CHAPTER 3

Results and Discussion

3.1 Characterization of the electrodes

3.1.1 Effect of material on electrode

Response of the electrodes based on the electrochemical reaction of $[Fe(CN)_6]^{3-/4-}$ solution were studied by cyclic voltammetry. Potential in the range of -1.0 to +1.0 V was applied to the electrode at a scan rate of 0.05 V min^{-1} and the obtained results are illustrated in Figure 3.1a. All of the electrodes can response to $[Fe(CN)_6]^{3-/4-}$ probe and show the anodic-cathodic peak currents. GO/SPCE provided higher peak current than bare SPCE because GO present a high specific surface area while anti-HIgG and HIgG are macro-molecules and they can inhibit mobility of redox probe toward the surface of the electrode so anti-HIgG/GO/SPCE and HIgG/anti-HIgG/GO/SPCE had lower peak currents than GO/SPCE, respectively [Zhang, 2016]. Thus, the results confirmed the immunointeraction between HIgG and anti-HIgG and anti-HIgG/GO/SPCE can be used as an immunosensing electrode for determination of HIgG. Fig. 3.1b shows linear relationship between the reduction-oxidation current and the square root of scan rate in the range of 10-100 mV s^{-1}, indicating that the electron transfer on the electrode was diffusion controlled process.

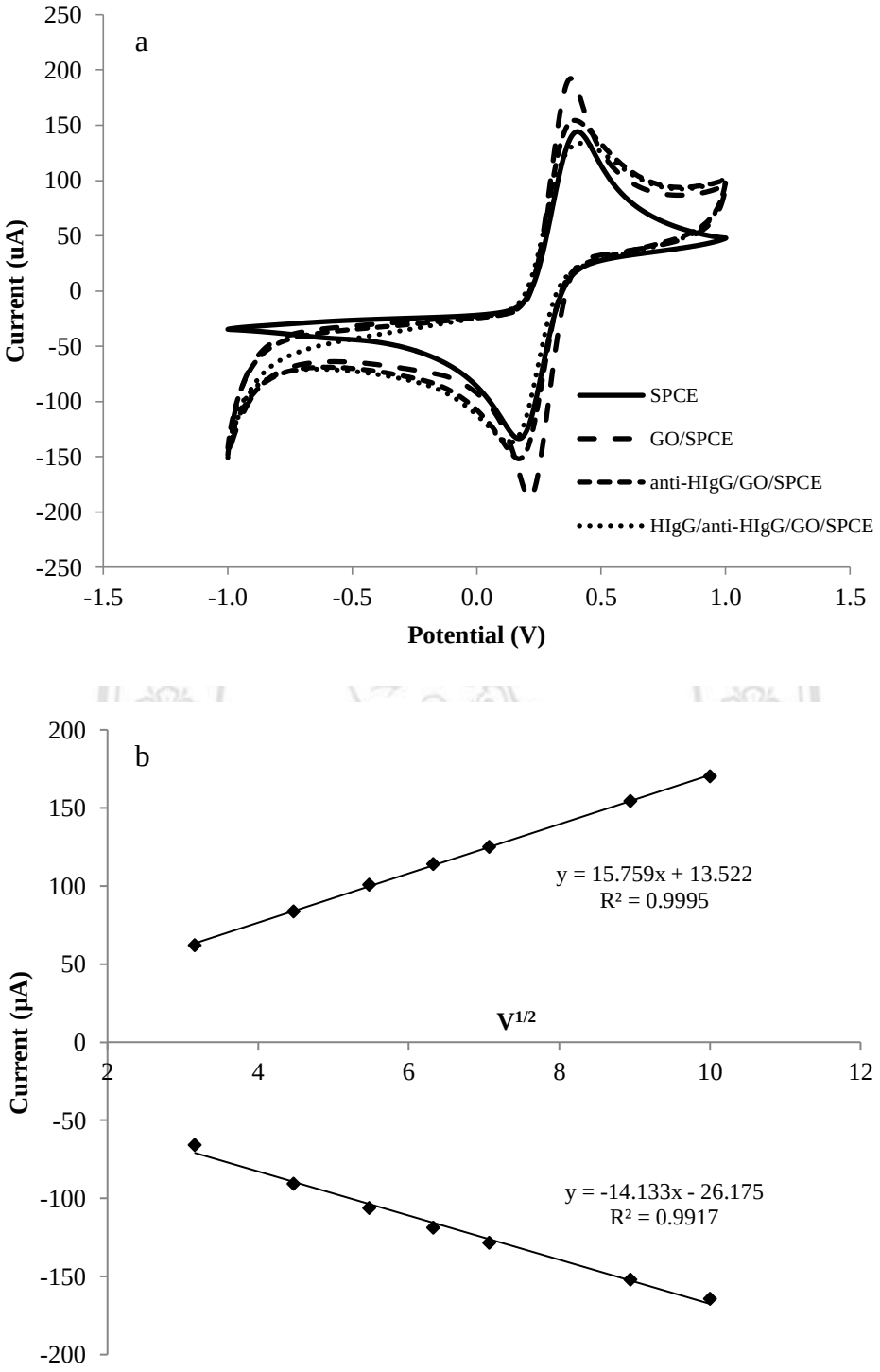

Figure 3.1 Cyclic voltammograms of 10 mM $[Fe(CN)_6]^{3-/4-}$ on various electrodes (a), the plot between the reduction/oxidation currents and the square root of scan rate in the range of 10-100 mV s^{-1} (b) of anti-HIgG/GO/SPCE.

3.1.2 Electrode morphologies

The scanning electron microscope was used to characterize the morphology of bare SPCE and the modified SPCE. The SEM image of bare SPCE (Figure 3.2a) indicated that the SPCE had a uniform surface. GO deposited on SPCE was seen in Figure 3.2b as a sheet shape covered the SPCE surface. The electrodes immobilized with anti-HIgG before and after binding with HIgG are illustrated in Figure 3.2c and Figure 3.2d, respectively. SEM images of Figure 3.2c and 3.2d showed bigger cluster than that in Fig. 3b because proteins on the surface is a macro biomolecule. In addition, the results of energy dispersive spectroscopy (EDS) as shown in Figure 3.2e - Figure 3.2g indicated the present of proteins (anti-HIgG and HIgG). From the result, the electrodes before and after immunointeraction with HIgG provided 6.10% and 7.83% of nitrogen element, respectively, while there is no nitrogen element present in GO/SPCE. Therefore the results confirmed that GO/SPCE was immobilized with anti-HIgG and binding of anti-HIgG/GO/SPCE with HIgG caused the increase in percentage of nitrogen element on the electrode.

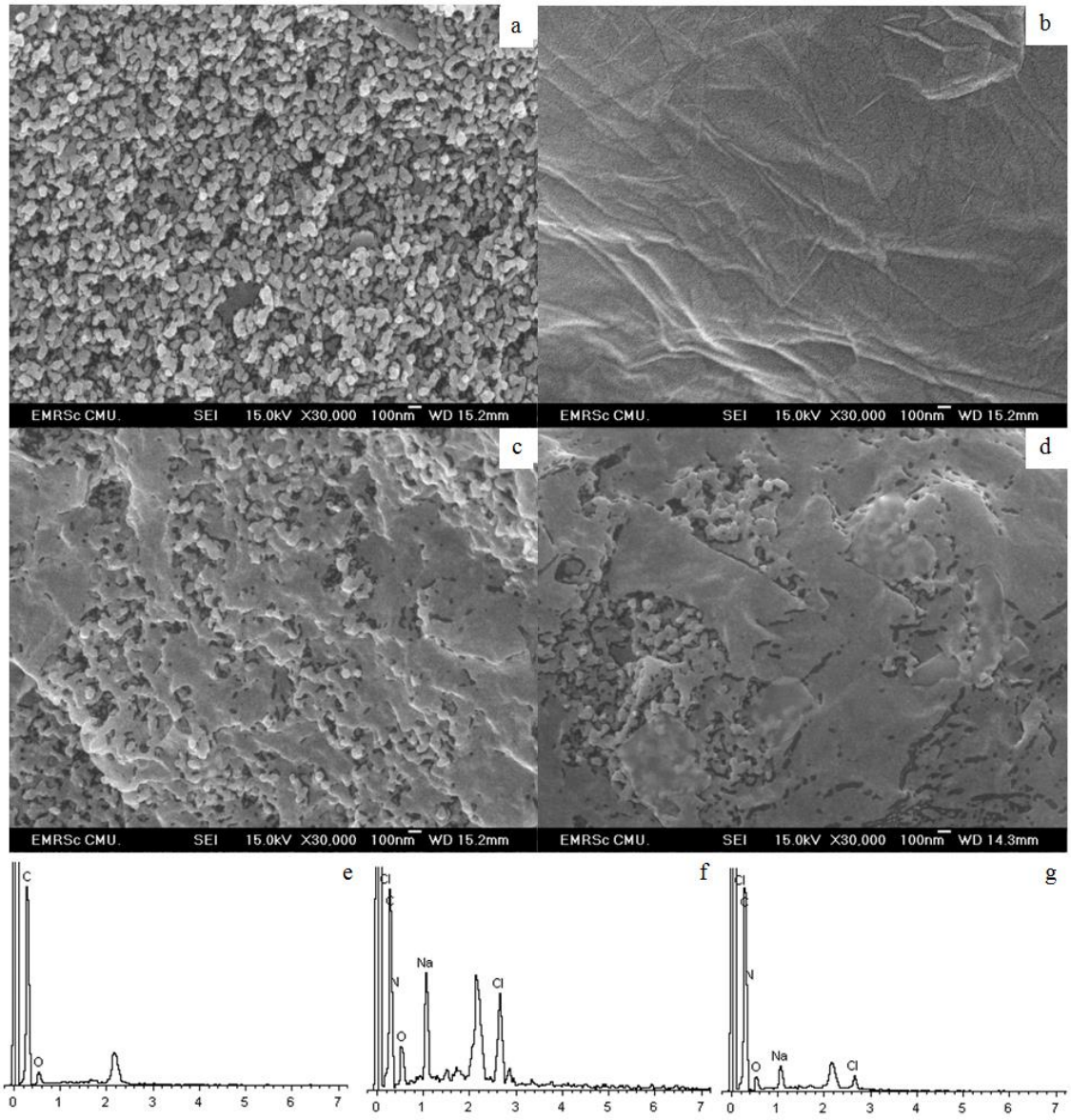

Figure 3.2 SEM images of SPCE (a), GO/SPCE (b), anti-HIgG/GO/SPCE (c) and HIgG/anti-HIgG/ GO/SPCE (d) and EDS spectra of GO/SPCE (e), anti-HIgG/GO/ SPCE (f) and HIgG/anti-HIgG/GO/SPCE (g).

3.2 Optimization of SIA system

The immunosensing electrode was prepared as described in section 2.2, and then utilized in a SIA-amperometric system. Various parameters of SIA system affecting sensitivity and precision of the method were studied.

3.2.1 Effect of flow rate

Flow rate controls dispersion of the solution zone therefore affect to shape of amperometric current signal such as peak height and peak area. Flow rate can be precisely controlled by a syringe pump that flow sample/reagent through the electrode surface. The dispersion of the sample zone is high at the very low or very high flow rate [Ruzicka, 1998]. In this experiment, flow rate in the range of 1-5 mL min^{-1} was studied. Signal profiles are shown in Appendix B. Peak heights obtained before and after immunointeraction were used to calculate decreasing current percentages. The comparison of results was presented in term of decreasing current percentage after immunointeraction occurred that shown in Figure 3.3. The results show that flow rate of 2 mL min^{-1} provide the highest change thus, flow rate of 2 mL min^{-1} was selected to study next parameter.

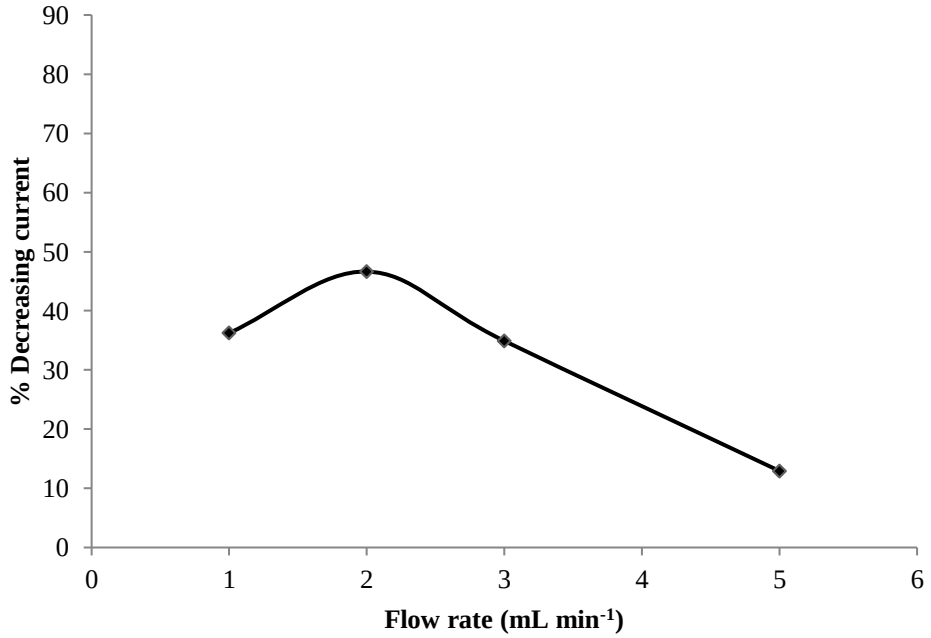

Figure 3.3 Effect of flow rate on 350 mV of applied potential, 10 mM of $[Fe(CN)_6]^{3-/4-}$ and 30 min of immunointeraction time at 4˚C.

3.2.2 Effect of applied potential

In this research, a home-made amperometer was used as a detector. A constant potential was applied to the working with respect to a Ag/AgCl reference electrode and the current flow between working and auxiliary electrode was measured. The applied potential affect to the reduction reaction of redox probes on the electrode surface because each substrate has specific potential of the reaction. The range of applied potential that selected from part of characterization of electrodes after immunointeraction was investigated between +300 and +500 mV. From the cyclic voltammogram of HIgG/anti-HIgG/GO/SPCE, it was found that the highest current was obtained at +362 mV but the applied potential that provided the highest current decreasing percentage was at +350 mV as shown in Figure 3.4 (The corresponded signal profiles are summarized in Appendix B). Moreover, the current gently reduced and invariable when potential was higher because of the background current of the electrode. Thus, applied potential of 350 mV was selected.

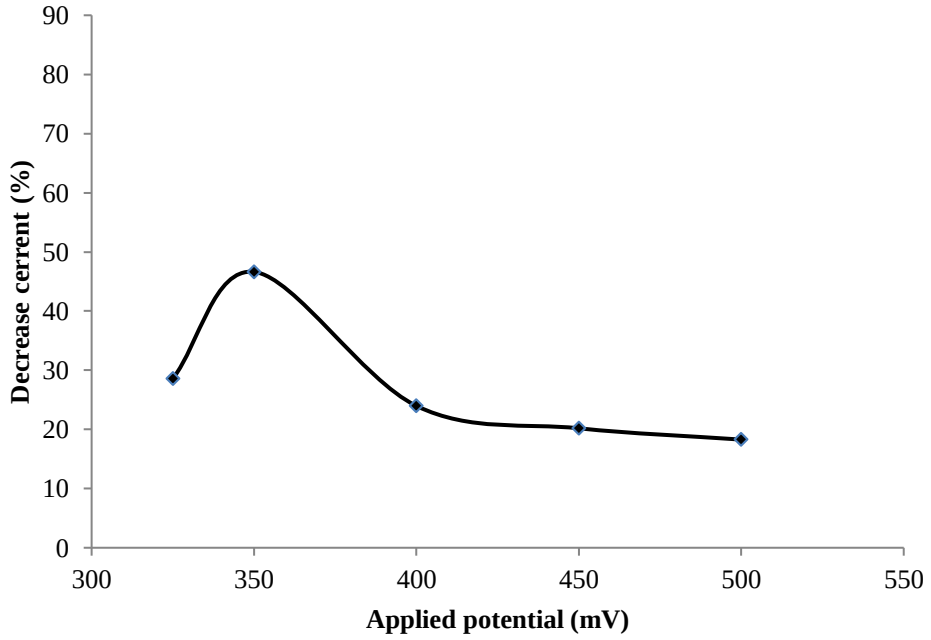

Figure 3.4 Effect of applied potential on 2 mL min^{-1} of flow rate, 10 mM of $[Fe(CN)_6]^{3-/4-}$ and 30 min of immunointeraction time at 4˚C.

3.2.3 Effect of concentration of $[Fe(CN)_6]^{3-/4-}$

This research used $[Fe(CN)_6]^{3-/4-}$ as a redox probe because the immuno-intereaction between anti-HIgG and HIgG did not produce any electroactive product but it can decrease electroactive site on the electrode surface that will reduce the electrochemical current of the redox probe. Moreover, $[Fe(CN)_6]^{3-/4-}$, a good redox probe, has fast reduction and oxidation reaction and provide higher sensitivity than other redox substances such as ascorbic acid, TMB, quinine and dopamine. Concentrations of $[Fe(CN)_6]^{3-/4-}$ were studied in the range of 5-15 mM and the result is shown in Figure 3.5 (The signal profiles are displayed in Appendix B). At low concentration, the low percentage of decreasing current was resulted because there is not enough mass transfer to electrode surface. Moreover, the results indicated the obvious reduction in response percentage higher than 10 mM too which may be due to the prevention of more mass transfer. When the amount of $[Fe(CN)_6]^{3-/4-}$ is highly at diffusion layer, it provided low current signal of I_0 and I (Appendix B) and decreasing current percentage is decreasing, respectively. Thus, concentration of $[Fe(CN)_6]^{3-/4-}$ of 10 mM was selected.

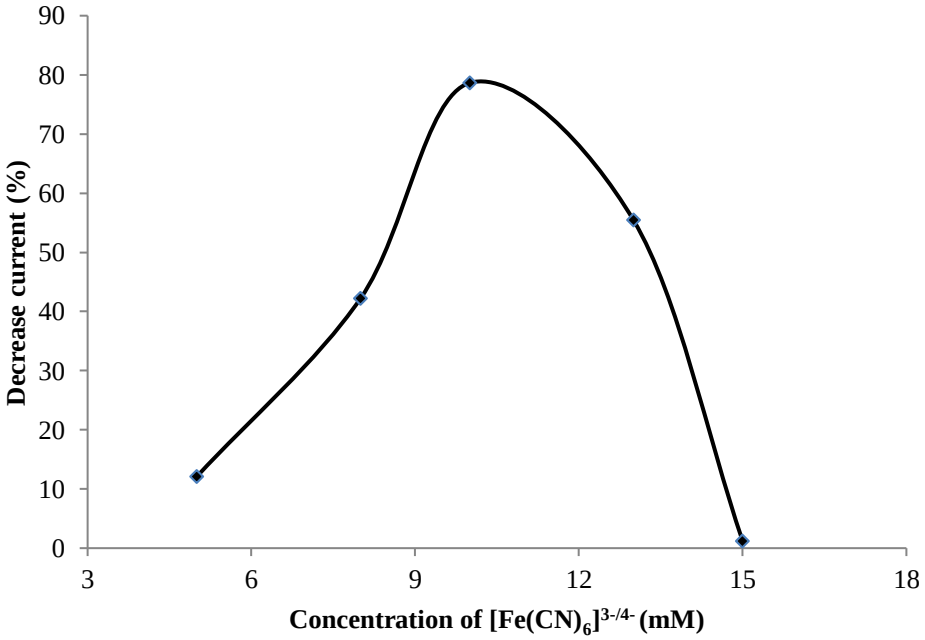

Figure 3.5 Effect of $[Fe(CN)_6]^{3-/4-}$ concentration on 2 mL min^{-1} of flow rate, 350 mV of applied potential and 30 min of immunointeraction time at 4˚C.

3.2.4 Effect of immunointeraction time

HIgG is one of protein which its stability and activity depends on temperature. Previous researches [1, 5, 8] suggested that the immunointeraction can be taken place at room temperature with reasonable sensitivity. Therefore in this work, the experiment was carried out at room temperature (25±2˚C) and the incubation time was studied in the range of 5-20 min. The result as shown in Figure 3.6 indicated that short incubation time gave low response percentage due to anti-HIgG and HIgG had low immunointeraction. The signal profiles are depicted in Appendix B. However, long incubation time provided low response percentage due to accumulation of HIgG that causes prevention of HIgG active site. Immunointeraction time of 10 min was selected as an optimum condition.

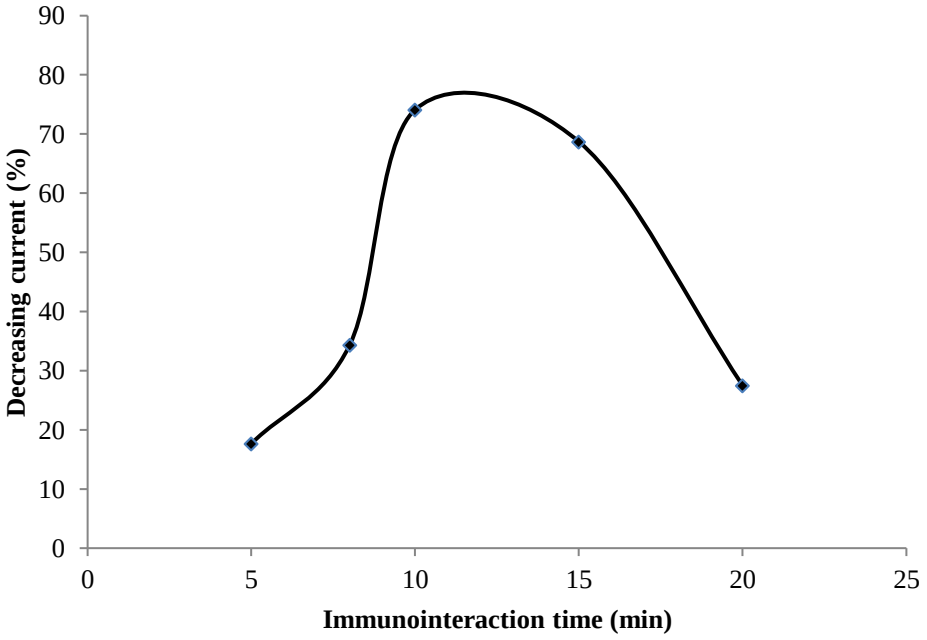

Figure 3.6 Effect of immunointeraction time on 2 mL min^{-1} of flow rate, 350 mV of applied potential and 10 mM of $[Fe(CN)_6]^{3-/4-}$.

3.3 Calibration of HIgG determination

The SIA-amperometric system was used for determination of HIgG. The following conditions selected from above studies were employed, i.e., flow rate of 2 mL min^{-1}, applied potential of +350 mV, 10 mM $[Fe(CN)_6]^{3-/4-}$, and 10 min of immunointeraction time. The proposed system provided the direct relationship of current decreasing percentage and concentration of HIgG as shown in Figure 3.7a. However, saturation of the response was observed at about 100 ng mL^{-1} HIgG which provided current decreasing of about 53%. Linear calibration graph was in the range of 2-100 ng mL^{-1} with detection limit (3SD) of 1.70 ng mL^{-1} (Figure 3.7b).

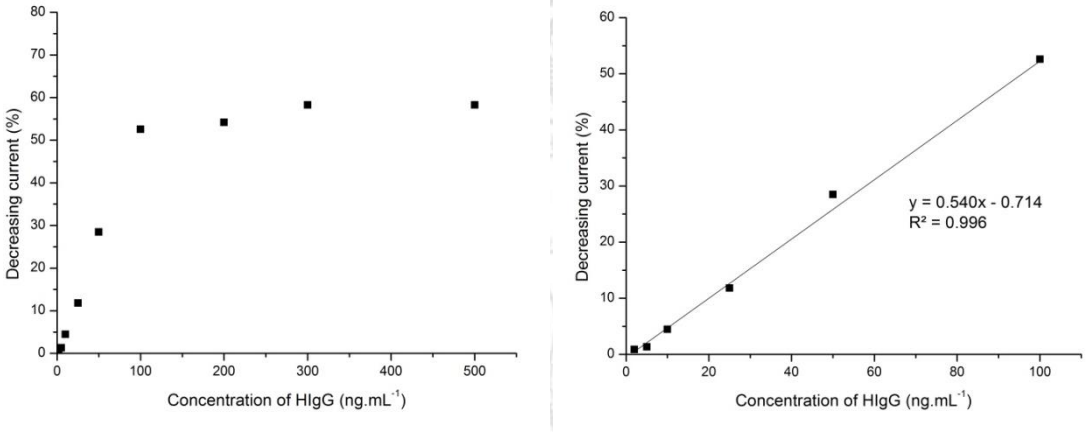

Figure 3.7 Relationship between decreasing current percentage and concentration of HIgG (a), and a calibration graph in the range of 2-100 ng mL^{-1} HIgG (b).

The results were compared with other methods from the literature as shown in Table 3.1. The analytical performances of the developed system are comparable to the similar immunosensor reported previously [Jumpathong, 2016]. This work provided advantages such as semi-automatic operation, using low cost and disposable electrode and simple amperometric detection system.

Table 3.1 Comparison of anti-HIgG/GO/SPCE immunosensor with other immunosensors for determination of HIgG.

Immunosensor	Detection Method	Linear range (ng mL^{-1})	LOD (ng mL^{-1})	Reference
Anti-IgG/CNFs-Chit/GCE	Amperometry	0.0001-5000	0.05	[Zhang, 2016]
Anti-HIgG/COOH-MWCNT NPs/Fe_3O_4 NPs/Au	CV	30-1000	25	[Zerei, 2012]
Anti-HIgG/OPPy-Au_{nano}/SPE	EIS	0.5-125.0	0.02	[Amouzadeh Tabrizi, 2016]
Anti-HIgG/GO/SPCE	DPV	2.5-100	1.99	[Jumpathong, 2016]
Anti-HIgG/rGO-MWCNT-Pd/GCE	SWV	0.01-25	0.0033	[Ahour, 2016]
Anti-HIgG/GO/SPCE	SIA-amperometry	2-100	1.70	This work

Abbreviations: HIgG, human immunoglobulin G; CNFs-Chit, carbon nanofibers-chitosan; COOH-MWCNT NPs, carboxyl group-multiwall carbon nanotubes; OPPy-Au_{nano}, overoxidized polypyrrole decorated with gold nanoparticle; GO, grapheme oxide; rGO-MWCNT-Pd, reduced graphene oxide–multiwalled carbon nanotube–palladium nanoparticles (NPs) nanocomposite; GCE, glassy carbon electrode; Au, gold electrode; SPE, screen printed electrode; SPCE, screen printed carbon electrode; CV, cyclic voltammetry; EIS, electrochemical impedance spectroscopy; DPV, differential pulse voltammetry; SWV, squarewave voltammetry; SIA, sequential injection analysis

3.4 Repeatability, reproducibility and stability of the immunosensor

The repeatability, reproducibility and stability were evaluated by analyzing decrease current percentage between before and after immunointeraction of anti-HIgG and HIgG of several sensing electrodes. Under the optimum conditions, the repeatability and reproducibility were estimated from the current response to 50 ng mL^{-1} HIgG. The results of the relative standard deviation (RSD) indicated 2.60% of repeatability (N=11) and 3.55% of reproducibility (N=7). Thus, the reproducibility of preparation of electrode and determination of HIgG was quite good.

The stability of immunosensor was examined. The immunosensor was dried and stored at 4˚C in refrigerator for different periods of time before use in analysis. Unfortunately, it was found that the current response decreased 50% in the next day, which may be caused by the aggregation of anti-HIgG [5, 15] due to high amount of anti-HIgG (1.6 µg) on electrode surface so immunosensor must be prepared freshly before used. However, SPCE bared immunosensor was made from low cost materials and can be disposable which is better than other electrodes such as glassy carbon electrode [Zhang, 2016; Zhang, 2016; Liu, 2015] and gold electrode [Zarei, 2012].

Comparison to the similar electrochemical immunosensor reported previously but operated in a batchwise manner and using a commercial differential pulse voltammetric analyzer as a detection device [Jumpathong, 2016], the SIA amperometric immunosensor offers more advantages such as less consumption of chemical reagents (25 mL vs 7.5 mL), shorter analysis time (60 min vs 20 min), manual operation vs semi-automatic operation, and the proposed system used inexpensive detection device.

3.5 Determination of HIgG in real samples

The proposed electrochemical immunosensor was investigated for its applicability to determine HIgG in real samples such as urine samples. Four urine samples were diluted with PBS (pH 7.4) (1:1) and analyzed by the developed method. They provided the current signal in the same level of background current of the anti-HIgG/GO/SPCE that indicated the HIgG presented at below the detection limit. The 1:1 diluted samples were spiked with certain concentration of HIgG and subjected to immunointeraction on electrode surface instead of standard HIgG (Figure 2.1) and evaluate the current signal

by SIA-amperometric system for three times. The percentage recoveries were calculated from calibration graph. The results found that percentage recoveries are in the range of 97.00 - 104.93 % as shown in Table 3.2 (The signal profiles are displayed in Appendix B). The substrates in urine sample that could interfere to the system were uric acid, ascorbic acid, dopamine, and glucose but they were non-specific adsorption on the electrode surface [Jumpathong, 2016]. When evaluated urine matrix influence from the method of standard additions by adding 10 - 100 ng mL^{-1} of standard HIgG in each samples, they were provided the sensitivity of calibration graph as shown in 0.461 - 0.539 of sensitivity and 0.119 - 0.453 ng mL^{-1} of HIgG concentration. The results as shown in Table 3.3 and the signal profiles are summarized in Appendix B). It indicated that the proposed method could be used for clinical diagnosis. The further analysis of more samples from a hospital is under investigation.

Table 3.2 Determination of HIgG percentage recoveries in human's urine samples.

Sample No.	Spiked (ng mL^{-1})	Found (ng mL^{-1})	Recovery (%)
1	5.00	4.85 ± 0.45	97.00
2	25.00	25.22 ± 0.68	100.87
3	50.00	52.46 ± 1.10	104.93
4	75.00	73.72 ± 2.43	98.29

Table 3.3 Determination of HIgG in human's urine samples by standard addition method.

Sample No.	Calibration graph	Found (ng mL^{-1})
1	y = 0.536x + 0.243	0.453
2	y = 0.539x + 0.064	0.119
3	y = 0.486x + 0.073	0.150
4	y = 0.461x + 0.186	0.403