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Original Article

Nanogold/graphene as sensing platform coupled with ferrocene/gold as signal amplifier for sandwich-like voltammetric immunosensor of human chorionic gonadotropin

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Abstract: An effective method to detect human chorionic gonadotropin (hCG) is of significance for early pregnancy testing and clinical diagnosis of non-pregnancy related diseases. Herein, a sensitive sandwich-like electrochemical immunosensor of hCG was constructed by introducing nanogold/graphene (Au/GNS) hybrids and ferrocene/gold nanoparticles (Fc/Au) as the sensing platform and signal amplifier, respectively. In this sensing platform, the Au/GNS hybrid was dedicated to increasing the loading capacity of primary antibody (Ab_1) and accelerate the electron transport, and Au is devoted to assembling secondary antibodies (Ab_2) and Fc derivative (Fc-SH) to fabricate signal amplifier (Ab_2-AuNPs-Fc). By selecting Fc as the signal probe, results revealed that peak currents increased when the special recognition among Ab_1, hCG, and Ab_2 occurred. A novel and sensitive sandwich-like immunosensor of hCG was thus constructed. After optimizing various testing conditions, a wide linearity from 0.005 to 7.0 ng mL^{-1} and a low detection limit of 1.0 pg mL^{-1} were achieved for hCG analysis, suggesting this method is useful for hCG analysis.

Keywords: Human chorionic gonadotropin, biomarker, hCG, pregnancy testing, electrochemical sensing

Introduction

hCG is a glycoprotein hormone composed of both an α-subunit and β-subunit. Commonly it is used as a marker for early pregnancy testing. hCG can be also used as a biomarker for many non-pregnancy diseases such as HIV/AIDS, breast cancer, testicular cancer, rheumatoid arthritis, prostate cancer, trophoblastic disease, and for Alzheimer's disease treatment [1-3]. Generally, after two months of pregnancy, the amount of hCG reaches a maximum (~1000.0 ng/mL). After that, the hCG concentrations become relatively constant. hCG concentrations of 1.0-10.0 ng/mL in human serum are a marker for a high risk of nonseminomatous testicular or ovarian tumor [4]. Normal concentration of HCG is under 1.0 ng/mL. Hence, developing an effective and sensitive method to detect hCG is very important for several purposes.

Currently there are many methods developed to detect hCG, including electrochemiluminescence immunoassay [5], colorimetric immunoassay [6], microtoroid optical resonators [1], photothermal immunoassay [7], hydrophilic interaction liquid chromatography-mass spectrometry [8] and electrochemical immunosensing. Among the above methods, electrochemical immunosensing has received great interest thanks to its high sensitivity, rapid response and simple operation [9-11]. For instance, Roushani and Valipour [10] constructed an antibody(Ab)/Pt nanoparticle/graphene-ionic liquid-chitosan immunosensor to detect hCG by using rutin as a probe, and the developed immunosensor is reproducible and stable. Xu's

group [11] developed an electrochemical immunosensor based on nanoporous AuAg alloy for hCG detection, and a detection limit of (LOD) 0.01 ng mL^{-1} and linearity of 0.05-35.0 ng mL^{-1} were obtained. Although these methods are of great significance, achieving effective and highly sensitive detection of hCG is still a challenge.

One of the key aspects of constructing a sensitive and reliable electrochemical immunosensor is to develop suitable materials as a sensing platform to improve conductivity and immobilize antibodies. In the past years, graphene (GNS)-based nanohybrids have captured considerable attention from materials scientists, chemists, and physicists alike [12, 13]. Generally, GNS is a 2D nanosheet of C atoms with hexagonal configuration [14, 15]. Owing to its large conductivity and surface area, GNS is applied widely in many fields, e.g., batteries, catalysis, supercapacitor and electrochemical sensing [16-19]. GNS is thus a suitable nanomaterial for constructing sensitive electrochemical immunosensors for hCG.

Recently, sandwich-like electrochemical immunosensors have attracted attention because of their advantages in signal amplification. For an effective sandwich-like immunosensor, besides suitable nanomaterials as sensing platform, another key element is to design a superior signal amplifier [20-22]. In this work, nanogold/graphene (Au/GNS) nanohybrids was first prepared, then the obtained Au/GNS nanohybrids were introduced to immobilize primary antibody (Ab_1) to capture hCG. Fc-SH and the secondary antibody (Ab_2) were assembled on the surface of Au nanoparticles (AuNPs) to fabricate a signaling amplifier (Ab_2-AuNPs-Fc). The results showed that a current response of Fc can be observed when the special immunoreaction of "Ab_1--hCG---Ab_2" occurred. With optimized conditions, the constructed sandwich-like electrochemical immunosensor shows superior analytical performance for hCG detection.

Materials and methods

Reagents and apparatus

Graphene oxide (GO) was synthesized with Hummers method [23]. hCG and its antibodies (Ab_1 and Ab_2), Bovine serum albumin (BSA) and gold chloride ($HAuCl_4$▪$4H_2O$) were purchased from Shanghai Lingcao Biotechnology Co., Ltd (China). Phosphate buffer solution (PBS) was prepared as the supporting electrolyte with KH_2PO_4 (0.1 M), K_2HPO_4 (0.1 M) and KCl (0.1 M). The electrochemical experiments were carried out at a CHI 660E Electrochemical Workstation (China). Transmission electron microscopy (TEM) (JEM-3010, JEOL) was used to study the morphologies and structures of Au/GNS and AuNPs. The Nyquist plots were measured in KCl and $[Fe(CN)_6]^{3-/4-}$ solution in the frequency range from 100 kHz to 0.1 Hz with a DC potential of 0.25 V and an amplitude of 5 mV. The software of Sigmaplot 14.0 was used to draw pictures.

Preparation of Au/GNS, AuNPs and Ab_2-AuNPs-Fc

The Au/GNS nanohybrids were prepared according to a previous method with some modification [24, 25]. Briefly, 25.0 mL of $HAuCl_4$ solution (0.2 mg mL^{-1}) was added in to 16.0 mL of GO (0.25 mg mL^{-1}) solution under stirring, which was further stirred for 1 hour to increase the interaction of the GO surface with gold ions. Then, 0.8 mL sodium citrate (0.2 mol L^{-1}) was added dropwise to the former mixture, and the formed mixture was heated for 2 hours at 70°C. Finally, the Au/GNS nanohybrids were obtained successfully by centrifugation, rinsing and vacuum-dried. Meanwhile, the similar processes were used to prepare AuNPs solution without GO.

For preparing Ab_2-AuNPs-Fc, 25.0 μL of Ab_2 was mixed into AuNPs solution of 1.0 mL and incubated at room temperature for 2 h, and then the Fc-SH solution (20 μL, 1.0 mM) was mixed with the Ab_2-AuNPs solution to form Ab_2-AuNPs-Fc.

Construction of hCG sandwich-like immunosensor

First, 8 μL Au/GNS suspension (0.25 mg mL^{-1}) was dropped on the surface of GCE and 3 μL Ab_1 (0.2 mg mL^{-1}) was incubated and immobilized. Next, the fabricated Ab_1/Au/GNS/GCE electrode was incubated in BSA solution for preventing the non-specific adsorption, and it was subsequently interacted with different concentrations of hCG at room temperature for capturing hCG. Finally, the formed hCG/BSA/Ab_1/Au/GNS/GCE was incubated with Ab_2-

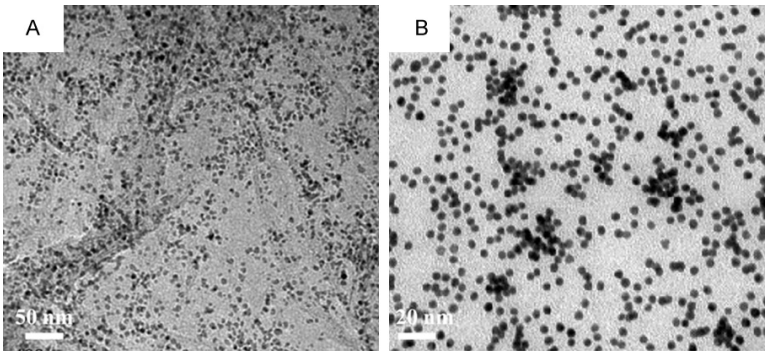

Figure 1. Characterization of Au/GNS and AuNPs. TEM images of Au/GNS (A) and AuNPs (B).

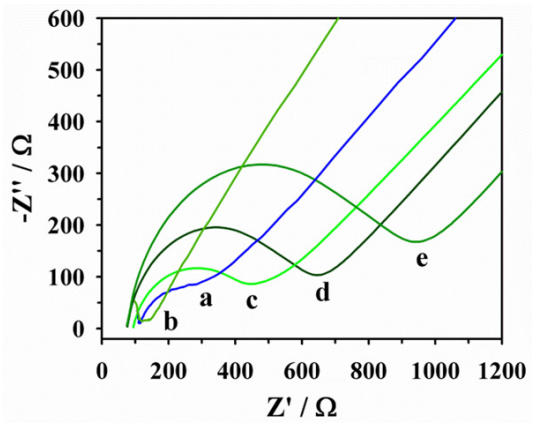

Figure 2. The conductive properties of various modified electrodes were assessed by EIS analysis. The EIS plots of GCE (a), Au/GNS/GCE (b), BSA/Ab_1/Au/GNS/GCE (c), hCG/BSA/Ab_1/Au/GNS/GCE (d) and Ab_2-AuNPs-Fc/hCG/BSA/Ab_1/Au/GNS/GCE (e).

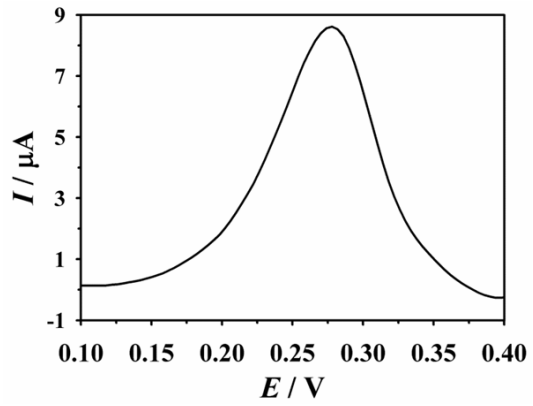

Figure 3. Feasibility study of the proposed sensor. DPV responses of Ab_2-AuNPs-Fc/hCG/BSA/Ab_1/Au/GNS/GCE in PBS; the concentration of hCG was 15.0 ng mL^{-1}.

AuNPs-Fc to fabricate a sandwich-like structure for sensing hCG electrochemically.

Results

Characterization of Au/GNS and AuNPs

The TEM images of Au/GNS hybrids and Au nanoparticles are shown in **Figure 1**. **Figure 1A** shows that a large amount of Au particles were dispersed on the GNS surface uniformly, and the Au content in the Au/GNS nanohybrids was evaluated to be about 6.3wt% through the analysis of ICP-MES. The uniform and abundant Au in Au/GNS were beneficial to immobilize Ab_1 and improve analytical performance. In addition, by measuring the Au diameter from the TEM image, the size value of Au particles was mainly distributed from ~3.0 and ~8.0 nm and the average diameter was ~5.5 nm. **Figure 1B** is a TEM image of AuNPs produced singly. It can be found that the AuNPs have a homogeneous size with an average diameter of ~5.0 nm by statistical investigation, which was applied to immobilize Ab_2 and Fc to form Ab_2-AuNPs-Fc.

Next, the EIS analysis of 5.0 mM $[Fe(CN)_6]^{3-/4-}$ solution containing 0.1 M KCl was used to assess the conductive properties of various modified electrodes, and the corresponding EIS plots are shown in **Figure 2**. The studies revealed that Au/GNS/GCE had a very small charge-transfer resistance (R_{CT}) that even could be neglected compared to the bare GCE, suggesting Au/GNS/GCE has prominent conductivity. When BSA/Ab_1, hCG and Ab_2-AuNPs-Fc were assembled continuously on Au/GNS/GCE, the *R*ct value at the related electrodes in ascending order was: Ab_2-AuNPs-Fc/hCG/BSA/Ab_1/Au/GNS/GCE > hCG/BSA/Ab_1/Au/GNS/GCE > BSA/Ab_1/Au/GNS/GCE > Au/GNS/GCE.

Electrochemical immunosensing of hCG

The electrochemical response of the immunosensor was studied in 0.1 M PBS through DPV. Results revealed a peak current of Fc observed at 0.27 V after the antigen-antibody recognition reaction between hCG/BSA/Ab_1/Au/GNS/GCE and Ab_2-AuNPs-Fc (**Figure 3**). In order to achieve sensitive analysis of hCG, several related conditions were optimized. **Figure 4** exhibits the corresponding results. As for the influence of PBS with different pH values, it was found that

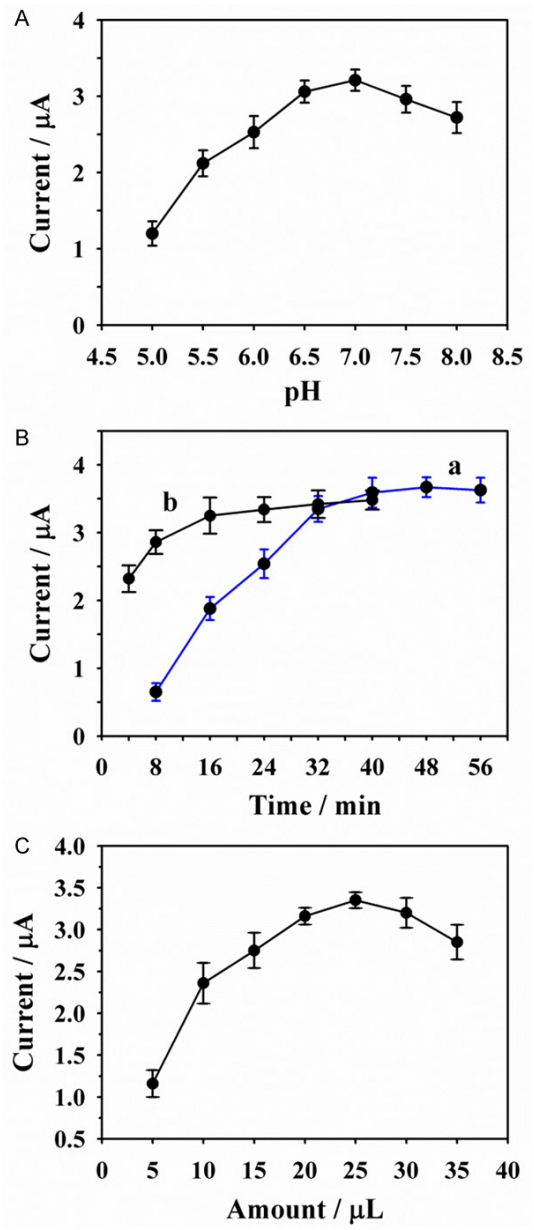

Figure 4. Optimization of experimental conditions. Influences of the (A) pH value of PBS; (B) interaction time of (a) BSA/Ab_1/Au/GNS/GCE in hCG solution and (b) hCG/BSA/Ab_1/Au/GNS/GCE in Ab_2-AuNPs-Fc solution; (C) amount of Ab_2. The concentration of hCG was 3.0 ng mL^{-1}.

the electrochemical response of Fc increased along with increasing of pH values from 5.0, reaching a maximum at 7.0 (**Figure 4A**), thus revealing a best pH value of PBS of 7.0. Next, the influences of the interaction time of BSA/Ab_1/Au/GNS/GCE in hCG solution and hCG/BSA/Ab_1/Au/GNS/GCE in Ab_2-AuNPs-Fc solution were assessed (**Figure 4B**). Results indicated that the optimum interaction time between BSA/Ab_1/Au/GNS/GCE and hCG was 40 min, while that was 16 min for hCG/BSA/Ab_1/Au/GNS/GCE interacting with Ab_2-AuNPs-Fc, hence 40 and 16 min were used respectively for the interactions of "Ab_1--hCG" and "hCG--Ab_2" in this study. Furthermore, the Ab_2 amount used in preparing Ab_2-AuNPs-Fc is another important factor for the analytical performance. It was thus also evaluated (**Figure 4C**), and the results showed that the electrochemical response of the immunosensor increased gradually with increasing of Ab_2 in the range from 5.0 to 25.0 μL, whereas the response showed a decrement when the Ab_2 amount increased further. This may bedue to the competitive immobilization from Fc and Ab_2 with AuNPs.

After optimizing various experimental parameters, the analytical performance of the developed immunosensor for hCG were evaluated through plotting the related calibration curve which included Fc response change upon the concentration change of hCG (**Figure 5**). The results showed that the DPV response of Fc increased linearly along with a hCG concentration increase from 0.005 ng mL^{-1} to 7.0 ng mL^{-1}; the linear equation is I_p(μA) =0.6427+0.8622*C* (ng mL^{-1}) (R^2=0.9986) and the LOD value is 1.0 pg mL^{-1} based on S/N=3.

Reproducibility, stability and selectivity

To investigate the reproducibility of the proposed sensor, the DPV responses of Fc at 8 independently fabricated Ab_2-AuNPs-Fc/hCG/BSA/Ab_1/Au/GNS/GCE were recorded (**Figure 6A**). Results showed that the relative standard deviation value of the DPV response was 3.23%. Next, for investigating the stability, the constructed Ab_2-AuNPs-Fc and BSA/Ab_1/Au/GNS electrode was preserved at 4°C. Analytical results exhibited that the electrochemical response of Fc almost did not decrease after storing three weeks (**Figure 6B**). These results indicated the developed immunosensor exhibited good repeatability and satisfactory stability. Then, the selectivity of the developed immunosensor was also investigated. This was carried out in the presence of various interfering components: haptoglobin, cortisol, dehydroepiandrosterone, uric acid, alpha fetoprotein, leptin, haptoglobin, carcinoembryonic antigen,

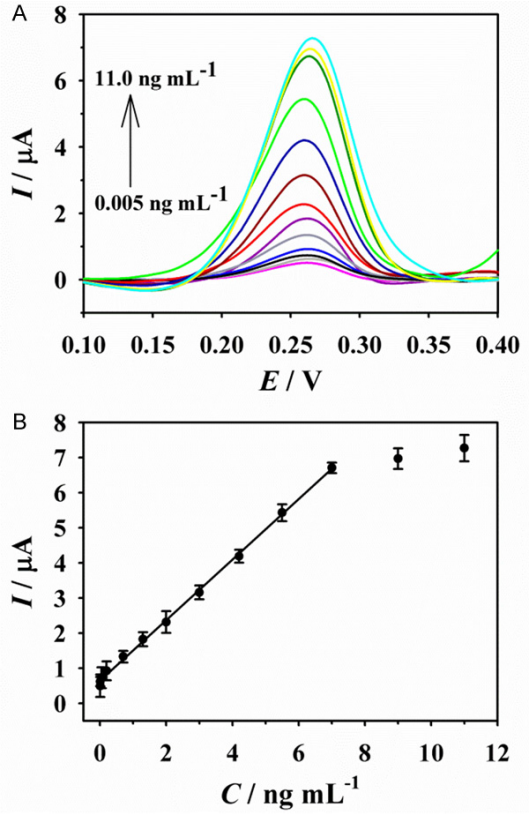

Figure 5. The analytical performance of the developed sandwich-like immunosensor. A. DPV responses of hCG with different concentrations in 0.1 M PBS (from the bottom up: 0.005, 0.01, 0.05, 0.2, 0.7, 1.3, 2.0, 3.0, 4.2, 5.5, 7.0, 9.0 and 11.0 ng mL^{-1}); B. Corresponding calibration curve for hCG detection.

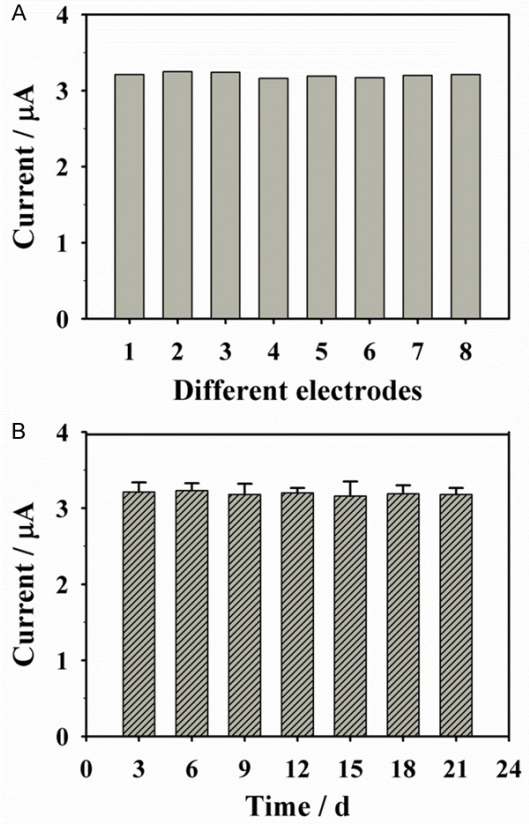

Figure 6. Reproducibility and stability of the immunosensor. The DPV peak current of Fc at independently fabricated Ab_2-AuNPs-Fc/hCG/BSA/Ab_1/Au/GNS/GCE (A) and stability of the immunosensor (B).

BSA, and ascorbic acid (20.0 times of hCG concentration). Results demonstrated there was almost no DPV signal change, suggesting that the fabricated immunosensor of hCG has desirable selectivity for detecting hCG.

Real sample analysis

In order to investigate the feasibility of the fabricated immunosensor in real application, the determination of hCG in the real human urine obtained from two donors was measured with standard addition method. By adding different concentrations of hCG into the urine sample, the DPV signals of Fc were measured by incubating the sensor in the urine, and the calculated recovery was used to evaluate the feasibility of the sensor. For comparison, ELISA was also used as a standard method to measure the hCG level. **Table 2** shows recoveries in the range from 94.5% to 98.3%; meanwhile, the levels of hCG in samples measured by the proposed immunosensor were close to those obtained by ELISA, indicating the proposed sandwich-like immunosensor can be applied to detect hCG in real urine samples.

Discussion

Developing an effective and sensitive method for detecting hCG is of great significance for clinical application [1-3]. We prepared Au/GNS nanohybrids and Fc/Au to construct an immunosensor for hCG (**Figure 7**). The TEM images of Au/GNS and AuNPs revealed that the AuNPs had a homogeneous size and dispersed uniformly on the surface of GNS, which is beneficial for immobilizing Ab_2. The EIS analysis was used to assess the conductive properties of various modified electrodes. Results showed that Au/GNS/GCE had prominent conductivity and the proposed immunosensor layers could be immobilized successfully by the proposed

Table 1. Comparison of proposed sandwich-like immunosensor to the existing sensors for detecting hCG

Electrode	Linear range [ng mL^{-1}]	LOD [pg L^{-1}]	Refs.
Ab/Pt nanoparticle/graphene-ionic liquid-chitosan/GCE	0.106-212.0; 212.0-35000.0	35.0	[10]
Ab/carbon nano-onions/AuNPs/polyethylene glycol/GCE	0.0000001-1.0	0.0001	[26]
Ab/porous carbons/SnS_2/AuNPs/GCE	0.5-50.0	6.4	[27]
Ab/AuNPs/carbon nanotubes microelectrode	0.01-2.0	2.4	[28]
Ab_2-AuNPs/hCG/AuNPs/GNS/SPE	0-0.5	5.0	[29]
Ab/AuNPs/cysteamine/AuNPs/GE	0.001-0.2; 0.5-60.7	0.3	[30]
Ab_2-AuNPs-Fc/hCG/BSA/Ab_1/Au/GNS/GCE	0.005-7.0	1.0	This work

Table 2. Detection of hCG in human urine samples

Sample	Added [ng mL^{-1}]	Found [ng mL^{-1}]	Recovery [%]	ELISA [ng mL^{-1}]
a	2.0	1.93	96.5	1.95
	4.0	3.88	97.0	3.85
	6.0	5.90	98.3	5.83
b	2.0	1.89	94.5	1.91
	4.0	3.92	98.0	3.95
	6.0	5.85	97.5	5.90

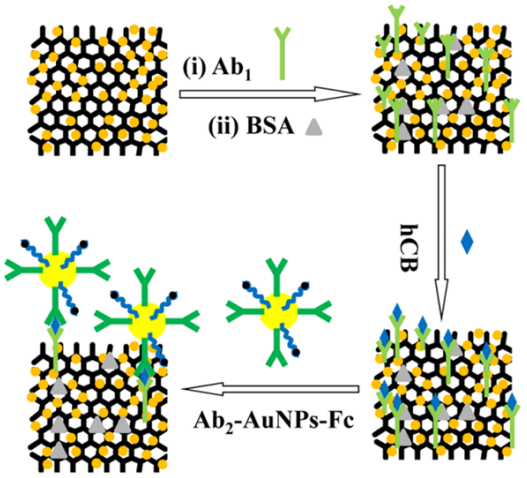

Figure 7. Illustrations for the construction of Au/GNS-based sandwich-like electrochemical immunosensor for hCG.

method. The feasibility study demonstrated that the developed sandwich-like immunosensing strategy for hCG was feasible. To obtain the optimal experimental conditions, several related factors were optimized. The results showed that the best pH value of PBS for the special recognition reaction between antigen-antibody was 7.0. Especially, **Figure 4B** shows that interaction times for "Ab_1--hCG" and "hCG--Ab_2" were 40 and 16 min respectively, indicating that it takes no more than 2 hours from receiving the samples to results output. In addition, the optimized Ab_2 amount used in preparing Ab_2-AuNPs-Fc was 25.0 μL.

Under optimized conditions, the analytical performances of the immunosensor for hCG were studied. To further evaluate the analytical performance of the immunosensor, the obtained LOD (1.0 pg mL^{-1}) and linear range (0.005 to 7.0 ng mL^{-1}) in this work were compared with previous studies [10, 26-30]. **Table 1** shows the analytical performances of the proposed electrochemical immunosensor exhibit wider linear range and lower LOD than most previous methods. In addition, according to reports in the relevant literature [1, 4, 31, 32], the hCG level of 1.0-10.0 ng/mL in human serum confers a high risk of nonseminomatous testicular or gonadal tumor. As for pregnancy, hCG can reach ~1000.0 ng/mL after two months of pregnancy. Obviously, the analytical performances of the proposed immunosensors for hCG can satisfy the demand for the early clinical diagnosis of pregnancy and non-pregnancy related diseases. It should be pointed out that the upper limit of the current method is lower than the range of other methods, which might induce a limitation that the real samples need to be diluted [33, 34]. In addition, the studies in reproducibility, stability, selectivity, and real application revealed that all the related results were satisfactory.

In short, a novel and highly sensitive sandwich-like electrochemical immunosensor for hCG detection was fabricated successfully through introducing Au/GNS nanohybrids and AuNPs-Fc respectively as the sensing platform and signal-

ing amplifier. After the optimization of various factors, the developed sandwich-like immunosensor of hCG exhibited an excellent analytical performance with wide linearity from 0.005 to 7.0 ng mL^{-1} and low LOD of 1.0 pg mL^{-1}. In addition, the fabricated sandwich-like immunosensor possessed good reproducibility, stability, and selectivity. This fabricated immunosensor for the determination of hCG has significant potential uses.

Disclosure of conflict of interest

None.

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