Regular Article

ANALYTICAL AND BIOANALYTICAL CHEMISTRY RESEARCH

Published by the Iranian Chemical Society

*Anal. Bioanal. Chem. Res.*, Vol. 4, No. 1, 79-90, June 2017.

# Immunoassay for Human Chorionic Gonadotropin Based on Glassy Carbon Electrode Modified with an Epitaxial Nanocomposite

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*(Received 1 June 2016, Accepted 22 December 2016)*

A highly sensitive electrochemical immunosensor was developed to detect hCG based on immobilization of hCG-antibody (anti-hCG) onto robust nanocomposite containing Gr, Chit, 1-methyl-3-octyl imidazolium tetra fluoro borate ionic liquid (IL) (Gr-IL-Chit). AuNPs were used to immobilize hCG antibody on the modified electrode. The amine groups of the antibody are covalently attached to AuNPs/Gr-IL-Chit nanocomposite. CV, EIS and SEM were employed to characterize the assembly process and the performance of the immunosensor. DPV and EIS studies demonstrated that the formation of antibody-antigen complexes decreased peak current and increased $R_{ct}$ of $[Fe(CN)_6]^{3-/4-}$ redox pair at the AuNPs/Gr-IL-Chit/GCE. The optimization of the pH of supporting electrolyte and the incubation time were studied in details. Because of the synergistic effect of IL, Chit and Gr and the unique properties of AuNPs, the obtained immunosensor exhibited a wide linear response to hCG in two ranges from 0.005-1.484 and 1.484-411.28 (mIU $ml^{-1}$). A relatively low detection limit of 0.0016 mIU $ml^{-1}$ (S/N = 3) was calculated from DPV. Satisfactory results were obtained for determination of hCG in human serum samples.

**Keywords:** Electrochemical immunosensor, Human chorionic gonadotropin, Nanocomposite, Impedance spectroscopy

## INTRODUCTION

Human chorionic gonadotropin (hCG) is a 37 kDa glycoprotein hormone. This is the first glycoprotein produced by trophoblasts of the placenta during pregnancy and is secreted by trophoblastic neoplasms and a variety of non-trophoblastic tumors [1]. The hCG molecule consists of two combined, dissimilar subunits designated alpha and beta. The beta subunit confers biological and immunological specificity to the entire hCG molecule by virtue of its unique amino acid sequence and content. The alpha subunit is essentially identical to the alpha subunit of the pituitary glycoprotein hormones: luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH) [2,3]. The appearance of hCG in urine or serum soon after conception and its rapid rise in concentration makes it an ideal indicator for the detection and confirmation of pregnancy. Thus, exact determination of the concentration of hCG in urine or serum plays an important role in monitoring of trophoblastic diseases in all modern immunological pregnancy tests [4]. Electrochemical immunosensors based on the specificity recognition of antigen and antibody is of great interest in clinical diagnosis. Electrochemical immunosensor is one of the most promising methods for detecting pathogenic biological species of clinical interest, for their low cost, fast response, simplicity, short response time, simple fabrication, small size, high sensitivity and a relatively low detection limit [5]. In order to increase sensitivity and selectivity of electrochemical immunosensing strategies, investigation of new composite materials has attracted widespread attention. Advances in nanotechnology have greatly influenced the field of electrochemical biosensors over the past few years. Much attention has been paid to the development of biocompatible and highly conductive nanomaterials for biosensing and biomedical applications, such as carbon

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nanotube, gold nanoparticles (AuNPs), graphene (Gr) and quantum dot. Among these nanomaterials, recently, Gr has been utilized in a number of forms for sensor and biosensor applications because of its interesting properties such as, large specific area, good conductivity and biocompatibility [6]. For immobilization of immunoreagent onto the electrode surface an effective and simple immobilization method is very important. Recently, organic-inorganic composite (or hybrid) materials have been one of the key research fields for investigation in today's material science. They combine the physicochemical attributes of components and improve their features. The use of ionic liquids (ILs) as binders with formation of IL-Gr paste due to the cation-cation-π interactions of ILs with Gr can prevent the aggregation of Gr [7]. Chitosan (poly-β-(1-4)-D-glucosamine), is a polysaccharide derived from deacetylation of chitin. It possesses many advantages such as excellent membrane-forming ability, high permeability towards water, good adhesion, biocompatibility, and high mechanical strength. Also, it has abundant reactive amino and hydroxyl functional groups; so, it has been widely used as an immobilization matrix for biofabrication [8]. Recently, some immunosensors based on various nanoparticles such as AuNPs, Ag nanoparticles and $TiO_2$ nanoparticles, *etc*., have been reported. AuNPs can firmly adsorb antibody because of their large specific surface areas, good biocompatibility and high surface free energies.

Herein, we design an electrochemical immunoassay using AuNPs/Gr-IL-Chit composite modified electrode, which constructs an effective antibody immobilization matrix and makes the immobilized immunocomponents possessing high stability and bioactivity. The performance of the developed hCG immunosensor is superior to that of other methods reported in the literature, especially in comparison with the reported LODs (Table 1). The immunosensor showed high sensitivity, and wide linear range owing to high surface to volume ratio and electronic structure of Gr, large-surface area of AuNPs causing large amounts of anti-hCGs immobilized on the electrode surface and covalent attachment of AuNPs with amin groups of Chit and anti-hCG. The resulting immunosensor is evaluated using Differential pulse voltammetry (DPV) and impedance spectroscopy (EIS), and utilized in the detection of hCG in biological samples.

## MATERIALS AND METHODS

### Reagents and Apparatus

Anti-hCG, hCG, $HAuCl_4$, sodium citrate ($Na_3C_6H_5O_7.2H_2O$), Chit, Gr, Bovine serum albumin (BSA), Progesterone and IL (1-methyl-3-octyl imidazolium tetra fluoro borate) were purchased from Sigma-Aldrich Co. LLC (USA). All other reagents with analytical grade such as glucose, ascorbic acid and NaOH were obtained from Merck or Fluka and used without further purification. All experiments were carried out at room temperature. Phosphate buffer solution (PBS, 0.1 M, pH = 7.4) containing 2.5 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl was used as a working solution. All aqueous solutions were prepared with deionized water. The real samples (human blood serum) were provided by a local clinical laboratory; a stock solution of human blood serum was diluted 50 times with PBS buffer (0.1 M), and then analyzed.

Cyclic voltammetry (CV), DPV and EIS experiments were performed with a μ-AUTOLAB electrochemical system type III and FRA board computer controlled Potentiostate/Galvanostate (Eco-Chemie, Switzerland) driven with NOVA software in conjunction with a conventional three electrode system with glassy carbon electrode (GCE) modified and unmodified 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 performed by scanning the potential from -0.1 to 0.7 V with modulation time of 50 ms and modulation amplitude of 25 mV. CV measurements were carried out from -0.2 to 0.6 V as initial and stop potential. EIS analysis was carried out with a bias potential of 0.2 V and a frequency range between 0.1 Hz and 100 kHz with signal amplitude of 5 mV. Nanocomposite and AuNPs were characterized by scanning electron microscopy (SEM). SEM images were recorded using a Vega-Tesacn electron microscope. The morphology of the Au nanoparticles was determined by a Hitachi H-800 transmission electron microscopy (TEM) at an operating voltage of 200 kV. A Metrohm model 780 pH/mV meters was used to measure the pH.

### Synthesis of Gold Nanoparticle

Gold nanoparticles were synthesized according to the

**Table 1.** Comparison of Different Immunosensors for Detection of hCG

| Modified Electrode | Method | LR[a] | LOD[b] | Ref. |
|---|---|---|---|---|
| Anti-hCG/nano-Au/MB[c]/GCE | CV | 1-1000 | 0.3 (mIU $ml^{-1}$) | [11] |
| Anti-hCG/nano-gold and CS hybrid film/GCE | AMP [m] | 0.2-1000 | 0.1 (mIU $ml^{-1}$) | [12] |
| Anti-hCG/GNPs[d]/pPA[e]/MWCNTs[f]/GCE | CV | 1-10, 10-160 | 0.3 (mIU $ml^{-1}$) | [5] |
| Anti-hCG/Pt–Au alloy nanotube array/GCE | AMP | 25-400 | 12 (mIU $ml^{-1}$) | [13] |
| Anti-hCG/gold nanotubesarray/GCE | AMP | 0.1-100 | 0.08 (mIU $ml^{-1}$) | [14] |
| Pt@MSN[g]/HRP[h]/$Ab_2$[i]/hCG Atigene/$Ab_1$/ TH[j]/Graphene/GCE | AMP | 0.01-12 | 7.50 (pg $ml^{-1}$) | [15] |
| Anti hCG/NPG[k]-Gs[l]/GCE | AMP | 0.5-40.00 | 0.034 (ng $ml^{-1}$) | [16] |
| Anti-hCG/Pd@SBA-15/TH/$HSO_3^-$GS/GCE | AMP | 0.01-16.00 | 8.60 (pg $ml^{-1}$) | [17] |
| Anti-hCG/Au@SiC–CS/GCE | DPV | 0.1-5,5-1000 | 0.042 (mIU $ml^{-1}$) | [18] |
| Anti-hCG/AuNPs/Gr-IL-Chit/GCE | DPV | 0.005-1.484, 1.484-411.28 | 0.0016 (mIU $ml^{-1}$) | This work |

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

references [9,10]. For a short time, at first, 500 ml $HAuCl_4$ 0.01% W/V solution was poured into a round-bottom flask equipped with a condenser and was heated and stirred until it reached boiling temperature. While stirring, 7.5 ml sodium citrate 1% was added to the solution. After 30 s, the solution turned into blue and after 70 s it turned into red. Boiling lasted for 10 min. Then, the heating was stopped and the solution was stirred for 15 min. The obtained solution was red and its particles were about 10 nm. After cooling, the solution was kept in refrigerator. The synthesized AuNPs were characterized by TEM (Fig. 1A).

## Preparation of Gr -IL- Chit Nanocomposite Modified Electrode

The GCE was polished with alumina powder, and then washed with deionized water and sonicated in ethanol, deionized water, respectively. The nanocomposite was prepared by mixing 1 µl of IL, 0.2 mg of Chit and 2 mg of Gr. The nanocomposite modified GCE was fabricated by casting nanocomposite onto the surface of a GCE and letting it stay at room temperature (for 6 h). The GCE modified with Gr-IL-Chit was washed with deionized water and then 8 µl of AuNPs solution was pipetted onto the surface of the modified electrode and after drying, the modified electrode was washed with distilled water. In this time the modified electrode can be used for electrochemical experiments. The composites of Gr with Chit and IL exhibited improved robustness and facilitated immobilization of antibodies. Gr was applied because of its particular properties, such as large surface area and high electrical conductivity. IL was used due to its ability in preventing the aggregation of Gr and improving properties of nanocomposite and Chit have been used as immobilization matrices for the immobilization of nanoparticle onto the surface of electrodes, since it has abundant reactive amino and hydroxyl functional groups.

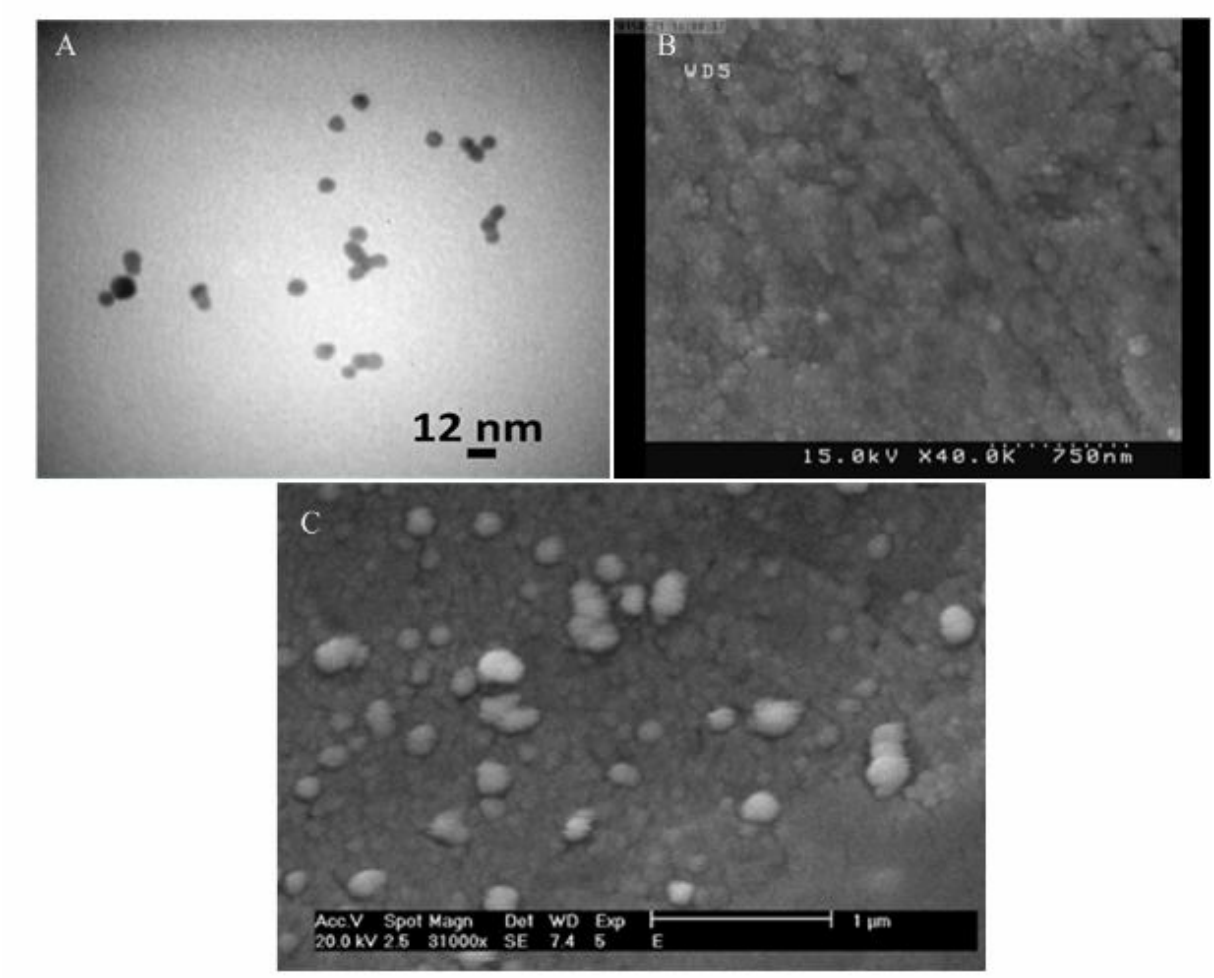



**Fig. 1.** A) TEM image of AuNPs A) SEM images of Gr-IL-Chit B) SEM images of AuNPs/Gr-IL-Chit.

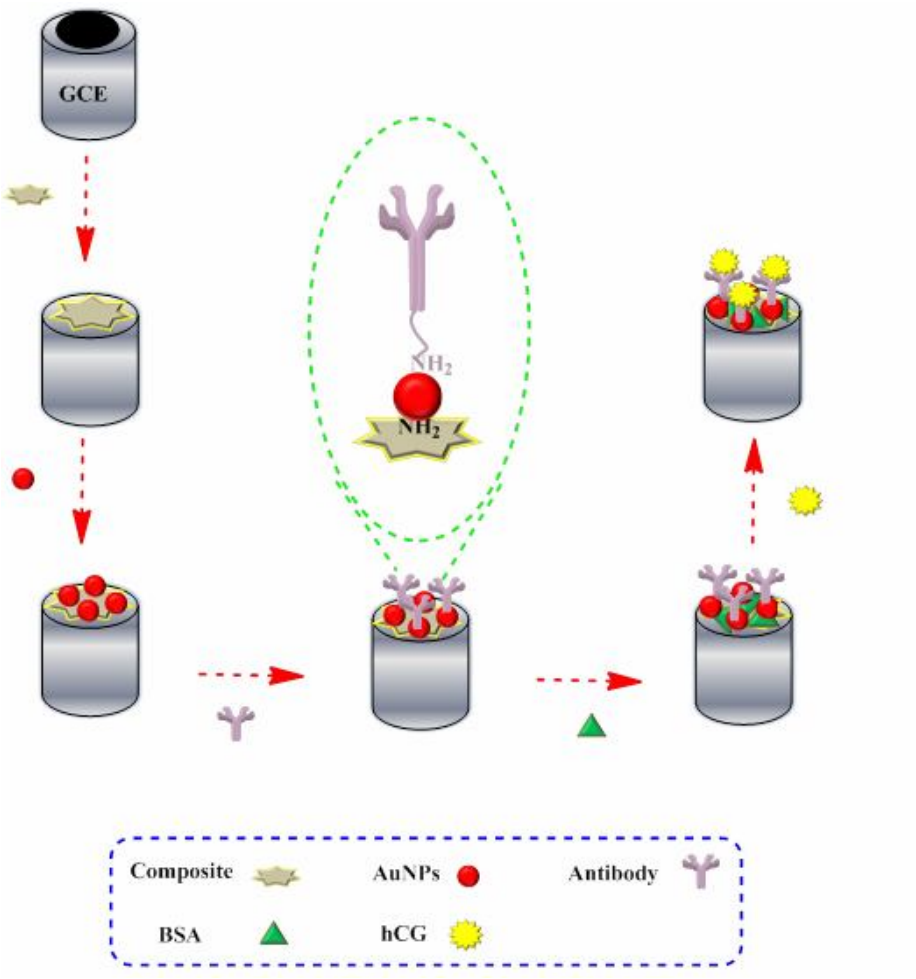
An interaction between AuNPs and amine group of chitosan resulted in a covalent immobilization of antibody molecules on the surface of the electrode.

## Fabrication of the Electrochemical Immunosensor

The GCE modified with AuNPs/Gr-IL-Chit was washed with water and immersed in PBS containing anti-hCG (1 mg $ml^{-1}$) at 4 °C for 12 h. At last, the resulting electrode was incubated in BSA solution (0.25% w/w) about 1 h in order to block possible remaining active sites and eliminate the risk of non-specific binding. The assemble process steps for the preparation of the immunosensor are shown in (Scheme 1). The prepared immunosensor was stored at 4 °C when not in use.

## Electrochemical Measurements

The formation of antigen and antibody complexes was performed by immersing the BSA/anti-hCG/AuNPs/Gr-IL-Chit/GCE into hCG solution for 50 min. After the specific reaction of antibody-antigen, the formed antigen-antibody immunocomplex on the electrode surface hindered the electron transfer toward the electrode surface, resulting in a decrease of electrochemical signal. The electrochemical measurements of the modified GCE were performed in 10 mM PBS (2.5 mM $Fe(CN)_6^{3-/4-}$ (the concentrations of $Fe(CN)_6^{4-}$ and $Fe(CN)_6^{3-}$ was 2.5 mM) + 0.1 M KCl, pH




*Scheme 1.* The schematic illustration of the stepwise immunosensor fabrication process

7.4)

## RESULT AND DISCUSSION

### Characterization of AuNPs/Gr-IL-Chit Modified GCE

The expected SEM images of GCE modified with, Gr-IL-Chits and AuNPs/Gr-IL-Chit is shown in (Fig. 1). A direct evidence for the attachment of AuNPs to the Gr-IL-Chit surface is given by the SEM tests. Compared with Fig. 1 C, Fig. 1B clearly shows that the AuNPs have decorated uniformly on the walls of Gr-IL-Chit. Furthermore, this uniform nanostructure provides an efficient electrode surface for loading anti-hCG (through the formation of covalent bond between Au and amine groups of the anti-hCG) and accelerating electron transfer.

### Electrochemical Characterization of Immunosensor

In this work Gr-IL-Chit nanocomposite was used for modification of the electrode and AuNPs were used to immobilize anti-hCG because it can be attached to $NH_2$ group of anti-hCG from one head, and to $NH_2$ group of Chitosan from another head existing in nanocomposite. Investigation of immunosensor was carried out by CV and EIS spectroscopy.

Cyclic voltammetry is an effective technique for probing

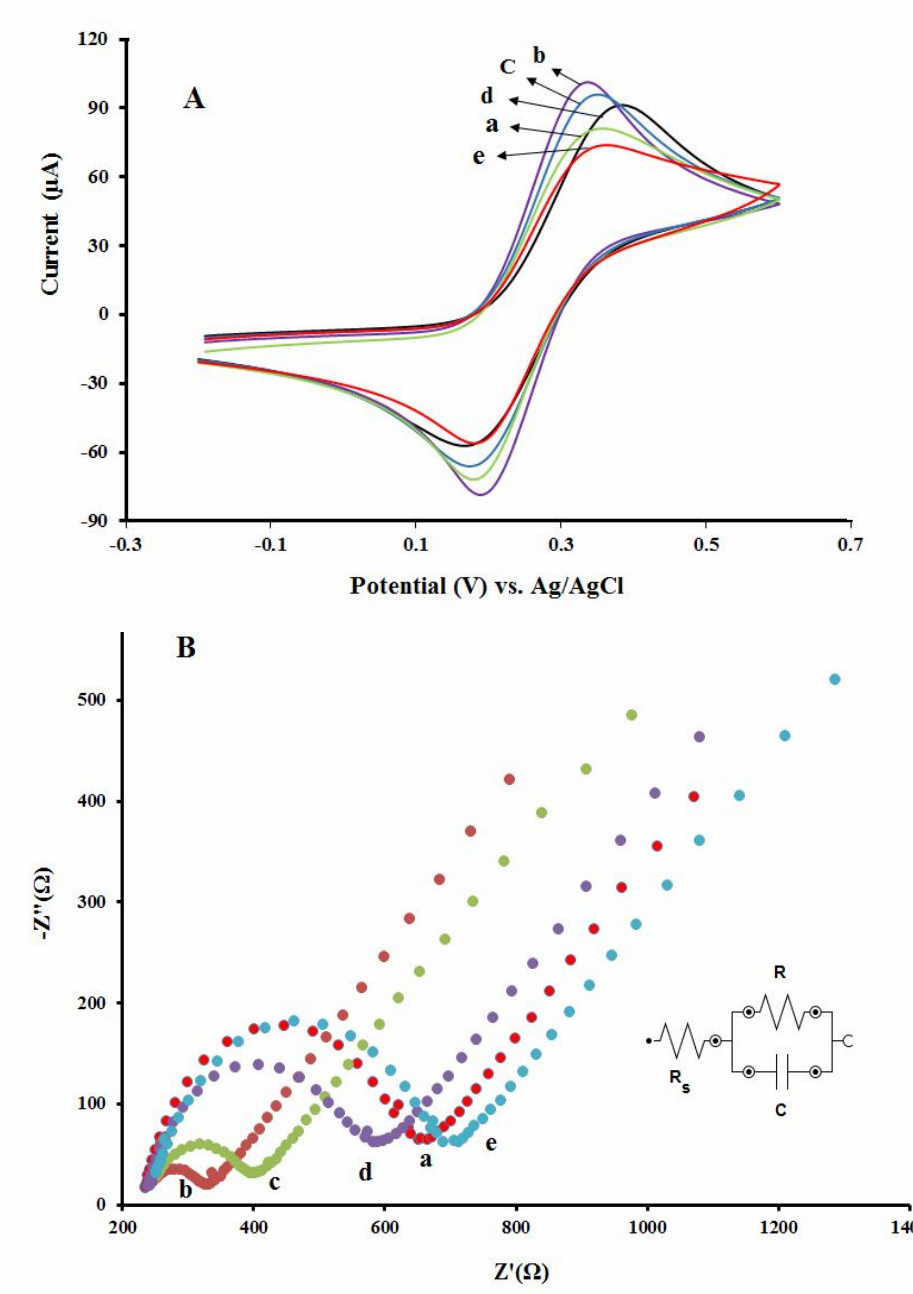



**Fig. 2. A)** Cyclic voltammograms of different electrodes in pH = 7.4 PBS solution containing 2.5 mM $[Fe(CN)_6]^{3-/4-}$ + 0.1 M KCl; scan rate, 50 mV $s^{-1}$. a) Bare GCE, b) Gr-IL-Chit/GCE, c) AuNPs/Gr-IL-Chit/GCE, d) Anti-hCG/AuNPs/Gr-IL-Chit/GCE, e) BSA-Anti hCG-AuNPs/Gr-IL-Chit/GCE. **B)** Nyquist plots for different electrodes in pH 7.4 PBS solution containing 2.5 mM $[Fe(CN)_6]^{3-/4-}$ + 0.1 M KCl. a) Bare GCE, b) Gr-IL-Chit/GCE, c) AuNPs/Gr-IL-Chit/GCE, d) Anti-hCG/AuNPs/Gr-IL-Chit/GCE e) BSA-Anti hCG-AuNPs/Gr-IL-Chit/GCE. The inset is the equivalent circuit of the immunosensor.

the feature of the modified electrode surface. The CV was carried out to investigate electrochemical behaviors after each assembly step. The CVs of the different modified electrodes in $[Fe(CN)_6]^{3-/4-}$ solution are presented in Fig. 2A. At the bare GCE, the $Fe(CN)_6^{3-/4-}$ redox label revealed reversible cyclicvoltammogram (voltammogram''a''). After modification of GCE with Gr-IL-Chit nanocomposite, the peak current increased greatly due to increase the effective surface area in the presence of Gr (voltammogram''b''). When AuNPs were immobilized on the electrode surface, the peak current decreased (voltammogram''c''). The AuNPs with negative charge on the electrode surface repel the negatively charged $[Fe(CN)_6]^{4/3-}$ anions; so, the response of redox probe was reduced and thereby led to enhanced electron-transfer resistance.

When anti-hCG was immobilized on the electrode surface the peak current clearly decreased. This result indicates the immobilization of anti-hCG on the electrode surface reducing the effective surface area and available active sites for electron transfer process (voltammogram''d''). Peak current decreased in the same way (voltammogram''e'') after BSA was used to block non-specific sites.

The immunosensor fabrication processes were also characterized by electrochemical impedance spectroscopy (EIS). Figure 2B shows the Nyquist plots during stepwise construction of immunosensor. The impedance spectra include a semicircle portion and a linear portion. The semicircle diameter at higher frequencies corresponds to the electron-transfer resistance ($R_{ct}$) which controls the electron transfer kinetics of the redox probe at the electrode interface. These results indicate very low resistance of nanocomposite modified electrode for redox probe. In this graph, we see that the EIS of the bare GCE displays a small semicircle at high frequencies and linear part at low frequencies (curve a, $R_{ct}$ = 429 Ω). After the bare electrode was modified with Gr-IL-Chit composite film, the resistance for the redox probe decreased (curve b, $R_{ct}$ = 78.2 Ω), indicating that Gr is an excellent electric conducting material accelerating the electron transfer. After dropping AuNPs on the surface of electrode, $R_{ct}$ increased because of its negative charge surface (curve c, $R_{ct}$ = 146.2 Ω). Subsequently, when the anti-hCG was placed on the surface of AuNPs, $R_{ct}$ increased dramatically (curve d, $R_{ct}$ = 333.5 Ω). This indicates that the antibody is immobilized on the electrode surface and further prevents the redox probe to the electrode surface. After incubating of immonosensor in BSA solution $R_{ct}$ increased further more in the same way (curve e, $R_{ct}$ = 427.3 Ω).

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 peak currents ($I_{pa}$) and cathodic peak currents ($I_{pc}$) 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

pH, incubation time in the hCG solution and also urea solution showed an important influence on the analytical performance of hCG immunosensor. In what follows, we investigated the importance of these factors.

## pH Optimizing

Since activities of biomolecules are pH dependent, we investigated the effect of this factor on the immunosensor response. The reduction peak current increased with increasing pH from 5 to 7.4 and then decreased as pH increased further. Therefore, a pH = 7.4 of the working buffer was applied for further experiments. This result is accordance with the fact that biological agents are more effective in an environment similar to that of the human body. The results are indicated in Fig. 4A.

## Optimization of Incubation Time

An important parameter for immuno-complex formation between antibody and antigen is the incubation time. The effect of incubation time on the immunoreaction is shown in Fig. 4, curves (B) and (C) showed respectively the change of current response with the increase in incubation time in hCG solution and urea solution. Based on the study of the incubation time, we found that current response of the immunosensor for 0.07 mIU $ml^{-1}$ hCG decreased with the increase of incubation time before 50 min and leveled off after 50 min. With the increase of hCG concentration,

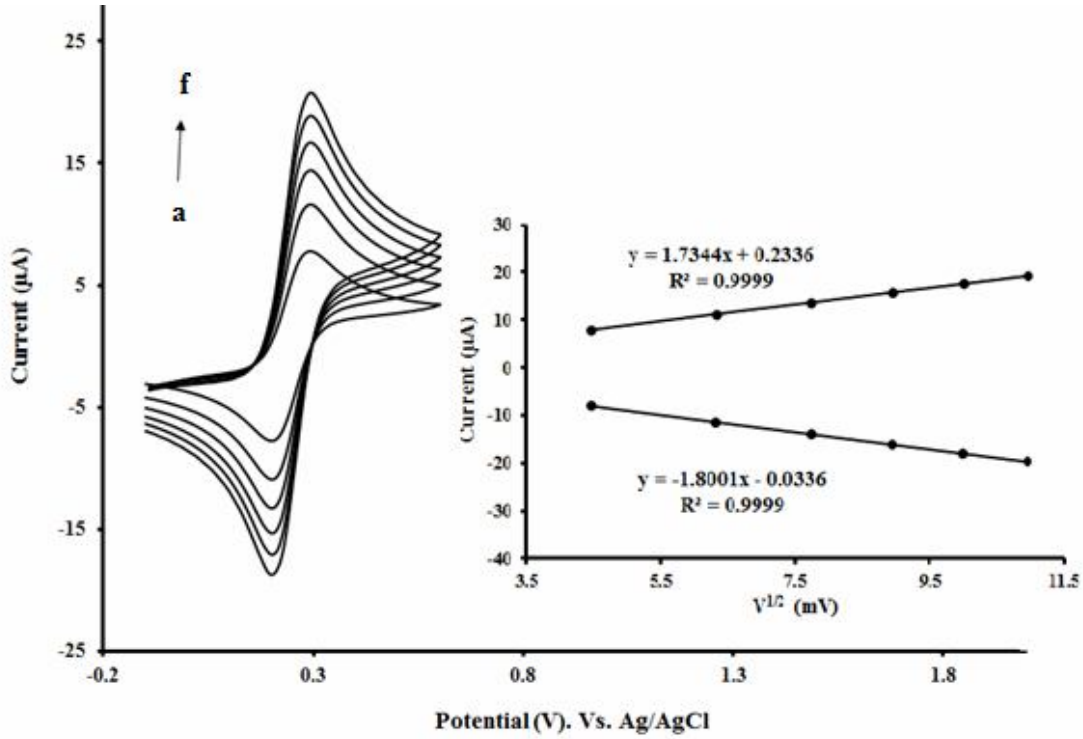



**Fig. 3.** CV studies of BSA/anti-hCG/Au/Gr-IL-Chit/GCE immunosensor at different scan rates (from a to f): 20, 40, 60, 80, 100 and 120 mV $s^{-1}$ in 2.5 mM $Fe(CN)_6^{3-/4-}$, 0.1 M KCl + 0.1 M PBS solution (pH = 7.4.), inset shows the plot of peak currents *vs.* $v^{1/2}$.

current response decreased because of the formed hCG/anti hCG complex acting as inert block layer and hindered the transfer of electrons toward the surface of the modified electrode. In addition, we examined incubation time in urea solution (4 M) and we found that increasing of incubation time in urea to 20 min caused an increase in current response (For breaking the link between hCG and anti-hCG) and for upper time, it remained stable.

## Calibration Curve of Immunosensor

Step by step, the standard solution of hCG at a known concentration was added into the incubation solution, and under optimal conditions the DPV of the immunosensor in the presence of different concentrations of hCG was performed in 0.1 M PBS solution containing 2.5 mM Fe $(CN)_6^{3-/4-}$. The hCG in solution interaction with anti-hCG immobilized on the surface of biosensor. The DPV peak current decreases with increasing the hCG concentration in the incubation solution (Fig. 5). The decrease of peak current was proportional to the concentration of hCG in two linear ranges from 0.005 to 1.484 mIU $ml^{-1}$, with a linear slope of 1.1331 µA/( mIU $ml^{-1}$) and correlation coefficient of 0.999 ($n$ = 3) and 1.484 to 411.28 mIU $ml^{-1}$ with a linear slope of 0.0037 µA/( mIU $ml^{-1}$) and correlation coefficient of 0.992 ($n$ = 8), inset in Fig. 5. A relatively low detection limit of 0.0016 mIU $ml^{-1}$ (S/N = 3) was calculated by DPV.

In this study, we also investigated the effect of hCG concentration on the immunosensor with EIS (LOD = 0.0009 mIU $ml^{-1}$). As illustrated in Fig. 6, increasing of hCG concentration leads to an increase in semi-circle diameter which indicates hCG interaction with anti-hCG. With increasing in concentration, more hCG will be placed on the surface, and this will consequently increase the resistance ($R_{ct}$). In other words, the impedance is influenced with the changes in amount, growth and morphological behavior of adherent substance. Therefore, this method can be proposed as an efficient way to monitor the formation of antigen-antibody interaction.

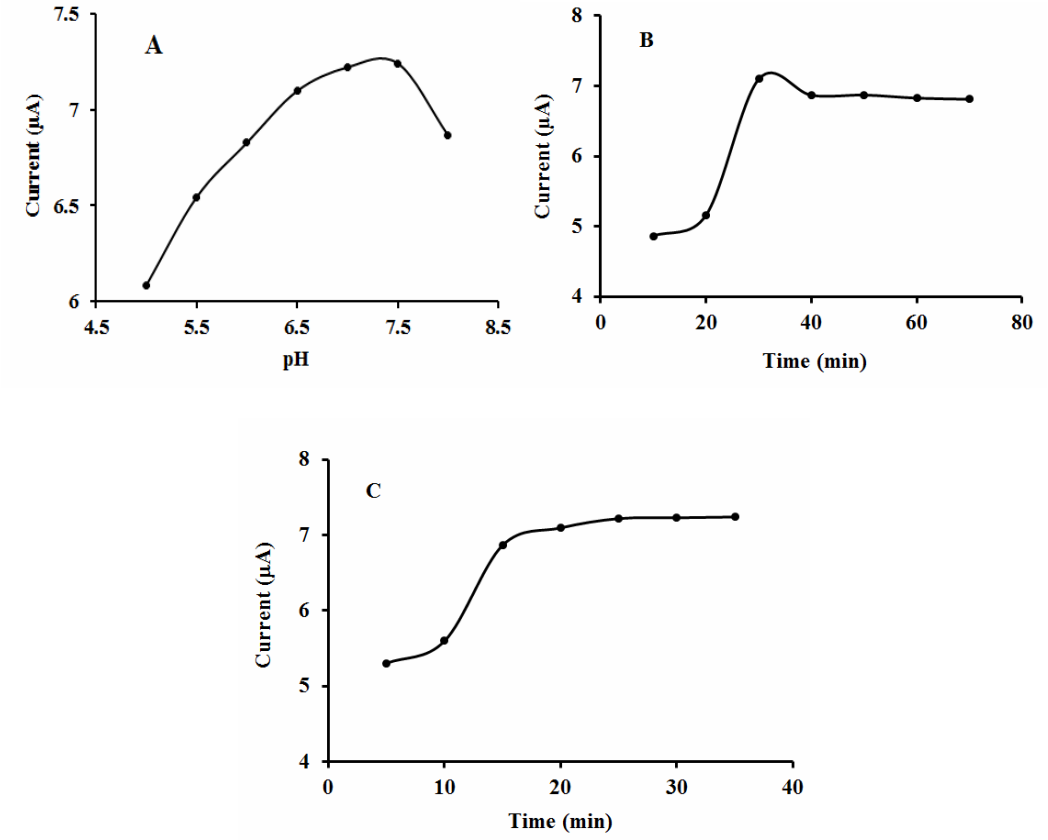



**Fig. 4.** Investigated three different parameters on the response of the immunosensor incubated with 0.07 mIU $ml^{-1}$ hCG in 2.5 mM $Fe(CN)_6^{4-/3-}$ solution containing 0.1 M KCl. A) Influence of the pH of the solution B) Dependence of peak currents on incubation time of immunoreaction and C) incubation time in Urea solution (4.0 M).

The analytical performance of the proposed immunoassay has been compared with other sensors reported previously (summarized in Table 1). The low detection limit and wide linear response to hCG for the proposed immunosensor is satisfactory. Acceptable factors affecting detection limit, sensitivity and linear range of the proposed immunosensor are: 1) High surface to volume ratio and electronic structure of Gr and unique features of IL such as cation-π intraction with Gr, 2) Large surface area of AuNPs causes the large amounts of anti-hCG immobilized on the electrode surface, 3) Covalent attachment of AuNPs with amin groups of Chit and anti-hCG lead to the more stability and repeatability in comparison with the adsorption method.

## Selectivity and Regeneration of Immunosensor

Selectivity is assessed by evaluating the effect of compounds present in the test solution, other than the target analyte (hCG), on the analytical response of a sensor device. Within the investigation of the selectivity of the immunosensors, it is found that the response of the sensor to hCG (0.6 mIU $ml^{-1}$) is significantly larger than its signal to some potentially interfering species such as ascorbic acid (AA), glucose (Gl), BSA, CEA and progesterone (PR) (60 mIU $ml^{-1}$) (Fig. 7).

An important factor in studies of antibody-antigen is regeneration of immunosensor. After using the immunosensor to detect hCG, the modified electrode was treated with 4 M urea solution for 20 min to dissociate

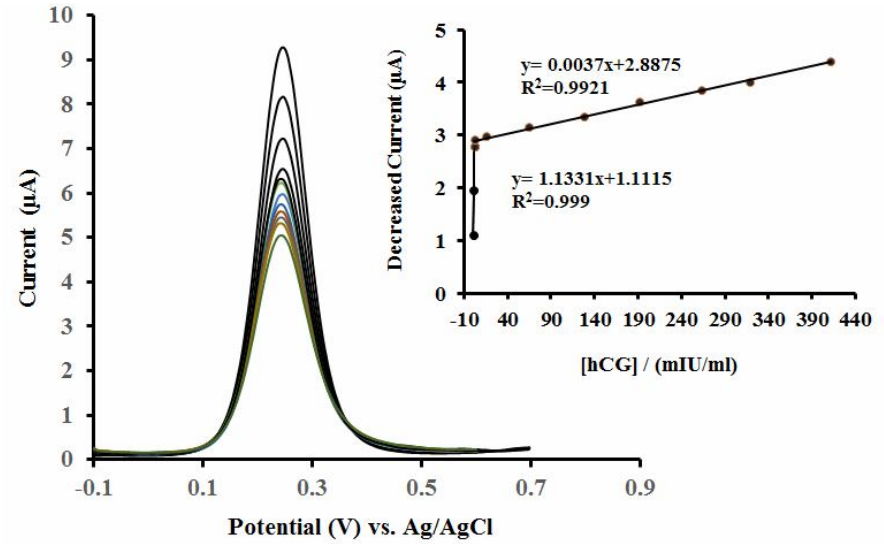



**Fig. 5.** Calibration curve for hCG determination (mIU $ml^{-1}$), from up to down: 0, 0.005, 0.0742, 1.484, 2.5, 14.84, 63.6, 127.2, 190.8, 262.88, 318 and 411.28 in 0.1 M pH = 7.4 PBS containing 2.5 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl. Pulse width: 0.05 S; amplitude: 0.025 V, Inset: Calibration plots of the reduction current *vs.* concentration of hCG.

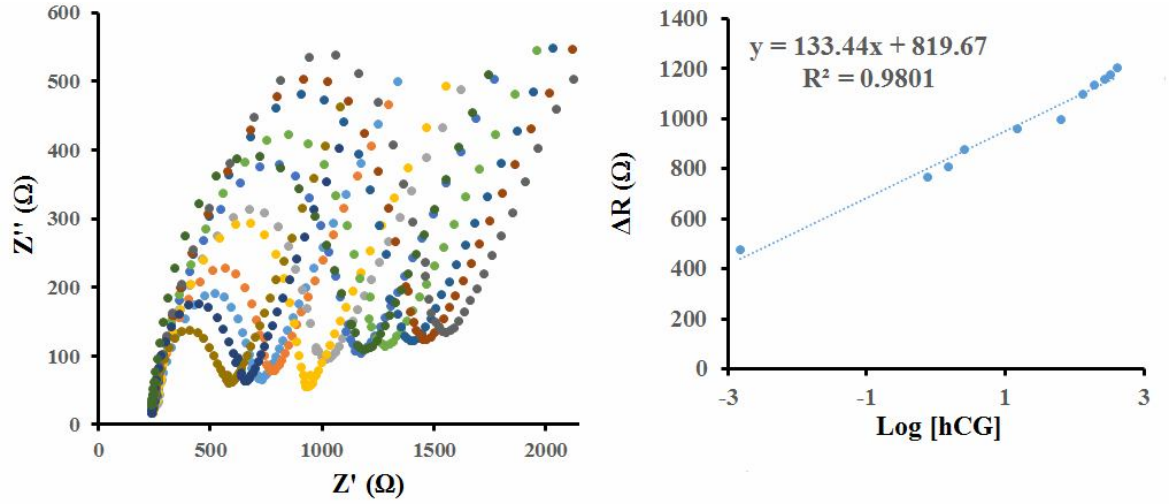



**Fig. 6.** Nyquist plots of the BSA/anti-hCG/AuNPs/Gr-IL-Chit/GCE Immonosensor, obtained in PBS pH = 7.4 containing 2.5 mM of $Fe(CN)_6^{3-/4-}$ previously incubated in increasing concentrations of hCG, from a to k (0, 0.005, 0.0742, 1.484, 2.5, 14.84, 63.6, 127.2, 190.8, 262.88 and 318 mIU $ml^{-1}$). Inset shows the plot of $\Delta R_{ct}$ *vs.* log[hCG].

Antigen-antibody linkage. The regenerated immunosensors were used to detect the same hCG concentration. The reproducibility of the electrode regeneration was examined by repeating the use-regeneration cycle six times. The RSD of 1.95% was obtained at the hCG concentration of 0.07 mIU $ml^{-1}$. This result indicates that the immunosensor

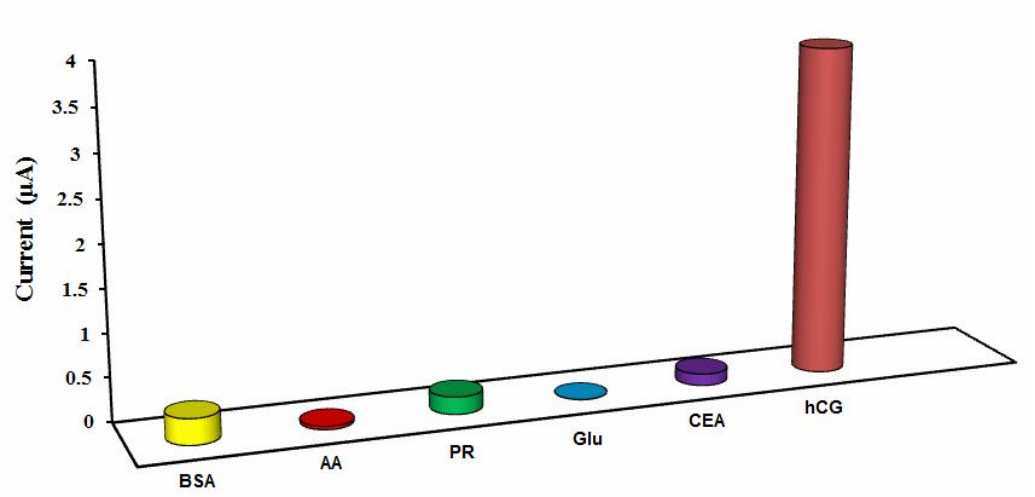



**Fig. 7.** Amperometric response of the immunosensor to interferents: glucose (Glu), carcinoembryonic antigen (CEA), ascorbic acid (AA), BSA and Progestron (Pr) (60 mIU $ml^{-1}$).

**Table 2.** hCG Detection (mIU $ml^{-1}$) in Serum Samples with a Developed Immunosensor

| Sample | hCG in serum | Added | Founded | Recovery (%) |
|---|---|---|---|---|
| | 0 | 19 | 19.5 ± 0.2 | 102.6 |
| A | 0 | 96 | 95.5 ± 0.1 | 99.4 |
| | 0 | 328 | 326.5 ± 0.2 | 99.7 |
| | 0 | 0.35 | 0.30 ± 0.02 | 95 |
| B | 0 | 1.5 | 1.45 ± 0.11 | 95 |
| | 0 | 3.5 | 3.4 ± 0.1 | 90 |
| | 0.53 | 1 | 1.55 ± 0.12 | 99.22 |
| C | 0.29 | 2 | 2.28 ± 0.14 | 99.56 |

could be regenerated and used again.

## Stability and Repeatability of Immunosensor

Stability of immunosensors is an important factor that shows their performance. In order to investigate this factor, during three weeks, the DPV of 10 mIU $ml^{-1}$ hCG solution was recorded, in every two days. After 21 days, it retained 85% of the initial response. The repeatability was evaluated via assaying 10 mIU $ml^{-1}$ hCG solution using the obtained immunosensor for 5 times and the relative standard deviation (RSD) was 1.22%. This result indicates satisfactory stability and repeatability of the immunosensor. Intra-electrode and inter-electrode coefficients of variation were used to investigate the reproducibility. The relative

standard deviation (RSD) of reproducibility was 3.3% for 5 measurements of hCG with the different immunosensors (Inter-electrode). Also for five times, the reproducibility of the immunosensor was estimated by determining hCG with one immunosensor (Intra-electrode) and RSD was calculated at 4.1%.

### Real Sample Analysis

Recovery experiments were performed by standard addition methods in spiked blood serum to evaluate the feasibility of the proposed immunosensor for real sample analysis. To this end, the human serum samples were ordered from a local clinical laboratory and subjected to an ultrafiltration by loading into a centrifugal filtration tube at 3000 rpm (30 min). Afterwards, the serum samples were diluted with PBS (0.1 M) and different concentrations of hCG were spiked to these samples. Finally, the serum hCG concentrations were detected with the calibration curve of the hCG immunosensors. The experimental results are shown in Table 2. They show an acceptable recovery. These results indicate that the system presented in this study can be valid for the analysis of hCG in biological fluids.

## CONCLUSIONS

In this work, we have designed a novel electrochemical immunosensor based on AuNPs/Gr-IL-Chit composite modified electrode for a rapid and sensitive immunoassay The results indicated that the AuNPs/Gr-IL-Chit based immunosensor can be used for detection of hCG at low detection limit. The detection limit, calculated from DPV, was 0.0016 mIU $ml^{-1}$ based on (S/N = 3). The immunosensor exhibited a wide linear response to hCG in two ranges from 0.005-1.484 mIU $ml^{-1}$ and 1.484-411.28 mIU $ml^{-1}$. In addition, the proposed sensitive approach holds great promise for the extended application in the fields of clinical diagnosis, bioaffinity assays and environmental monitoring.

## ACKNOWLEDGMENTS

The financial suppot of Ilam University is gratefully acknowledged.

## REFERENCES

[1] T. Fang, Y. Feng, J. Huangxian, Biosens. Bioelectron. 22 (2007) 2945.

[2] F.J. Morgan, Endocrinology 88 (1971) 1045.

[3] O.M. Bahl, R.B. Carlsen, R. Bellisario, N. Swaminathan, Biochem. Biophys. Res. Commun. 48 (1972) 416.

[4] H. Lund, S.B. Torsetnes, E. Paus, K. Nustad, L. Reubsaet, T.G. Halvorsen, J. Protein. Res. 8 (2009) 5241.

[5] J. Wang, R. Yuan, Y. Chai, S. Cao, S. Guan, P. Fu, Li. Min, Biochem. Eng. J. 51 (2010) 95.

[6] Q. Wei, R. Li, B. Du, D. Wu, Y. Han, Y. Cai, Y. Zhao, X. Xin, H. Li, M. Yang, Sens. Actuators, B 153 (2011) 256.

[7] J.Y. Sun, K.J. Huang, S.F. Zhao, Y. Fan, Z.W. Wu, Bioelectrochemistry 82 (2011) 125.

[8] K.J. Huang, D.J. Niu, W.Z. Xie, W. Wang, Anal. Chim. Acta 659 (2010) 102.

[9] G. Frens, Nature Phys. Sci. 241 (1973) 20.

[10] W.S. Sutherland, J.D. Winefordner, J. Colloid Interface Sci. 148 (1992) 129.

[11] R. Chai, R. Yuan, Y.Q. Chai, C.F. Ou, S.R. Cao, X.L. Li, Talanta 74 (2008) 1330.

[12] G.M. Yang, Y.B. Chang, H. Yang, L. Tan, Z.S. Wu, X.X. Lu, Y.H. Yang, Anal. Chim. Acta 644 (2009) 72.

[13] M. Tao, X.F. Li, Z.S. Wu, M Wang, H. Mei, Y.H. Yang, Clin. Chim. Acta 412 (2011) 550.

[14] G.M. Yang, X.Y. Yang, C.Y. Yang, Y.H. Yang, Colloids Surf. A: Physico Chem. Eng. Asp, 389 (2011) 195.

[15] Q. Wei, R. Li, B. Du, D. Wu, Y. Han, Y. Cai, Y. Zhao, X. Xin, H. Li, M. Yang, Sens. Actuators, B 153 (2011) 256.

[16] R. Li, D. Wu, H. Li, C. Xu, H. Wang, Y. Zhao, Y. Cai, Q. Wei, B. Du, Anal. Biochem. 414 (2011) 196.

[17] D. Wu, Y. Zhang, L. Shi, Y. Cai, H. Ma, B. Du, Q. Wei, Electroanalysis 25 (2013) 427.

[18] L. Yang, H. Zhao, Sh. Fan, Sh. Deng, Q. Lv, J. Lin, C.P. Li, Biosens. Bioelectron. 57 (2014) 1996.