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Electrochemical Immunoassay of Human Chorionic Gonadotrophin Based on Its Immobilization in Gold Nanoparticles-Chitosan Membrane

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Abstract
A human chorionic gonadotrophin (hCG) doped gold nanoparticles – chitosan membrane was prepared for forming an immunoconjugate of horseradish peroxidase labeled hCG antibody and hCG on glassy carbon electrode. The nanoparticles provided a congenial environment of the adsorbed proteins. Thus, the immobilized HRP-labeled immunoconjugate showed good enzymatic activity for the oxidation of *o*-phenylenediamine by H_2O_2. With a competitive mechanism, an amperometric method for immunoassay of hCG up to 30 mIU mL^{-1} with a relatively low detection limit of 0.26 mIU mL^{-1} at 3σ was developed. The hCG immunosensor showed good precision, high sensitivity, acceptable stability and reproducibility.

Keywords: Immunosensors, Immunoassay, Gold nanoparticles, Chitosan, Human chorionic gonadotrophin

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1. Introduction

During the past decades, immunoassays using the specific antigen – antibody immunoconjugates for analytical purposes have been extensively developed in many fields including food industry [1, 2], environmental protection [3, 4] and clinical control [5 – 7]. In order to avoid the utilization of radioisotopic labels with their apparently associated related problems such as disposal and operation safety, many electrochemical immunoassay systems combine the features of fast, sensitive, inexpensive and miniaturizable electrochemical measurement systems with the exquisite specific immunoassay procedures have been widely used for determination of clinically important substances [8 – 10]. In those electrochemical immunoassays, labeled immunoconjugate species immobilized on electrode surface through immunoreaction are often used as necessary to produce electrochemically active species or amplify electrochemical signal for the measurements [11 – 15].

As far as the immobilization of labeled immunoconjugate species and electrochemical transducers, solid supports such as carbon paste [16], gold [17], glassy carbon electrode (GCE) [10], spectrographic graphite [15] and screen-printed electrode [18] are usually used. However, a simple and stable enzyme immobilization approach on various interfaces to avoid the denaturation of immobilized bioconjugates has been a long-standing goal for the development of immunosensors.

Colloidal gold nanoparticles are of special interest, which have been extensively used as a matrix and cytochemical label for the immobilization and study of macromolecules. However, the process of dispersing nanoparticles in a polymer matrix is problematic, as it is necessary that the nanoparticles and the host matrix should be chemically compatible [19]. Here we adopted chitosan, (1,4)-α-amino-2-deoxy-β-D-glucan, to construct a gold nanoparticles modified membrane (nano-Au-CS) for loading of protein. Chitosan is a kind of attractive biocompatible matrix for the immobilization of various biomolecules due to its unusual combination of desirable properties including excellent membrane-forming ability, good adhesion, low cost, non-toxicity, high mechanical strength and hydrophilicity [20]. The presence of reactive amino and hydroxyl functional groups on the chitosan molecules results in their high susceptible property to chemical modifications. Particularly, its positively charged nature has a high affinity for adsorption of colloidal gold nanoparticles [21], which can provide an efficient scaffold of nanoscale dimensions with an orderly structural organization of a large variety of nanoparticles. The formed nano-Au-chitosan assembly can provide a suitable microenvironment similar to the native environment of bioconjugates in electrochemical immunoassay.

In this paper, we used human chorionic gonadotrophin (hCG), a 37 kDa glycoprotein hormone [9], as a model biomolecule to examine the application of the constructed gold nanoparticles modified chitosan membrane in electro-

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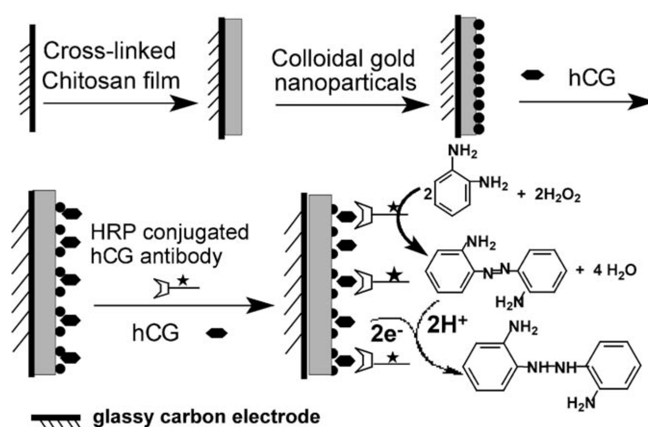
chemical immunoassay. HCG is an important indication of pregnancy as well as a carbohydrate tumor marker related to trophoblastic diseases. Thus, the detetction of hCG in urine or serum has been widely employed in clinical situations. Several immunosensors for hCG have been prepared [14, 22]. A separation-free sandwich-type enzyme immunoassay can detect hCG down to 2.5 mIU mL^{-1} [14]. Recently, a number of immunoassay kits and new methods for hCG determinations have also been reported [9,23–26]. In this work the nano-Au-chitosan scaffold proposed a sensitive electrochemical immunoassay for serum hCG with a detection limit of 0.26 mIU mL^{-1} at 3σ, which was below the threshold value of 14.3 mIU mL^{-1} hCG in serum for reliable pregnancy diagnosis [27]. Compared with some other reports, this novel detection strategy exhibited an acceptable accuracy and appeared to be practical, convenient and significant in clinic diagnoses.



Scheme 1. Schematic diagram of the hCG immunosensor based on electrochemical detection using *o*-phenylenediamine as an electron transfer mediator.

2. Experimental

2.1. Materials and Reagents

All reactions were carried out under an atmosphere of nitrogen. hCG and CA 19-9 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Diagnostic Products (DPC, USA). The ELISA kits consisted of a series of hCG standard solutions with different concentrations from 0 to 300 mIU mL^{-1}, a stock solution of HRP labeled hCG monoclonal antibody from goat. *o*-phenylenediamine and H_2O_2 with analytical grade were from the Shanghai Biochemical Reagent Company (China). Bovine serum albumin was the product of Sigma Chemical Company (St. Louis, MO USA). Phosphate buffer solution (PBS 0.1 M, pH 7.0) was used as the electrolyte in the measuring system. The dilute solution of enzyme conjugate contained 0.04% bovine serum albumin, 1.0 mM ethylenediamine tetraacetic acid and 0.1 M PBS. Mos-butyrylchitosan was prepared and characterized by Nanjing University. All other reagents were of analytical grade. All solutions were made up with deionized water of 18 MΩ purified from a Milli-Q purification system.

2.2. Measurements

Electrochemical measurements were performed on a BAS-100B electrochemical analyzer (Bioanalytical Systems Inc, USA) with a conventional three-electrode system comprising platinum wire as auxiliary electrode, saturated calomel electrode (SCE) as reference electrode and a HRP-anti-hCG/hCG/nano-Au-CS/GCE as working electrode. The electrochemical detection solution was 1.0 mM pH 7.0 PBS containing 2.0 mM *o*-phenylenediamine and 2.0 mM H_2O_2, which was deaerated thoroughly with highly pure nitrogen for 5 min and maintained in nitrogen atmosphere at $20 \pm 0.5\,^{\circ}C$. In presence of HRP immobilized on GCE surface, the electroactive species, 2,2′-diaminoazobenzene, was firstly produced. The mechanism of enzymatic and electrode reactions could be expressed as shown in Scheme 1 [28]. The differential pulse voltammetric (DPV) measurements were performed from -300 to -800 mV with the pulse amplitude of 50 mV and the pulse width of 50 ms.

UV-vis absorbance spectroscopy was carried out using a UV-2201 spectrophotometer (Shimadzu; Kyoto Japan). IRMA procedure was carried out with a FMJ-182 immunoradiometric α-counter according to the instructions and assay procedure in the operator's manual.

2.3. Preparation of Colloidal Gold Nanoparticles

All glassware used in this procedure was cleaned in freshly prepared 3:1 HNO_3.HCl, rinsed thoroughly in H_2O and dried prior to use. According to our previous results [29], colloidal gold nanoparticles of 24-nm-diameter were prepared by adding 0.5 mL of 1% trisodium citrate solution to 50 mL boiling solution of 0.01% $HAuCl_4$ (Aldrich, USA), which was maintained at boiling point for 15 min and stirred for another 15 min after removing the heating source.

2.4. Construction of Gold Nanoparticles Modified Chitosan Membrane

The hydrolysis of 1.0 wt.% Mos-butyrylchitosan was performed in 0.05 M acetic acid (Scheme 2). Then the mixture of 5 μL was dropped onto a pretreated GCE for preparation of chitosan membrane.

Prior to modification, the GCE (4 mm diameter) was polished to a mirror using 0.3 and 0.05 μm alumina slurry (Beuhler) followed by rinsing thoroughly with doubly distilled water. After sonicated successively in 1:1 nitric acid, acetone and doubly distilled water, the electrode was pretreated electrochemically by applying a potential of $+1.75$ V in 0.1 M pH 5.0 PBS for 300 s, and scanned between $+0.3$ and $+1.3$ V then $+0.3$ and -1.3 V until a steady-state

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Scheme 2. Preparation of cross-linked chitosan membrane on glassy carbon electrode.

current – voltage curve was observed [30]. This process led to the formation of hydroxyl groups on GCE surface and increased surface hydrophilicity of GCE [30, 31]. These groups could react with the hydrolysis product of 1.0 wt.% Mos-butyrylchitosan to form a cross-linked Mos-butyryl-chitosan membrane [32], as shown in Scheme 2, which was then soaked in prepared colloidal gold solution for 30 min to form nano-Au-chitosan assembly (nano-Au-CS/GCE).

2.5. Formation of Immobilized Immunoconjugate

HCG was firstly immobilized on nano-Au-CS/GCE by dropping 3 μL 300 mIU mL^{-1} hCG standard solution. After evaporation of water the electrode was rinsed thoroughly with pH 7.0 PBS and dried for an hour. The obtained hCG immunosensor was then refrigerated at 4 °C prior to electrochemical immunoassay. The competitive immuno-reaction was accomplished on the immunosensor surface. Firstly the stock solution of HRP labeled hCG antibody was diluted with the dilute solution to a total volume of 80 μL in a volume ratio of 3:1 (volume fraction = 0.75). 5 μL hCG standard or sample solution was then added in the diluted enzyme conjugate solution to prepare the incubation solution. After the immunosensor was incubated in the solution at 33 °C for 50 min and washed carefully with twice distilled water, the immobilized immunoconjugate layer was formed on the immunosensor, named as HRP-anti-hCG/hCG/nano-Au-CS/GCE. The amount of the immobilized immunoconjugate depended on the concentration of hCG in standard or sample solution.

3. Results and Discussion

3.1. Electrochemical Characterization of Electrode Surface

No amperometric response was observed at GCE, pre-treated GCE, hCG/nano-Au-CS/GCE and HRP-anti-hCG/hCG/nano-Au-CS/GCE in the work potential range in 0.1 M pH 7.0 PBS (curves a, b, c and d in Fig. 1). After electrochemical pretreatment, the GCE showed a greater charging current (curve b in Fig. 1). The immunosensor showed a relatively low background current (curve c in Fig. 1), which slightly increased after immunoreaction. After incubation, hCG antigen adsorbed on the nano-Au-Chitosan scaffold bound with HRP labeled hCG antibody, leading to the immobilization of immunoconjugates. When the solution contained *o*-phenylenediamine and H_2O_2, the cyclic voltammogram of obtained HRP-anti-hCG/hCG/nano-Au-CS/GCE showed a pair of stable and well defined redox peaks at 100 mV s^{-1} (curve f in Fig. 1), while no change was observed at hCG/nano-Au-CS/GCE (curve e in Fig. 1). The anodic and cathodic peak potentials were − 0.545 and − 0.610 V (vs. SCE), respectively (curve f in Fig. 1), corresponding to the redox of 2,2′-diaminoazobenzene, the enzymatic product [16]. With the increasing scan rate the separation of peak potentials (ΔE_p) increased, and both the anodic and cathodic peak currents were proportional to the square root of scan rate ($v^{1/2}$) (inset in Fig. 1). Thus, the oxidized and reduced forms of 2,2′-diaminoazobenzene did not adsorb on electrode surface. The immunosensor based on modified chitosan membrane without the presence of gold nanoparticles did not show observable electrochemical response due to the less adsorption of hCG imunoconjugates on the chitosan membrane.

3.2. Optimization of Colloidal Gold Nanoparticles Adsorption

The freshly prepared colloidal Au solution showed a maximum absorbance at 530 nm (curve a in Fig. 2), which was the characteristic absorbance of unaggregated colloidal gold nanoparticles [23]. After the CS/GCE was soaked in the solution for 10 min, the maximum absorbance decreased greatly (curve b in Fig. 2), indicating that some of the colloidal Au nanoparticles were adsorbed on the CS/GCE by strongly electrostatic interaction between protonated amino groups of cross-linked CS membrane and nanoparticles. With an increasing soaking time the maximum

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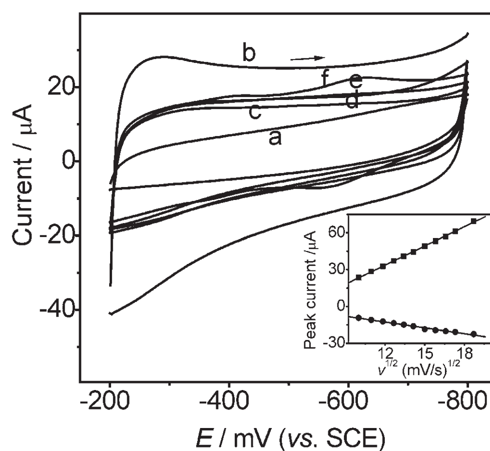

Fig. 1. Cyclic voltammograms of a) GCE, b) pretreated GCE, c) hCG/nano-Au-CS/GCE, and d) HRP-anti-hCG/hCG/nano-Au-CS/GCE in 0.1 M pH 7.0 PBS, e) (c) + 2.0 mM H_2O_2 + 2.0 mM *o*-phenylenediamine, f) (d) + 2.0 mM H_2O_2 + 2.0 mM *o*-phenylenediamine. Scan rate: 100 mV s^{-1}. Inset: plots of cathodic and anodic peak currents of (f) vs. $\nu^{1/2}$.

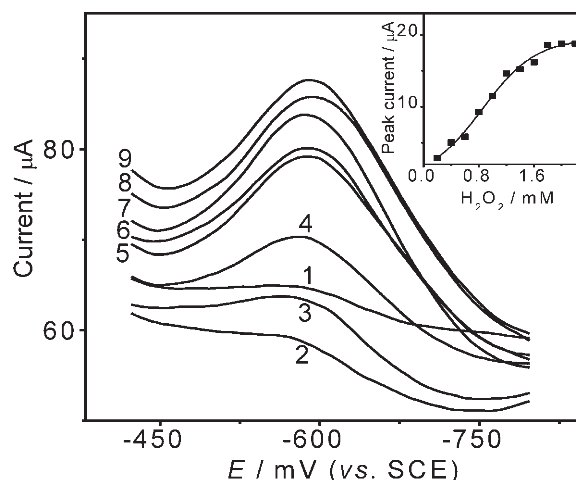

Fig. 3. DPV of hCG/nano-Au-CS/GCE in 0.1 M pH 7.0 PBS + 2.0 mM *o*-phenylenediamine without H_2O_2 (1) and with successive addition of 0.2, 0.4, 0.6, 0.8, 1.2, 1.6, 2.0, and 2.2 mM H_2O_2 (from 2 to 9). Inset: plot of peak current vs. H_2O_2 concentration.

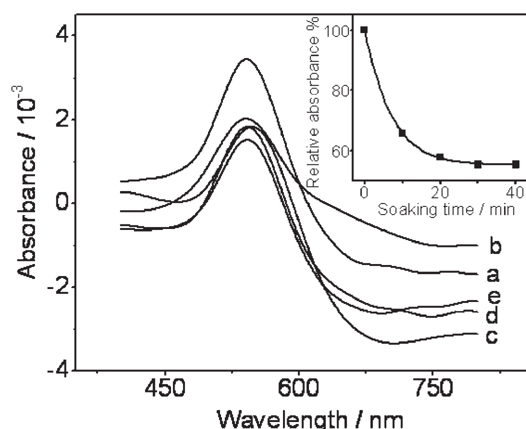

Fig. 2. UV-vis spectra of colloidal gold solution after soaking CS/GCE for a) 0, b) 10, c) 20, d) 30, and e) 40 min. Inset: plot of absorbance ratio of colloidal gold solution after soaking CS/GCE to initial colloidal gold solution vs. soaking time.

absorbance further decreased to reach a plateau after 30 min (inset in Fig. 2), which was selected as optimum time to prepare Au-CS/GCE.

3.3. Optimal Conditions for Enzymatic Reaction

With an increasing H_2O_2 concentration in detection solution the DPV peak current of HRP-anti-hCG/hCG/nano-Au-CS/GCE (no hCG in the incubation solution) increased and then reached a maximum value (Fig. 3), displaying a shape of Michaelis – Menten's curve. At low H_2O_2 concentration, both *o*-phenylenediamine and immobilized HRP were in excess, the rate producing 2,2′-diaminoazobenzene only depended on the concentration of H_2O_2. Thus, its reduction current was proportional to H_2O_2 concentration. When the concentration of H_2O_2 was higher than 2.0 mM, the enzymatic reaction rate became dependent on the amount of the immobilized HRP, resulting in a constant peak current. Thus 2.0 mM H_2O_2 was used for the activity determination of HRP in a competitive immobilized immunoconjugate. The apparent Michaelis – Menten constant, K_M^{app}, a reflection of both the enzymatic affinity and the ratio of microscopic kinetic constants, could be obtained to be 4.5 ± 0.8 mM from the electrochemical version of the Linweaver – Burk equation [33].

The amperometric response of the enzymatic reaction product was related to the solution pH. The relationship between the DPV peak current of 2,2′-diaminoazobenzene and the pH of detection solution indicated that the optimal pH range was between 7.0 and 7.2 with the maximum response at pH 7.0 (inset A in Fig. 4), which was just the optimum pH value for living organisms and usually also used for the binding of enzyme labeled conjugate with antigen. Consequently, pH 7.0 PBS was selected for enzymatic reaction. According to the dependence of formal potential on solution pH (inset B in Fig. 4B), the electrode process involved the participation of proton. Furthermore, the plot of formal potential versus pH showed a slope of 51.5 ± 0.97 mV pH^{-1} in the pH range of 4.3 – 9.2, which was coincident with the behavior of a two-electron and two-proton electrode process.

3.4. Optimization of Immunoreaction Conditions

The factors that influenced the immunoreaction included incubation temperature, incubation time and the concentration of HRP labeled hCG antibody in the incubation solution. Figure 5A shows the effect of incubation temperature on amperometric response of obtained immunoconjugate. The maximum response occurred at an incubation

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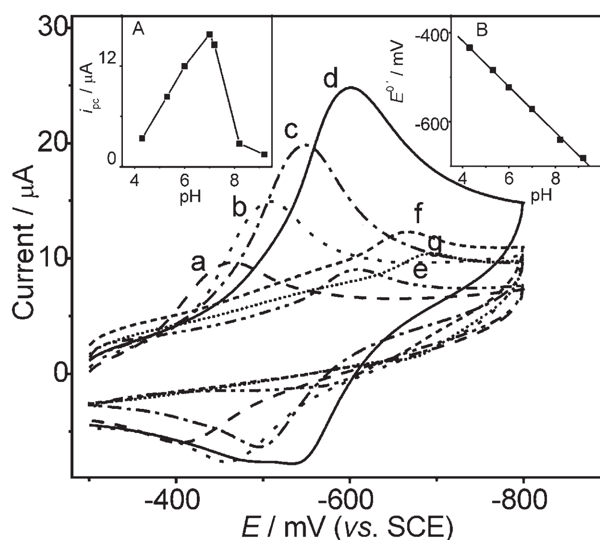

Fig. 4. Cyclic voltammograms of HRP-anti-hCG/hCG/nano-Au-CS/GCE in 0.1 M pH 7.0 PBS + 2.0 mM *o*-phenylenediamine + 2.0 mM H_2O_2 at pH 4.3, 5.3, 6.0, 7.0, 7.2, 8.2 and 9.2 (from a to g) at 100 mV s^{-1}. Inset: A) dependence of cathodic peak current on solution pH and B) effect of pH on formal potential.

temperature of 33 °C. The lower responses at other temperatures were attributed to the low immunoreaction rate at temperatures lower than 33 °C and the denaturation of labeled enzyme or immunoreagents at temperatures higher than 33 °C. At the temperature of 33 °C, the amperometric response of obtained immunoconjugate increased with an increasing incubation time, and reached a maximum at 50 min (Fig. 5B). Longer incubation time did not improve the response.

The effect of the volume fraction of HRP-anti-hCG of given concentration in incubation solution on DPV peak current was shown in Figure 5C. With the increasing volume fraction, the peak current increased and then trended to a constant value at the volume fraction of 0.75. Thus the incubation solution of 80 μL was prepared by mixing 60 μL HRP-anti-hCG stock solution and 20 μL dilute solution. The optimal incubation conditions were in HRP-anti-hCG solution diluted in a dilute ratio of 3:1 at 33 °C for 50 min to form HRP-anti-hCG/hCG/nano-Au-CS/GCE.

3.5. HCG Immunosensor Calibration Curve of Electrochemical Response

A competitive assay configuration was applied for determination of hCG sample. The standard solution of 5 μL hCG was firstly mixed with 80 μL incubation solution. The hCG in solution competed with the hCG in hCG/nano-Au-CS/GCE to bind the limited binding sites of the HRP labeled hCG conjugate. As expected for a competitive mechanism, the DPV peak current of the obtained HRP-anti-hCG/hCG/nano-Au-CS/GCE in detection solution containing H_2O_2 and *o*-phenylenediamine decreased with an increasing hCG concentration in the incubation solution (Fig. 6). According to the method reported previously [34] for a competitive immunoassay, a logistic four-parameter model was used to fit the calibration plot. The calibration range for amperometric immunoassay of hCG was up to 30 mIU mL^{-1} (inset in Fig. 6). The detection limit was 0.26 mIU mL^{-1} at 3σ, which was relatively lower than those of 2 [25], 2.5 [14], 11.2 [9] and 100 mIU mL^{-1} [35] reported with immunosensors or heterogeneous hCG immunoassays using electrochemical analytical principle. Thus, this proposed method could be used for detection of low level of hCG in serum sample. Higher serum hCG levels could be detected with an appropriate dilution.

3.6. Specificity, Reproducibility and Stability of the hCG Immunosensor

The specificity of proposed hCG immunosensor was examined by detecting the amperometric response to an incubation solution containing 5 mIU/mL carbohydrate antigen 19–9 (CA19–9), 5 mIU/mL hCG and the diluted enzyme conjugate. No difference of currents was observable in comparison with the result obtained in presence of only 5 mIU/mL hCG and the diluted enzyme conjugate. The

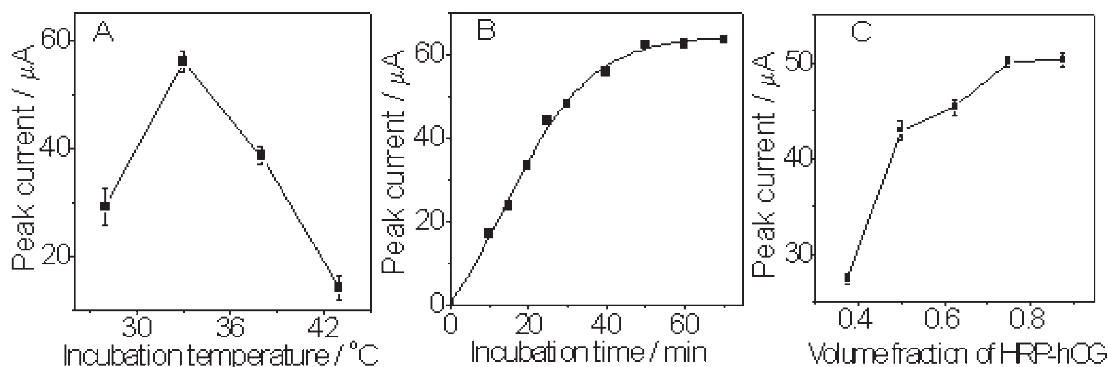

Fig. 5. Effects of A) incubation time, B) incubation temperature, and C) volume fraction of HRP-hCG conjugate in incubation solution on DPV peak current of HRP-anti-hCG/hCG/nano-Au-CS/GCE in 0.1 M pH 7.0 PBS containing 2.0 mM H_2O_2 and 2.0 mM *o*-phenylenediamine.

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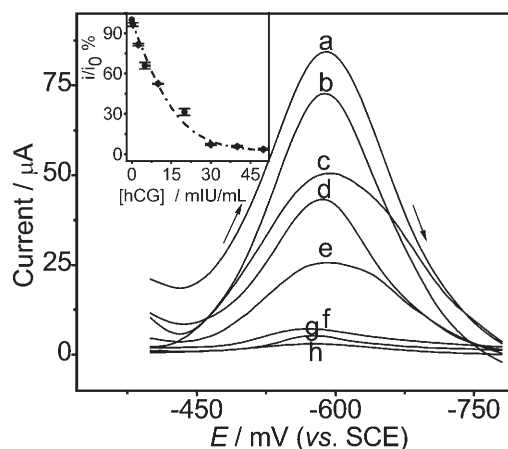

Fig. 6. DPV of 0.1 M pH 7.0 PBS + 2.0 mM *o*-phenylenediamine + 2.0 mM H_2O_2 at HRP-anti-hCG/hCG/nano-Au-CS/GCE obtained by incubating immunosensor in incubation solutions containing 60 μL HRP labeled hCG antibody and a) 0, b) 0.5, c) 5.0, d) 10.0, e) 20.0, f) 30.0, g) 40.0, and h) 50.0 mIU mL^{-1} hCG at 33 °C for 50 min. Inset: calibration for hCG determination fitted with a logistic four-parameter model. i/i_0 is the ratio of DPV peak currents at HRP-anti-hCG/hCG/nano-Au-CS/GCEs obtained by incubating immunosensors in incubation solutions in presence to absence of hCG sample.

increase of CA19 – 9 concentration to some extent did not lead to significant change of the current. After the immunosensor was used for detection of 5 mIU/mL hCG, the enzyme conjugate could be stripped from the formed immunocomplex by rinsing the immunosensor with pH 2.0 glycine-HCl. The regenerated immunosensor was incubated again in the incubation solution containing 5 mIU/mL hCG and the diluted enzyme conjugate. The formed immunocomplex showed 96% of the initial amperometric response to the detection solution. Thus, the immunosensor had a good selectivity to hCG as well as an acceptable regeneration efficiency.

The intra-assay precision of the immunosensor was examined by assaying the hCG levels of two sera with a successive detection for five times. The variation coefficients of intra-assay with this method were 3.7% and 7.9% at the hCG concentrations of 5 and 20 mIU mL^{-1}, respectively. The inter-assay precision, or the fabrication reproducibility, was estimated by determining in duplicate the hCG level in one serum sample with three immunosensors made at the same electrode independently. The inter-assay precision of this method was 5.1% at the hCG concentration of 10 mIU mL^{-1}, showing an acceptable reproducibility.

The biosensor lost its sensitivity rapidly if stored in air. However, if the immunosensor was stored in pH 7.0 PBS at 4 °C, it retained 90% of its initial current after a storage period of 30 days. This indicated that colloidal gold nanoparticles adsorbed on cross-linked chitosan were very efficient for retaining the bioactivity of immunoreagent and preventing the immunoreagent from leaking out because of the covalent interaction between colloidal gold nanoparticles and mercapto or primary amine groups in biological molecules.

3.7. Detection of Serum hCG Levels

The serum hCG levels in a series of samples were detected using the proposed hCG immunosensor. From the standard curve and the DPV peak currents of the hCG immunosensors incubated in the incubation solutions containing the obtained samples under the optimal conditions, we can see that the result detected by the proposed method is agreed with those obtained by ELISA method. Thus, the proposed method could be satisfactorily applied to the clinical determination of the hCG level in human serum.

4. Conclusions

Colloidal gold nanoparticles were demonstrated to be stable in chitosan matrix with an orderly structural organization as well as high densities. This provided a facile approach for immobilization of immunoconjugates. Under the catalysis of the formed immobilized HRP, the amperometric response of enzymatic reaction product was used for determination of serum hCG level without the requirement of separation or washing steps. The immunosensor showed good precision and acceptable sensitivity, reproducibility and storage stability, which were due to the advantages of colloidal gold nanoparticle – chitosan biopolymer matrix, including its good biocompatibility and the strong interaction between nano-Au and immobilized hCG immunoconjugates. Furthermore, the good assembly and ordered distribution of gold nanoparticles in chitosan membrane increased the immunoconjugate loading and simplified the fabrication process of immunosensor.

5. Acknowledgements

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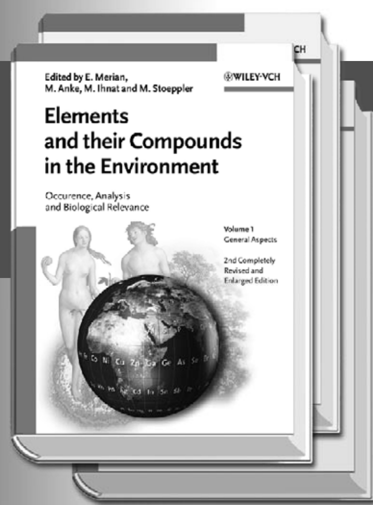

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