrate [1]. Signal amplification is a main concern for detecting target molecules at quite low concentration [11]. For suitable immobilization of antibody molecules in fabrication of electrochemical immunosensor, several nanomaterials such as gold nanoparticles [12], platinum nanoparticle [13], TiO_2 [14], and palladium [15] which have shown their potential in the fabrication of these immunosensor, have been used as matrices to improve the stability of the sensor probe. Pt nanoparticles (PtNPs) possess unique properties such as excellent conductivity, and large surface area, which

can be used to enhance electron transfer. As a traditional electrochemical technique, electrodeposition is a useful approach to prepare metal nano crystals, with the advantages of being straight forward, not requiring templates, and conferring easy shape control of metal particles [16,17]. Several methods for the synthesis of Pt nanoparticles have been reported. Among these methods, the most quoted is the deposition-precipitation. Deposition-precipitation is the most commonly used procedure to synthesize PtNPs.

This paper reports the development of a hCG immunosensor based on the deposition of PtNPs on the surface of GE and covalent attachment of cysteamin as a linker. The prepared unique platform can increase the effective electrode surface area and load more antibodies, accelerate the electron transfer significantly and subsequently, improve the electrochemical signal.

In this method, cysteamine is covalently attached to the PtNPs-coated surface (PtNPs/GE) by using cysteamine followed by AgNPs as a linker agent. By hCG incubation and upon the formation of the antigen/antibody complex on the modified electrode surface, the electron transfer characteristic of $[Fe(CN)_6]^{3-/4-}$ as the electrochemical probe changes; this can be monitored by the differential pulse voltammetry technique. This fabrication method is simple and may provide many potential applications for the ultrasensitive detection of different biomolecules.

EXPERIMENTAL

Reagents and Apparatus

$AgNO_3$, sodium borohydride ($NaBH_4$), sodium citrate ($Na_3C_6H_5O_7.2H_2O$), K_2PtCl_6, anti-hCG, hCG and Bovine serum albumin (BSA) were purchased from Sigma-Aldrich Co. LLC (USA). All other reagents with analytical grade such as glucose, histidine, trypsin and NaOH were obtained from Merck or Fluka and used without further purification. The real samples (human blood serum) were provided by a local clinical laboratory and were diluted 50 times with phosphate buffered solutions (PBS, 0.1 M, pH = 7.5) and then analyzed. PBS was prepared using 0.1 M Na_2HPO_4 and was set by HCl solution. All solutions with various concentrations were prepared by direct dissolution in 0.1 M PBS in pH: 7.5. For the electrochemical impedance spectroscopy (EIS) experiments, a solution containing 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ at a ratio of 1:1 and 0.1 M KCl as a redox probe was applied.

Cyclic voltammetry (CV) and impedance spectroscopy (EIS) were conducted with a μ-AUTOLAB electrochemical system type III and FRA2 board computer controlled Potentiostat/Galvanostat (Eco-Chemie, Switzerland) driven with NOVA software in conjunction with a conventional three electrode system with GE as the working electrode, a platinum wire as the counter electrode, and an Ag/AgCl (satd 3.0 M KCl) as the reference electrode. The DPV measurements were carried out by scanning the potential of 0 to 0.5 V with modulation time of 50 ms and modulation amplitude of 25 mV. CV measurements were taken from -0.3 to 0.8 V as initial and stop potential. The impedance analysis was performed in a frequency range of 0.1 Hz to 100 kHz with a modulation voltage of 5 mV. Scanning electron microscopy (SEM) images were obtained by using scanning electron microscopy (Philip's Company, Netherlands). The morphology of the AgNPs was determined by a Hitachi H-800 transmission electron microscopy (TEM) at an operating voltage of 200 kV, and energy-dispersive X-ray (EDS) spectra were performed with a Philips CM-200-FEG electron microscopy operating at 200 kV (accelerating voltage).

A Metrohm model 780 pH/mV meters was used to measure the pH. All experiments were carried out at room temperature.

Synthesis of Ag Nanoparticle

AgNPs were synthesized based on the reported procedure [18] with modifications: in short, 20 ml of 1.0 mM $AgNO_3$ aqueous solution was mixed with 0.5 ml of 0.1 M sodium citrate solution and then 1 ml of 3.5 mM $NaBH_4$ (freshly prepared) was added to the above mixture dropwise under vigorous stirring. After reaction under vigorous stirring for 2 h, the product was stored in a brown bottle at room temperature for 24 h prior to further use. A transparent yellow homogenous colloidal solution of AgNPs was obtained. The synthesized AgNPs were characterized by TEM (Fig. 1A).

Fabrication of the Immunosensor

Prior to the fabrication of the immunosensor, the bare

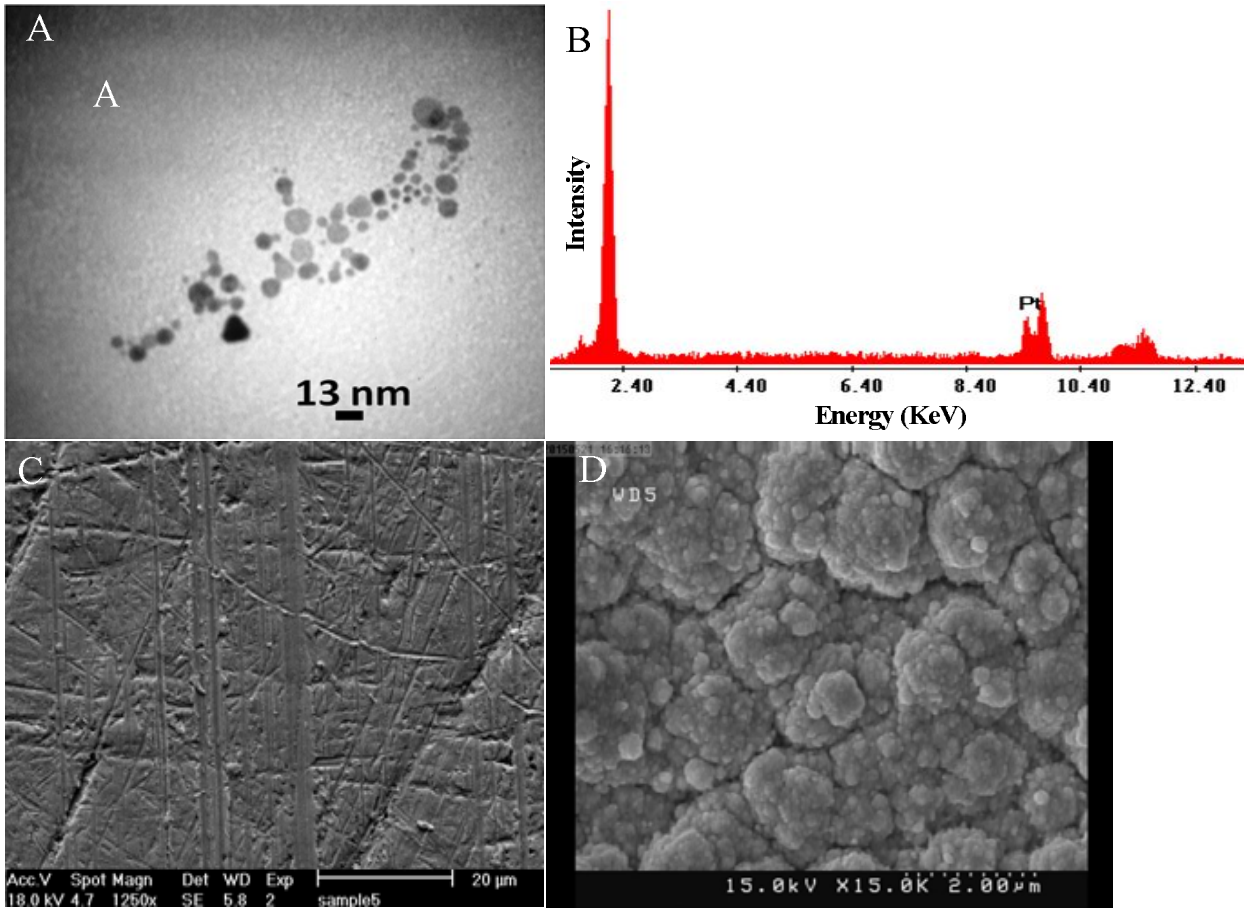
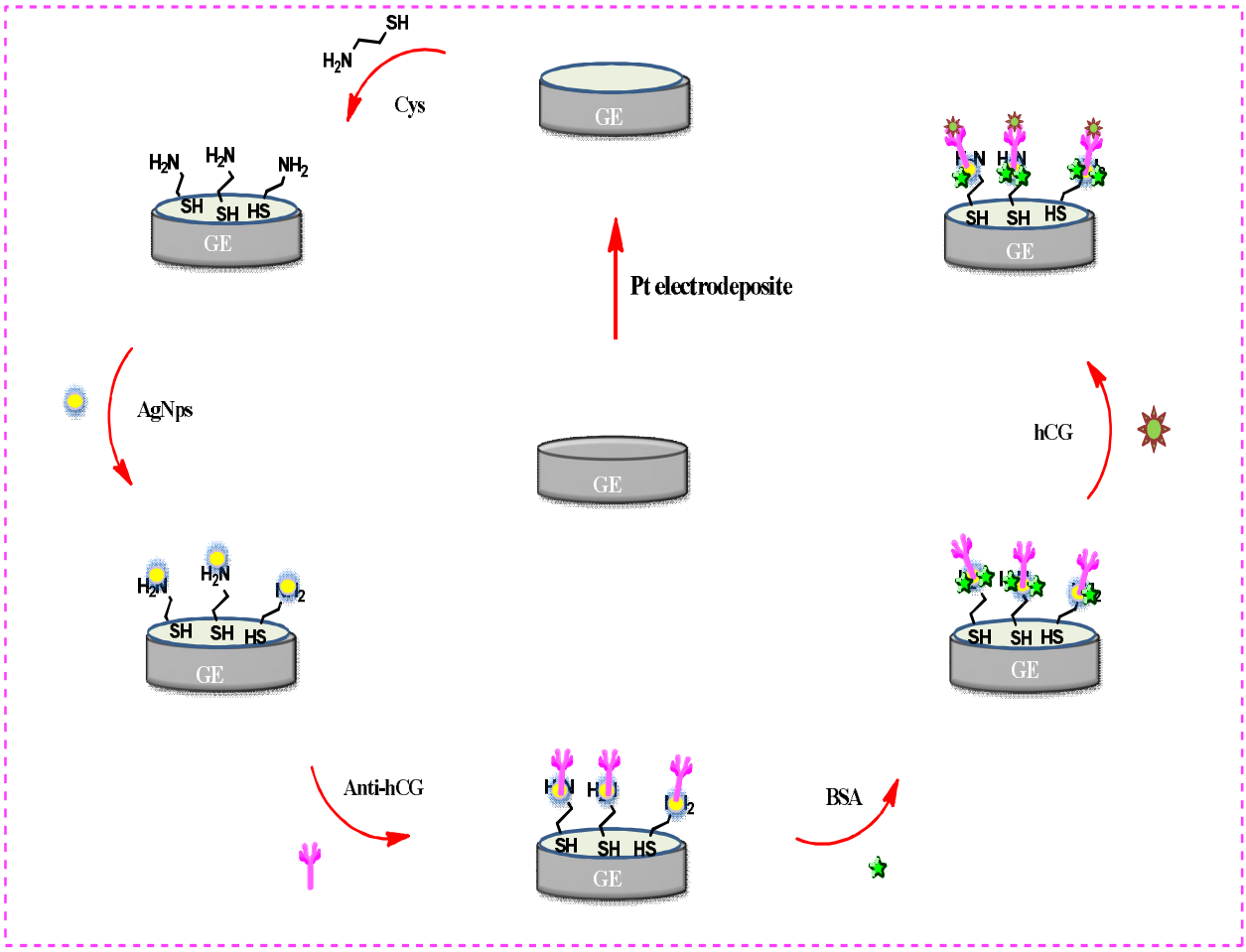


Fig. 1. A) TEM image of AgNPs, B) EDS image of PtNPs/GE C) SEM image of bare GE and D) SEM image of PtNPs/GE.

gold electrode (GE) was well polished with 0.3 and 0.05 µm alumina powder and cleaned ultrasonically in anhydrous ethanol. Then, the GE was subjected to electrochemical cleaning fluid (0.5 M H_2SO_4) at the scanning potential between 0.0 and 1.6 V at the scan rate of 100 mVs^{-1} until the stable cyclic voltammogram of a clean gold electrode was obtained. Initially, for the fabrication of the electrochemical immunosensor the pretreated GE was modified with PtNPs. Electrodeposition of platinum on gold electrode was carried out in an electroplating bath. The composition of the electroplating bath consisted of 1.3 mM H_2PtCl_6 and 0.5 M H_2SO_4, making a total volume of 10 ml. The gold electrode was immersed in the plating bath and a constant potential of -0.25 V was applied for 5 min under gentle stirring condition [19]. In the next step, 10 µl 10 mM Cys was dropped on the surface of electrode for 4 hours at room temperature. In order to remove the physically and weakly adsorbed Cys, the Cys/PtNPs/GE was rinsed with deionized water. Afterwards, 8 µl of the synthesized AgNPs was dropped onto the Cys layer of the modified electrode at 4 °C for 12 h. Following that, 3 µl PBS containing anti-hCG (1 mg ml^{-1}) was dropped onto the surface of AgNPs/Cys/PtNPs/GE that was prepared overnight to immobilize anti-hCG molecules on the surface of AgNPs by chemisorption between AgNPs and amine groups of anti-hCG. Finally, the antibody modified electrode (anti-hCG/AgNPs/Cys/PtNPs/GE) was soaked in 25% BSA at room temperature for 3 h in order to block nonspecific



Scheme 1. The schematic illustration for fabrication of the electrochemical immunosensor

binding sites and avoid the nonspecific adsorption. The prepared immunosensor was stored at 4 °C. The preparation steps for the immunosensor are shown in Scheme 1.

Electrochemical Measurements

Electrochemical experiments containing a three electrode arrangement were performed in a conventional electrochemical cell. The potential was swept from 0 to 0.5 V (*vs*. Ag/AgCl) in 10 ml of pH 7.5 PBS containing 2.5 mM K_3[Fe(CN)6]/K4[Fe(CN)6] (1:1) and 0.1 M KCl at room temperature. By immersing the BSA/anti-hCG/AgNPs/Cys/PtNPs/GE in hCG solution for 50 min, antigen and antibody complexes are performed with increase in hCG concentration and the current peak is further reduced.

RESULTS AND DISCUSSION

Characterization of the Deposited PtNPs on the GE

Surface elemental analysis of PtNPs/GE was studied by energy dispersive X-ray (EDX) technique. As shown in Fig. 1B, EDX spectrum confirms the presence of Pt on the GE surface. Under the deposition conditions, the morphologies of the PtNPs electrodeposited on the GE were characterized with SEM images. The SEM micrograph in Fig. 1D as compared with Fig. 1C (bare GE) indicates that an appropriate layer of PtNPs is formed on the surface of the GE. As seen, the SEM images show a uniform coverage of the PtNPs with small particle sizes on the surface of GE.

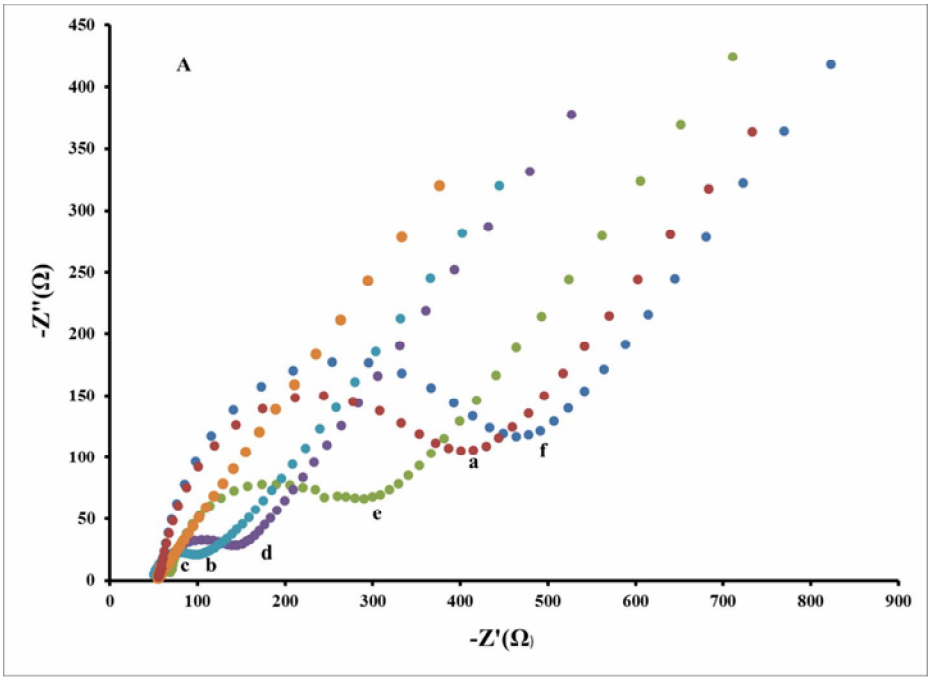


Fig. 2. A) Typical EIS studies of $[Fe(CN)_6]^{3-/4-}$ (5 mM, KCl 0.1 M) in 0.1 M PBS (pH = 7.5) at (a) GE (b) PtNPs/GE (c) Cys/PtNPs/GE (d) AgNPs/Cys/PtNPs/GE (e) anti-hCG/AgNPs/Cys/PtNPs/GE f) BSA/anti-hCG/AgNPs/Cys/PtNPs/GE. B) Recorded CVs for the different steps of the modified electrode: (a) to (f) are the same as (A). C) Recorded DPVs for the different steps (a) to (f) are the same as (A) and (B).

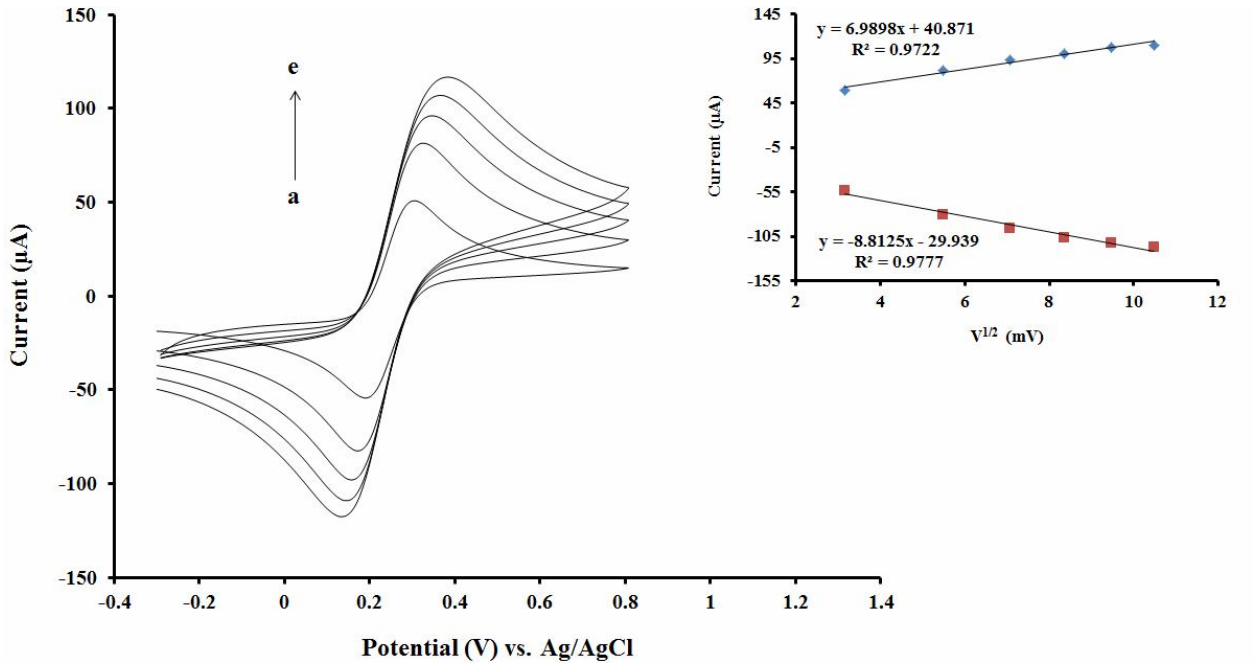


Fig. 3. Investigated three different parameters on the response of the immunosensor incubated with 4.5 ng ml^{-1} hCG in 2.5 M $[Fe(CN)_6]^{3-/4-}$ solution containing 0.1 M KCl. A) Influence of the pH of the solution, B) dependence of DPV peak currents on incubation time of immunoreaction, and C) incubation time in urea.

Electrochemical Characterization of the Stepwise-modified Electrode

EIS, CV and DPV have been employed to monitor the stepwise fabrication processes of the immunosensor using $[Fe(CN)_6]^{3-/4-}$ as redox probe. We utilized EIS to monitor the impedance change of the electrode after each modification step. EIS is a characterization technique providing electrical information in the frequency domain [20]. Figure 2A shows the observed Nyquest plots during immunosensor preparation process. In this graph, we see the EIS of the bare GE (curve a). After the electrodeposition of K_2PtCl_6, the resistance for the redox probe obviously decreased (curve b), and by dropping 10 µl Cys on the surface of PtNPs/GE, resistance of the electrode decreased (curve c). This is attributed to the electrostatic interaction between the terminal positively charged amine moieties and the negative charge of $[Fe(CN)_6]^{(3-/4-)}$ [21]. When AgNPs dropped on the surface of electrode, AgNPs with negative charge on the electrode surface repelled the negatively charged $[Fe(CN)_6]^{3-/4-}$ anions to the extent that the response of redox probe was reduced and thereby leading to enhanced electron-transfer resistance (curve d). In the next step, anti-hCG was immobilized on the electrode surface and R_{ct} clearly increased (curve e), which is attributed to reducing the effective surface area and available active sites for electron transfer process by protein [22]. After incubating immonosensor in BSA solution, R_{ct} likewise increased further more (curve f).

Also, the immunosensor fabrication process was investigated step by step by recording CVs of the modified electrode in the $[Fe(CN)_6]^{3-/4-}$ solution. Figure 2B shows the CVs of the electrode modification processes. First, a CV of the bare electrode was obtained (curve a). After the electrodeposition of K_2PtCl_6, the peak current increased (curve b). By dropping 10 µl Cys on the surface of PtNPs/GE, the peak current increased (curve c). The peak current decreased when AgNPs was dropped on the surface of electrode, (curve d). In the next step, anti-hCG was

immobilized on the electrode surface and the peak current clearly decreased (curve e). In the end, after that BSA was used, peak current decreased (curve f).

The CVs of the obtained immunosensor in PBS at different scan rates were investigated. Useful information involving electrochemical mechanism can be acquired from this investigation. As shown in Fig. 3, both anodic peaks current (I_{pa}) and cathodic peak current (I_{pc}) increased with the increase of scan rate. In addition, the peak currents of the immunosensor were directly proportional to the square root of the scan rate (insert in Fig. 3), suggesting that the electrode reaction is a diffusion-controlled electrochemical process.

Optimization of Experimental Conditions

The electrochemical performance of the immunosensor is determined by many factors, such as pH, incubation time in the hCG solution and urea solution. pH is an important parameter which greatly affects the analytical performance of the developed immunosensor. In this study, we noticed a significant impact of pH on the analytical performance of hCG immunosensor. As shown in Fig. 4A, the reduction peak current increased with increasing pH from 5.5 to 7.5 and then decreased as pH increased further. Therefore, the hCG solution with pH = 7.5 was applied for further experiments. The incubation time is an important parameter for proper formation of antigen-antibody complex which greatly affects the analytical performance of the immunosensor. Based on the study of the effect of incubation time according to Fig. 4B, for 4.5 ng ml^{-1} hCG, current response of the proposed immunosensor decreased rapidly to 50 min and then reached a plateau. Thus, 50 min was chosen for binding of hCG to the antibody modified electrode as the optimal in the following experiments**.** In addition, incubation time in urea solution was examined; and we found that if the incubation time in urea is increased to 25 min, the current response would correspondingly increase, whereas for a longer incubation time the current response would remain stable (Fig. 4C).

Calibration curve of Immunosensor

The calibration graph for the determination of hCG was obtained using BSA/Anti-hCG/AgNPs/Cys/PtNPs/GE as an immunosensor. Under optimal conditions, the immunosensor was incubated in hCG solution with different concentrations for 50 min, and then DPV measurements were performed in PBS (pH 7.5, 2.5 mM $[Fe(CN)_6]^{3-/4-}$) at 25 °C. With increase of hCG concentration, current response decreased because the formed hCG/anti hCG complex, acting as an inert block layer, hindered the transfer of electrons toward the surface of the modified electrode. The DPV detection was based on the change in the DPV currents response (ΔI) before and after the antigen-antibody reaction. The calibration curve of the immunosensor to different concentrations of hCG is shown in inset in Fig. 5. For the concentration range of 0.007 to 1.11 ng ml^{-1}, the regression equation was I (μA) = 1.9607 [hCG] (ng ml^{-1}) + 0.9578 (R^2 = 0.9905) and the LOD was estimated at 2 pg ml^{-1} (based on signal/noise [S/N] = 3). For higher concentrations from 1.11 to 68 ng ml^{-1}, the regression equation was I (μA) = 0.0563 [hCG] (ng ml^{-1}) + 3.1972 (R^2 = 0.9795) (inset in Fig. 5).

The performance of the developed immunosensor was superior to that of the other electrochemical immunosensors reported in the related literature, especially in comparison to the simple fabrication process and the use of cost-effective and biocompatible compounds. Acceptable factors that can affect the sensitivity and linear range of the proposed immunosensor are: 1) Large surface area of PtNPs causes the large amounts of anti-hCG immobilized on the electrode surface. 2) Covalent attachment of AgNPs with amin groups of anti-hCG leads to the more stability and repeatability in comparison to the adsorption method. 3) Cysteamine self-assembled monolayer was chemisorbed spontaneously on Pt substrates *via* the Pt-S bond. 4) PtNPs joint to AgNPs via Cys, increase the immobilized amount of antibodies on the electrode results in an increase in the sensitivity of the sensor. The performance of the developed hCG immunosensor was superior to that of other methods reported in the literature, especially in comparison with the reported LODs (Table 1).

Selectivity and Regeneration of Immunosensor

To test the selectivity of the prepared immunosensor for hCG analysis, some molecules including glucose, histidine, trypsin and progesterone were used confirming that the change in DPV resulted only from the specific binding of hCG antibody and its specific antigen. Compared with the

result obtained in the presence of hCG antigen, the peak current had no significant change (Fig. 6). Obviously, the developed strategy has a sufficient specificity to detect the hCG against the other interference substrates and this can be attributed to the fact that interaction between anti-hCG and hCG is based on the specific recognition between them but not on nonspecific adsorption. This indicates a good specificity of the fabricated immunosensor.

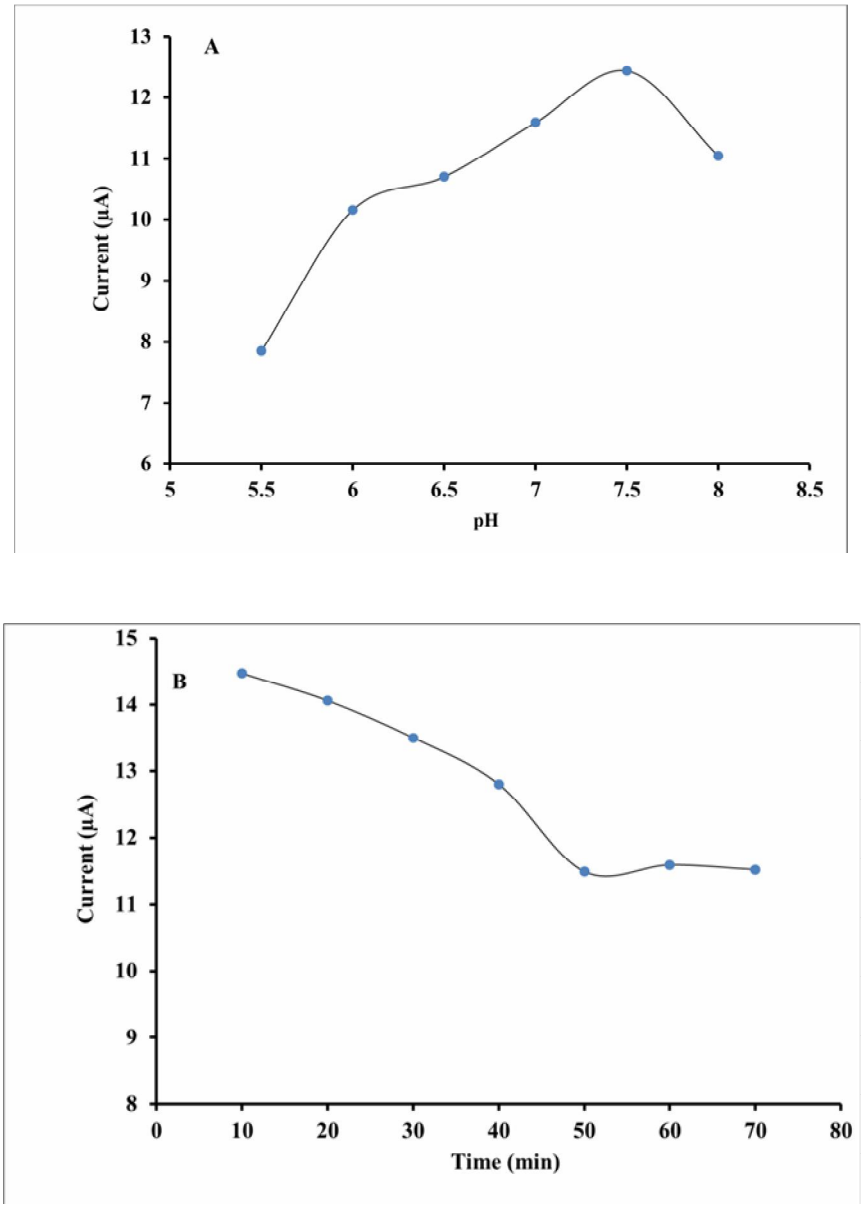


Fig. 4. DPV curves of the proposed immunosensor after incubating with different hCG solutions with concentrations of 0, 0.007, 0.074, 0.148, 0.444, 1.11, 4.44, 8.88, 16.3, 23.6, 45.8 and 68 ng ml^{-1} (from top to bottom) in $[Fe(CN)_6]^{3-/4-}$ as electrolyte solution. Inset: Calibration plots of the reduction current versus concentration of hCG.

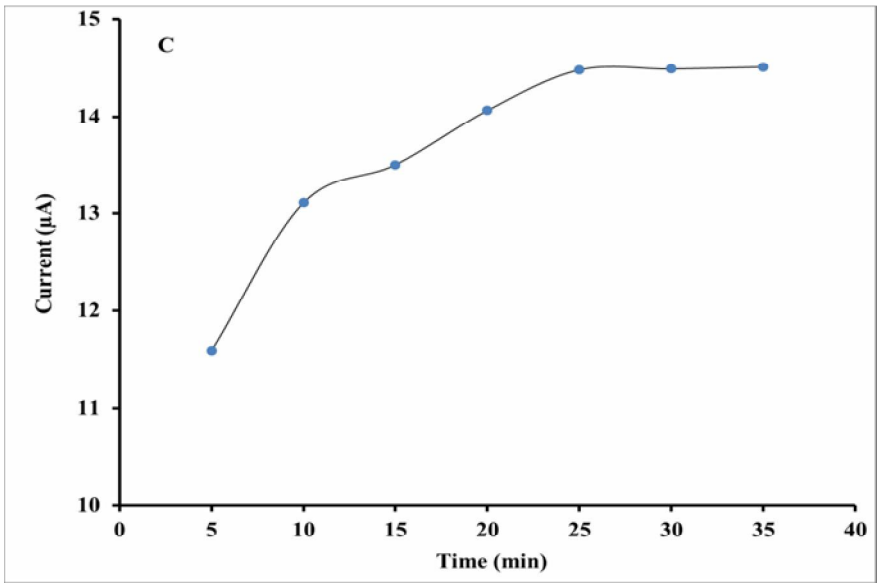


Fig. 4. Continued.

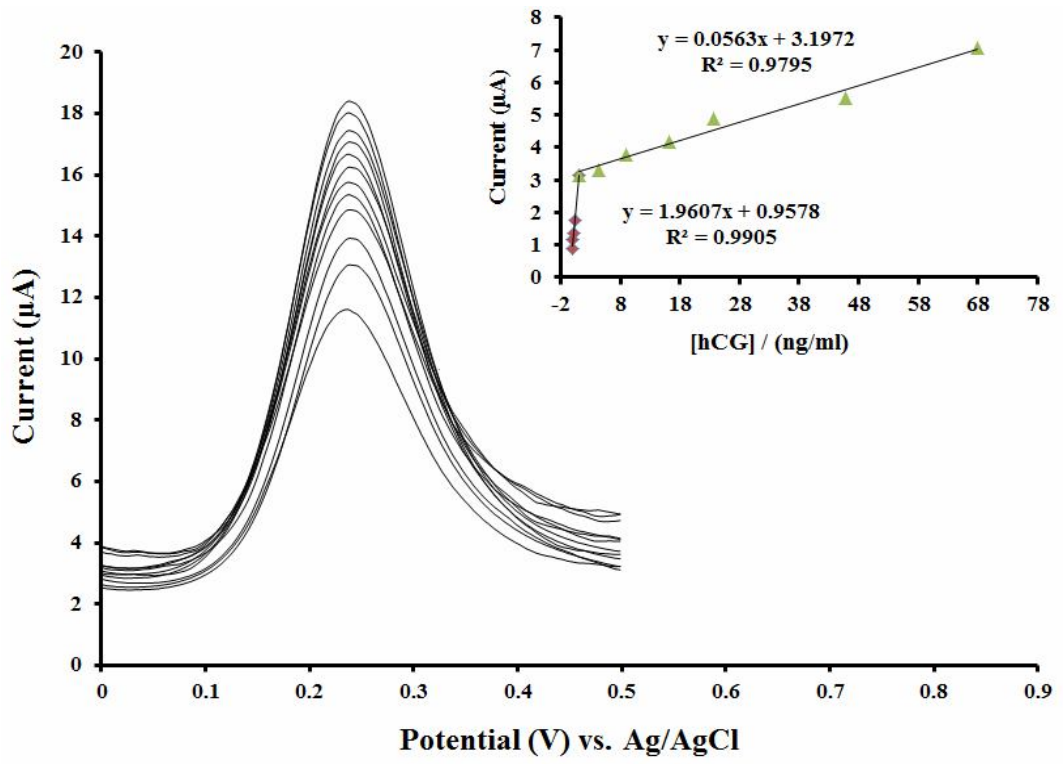


Fig. 5. Bar chart of the changes of immunosensor response after incubation with 45 ng ml^{-1} hCG and some biomoleculs: glucose (Glu), histidin (Hist), trypsin (Tryp) and progestron (Pr) (4.5 µg ml^{-1}).

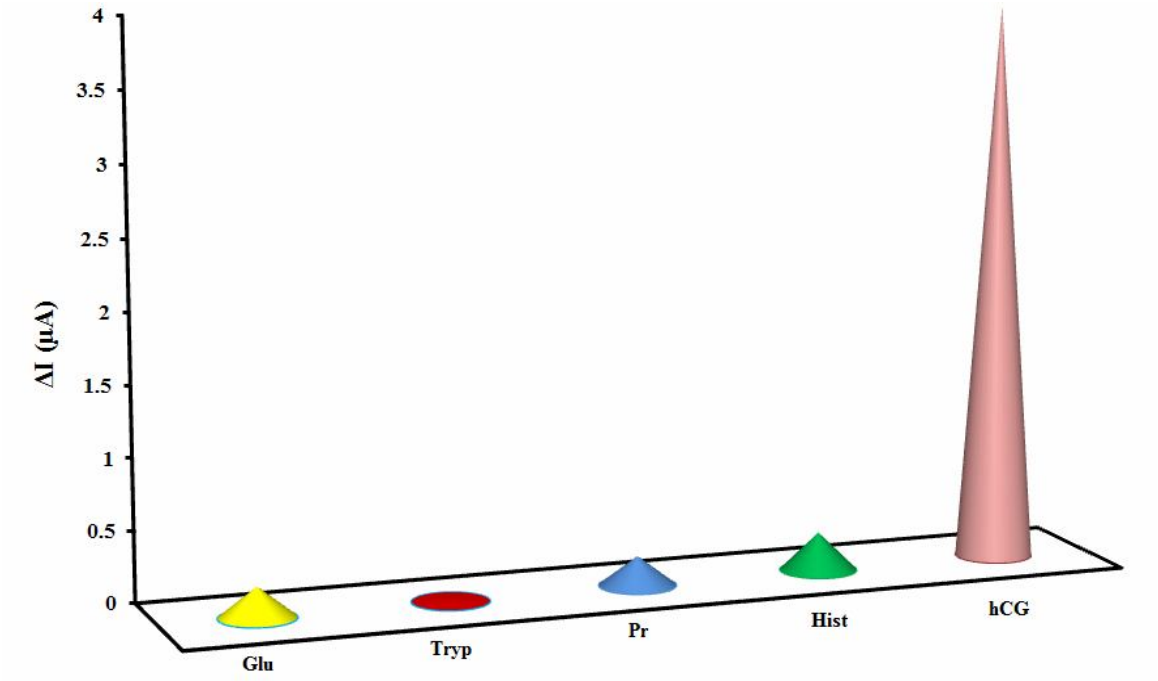


Fig. 6.

Table 2. Detection of hCG in Real Samples with Two Methods

Samples	Proposed immunosensor ($ng\ ml^{-1}$)	Reference method, ELISA ($ng\ ml^{-1}$)
1	45.5 ± 0.15	45
2	8.8 ± 0.2	9
3	60 ± 0.1	61

Regeneration of immunosensor is an important factor in studies of antibody-anti gene. Thus, in order to separate antigen-antibody linkage after each utilization of the immunosensor in detecting hCG, the immunosensor was treated with incubating it in 4 M urea solution for 25 min.

Analysis of the Real Samples

To test the validity of our proposed immunosensor in the clinical sample analysis, three serum samples were used as real samples that were diluted with the phosphate buffer (pH 7.5). The current response was measured for each diluted

sample. The amount of hCG was examined by the proposed immunosensor and ELISA method. The results for the determination of hCG in real samples are summarized in Table 2. The detected result revealed that this method can be used for the measurement of hCG in clinical routine test.

CONCLUSIONS

An ultrasensitive and selective electrochemical immunosensor has been developed for the detection of tumor marker hCG. The presence of PtNPs in the deposited surface of GE, covalent attachment of cysteamine on the PtNPs and functionalization of the anti-hCG with AgNPs lead to the amplification of the electrochemical signal. The immunosensor also has the advantages of low cost, high sensitivity, and good reproducibility which makes it a promising tool for clinical research and diagnostic application.

ACKNOWLEDGEMENTS

The financial support of Ilam University is gratefully acknowledged.

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