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Using Boehmite Nanoparticles as an Undercoat, and Riboflavin as a Redox Probe for Immunosensor Designing: Ultrasensitive Detection of Hepatitis C Virus Core Antigen

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In this study, a label-free electrochemical immunosensor for ultrasensitive detection of Hepatitis C virus core antigen in serum samples was fabricated using a simple approach. In this method, a low-cost and sensitive immunosensor was fabricated based on a boehmite nanoparticles (BNPs) modified glassy carbon. The BNPs provide a specific platform with an increased surface area which is capable of loading more antibody molecules as a receptor element of Hepatitis C virus core antigen on the electrode surface. It seems that BNP/ antibody conjugate may be an ideal platform for the development of an efficient immunosensor. It is worth noting that the proposed nanoimmunosensor combines the advantages of the elimination of another substrates in modifying the electrode and reducing the length of the modifying process. The experimental parameters, such as pH and incubation of time were optimized. Under the optimal conditions for modified glassy carbon electrode, a linear relationship was realized in the range of 0.08-110 pg ml^{-1}, with the detection limit of 10 fg ml^{-1}. The proposed method was applied to the determination of Hepatitis C virus core antigen in serum samples with the desirable results.

Keywords: Boehmite nanoparticles, Hepatitis C virus core antigen, Riboflavin, Immunosensor

INTRODUCTION

Here, we are interested in developing an electrochemical immunosensor for the detection of hepatitis C virus (HCV). Hepatitis C is an infection caused by the hepatitis C virus (HCV) that attacks the liver and leads to inflammation. There are a number of diagnostic tests for hepatitis C virus infection including detection of anti-HCV antibody and quantification of HCV RNA. Detection of anti-HCV antibody cannot distinguish between a current or past infection because people will retain anti-HCV antibodies for life once they are exposed to HCV. In addition, testing anti-HCV antibody might provide false negative results because it takes 45-68 days to develop anti-HCV antibody post HCV infection. On the other hand, detection of HCV RNA can distinguish between a current or past infection. However, detection of HCV RNA could provide false positive results due to contamination. It is also expensive and labor-intensive for routine use. It has been reported that HCV core antigen can be detected in the serum for most patients during the acute infection. HCV core antigen levels correlate well with HCV RNA levels and may consequently be an indirect marker of HCV replication as a low-cost alternative for diagnosis of HCV acute infection [1]. HCV Core Antigen testing can be utilized to: 1) identify HCV infection in seronegative individuals (pre-seroconversion window period detection), 2) identify seropositive individuals who are actively infected with HCV, 3) as a complementary test to HCV NAT to monitor antiviral therapy. Thus, accurate and sensitive diagnosis of HCV core antigen in blood samples during the early stages of infection is very important.

There are various methods used to detect HCV core antigen [2-8]. Among them, electrochemical immunosensors, based on the specific molecular

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Table 1. Comparison of Different Immunosensors for Detection of HCV Core Antigen

Modified Electrode	Method	LR [a]	LOD [b]	Ref.
Ab1/AuNPs/GMCs-MB[c]/GE[d]	DPV	0.25-300 pg ml^{-1}	0.01 pg ml^{-1}	[14]
AuNPs/ZrO_2-Chits/GCE	DPV	2 -512 ng ml^{-1}	0.17 ng ml^{-1}	[1]
Ab/AgNPs/SH-GQD/GCE	DPV	0.05 to 60 pg ml-¹	3 fg ml^{-1}	[8]
BSA/Ab/BNP/GCE	DPV	0.08 to 110 pg ml-¹	10 fg ml^{-1}	This work

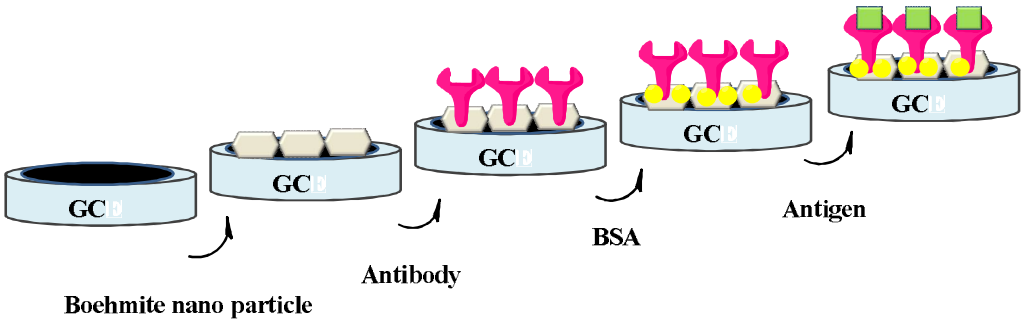
recognition of antigen by its antibodies, have created a high interest in clinical diagnostics and will be expected to provide fast detection of HCV core antigen. Based on our knowledge, there are a few reports using electrochemical immunosensor (as listed in Table 1) for the detection of HCV core antigen. Thus it is valuable to explore the electrochemical immunosensors to detect HCV core antigens for the clinical applications.

Nanoparticles have been used extensively for modeling the electrochemical immunosensors. Nanoparticle research is currently an area of intense scientific interest due to a wide variety of potential applications in optical, electronic fields and biomedical. Nanoparticles can also contribute to lighter, stronger and cleaner surfaces. They have a large surface enabling them to adsorb, bind and carry other compounds such as probes and proteins.

Nanostructured metal oxides belong to the class of nanomaterials that are used in the manufacture of immunosensors. Nanostructured metaloxides have attracted plentiful attention of the researchers owing to their adsorption capabilities, effective surface area for biomolecule immobilization with desired orientation and better conformation leading to high biological activity of the immobilized biosensing molecules [9]. Aluminium oxide (a kind of metaloxides) is one of the most stable inorganic materials and are generally inexpensive, non-toxic, and resistant to the chemical cleaning agents.

Boehmite is an aluminium oxide hydroxide (γ-AlOOH) particle, containing hydroxyl groups attached to its surface. Boehmite has excellent properties such as chemical resistance, good mechanical strength, high hardness, transparency, high abrasive and corrosion resistance [10].

Here, we propose a boehmite nanoparticles modified glassy carbon as a transducer for development of label-free electrochemical immunosensor for ultrasensitive detection of Hepatitis C virus core antigen in serum samples. The immunosensor displayed a good selectivity when challenged with Hepatitis C virus core antigen. To the best of knowledge this is the first report on the application of boehmite nanoparticles based immunosensor for Hepatitis C virus core antigen. On the other hand, using riboflavin as a probe in immunosensing Hepatitis C virus core antigen has been reported in a few publications. Riboflavin is oxidized in negative potential range, where the number of interfering species for riboflavin is much fewer. This is one of advantages of riboflavin compared to other probes. In this method the modified electrode was fabricated by using a simple approach. It is worth noting that the proposed nano-immunosensor combines the advantages of the elimination of another substrates in modifying the electrode and reducing the length of the modifying process. The proposed method has a great potential for recognizing an accurate, rapid and low cost technique of Hepatitis C virus core antigen determination. The use of a boehmite nanoparticles film as an efficient platform may provide many potential applications for various diagnostic targets in designing the immunosensors.



Scheme 1. Schematic illustration of the proposed electrochemical immunosensor for HCV core antigen detection

EXPERIMENTAL

Materials and Instrumentation

All chemicals used in the study were of analytical reagent grade purity. Antigen and antibody $Al(NO_3)_3.9H_2O$, riboflavin, NaOH, and all other reagents were ordered from commercial suppliers (Sigma-Aldrich, Merck or Fluka Company).

All electrochemical experimental data including differential pulse voltammetry (DPV), cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were carried out with a µ-AUTOLAB electrochemical system type III and FRA2 board computer controlled Potentiostate/Galvanostate (Eco-Chemei, Switzerland) driven with NOVA software. A three-electrode system including a saturated Ag/AgCl electrode as a reference electrode modified, GCE as a working electrode and a platinum wire as a counter electrode were used. The DPV measurements were carried out by scanning the potential of -0.50 to -0.25 V with modulation amplitude of 25 mV and modulation time of 50 ms. EIS analysis was carried out with a bias potential of 0.2 V and a frequency range between 0.1 Hz and 100 kHz with signal amplitude of 5 mV. A Metrohm model 780 pH/mV meters was used to measure the pH.

Synthesis of Boehmite Nano Particles (BNP)

The boehmite nanoparticles (BNPs) were synthesized in the laboratory according to a previous report by some modification [11]. In brief, 0.67 g ml^{-1} of $Al(NO_3)_3.9H_2O$ and solutions of 0.13 g ml^{-1} of NaOH and in distilled water were prepared. The sodium hydroxide solution was then added to the aluminum nitrate solution under vigorous stirring for 17 min. The resulting milky solution was sonicated in the ultrasonic bath for 3 h at a temperature of 25 °C. The resulted precipitates were filtered and washed by distilled water and were kept in the oven at 220 °C for 4 h.

Fabrication of the Immunosensor

Before fabrication, the GCE with a diameter of 3 mm was polished with 0.3 and 0.05 µm alumina powder slurry sequentially to remove organic matter until a mirror-like surface was obtained, and then, thoroughly cleaned with deionized water. The cleaned electrode was covered by 10 µl of BNP (dissolved in ethanol and water) and dried in the room temperature. After drying, the modified electrode was washed with deionized water and prepared to the next step. Subsequently, solution of antibody (anti-HCV) was dropped onto the surface of the modified GCE in order to immobilize anti-HCV molecules on the surface of BNP by chemisorption between BNP and amino groups of anti-HCV core antigen. The modified GCE was washed with deionized water and then 10 µl of BSA (10%) solution was pipetted onto the surface of the modified electrode to reduce the available active sites and effective surface area and avoid non-specific adsorption (Scheme 1).

Measurement Method

Electrochemical experiments containing a three-electrode arrangement were performed in a conventional electrochemical cell. The potential was swept from -0.50 to -0.25 V (*vs*. Ag/AgCl) in 10 ml of pH 7.5 PBS containing 0.5 mM riboflavin at room temperature. By casting HCV

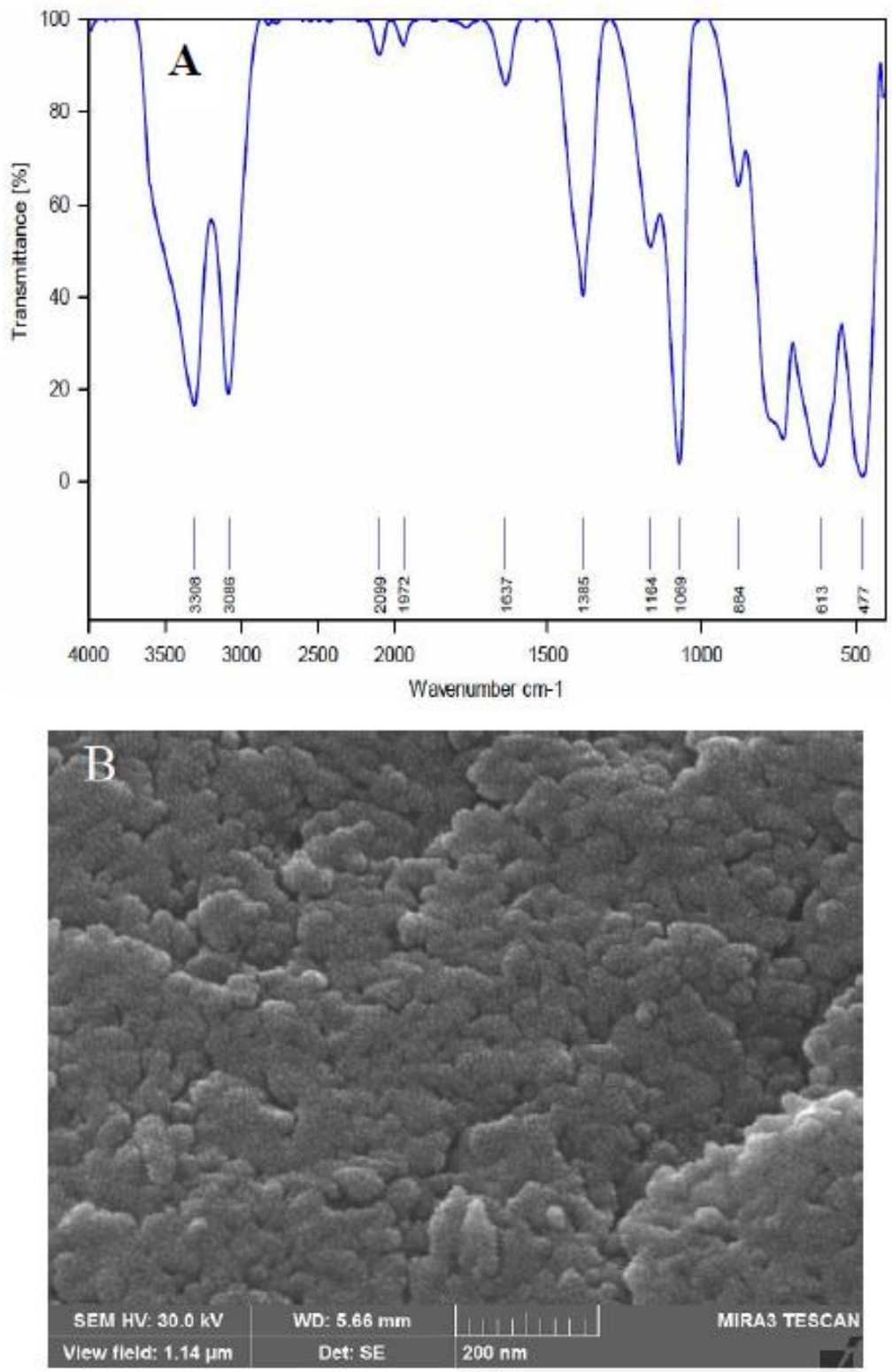


Fig. 1. (A) FTIR spectra of the Boehmite nanoparticles (BNP) and (B) SEM image of Boehmite nanoparticles.

core antigen solution on the BSA/anti-HCV/V_2O_5/GCE for 30 min the formation of antigen and antibody complexes was performed. Antigen and antibody complexes are formed moreover with increase in HCV core antigen concentration. After the specific reaction of antigen-antibody, the formed antigen-antibody immunocomplex on the electrode surface hindered the electron transfer toward the electrode surface, resulting in a decrease of electrochemical signal.

RESULTS AND DISCUSSION

Characterization of Boehmite Nanoparticles

As shown in Fig. 1A, the FTIR spectrum of boehmite nanoparticles is well-matched with the corresponding spectrum of the reported work in the literature [11]. The two strong bands at frequency peaks of 3086 cm^{-1} and 3308 cm^{-1} correspond to the stretching frequency of Al-OH. The frequency peak of 1637 cm^{-1} corresponds to absorbed water in the crystal structure. The frequencies 1069 cm^{-1} and 1164 cm^{-1} are related to the symmetrical bending vibrations of hydrogen bands Al-OH···HO-Al and the frequencies 613 cm^{-1} and 477 cm^{-1} also refer to Al-O-Al vibration modes [12]. The other peaks presented in the spectra belongs either to nitrate impurity or to combinations, differences and overtones. Figure 1B shows the SEM image of uniform boehmite nanoparticles.

Electrochemical Characterization of the Stepwise-modified Electrode

Electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) were used for characterization of the surface features of the modified electrodes at each immobilization step. EIS is a suitable method for monitoring the changes of the surface features during the fabrication process of electrochemical immunosensor. In this paper, EIS was also used to characterize fabrication process of the immunosensor. Figure 2A shows the Nyquist plots of EIS in the process of modifying electrode recorded from 1 to 10^5 Hz at 0.2 V in a solution containing 0.1 mM KCl and 2.5 mM $[Fe(CN)_6]^{3-/4-}$. Figure 2A shows the EIS of $Fe(CN)_6^{3-/4-}$ at the bare GCE (curve a), BNP (curve b), Ab/BNP/GCE (curve c), and BSA/Ab/BNP/GCE (curve e), respectively. First, the impedance of the bare electrode (GCE) was obtained (curve a). Second, after casting BNP on the surface of electrode, R_{ct} decreased, suggesting that nanocomposite enhances the electrode conductivity and electron transfer between the redox probe and the electrode surface due to increasing effective surface area in the presence of BNP (curve b). In the next step, Ab was immobilized on the electrode surface and R_{ct} clearly increased (curve c), this is attributed to reducing the available active sites for electron transfer process by protein. R_{ct} likewise increased further more when the immunosensor was incubated in BSA solution (curve d).

The well-defined cyclic voltammetric curve of bare GCE (curve a, Fig. 2B) shows reversible redox behavior owing to flexible electron transfer between $[Fe(CN)_6]^{3-}$ and GCE. On treatment with BNP, the magnitude of peak current is found to increase (curve b). After the fabrication of BNP film on GCE, BNPs not only have excellent adsorption abilities to increase the effective area of electrode surface in response to $[Fe(CN)]^{3-}$, but also have good enrichment effect and high surface/volume ratio to $[Fe(CN)]^{3-}$, leading to the increased current in the case of BNP/GCE (curve b) contrasted to the bare GCE. In the next step, anti-HCV was immobilized on the electrode surface and the peak current clearly decreased (curve c). This is attributed to reduce in effective surface area and available active sites for electron transfer process by protein [13]. Finally, peak current decreased in the same way (curve d) after using BSA to block nonspecific sites.

Optimization of Method

The pH value of solution and the incubation time are of vital importance for the analytical performance of immunosensors. Thus, we checked the importance of these factors. The effect of pH on the peak current was studied in the pH range of 5.5-8.0 (Fig. 3A). It was found that the peak current increased when the pH value increased from 5.5 to 7.5. When the pH value was above 7.5, the response decreased with the increasing of pH value. Therefore, a pH 7.5 of the working buffer was applied for further experiments. Solution pH has great effects on the electrochemical performance of the immunosensor so that in strong acidic and alkaline solutions the amperometric signal decreased. For detection of the sufficient recognition time of target protein, the incubation time was investigated.

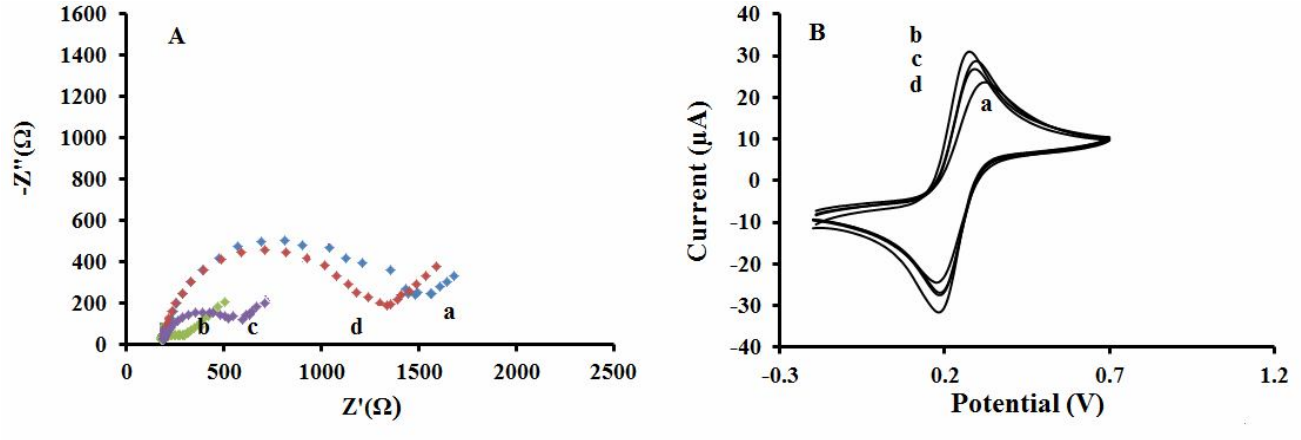


Fig. 2. (A) Recorded EIS for the different steps of the modified electrode: (2.5 mM $[Fe(CN)_6]^{3-/4}$, KCl 0.1 mM), in 0.1 mM PBS (pH = 7.5) at (a) GCE, (b) BNP/GCE, (c) Ab/BNP/GCE, (d) BSA/Ab/BNP/GCE (B) typical CV studies of $[Fe(CN)_6]^{3-/4}$ (a) to (e) are the same as (A).

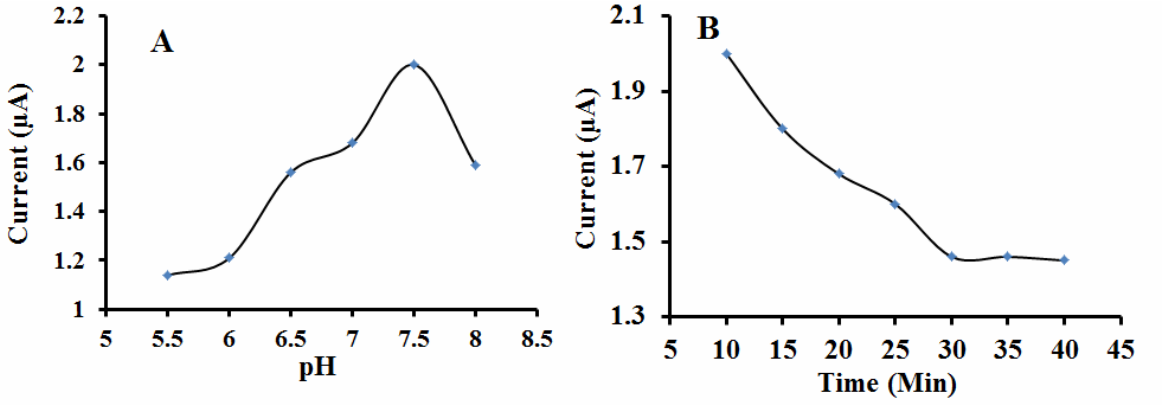


Fig. 3. Effect of (A) the pH value, (B) the incubation time of the antibodies and antigens.

It was found that the peak current intensity decreased as the incubation time increased from 10 to 30 min, and leveled off after 30 min. Thus 30 min was chosen as the optimal incubation time (Fig. 3B).

Analytical Performance

Under optimal conditions, the immunosensor was incubated in HCV solution of different concentrations for 30 min, and then differential pulse voltammetry (DPV) measurements were performed in 0.1 M phosphate buffered solution (pH 7.5) containing 0.5 mM riboflavin at 25 °C. The DPV detection was based on the change in the current response (ΔI) before and after antigen-antibody reaction. The peak currents decreased with increasing the concentration of HCV core antigen, pertaining/concerning to the formation of increasing the number of antigen/antibody complex. A linear relationship between the signal intensity and the logarithm value of different concentrations of HCV core antigen was obtained in the range of 0.08-110 pg ml^{-1} (Fig. 4). The regression equation between the current response and the HCV concentration ranging from 0.08-110 pg mL^{-1} was found; ΔI (µA) = 0.1188 log C + 0.5508 and the correlation coefficient R was 0.993. The limit of detection was calculated as 10 fg ml^{-1}. Each point of the calibration curve corresponds to the mean value obtained from 3 measurements, and the error bars

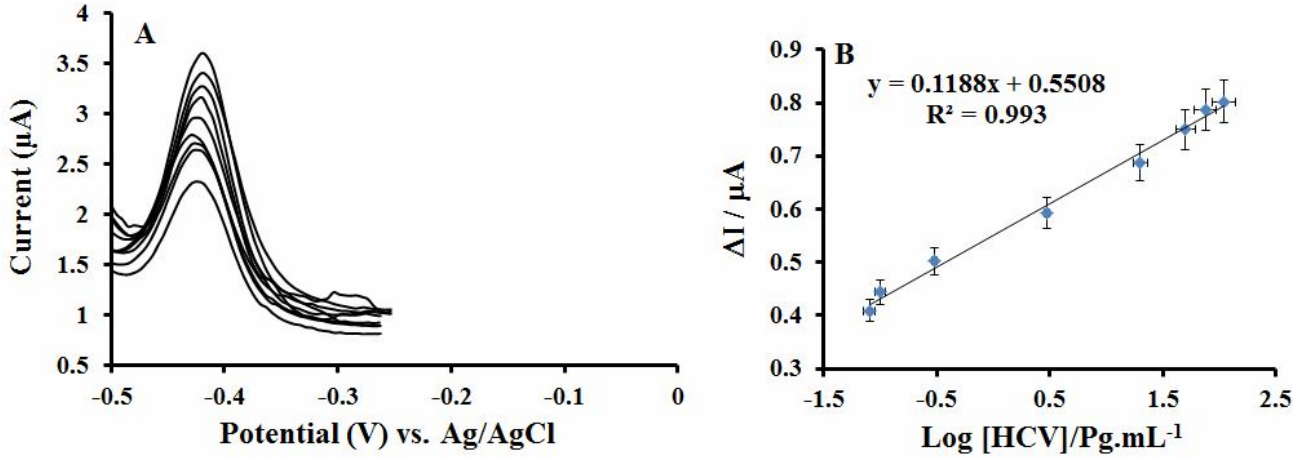


Fig. 4. (**A**) DPV responses of the immunosensor with increasing concentrations of HCV core antigen (from up to down: 0, 0.08, 0.1, 0.3, 3, 20, 50, 75 and 110 pg ml^{-1}), and (B) calibration curve of the peak current response versus the logarithm of HCV core antigen concentration. The parameters of DPV are as follows: initial potential -0.5 V, final potential -0.25 V, amplitude 25 mV, and modulation time of 50 ms.

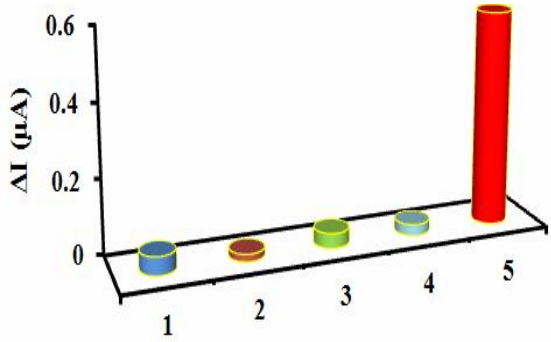


Fig. 5. Amperometric response of the immunosensor to interfere: (1) progestron (2) glucose (3) BSA (4) HCG, and (5) HCV.

show the standard deviations of the measurements. The performance of the immunosensor was superior to that of the other immunosensors reported in the related literature (Table 1), especially in comparison to simple fabrication process. Furthermore, the use of cost effective compounds are the major advantages in comparison to different HCV core antigen immunosensors.

Reproducibility, Stability and Sensitivity

Intra-electrode and inter-electrode coefficients of variation were used in the investigation of reproducibility. The relative standard deviation (RSD) of reproducibility was 2.5% for 5 measurements of HCV core antigen with different immunosensors (Inter-electrode). Also, for five times, the reproducibility of the immunosensor was estimated by HCV determination with one immunosensor (Intra-electrode) and RSD was calculated at 3%.

In the practical applications, the stability of the immunosensor is also a key factor. Thus, the immunosensor was stored at refrigerated conditions (4 °C) and used periodically for the analysis of HCV core antigen at an interval of 5 days. After 25 days, no apparent change was found toward HCV core antigen with similar concentration and retained more than 85% of the initial response.

Table 2. Detection of HCV Core Antigen in Real Samples

Added ($pg\ ml^{-1}$)	Founded ($pg\ ml^{-1} \pm$ RSD%)	Recovery (%)
5	4.85 ± 0.30	97.00
15	14.70 ± 0.30	98.00
50	50.90 ± 0.20	101.8
100	99.50 ± 0.10	99.5

For evaluating the selectivity of the HCV core antigen immunosensor in human serum sample, the DPV responses were recorded for some molecules such as progesterone, glucose, bovine serum albumin (BSA) and HCG. When HCV core antigen and 500 times of interfering substances were incubated on to the immunosensors respectively, no significant change was found compared with the initial response of the immunosensor with HCV core antigen only. These results are in agreement with the fact that binding event between Ab and antigen is based on a selective recognition between them, but not on nonspecific adsorption. Figure 5 suggests a good specificity of the fabricated immunosensor.

Real Sample

The immunosensor was tested in order to confirm the accuracy and precision. The immunosensor was used to detect the recoveries of different concentrations of HCV core antigen in human serum samples. Human serum samples were ordered from a local clinical laboratory and subjected to ultrafiltration by loading into a centrifugal filtration tube at 3000 rpm (30 min). Afterwards, the serum samples were diluted 50 times with PBS (0.1 M) and different concentrations of HCV were spiked to these samples. As can be seen, the recovery was in the range from 97-101.8% and the RSD was in the range from 0.1% to 0.3%, indicating that the precision of this method is good (Data are shown in Table 2).

CONCLUSIONS

The use of boehmite nanoparticles (BNPs) to increase the loaded antibody, and enhance the electrochemical response signal for protein analysis, has effectively improved the sensitivity of the electrochemical immunosensor. BNPs were successfully casted on the working electrode and antibody has been loaded on the electrode surface via the interaction between NH_2 group of antibody and Al. Using the boehmite nanoparticles (BNPs) to bring more antibody/antigen complexes significantly increases the sensitivity of the immunosensors. The modified electrode not only provides a new method for Hepatitis C virus core antigen detection, but also expands the application of the BNPs to design some immunosensors. The developed method provides a promising nanoparticles and riboflavin as a universal redox probe for different analytes.

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