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Layer-by-layer assembly of gold nanoparticles and cysteamine on gold electrode for immunosensing of human chorionic gonadotropin at picogram levels

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ABSTRACT

The development of an electrochemical immunosensor for the detection of human chorionic gonadotropin (hCG) is described with a limit of detection as low as 0.3 pg mL^{-1} in phosphate buffer. In this immunosensor, cysteamine (Cys) and gold nanoparticles (AuNPs) were used to immobilize an anti-hCG monoclonal antibody onto a gold electrode (GE). The structure of AuNPs has been confirmed by EDS, SEM, and TEM analysis. Due to the large specific surface area and excellent electrical conductivity of AuNPs, electron transfer was promoted and the amount of hCG antibody was enhanced significantly. A systematic study on the effects of experimental parameters such as pH, incubation time in the hCG solution and urea solution used for experiments on the binding between the immobilized antibody and hCG has been carried out. Under optimal experimental parameters, differential pulse voltammetry (DPV) signal changes of the $[Fe(CN)_6]^{3-/4-}$ are used to detect hCG with two broad linear ranges: 0.001 to 0.2 and 0.2 to 60.7 ng mL^{-1}. The LOD value proves more sensitive in comparison with previously reported methods. The prepared immunosensor showed high sensitivity and stability. In addition, the immunosensor was successfully used for the determination of hCG in human serum.

1. Introduction

Human chorionic gonadotropin (hCG) is a 37 kDa glycoprotein hormone. 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) [1–3]. HCG is a well-known and important diagnostic marker of pregnancy, as well as acting a tumor marker for certain cancers [4]. Thus, exact calculation of the concentration of hCG in urine or serum plays an important role in monitoring trophoblastic diseases in all modern immunological pregnancy tests [5] and cancer monitoring. The interactions between an antibody and an antigen are known to be very specific chemical reactions. Such a specific molecular recognition of antigens by antibodies has been exploited in immunoassays to develop highly selective detection methods in many clinical analyses and medical diagnostics as well as for environmental monitoring [6]. During recent years, conventional diagnostic methods, such as enzyme-linked immunosorbent assay, chemiluminescence, surface Plasmon resonance, and quartz crystal microbalance, have been the main methods used for detection hCG. Compared with conventional immunoassays, electrochemical immunoassay has exhibited several advantages, including simplicity of instrument, low cost, feasibility of miniaturization, and subsequent portability [7]. In the process of the design and fabrication of highly sensitive electrochemical immunosensors, signal amplification, antibody immobilization and noise reduction are the crucial steps. Among the various immobilization methods, the traditional method was to form covalent couplings, which provides stable and strong bindings between the desired biomolecule and the electrode substrate [8]. Signal amplification is a main concern for detecting target molecules at quite low concentration [9].

Many kinds of nanomaterial, including noble metal nanoparticles such as gold nanoparticles [10], platinum nanoparticle, and palladium [11–13], silver nanoparticle [14], TiO_2 [15], cerium oxide nanowires [16], and graphene have been developed to amplify electrochemical signal in order to improve the sensitivity of electrochemical immunosensor. Gold nanoparticles (AuNPs) are well known nanomaterials because of their large specific surface area, strong adsorption ability, well suitability, and good conductivity [17]. It can strongly interact with biomaterials and has been utilized as an intermediator to immobilize antibody to efficiently retain its activity and to enhance current response in the construction of immunosensor [18]. In this study, AuNPs were deposited onto the surface of bare gold electrode to enlarge the electrochemically active sites. The

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deposition of neatly arranged AuNPs on the surface of a GE increases the effective electrode surface and the sensitivity of electrochemical analyses, and provides a unique platform for increased loading of molecules.

In this strategy, amino group of cysteamine is covalently attached to the AuNP-coated surface (AuNPs/GE) via the formation of a stable self-assembled monolayer (SAM). And the amino groups array exposed on the electrode surface was used to anchor AuNPs. By adding hCG as the target and upon the formation of the hCG/anti-hCG complex on the electrode surface, the electron transfer characteristics of $[Fe(CN)_6]^{3-/4-}$ as the electrochemical probe, change; this can be monitored by the differential pulse voltammetry (DPV) technique. Using AuNPs/Cys/AuNPs as an efficient interface lead to a simple and economic method with higher sensitivity and a wider concentration range for detection of hCG. Acceptable factors that can affect sensitivity and linear range of the proposed immunosensor are: 1) Large surface area of AuNPs that causes the large amounts of anti-hCG immobilized on the electrode surface 2) Covalent attachment of AuNPs with amino groups of anti-hCG lead to more stability and repeatability in comparison to the adsorption method.

2. Experimental

2.1. Reagents and apparatus

Anti-hCG (1 mg ml^{-1}), hCG (37 kDa), HAuCl4, sodium citrate ($Na_3C_6H_5O_7.2H_2O$), Bovine serum albumin (BSA) were purchased from Sigma-Aldrich Co. LLC (USA). All other reagents with analytical grade such as glucose and NaOH were obtained from Merck or Fluka and used without further purification. The real samples (3 human serum samples from different people) were provided by a local clinical laboratory and subjected to ultrafiltration by loading into a centrifugal filtration tube at 2800 rpm for 30 min. Stock solution of human blood serum was diluted 50 times with phosphate buffer (0.1 mol L^{-1}), and then analyzed. Phosphate buffer was prepared using 0.1 mol L^{-1} Na_2HPO_4. All hCG solutions with various concentrations and other solutions were prepared by direct dissolution in 0.1 mol L^{-1} phosphate buffered, pH: 7.5. Phosphate buffered, 0.1 mol L^{-1}, pH = 7.5, containing 3.5 mmol L^{-1} $Fe(CN)_6^{3-/4-}$ was used as working solution. For the electrochemical impedance spectroscopy (EIS) experiments, a solution containing 5 mmol L^{-1} $Fe(CN)_6^{3-/4-}$ at a ratio of 1:1 and 0.1 mol L^{-1} KCl was applied. All experiments containing CV, DPV, and EIS were performed with a μ-AUTOLAB electrochemical system type III. In addition a FRA board computer controlled Potentiostate/Galvanostate (Eco-Chemie, Switzerland) driven with NOVA software in conjunction with a conventional three electrode system with GE as the working electrode was used and a platinum wire as the counter electrode, and an Ag/AgCl (satd 3.0 mol L^{-1} KCl) as the reference electrode. The DPV measurements were carried out by scanning the potential of 0 to 0.5 V with modulation time of 50 ms and modulation amplitude of 25 mv. CV measurements were taken from −0.4 to 1.0 V as initial and stop potential. The impedance analysis was performed with the frequency range of 0.1 Hz to 100 kHz with a modulation voltage of 5 mV. Scanning electron microscopy (SEM) images were obtained by using scanning electron microscopy (Philip's Company, Netherlands). The morphology of the Au nanoparticles was determined by a Hitachi H-800 transmission electron microscopy (TEM) at an operating voltage of 200 kV. Energy-dispersive X-ray spectroscopy (EDS) spectra were performed with a Philips CM-200-FEG electron microscopy operating at 200 kV (accelerating voltage). A Metrohm model 780 pH/mV meters was used to measure the pH. All experiments were carried out at room temperature.

2.2. Synthesis of Au nanoparticle

Gold nanoparticles were synthesized according to the reported procedure [19,20]. Synthesis was conducted in four stages: i) At first, 500 mL HAuCl4 0.01% W/V solution was poured into round-bottom flask equipped with a condenser and was heated and stirred until it reached boiling temperature. ii) While stirring, 7.5 mL sodium citrate 1% was added to the solution. iii) After 30 s, the solution turned into blue and after 70 s it turned into red. iv) Boiling lasted for 10 min. v) The heating was stopped and the solution was stirred for 15 min. The obtained solution was red and its particles were about 12 nm. After cooling, the solution was kept in refrigerator. The synthesized AuNPs were characterized by TEM (Fig. 2A).

2.3. Fabrication of the immunosensor

The GE was polished to a mirror finish with 0.3 and 0.05 μm alumina slurry, and then thoroughly washed ultrasonically in ethanol and ultrapure water. Then, the gold substrate was cleaned in piranha solution (1:3 mixture of 30% H_2O_2–H_2SO_4 conc.) for approximately 3–5 min. Afterward; the substrates were washed several times with deionized water. Fig. 1 displays the stepwise procedure for the synthesis of the immunosensor. i) The formation of AuNPs on the GE was carried out by cyclic voltammetry (CV) scanning from 0.7 V to 0.0 V, vs. Ag/AgCl, in 0.1 mol L^{-1} KCl and 2 mol L^{-1} HCl solution containing 250 mg L^{-1} $HAuCl_4$ at a scan rate of 50 mV s^{-1} for 20 cycles [21] the obtained electrode was taken out and rinsed with water. ii) 10 μL of 10 mmol L^{-1} Cys was dropped on the surface of electrode for 4 h at room temperature. In order to remove the physically and weakly adsorbed Cys, the Cys/AuNPs/GE was rinsed with deionized water. iii) 8 μL AuNPs were dropped onto the cysteamine layer modified electrode at 4 °C for 12 h. v) 3 μL PBS containing anti-hCG (1 mg mL^{-1}) was dropped onto the surface of AuNPs/Cys/AuNPs/GE that was prepared overnight to immobilize anti-hCG molecules on the surface of AuNPs. By chemisorption between AuNPs and amine groups of anti-hCG. vi) Finally, the antibody modified electrode (anti-hCG/AuNPs/Cys/AuNPs/GE) was soaked in 25% BSA at room temperature for 3 h in order to block nonspecific binding sites and avoid the nonspecific adsorption. The prepared immunosensor was stored at 4 °C.

2.4. Electrochemical measurements

Electrochemical experiments containing a three electrode arrangement were performed in a conventional electrochemical cell. The potential was swept from 0 to 0.5 V (vs. Ag/AgCl) in 10 mL of pH 7.5 phosphate buffer containing 3.5 mol L^{-1} K_3[Fe(CN)6]/K4[Fe(CN)6] (1:1) and 0.1 mol L^{-1} KCl at room temperature. By immersing the BSA/anti-hCG/AuNPs/Cys/AuNPs/GE in hCG solution for 40 min, antigen and antibody complexes are performed moreover with increase in hCG concentration and the current peak is further reduced.

3. Results and discussion

3.1. Characterization of the deposited AuNPs on the GE

Under the deposition conditions, the morphologies of the AuNPs electrodeposited on the GE were characterized with SEM images. The SEM micrograph in Fig. 2B (bare GE) as compared with Fig. 2C, indicates that an appropriate layer of AuNPs is formed on the surface of the GE. As seen, the SEM images show a uniform coverage of the AuNPs with small particles sizes on the surface of GE. In order to have more information about the chemical surface composition of the modified AuNPs/GE, the EDS pattern of the electrode surface was obtained. It is well established that EDS penetrates much more deeply and hence gives the bulk materials composition. As shown in Fig. 2D, EDS spectrum confirms the presence of Au on the GE surface.

3.2. Electrochemical characterization of the stepwise-modified electrode

EIS, CV, and DPV have been employed to monitor the stepwise fabrication processes of the immunosensor using $[Fe(CN)_6]^{3-/4-}$ as redox