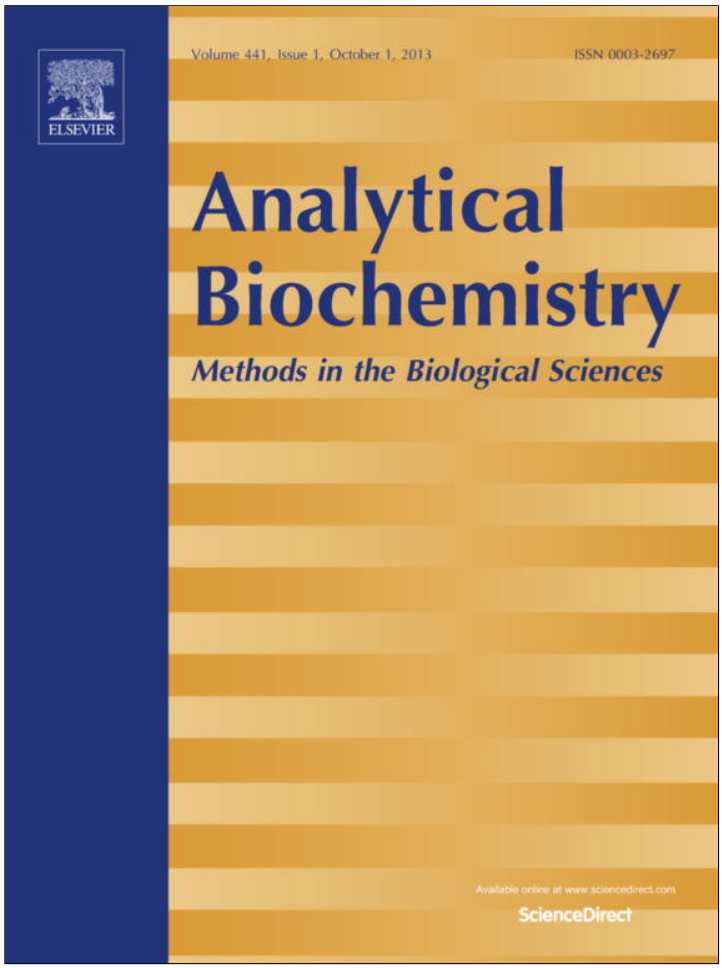

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Magnetic nanoparticle-based immunosensor for electrochemical detection of hepatitis B surface antigen

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ABSTRACT

An electrochemical immunosensor was developed for the detection of hepatitis B surface antigen (HBsAg). The biotinylated hepatitis B surface antibody was immobilized on streptavidin magnetic nanoparticles and used for targeting the HBsAg. By the addition of horseradish peroxidase conjugated with secondary antibody (HRP–HBsAb), a sandwich-type immunoassay format was formed. Aminophenol as substrate for conjugated HRP was enzymatically changed into 3-aminophenoxazone (3-APZ). This electroactive enzymatic production (3-APZ) was transferred into an electrochemical cell and monitored by cyclic voltammetry. Under optimal conditions, the cathodic current response of 3-APZ, which was proportional to the HBsAg concentration, was measured by a glassy carbon electrode. The immunosensor response was linear toward HBsAg in the concentration range from 0.001 to 0.015 ng/ml with a detection limit of 0.9 pg/ml at a signal/noise ratio of 3.

Specific affinity between antibody and corresponding antigen, so-called immunoassay, provides a promising analytical method for clinical assay and biochemical analysis [1–4]. During recent years, conventional diagnostic methods, such as enzyme-linked immunosorbent assay (ELISA),[1] microparticle enzyme immunoassay, and radioimmunoassay, have been the main methods used for detection of different viruses such as human hepatitis B virus (HBV) [5]. HBV is one of the most common contagious diseases that cause cirrhosis, chronic hepatitis, and primary liver cancer. However, these methods are often time-consuming and complicated, and they require advanced and costly instrumentation. Conversely, electrochemical immunosensors have offered several benefits, including easy-to-use, high-sensitivity, low-cost, and inherent miniaturization [6–11]. To obtain highly sensitive immunosensors, various labels such as nanomaterials [12,13], fluorescent compounds [14], quantum dots [3,4], and enzymes [15–17] have been developed to monitor antibody–antigen interaction. The most common label employed for signal amplification is enzyme [18–20]. A series of enzyme labels could be used, including glucose oxidase [21], alkaline phosphatase [22–27], esterase [28], and horseradish peroxidase (HRP) [29,30]. HRP-labeled antibody is popular due to its small size and high stability to the chemical modifications. There are several substrates for electrochemical HRP-labeled bioassays, including *o*-phenylenediamine dihyrochloride [31], hydroquinone [32], tetramethylbenzidine [33], and osmium complex [34]. In addition, phenol-based substrates such as *m*-aminophenol [35], *p*-aminophenol [36], and *o*-aminophenol (o-AP) [37] have been reported. So far, these substrates have been used for determination of *Brucella melitensis* [38], transferrin [39], *Schistosoma japanicum* antigen [40], and complement 3 in human serum [41].

Magnetic nanoparticles (MNPs) are widely used as a carrier of antibody in clinical laboratories and have become one of standard formats in high-throughput assay [42]. MNPs allow several benefits, including (i) easy separation and localization of targeted molecules in the reaction mixture by an external magnet, (ii) extremely high surface/volume ratio to enhance the amounts of loaded biomolecules, (iii) fast reaction between antigen and antibody, and (iv) easy miniaturization [43].

In the current study, an electrochemical immunosensor was designed and developed for detection of hepatitis B surface antigen (HBsAg). HBsAg was targeted by formation of a sandwich-type immunocomplex on MNPs. Then, aminophenol as substrate for HRP, conjugated on secondary antibody, was enzymatically changed into 3-aminophenoxazone (3-APZ). This electroactive enzymatic product (3-APZ) was transferred into an electrochemical cell and, apart from sandwich complex, was monitored by cyclic

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[1] *Abbreviations used:* ELISA, enzyme-linked immunosorbent assay; HBV, hepatitis B virus; HRP, horseradish peroxidase; o-AP, *o*-aminophenol; MNP, magnetic nanoparticle; HBsAg, hepatitis B surface antigen; 3-APZ, 3-aminophenoxazone; b-HBsAb, biotinylated hepatitis B surface antibody; HRP–HBsAb, HRP-conjugated HBsAb; PBS, phosphate buffer solution; BSA, bovine serum albumin; BR, Britton–Robinson; GC, glassy carbon; Ag/AgCl, silver/silver chloride; CV, cyclic voltammogram.

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voltammetry. In an optimal condition, the characteristics of electrochemical sensor such as sensitivity, detection limit, stability, and specificity were investigated in detail.

Materials and methods

Chemicals and materials

Biotinylated hepatitis B surface antibody (b-HBsAb), HRP-conjugated hepatitis B surface antibody (HRP–HBsAb), and HBsAg standard stock solution (50 ng/ml) were obtained from Pishtaz Teb (Tehran, Iran) and diluted in phosphate buffer solution (PBS). The streptavidin-coated MNPs in phosphate/glycine buffer (pH 7.4), containing bovine serum albumin (BSA) and sodium azide (0.09%) as stabilizer and H_2O_2 (30%) (w/w), were purchased from Merck and used without further purification. o-AP was obtained from Sigma. PBS (pH 7.4) was prepared by dissolving NaCl (8.0 g), Na_2HPO_4 (1.15 g), KH_2PO_4 (0.2 g), and KCl (0.2 g) in 1000 ml of double distilled water. The working substrate solution was prepared daily with Britton–Robinson (BR) solution containing 2 mM o-AP and 0.01 M H_2O_2. Washing buffer was prepared by dissolving 0.05% Tween 20 in PBS. All solutions were prepared in double distilled water.

Apparatus

All electrochemical experiments were performed using a potentiostat/galvanostat system (model 263-A, EG&G, USA) equipped with the Power Suite software package. Electrochemical studies were carried out at room temperature using a three-electrode system consisting of a bare glassy carbon (GC) working electrode, a KCl saturated silver/silver chloride (Ag/AgCl) reference electrode (both from Azar Electrode, Uromia, Iran), and a platinum wire as an auxiliary electrode.

Conjugation of b-HBsAb to MNPs

Initially, 100 μl of the streptavidin-coated MNPs was suspended in phosphate/glycine buffer (pH 7.4) containing BSA and sodium azide while the solution was shaking vigorously. Then, 100 μl of b-HBsAb was added to the suspension, and the mixture was shaken slowly to prevent sedimentation of the streptavidin-coated MNPs. Thereafter, the suspension was washed three times with washing buffer. Finally, HBsAb/MNP conjugate (composed of b-HBsAg with streptavidin-coated MNPs) was separated from the suspension using an external magnet. Based on our experience, it seems that approximately 80 μg of b-HBsAb is generally optimal for conjugating with 1 mg of streptavidin-coated MNPs.

Capturing target via immunosensing complex

The HBsAb/MNP conjugate was resuspended in 100 μl of PBS (pH 7.4) containing HBsAg. After 30 min of incubation with delicate shaking at 37 °C, HBsAg was captured with the HBsAb/MNP conjugate separated magnetically. Then, it was washed three times with washing buffer to remove any unbound HBsAg. Thereafter, the MNP–HBsAb/HBsAg conjugate was resuspended in a mixture of PBS (pH 7.4) containing HRP–HBsAb (1:5000 dilutions) and then incubated for 30 min at 37 °C while it was shaking mechanically. The MNP–HBsAb/HBsAg/HBsAb–HRP complex was then separated by using an external magnet and washed three times with washing buffer. Finally, the complex was resuspended in 500 μl of PBS and stored at 4 °C (Scheme 1).

Electrochemical measurement

The sandwich complexes containing MNP–HBsAb/HBsAg/HBsAb–HRP were formed using different concentrations of HBsAg. Afterward, in the presence of working substrate solution, the HRP enzymatic product of sandwich complexes was formed in PBS (pH 6.5) at 37 °C. Then, the enzymatic product (900 μl) was transferred into the electrochemical cell and measured using a three-electrode cell system. Finally, using a naked GC electrode, the cyclic voltammogram (CV) of 3-APZ as enzymatic product was recorded in the potential range from 0.1 to −0.5 V (vs. Ag/AgCl) at a scan rate of 50 mV/s.

Results and discussion

Detection of HBsAg by cyclic voltammetry

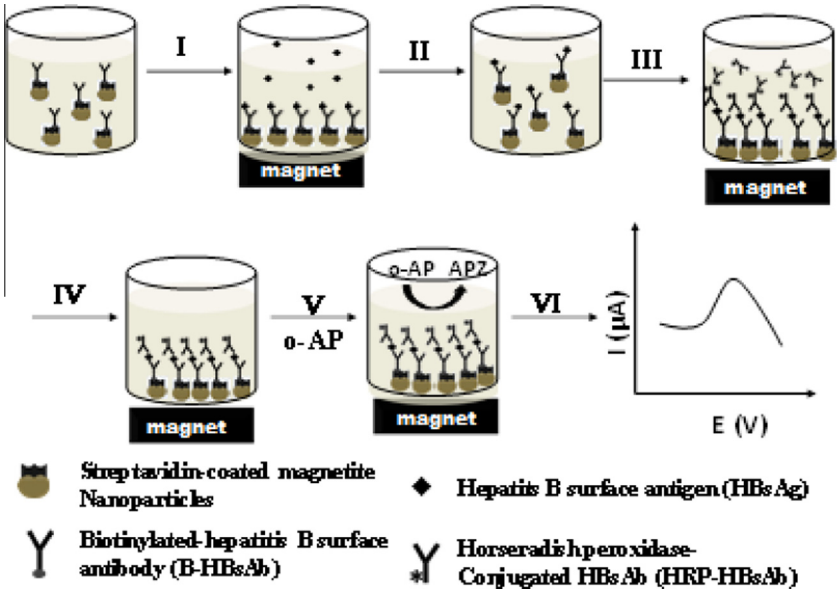
The direct linking of biotinylated antibody to the streptavidin-coated MNPs provides a right orientation of antibody to free antigen. HRP–HBsAb was used as a tracer, and o-AP and H_2O_2 were used as enzyme substrates. HRP is known to catalyze redox reactions of the following type:

$$S(\text{red}) + H_2O_2 \rightarrow P(\text{ox}) + H_2O, \quad (1)$$

where S(red) refers to the reduced form of substrate that is oxidized to product of P(ox). But in the current study, enzyme-labeled antibody (HRP–HBsAb) in BR buffer and in the presence of H_2O_2 is expected to catalyze the oxidation of o-AP and produce the electroactive enzymatic product of 3-APZ [44]. Then, 3-AZP could be reduced at GC electrode during the cyclic voltammetry. According to the Michaelis–Menten kinetic model in the saturated substrate condition, the product concentration depends on enzyme concentration (HRP–HBsAb). However, HRP–HBsAb concentration is limited by the amount of HBsAg. In other words, the resulting reduction current output is proportional to the amount of HBsAg in the sample.

Cyclic voltammetry of 3-APZ was performed at naked GC electrode in the potential range from 0.10 to −0.50 V, at a scan rate of 50 mV/s, in BR buffer. H_2O_2 showed no voltammetric peak in this potential window (Fig. 1, curve a). However, in the presence of o-AP in BR buffer, a couple of reduction and oxidation peak currents were observed at −0.25 and −0.23 V versus Ag/AgCl (Fig. 1, curve b) with peak separation (ΔE_p) and formal potential (E°) of 0.02 and −0.24 V, respectively. This redox reaction could be assigned to 3-APZ, which was produced by either auto-oxidation or HRP catalytic oxidation of o-AP [45,46]. As seen in curve c of Fig. 1, by the addition of H_2O_2 to o-AP, no change in redox peak current was observed. But by the addition of HRP–HBsAb (20 μl) to the substrate solution (H_2O_2 and o-AP), the redox current drastically increased with a slight shift in both cathodic and anodic peak potentials (E_{pc}: −0.28; E_{pa}: −0.25; E°: −0.265 V) (Fig. 1, curve d). This shift could be due to the accumulation of the electroactive product at electrode surface. Using different concentrations of HBsAg (0, 15, 20, and 40 ng.ml^{-1}), sandwich complexes of MNP–HBsAb/HBsAg/HBsAb–HRP were formed. Detection of HBsAg was performed by measuring the cathodic peak intensity. As depicted in Fig. 2, the current intensity was increased by increasing the HBsAg concentrations.

o-AP was used as mediator for the electron transfer between HRP and H_2O_2 [37]. It has been shown that by electro-oxidation of o-AP, *o*-quinone imine is formed through formation of o-AP$^{\cdot+}$ radical cations, a reactive and soluble intermediate. Finally, by the addition and tautomerization reactions, with the cyclic dimer of 3-APZ resulting as the major product [45], it could undergo a reversible redox reaction [46] (Scheme 2).



Scheme 1. Steps for sandwich preparation and electrochemical immunoassay: (I) addition of HBsAg to MNP/HBsAb; (II) washing and separation of unbound HBsAg magnetically; (III) sandwich formation by addition of HRP–HBsAb to MNP–HBsAb/HBsAg; (IV) washing and separation of unbound HRP–HBsAb magnetically and resuspension of complex in fresh PBS; (V) addition of o-AP to sandwich complex to produce 3-APZ; (VI) separation of sandwich complex magnetically and transferring 3-APZ to electrochemical cell for signal recording.

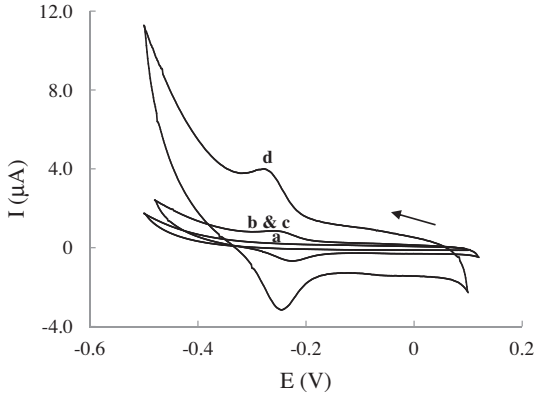


Fig.1. CVs obtained in the solution containing 0.01 M H_2O_2 (a), 2 mM o-AP (b), mixture solution of H_2O_2 (0.01 M) and o-AP (2 mM) (c), and mixture solution of H_2O_2 (0.01 M), o-AP (2 mM), and HRP–HBsAb (dilution of 1:5000, v/v) (d) at naked GC electrode. The experiments were carried out in 0.01 M BR buffer solution (pH 6.5). The scan rate was 50 mV/s.

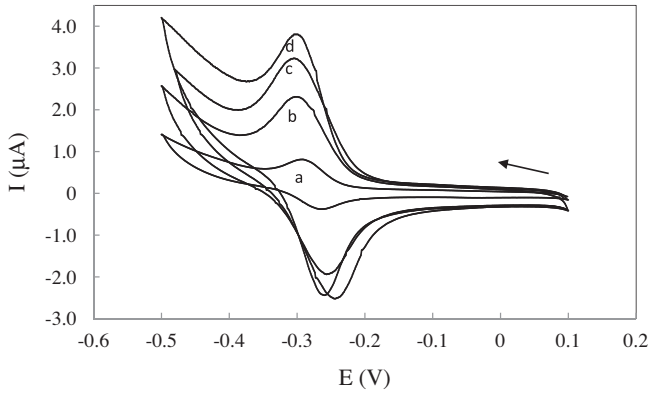


Fig.2. CVs of 3-APZ on a naked GC electrode. 3-APZ samples produced in the presence of sandwich complexes (MNP–HBsAb/HBsAg/HBsAb–HRP) using different concentrations of HBsAg: (a) 0 ng/ml; (b) 15 ng/ml; (c) 20 ng/ml; (d) 40 ng/ml. 3-APZ samples were transferred to the electrochemical cell for cyclic voltammetry. The samples were prepared in BR buffer solution (pH 6.5) containing 2 mM o-AP and 0.01 M H_2O_2. The scan rate was 50 mV/s.

Optimization of experimental parameters

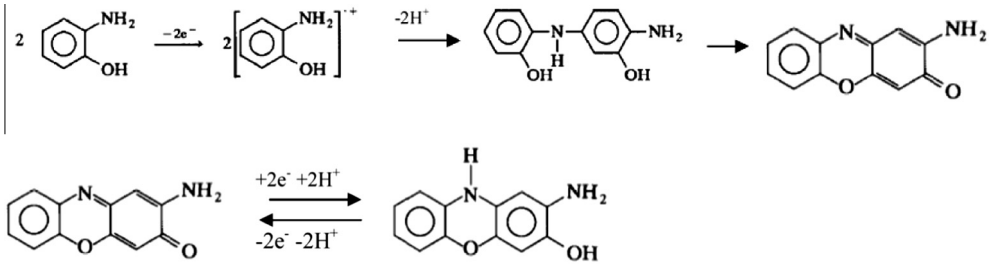
pH Effect

The sensitivity of the immunosensor is affected by pH at two steps. First is the measurement of cathodic current produced by 3-APZ as signal source for the immunosensor. To obtain the best sensitivity, 3-APZ as enzyme reaction product was electrochemically measured at different pHs (ranging from 5.2 to 7.0) in 0.01 M BR buffer containing H_2O_2, o-AP, and HRP–HBsAb. As shown in Fig. 3, increases in pH (from 5.2 to 7.0) caused a negative shift in both anodic and cathodic peak potentials. By increasing pH from 5.2 to 7.0, the E° value of 3-APZ was found to be linearly decreased with a slope of −57.4 mV/pH (Fig. 3, inset). This result was close to the expected theoretical value of −57.8 mV pH^{-1} for a reversible proton-coupled single electron transfer process. This indicates that one proton participated in the electron transfer process between the electrode and 3-APZ. Obviously, the maximum peak currents are observed at a pH value of 6.5. This shows that the HRP–HBsAb has its highest bioactivity at around pH 6.5.

The pH of antigen–antibody interaction is another important factor that affects sensitivity of the immunosensor. In Fig. 4, the immunosensor responses are compared while the immunoreaction takes place at different pHs in 0.01 M BR buffer (containing H_2O_2, o-AP, and HRP–HBsAb). As seen, the reaction of antigen and antibody takes place with a smooth change over pHs ranging from 6.5 to 9.0 and performs excellently at an optimal pH of 7.5. Thus, pHs of 7.5 and 6.5 were chosen as optimal pH for incubating and electrochemical steps, respectively.

Temperature effect

The immunosensor response was also compared in the temperature range from 4 to 50 °C. As shown in Fig. 5, by increasing the temperature up to 37 °C, the response signal was increased. At temperatures above 37 °C, the response signal deteriorated. This



Scheme 2. Top: Production of 3-APZ from auto-oxidation of o-AP monomers. Bottom: Reversible redox reaction of 3-APZ.

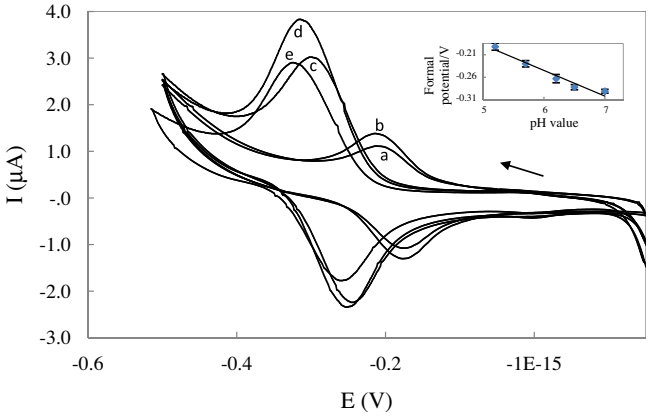


Fig.3. CVs of 3-APZ at different pHs. The naked GC electrode was placed in the BR buffer solution with different pHs of 5.2 (a), 5.7 (b), 6.0 (c), 6.5 (d), and 7.0 (e) containing 2 mM o-AP, 0.01 M H_2O_2, and HRP–HBsAb (dilution of 1:5000, v/v). The scan rate was 50 mV/s. The inset shows the plot of formal potential versus pH.

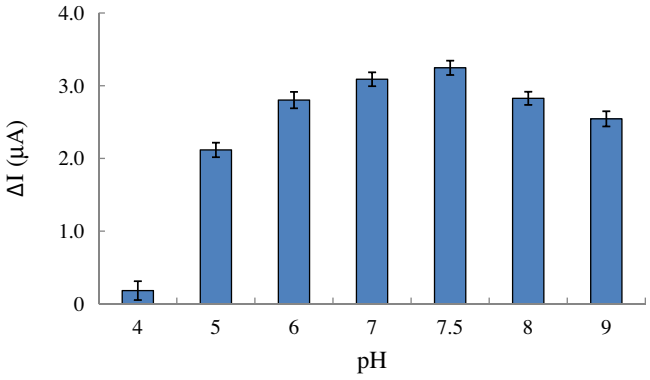


Fig.4. Dependency of antigen–antibody interaction on pH. Each column (mean value of three replicate measurements) shows cathodic current obtained by cyclic voltammetry of 3-APZ on naked GC electrode. The complex of MNP/HBsAb/HBsAg was incubated with HRP–HBsAb for 30 min at different pHs (4.0–9.0). The interaction was carried out in 0.01 M PBS in the presence of 2 mM o-AP and 0.01 M H_2O_2 solution.

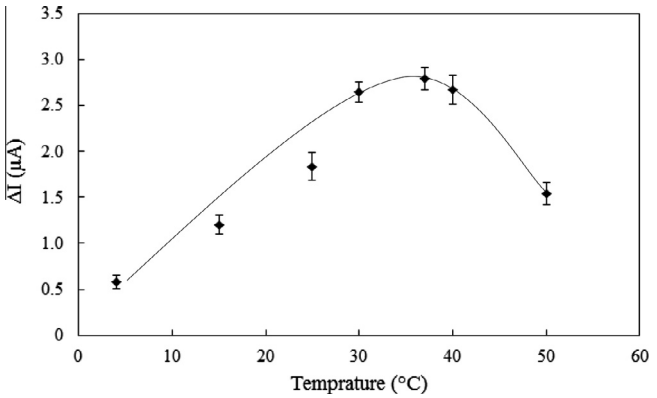


Fig.5. Dependency of antigen–antibody interaction on temperature. Each point (mean value of three replicate measurements) shows cathodic current obtained by cyclic voltammetry of 3-APZ on naked GC electrode. The complex of MNP–HBsAb/HBsAg was incubated with HRP–HBsAb for 30 min in 0.01 M PBS (pH 7.4) at different temperatures (4, 15, 25, 30, 37, 40, and 50 °C) in the presence of 2 mM o-AP and 0.01 M H_2O_2.

could be due to the effect of high temperature on the equilibrium constant of antigen–antibody interaction and the activity of biomolecules. Therefore, to obtain the maximum response of the immunosensor, 37 °C was chosen as the optimized temperature.

Incubation time

The influence of incubation time on the immunosensor response was studied in the time periods from 5 to 50 min for interaction between HBsAg and either MNP/HBsAb or HRP–HBsAb. As shown in Fig. 6A, the maximum cathodic current response was obtained after 30 min of incubation of primary antibody (HBsAb) and HBsAg, and then the current reached a plateau. In addition, Fig. 6B shows that the current response for secondary antibody (HRP–HBsAb) binding to HBsAg increases rapidly with incubation time up to 20 min and then tends to be stable. Therefore, an incubation time of 30 min was employed as the optimal incubation time for both primary and secondary antibodies and antigen.

HRP–HBsAb concentration effect

Because the assay method involved a sandwich format between MNP–HBsAb/HBsAg complex and HRP-labeled secondary antibody (HRP–HBsAb), the amount of the secondary antibody plays an important role in obtaining the best response. To optimize the concentration, the complex (MNP–HBsAb/HBsAg) was incubated with various volumes of HRP–HBsAb (0.004 mg/ml) for 30 min, and CVs were recorded. As shown in Fig. 7, with increases in the amount of HRP–HBsAb up to 65 μl, the response was increased to its extreme value.

Calibration curve

Under the optimized experimental conditions, the immunosensor was used to detect HBsAg. Experiments were carried out in working buffer solution containing 2 mM o-AP and 0.01 M H_2O_2. Fig. 8 shows the CVs of the immunosensor when it was incubated in HBsAg with different concentrations. The baseline was obtained when the immunosensor was incubated without any HBsAg in working buffer solution (Fig. 8A). Then, with the increase of HBsAg concentration, the current response increases significantly due to the formation of MNP–HBsAb/HBsAg immunocomplex and then the capturing of HRP–HBsAb. As expected in the sandwich format

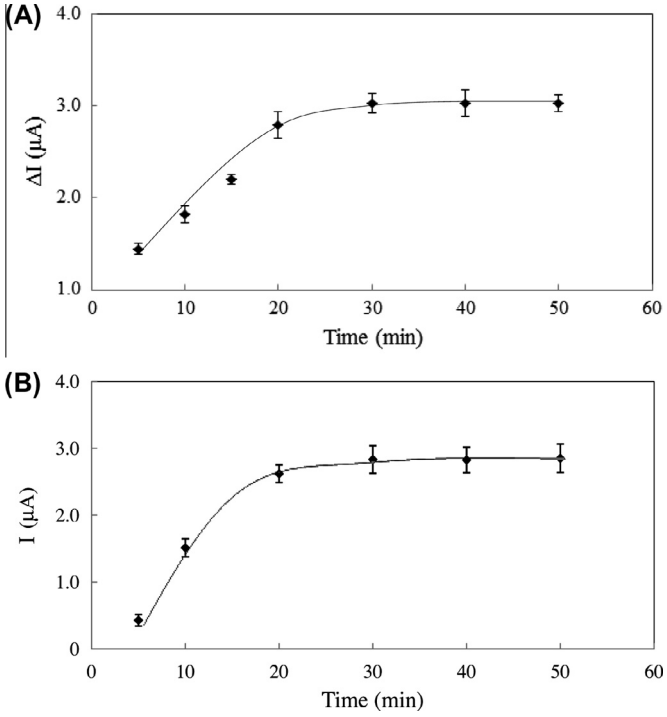


Fig.6. Optimization of incubation time for interaction of primary (A) and secondary (B) antibody with antigen. Each point (mean value of three replicate measurements) shows cathodic current obtained by cyclic voltammetry of 3-APZ on naked GC electrode. In panel A, the MNP–HBsAb was incubated with HBsAg at different times, and then the complex was incubated with HRP–HBsAb for 30 min. In panel B, the complex of MNP–HBsAb/HBsAg was incubated with HRP–HBsAb at different times. The interaction was carried out in 0.01 M PBS (pH 7.4) at 37 °C in the presence of 2 mM o-AP and 0.01 M H_2O_2 solution.

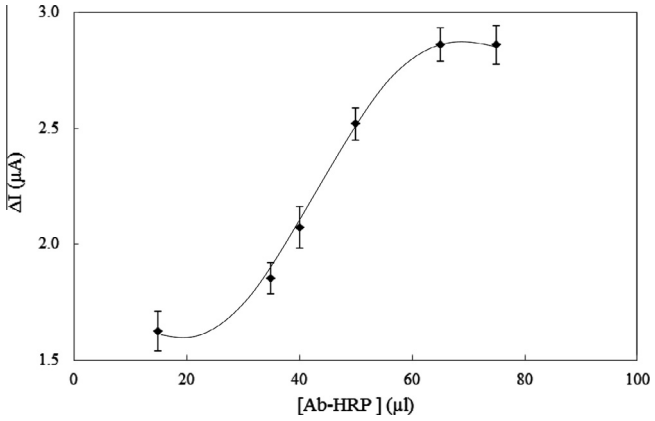


Fig.7. Optimization of secondary antibody volume. Each column (mean value of three replicate measurements) shows cathodic current obtained by cyclic voltammetry of 3-APZ on naked GC electrode. The MNP/HBsAb/HBsAg was incubated with different volumes of HRP–HBsAb (15, 35, 40, 50, 65, and 75 μl) from the same batch (dilution of 1:5000, v/v) for 30 min. The interaction was carried out in 0.01 M PBS (pH 7.4) in the presence of 2 mM o-AP and 0.01 M H_2O_2 solution. Ab, antibody.

immunoassay, the current response of 3-APZ was proportional to the amount of enzyme labeled on secondary antibody, which is proportional to the amount of targets present in sample solution. As shown in Fig. 8B, by changing the HBsAg concentration in the range from 0.001 to 0.015 ng/ml, the cathodic peak current was linearly increased with a detection limit of 0.9 pg/ml at a signal/noise ratio of 3.

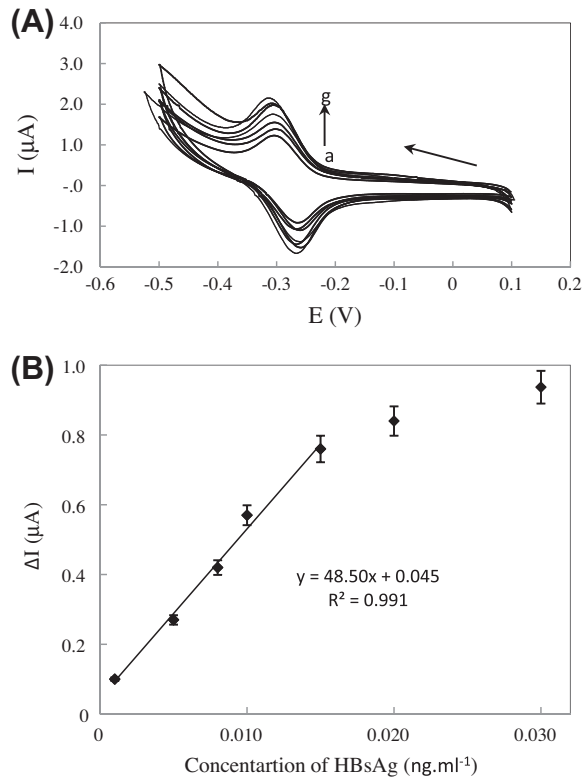


Fig.8. (A) CVs of 3-APZ on naked GC electrode. The cycles from a to g represent the HBsAg concentrations of 0.001, 0.005, 0.008, 0.010, 0.015, 0.020, and 0.030 ng.ml^{-1}, respectively. The samples were measured in BR buffer solution at pH 6.5 and in the presence of 2 mM o-AP and 0.01 M H_2O_2. (B) Calibration curve for determination of HBsAg. ΔI is the difference between the cathodic currents at each HBsAg concentration and background current.

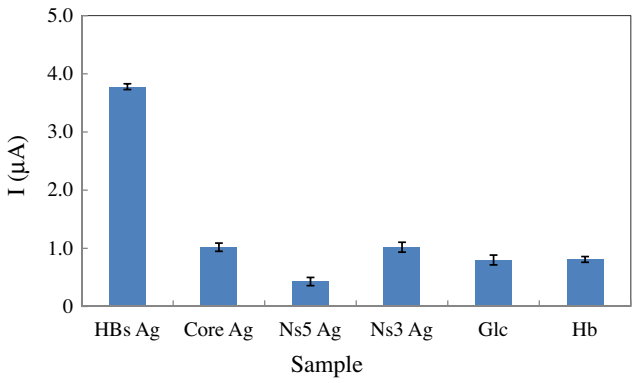


Fig.9. Specificity of proposed immunosensor toward HBsAg and different biomolecules (50 ng/ml), including hepatitis C antigens (core Ag, Ns5 Ag, and Ns3 Ag), glucose (Glc), and hemoglobin (Hb).

Specificity and stability of immunosensor

The response of the immunosensor toward HBsAg was compared with the responses obtained with other materials that are commonly present in human serum. The immunosensor was exposed to glucose, hemoglobin, and the structural proteins made by hepatitis C virus comprising core protein and nonstructural proteins, including NS3, NS4, and NS5 in the same concentration (50 ng.ml^{-1}). As demonstrated in Fig. 9, a higher selective and repeatable response was observed for HBsAg relative to the response currents recorded for the other interfering samples.

Table 1
Comparison of analytical parameters obtained by current work and those reported in the literature.

Detection method	Linear range ($ng.ml^{-1}$)	Detection limit ($pg.ml^{-1}$)	Reference
Potentiometry	4–960	1900	[47]
Potentiometry	4–800	1300	[48]
Electrochemical impedance spectroscopy	20–160	7800	[49]
Electrochemical impedance spectroscopy	2.6–153.6	1300	[50]
Anodic stripping voltammetry	0.1–1500	87	[51]
Cyclic voltammetry	0.5–650	100	[52]
Cyclic voltammetry	1.5–400	500	[53]
Chronoamperometry	5–65 (mIU/ml)	5 (mIU/ml)	[54]
Amperometry	2–300	0.42	[55]
Differential pulse voltammetry	0.01–1.0	2.3	[56]
Cyclic voltammetry	0.001–0.015	0.9	This work

Table 2
Comparison of biosensor responses and results obtained by conventional ELISA method for determination of HBsAg in real human serum samples.

Specimen	Immunosensor response[a] ($ng.ml^{-1}$)	ELISA[b] ($ng.ml^{-1}$)
1	0.003 ± 0.40	0.005
2	0.012 ± 0.20	0.010
3	0.016 ± 0.06	0.015

[a] Results are the mean values of three replicated measurements.
[b] Results obtained by conventional ELISA method [57] and divided to dilution factor to be comparable with immunosensor responses.

To investigate the stability of the immunosensor, the proposed complex (MNP–HBsAb/HBsAg/HBsAb–HRP) was stored in PBS (pH 7.4) at 4 °C and the cathodic peak current of 3-APZ was recorded. It retained 82% of its initial response after a storage period of 15 days.

Finally, the analytical characteristics of the developed HBsAg immunosensor were compared with those of the other HBsAg immunosensors reported in the literature [47–56] (Table 1). Despite the advances reported (in the references of Table 1), it is still a challenge to find the most sensitive and fastest approaches that do not require tedious and time-consuming sample preparation and cleanup steps. In the current work, MNPs were used as a platform for oriented immobilization of antibody. The magnetic beads provided an important advantage in that 3-APZ (the source of electrochemical signal) can be separated from the complex matrix of the sample (MNP–HBsAb/HBsAg/HBsAb–HRP). This, of course, could improve the sensitivity of the assay. The proposed strategy could also provide some other advantages such as easy cleanup of GC electrode surface, shorter time for analysis, and lower detection limit. Besides, o-AP as substrate for HRP provides better specificity for HRP assay compared with other electrochemical substrates. The favorable redox characteristics of the enzymatic product (3-APZ) provide a facile electrochemical strategy for the detection of HBsAg. Therefore, as compared in Table 1, the detection limit and linear range of the immunosensor were improved satisfactorily.

Determination of HBsAg in real human serum

To investigate the feasibility and reliability of the proposed electrochemical immunosensor for clinical analyses, three human serum specimens were diluted in PBS (pH 7.4) and examined by the proposed immunosensor. The results were compared with the data obtained by the standard ELISA method. Table 2 shows that the results obtained by the electrochemical immunosensor are in agreement with those acquired by the ELISA method [57]. This indicates that the data obtained by the immunosensor are acceptable and feasible for HBsAg determination in clinical immunoassays.

Conclusion

A simple electrochemical immunosensor has been proposed for fast and sensitive detection of HBsAg. In designing the immunosensor, an essential step was the immobilization of primary antibody on the MNPs as special carrier. It is believed that the direct linking of biotinylated antibody to the streptavidin coated on the carrier provides a right orientation of antibody to free antigen binding sites [58]. This is why the sensitivity and detection ability of the biosensor could be increased by using nanoparticles [59,60]. On the other hand, under the catalytic effect of HRP–HBsAb, o-AP was changed into 3-APZ, a sufficient and electroactive tracer for HBsAg. Thus, the presented immunosensor showed an efficient strategy for clinical immunoassay with easy separation steps by using an external magnet for collection of immune complex (MNP–HBsAb/HBsAg/HBsAb–HRP) and is well suitable for high-throughput biomedical sensing.

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