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Gold nanoparticles based sandwich electrochemical immunosensor

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ABSTRACT

In this report we have used gold nanoparticles (AuNPs) to covalently attach an antibody (Ab_1) using a spacer arm. The AuNPs/Ab_1 modified gold electrode was used for a sandwich electrochemical immunoassay. The detection was done using cyclic voltammetry and impedance measurements using Horse Radish Peroxidase (HRP) as enzyme label on secondary antibody (Ab_2) and 3,3′, 5,5′-tertramethyl benzidine (TMB) as an electroactive dye. The cyclic voltammetric experiments showed three clear peaks at potentials 154 mV, −33 mV and −156 mV. There was an increase in the both anodic and cathodic current values for the peak at potential −33 mV, when H_2O_2 was added and the other peaks observed at potential 154 mV and −156 mV resulted due to the oxidation and reduction of TMB. The detection limit of this electrode was 2 ng/mL or 10 pg/5 μL of the analyte. The electrochemical impedance spectroscopy studies demonstrate that the formation of antigen–antibody complexes increases the series resistance and thus confirms the assembly on the electrode. This study showed that AuNPs was efficient in preserving the activity and orientation of the antibody and it can form a major platform in many clinical immunoassays.

1. Introduction

In recent years, the field of electrochemical biosensors design has been capable of providing better analytical characteristics in terms of sensitivity, selectivity reliability, ease of use and low cost. Among the various types of electrochemical sensors, immunosensors are attractive tools for the detection of analytes based on the binding of antibodies with antigens. Unlike other techniques, electrochemical methods are not usually affected by sample impurities and the equipment required is relatively simple and are of low cost. Most of the studies in immunosensors concentrate on the electrochemical detection using labeled immunoagent (enzymes are most commonly been used as the labels). The achievement of high sensitivity requires different amplification platforms and processes. It has been shown that, we can enhance the sensitivity of the signal and the detection ability (Storhoff et al., 1998; Hu et al., 2003; Kumar et al., 2008; Parak et al., 2003; Tkachenko et al., 2003) of the biosensing devices by using nanoparticles in their construction. Although different types of nanoparticles have been used earlier for the construction of electrochemical sensors, but the use of gold nanoparticles (AuNPs) allows enhanced analytical detection with respect to others. Further flow through system can be used to enhance the binding of the antigen to the given antibody.

The ability of AuNPs to provide a stable immobilization of biomolecules, in order to retain their bioactivity is a major advantage for the preparation of sensors (it is also an highly conducting material with a high surface to volume ratio). Various approaches like, layer by layer self assembly using nafion (Tang et al., 2004a), dithiothreitol (Chatrathi et al., 2007), cysteamine (Wang et al., 2004a), 4-aminothiophenol (Wang et al., 2004b), polyvinyl butyral (Tang et al., 2004b) have been used earlier for the construction of immunosensors. In the recent years construction of immunosensors using AuNPs have received significant attention. Antibodies were also used in sol–gel matrix (Wu et al., 2005) and carbon paste electrode (Dana et al., 2007) immunosensors. Some of the earlier works haves demonstrated the direct assembly of antibody (Chen et al., 2007), antigen (Huang et al., 2007) onto the electrode. Taking an account of the advantages and methods used for gold nanoparticles modified electrode preparation, we have prepared modified gold nanoparticles surface by the direct covalent linking of the antibody to the nanoparticles and assembling them onto the electrode surface. In the current study, we have shown that direct linking of antibody to the AuNPs using covalent methods can be used for the development of immunosensors. This kind of approach provides a right orientation of the antibody with the free antigen binding sites for further immunoreaction without affecting the structure and function of the antibody. The AuNPs also helps in better immobilization of the molecules onto the electrode preventing them from dissolving back into the bulk solution.

Horse Radish Peroxidase (HRP) has been used for the detection purpose in this study because of its small size and high stability to

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the chemical modifications. Peroxidases are enzymes of the class **EC 1.11**, which are defined as oxidoreductases that use hydrogen peroxides as electron acceptor. Shown below is the reaction mechanism for the enzymatic catalytic cycle of HRP (Ruzgas et al., 1996).

$$\text{Native peroxidase}\,(Fe^{3+}) + H_2O_2 \rightarrow \text{Compound-I} + H_2O \quad (1a)$$

$$\text{Compound-I} + AH_2 \rightarrow \text{Compound-II} + AH* \quad (1b)$$

$$\text{Compound-II} + AH_2 \rightarrow \text{Native peroxidase}\,(Fe^{3+}) + AH* + H_2O \quad (1c)$$

The first reaction (1a) involves the two-electron oxidation of the ferriheme prosthetic group of the native peroxidase by H_2O_2 (or organic hydroperoxides). This reaction results in the formation of an intermediate, compound-I (oxidation state +5), consisting of oxyferryl iron (Fe(IV) 0 = O) and a porphyrin π cation radical. In the next reaction (1b), compound-I loses one oxidizing equivalent upon one-electron reduction by the first electron donor AH_2 and forms the compound-II (oxidation state +4). The later in turn accepts an additional electron from the second donor molecule AH_2 in the third step (1c), whereby the enzyme is returned to its native resting state, ferriperoxidase.

3,3′, 5,5′-Tertramethyl benzidine or TMB is a chromogenic substrate used in the staining procedure of immunohistochemistry and also as a visualizing agent in ELISA. In solution, TMB forms a blue product when allowed to react with peroxidase enzyme. The resulting color change can be read at 370 nm or 655 nm. The reaction is stopped using acid and the color changes to yellow and can be read at 450 nm.

2. Materials and methods

2.1. Materials and instrumentation

We have used anti-human serum albumin (HSA) (Ab_1), HSA (Ag) and HRP labeled antibody (Ab_2) from Bangalore Genei (India) and *N*-Ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was purchased from Sigma. Gold chloride [$AuCl_4$, $4H_2O$, Au% > 49%] and all other chemicals were of the analytical grade and were used without further purification. All the solutions were prepared using double distilled water.

The absorption spectrum of the samples was recorded in UV-1601 PC (Shimadzu). Electrochemical measurements were performed using CH Instruments 660A with three electrode system, comprising of platinum auxiliary electrode, an Ag/AgCl reference electrode and a gold electrode (as the working electrode). The 1:1 ratio of 0.1 M phosphate buffer (PB) pH 7.0 and 0.1 M KCl was used as electrolyte for all the measurements.

TEM pictures of gold nanoparticles were taken at IICT (Hyderabad).

2.2. Preparation of glutathione coated AuNPs

All the glassware used for the preparation were soaked in freshly prepared HNO_3/HCl mixture, rinsed thoroughly with distilled water and dried. In a typical preparation of gold colloid, glutathione was used for the capping of gold nanoparticles. The gold colloid was prepared by dissolving aurate salt (400 μmol) in double distilled water (100 mL) and then freshly prepared NaBH4 solution (66 μmol, 400 μL) was added dropwise with stirring. The solution was stirred for around 5 min. The prepared AuNPs were stored at 4 °C in refrigerator for further use.

2.3. Preparation of glutathione capped AuNPs

To the gold colloid solution (25 mL; 9 μmol) glutathione (20 mg; 65 μmol) was added and was stirred for 10–15 min. After the stirring was complete the mixture was centrifuged at 4500 rpm to separate the capped AuNPs. The pellet obtained was resuspended in 1 mL of phosphate buffer (pH 7).

2.4. Preparation of AuNP–antibody conjugate

2.4.1. AuNP–antibody was prepared in two steps

Glutathione capped AuNPs were first activated by adding 58 mM EDC (300 μL) in 0.1 M pH 5.0 MES buffer (3 mL) and the solution was kept at 4 °C for 1.5 h on a rocker. The solution was next centrifuged at 15,000 rpm for 1 h to separate activated AuNPs and the pellet was resuspended in 0.1 M phosphate buffer. 50 μL of 500 μM 1,6-diaminohexane (DAH) was added to the activated AuNPs and mixed for 30 min at room temperature and the reaction mixture was again centrifuged and the pellet obtained was redissolved in phosphate buffer.

In the first step 500 μL of 10 μg/mL primary antibody was added to pH 5.0 MES buffer (3 mL) and 58 mM EDC (300 μL) was added and the reaction mixture was kept at 4 °C for 1.5 h on a rocker. The activated primary antibody was added to AuNPs/DAH and the reaction mixture was kept at 4 °C for 6 h. The coupled AuNP–antibody (AuNPs/Ab_1) conjugate was separated by centrifugation (Scheme 1). Scheme 1 (upper part) shows the broad outline of the preparation of the modified gold nanoparticles.

2.5. Antibody immobilization and immunoassay procedure

AuNPs/Ab_1 was immobilized (by adsorption) onto a gold electrode. 5 μL of AuNPs/Ab_1 solution was spread onto the cleaned and polished gold electrode and the electrode was incubated at 4 °C. After the incubation, the electrode was rinsed with distilled water and the electrode was blocked with 1% BSA + 0.05% tween for 30 min at room temperature. The electrode was again rinsed with distilled water to remove any residuals. The AuNPs/Ab_1 electrode was then incubated with a 5 μL of antigen for 30 min at room temperature. After the binding of antigen, the electrode was incubated in 50 μL of 2 mg/mL Ab_2/HRP conjugate. Finally the electrode was washed to remove unbound conjugate. Scheme 1 (lower part) shows the schematic representation of the immobilization procedure. We would like to mention here that the gold electrode is basically "painted over" with a suspension of modified gold nanoparticles.

2.6. Measurement procedure

The modified electrode was then placed in electrochemical cell containing 2.0 mL of 0.1 M PB (pH 7.0) and 0.1 M KCl. TMB (15 μM) was used as electron mediator and H_2O_2 (20 μM) was used as substrate. The cyclic voltammetry measurements were done at the potential range of 450 mV to −300 mV at a scan rate of 10 mV/s and Ag/AgCl as the reference electrode. The impedance measurements were done at the frequency range of 0.1 Hz to 1.0×10^{-5} Hz at room temperature (Z_{re} vs. Z_{im} at 160 mV vs. Ag/AgCl reference electrode). All the electrochemical measurements were performed at room temperature.

3. Results and discussions

3.1. UV–vis spectroscopy studies

The gold nanoparticles synthesized by borohydride reduction of aurate salt are relatively monodisperse in colloidal solution, which is confirmed by a single peak in the absorbance spectra