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To cite this article: Quang Huy Tran *et al* 2012 *Adv. Nat. Sci: Nanosci. Nanotechnol.* **3** 015012

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Development of electrochemical immunosensors based on different serum antibody immobilization methods for detection of Japanese encephalitis virus

Quang Huy Tran^{1,2}, Thi Hong Hanh Nguyen¹, Anh Tuan Mai²,
Thi Thuy Nguyen², Quang Khue Vu³ and Thi Nga Phan¹

¹ National Institute of Hygiene and Epidemiology (NIHE), 1 Yersin, Hanoi, Vietnam

² International Training Institute for Materials Science (ITIMS), Hanoi University of Science and Technology (HUST), 1 Dai Co Viet, Hanoi, Vietnam

³ Bac Ninh Vocational College of Technology and Economics, Bac Ninh City, Vietnam

E-mail: huytq@nihe.org.vn and nghhanh@nihe.org.vn

Received 6 November 2011

Accepted for publication 6 January 2012

Published 6 March 2012

Online at stacks.iop.org/ANSN/3/015012

Abstract

This paper describes the development of electrochemical immunosensors based on human serum antibodies with different immobilization methods for detection of Japanese encephalitis virus (JEV). Human serum containing anti-JEV antibodies was used to immobilize onto the surface of silanized interdigitated electrodes by four methods: direct adsorption (APTES-serum), covalent binding with a cross linker of glutaraldehyde (APTES-GA-serum), covalent binding with a cross linker of glutaraldehyde combined with anti-human IgG (APTES-GA-anti-HIgG-serum) and covalent binding with a cross linker of glutaraldehyde combined with a bioaffinity of protein A (APTES-GA-PrA-serum). Atomic force microscopy was used to verify surface characteristics of the interdigitated electrodes before and after treatment with serum antibodies. The output signal of the immunosensors was measured by the change of conductivity resulting from the specific binding of JEV antigens and serum antibodies immobilized on the electrodes, with the help of horseradish peroxidase (HRP)-labeled secondary antibody against JEV. The results showed that the APTES-GA-PrA-serum method provided the highest signal of the electrochemical immunosensor for detection of JEV antigens, with the linear range from 25 ng ml⁻¹ to 1 µg ml⁻¹, and the limit of detection was about 10 ng ml⁻¹. This study shows a potential development of novel electrochemical immunosensors applied for virus detection in clinical samples in case of possible outbreaks.

Keywords: electrochemical immunosensors, serum antibody, protein A, virus detection

Classification numbers: 2.05, 6.09, 6.10

1. Introduction

Japanese encephalitis virus (JEV) is a leading cause of childhood encephalitis in Asia. It has high mortality and high risk for subsequent infections. It is the most significant mosquito-borne viral encephalitis in several Asian countries. Twenty-five countries are at risk from Japanese encephalitis

(JE), and approximately three billion people, including more than 700 million children under the age of 15 live in risk areas [1, 2]. Several conventional diagnostic methods have been developed for detection of JEV infection such as: immunoglobulin M (IgM) assay, plaque reduction neutralization test, reverse transcription polymerase chain reaction (RT-PCR) or virus isolation [2]. However, these

developed diagnosis techniques require a pre-treatment sample, biological products, and time-consuming analysis to yield an answer. Therefore, the development of rapid diagnostic tests always plays a crucial role for the control and prevention of JE outbreak.

In recent decades, biosensors/biochips have been envisaged to compensate and complement conventional diagnostic methods due to their easy operation and transport; they require no expensive reagents and provide results in a few minutes [3–5]. Among them, immunosensors based on electrochemical detection have the advantage of being highly sensitive, rapid, inexpensive and highly amenable to micro-fabrication and it is also easy to measure the changes in electrical/electrochemical properties resulting from the antigen-antibody reaction on the surface of the sensor [4, 5].

However, most immunosensors normally use purified antibodies as probes for detecting pathogens [6]. In outbreaks, it is not easy to dispose of specific antibodies against these pathogens, especially against unknown pathogens within a short period of time and screened human serum becomes an effective choice to develop serum antibody-based biosensors for preliminary pathogen screening.

A major problem in the development of immunosensors is to overcome the complexity of binding antibody to the sensor surface. In fact, the immobilization of antibody is a key for the success of electrochemical immunosensors in virus detection. So, antibodies need to be immobilized onto surfaces with a high density and good orientation in order to detect viral antigens easily with the usage of a small volume of sample solution. The influence of nonspecific bindings should be minimized to improve the detection performance. Several immobilizing methods of antibody onto the sensor surfaces were proposed such as physical adsorption, entrapment in a gel, covalent binding, cross-linking, electrochemical polymerization and conducting polymers [6–10]. In spite of these studies, there is no standard immobilization with results that can be reproduced in order to use them to evaluate new methods [11]. This is because all methods depend on the surface of developed sensors as well as the nature, origin and history of the used antibody. In our study, a suitable serum antibody immobilization method will offer a high throughput, high ratio of signal per noise, reproducible results, high sensitivity and specificity.

This study aims to compare the detection performances of electrochemical immunosensors developed using different serum antibody immobilization methods for detection of Japanese encephalitis virus in solution. The use of interdigitated sensors designed with two separate micro-electrode regions of the working and reference electrodes was convenient for electrochemical measurements, and the change in conductivity caused by the binding of JEV antigens to working electrodes, under the help of horseradish peroxidase (HRP)-labeled secondary antibody against JEV resulting in the difference of the output signals.

2. Experimental

2.1. Materials and reagents

Human serum containing antibodies against JEV (tested for non-cross reactivity with other *flaviviruses* by

IgM antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA) kits), inactivated JEV, horseradish peroxidase-labeled anti-JEV antibody (Ab-HRP), Dengue virus (Dengue antigens) and healthy mouse serum were provided by the National Institute of Hygiene and Epidemiology (NIHE) of Vietnam. These biological products were stored at -20°C before use.

Goat anti-human immunoglobulin G polyclonal antibody (anti-HIgG), bovine serum albumin (BSA), protein A (PrA), 3-aminopropyl-triethoxy-silane (APTES), glutaraldehyde (GA), potassium iodide (KI), sodium chloride (NaCl) and hydrogen peroxide (H_2O_2) were purchased from Sigma, USA. All other chemicals were of analytical grade.

The interdigitated sensors were designed and fabricated at the Hanoi University of Science and Technology (HUST). The fingers of interdigitated electrodes were $10\text{ }\mu\text{m}$ wide and their gap size was $10\text{ }\mu\text{m}$, by sputtering 10 nm Ti and 200 nm Pt on a 100 nm thermally thick silicon dioxide (SiO_2) layer grown on top of a silicon wafer. The detailed configuration of this sensor and a diagram of measuring principle have been described in previous publications [3, 10].

2.2. Serum antibodies immobilization methods

Sensors were immersed in KOH/MeOH (1 : 1) solution for 30 min for surface cleaning and functionalization. These sensors were then rinsed in de-ionized (DI) water and nitrogen-dried. The silanization process was conducted in 5% APTES/MeOH for 1 h to create amino groups (NH_2), allowing binding between the antibodies and interdigitated surface. A drop of acetic acid was added during the silanization to orientate amino groups outward from the interdigitated surface. Sensors were then washed three times in DI water, nitrogen-dried and annealed thermally at 120°C for 6–8 min to completely remove excess water molecules on the surface [10]. The silanized sensors were kept in a dry box at room temperature before use.

In this work, four serum antibody immobilization methods were performed on the interdigitated electrodes to develop immunosensors for optimizing conditions of JEV detection.

2.2.1. APTES-serum antibody (APTES-serum). Silanized sensor was incubated in 1 mg ml^{-1} JEV serum antibodies for 1 h, respectively. The unsaturated and non-specific binding sites on the surface were blocked with 2% BSA in phosphate buffer saline (PBS) for 30 min, washed with PBS (pH 7.0), and air-dried.

2.2.2. APTES-glutaraldehyde-serum antibody (APTES-GA-serum). The silanized sensor was dipped in 5% glutaraldehyde solution for 30 min. The sensor was washed three times in DI water, followed by incubation in 1 mg ml^{-1} JEV serum antibodies for 1 h, respectively. The unsaturated and non-specific binding sites on the surface were blocked with 2% BSA/PBS for 30 min, washed with PBS (pH 7.0) and air-dried.

2.2.3. APTES-glutaraldehyde-antihuman IgG-serum antibody (APTES-GA-antiHlgG-serum). The silanized sensor was dipped in 5% glutaraldehyde for 30 min and washed in DI water three times. Next, 1 mg ml goat anti-human immunoglobulin G polyclonal antibody was added to the surface for 30 min, then washed in PBS (pH 7.0) followed by incubation in 1 mg ml JEV serum antibodies for 1 h, respectively. The unsaturated and non-specific binding sites on the surface were blocked with 2% BSA/PBS for 30 min, washed in PBS (pH 7.0) and air-dried.

2.2.4. APTES-glutaraldehyde-protein A-serum antibody (APTES-GA-PrA-serum). The silanized sensor was dipped in 5% glutaraldehyde for 30 min and washed in DI water three times. Next, 5 μ l of PrA solution [1 mg PrA/1 ml PBS (pH 7.0)] was added to the surface for 30 min, then washed in PBS (pH 7.0) followed by incubation in 1 mg ml⁻¹ JEV serum antibodies for 1 h, respectively. The unsaturated and non-specific binding sites on the surface were blocked with 2% BSA/PBS for 30 min, washed with PBS (pH 7.0) and air-dried.

The surface morphology of the interdigitated electrodes before and after immobilization methods was observed by atomic force microscopy (Multimode, Veeco). Other surface characteristics and detailed immobilization methods have been reported in our previous publication [10].

2.3. Principle and measurements

Prior to measurement, immunosensors were incubated with JEV antigens diluted for 45 min, then washed carefully in PBS followed by incubation in 0.5 μ g ml⁻¹ horseradish peroxidase-labeled-JEV antibody for 45 min. A potential of 100 mV with a fixed frequency of 10 kHz was applied to electrodes using an RS830 Lock-in amplifier (Stanford Research Systems, USA) for measurements and the voltage drop across two 1 k Ω resistors. Conductimetric measurements were then performed at room temperature (27 $^{\circ}$ C \pm 2 $^{\circ}$ C) by immersing the immunosensors in glass cells, each cell containing a total volume of 200 μ l of 0.02 M PBS (pH = 7.0) containing 0.05 M KI, 80 μ M H₂O₂ and 0.15 M NaCl. The differential signal between working electrodes and reference electrodes was calculated from $\Delta S = S_w - S_r$, where S_w is the conductive value obtained on working electrodes resulting from JEV antigens-serum antibody binding, and S_r is the conductive value obtained on reference electrodes resulting from the free binding or non-specific bindings. The principle of conductimetric measurements is illustrated via the method of APTES-GA-PrA-serum in figure 1.

For specificity, the non-specific reactions were tested using a closely related viral antigen-Dengue virus and healthy mouse serum and BSA under the same conditions as for JEV antigens.

3. Results and discussion

3.1. Morphology of the interdigitated electrodes

AFM was used to investigate the change in morphology of interdigitated electrodes before and after treatment with serum

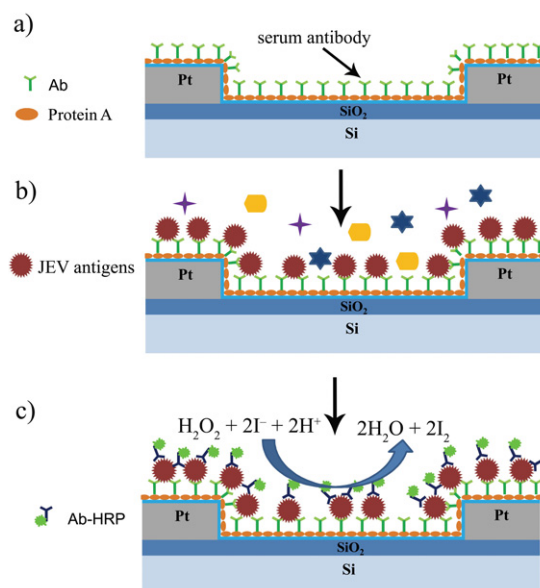


Figure 1. The principle of conductimetric measurements of electrochemical immunosensors based on serum antibody immobilization via protein A (APTES-GA-PrA-serum method) immobilization of serum antibody, (b) incubation with JEV antigens and (c) incubation with a secondary anti-JEV antibody conjugated with horseradish peroxidase.

antibodies via different immobilization methods. Figure 2(A) shows the surface of bare electrodes before silanization with uniform grains and defined boundaries. The mean surface roughness is determined as approximately 1.2 ± 0.5 nm in height. The characteristic AFM image of the surface after silanization with APTES becomes more uniform (figure 2(B)), and the average surface roughness decreases to 0.6 ± 0.08 nm in height due to the formation of silanized layer on the electrodes. This is because APTES molecules have been deposited into the gaps in-between the grains to fill some of the grain boundaries resulted in the overall smoothing of the surface [12]. After treatments with serum antibodies, the surface of the electrodes have become rougher depending on the immobilization method (figures 2(C)–(F)). Figure 2(C) shows the rough surface of the electrode resulting from the APTES-serum method. This image reveals big grains of 3–4 nm in height, and may come from the direct covalent binding or physical adsorption of the random clusters of serum antibodies on the electrode tested. Figure 2(D) shows that the cross-linker of GA could help to improve the binding of serum antibodies over the surface of the electrode, and the size of grains to decrease [13]. However, this method could not help to select specific antibodies against JEV in serum for the immobilization on the electrode, because GA would bind both serum antibodies and other proteins remaining in serum and lead to the formation of clusters. Figure 2(E) shows the change in geography of the surface when using antiHlgG to select IgG molecules in serum for the development of the immunosensor, and there are lots of small peaks like waves on the electrode. However, a drawback of antiHlgG mediated immobilization is the lack of control on the orientation of antiHlgG itself; it may lead to the disorder in orientation of serum antibodies. Therefore, another advanced method has been developed to achieve higher orientation

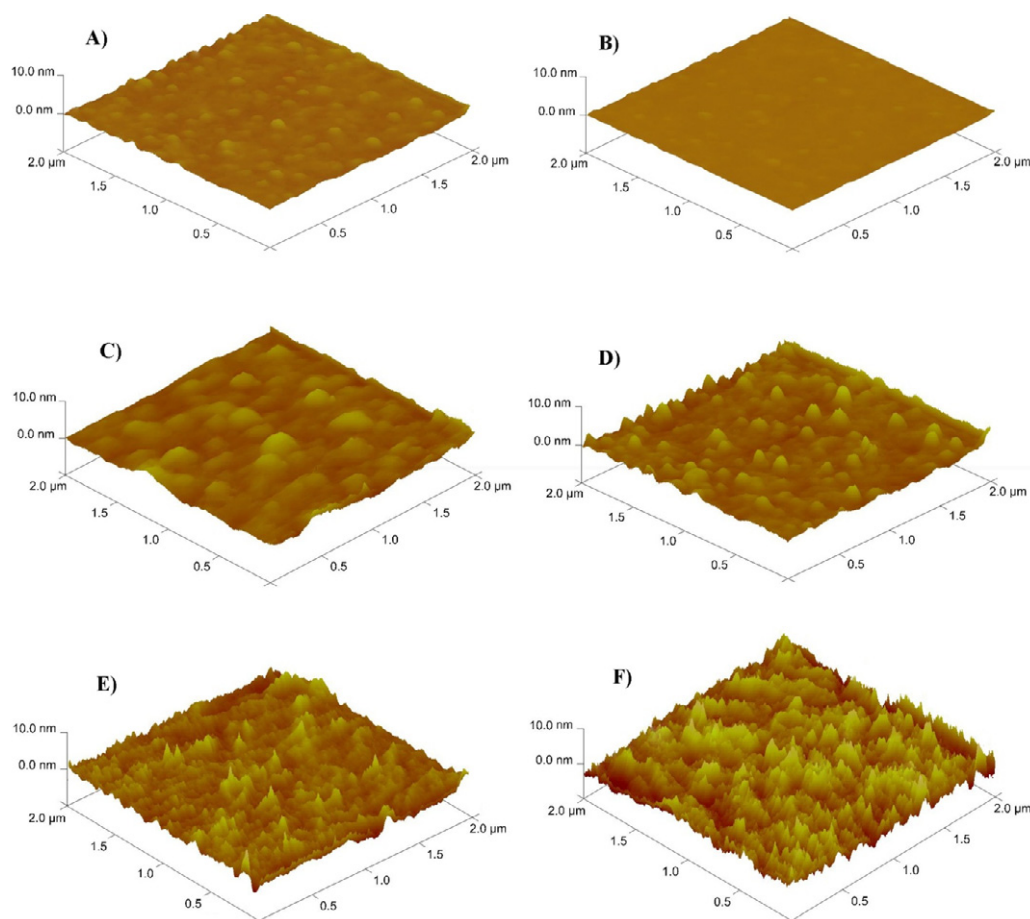


Figure 2. AFM images of the sensor surfaces: (A) before silanization; (B) after silanization with APTES; (C) APTES-serum method; (D) APTES-GA-serum method; (E) APTES-GA-antiHlgG-serum method; (F) APTES-GA-PrA-serum method.

control upon immobilization. Figure 2(F) shows many shape peaks arranged on the surface of the electrode when using protein A to bind and orient serum antibodies. In fact, IgG molecules are the main immunoglobulins, constituting 75% of the total immunoglobulins in human serum [14]. They are also major factors responsible for the detection of antigens in immunosensor applications. In these experiments, PrA was used to immobilize serum antibodies on the silanized surface. PrA can bind with high affinity to immunoglobulins (Ig), especially to the Fc region of human IgG1 and IgG2, binds with moderate affinity to human IgM, IgA and IgE, but not react with human IgG3, IgD or other proteins in human serum. This binding of PrA to immunoglobulin molecules does not influence their binding sites to antigens [15]. Moreover, PrA is also often immobilized onto a solid support and used as a reliable method for purifying total IgG from crude protein mixtures [16]. Hopefully, PrA would be the best choice for selection and orientation of IgG antibodies immobilized on the electrode, and lead to the possibility of serum antibodies immobilized to detect JEV antigens significantly in solution.

3.2. Response mechanism of the electrochemical immunosensor

In the configuration of electrochemical immunosensors, the specific binding of serum antibodies immobilized on the electrode and JEV antigens detected in the solution would

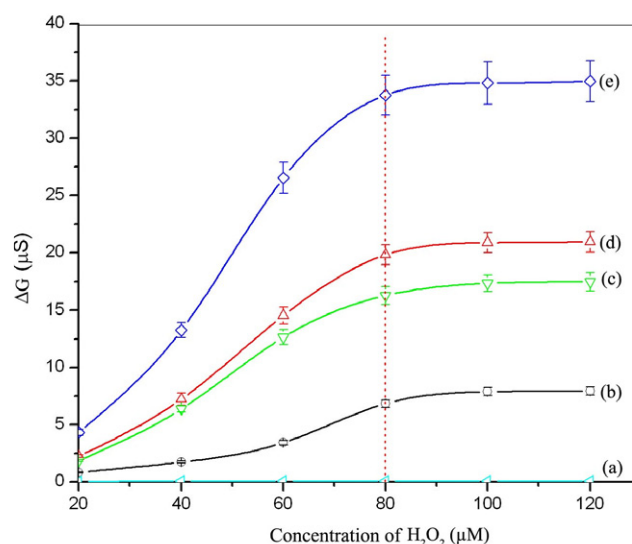


Figure 3. Bioactivity of the carried HRP versus concentration of H_2O_2 measured in 0.02 M PBS (pH 7.0) containing 0.05 M KI and 0.15 M NaCl using immunosensors developed after incubation with 50 ng ml^{-1} JEV antigens: (a) incubation with healthy mouse serum, (b) APTES-serum method, (c) APTES-GA-antiHlgG-serum method, (d) APTES-GA-serum method and (e) APTES-GA-PrA-serum method. Error bars were calculated from the mean values, $n = 3$.

lead to binding with the secondary anti-JEV antibody labeled HRP by the 'sandwich' mechanism. This configuration was similar to an enzyme immune-bioassay format to detect JEV

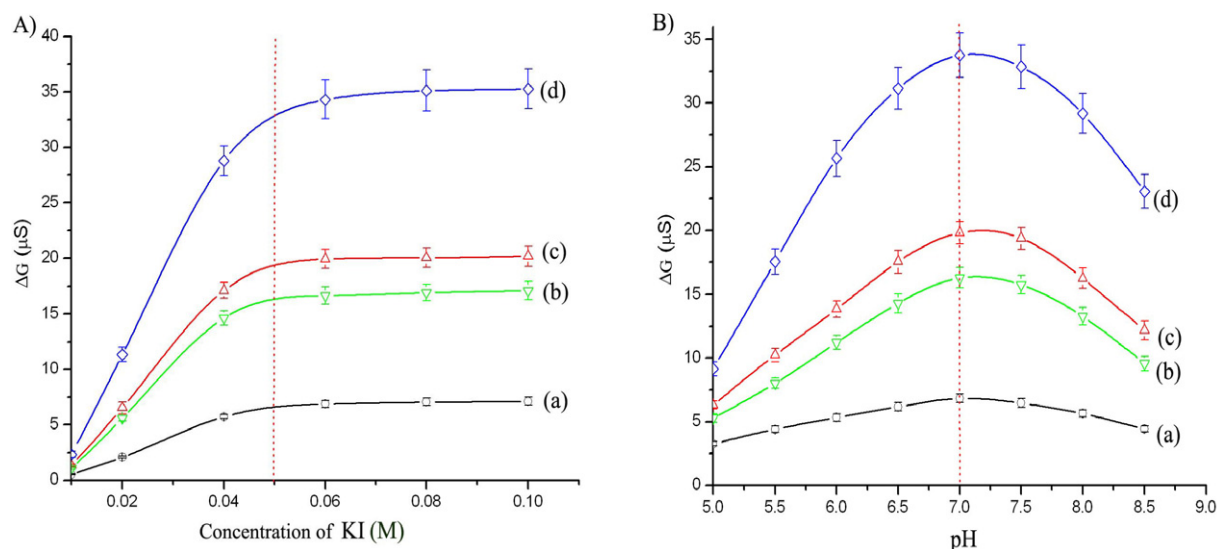
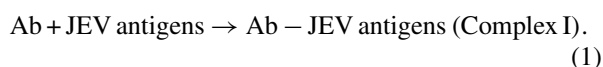


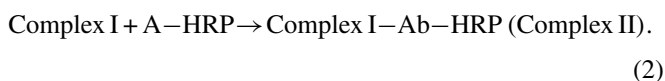
Figure 4. The influence of KI concentration (A) and pH of detection solution (B) for immunosensors developed: (a) APTES-serum method; (b) APTES-GA-antiHlgG-serum method; (c) APTES-GA-serum method and (d) APTES-GA-PrA-serum method. Error bars were calculated from the mean values, $n = 3$.

antigens indirectly in solution. Thanks to HRP molecules, most enzymatic reactions were accompanied by changes in the free iodine concentration when KI was used as a supporting electrolyte [17, 18]. The process of reactions is as follows:

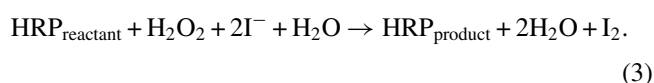
– Immunoreactions:



Complex I reacted with HRP labeled-anti-JEV antibody:



– Conductimetric measurement:



3.3. Optimization of experimental conditions

Figure 3 describes the change in conductivity versus the concentration of H_2O_2 for immunosensors developed using four serum antibody immobilization methods, and after incubation with $50 \mu\text{g ml}^{-1}$ JEV antigens. These conductimetric measurements of immunosensors were performed in 20 min after immersion in 0.02 M PBS (pH 7.0) containing 0.05 M KI and 0.15 M NaCl. The results revealed that the conductivity of the immunosensors developed increased significantly, and depended on the serum antibody immobilization method and concentration of H_2O_2 (figures 3(b)–(e)), whereas it did not recognize any change in conductivity of the immunosensors incubated with BSA, healthy mouse serum or Dengue antigens instead of JEV antigens (figure 3(a)). This clarified that all immunosensors developed could detect and bind specifically with JEV antigens and not be influenced by interference. The highest conductivity was recognized by the immunosensors

developed using the APTES-GA-PrA-serum method (figure 3e). These results also revealed that with increasing H_2O_2 concentration, the conductivity response increased and reached a maximum response at the concentration of $80 \mu\text{M}$ for all immunosensors developed. So, H_2O_2 concentration of $80 \mu\text{M}$ was optimal for measurements.

The detection performance of the immunosensors was not only dependent on H_2O_2 , but also KI concentrations, pH of the detection solution, time and temperature of the JEV antigens–serum antibody binding. After incubation with 50 ng ml^{-1} JEV antigens, the immunosensors developed were immersed in 0.02 M PBS (pH 7.0) containing $80 \mu\text{M}$ H_2O_2 and 0.15 M NaCl. When the concentration of KI increased, the conductivity increased linearly and reached a plateau at 0.05 M (figure 4(A)). So, 0.05 M KI was selected for conductimetric measurements.

The influence of pH of the detection solution was also investigated. Because most enzymes show good activity in only a limited range of pH, the solution pH of catalytic reaction influences the response of the product of enzymatic catalysis. After incubation with 50 ng ml^{-1} JEV antigens, the developed immunosensors were immersed in 0.02 M PBS containing 0.05 M KI, $80 \mu\text{M}$ H_2O_2 and 0.15 M NaCl resulted in the highest conductive responses in the pH range of 6.8–7.4, exhibiting a maximum activity of enzymatic catalysis (figure 4(B)). Thus, the value of pH 7.0 was selected for all measurements.

The study aims to investigate the detection performance of electrochemical immunosensors developed using four immobilization methods of serum antibodies to detect JEV antigens, the formation of immunocomplex on the electrode surfaces was carried out in the same condition. The time and temperature of incubation between JEV antigens and immobilized serum antibodies were referenced from recent publications [17, 18] for testing in laboratory condition (temperature range of $25\text{--}32^\circ\text{C}$ and incubation time of 30–60 min). The experimental results showed that the detection performance of all developed immunosensors

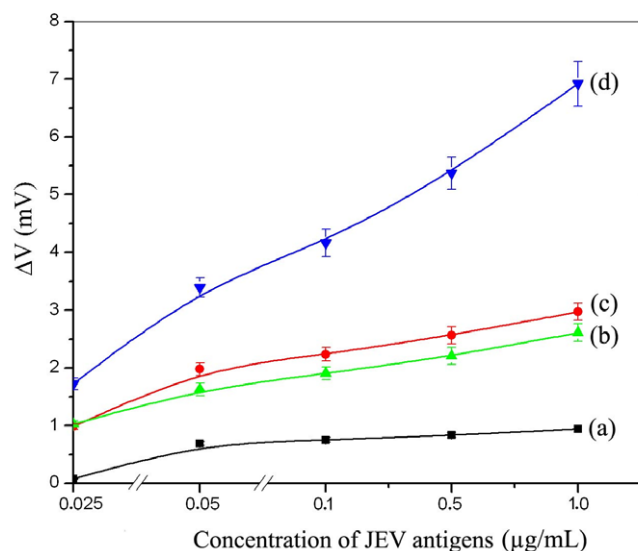


Figure 5. The differential voltage output versus concentration of JEV antigens from immunosensors developed using different immobilization methods of serum antibodies: (a) APTES-serum method, (b) APTES-GA-antiHlgG-serum method, (c) APTES-GA-serum method and (d) APTES-GA-PrA-serum method. Error bars were calculated from the mean values, $n = 3$.

was stable in these conditions. In fact, the temperature of $27 \pm 2^\circ \text{C}$ and incubation time of 45 min were selected for most measurements.

3.4. Detection of JEV antigens

The detection signals have been found stable for 20 min, since immersion of immunosensors developed in 0.02 M PBS (pH 7.0) containing 0.05 M KI, 80 μM H_2O_2 and 0.15 M NaCl. The change in conductivity comes from the working electrodes in comparison with reference electrodes, these signals could be converted into the differential voltage in the output of RS 830 Lock-in amplifier. Figure 5 shows the differential voltages of immunosensors developed by different concentration of JEV antigens in 0.02 M PBS (pH 7.0) containing 0.05 M KI, 80 μM H_2O_2 and 0.15 M NaCl. Figure 5(d) shows that the electrochemical immunosensor developed using the APTES-GA-PrA-serum method obtained the highest signal for detection of JEV antigens in the linear range from 25 ng ml^{-1} to 1 $\mu\text{g ml}^{-1}$, and the limit of detection (LOD) was about 10 ng ml^{-1} [19].

This demonstrated that the use of protein A is the best choice for selection and orientation of serum antibodies immobilized on the electrodes for the development of electrochemical immunosensors in virus detection.

4. Conclusion

This study presented the development of electrochemical immunosensors based on serum antibodies via four

immobilization methods to detect JEV antigens. The detection process was carried out indirectly by conductimetric measurements in 0.02 M PBS (pH 7.0) containing 0.05 M KI, 80 μM H_2O_2 and 0.15 M NaCl with the support of HRP-labeled secondary antibody against JEV. The results showed that the electrochemical immunosensor developed using the APTES-GA-PrA-serum method obtained the highest signal for detection of JEV antigens in the linear range from 25 ng ml^{-1} to 1 $\mu\text{g ml}^{-1}$, and the limit of detection was about 10 ng ml^{-1} . The study showed a potential development of useful and powerful devices for virus detection from clinical samples in case of possible outbreaks.

Acknowledgments

This work was financially supported by Vietnam's National Foundation for Science and Technology Development (NAFOSTED), project code: 106.16.181.09.

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