

## Full Paper

# Gold Nanoparticles Doped Three-Dimensional Sol-gel Matrix for Amperometric Human Chorionic Gonadotrophin Immunosensor

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Received: March 13, 2006

Accepted: June 2, 2006

## Abstract

A novel electrochemical immunosensor for human chorionic gonadotrophin (hCG) was proposed by immobilization of hCG in gold nanoparticles doped three-dimensional (3D) sol-gel matrix and an interfacial competitive immunoreaction. The 3D organized composite structure was prepared by assemble of gold nanoparticles into a hydrolyzed (3-mercaptopropyl)-trimethoxysilane sol-gel matrix, which showed good biocompatibility. After the interfacial competitive immunoreaction the formed HRP-labeled immunoconjugate showed good enzymatic activity for the oxidation of *o*-phenylenediamine by H<sub>2</sub>O<sub>2</sub>. With a competitive format, a method comprising of *o*-phenylenediamine-H<sub>2</sub>O<sub>2</sub>-immobilized HRP labeled hCG immunoconjugate system for immunoassay of hCG from 5.0 to 30.0 mIU mL<sup>-1</sup> was developed. The immunosensor showed good precision, high sensitivity, acceptable stability and reproducibility and could be used for detection of hCG in human serum with the consistent results in comparison with those obtained by a commercial analyzer.

**Keywords:** Immunosensors, Immunoconjugate, Gold nanoparticles, Sol-gel, Human chorionic gonadotrophin

DOI: 10.1002/elan.200603575

## 1. Introduction

In recent years, there has been considerable interest in the methodological development of immunoassay or immunosensor due to the specific antigen-antibody (Ag-Ab) recognition on various transducers [1–4] for different analytical purposes [5–12]. Compared with the common optical detection methods, electrochemical immunoassays and immunosensors which are useful in the case of point-of-care testing and in vivo monitoring appear to provide a promising platform with a wide application, especially for determination of clinically important substances [2, 8, 13], due to the simple and fast analytical process and inexpensive and miniaturizable instrumentation [14, 15].

In the field of electrochemical biosensors, stability or activity of the immobilized biocomponents on solid support has been a long-standing goal. Among various immobilized matrix adopted, sol-gel processing offers a facile route to demonstrate favorable merits such as tunable porosity and high thermal stability of inorganic materials, which are suitable for the encapsulation of a variety of biocomponents [16–20]. Furthermore, sol-gel-derived inorganic materials are attracting more attention for fabrication of biosensors or immunosensors [2, 21, 22]. A gold nanoparticles imbedded porous sol-gel composite network has been demonstrated for glucose sensing [19].

Colloidal gold nanoparticles are of special interest in the wide application of molecular self-assembly matrix for loading of biomolecules [23–25] and for electron conducting pathway [26]. Thus, the gold nanoparticles doped sol-gel hybrid materials have been extensively studied in the biosensor field [27–30]. Here by utilization of (3-mercaptopropyl)-trimethoxysilane (MPS) sol-gel (SG) as a functional sol-gel precursor containing multiple thiol and silane groups to fabricate an uniform and extensive silicate functional matrix [19, 27], an efficient three-dimensional scaffold (nano-Au-MPS scaffold) was prepared with an orderly structural organization of a large variety of nanoparticles on pretreated glassy carbon electrode. Thus, a novel immunosensor for human chorionic gonadotrophin (hCG) was developed based on this self-assembly of hydrophilic interfacial structure with a high retainment of stability and bioactivity of the immobilized immunocomponents.

The determination of hCG in human urine or serum is important for screening and monitoring of trophoblastic cancers and employed in modern immunological and pregnancy tests. Some electrochemical immunosensors for hCG have been prepared [31, 32]. Various immunoassay kits and methods for hCG analyte have also been proposed [13, 33–37]. This work used the nano-Au-MPS scaffold to develop an electrochemical immunoassay for serum hCG with a detection limit of 1.4 mIU mL<sup>-1</sup> at 3 $\sigma$ , which was below the threshold value of 14.3 mIU mL<sup>-1</sup> hCG in serum

for reliable pregnancy diagnosis [38]. Compared with some other reports, the novel detection strategy adopted three-dimensional sol-gel network by hydrolysis of silicon alkoxide and self-assembling nanoparticles to construct a uniform structure for effective loading of immunoconjugates and offered a versatile, practical and convenient protocol in clinic diagnoses.

## 2. Experimental

### 2.1. Materials and Reagents

(3-Mercaptopropyl)-trimethoxysilane (MPS) and bovine serum albumin (BSA) were the products of Sigma (St. Louis, MO, USA). Carbohydrate antigen 19-9 (CA 19-9) and hCG enzyme-linked immunosorbent assay (ELISA) kits were purchased from BioCheck, Inc. (Foster city, USA). The ELISA kits consisted of a series of hCG standard solutions at 0, 5, 20, 50, 150 and 300 mIU mL<sup>-1</sup> (IU stands for international unit that was given by the kits), an enzyme conjugate reagent of HRP-labeled hCG monoclonal antibody (HRP-anti-hCG) from mouse and a hCG zero buffer for in vitro diagnostic incubation. 0.1 M pH 7.0 phosphate buffer solution (PBS) was used as the electrolyte in the measuring system. The dilute solution of hCG standard or sample solution was 0.1 M pH 7.0 PBS containing 0.04% BSA and 1.0 mM ethylenediamine tetraacetic acid. All other reagents were of analytical grade. All solutions were made up with deionized water.

### 2.2. Apparatus

UV-vis absorbance spectroscopy was carried out using a UV-2201 spectrophotometer (Shimadzu; Kyoto Japan). The Access analyzer (Beckman Coulter, Inc., Chaska, USA) was

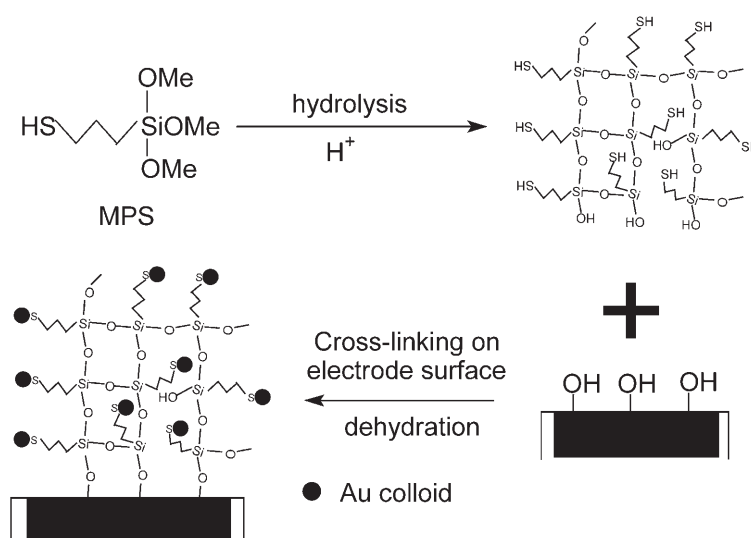
used to detect hCG in human sera by an automated chemiluminescent immunoassay. Briefly, the serum sample and the limited hCG conjugated with alkaline phosphate were added to a reaction vessel coated with polyclonal goat antibody specific for human hCG. After washing to remove the unbound conjugate, a chemiluminescent substrate was added and the resulting light was recorded with a luminometer.

### 2.3. Preparation of Silica Sol-gel and hCG Immunosensors

Silica sol-gel stock solution was prepared by mixing MPS with deionized water at a molar ratio of 1:4, ethanol at 10% (v/v) and 0.1 mol L<sup>-1</sup> hydrochloric acid at 3.3% (v/v) and sonicating the homogeneous mixture for 30 min until a pellucid solution was obtained. The solution was stored at room temperature for 2–3 h [28, 29] to obtain silica sol.

Colloidal gold solution was prepared by adding 0.5 ml 1% trisodium citrate solution to 50 mL boiling solution of 0.01% HAuCl<sub>4</sub> (Aldrich, USA), which was maintained at boiling point for 15 min and stirred for another 15 min after removing the heating source. The glassy carbon electrode (GCE, 3 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 current-voltage curve was observed [39]. This process led to the formation of hydroxyl groups on GCE surface and increased surface hydrophilicity of GCE [39, 40].

3 μL hydrolyzed MPS (silica sol) was firstly assembled on a pretreated GCE to form a cross-linked MPS (silica gel) membrane (Scheme 1), which was full of thiol-functional



Scheme 1. Preparation of gold nanoparticles doped MPS membrane on glassy carbon electrode.

groups [27]. The cross-linked gel membrane modified GCE was soaked in prepared colloidal gold solution for 10 min to form nano-Au-MPS assembly (nano-Au-SG/GCE).

The immobilization of hCG was accomplished by dropping 3  $\mu\text{L}$  300 mIU  $\text{mL}^{-1}$  hCG standard solution on nano-Au-SG/GCE. After evaporation of water, hCG molecules were strongly adsorbed in the network to form a stable immunosensor. The immunosensor was rinsed thoroughly with pH 7.0 PBS, dried for an hour, and then refrigerated at 4 °C prior to electrochemical immunoassay.

## 2.4. Formation of Immobilized Immunoconjugate

The competitive immunoreaction 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  $\mu\text{L}$  in a volume ratio of 3:1 (volume fraction = 0.75). 5.0  $\mu\text{L}$  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 30 °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-SG/GCE. The amount of the immobilized immunoconjugate depended on the concentration of hCG in standard or sample solution.

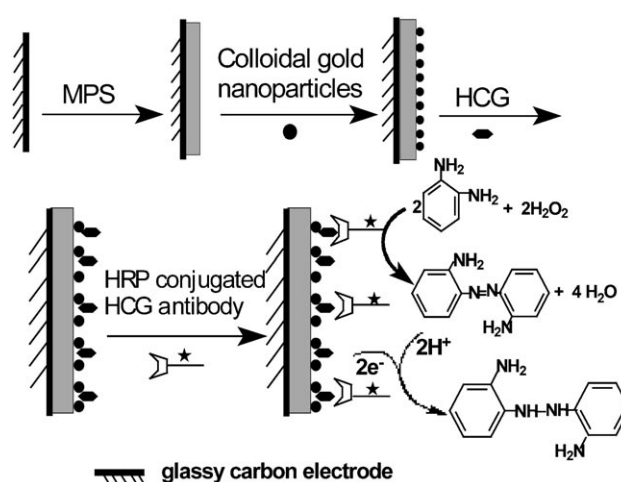
## 2.5. Electrochemical Measurement

Voltammetric 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 modified glassy carbon electrode (GCE) as working electrode. The electrochemical detection solution was 1.0 mM pH 7.0 PBS containing 1.0 mM *o*-phenylenediamine and 3.0 mM  $\text{H}_2\text{O}_2$ , which was deaerated thoroughly with highly pure nitrogen for 5 min and maintained in nitrogen atmosphere at room temperature. 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 2 [41]. 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.

## 3. Results and Discussion

### 3.1. Electrochemical Behaviors of Modified Electrodes

No amperometric response was observed at both bare GCE, pretreated GCE and HRP-anti-hCG/hCG/nano-Au-SG/GCE in the working potential range in 0.1 M pH 7.0 PBS



Scheme 2. Schematic diagram of the hCG immunosensor based on electrochemical detection using *o*-phenylenediamine as an electron transfer mediator.

(curves a, b and c in Fig. 1). After electrochemical pretreatment the GCE showed a relatively lower charging current due to the active surface of GCE [39, 40] (curve b in Fig. 1). When the solution contained *o*-phenylenediamine and  $\text{H}_2\text{O}_2$ , the cyclic voltammogram of HRP-anti-hCG/hCG/nano-Au-SG/GCE at 100  $\text{mV s}^{-1}$  showed a pair of stable and well defined redox peaks, the anodic and cathodic peak potentials were  $-0.559$  and  $-0.607$  V (vs. SCE), respectively (curve d in Fig. 1), corresponding to the redox of 2,2'-diaminoazobenzene, the enzymatic product [41].

With the increasing scan rate the separation of peak potentials ( $\Delta E_p$ ) increased (Fig. 2), and both the anodic and cathodic peak currents were proportional to the square root of scan rate ( $v^{1/2}$ ) (inset in Fig. 2). Thus, the oxidized and reduced forms of 2,2'-diaminoazobenzene did not adsorb on electrode surface. Here the colloidal gold nanoparticles could provide a congenial microenvironment similar to that of redox protein in a native system and allow the protein molecules more freedom in orientation [42], thus facilitated close approach of the protein to the sensing surface, reduced the insulating property of the protein shell for the direct electron transfer, and could be thought of as “electron antennae” to funnel electrons between the electrode and the immobilized HRP [23, 26, 42, 43], which provides stereo attaching sites for gold nanoparticles and an ideal platform for the immobilization of hCG molecules.

### 3.2. UV-Vis Spectrum of MPS Sol and Colloidal Gold Solution

MPS sol did not show any absorption (curve a in Fig. 3). The freshly prepared colloidal Au solution showed a maximum absorbance at 525 nm (curve b in Fig. 3), which was the characteristic absorbance of unaggregated colloidal gold nanoparticles [33]. After the colloidal Au solution was mixed with the MPS sol, the maximum absorbance decreased slightly (curve c in Fig. 3). The similarity of the

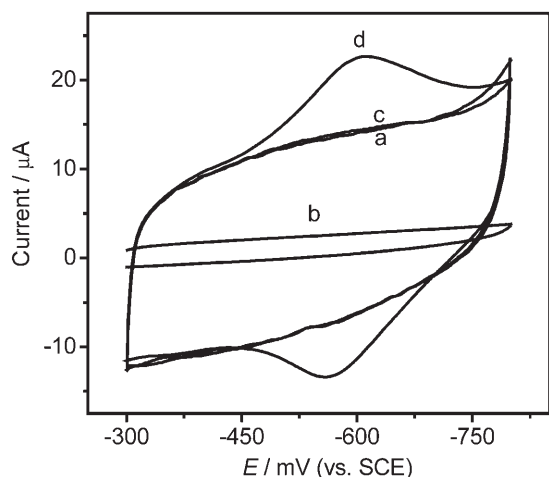


Fig. 1. Cyclic voltammograms of a) GCE, b) pretreated GCE, and c) HRP-anti-hCG/hCG/nano-Au-SG/GCE in 0.1 M pH 7.0 PBS, d) (c) + 3.0 mM  $\text{H}_2\text{O}_2$  + 1.0 mM *o*-phenylenediamine. Scan rate:  $100 \text{ mV s}^{-1}$ .

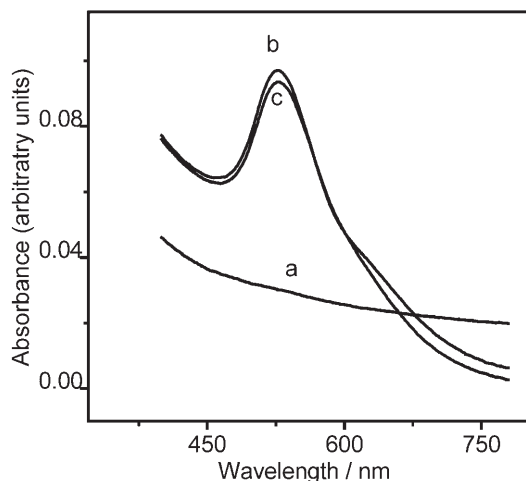


Fig. 3. UV-vis spectra of a) 20% silica sol solution, b) 20% (v/v) colloidal Au solution, and c) mixture of silica sol and colloidal Au at a volume ratio of 1:1.

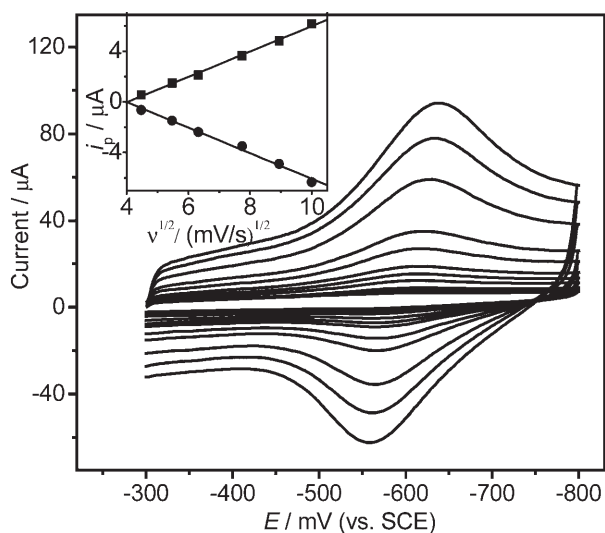


Fig. 2. Cyclic voltammograms of HRP-anti-hCG/hCG/nano-Au-SG/GCE in 0.1 M pH 7.0 PBS containing 1.0 mM *o*-phenylenediamine and 3.0 mM  $\text{H}_2\text{O}_2$  at 20, 30, 40, 60, 80, 100, 150, 200, 300, 400 and 500  $\text{mV s}^{-1}$  (from internal to external). Inset: plots of cathodic and anodic peak currents vs.  $v^{1/2}$ .

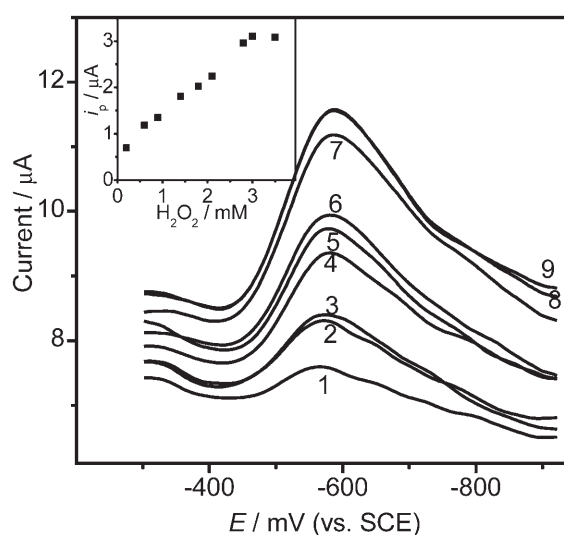


Fig. 4. DPV of HRP-anti-hCG/hCG/nano-Au-SG/GCE in 0.1 M pH 7.0 PBS + 1.0 mM *o*-phenylenediamine without  $\text{H}_2\text{O}_2$  (1) and with successive addition of 0.2, 0.6, 0.9, 1.4, 1.8, 2.1, 2.8, 3.0, and 3.5 mM  $\text{H}_2\text{O}_2$  (from 2 to 9). Inset: plot of peak current vs.  $\text{H}_2\text{O}_2$  concentration.

adsorption spectra of the mixture solution and the colloidal Au solution indicated clearly that the dispersion of gold nanoparticles was maintained in the MPS sol [27] and could be applied to the construction of the modified film on the electrode surface.

### 3.3. Optimal Conditions for Enzymatic Reaction

With an increasing  $\text{H}_2\text{O}_2$  concentration in detection solution the DPV peak current of HRP-anti-hCG/hCG/nano-Au-SG/GCE (no hCG in the incubation solution) increased and then reached a maximum value (Fig. 4), displaying a shape of Michaelis-Menten's curve. At low  $\text{H}_2\text{O}_2$  concentration,

both *o*-phenylenediamine and immobilized HRP were in excess, the rate producing 2,2'-diaminoazobenzene only depended on the concentration of  $\text{H}_2\text{O}_2$ . Thus, its reduction current was proportional to  $\text{H}_2\text{O}_2$  concentration. When the concentration of  $\text{H}_2\text{O}_2$  was higher than 3.0 mM, the enzymatic reaction rate became dependent on the amount of the immobilized HRP, resulting in a constant peak current. Thus 3.0 mM  $\text{H}_2\text{O}_2$  was used for the activity determination of HRP in a competitive immobilized immunoconjugate. The apparent Michaelis-Menten constant ( $K_M^{\text{app}}$ ), a reflection of both the enzymatic affinity and the ratio of microscopic kinetic constants, could be obtained to be  $3.1 \pm 0.1 \text{ mM}$  from the electrochemical version of the Linweaver-Burk equation [44].

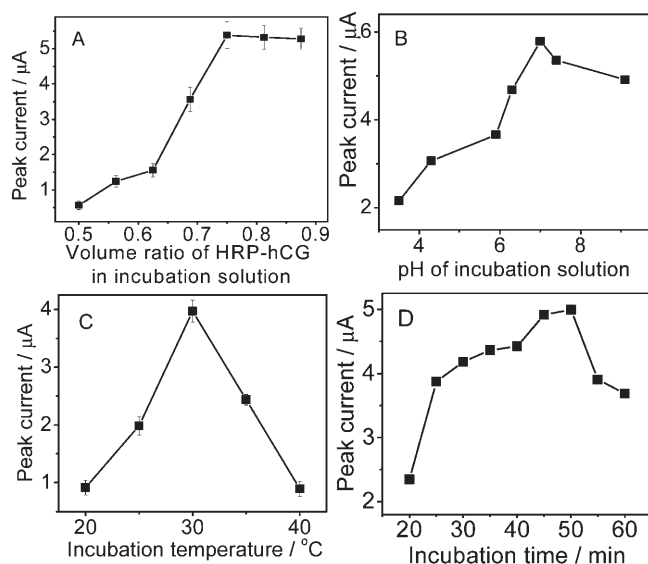


Fig. 5. Effects of volume fraction of HRP-anti-hCG in incubation solution (A), pH of incubation solution (B), incubation temperature (C), incubation time (D) in 0.1 M pH 7.0 PBS on DPV peak current of the immunosensor with 1.0 mM *o*-phenylenediamine and 3.0 mM H<sub>2</sub>O<sub>2</sub> in the detection solution.

### 3.4. Optimization of Immunoreaction Conditions

The concentration of HRP-labeled hCG antibody in the incubation solution was an important parameter which indicated the surface character of the immunosensors. The effect of the volume fraction of HRP-anti-hCG of given concentration on DPV peak current at a fixed total volume was shown in Figure 5A. With the increasing amount of HRP-anti-hCG, the peak current increased and reached a maximum value at the volume fraction of 0.75 (3:1, V/V). Thus the incubation solution of 80 μL was prepared by mixing 60 μL HRP-anti-hCG stock solution and 20 μL dilute solution.

Other factors that influenced the immunoreaction for immunoassay of analyte included pH of incubation solution, incubation temperature and incubation time in the incubation solution. The relationship between the catalytic peak current of HRP-anti-hCG modified electrode to H<sub>2</sub>O<sub>2</sub> and *o*-phenylenediamine system and pH of incubation solution indicated the optimal pH range was between 6.3 and 7.4, with the maximum response at pH 7.0 (Fig. 5B), which was close to the pH value usually used for the binding between antigen and its enzyme conjugate. So pH 7.0 PBS was selected for immunoassay.

Figure 5C shows the effect of incubation temperature on amperometric response of obtained immunoconjugate. The maximum response occurred at an incubation temperature of 30 °C. The lower responses at other temperatures were attributed to the low immunoreaction rate at temperatures lower than 30 °C and the denaturation of labeled enzyme or immunoreagents at temperatures higher than 30 °C. At the temperature of 30 °C, the amperometric response of ob-

tained immunoconjugate increased with an increasing incubation time, and reached a maximum at 50 min (Fig. 5D). Longer incubation time did not improve the response.

### 3.5. Amperometric Response of Immunosensor to hCG Concentration

Under optimal incubation conditions, the hCG molecules in incubation solution occupied the limited binding sites of the HRP-anti-hCG to form immunocomplex. As expected for a competitive mechanism, the DPV peak current of the obtained HRP-anti-hCG/hCG/nano-Au-SG/GCE in the detection solution containing H<sub>2</sub>O<sub>2</sub> and *o*-phenylenediamine decreased with an increasing hCG concentration in the incubation solution. With the method reported previously for a competitive immunoassay, a logistic four-parameter model was used to fit the calibration plot [45], resulting in a detection range for electrochemical immunoassay of hCG from 5.0 up to 30.0 mIU mL<sup>-1</sup> (Fig. 6). The detection limit was calculated to be 1.4 mIU mL<sup>-1</sup> at 3σ. Although the detection limit was the same as that in our previous report based on direct electrochemistry of immobilized horseradish peroxidase [37], the detectable range was wider than that up to 12.5 mIU mL<sup>-1</sup>. The proposed detection limit was relatively lower than those of 2 mIU mL<sup>-1</sup> [34], 2.5 mIU mL<sup>-1</sup> [32], 11.2 mIU mL<sup>-1</sup> [13] and 100 mIU mL<sup>-1</sup> [46] reported with heterogeneous hCG immunoassays based on electrochemical analytical principle. Thus, the proposed method could be used for detection of low level of hCG in serum sample. Higher serum hCG levels could be determined with an appropriate dilution. Another method for determination of high serum hCG level was to increase the concentration of free antibody in solution and the binding capacity of the immobilized immunoreagent by increasing the concentration of hCG used for immunosensor preparation.

### 3.6. Specificity, Reproducibility and Stability of the hCG Immunosensor

Using an incubation solution containing 10 mIU/ml CA19-9 and 10 mIU/mL hCG, the specificity of the proposed hCG immunosensor was examined by detecting the amperometric response. No difference of current was observable in comparison with the result obtained in presence of only hCG. The increase of CA19-9 concentration to some extent did not lead to a significant current change. Furthermore, after the immunosensor was incubated in 10 mIU/ml hCG followed by rinsing with stripping buffer of pH 2.0 glycine-HCl to remove the hCG antibody from the Ag-Ab immunocomplex, and the same immunosensor was incubated again in the incubation solution containing 10 mIU/mL hCG, the amperometric signal obtained in the detection solution containing H<sub>2</sub>O<sub>2</sub> and *o*-phenylenediamine restored the 95% of the initial value. Thus, the immunosensor had a

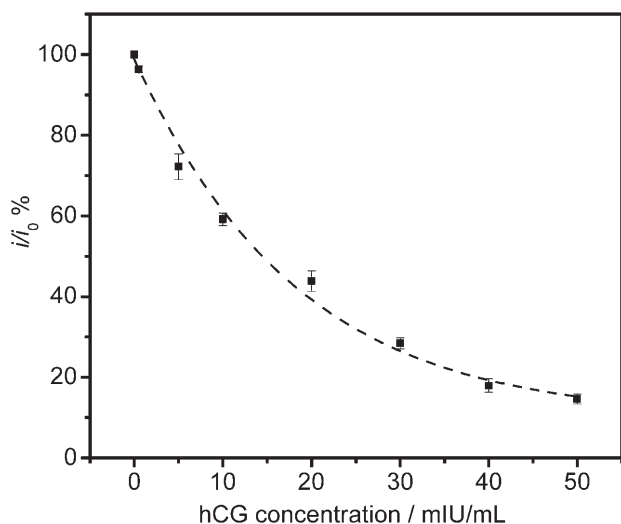


Fig. 6. Calibration for hCG determination fitted with a logistic four-parameter model.  $i/i_0$  is the ratio of DPV peak currents at HRP-anti-hCG/hCG/nano-Au-SG/GCEs obtained by incubating immunosensors in incubation solutions in presence to absence of hCG sample.

good selectivity to hCG as well as an acceptable regeneration efficiency.

The intra-assay precision of the immunosensor was examined by successively assaying the hCG levels of two sera for five times. The variation coefficients of the results with this method were 9.6% and 9.5% at the hCG concentrations of 5 and 20 mIU mL<sup>-1</sup> in serum samples, 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 5.9% at the hCG concentration of 10 mIU mL<sup>-1</sup>, showing acceptable fabrication reproducibility.

The performance stability of the biosensor was examined by storage in air and in pH 7.0 PBS, separately. The biosensor lost its sensitivity rapidly if stored in air. However, when the immunosensor was stored in pH 7.0 PBS at 4 °C, it retained 90% of its initial current after a storage period of three weeks. This indicated that the colloidal gold nanoparticles/sol-gel composite structure was very efficient for retaining the bioactivity and electrical communication ability of the immobilized enzyme conjugate, and preventing the biomolecules from leaking out because of the strong interaction between colloidal gold nanoparticles and mercapto or primary amine groups in these biomolecules.

### 3.7. Clinical Application of the hCG Immunosensor

After five human serum samples from different diagnostic patients were diluted with the appropriate volumes of dilution solution, the serum hCG concentrations were detected with the calibration curve of the hCG immuno-

Table 1. Comparison of serum hCG levels from diagnostic patients determined using two methods.

Serum samples	1	2	3	4	5
This method (mIU/mL)	65	102	192	216	496
Access (mIU/mL)	73.3	119.9	162.7	255.4	424.8
Relative deviation (%)	-11.3	-14.9	18	-15.4	16.7

sensors. In comparison, the concentrations were detected with Access analyzer, a commonly used analyzer in clinic laboratory. The results were listed in Table 1. The relative deviations between two methods were in the range of -15.4%–18%.

## 4. Conclusions

A novel amperometric immunosensing method for hCG in serum samples by encapsulating hCG immunoconjugates into a nano-Au/sol-gel composite structure on glassy carbon electrode. Such composite matrix is effective for retaining the bioactivity of immobilized biomolecules and supplied a facile methodology for immobilization of immunoconjugates. Under optimal conditions, the proposed immunosensor can be applied to the determination of serum hCG. The proposed immunosensor shows good precision and acceptable sensitivity, fabrication reproducibility and storage stability, which could be readily extended toward the measurement of other clinically important tumor markers.

## 5. Acknowledgements

This work was supported by the National Science Fund for Distinguished Young Scholars (20325518), Creative Research Groups (20521503) and the Key Program from the National Natural Science Foundation of China (20535010).

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