Int. J.Nano Dimens. 4(1): 69-76, Summer 2013
ISSN: 2008-8868

Contents list available at **IJND**

International Journal of Nano Dimension

Journal homepage: *www.IJND.ir*

Gold nano-particles as electrochemical signal amplifier for immune-reaction monitoring

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Received 10 August 2012
Accepted 15 November 2012

ABSTRACT

A new signal amplification strategy based on simultaneous application of gold nanoparticles (AuNPs) and horseradish peroxidase (HRP) was employed to improve the sensitivity of an electrochemical immunoassay for detection of human IgG (hIgG), as a model antigenic protein. This immunoassay system was fabricated on magnetic carboxyl-functionalized multi-walled carbon nanotubes (COOH-MWCNT/Fe_3O_4 nanocomposite). The COOH-MWCNT/Fe_3O_4 nanocomposite was constructed by chemical co-precipitation of Fe^{2+} and Fe^{3+} in alkaline solution in the presence of the COOH-MWCNT. Goat anti-human IgG (anti-hIgG) was covalently bound to the carbon nanotubes and the resulting anti-hIgG bearing nanocomposite was immobilized on the surface of a gold electrode using a permanent magnet. Human IgG (hIgG) as an antigen was detected electrochemically using a secondary HRP-conjugated anti-hIgG immobilized noncovalently on the surface of AuNPs. Electrochemical detection of hIgG was performed in the presence of H_2O_2 and KI as substrates of HRP. When the concentration of antigen was 200 ng/ml, the sandwich arrangement with AuNPs amplified electrochemical response 56 µA more than a same sandwich arrangement without AuNPs. Binding of HRP-conjugated anti-hIgG to AuNPs was studied by UV–Visible and fluorescence spectrophotometry. The spectrophotometric data showed that binding of antibody molecules to AuNPs occurred without any significant structural changes.

Keywords: *Signal amplification; Gold nanoparticles; Electrochemical immunoassay; Human IgG; COOH-MWCNT/Fe_3O_4 nanocomposite.*

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INTRODUCTION

Immunoassay is a selective, sensitive, versatile and reliable approach in clinical diagnosis [1-3]. Conventional immunoassay methods such as enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) have some limitations such as expensive instrumentation, radiation hazards, time-consuming steps and complicated procedures [4].

On the other hand, electrochemical immunosensor technology has recently received increasing attention for clinical diagnosis due to several benefits including simplicity of use, speed to arise to a result, low cost, continuous monitoring, simple instrumentation, compatibility for miniaturization, mass-fabrication and easy signal amplification [5-8].

Electrochemical immunosensors are usually fabricated by immobilizing immunoreagent component on the surface of an electrode and their responses are based on the specific reaction of the antibody and antigen [5-6].

Disease biomarkers and biological agents are often present at ultra-low levels, therefore development of new methods to increase sensitivity of electrochemical immunosensors is still of great interest. Signal amplification and noise reduction are crucial for obtaining low detection limits in clinical immunoassays [9]. Enzymes, electroactive molecules, redox complexes, and metal ions have been used conventionally as label in amplification strategies. But, in the recent years, introduction of nanomaterials as label has opened new horizons for signal amplification in electrochemical immunoassays. Beyond it, simultaneous use of enzymes (especially HRP) and nanomaterials as a new generation of labels has recently received increasing attention. In this case, nanomaterials are used as a carrier to load HRP or HRP-conjugated antibodies for enhancement of electrochemical signal compared to a system consisting only of a single-enzyme label [10]. Tang et al. developed a new signal amplification strategy based on thionine (TH)-doped magnetic gold nanospheres labeled with HRP-bound anti-carcinoembryonic antigen (anti-CEA) for electrochemical detection of CEA with a detection limit of 0.01 ng mL^{-1}[9]. Wang et al. used numerous HRP loaded on CNT as a label for sandwich type ultrasensitive electrochemical immunoassay of IgG, and a detection limit of 500 fg mL^{-1} was obtained [11]. The gold nanoparticle (Au NPs) is an ideal label in electrochemical immunosensing due to its inherent advantages, such as easy preparation and good biocompatibility [12-13].

In the present work, we used Au NPs to amplify the electrochemical signal to monitor a sandwich type immunoreaction. Au NPs were applied as carriers for loading of HRP-conjugated anti-hIgG (AuNPs- anti-hIgG -HRP conjugate). The sandwich type immunoreaction was designed as follow: AuNPs- anti-hIgG -HRP conjugate /IgG/anti- hIgG/COOH- MWCNT/nano- Fe_3O_4 composite. Electrochemical detection of hIgG was performed in the presence of H_2O_2 and KI as substrates of HRP.

EXPERIMENTAL

Materials

Polyclonal goat anti-human IgG (anti-hIgG) and HRP-conjugated goat anti-hIgG (anti-hIgG-HRP) were obtained from Razi Biotech Co. (Kermanshah, Iran). Human tetanus immunoglobulin G (hIgG) was purchased from CSL Behring GmbH (Germany). MWCNTs (purity>95 wt%, outer diameter: 8-15 nm, inner diameter: 3-5 nm, and length: ~50 μm) were provided by Times Nano Company (Chengdu, China). Hydrogen tetrachloroaurate ($HAuCl_4 \cdot 3H_2O$), trisodium citrate, Nitric acid, Tween 20, potassium iodide, ammonia (NH_3), N-(3-Dimethylaminopropyl)-N′-ethyl carbodiimid hydrochloride (EDC), potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), The ammonium ferric sulfate dodecahydrate, ($NH_4Fe(SO_4)_2.12H_2O$) and ammonium ferrous sulfate hexahydrate, ($(NH_4)_2FeSO_4.6H_2O$, were purchased from Merck (darmstadt, Germany) and used as delivered. N-Hydroxy-succinimide (NHS), 2-(N-morpholino) ethanesulfonic acid (MES) and bovine serum albumin (BSA) were from Sigma-Aldrich Chemical Co. (St. Louis, MO). Poly (tetra-fluoro ethylene) (PTFE) membrane with 0.45 μm in pore size was purchased from Schleicher & Schüll (Germany). All other chemicals were purchased from Merck and used without further purification. A 50 mM phosphate-buffered saline (PBS), pH 7.2, containing 1% (w/v) BSA was used as blocking buffer. Washing solution was prepared by dissolving 0.05% (v/v) Tween-20 in PBS. Various concentrations of hIgG and anti-hIgG were prepared by diluting the stock solutions in the PBS. All experiments were carried out at room temperature (25 ± 2 ^oC).

Apparatus

All electrochemical experiments were performed using a computerized Potentiostat/Galvanostat (model 263-A, EG&G, USA) equipped with Power Suite software package. A single-compartment three-electrode cell (volume 300 µL) was employed for electrochemical studies. A gold plate working electrode (2 mm diameter) equipped with a permanent magnet, a saturated silver/silver chloride (Ag/AgCl) reference electrode containing 3 M KCl (Azar electrode, Iran), and a platinum wire auxiliary electrode were employed for all electrochemical experiments. All potentials were measured and reported versus the Ag/AgCl reference electrode. The morphology of the modified MWCNTs was characterized using a Hitachi, model s-4160 (Japan) field emission scanning electron microscopy (FESEM) at 15 kV. UV-Vis spectrophotometric measurements were carried out using a UV3100 Shimadzu spectrophotometer (Japan). Fluorescence spectroscopy was performed by using a Hitachi MPF-4 spectrofluorimeter (Japan).

Preparation of COOH-MWCNT/Fe_3O_4 nanocomposite

Functionalization of MWCNTs with carboxyl groups was performed according to Rahimi et al. report [14]. In brief, MWCNT samples were dispersed in nitric acid (35%) and sonicated in a bath sonicator (30 W, 40 kHz) for 5 h. Functionalized MWCNT was filtered using a 0.45 µm hydrophilized PTFE membrane. Obtained MWCNT mat was washed with deionized water to remove residual acid, followed by drying under infra red lamp.

For preparation of The COOH-MWCNT/Fe_3O_4 nanocomposite according to Qu et al. procedure [15], 10 mg of purified COO-MWCNT was suspended in 2 mL of a solution containing (4.33 mM) $(NH4)_2$ $Fe(SO_4).6H_2O$ and 25 mg $NH_4Fe(SO_4)_2.12H_2O$ (8.66 mM). The mixture was sonicated for 10 min at 50°C. Then, under sonication condition, 10 mL of NH_3 aqueous solution (8 M) was added dropwise to the mixture. In order to precipitate Fe_3O_4, the pH of the final mixture should be in the range of 11-12. Finally, to remove the impurities such as sulfate, ammonia and excess of OH^-, the prepared magnetic nanocomposite (COOH-MWCNT-Fe_3O_4) was washed with water thoroughly with the aid of a permanent magnet.

Immobilization anti-hIgG on magnetic nanocomposite

Covalent attachment of anti-hIgG (capturing antibody) to COOH-MWCNT-Fe_3O_4 nanocomposite was carried out using the method introduced by Jiang et al. [16] with little modification. In the first step, 0.7 mg magnetic nanocomposite was suspended in 1.5 ml of MES buffer (50 mM, pH 6.1). Then 0.5 mL from 50 mg/ml NHS in MES buffer solution was added to the suspension and the mixture was sonicated for 10 minute. Under fast stirring, 0.3 ml fresh EDAC (10 mg/mL) prepared in the MES buffer was added quickly, and the mixture was continuously stirred at room temperature for 45 min. To remove the excess of EDAC, NHS and byproduct urea, the magnetic nanocomposite was washed with MES buffer three times using a permanent magnet. In the second step, 25 µL of anti-hIgG (1 mg/ml) in PBS buffer (pH 7.2) was added to the 2.5 ml of nanocomposite suspension in 50 mM MES buffer (pH 6.1). The mixture was shacked at 220 rpm for 2 hours. During this step, the activated carboxyl groups on MWCNTs react with terminal amino groups of the antibody giving rise to stable amide bonds. Finally, to remove the loosely bound antibody molecules, magnetic nanobiocomposite was washed with MES buffer three times. To block nonspecific binding sites, the anti-hIgG/MWCNT-Fe_3O_4 composite was treated with blocking buffer and washed with washing buffer. The obtained magnetic nanobiocomposite was dispersed in 50 mM MES buffer and stored at 4°C until use.

Synthesis of Au nanoparticles

Colloidal gold nanoparticles (Au-NPs) were prepared via the reduction of Au salt by a strong reducing agent according to the previous literature [17]. Briefly, 0.5 mL of 1% (w/v) sodium citrate solution was added to 50 mL of 0.01% (w/v) $HAuCl_4 \cdot 3H_2O$ with constant stirring and then solution was heated up to 60°C. After 15 min, when the solution's color changed from light yellow to wine red, the solution was cooled down. The prepared AuNPs were stored in dark glass bottles at 4°C.

Conjugation of AuNPs with anti-hIgG-HRP (AuNPs-Ab-HRP conjugate)

For conjugation, 150 µL of synthesized AuNPs colloidal solution was diluted to 700 µL with phosphate buffer solution (PBS, pH 7.2) and then 2 µL of HRP-conjugated anti hIgG was added to it. The mixture was continuously stirred for 3 hours at room temperature. Then, for removing of unbounded antibody molecules, the mixture was centrifuged at 4000 rpm for 15 min and the precipitate was re-dispersed in 3 mL of PBS. BSA solution (4% w/v) was used to block the nonspecific binding sites. After washing, the AuNPs-Ab-HRP conjugate was resuspended in 20 mL of PBS to provide a final dilution 1:10000 (v/v) of Ab-HRP in same concentration of previous reported work [18]. The AuNPs-Ab-HRP conjugate was stored at 4°C before use.

Electrode preparation and measurement

Schematic diagram of the prepared electrode is shown in Figure 1. At first, the anti-hIgG/COOH-MWCNT-Fe_3O_4 bio-nano-composite was fixed on the working gold plate electrode using a permanent magnet. Then, the electrode was incubated for 1 hr with 80 µL of different concentrations of hIgG in PBS solution containing 1% BSA. After washing with washing buffer, the electrode was incubated with 80 µL of AuNPs-Ab-HRP conjugate (dilution 1:10000) in PBS containing 1% BSA for 1 hr, followed by three times washing. The electrochemical measurement of the modified electrode was performed using cyclic voltammetric method in 300 µL PBS (50 mM, pH 7.2) containing 2 mM H_2O_2 and 3 mM KI. The background response was recorded in the same condition but without incubating the electrode with hIgG.

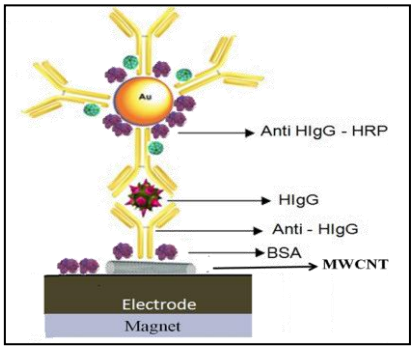

Fig. 1. Diagram of the sandwich type electrochemical immunoassay.

RESULTS AND DISCUSSION

Characterization of the magnetic nano-composite on Au electrode

The morphology of functionalized carbon nanotubes was studied using FESEM. Figure 2a demonstrates the morphology of COOH-MWCNTs/Fe_3O_4 nanocomposite absorbed on Au electrode. As seen, treatment of MWCNTs with nitric acid and subsequent sonication did not affect severely the physical structure of MWCNTs so that they could conserve their tube-shaped structure perfectly. Figure 2b shows the FESEM image of anti-hIgG immobilized on the magnetic nanocomposite. The diameter of MWCNT in Figure 2b is larger than those in Figure 2a due to antibody immobilization. To confirm the immobilization of antibody molecules on nanocomposite, a control experiment without EDAC and NHS was carried out. In this case, the nanocomposites were rather clean under electron microscopy.

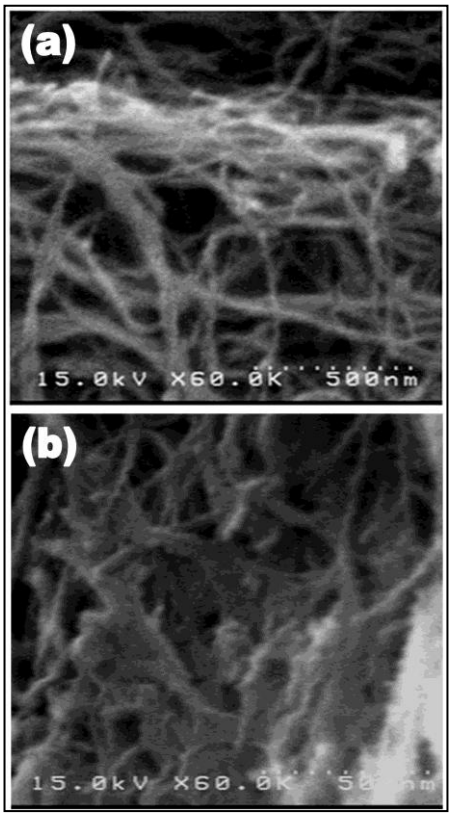

Fig. 2. FESEM images of (a) COOH-MWCNT/Fe_3O_4 nanocomposite and (b) anti-hIgG/COOH-MWCNT-Fe_3O_4 nanobiocomposite. Accelerating voltage and magnification were 15 kV and ×60000, respectively.

Spectrophotometric characterization

UV–Vis absorption spectra of AuNPs and AuNPs-Ab-HRP were recorded with a UV–Vis spectrophotometer. The fresh AuNPs solution without any further treatment showed a peak at 524 nm (Figure 3a) which confirm the formation of gold nanoparticles in the colloidal solution [19]. After incubating AuNPs with HRP-conjugated anti-hIgG a significant red shift (λ_{max} of 542 nm) was seen (Figure 3b). The observed red shift may be due to an interaction between AuNPs and antibody molecules [9, 19].

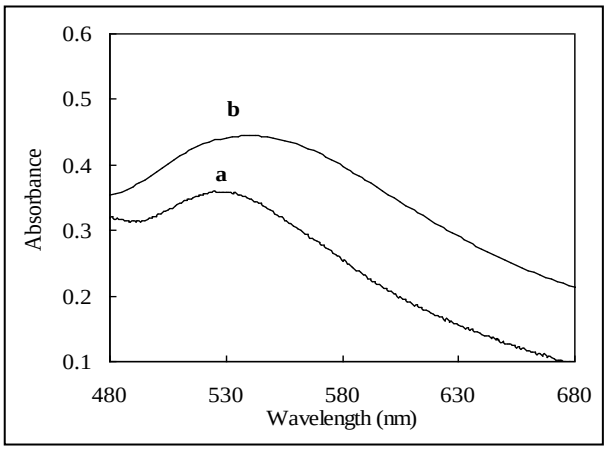

Fig. 3. UV-Vis absorption spectra of (a) synthesized AuNPs colloidal solution and (b) AuNPs-labeled HRP-conjugated antibody.

To confirm the attachment of antibody molecules to AuNPs and also structural characterization of attached antibodies, intrinsic fluorescence emission test of tryptophan at the excitation wavelength of 280 nm was performed. The intrinsic emission of tryptophan is between 300-360 nm. The emission spectra of AuNPs, HRP-conjugated anti-hIgG and AuNPs- anti-hIgG-HRP conjugate, are shown in Figure 4. In the region of 300-360 nm, AuNPs did not showed any detectable emission peak. HRP-conjugated anti-hIgG showed a peak at 336 nm which is related to the intrinsic emission of tryptophan residues in HRP and anti-hIgG molecules. At the presence of AuNPs quenching of the protein tryptophan fluorescence was seen (Figure 4d). Significant quenching of tryptophan fluorescence reveals the interaction of HRP-conjugated anti-hIgG with AuNPs. No considerable red or blue shift in the spectrum of HRP-conjugated anti-hIgG was seen in the presence of gold nanoparticles. Thus, it is concluded that antibody molecules interact with AuNPs without evident structural changes [20-22].

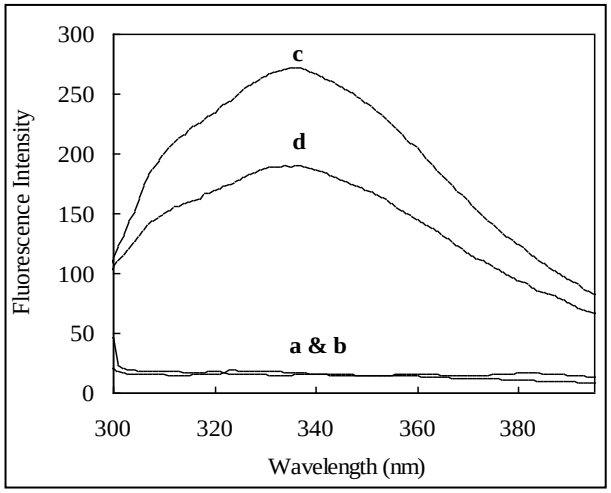

Fig. 4. Intrinsic Fluorescence spectrum of (a) 50 mM PBS (pH-7.2), (b) AuNPs colloidal solution diluted in the PBS, (c) HRP-conjugated anti-hIgG in the PBS and (d) AuNPs-labeled HRP-conjugated anti-hIgG in the PBS.

Electrochemical behavior of anti-hIgG on MWCNT/Fe3O4 nanocomposites

Cyclic voltammetry of ferricyanide as a marker was used as a convenient tool for monitoring the barrier effect of the modified electrode. The change of electrode behavior after each modification step was investigated using this marker. The cyclic voltammograms (CVs) of $[Fe(CN)_6]^{4-}/[Fe(CN)_6]^{3-}$ (2mM) in 0.1 M KNO_3 (supporting electrolyte) on the modified electrodes are shown in Figure 5. Curve (a) shows the CV of $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ redox couple at a bare Au electrode. When COOH-MWCNT/Fe_3O_4 nanocomposite was fixed on the Au electrode surface, the CV of redox couple was increased (curve b) in comparison to the bare electrode (curve a) due to the high conductivity of MWCNTs. Proteins can act as an insulating layer, thus immobilization of anti-hIgG on COOH-MWCNTs/Fe_3O_4 nanocomposite could decrease the current of redox peaks (curve c). In the same manner, blocking of the modified electrode with BSA, addition of antigen (hIgG) and finally addition of AuNPs-anti-hIgG -HRP conjugate (sandwich assembly), were further reduced anodic and cathodic peaks (curves d-f).

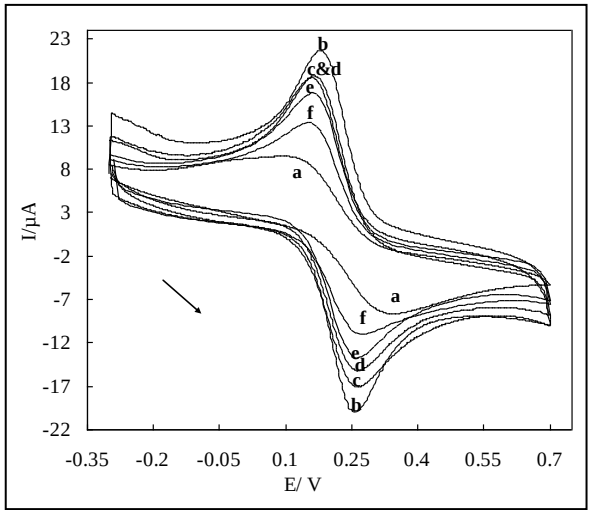

Fig. 5. CVs of different electrodes in $[Fe(CN)_6]^{4-/3-}$ solution (2 mM) prepared in KNO_3 (0.1 M): (a) bare Au, (b) COOH-MWCNT-Fe_3O_4/Au, (c) anti-hIgG/COOH-MWCNT-Fe_3O_4/Au, (d) anti-hIgG/COOH-MWCNT-Fe_3O_4/Au blocked with BSA 1% (w/v), (e) hIgG -anti-hIgG/COOH-MWCNT-Fe_3O_4/Au blocked with BSA, (f) same electrode as (e) but incubated with HRP-conjugated anti-hIgG [dilution of 1/10000 (v/v)] for 1 hr. The scan rate was 50 mV s^{-1}.

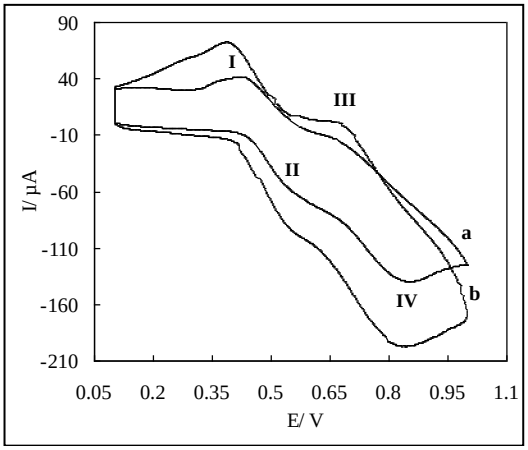

Fig. 6. CVs of hIgG/anti-hIgG/COOH-MWCNT-Fe_3O_4/Au electrode incubated with (a) HRP-conjugated anti-hIgG, (b) AuNPs-labeled HRP-conjugated hIgG in 0.05 M PBS (pH 7.2) containing 2 mM H_2O_2 and 3 mM KI. The scan rate was 50 mV s^{-1}.

Comparison of Electrochemical Responses

Previously we proposed a sandwich electrochemical immunoassay based on a magnetic nanocomposite for detection of hIgG as an antigenic protein [18]. In the current investigation, a comparative study of the amperometric responses of immunoreactions was carried out between bare HRP-conjugated anti-hIgG and AuNPs -labeled HRP-conjugated anti- hIgG as the secondary antibodies in the sandwich immune-reactions. Figure 6 shows the cyclic voltammograms of the HRP-Ab-Ag-Ab/MWCNT-Fe_3O_4/Au electrode and HRP- Ab- Au NPs – Ag – Ab / MWCNT Fe_3O_4/Au electrode. One can find that by using AuNPs-labeled HRP-conjugated anti-hIgG, a much greater amperometric change (about 56 µA) at 0.85 V peak (vs Ag/AgCl) is obtainable in comparison to sandwich immune-reaction without AuNPs. The peak at 0.85 V is related to oxidation of I^- to I^2 [18]. HRP catalyses the oxidation of I^- in the presence of H_2O_2 as follows [22]:

$$HRP\ (Fe^{3+}) + H_2O_2 \rightarrow Compound\ (I) + H_2O \qquad (1)$$

$$Compound\ (I) + 2\ I^- \rightarrow HRP\ (Fe^{3+}) + I_2 \qquad (2)$$

This observation shows that AuNPs can increase the surface coverage of HRP-conjugated anti-hIgG and subsequently leads to an amplified signal output due to more oxidation of I^- to I^2. The above results apparently suggest that AuNPs-labeled HRP-conjugated antibody molecules could have a better performance for detection of antigenic molecules in comparison to bare HRP- conjugated antibody molecules. According to our results, the proposed immune-reaction may also have a potential to improve analytical parameters such as antigen detection limit.

Determination of non-specific binding

Non-specific binding of antigens or detecting antibodies to unoccupied spaces on the surface of MWCNT-Fe_3O_4 nanocomposites and/or AuNPs during consecutive steps of the immune-reaction could be detrimental to the specificity and sensitivity of the assay. Two possible non-specific bindings can occur in the proposed immune-reaction: binding of AuNPs-labeled HRP-conjugated anti-hIgG to the surface of modified electrode and binding of anti-hIgG (capturing antibody) to AuNPs. We minimized these non-specific bindings to the surfaces by blocking unoccupied binding sites with BSA.

Figure 7 shows CVs of anti-hIgG/COOH-MWCNT-Fe_3O_4/Au electrode incubated with (a) BSA (1%) only, (b) BSA (1%) and HRP-anti-hIgG-AuNPs conjugates that treated with BSA (4%), respectively in presence 0.05 M PBS (pH 7.2) containing 2 mM H_2O_2 and 3 mM KI. In fact, the

control experiment (b) doesn't have any two non-specific binding conditions that mentioned above. As seen in voltammograms, CVs of both of modified electrode (a, b) don't show significant difference in anodic peak (III-IV), even at others peaks.

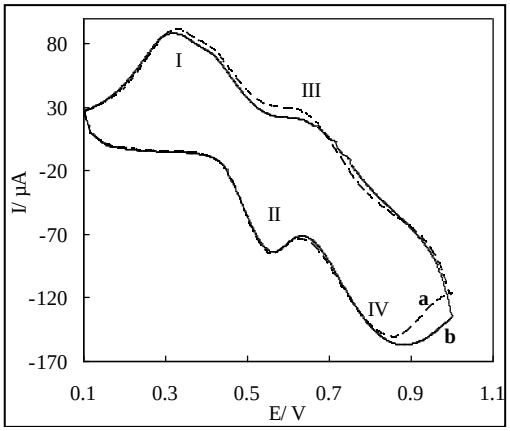

Fig. 7. CVs of anti-hIgG/COOH-MWCNT-Fe_3O_4/Au electrode in the presence of 0.05 M PBS (pH 7.2) containing 2 mM H_2O_2 and 3 mM KI incubated with (a) BSA (1%), (b) BSA (1%) and HRP-anti-hIgG-AuNPs conjugates which are treated with BSA (4%), respectively. The scan rate was 100 mV s^{-1}.

Three possible explanations may contribute to this result. First, because of same electrochemical response in CVs of a &b, it shows that treated anti-hIgG/ MWCNT-Fe_3O_4 with BSA (1%) and anti-hIgG-HRP-AuNPs with BSA (4%) is sufficient for blocking non specific binding. Second, it validates which signal amplification exhibited in Figure 6 is not related to non-specific binding of Au-Ab-HRP on modified electrode. Third, the proposed method could have potential to prevent other protein and biomolecules in a real sample.

CONCLUSIONS

We have proposed a novel strategy for developing an electrochemical signal amplification method in sandwich immune-reactions based on covalently immobilized antibody on the COOH-MWCNTs/Fe_3O_4 nanocomposite and AuNPs-labeled HRP-conjugated antibody. The results of our study showed that combination of HRP-conjugated antibodies and AuNPs could amplify the current signal compared to conventional methods which are based on enzyme technology. Using the proposed signal amplification, a lower detection limit in compared to our previous report [18] is probably obtainable in electrochemical immunoassay. The designed immune-reaction could be developed for the construction of different electrochemical immunosensors for clinical applications.

ACKNOWLEDGEMENTS

Financial supports provided by the Research Council of the University of Tehran, Iran is gratefully appreciated.

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Cite this article as: H. Zarei *et al.*: Gold nano-particles as electrochemical signal amplifier for immune-reaction monitoring.
Int. J.Nano Dimens. 4(1): 69-76, Summer 2013