Final Report

Project Title Design and Development of Graphene Electrode Bio-Platforms for Immunosensing

By Assistant Professor Dr. Kontad Ounnunkad

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Project Title **Design and Development of Graphene Electrode Bio-Platforms for immunosensing**

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Abstract:

Monitoring human immunological responses due to infections plays the important role in disease diagnosis, including early human immunodeficiency virus (HIV) infection and cancers. Such immune responses could be investigated by using changes in levels of cytokines or biomarker proteins in human plasma such as very high levels of two cytokines, i.e. interleukin-15 (IL-15) and interferon-α (IFNα), resulted from the HIV cytokine storm due to the acute HIV infection. Studies of cytokine production behaviour would predict the stage of HIV disease progression. The immunoassay requires a special tool having very high selectivity and high sensitivity due to many protein interferences in the human plasma. Consequently, immunosensor, one of the most important biosensors, has been widely used for detection and screening of the biomarker proteins because of the specific reaction of antigen-antibody pair, possessing high accuracy. Although immunosensor reveals the best benefits, the sensitivity and detection limit can be suppressed by low loading of active biomolecules and improper biomolecular arrangement on immunosensing surfaces. In nanobiotechnology, usage of graphene/graphene oxide as electric nanocarrier has permitted the development of structures of immunosensing surfaces in which the improved antibody loading, good molecular recognition and electron transport property provide fast response, signal enhancement, high stability, and high sensitivity for electrochemical detection. Therefore, this research aims to design, develop and produce immunosensing platforms based on highly conductive graphene/graphene oxide nanohybrids. The platforms were evaluated through high selectivity and sensitivity, low limit of detection and good stability. The efficiency of the determination of

selected cytokines due to relationships of structure and molecular recognition were investigated. The designated platforms were conducted with the optimised testing condition. Our proposed approach also employed human immunoglobulin G (IgG) as a model protein as well as interleukin-15 (IL-15) for the further development of immunosensors for detection of other cytokines.

Keywords: Graphene, Graphene oxide, Electrochemistry, Electrochemical immunosensor, Biomarker, Screen-printed electrode, Modification, Label-free detection

1. Abstract

In Thai:

งานวิจัยนี้ได้ทำการพัฒนาอิมมูโนเซนเซอร์อย่างง่าย มีราคาถูกชนิดแบบไม่ติดฉลาก เพื่อเป็นพื้นฐานในการพัฒนาเซนเซอร์ต่อไป โดยงานวิจัยได้ทำการใช้ขั้วพิมพ์สกรีนที่ดัดแปรด้วยสารกราฟีนออกไซด์ ซึ่งให้ค่าการนำไฟฟ้าที่ดีและสามารถที่จะคอนจูเกทกับแอนติบอดี้ผ่านหมู่ฟังค์ชั่นคาร์บ๊อกซิลิกสำหรับการตรวจวัดสารไบโอมาร์คเกอร์ ในงานวิจัยนี้ได้พัฒนาระบบการวัดสารอินเทอลูคิน-15 เป็นครั้งแรก ได้ค่าขีดจำกัดของการตรวจวัด เท่ากับ 3.51 $ng.mL^{-1}$ และมีความว่องไวในการตรวจวัดเบื้องต้น 0.5655 $\mu A.cm^{-2}.mL.ng^{-1}$ ซึ่งสำหรับการพัฒนาอย่างง่ายนี้ ก็ยังให้ความจำเพาะเจาะจงและความเสถียรในการตรวจวัด ดังนั้นจะเห็นได้ว่า อิมมูโนเซนเซอร์อย่างง่ายนี้ น่าจะนำมาใช้วิเคราะห์สารอินเทอลูคิน-15 ในตัวอย่างจริงได้ อย่างไรก็ตามอิมมูโนเซนเซอร์ควรที่จะนำมาพัฒนาต่อเพื่อให้ได้ความว่องไวและขีดความสามารถในการวัดที่ต่ำสุดที่ดีขึ้นและสามารถวัดสารอินเทอลูคิน-15 ในระดับที่ต่ำมากๆ ได้ (ต่ำกว่า 1 pg mL^{-1}) เพื่อเหมาะสมสำหรับการตรวจวัดการติดเชื้อเอชไอวี-1 และการติดตามระยะการติดเชื้อเพื่อให้คนไข้ได้รับการรักษาอย่างถูกต้องและเหมาะสม

In English:

A simple, label-free and cost-effective electrochemical immunosensor based on graphene oxide film-modified screen-printed carbon electrode for the detection of interleukin-15 was reported for the first time. The electroactivity of the screen-printed carbon electrode was greatly improved by the carboxylic group-containing graphene oxide. The simple immunosensor showed a satisfactory detection limit of 3.51 $ng.mL^{-1}$ and a sensitivity of 0.5655 $\mu A.cm^{-2}.mL.ng^{-1}$. Moreover, the immunosensor exhibited a good selectivity and stability. The proposed immunosensor would thus be applied as a promising candidate for the determination of interleukin-15 in real samples. However, this preliminary research for immunosensing of interleukin-15 requires the further development in order to obtain better device performances

such as higher sensitivity and lower limit of detection in trace amount, which will ultimately be beneficial for the determination of HIV-1 acute infection and pathogenesis.

2. Executive Summary

Biofunctionalising the simple and disposable graphene oxide-modified screen-printed carbon electrode with anti-interleukin-15 antibodies for label-free electrochemical detection of interleukin-15, a proposed biomarker of HIV early infection, has been successfully demonstrated for the first time. In order to both improve the electrochemical reactivity and introduce the carboxylic functional groups on the surface of screen-printed carbon electrode, the high quality graphene oxide was employed for the modification of screen-printed carbon electrode. With a simple modification of screen-printed carbon electrode, the device exhibited satisfactory sensitivity, selectivity, stability, reproducibility and regenerability. The immunosensor showed a detection limit of 3.51 ng.mL^{-1} and a sensitivity of 0.5655 μA.cm^{-2}.mL.ng^{-1}. The simply constructed immunosensor thus rendered promising device performances towards immunoreaction on the surface of electrode.

3. Objectives

This work involves the research on design and development of optimised nanostructures of graphene/graphene oxide electrode platforms with high charge transport properties and good connection with active biomolecules, i.e. antibodies. The immunosensing test of such platforms fabricated will be demonstrated for detection of selected cytokine (interleukin-15, IL-15).

3.1 To synthesise and characterise high quality graphene/graphene oxide possessing high conductivity

3.2 To design and fabricate graphene/graphene oxide electrode platforms and to optimise structure of the electrochemical platforms

3.3 To immobilise antibody on superior electrochemical graphene/graphene oxide platforms, to optimise graphene/graphene oxide-biomolecule interfaces, and to characterise the electrochemical immunosensing platforms

3.4 To investigate the immunosensing electrode for the detection of cytokine and to optimise conditions according to fast responses, good electrode kinetics, low limit of detection, high stability and high sensitivity

3.5 To study the experimental parameters or factors which affect the efficiencies of the immunosensors for determination of cytokine

Note: The outputs of the all objectives will be given in Appendix section

4. Research methodology

4.1 Introduction

Interleukin-15, a member of the common **γ**-chain family of cytokines, has a major role in the immune response during the HIV-1 infection (Lim et al. 2013; Mastroianni et al. 2004; Naora, and Gougeon 1999). As compared to that of a healthy individual, a restrictive production of interleukin-15 has been found in AIDS-stage patients, and might stem from the impairment of immune responses against HIV (d'Ettorre et al. 2006; Mastroianni et al. 2004), considerably resulting in low levels of interleukin-15. Interestingly, a rapid increase in the level of interleukin-15 has been observed during the acute infection (Stacey et al. 2009). Interleukin-15 could thus be proposed and validated as a potential biomarker for the evaluation of the stages of HIV infection (Mastroianni et al. 2004). Importantly, the early detection of the infection, which is defined by elevation of the concentration of interleukin-15 in the blood, could represent one of the most promising approaches to monitor HIV pathogenesis and for the treatment, hence prolonging the survival rate of the HIV-infected patients. The precise and sensitive determination of the viral infection-related proteins is very important for the better clinical prognosis. In general, the technique used for a quantitative determination of the biomarkers is usually immunoassay, which renders a promising analysis with adequate selectivity and sensitivity.

Many traditional immunoassays, based on antigen-antibody binding detection, have been employed for the determination of biomarker proteins, such as enzyme-linked immunosorbent assay (ELISA) (Pålsson et al. 2000), chemiluminescence immunoassays (Tanaka, and Matsunaga 2000), and surface plasmon resonance (SPR) immunoassays (Dong et al. 2008). Although these

methods have high sensitivity and accuracy, they are time-consuming, expensive, and complicated. To overcome the above drawbacks, electrochemical immunosensors have been used widely and gaining increasing attention continuously. They combine the remarkable specificity of the traditional methods with the lower limits of detection, as well as with the lower cost of the electrochemical detection system. They also, in particular, offer high sensitivity and compatibility for miniaturization (Okuno et al. 2007; Tang et al. 2007). In comparison with many optical based techniques, the electrochemical immunoassays are not affected by coloured and turbid samples, nor by the fluorescing compounds present in the biological samples (Hu et al. 2003). In order to construct the electrochemical immunosensors, capturing antibodies are generally immobilised onto an electrode surface, which can provide a precise and fast detection of the related antigen, namely protein biomarkers, owing to their unique combination of exquisite specific antigen-antibody interaction and sensitive electrochemical transduction (Liu, Duckworth, and Wong 2010; Pandiaraj et al. 2014). The devices have been developed by an electrochemical detection of labelled immunoagent because there are no detectable redox responses from most of the target proteins (Bahadır, and Sezgintürk 2015). Consequently, they require the use of some external mediators or labelling either antigen or antibody to generate signal by their electron-transfer process, corresponding to the amount of the antibody-antigen compounds or immunocomplexes, which refer to the concentration of the biomarkers. However, all of these immunoassays usually involve complicated and elaborated architectures and procedures, leading to inconvenience of use (Liu, and Lin 2007). In addition, the labelling with enzymes or redox agents might affect the antigen-antibody binding efficiency and the enzymes generally are highly environmental

sensitive, in turn influencing the sensitivity and detection limit of the biosensors. In order to avoid this complexity, the electrochemical immunosensors have been developed with no labeling (Bangar et al. 2009; Li et al. 2013). Detection of the immune reactions is carried out by a determination of a change in physical properties at the immunoelectrode surface. The quantitation of the formation of resultant antibody-antigen complexes can be performed using the recording of amperometric, potentiometric, impedimetric or conductometric signals.

Over the last few decades, screen-printed carbon electrodes have been popularly employed for various electrochemical measurements (Couto, Lima, and Quinaz 2016). The screen-printed carbon electrodes offer low background current, readily renewable surface and wide potential window. They are relatively inexpensive, versatile and chemically inert. However, the development of label-free immunosensors based on screen-printed carbon electrodes has been limited by their low electroreactivity, low sensitivity and high limit of detection. In order to avoid these disadvantages of the screen-printed carbon electrodes, the enhancement of their electroactivity is required. Therefore, the label-free immunosensors based screen-printed carbon electrodes have been developed by a modification with electroactive nanomaterials and high surface area nanoparticles, especially nanocarbons that can be coupled with antibodies. The covalently bound antibodies have an ability to capture biomarker proteins efficiently, which inhibit the electron transfer process of redox probe at the electrode due to its surface coverage by the biomarkers, thus resulting in the reduction of the redox signal. The changes in redox response as analytical signals come along with the levels of immunoreaction or the amount of the captured biomarkers on the electrode. In addition, nanocarbons such as graphene (Couto, Lima, and Quinaz 2016), graphene oxide (Tertiş et al. 2015), and carbon

nanotube (Upan et al. 2016), possessing large specific surface areas, good electrical conductivities, and high thermal and chemical stabilities have been found in the modification in order to construct electrochemical sensors. Interestingly, though having lower conductivity due to the destruction of its π-conjugation structure, graphene oxide provides carboxylic groups (-COOH), on which antibodies can be covalently immobilised. This allows the use of graphene oxide for a construction of biosensors with specific antibodies conjugated on graphene oxide nanoparticles. As shown in previous study (Jumpathong, Jakmunee, and Ounnunkad 2016), graphene oxide with high quality can improve the electrochemical properties as well as immobilise the anti-human immunoglobulin G molecules for construction of biosensor. The biosensor developed showed good performances for the detection of human immunoglobulin G at the physiological level.

In this work, the analytical performance of a simple, disposable, and cost-effective immunosensing platform based on a graphene oxide-modified screen-printed carbon electrode for detection of interleukin-15 was reported and described for the first time. The label-free detection of Interleukin-15, which could be a predictive biomarker for the analysis of HIV infection, was investigated by the developed electrochemical bioplatform employing a response of liquid-phase $[Fe(CN)_6]^{4-/3-}$ reaction (1:1 molar ratio) as the signal. Graphene oxide used for the surface modification in this study is of high quality, which gave rise to both the improvement of the electroactivity of screen-printed carbon electrode and the effective anchoring of the antibodies by covalent bonding, resulting in acceptable stabilities. The conditions of the attachment of the active proteins onto the screen-printed carbon electrode

were studied and optimised in order to obtain the best analytical response. The reduction of electrical current as a function of the restriction of the redox process due to surface coverage by immunocomplexes was employed to quantitate the interleukin-15 concentration. The proposed label-free immunosensors preliminarily revealed satisfactory device properties including acceptable sensitivity, selectivity, reproductivity, linearity, regenerability, detection limit, and stability. The simple graphene oxide-modified screen-printed carbon electrode-based electrochemical platform is thus a promising candidate for further development of better immunosensors.

5. Experimental Details

5.1 Reagents and Materials

All chemicals were of analytical grade and were used as received. Natural graphite powder (SP-1) was purchased from Bay Carbon Inc. (Michigan, USA). Carbon ink (Acheson Electrodag PR-406) was purchased from Henkel (USA). Anti-human interleukin-15 monoclonal antibody (~13 kDa, clone 1H3) was achieved from Merck Millipore (Germany). Interleukin-15, potassium hexacyanoferrate(III) ($K_3Fe(CN)_6$), potassium hexacyanoferrate (II) trihydrate ($K_4Fe(CN)_6 \cdot 3H_2O$), ascorbic acid, uric acid, glucose, bovine serum albumin and PBS tablets (pH 7.4) were purchased from Sigma-Aldrich (USA). 1-Ethyl-3-(3-dimethylamino-propyl) carbodiimide and N-hydroxysuccinimide were obtained from Merck (Germany). Disodium hydrogen phosphate, sodium dihydrogen phosphate, sodium hydroxide and sodium chloride were purchased from Carlo Erba Reagent (Italy). A graphene oxide powder for this study was prepared from a natural

graphite powder via a liquid-phase exfoliation process as previously reported (Pothipor et al. 2015). All aqueous solutions were prepared using deionised water.

5.2. Apparatus

A plasma cleaner (PDC-32G, Harrick Plasma, USA) was used to treated screen printed carbon electrode before being modified. Electrochemical measurements were carried out using a μAutolab type II (Eco Chemie, Utrecht, Netherlands). Three-electrode system was used for all electrochemical experiments, which consisted of a Ag/AgCl (3 M NaCl) reference electrode, a platinum wire auxiliary electrode, and a screen-printed carbon electrode as a working electrode.

5.3 Fabrication and Modification of Screen-Printed Carbon Electrode

Home-made screen printed carbon electrodes used as electrochemical sensing platforms were fabricated employing a carbon ink and a polyvinyl chloride sheet was used as a substrate. Briefly, the ink was screen-printed onto the polyvinyl chloride substrate and then the screen printed electrodes were incubated at 150 °C in oven for 1 h. The fabrication process was performed twice to obtain the greater film connection of carbon particles on the substrate, thus possessing the good conductivity. Before being modified, screen printed carbon electrodes were treated in a chamber of plasma cleaner under the optimum condition. Briefly, the screen printed carbon electrodes were placed in the chamber of the plasma cleaner. The chamber was evacuated to 0.15 torr and then backfilled with air. After that plasma was generated at low frequency RF and operated at pressure 0.4 torr for 60 s. The electrodes except working area

were well-covered with nail vanish before use. The screen-printed carbon electrode was modified with 2 µL of 3 mg.mL^{-1} graphene oxide dispersion solution twice in order to obtain better electrochemical responses that were evidenced by the cyclic and differential pulse voltammograms as seen in Figure 1 and Figure 2, respectively. After the modification with graphene oxide, the modified screen printed carbon electrode was dried at room temperature.

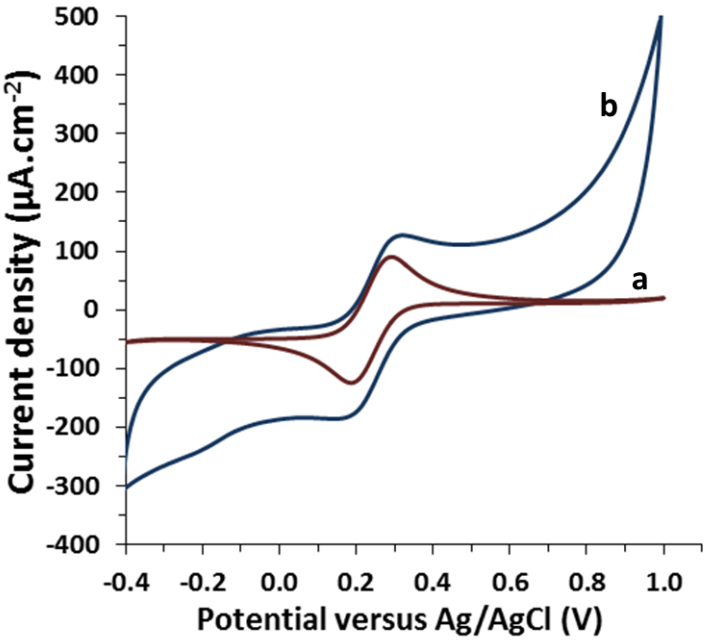

Figure 1 Cyclic voltammograms of $[Fe(CN)_6]^{4-/3-}$ process at (a) bare and (b) graphene oxide-modified screen-printed carbon electrodes in phosphate buffered saline (pH 7.4) containing 5.0 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ (1:1 molar ratio) and 0.1 M NaCl at scan rate of 50 mV.s^{-1}

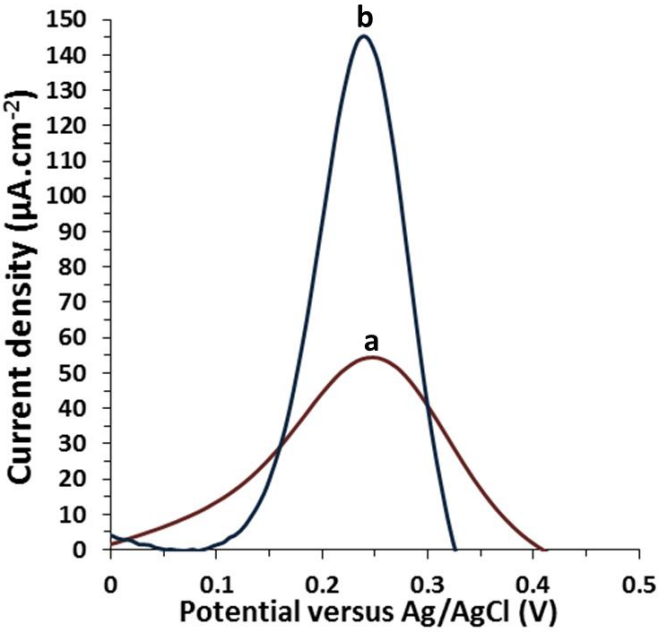

Figure 2 Differential pulse voltammetric signals of $[Fe(CN)_6]^{4-/3-}$ process at (a) bare and (b) graphene oxide-modified screen-printed carbon electrodes in phosphate buffered saline (pH 7.4) containing 5.0 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ (1:1 molar ratio) and 0.1 M NaCl under step potential of 5mV, modulation amplitude of 50 mV, and modulation time of 20 ms at scan rate of 10 $mV.s^{-1}$

5.4 Preparation of Immunosensor

A 10 μL of mixed solution containing 0.4 M 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide and 0.1 M N-hydroxysuccinimide with a volume ratio of 1:1 was dropped onto the surface of the modified screen printed carbon electrode and the modified electrode was then incubated at 4°C for 30 min. The activation of carboxylic groups available on the graphene oxide particles was obtained. After the electrode was washed with phosphate buffered saline (pH 7.4) several times and dried, 20 μL of 20 μg mL^{-1} anti-human interleukin-15 antibody solution was coated

onto the activated surface at 4°C to obtain a covalent binding of the antibodies. The electrode with the immobilised antibodies was rinsed with the phosphate buffer several times and nonspecific binding sites were then blocked via casting with 20 μL of 0.5% bovine serum albumin solution onto the surface at the same temperature for 30 min. After the above electrode was rinsed with phosphate buffer saline again several times and air-dried, 20 μL of different concentrations of human interleukin-15 were subsequently added onto the immunoelectrode surface to obtain the calibrating data. Between the incubation with each interleukin-15 solution, the surface of immunosensing electrode was regenerated using a 5.0 mM NaOH solution for 2 min as reported elsewhere (Chaocharoen et al. 2015; Dostálek et al. 2006). Higher concentration of the alkaline base solution and longer period for regeneration, which were also studied in this work, destroyed the electrode activity, thus resulting in no rebinding of interleukin-15 (data not shown) afterwards. The electrochemical responses of the electrode are represented in Figure 3. As seen in Figure 3, the reduction of current in the differential pulse voltammetry is related to the step-by-step construction of the immunoelectrode. After being washed with phosphate buffer and dried, the electrode was stored at 4°C for subsequent use.

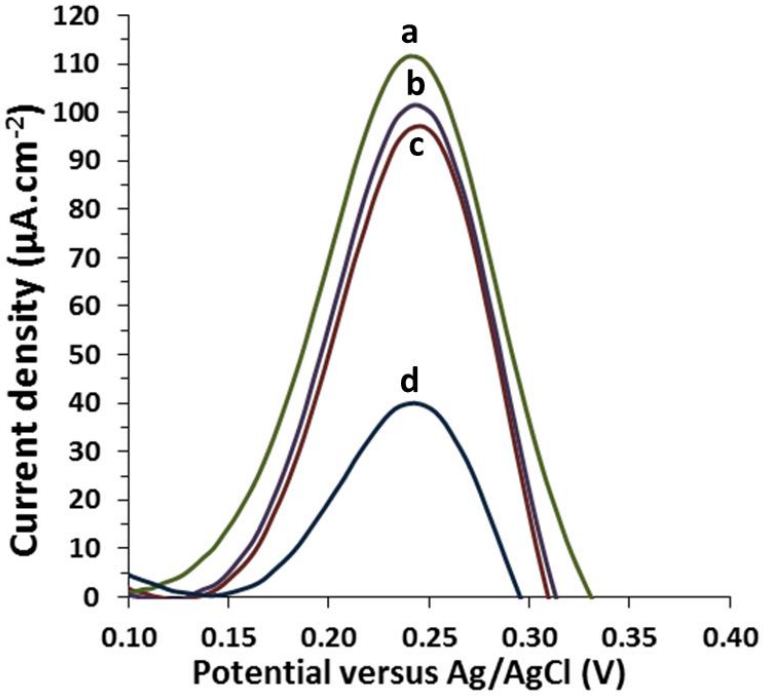

Figure 3 Differential pulse voltammetric signals recorded after (a) modification with graphene oxide onto screen-printed carbon electrode, (b) activation of graphene oxide-modified screen-printed carbon electrode by a mixed solution of 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (0.4 M) and N-hydroxysuccinimide (0.1 M) with a volume ratio of 1:1, (c) immobilisation of anti-interleukin-15 (20 $\mu g.mL^{-1}$), and (d) binding with 100 $ng.mL^{-1}$ interleukin-15.

5.5 Electrochemical Characterization

Electrochemical detections were performed using 5.0 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ (1:1 molar ratio) solution containing phosphate buffered saline (pH 7.4) and 0.1 M NaCl. Differential pulse voltammetry was used to study the optimisation of conditions and the detection of signals resulted from the immunocomplex with different concentrations of interleukin-15. The

electrochemical properties of the electrodes were also investigated by cyclic voltammetry. Differential pulse voltammetric parameters were step potential of 5 mV, modulation amplitude of 50 mV, modulation time of 20 ms, and scan rate of 10 $mV.s^{-1}$.

6. Results and Discussion

6.1 Fabrication of Electrodes and Reproducibility

Electrochemical properties of the graphene oxide-modified screen-printed carbon electrode in comparison with that of the bare screen-printed carbon electrode were investigated by cyclic voltammetry (Figure 1) and differential pulse voltammetry (Figure 2). Figure 2 shows the measured differential pulse voltammetric current of the screen-printed carbon electrodes in phosphate buffered saline (pH 7.4) containing 5.0 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ (1:1 molar ratio) and 0.1 M NaCl. The differential pulse voltammetric response of the $[Fe(CN)_6]^{4-/3-}$ process at a screen-printed carbon electrode was improved by the modification with graphene oxide, which the current was increased for approximately three times, indicating that graphene oxide could facilitate heterogeneous electron transfer between screen-printed carbon electrode and the electrolyte. In addition, graphene oxide improved the specific surface area of screen-printed carbon electrode. This current enhancement also correlates to the cyclic voltammetric result (Figure 1). The graphene oxide used in this study would be of good quality with a provision of oxygenic groups and sufficient electroreactivity. In order to obtain the immunoelectrode, the electrochemical differential pulse voltammetric signal at the screen-printed carbon electrode, which is related to each step of the electrode treatment, was found to be reduced due to the surface coverage as established in Figure 3. As seen in this figure, the electroreactivity at the

electrode was reduced with a lowered differential pulse voltammetric current, caused by covering the electrode surface with nonconducting molecules from each step. Of more interest is the restriction of the redox process at the immunoelectrode after capturing the interleukin-15, thus resulting in the reduction of the current. The difference in the differential pulse voltammetric currents before and after incubation with interleukin-15 solution is proportional to its concentration. On the basis of change in the electrochemical response above, a label-free electrochemical immunosensor for the detection of human interleukin-15 had been fabricated and readily employed to obtain calibration data for this work. After regeneration of the biosensor surface with an alkaline base solution, the interaction of antibody-antigen pair on surface was again carried out by the incubation with another interleukin-15 solution. The electrode using the graphene oxide-modified screen-printed carbon electrode was reproduced, giving no significant difference of the redox response at eight electrodes (Figure 4). The fabrication parameters and operating conditions for immunosensing were further optimised to illustrate the best differences of differential pulse voltammetric current response after the immunoreaction.

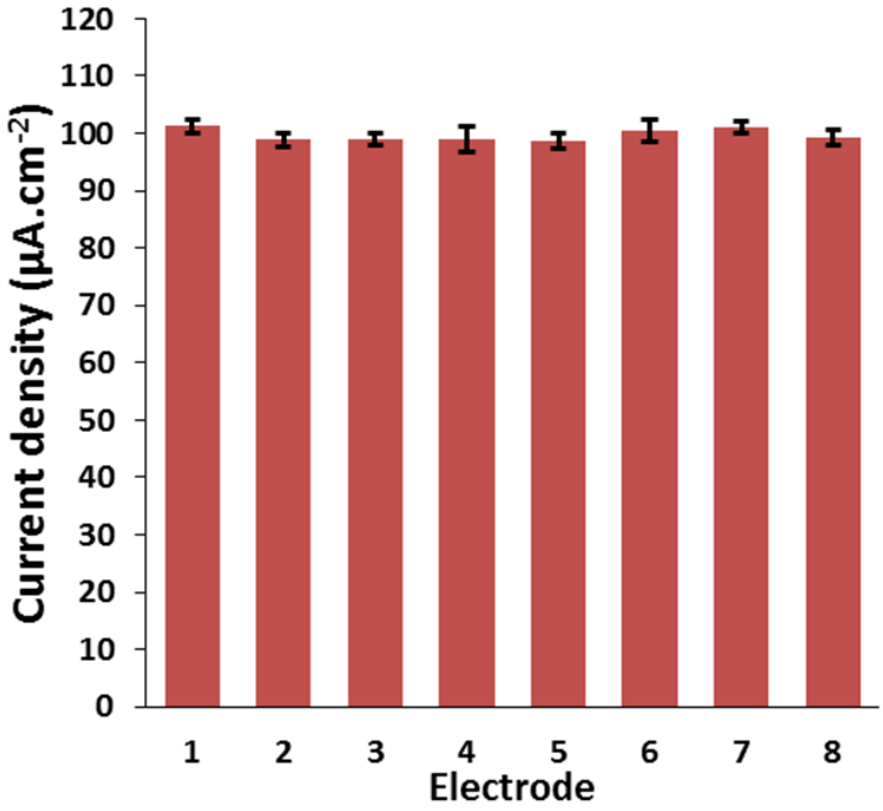

Figure 4 Differential pulse voltammetric currents of eight fabricated electrodes in phosphate buffered saline (pH 7.4) containing 5.0 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ (1:1 molar ratio) and 0.1 M NaCl.

6.2 Effect of incubation times

To obtain the best signal due to the highest protein loading, the effect of immobilisation time of the antibodies on the current response difference of the immunosensor was examined as shown in Figure 5. Chemical coupling of the antibodies onto the electrode surface was performed in the incubation period range of 10-60 min. The loading of the anti-interleukin-15 antibodies was monitored by differential pulse voltammetric method towards the immunoreaction causing the resultant current. It is plausible that the reduction of the redox

response referred to the amount of the immunoreaction as well as the amount of anti-interleukin-15 antibody loaded onto the electrode surface. The 20 $\mu g.mL^{-1}$ anti-interleukin-15 solution was used for the optimisation. After binding with the same concentration of 10 $ng.mL^{-1}$ interleukin-15 solution for 30 min, the decreased differential pulse voltammetric signal (*J*, current density) of the redox probe at the biosensor as a function of the period was observed. From the incubation time of 40 min, the current appeared to be constant. On the other hand, Figure 5 shows the difference of the differential pulse voltammetric currents between before and after the immunoreaction that tends to increase and then reaches a constant value after the incubation time of 40 min, suggesting that at this period the coupling reaction on the surface could be complete and the proper orientation of the anti-interleukin-15 antibodies could be available on the surface. It is noted that the electrode surface was saturated by the immobilised antibodies. The highest affinity towards interleukin-15 due to greatest amount of the antibodies on the surface of screen-printed carbon electrode would be obtained at this incubation period. Longer incubation time has no significant effect on the analytical current (ΔJ). Therefore, the period of 40 minutes as the optimised incubation time for immobilisation of anti-interleukin-15 was employed in this study. Moreover, the influence of incubation time on the immunochemical reaction attributing to the observed current response was also investigated. The immunoelectrode was incubated with a constant concentration of 50 $ng.mL^{-1}$ interleukin-15 solution for different time intervals. The reaction of antibody (anti-interleukin-15)-antigen (interleukin-15) pair on the immunoelectrode was found by the reduction of the differential pulse voltammetric current density (*J*). As demonstrated in Figure 6, the current

density difference (ΔJ) increases with increasing incubation time and reaches a plateau at the incubation period of 30 min, indicating the complete formation of the immunocomplex on the biosensor. In addition, capture efficiency of antigens in the proposed immunosensor was saturated after this interval. Thus, the incubation time of 30 min was adopted in this work.

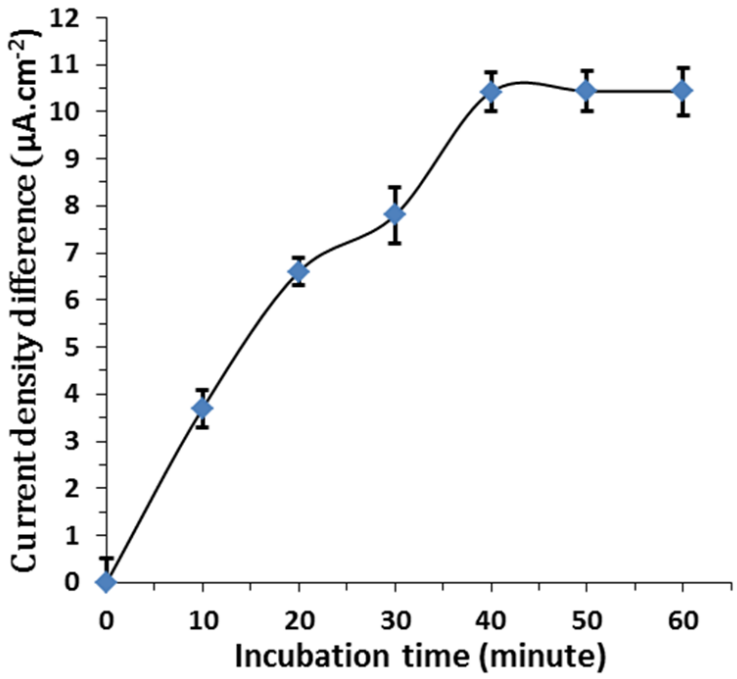

Figure 5 Effect of the incubation time for immobilisation of anti-interleukin-15 antibodies (20 μg.mL^{-1}) on differential pulse voltammetric analytical responses after binding of interleukin-15 antigens (10 ng.mL^{-1}) for 30 minutes.

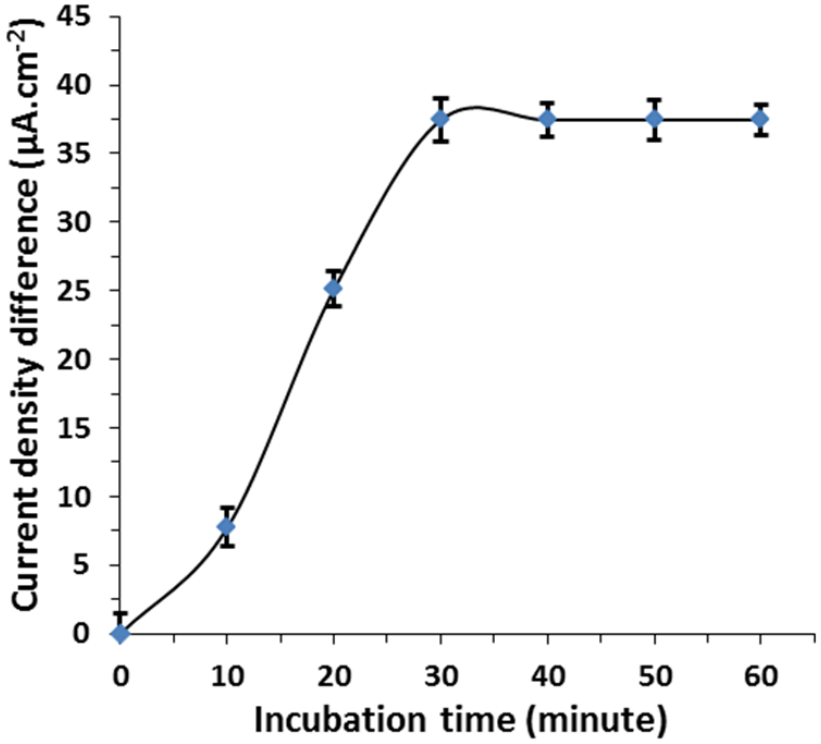

Figure 6 Effect of the incubation time for capturing interleukin-15 antigens on differential pulse voltammetric analytical signals determined after binding of interleukin-15 antigens (50 ng.mL^{-1}) by screen-printed carbon electrode functionalised with anti-interleukin-15 antibodies (20 μg.mL^{-1}) for 40 minutes.

6.3 Effect of pH and Surface Regenerability

Another main experimental condition, pH of the operating buffered electrolyte, which could affect the performance and the electrochemical behaviour of the immunosensor, was optimised by differential pulse voltammetric measurement. As shown in Figure 7, the effect of pH on the peak current of the immunosensor was studied. It was found that the differential pulse voltammetric response increased from pH 6.2 to 7.4 to reach maximum and then decreased from pH 7.4 to 7.8 whilst the differential pulse voltammetric peak position was

proportionally shifted to lower potential. Therefore, the conformation or the proper orientation of the anti-interleukin-15 antibody molecules on the electrode surface greatly depended on working pH, in which pH 7.4 would exhibit the best molecular recognition of the immunoproteins. The mechanism is proposed here that the biomolecules could adsorb H+ or OH- at different pH values, presumably through their amino acid side chains, thus resulting in the different electrochemical responses (Sun, Qiao, and Wang 2013) and device sensitivity. Moreover, activity loss and structural denaturation are generally found in biomolecules at significantly higher or lower pH than physiological level (Liu et al. 2015). Therefore, pH 7.4, a typical level in the human blood, would mediate the best ability for capturing the antigens in immunosensor. Hence, with a consideration of the response and the activity of the antibodies, the pH 7.4 of the phosphate buffered saline solution containing 0.1 M NaCl and 5 mM $[Fe(CN)_6]^{4-}/[Fe(CN)_6]^{3-}$ (1:1) was chosen for this study. Figure 8 shows the investigation of the regeneration of the electrode surface after binding with incubation in 25 $ng.mL^{-1}$ interleukin-15 solution for the immunosensor. With the formation of immunocomplex on the electrode surface, the measured differential pulse voltammetric current of $[Fe(CN)_6]^{4-/3-}$ process was approximately reduced from 105 to 85 $\mu A.cm^{-2}$. To remove the captured antigens, the electrode was treated with the alkaline base solution. Consequently, the differential pulse voltammetric current for the redox process was undertaken again, giving the value back to the starting point. The rebinding and removal of the antigens were successfully performed several times, giving no significant change in the current density difference due to occurrence of the

antibody-antigen complex. This evidence above suggests that the proposed immunosensor can be regenerated, thus revealing its great reusability.

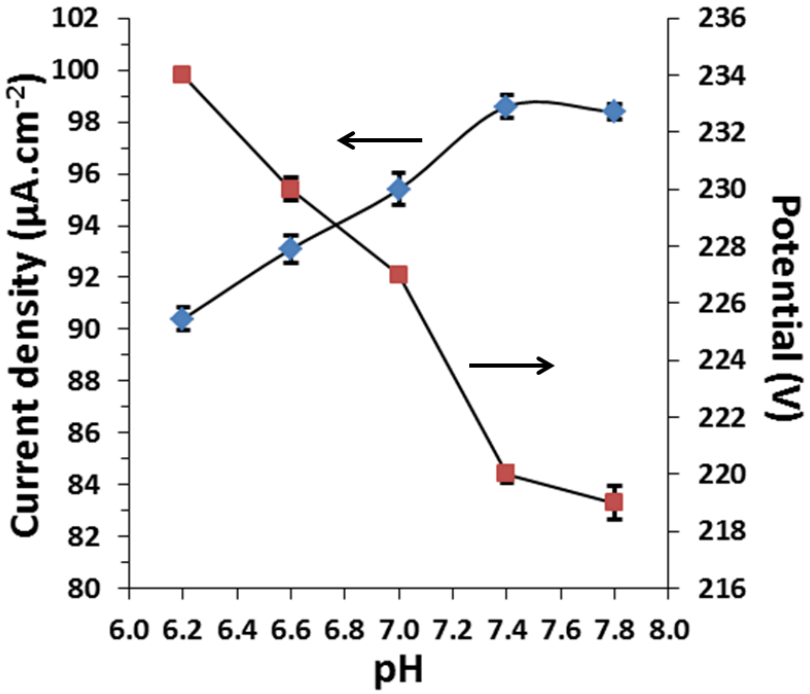

Figure 7 Effect of the operating pH on differential pulse voltammetric signals of the immunosensor in phosphate buffered saline (pH 6.2-7.8) containing 5.0 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ (1:1 molar ratio) and 0.1 M NaCl.

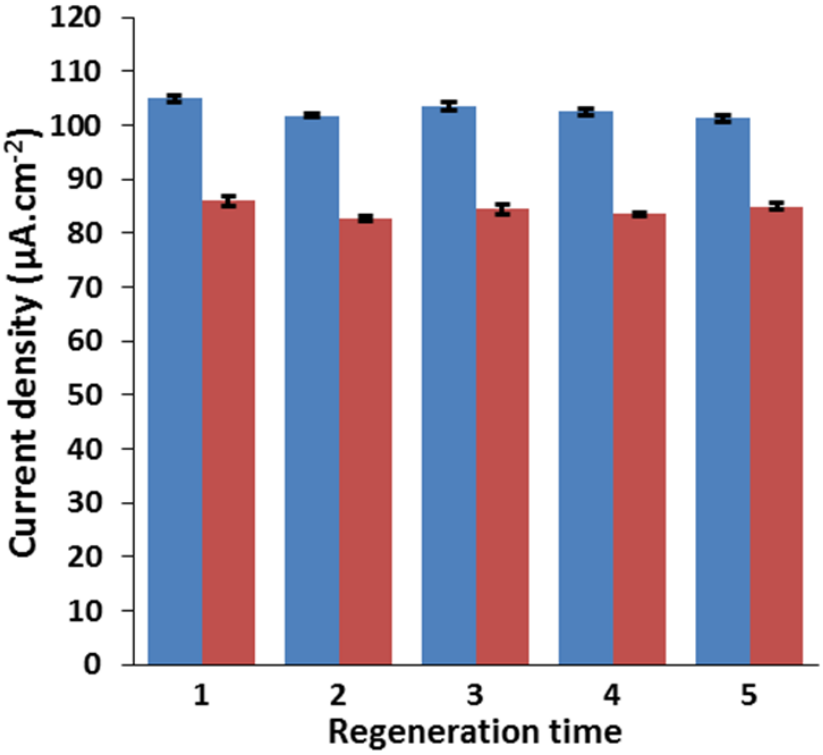

Figure 8 Differential pulse voltammetric signals recorded after (red) binding with 25 ng.mL^{-1} interleukin-15 solution and (blue) regeneration with NaOH solution.

6.4 Calibration curve, Linearity, Sensitivity, and Limit of Detection

Under the optimised experimental conditions, differential pulse voltammograms from detection of different concentrations of interleukin-15 were obtained and found to decrease systematically (Figure 9). Figure 9 shows that the peak currents decreased with increasing the concentration of interleukin-15. The current density difference (ΔJ) of the immunosensor, which is corresponding to this figure, was found to be proportional to the interleukin-15 concentration in range from 5 ng.mL^{-1} to 100 ng.mL^{-1} with a linear efficiency of 0.9987. The observable linear regression equation is ΔJ (µA.cm^{-2}) = 0.5655[interleukin-15](ng.mL^{-1}) + 1.2442 and a detection limit is calculated to be 3.51 ng.mL^{-1}. The observed sensitivity was found to be

0.5655 μA.cm^{-2}.mL.ng^{-1}. It is hence noted that the graphene oxide-modified screen-printed carbon electrode is sufficient for fabrication of the electrochemical device. The immunosensor fabricated in this study presents its simplicity and low cost and consumes less chemicals. As demonstrated with the early HIV-1 infection, the median baseline and highest recorded levels of interleukin-15 were observed at the concentrations of 1.9 and 3.6 pg.mL^{-1} (Stacey et al. 2009). The preconcentration process would be required to detect interleukin-15 by the immunosensor in the present study. Moreover, in this study the label-free immunosensor for a detection of human interleukin-15 based on measurement of the electrochemical response has been investigated here for the first time. As compared with the analytical performances of the immunosensor fabricated using the same device structure for detection of another molecule, i.e. acetaminophen (Tertiş et al. 2015), it was found that the proposed immunosensor exhibited approximately 7-fold lower in the detection limit and 136-fold higher in the device sensitivity. Although both devices have different mechanisms in their signal generation, the immunosensor in this study is significantly better and could be applied to determination of other analytes. Furthermore, for the detection of interleukin-15, it needs the further development in order to operate at lower detection limit and higher sensitivity, preferably as good as those of the commercial ELISA kit in order to investigate the crucial levels or changes of interleukin-15 during the early HIV-induced cytokine storm (Stacey et al. 2009) or during its restrictive production observed in AIDS stages (d'Ettorre et al. 2006). Its performances could be improved by incorporation with high surface area nanomaterials, which facilitate electrochemical process at the electrode surface (Samanman et al. 2015).

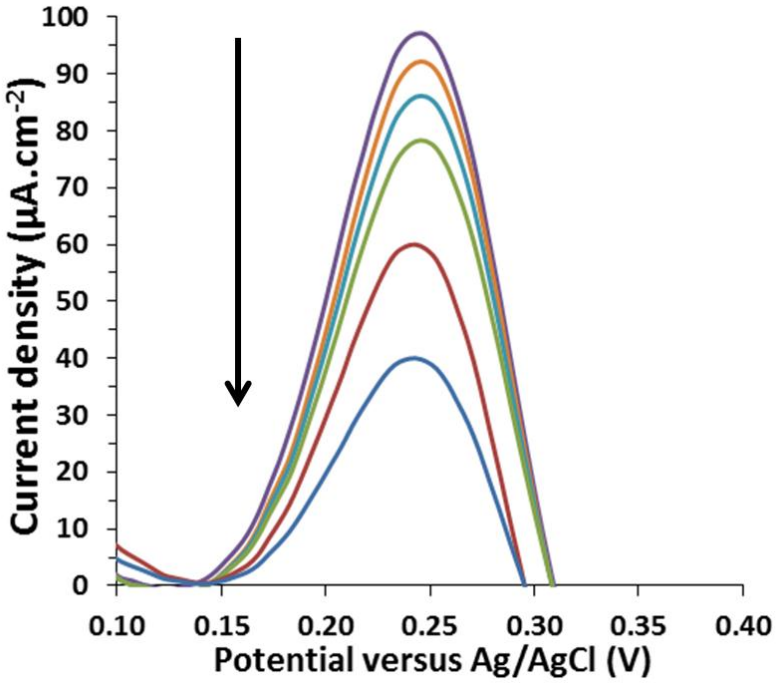

Figure 9 Differential pulse voltammetric responses of $[Fe(CN)_6]^{4-/3-}$ process at the immunosensing electrode after incubation with different concentrations of interleukin-15: 0, 5, 10, 25, 50, 100 $ng.mL^{-1}$.

6.5 Selectivity and Interference Study

The selectivity of the immunosensors was also tested as shown in Figure 10. In real applications, the determination of the biomarkers or human interleukin-15 with no sample separation (in order to remove the interferences) is greatly important to reach fast sample analysis. The electrochemical responses of the immunosensors towards nonspecific adsorptions of the interferences such as uric acid, ascorbic acid, bovine serum albumin and glucose were investigated. The immunosensor was incubated in 50 $ng.mL^{-1}$ interleukin-15 containing high concentration of the potential coexisted species (0.10 mM) or 0.1 $mg.mL^{-1}$ BSA. It was found that these possible interferences at the high concentration had almost no significant influence

on the detection of interleukin-15. The results suggested that the immunosensor displayed a good specificity for the determination of interleukin-15. The variation of the response due to the interferences was less than 5.7% of current response in no interferences, suggesting that the selectivity of the immunosensors was acceptable.

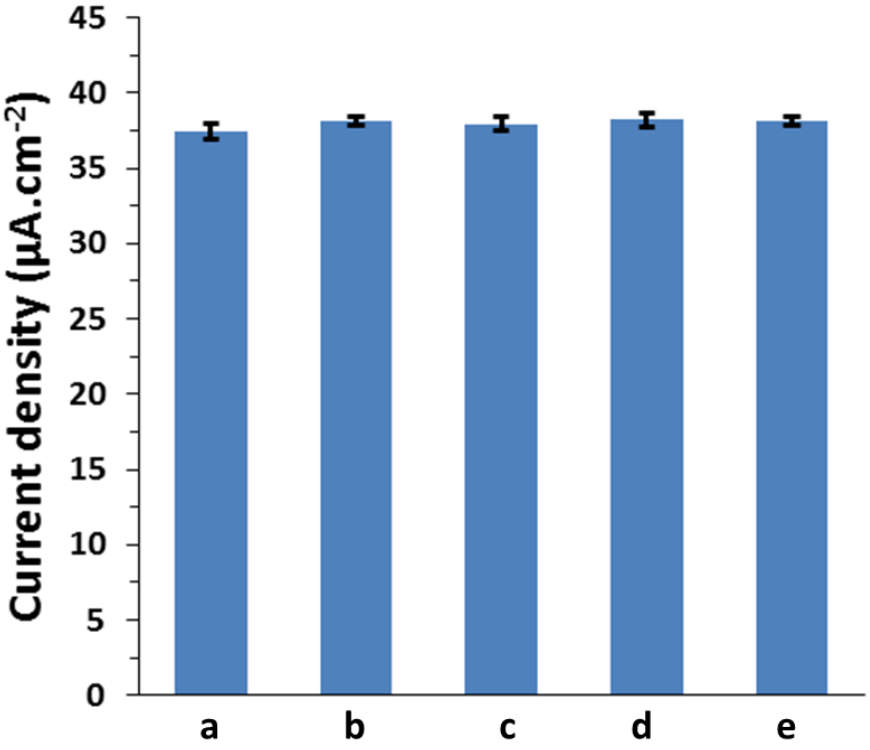

Figure 10 Differential pulse voltammetric responses of $[Fe(CN)_6]^{4-/3-}$ process at the immunosensor after incubation with (a) 50 ng.mL^{-1} interleukin-15, (b) 50 ng.mL^{-1} interleukin-15 + 0.10 mM uric acid, (c) 50 ng.mL^{-1} interleukin-15 + 0.10 mM ascorbic acid, (d) 50 ng.mL^{-1} interleukin-15 + 0.10 mg.mL^{-1} bovine serum albumin, (e) 50 ng.mL^{-1} interleukin-15 + 0.10 mM glucose.

7. Conclusions

A simple, label-free and cost-effective electrochemical immunosensor based on graphene oxide film-modified screen-printed carbon electrode for the detection of interleukin-15 was reported for the first time. The electroactivity of the screen-printed carbon electrode was greatly improved by the carboxylic group-containing graphene oxide. The simple immunosensor showed a satisfactory detection limit of 3.51 $ng.mL^{-1}$ and a sensitivity of 0.5655 $\mu A.cm^{-2}.mL.ng^{-1}$. Moreover, the immunosensor exhibited a good selectivity and stability. The proposed immunosensor would thus be applied as a promising candidate for the determination of interleukin-15 in real samples. However, this preliminary research for immunosensing of interleukin-15 requires the further development in order to obtain better device performances such as higher sensitivity and lower limit of detection in trace amount, which will ultimately be beneficial for the determination of HIV-1 acute infection and pathogenesis.

8. References

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9. Appendix

1. publications from this project

Chamari Pothipor, Nawee Kungwan, Jaroon Jakmunee, Kontad Ounnunkad*, A Disposable and Flexible Graphene Electrode Fabricated by Inkjet Printing of an Aqueous Surfactant-free Graphene Oxide Dispersion, Chemistry Letters 44[6] (2015) 800–802, IF = 1.550 (2015)

Watthanachai Jumpathong, Jaroon Jakmunee, Kontad Ounnunkad*, A Sensitive and Disposable Graphene Oxide Electrochemical Immunosensor for Label-free Detection of Human Immunoglobulin G, Analytical Sciences 32 (2016) 323-328, IF = 1.174 (2015)

Poachanee Norfun, Watthanachai Jumpathong, Nawee Kungwan, Jaroon Jakmunee, Kontad Ounnunkad*, Electroanalytical Application of Screen-printed Carbon Electrode Modified with Conductive Graphene Oxide Poly(acrylic acid) Film for Label-free Detection of Human Immunoglobulin G, Chemistry Letters 45[12] (2016) 1444–1446, IF = 1.550 (2015)

Poachanee Norfun, Nuttee Suree, Nawee Kungwan, Winita Punyodom, Jaroon Jakmunee, Kontad Ounnunkad*, Electrochemical Detection of Human Interleukin-15 Using a Graphene Oxide-Modified Screen-Printed Carbon Electrode, Analytical Letters 50[7] (2017) 1-14, IF = 1.088 (2015)