Chiang Mai J. Sci. 2020; 47(3) : 530-541
http://epg.science.cmu.ac.th/ejournal/
Contributed Paper

A Poly(pyrrole-3-carboxylic acid) Thin Film Modified Screen Printed Carbon Electrode as Highly Sensitive and Selective Label-free Electrochemical Immunosensing Platform

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Received: 23 July 2019
Revised: 8 October 2019
Accepted: 29 October 2019

Abstract

Giving good stability and sensitivity of the low-cost electrochemical immunosensor is achieved using a conducting poly(pyrrole-3-carboxylic acid) (PP3C) film on a screen-printed carbon electrode (SPCE). The PP3C film offers high density surface carboxylic groups, on which can be covalently conjugated with capturing antibodies, and improves electrode reactivity. Our simple immunosensor exhibitessatisfactory sensitivity, selectivity, stability, reproducibility, and regenerability for the detection of human immunoglobulin G (IgG). The proposed sensitive immunosensor provides a detection limit of 0.120 ng mL^{-1} and a sensitivity of 3.61 μA cm^{-2} mL ng^{-1}, which thus is promising for clinical applications.

Keywords: poly(pyrrole-3-carboxylic acid), immunosensor, screen-printed carbon electrode, label-free detection, immunoglobulin G

1. Introduction

Clinical diagnosis for the prevention and proper treatment of diseases and virus infections requires fast analytical procedure and its accuracy [1, 2]. There are many diagnosis tools for assays of the potentially clinical biomarkers such as electrochemical immunosensors [3, 4], electrochemiluminescence immunosensor [5], and colorimetric immunosensor [6]. Because of the high sensitivity, low detection limit, and simplicity, electrochemical analytical devices have been paid considerable attention [1, 2, 7]. In recent decades, screen-printed electrodes based carbon materials have been commonly used for various electrochemical detections [8-11]. Screen-printed carbon electrodes (SPCEs) offer a low background current, a readily renewable surface, and a wide potential window [8, 9]. They are relatively inexpensive, versatile, and chemically inert [8, 9]. However, the development of sensors based on SPCEs has been limited by their low electroreactivity, low sensitivity, and

high limits of detections (LODs). To avoid these drawbacks of the SPCEs, the enhancement of their electroactivity is required. This has used the modification with active nanomaterials such as nanocarbons and conducting polymers [10, 11].

Electrochemical immunosensors have been used to specifically measure biological substances or biomarkers elevated by the diseases and virus infections [1, 2] Immunosensors generally need signal amplification in order to generate the analytical response, monitoring the specific reaction of antigen-antibody pair. Since a multistep procedure is no longer required in terms of both time and chemical consuming, label-free immunosensors can directly detect the change of electrochemical signal resulted from the blockage of redox process at the electrode surface due to different surface coverage by non-conducting molecules [10, 11]. Moreover, the immunosensors based on SPCEs have been developed by modification with a wide range of the high-surface-area electroactive nanoparticles, coupled with antibodies [10]. In our study, we employed human immunoglobulin G (IgG) as a biomarker model to develop the immunosensors without the labeling step. Human IgG, the most abundant type of immunoglobulin antibodies, is an important component in the immune system that protects against bacterial and viral infections for our body [10, 11]. IgG is found in blood of normal adults at the concentration range of 9.40-15.10 mg mL^{-1} [10]. IgG can identify and form a compound with antigen specifically, which then the swallowed compound by the phagocytes would lose toxicity and pathogenicity. Moreover, the determination of its level can be used as the biomarker of many diseases such as Alzheimer's disease, inflammatory bowel disease, autoimmune disease, liver disease, cancers, and infectious diseases [10]. Thus, the human IgG assay in human body is necessary in clinical diagnosis.

With no tagging, the electrochemical immunosensor constructed in the study was developed to specifically detect human IgG, which would provide an accessible method for assaying the IgG concentration in human fluids. Here, the study of analytical performances and fabrication of a simple, disposable, and cost-effective immunosensor based on a poly(pyrrole-3-carboxylic acid) (PP3C)-modified SPCE for the detection of IgG is reported. We employed PP3C to modify the SPCE surface for enhancement of the electrode reactivity and covalent binding of anti-human IgG via an amide linkage, giving stability of the measurement. PP3C is one of the conducting polypyrrole derivatives, which possesses high density surface carboxyl groups [12], thus offering high loading of anti-IgG. This would result in high sensitivity and selectivity of the immunosensors. PP3C has been widely employed in developing many sensors such as chemical sensor [13, 14], plasmonic immunosensors [12, 15], impedance immunosensor [16] and electrochemical-surface plasmon dual biosensor [17]. PP3C offers high catalytic activity giving satisfactory selectivity in the chemical detection [13, 14] while using PP3C as a platform provides great performances in the immunosensing detection which the sensitivity of the assay can be electrochemically tunable [12, 15] and good reproducibility and excellent stability can be achieved [16]. In our study, the label-free immunosensor based on PP3C film preliminarily revealed satisfactory properties including acceptable sensitivity, selectivity, reproductivity, linearity, regenerability, detection limit, and stability. The simple PP3C-modified SPCE is thus promising for further development of more sophisticated immunosensors.

2. MATERIALS AND METHODS

2.1 Chemicals and Materials

All chemicals used in this study are of analytical grade. Potassium hexacyanoferrate(II) trihydrate ($K_4[Fe(CN)_6]\cdot 3H_2O$), pyrrole-3-carboxylic acid (P3C, ≥ 96%), anti-human immunoglobulin G (Anti-IgG), human immunoglobulin G (IgG), phosphate buffered saline tablets (PBS, pH 7.4), bovine serum albumin (BSA), myoglobin from human heart, interleukin-15 (IL-15), L-cysteine,

ascorbic acid (AA), uric acid (UA) ,and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich, USA. Sulfuric acid (H_2SO_4) and potassium ferricyanide ($K_3[Fe(CN)_6]$) were ordered from Lab Scan, Poland. An Acheson Electrodag PR-406 carbon ink was purchased from Henkel, USA. 1-Ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were obtained from Merck, Germany. All aqueous solutions were prepared using a Milli-Q water. An Ag/AgCl (3.0 M NaCl) reference electrode was bought from BASi, USA and platinum (Pt) wire was ordered from the Nilaco Corporation, Japan. SPCEs were in-house prepared according to our process [10, 11].

2.2 Instrumentation

SPCEs used for fabrication of the immunosensor were treated in a plasma cleaner (PDC-32G, Harrick Plasma, USA). Differential pulse voltammetry (DPV) and cyclic voltammetry (CV) were carried using a conventional three-electrode electrochemical cell connected with the μAutolab type II Potentiostat/Galvanostat while electrochemical impedance spectroscopy (EIS) was performed using an electrochemical impedance analyzer (Z100 EDAQ potentiostat, Australia). An Ag/AgCl reference electrode, Pt wire counter electrode, and SPCEs were used for the electrochemical studies. The morphologies of samples were studied using a scanning electron microscope (SEM, JOEL, JSM-6335F).

2.3 Immunosensor Fabrication and Assay

Our immunosensor was constructed as follows. Firstly, the SPCEs were in-house fabricated employing a carbon ink and a poly(vinyl chloride) sheet was used as a substrate [11]. Before use, SPCEs were treated in plasma cleaner under an optimum condition for 60 s [11]. PP3C film was then deposited onto a treated SPCE by the electropolymerization of pyrrole-3-carboxylic acid (P3C) in an acidic solution of H_2SO_4 [12]. CV was employed for the polymerization using the conventional three-electrode electrochemical cell composed of Ag/AgCl reference, Pt wire counter, and screen-printed electrodes. The construction steps of immunosensor are presented in Figure 1. Briefly, poly(pyrrole-3-carboxylic acid)(PP3C) modified SPCEs were incubated with a coupling reagent consisting of 0.40 M EDC and 0.10 M NHS (a ratio of 1:1 v/v). After washing with Milli-Q water several times, anti-IgG antibody solution was dropped onto the treated electrode surface and then the surface was rinsed with Milli-Q water several times. To quantitate the IgG concentration, the responses of 5.0 mM $[Fe(CN)_6]^{4-/3-}$ (1:1 molar ratio) solution, prepared from $K_4[Fe(CN)_6]\cdot 3H_2O$ and $K_3[Fe(CN)_6]$, were recorded at the immunosensing electrode after incubation with different concentrations of human IgG. For detection of IgG in human serum, standard IgG solutions with different concentrations containing 2-fold diluted human serum were prepared for assay and % recovery study. The solutions were prepared by mixing PBS containing IgG and as-achieved human serum (1:1 volume ratio). For the assay, 2-fold diluted human serums with and without added IgG were incubated on the immunosensing electrodes and then the blockage of the redox process was studied. The 2-fold diluted human serum was also used as a blank.

3. Results and Discussion

3.1 Properties of PP3C-modified SPCEs

Figure 2(a) shows the typical electropolymerization profile of 0.10 M P3C in 0.50 M H_2SO_4 solution onto a treated SPCE at a scan rate of 20 mV s^{-1} [12]. The polymerization using CV was performed by a scan potential range of 0.0-1.0 V. With increasing the number of polymerization cycles, the oxidation current is decreased due to the reduced P3C concentration under the polymerization [12]. The number of electropolymerization cycles was optimized to find the best redox response, which was assessed by DPV of 5.0 mM $[Fe(CN)_6]^{4-/3-}$ solution containing 0.01 M PBS. Figure 2(b) shows

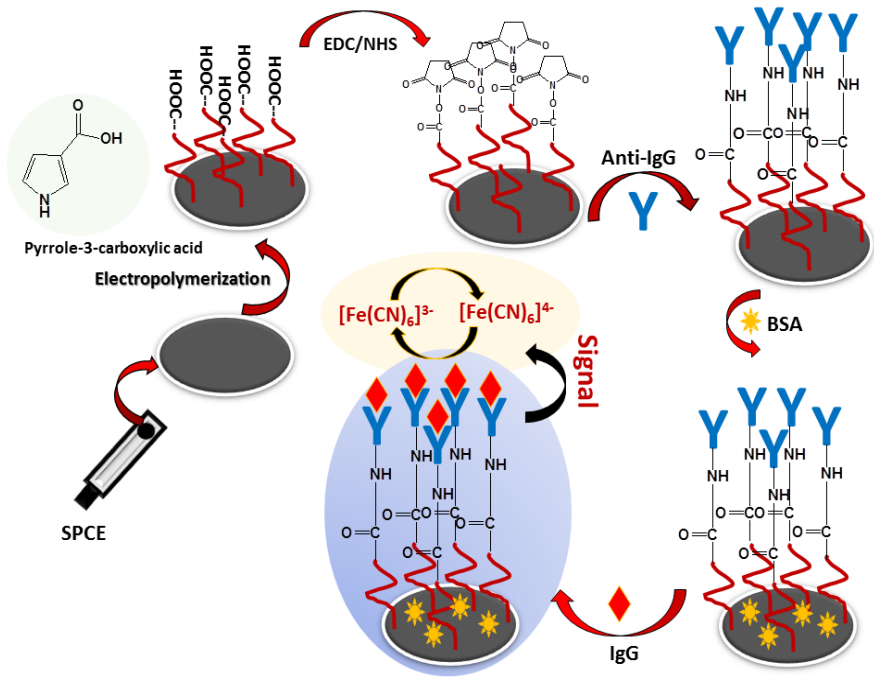

Figure 1. Schematic diagram of the label-free immunosensor based on a PP3C thin film.

DPV responses of the $[Fe(CN)_6]^{4-/3-}$ process (1:1 molar ratio) at PP3C film-modified SPCEs with different numbers of the polymerization cycles. The DPV current was increased with increasing the polymerization cycle, reached a highest current at 10 polymerization cycles, and then reduced. At 20 polymerization cycles, the current response of the $[Fe(CN)_6]^{4-/3-}$ process at PP3C-modified electrode was lower than that of bare SPCE due to low reactivity of highly carboxylated PP3C. At 10 polymerization cycles, the current was improved about 85.18%, which the PP3C film would be expected to give the best sensitivity of the immunosensor. The polymerization with 10 cycles was chosen for further construction of immunosensor. The electrochemical property of the optimized PP3C film is investigated using CVs of $[Fe(CN)_6]^{4-/3-}$ process at different scan rates (Figures 2(c) and 2(d)). It is found that the anodic and cathodic peak currents are observed as functions of square root of the scan rate, which show linearity. This suggests that the mechanism of redox probe at the electrode is diffusion-controlled and the PP3C film had a sufficient electrochemical reactivity for the device development.

In addition, EIS spectra of bare and PP3C film-modified SPCEs were recorded in in 5.0 mM $[Fe(CN)_6]^{4-/3-}$ solution as shown in Figure 3. The semicircle of SPCE is reduced due to deposited PP3C film (10 cycles). This suggests that the electron transfer at electrode is improved with the polymer film. Therefore, the PP3C film-modified SPCE is expected to give the good properties of the immunosensor. The morphology of PP3C film was studied using a SEM as shown in Figure 4. Bare SPCE (Figure 4(a)) revealed agglomerate carbon particles on the surface while smooth surface was observed on PP3C film-modified SPCE (Figure 4(b)), indicating PP3C film covering the SPCE surface. Moreover, as shown in Figure 4(c), the reproducibility of the electrodes coated with PP3C film at the optimized condition is examined using DPVs of $[Fe(CN)_6]^{4-/3-}$ process. No significant change in

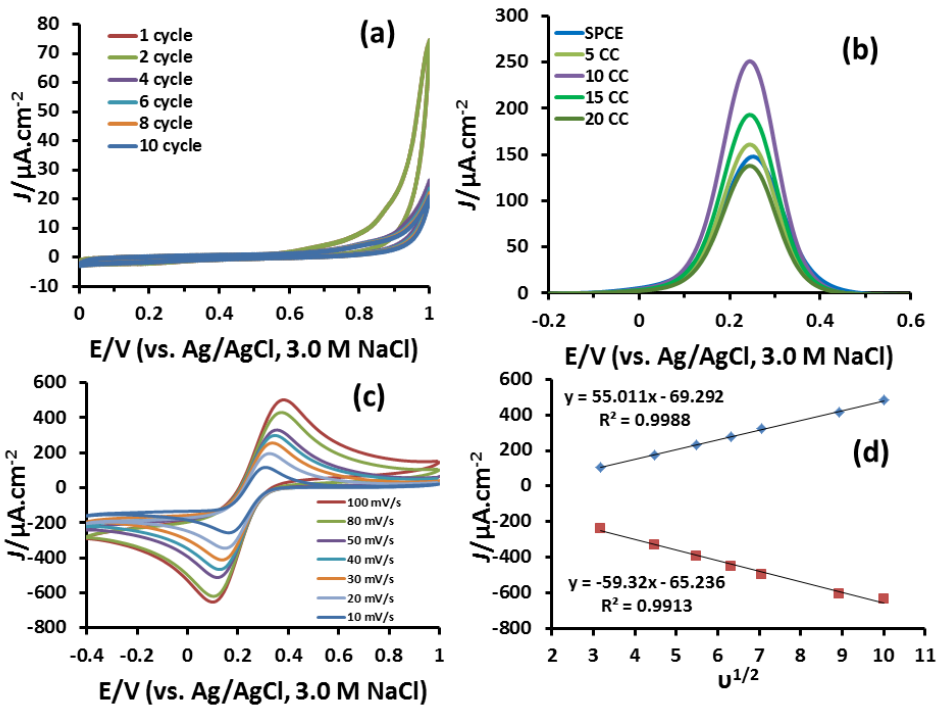

Figure 2. CVs for electropolymerization of 0.10 M P3C in 0.50 M H_2SO_4 onto SPCE at a scan rate of 20 mV s^{-1} (a), DPV responses obtained for $[Fe(CN)_6]^{4-/3-}$ process at SPCEs modified with PP3C films with different numbers of polymerization cycles (b), CVs of $Fe(CN)_6]^{4-/3-}$ process at a PP3C film-modified SPCE (10 polymerization cycles) at different scan rates (c) and plots of anodic and cathodic peak currents versus square root of scan rate (d).

current response for eight individual electrodes was observed. Therefore, our strategy for preparing the film is reproducible (0.44 %RSD).

3.2 Optimization of Experiment Conditions

The immunosensor was fabricated employing the PP3C film-modified SPCE obtained from the study above, regarding to Figure 1. Anti-human IgG was covalently immobilized onto the film by incubation of the electrode with a coupling reagent prepared from EDC and NHS. After the electrode was incubated with each reagent or each chemical for each step of immunosensor fabrication and measurement, the electrode was washed with deionized water several times. The conditions for the attachment of the active proteins, namely anti-IgG on the PP3C film-modified SPCE were optimized to obtain the maximum analytical response. The PP3C film-modified SPCE was incubated with 20 mL of a coupling agent at a temperature of 4°C for 30 min. Consequently, the activated electrode was then incubated with 20 mL of anti-human IgG solution for covalent attachment via amide linkage onto PP3C film. Anti-IgG solution was prepared using phosphate buffered saline (PBS, pH 7.4). The anti-IgG loading onto the electrode was studied, which the higher loading would be expected to offer the higher sensitivity caused from higher amount of the binding sites [11, 18, 19]. At an incubation time of 30 min and the same temperature, the concentration of anti-human IgG used for the incubation is varied from 20 mg mL^{-1} to 100 mg mL^{-1} (Figure 5(a)). It is found that the current is

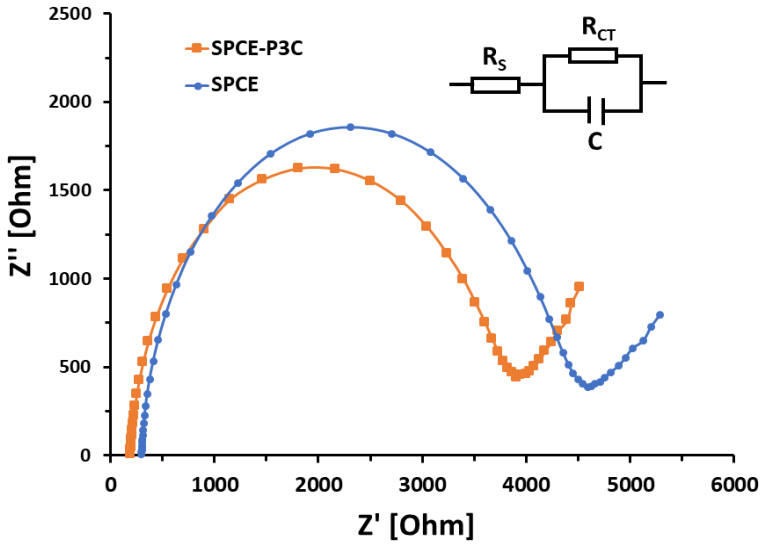

Figure 3. EIS spectra of bare and PP3C-modified SPCEs in 0.010 M phosphate buffer pH 7.4 containing 5.0 mM of $[Fe(CN)_6]^{4-/3-}$ at the frequency range from 0.10 Hz to 10 kHz and amplitude of 0.010 V.

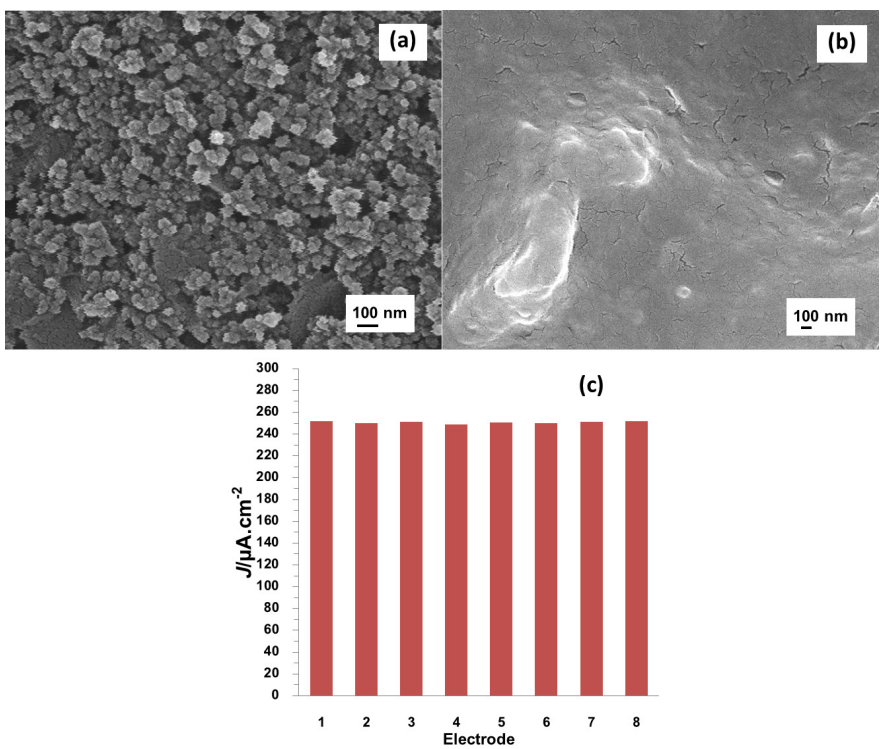

Figure 4. SEM micrographs for bare SPCE (a) and PP3C film-modified SPCE (b) (10 polymerization cycles) and reproducibility of PP3C film-modified SPCE (number of polymerization cycles of 10).

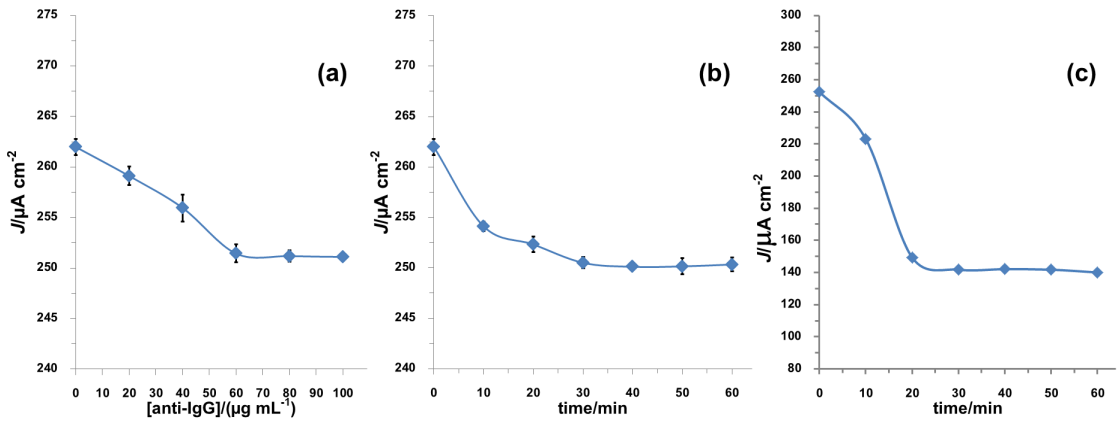

Figure 5. Effects of anti-IgG concentration (a), of incubation time for covalent binding of anti-IgG onto the electrode surface based on PP3C film (b), and of incubation time for capturing human IgG (anti-IgG concentration of 60 mg mL^{-1}) on DPV response for $[Fe(CN)_6]^{4-/3-}$ redox process (c).

reduced due to surface coverage by non-conducting anti-IgG molecules and starts constant at the concentration of 60 mg mL^{-1}. At this point the loading of anti-IgG is saturated, which means the highest loading of anti-IgG and the binding process of anti-IgG antibodies cannot further occur [11, 18]. Therefore, anti-IgG concentration of 60 mg mL^{-1} is chosen for next study. The remaining binding sites of the immunoelectrode were blocked by incubation with 20 mL of 0.5%w/v BSA solution at 4°C for 30 min in order to eliminate nonspecific binding [18]. In addition, the suitable incubation time of anti-IgG binding on the electrode surface is investigated to be 30 min (Figure 5(b)). Therefore, for immobilization of anti-IgG in the subsequent sensing study, the anti-IgG concentration of 60 mg mL^{-1} and the incubation period of 30 min are used for further study. Before capturing target human IgG, the DPV signal of $[Fe(CN)_6]^{4-/3-}$ system at immunoelectrode was recorded and then rinsed with deionized water several times. After the immunoelectrode was incubated with 20 μL of human IgG solution at the certain time, the immunoreaction degree was monitored using the DPV response from a $[Fe(CN)_6]^{4-/3-}$ process at the immunoelectrode surface [11]. The reduction of the current due to more restriction of the heterogeneous redox process by nonconducting human IgG molecules (25.0 ng mL^{-1}) captured on the electrode surface was observed. Figure 5(c) shows the study of incubation time of IgG (ranging 10-60 min), influencing the immunoreaction degree [11, 18]. With increasing the incubation time, the current is decreased until 30 min and then become constant, referring to the complete immunoreaction or reaching the highest immunoreaction degree. This suggests that the incubation period at least 30 min for capturing human IgG by the immunoelectrode is required in order to complete the immunoreaction. The immunoreaction amount with respect to the IgG concentration is also observed, which the reduction of electrical current, as a function of the restriction of the redox process due to surface coverage by the forming immunocomplexes, can be used to quantitate the IgG concentration. A pH value of the working solution is one of the important factors affecting the activity of the immobilized anti-IgG as well as immunosensor performances [11, 18, 19]. As shown in Figure 6(a), the effect of pH on the response of the immunosensor is studied in the working solutions at a pH range of

6.2-7.8. The optimal pH giving highest baseline current, which refers to the proper orientation of the anti-IgG, is 7.4 (Figure 6(a)). It is noted that at this pH, anti-IgG presents ability to capture IgG sufficiently. Thus, pH 7.4 is chosen for subsequent experiments.

3.3 Reusability, Stability and Selectivity

Reusability of the immunosensor was also studied via regeneration of the sensor surface by incubation with 5.0 mM NaOH solution for 2 min. As started from the sensor with captured IgG molecules (25 ng mL^{-1}), the current was reduced from approximately 255 to 145 μA cm^{-2}. The regeneration was tested for five times as shown in Figure 6(b). After treated with the alkaline base solution, the redox response is back to the baseline current (255 μA cm^{-2}) whilst after reformation of immunocomplexes occurred on the regenerated electrode surface, again the redox response is decreased to 145 μA cm^{-2}. Five sets of this study give no significant change in the current density difference (analytical signal). Actually, the regeneration of this sensor can be performed several times and thus the calibration curve can be constructed using an immunoelectrode. Moreover, the immunosensors showed stability with no obvious change in current DPV response for two weeks after the electrodes were stored at 4°C (96% of the initial analytical response). Selectivity is another important factor for evaluating the performance of our immunosensor. Figure 6(c) shows the investigation of nonspecific adsorptions of possible interferences involving redox and non-redox species/proteins such as myoglobin, interleukin-15 (IL-15), BSA, L-cysteine, ascorbic acid (AA), and uric acid (UA). As depicted from Figure 6(c), with the 4000-fold higher concentration for all interferences except myoglobin (4-fold higher), the 21-fold higher analytical signal for IgG detection (25 ng mL^{-1}) is observed as compared to those of others. This indicates that our immunosensor possesses a high selectivity in detecting human IgG.

3.4 Detection of Human Immunoglobulin G

Under the optimized conditions, the performance of the immunosensor was investigated as illustrated in Figure 7. The DPV response decreased gradually with the increase of the concentration of IgG. A good linear relationship between the intensity of current density difference (ΔJ) and the IgG concentration from 0.5 to 50 ng mL^{-1} with a detection limit of 0.120 ng mL^{-1} (S/N = 3) is observed. The linear equation is ΔJ (μA cm^{-2}) = 3.6061[IgG] (ng mL^{-1}) + 7.8096 (Figure 7(b)) and the sensitivity was 3.6061 μA cm^{-2} mL ng^{-1}. Therefore, the immunosensor fabricated using the PP3C-modified SPCE offers its simplicity, low cost, and consumption of few chemicals, which is sufficient for detection of IgG at physiological level. Compared with previous reports illustrated in Table 1, the proposed immunosensor based on the PP3C film shows a satisfactory detection range and detection limit. Although Entries 1, 3, and 4 provide low limits of detection, they consume the costly chemicals and complex molecules. Moreover, they also consume costly noble metals and Entries 3 and 4 reveal narrow dynamic ranges. Our immunosensor based on the PP3C film-modified disposable SPCE shows the comparable performances with Entry 5 whilst Entries 6, 7, and 8 show higher limits of detection and wider dynamic ranges. As compared to the strategy of Entry 7, there is more complexity in this assay. Although above three studies reveal wider calibration ranges, our strategy is acceptable for detection of IgG in normal and cut-off levels in human body. Although Entry 9 successfully demonstrates the application of microfluidic system to electrochemical immunosensor, it presents wider dynamic range and higher detection limit (*ca.* 14 times). Entry 10 demonstrates use of a polyaniline derivative containing amino group and shows comparable performances with those of our proposed immunosensor. Entry 10 shows a linear dynamic range versus log [IgG] whilst the present work shows a linear dynamic range versus [IgG]. Furthermore, to validate the reliability and

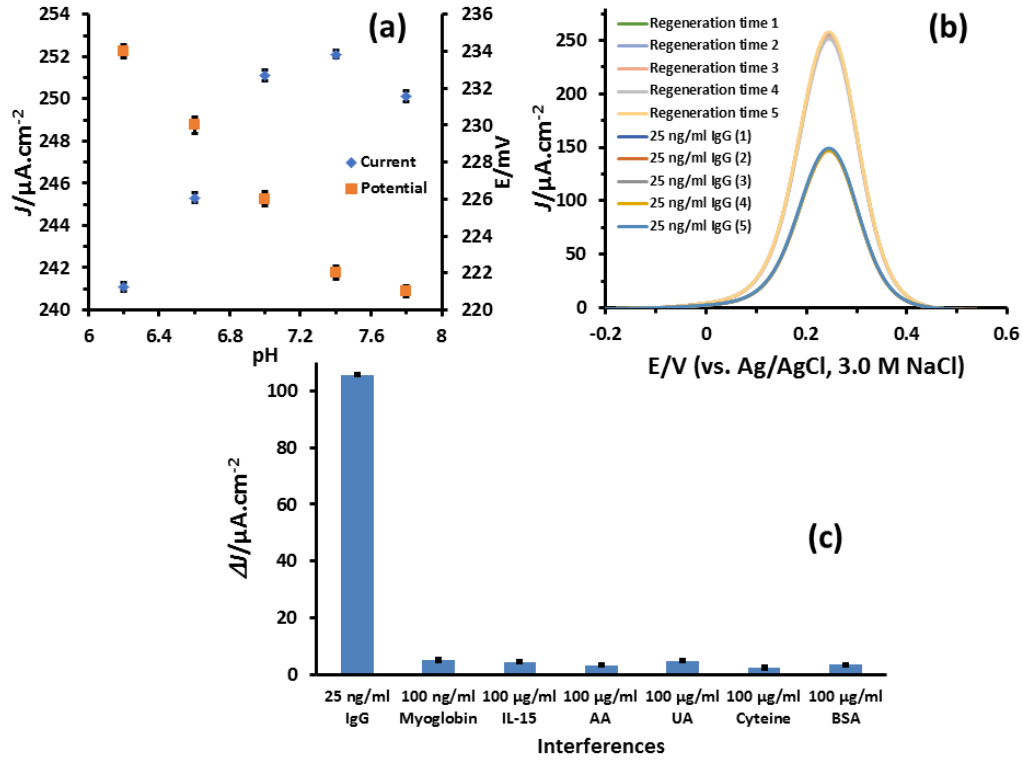

Figure 6. effect of pH on the DPV redox response at the immunoelectrode (a), regeneration of the immunosensor: recorded redox responses at the immunoelectrode after human IgG removal with 5.0 mM NaOH solution for 2 min (higher current) and human IgG rebinding (lower current) (b), and interference study of immunosensor based on PP3C film-modified SPCE (c).

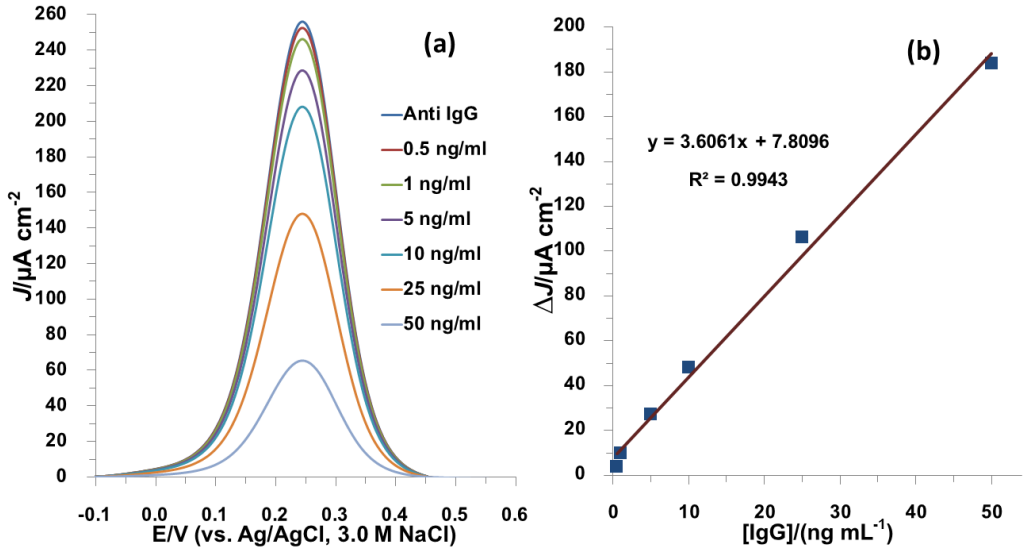

Figure 7. DPV responses for the different concentrations of human IgG (a) and calibration curve (b).

Table 1. Comparison of the analytical performances of proposed electrochemical immunosensor with other IgG immunosensors.

Immunosensor	Method	Linear range (ng mL^{-1})	Detection limit (ng mL^{-1})	Ref.
anti-IgG/AMPPH-AuNPs/GCE	DPV	0.1-5, 5-100	0.08	[20]
anti-IgG/AuNPs/PTH-MB/AuE	DPV	10-10000	3	[21]
anti-IgG/NH_2-CNFs-PAMAM/GCE	LSV	0.000001-1.0	0.0000005	[22]
anti-IgG/rGO-MWCNT-Pd/GCE	SWV	0.01-25	0.0033	[19]
anti-IgG/AuNPs/L-cysteine/AuE	DPV	0.82 - 90	0.25	[23]
anti-HIgG/PANI NNWs	CON	5-100, 100-3000	3	[24]
protein A/AuE	EIS	10 -1000	5	[25]
anti-IgG/COOH–MWCNT/Fe_3O_4/AuE	CV	30 - 1000	25	[26]
anti-IgG/GO/SPCE	SIA-amp	2-100	1.7	[10]
anti-IgG/P2ABA/SPCE	DPV	1.5-50	0.15	[11]
anti-IgG/PP3C/SPCE	DPV	0.50-50	0.12	This work

anti-immunoglobulin G antibody (anti-IgG), 4-amino-1-(3-mercapto-propyl)-pyridine hexafluorophosphate (AMPPH), gold nanoparticles (AuNPs), methylene blue (MB), polythionine (PTH), amine-terminated carbon nanofibers-polyamidoamine dendrimer (NH2-CNFs-PAMAM), reduced graphene oxide (rGO), polyaniline (PANI), nanowires (NNWs), multiwalled carbon nanotube (MWCNT), glassy carbon electrode (GCE), gold electrode (AuE), differential pulse voltammetry (DPV), electrochemical impedance spectroscopy (EIS), square wave voltammetry (SWV), cyclic voltammetry (CV), conductivity measurement (CON), linear sweep voltammetry (LSV), sequential injection analysis-amperometry (SIA-amp), poly(2-aminobenzylamine) (P2ABA), poly(pyrrole-3-carboxylic acid) (PP3C), screen-printed carbon electrode (SPCE).

Table 2. Recovery study of the detection of human IgG in human serum by our proposed immunosensor.

Added (ng mL^{-1})	Found (ng mL^{-1})	% Recovery	% RSD
1.0	1.03 ± 0.02	103.0	1.55
5.0	5.02 ± 0.15	100.4	3.05
10	10.10 ± 0.24	101.0	2.40
25	25.05 ± 0.47	100.2	1.87

accuracy of our proposed strategy, the detection of human IgG spiked in a 2-fold diluted human serum is demonstrated using our immunosensor. As seen in Table 2, the assays in four spiked samples are investigated. Satisfied recoveries (100.4-103.0%) are observed, indicating good reliability and applicability of the immunosensor. As a result, our immunosensor is a promising candidate in the clinical diagnosis application and can be further developed for others in detection of other proteins.

4. Conclusions

A simple, sensitive and cost-effective electrochemical immunosensor based on electrochemically synthesized PP3C film-modified SPCE for the detection of human IgG has been successfully demonstrated without the labeling step.

PP3C film used for the surface modification gave rise to the great improvement in the electroactivity of SPCE and the effective anchoring of the anti-IgG antibodies by covalent bond, resulting in acceptable stability. The immunosensor showed a satisfactory detection limit of 0.120 ng mL^{-1}, a dynamic range of 0.50-50 ng mL^{-1}, and a good sensitivity of 3.6061 μA cm^{-2} mL ng^{-1}. Moreover, the immunosensor exhibited good selectivity. The reported immunosensor is therefore a promising candidate for the determination of human IgG in real samples for diseases and infection diagnosis and pathogenesis.

Acknowledgements

The present work is supported by Department of Chemistry, Graduate School and Center of Excellence for Innovation in Chemistry (PERCH-CIC), Siam-Franco Mobility Project 2016-2017, Research Center on Chemistry for Development of Health Promoting Products from Northern Resources, Chiang Mai University. We also wish to thank Center of Excellence in Materials Science and Technology, Chiang Mai University for the support under the administration of Materials Science Research Center, Faculty of Science, Chiang Mai University.

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