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Electrochemical immunosensor for hepatitis B detection

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ABSTRACT

Nowadays, hepatitis B (HB) virus infects a large number of people worldwide and many of them suffer from chronic infection which requires continuous monitoring of the disease status. However, the current detection method is costly as it is based on the conventional ELISA technique thus patients may not get proper treatment. This study proposed a new, low cost and simple immunoassay for hepatitis B detection via Electrochemical Impedance Spectroscopy (EIS) technique. Direct ELISA, a conventional immunoassay, was performed on a commercial screen-printing carbon electrode instead of a 96-well plate. Immobilizing agent, 1-pyrenebutanoic acid, succinimidyl ester, was used to immobilize HBs antibody effectively on the electrodes. The results showed that our electrochemical immunosensor can detect serological marker of hepatitis B which is HBs antigen with the range of measurement (5 pg/ml to 1000 pg/ml) and the usage of lower cost disposable electrode. This work is expected to reduce the cost of detection allowing infection to be identified and continuously monitored thus patients can obtain the proper treatment and punctually preclude the disease transmission.

1. INTRODUCTION

World Health Organization (WHO) reported that approximately 2 billion people are exposed to hepatitis B while 240 million people have developed into chronic hepatitis B (World Health Organization, 2015). Amongst these patients, many of them do not show the symptom thus become carrier of the virus which transmit to others. This greatly increases the risk of spreading the infection. Therefore, biosensors for hepatitis B detection using various techniques have been developed to identify the infection allowing the patients to obtain the proper treatment and control the transmission of the disease. Screen-printing technology has become popular, as it is a simple technique used for electrodes fabrication. These electrodes can be used as a transducer in

biosensor (Tudorache, 2007). It offers several advantages such as low cost, design variety, good reproducibility (Rogers, 2006). Nevertheless, the major bottleneck for applying this technique as immunosensors is the ability to immobilize antibody molecules. Moreover, Electrochemical Impedance Spectroscopy (EIS) is an interesting option as a biosensor since it is label-free, low destructive of biomolecule activities and highly sensitive (Patolsky, 1999). In this assay, a label-free impedimetric immunosensor for hepatitis B detection was developed using a commercial carbon screen-printed three electrodes system. The HBs antibody was immobilized onto the electrodes using 1-pyrenebutanoic acid, succinimidyl ester as a functional molecule. The experimental results showed that this electrochemical immunosensor can detect HBs, one of the serological markers of hepatitis B infection, with good sensitivity.

2. EXPERIMENTAL

2.1 Reagents and apparatus

1-pyrenebutanoic acid succinimidyl ester, ethanolamine, and dimethyl sulfoxide dehydrated (DMSO) were purchased from Sigma Aldrich. Bovine serum albumin (BSA) was purchased from Bio basic Canada Inc. Monoclonal anti-hepatitis B virus surface antigen antibody [hb12] (ab2039) and recombinant hepatitis B surface antigen ad protein (ab193473) (HBsAg) were purchased from Abcam. The reagents were used the highest commercially available purity. All solutions were prepared with deionized water (DI) of resistivity no less than 18 MΩcm. The screen-printed carbon electrode (SPCE) were obtained from Quasense Co., Ltd. The disposable electrodes were fabricated by screen-printing technology and designed as a system with three electrodes containing carbon ink working, carbon ink counter and Ag/AgCl ink reference electrodes. Surface area of the working electrode is 7.07 mm2. An AutoLab PGSTAT128N system (EcoChemie B.V., Ultrecht, The Netherlands) was used to perform EIS measurements.

2.2 Conjugation of antibody protein on electrode substrate (Lein, 2011).

Conjugation of antibody on the electrode was performed as previously described (Lein, 2011). Briefly, stock solution (a) was prepared by diluting antibody in 10 mM carbonate buffer (pH=8.2) to the final concentration of 20 µg/ml (1:100 original stock). Stock solution (b) was prepared by diluting 10 mg of 1-pyrenebutanoic acid succinimidyl ester in 1 ml of mixed solution of DMSO:DI (70%:30%). Then, solution (a) and (b) were mixed using ratio of 10:1 (v/v). During conjugation, the mixture was shaken at room temperature (RT) for 3 hours. Finally, the conjugated mixture was kept at 4°C until use.

2.3 The fabrication of immunosensors

To fabricate the immunosensor, 5 µl of conjugated mixture was dropped on the working electrode surface and incubated at 40°C for 18 hours. Next, the modified

electrodes were rinsed with washing solution composed of 0.05% tween in 10 mM phosphate buffer (pH 7.4) followed by deionized water and dried gently with purified nitrogen gas. To prevent non-specific adsorption, 8 µL of 100 mM ethanolamine was dropped on the modified electrode. After that, the modified electrodes were rinsed again with the same washing solution, deionized water and dried with purified nitrogen gas. Finally, the fabricated electrodes were used to measure the antigen by dropping 5 µL of mixtures containing various concentrations of HBsAg.

3. RESULTS AND DISCUSSION

3.1 Cyclic voltammetry studies

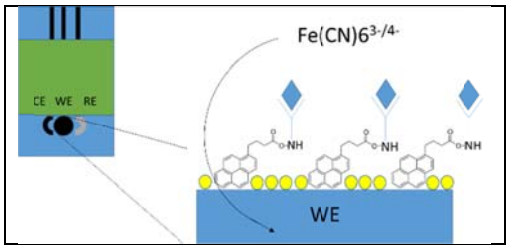


Fig.1 Schematic of an electrochemical diagnostic for immunosensors

The fabrication of immunosensor was performed in Fig. 1. After the solution (a) and solution (b) were mixed together, the amine group of antibodies was functioned with the solution (b) to form amide bonds for the protein conjugation. Then, this mixture was strongly immobilized on the electrode surface because the pyrenyl groups was occurred the π-stracking via the basal plane of carbon electrode (Katz,1994, Jaegfeldt, 1983). And the blocking reagent was added on the remaining area of electrode in order to prevent non-specific adsorption of the abundance antigens and the other impurities. Thus, the electrode surface becomes to be reactive again during the specified antibodies was conjugated with the only target antigens.

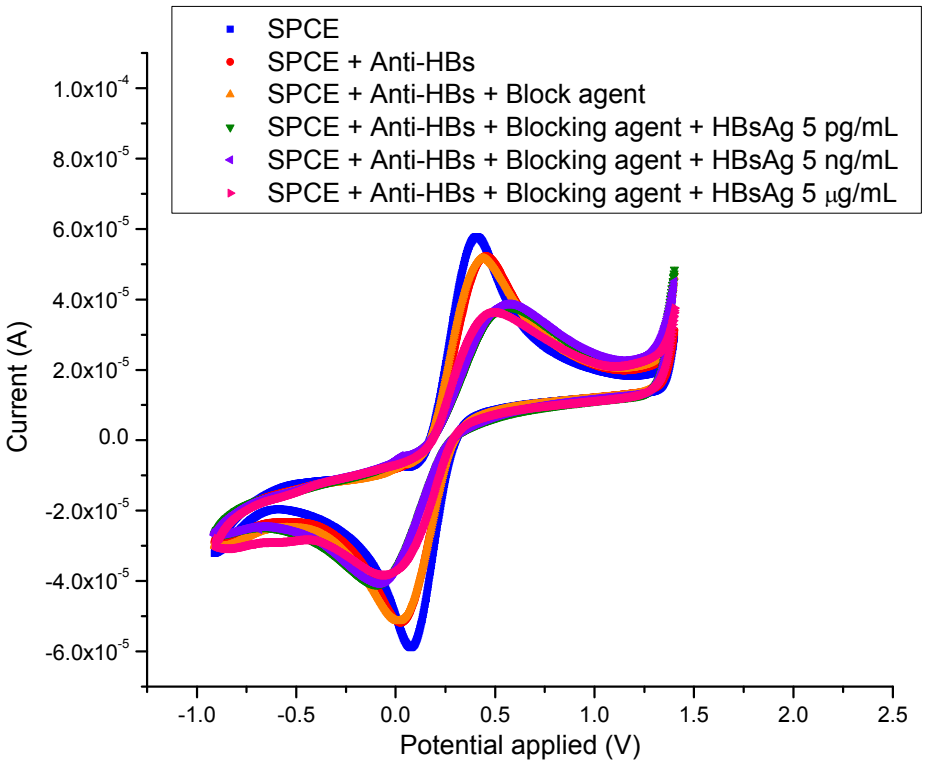
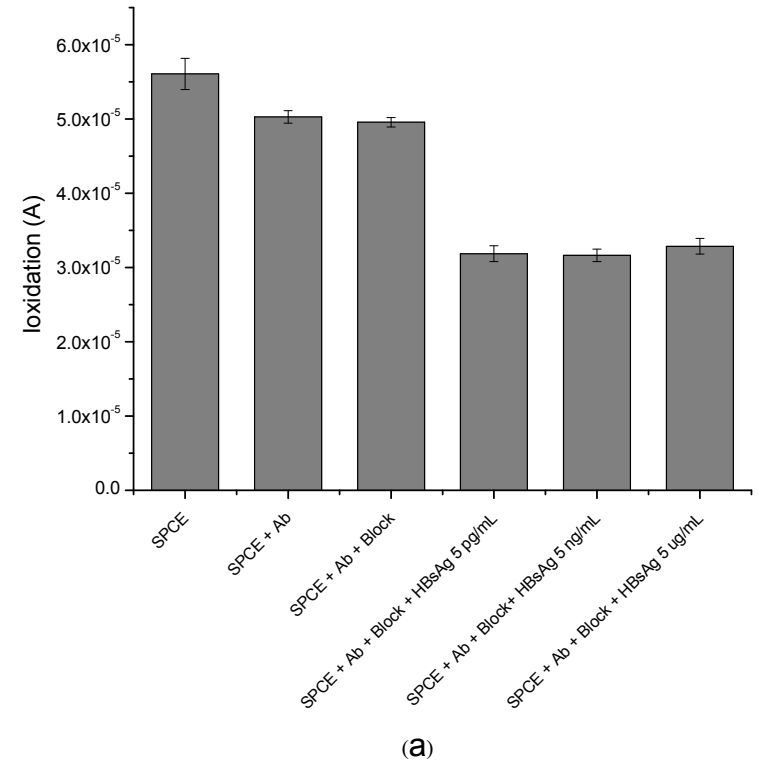
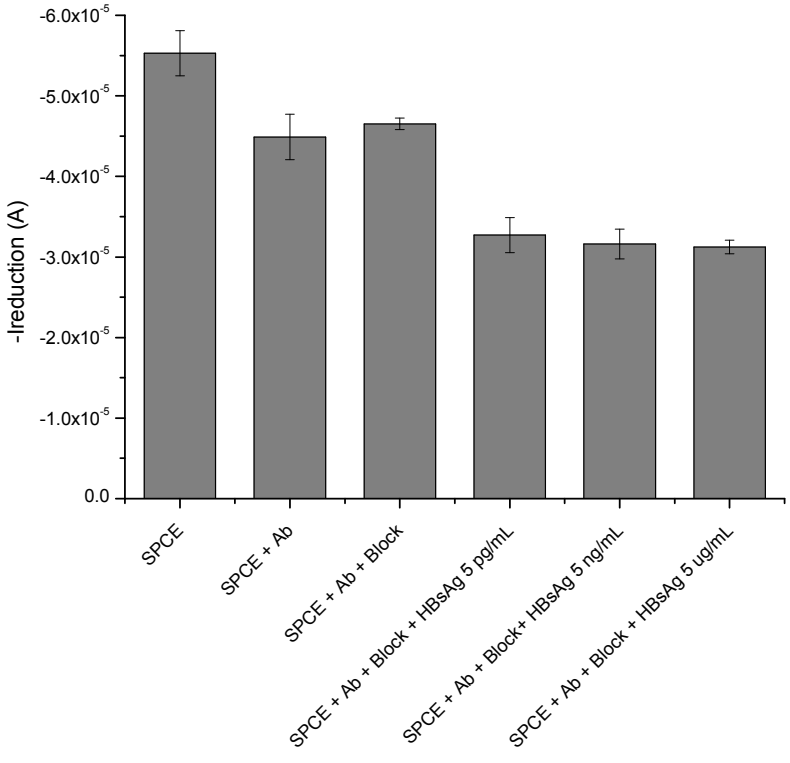


Fig. 2 The cyclic voltammogram of $Fe(CN)_6^{3-,4-}$ on the difference electrode processes. There are the step processes of the bare SPCE (blue), SPCE modified with antibodies (red), SPCE modified with antibodies and blocking agent (orange), and SPCE modified with antibodies and blocking agent tested with varying HBsAg concentrations as 5 pg/ml (green), 5 ng/ml (violet), and 5 μg/ml (pink), respectively.

In order to optimize the fabrication process, cyclic voltammetry was used to study the redox current response of $Fe(CN)_6^{3-,4-}$ on each modified surface. Fig. 2 shows the cyclic voltammograms of different electrode processes while Fig. 3 shows the current response of different electrode conditions. The results from both figures showed that the bare electrode (SPCE) gave the highest current response. The current response decreased when the electrode was modified such that more molecules were adsorbed onto the surface, for example, the antibody and blocking molecules were adsorbed on the electrode surface. Therefore, the resistance increases with increasing protein adsorption as shown in Fig. 4 (n=3).



(a)



(b)

Fig. 3 The relationship of the oxidation (a) and reduction (b) current responses considered at different steps of electrode modification.

Fig. 2 shows the cyclic voltammogram of $Fe(CN)_6^{3-,4-}$ when various concentrations of antigen were added, i.e., 5 pg/ml, 5 ng/ml, and 5 μg/ml, respectively. The current response of redox peak decreased with higher concentration of antigen because antigen molecules bound to antibodies on the electrode surface inhibiting the redox species to diffuse to the resistance films which occurred as the layer-by-layer process. Thus, the high-resistance surface was created with increasing number of antigen molecules.

3.2 Impedance detection of hepatitis B antigen

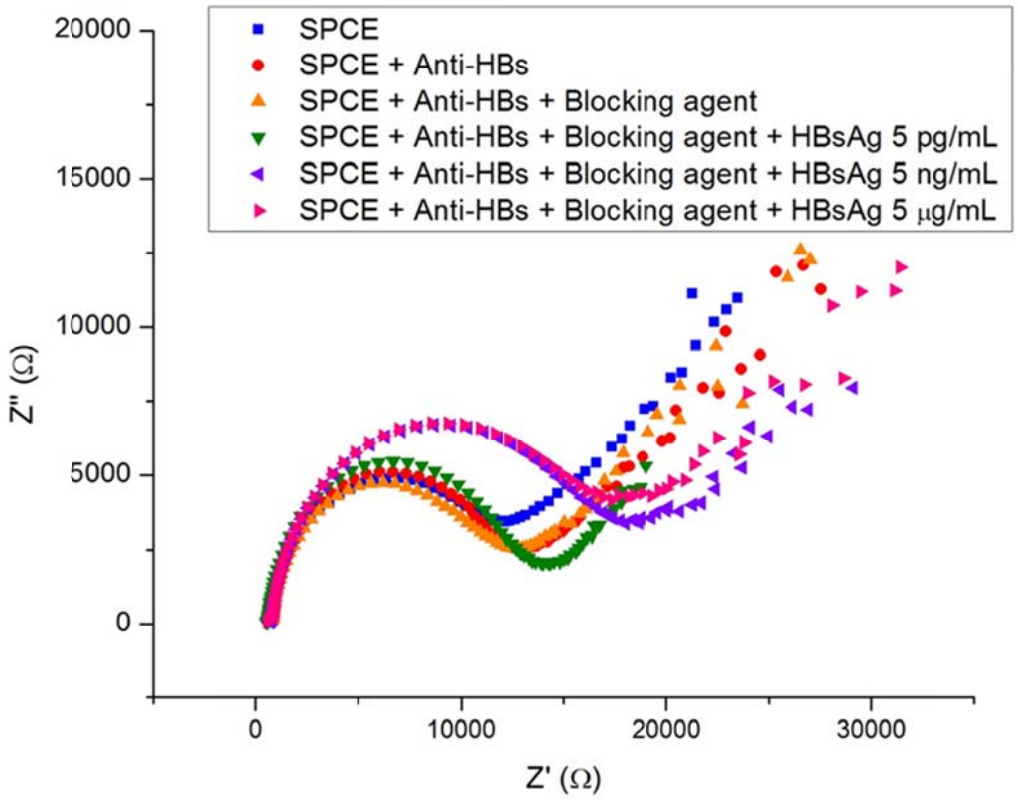


Fig. 4 Nyqiust plots observed at different conditions of SPCE modifications. Blue, red, orange, green, violet and pink colors represent bare SPCE, SPCE modified with antibodies, SPCE modified with antibodies and blocking agent, SPCE modified with antibodies and blocking agent tested with varying HBsAg concentrations as 5 pg/ml, 5 ng/ml, and 5 μg/ml, respectively.

Fig. 4 shows the nyquist plots of $Fe(CN)_6^{3-,4-}$ when 5 pg/ml, 5 ng/ml, and 5 μg/ml of HBsAg were added to the functional electrode. This experiment also compared impedance spectra of the modified electrode when only antibodies were embedded to

that of when blocking agent was included. In table 1, the impedance spectra were fitted with the curve using Randles equivalent circuit (Randles,1947). The equivalent circuit elements followed the theory of nonfaradaic interface (Kasemo, 2002). For an electrochemical impedance sensor, the AC signal is applied to the electrode from the high frequencies to low frequencies (10000 Hz to 10 Hz). At low frequencies, the charge of redox species represents only the diffusion process. The impedance response value is small. Thus, results from lower hertz were neglected from the information data. At high frequencies, the mass transfer interface depends on the attached redox species at the approached electrode interface (in the outer Helmholtz plane's region (OHP)) (Zhang, 2009). which means that $Fe(CN)_6^{3-,4-}$ was balanced at the modified electrode. Here the semicircles showing impedance spectra are increasing as each surface modification occurred because of binding of more antigen molecules to the antibodies adsorbed on the electrode surface. For the double layer charges (C_{dl}), they were lowest at the bare SPCE and increased with higher barrier molecules due to surface modification.

Table 1 Impedance parameters obtained from the impedance spectra fitting curve in Fig. 4.

conditions	Fitting parameters (n=3)		
	R_s (Ω)	R_{ct} (kΩ)	C_{dl} (nF)
1.SPCE	795.89±109.92	11.47±0.32	140.63±11.89
2.SPCE+Ab	736.01±61.27	11.66±0.02	187.02±119.08
3.SPCE+Ab+blocking agent	622.49±119.08	11.60±0.675	265.69±71.91
4.SPCE+Ab+Ag (5pg/ml)	527.75±27.11	12.56±0.35	372.35±26.11
5.SPCE+Ab+Ag(5ng/ml)	643.45±35.89	17.21±0.23	468.24±36.27
6.SPCE+Ab+Ag (5 μg/ml)	644.77±181.62	18.12±1.10	468.69±38.42

Fig. 5 shows R_{ct} plots when different concentrations of HBsAg were tested (5 pg/mL,100 pg/mL, 500 pg/mL, 1 ng/mL, 5 ng/mL and 50 ng/ml respectively). The charge transfer impedance (R_{ct}) is proportional to the concentrations of antigen. The measured impedance values were observed at the redox probe which corresponds to the mass transfer diffusion effects of $Fe(CN)_6^{3-,4-}$. Hence, binding of antigen molecules to the antibodies adsorbed on the electrode surface will inhibit the redox species from diffusing to the electrode surface because the antigen molecules form physical barrier on the electrode surface. The results showed the measurements at various HBsAg concentrations ranging from 5 pg/ml to 50 ng/ml (n=3) while the inset shows the results of concentrations ranging from 5 pg/ml to 1000 pg/ml (n=3) in logarithmic scale.

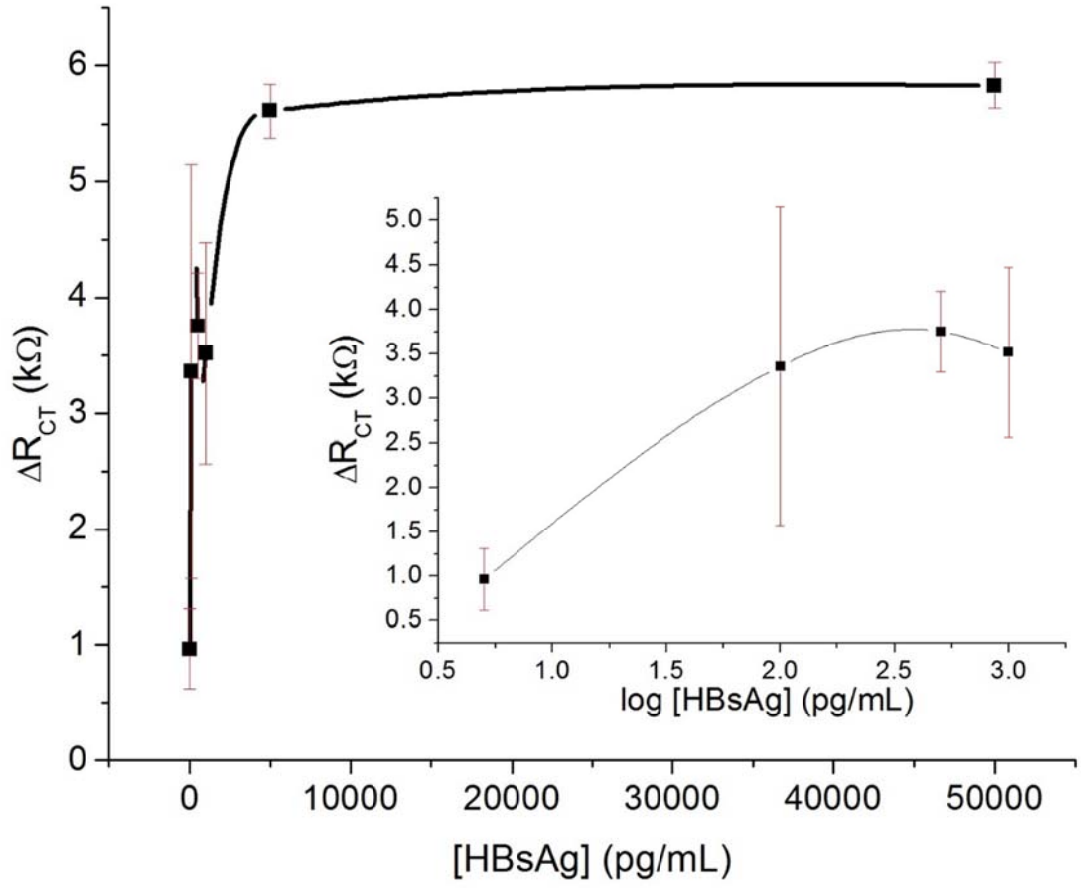


Fig.5 the calibration curves obtained from ΔR_{ct} values of the antigen concentrations ranging from 5 pg/ml to 50 ng/ml (n=3). Inset shows ΔR_{ct} values of the antigen concentrations ranging from 5 pg/ml to 1000 pg/ml (n=3) in logarithmic scale

4. CONCLUSIONS

The results showed successful application of an impedance sensor for HBsAg detection at 5 to 1000 pg/ml. This study reported a great technique for immobilization of antibody on a disposable electrode which can be applied to a wide range of immunosensors. It is simple and very specific method due to the use of antibody which is very specific to its cognate antigen. We proved the feasibility of this electrode modification by studying cyclic votammetry. When the antigen was added on the modified electrode, the current response decreased with the higher antigen concentration following the double layer interface theory. It could be implied that the impedance spectra increased with the higher resistance interface. Therefore, this method represents a simple technique for antigen measurement using label-free impedimetric immunosensors.

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