CHAPTER 2

Experimental

2.1 Chemicals

All chemicals were of analytical reagent grade and prepared by dissolving in PBS and deionized (DI) water obtained from a Millipore water purification system (Millipore, Sweden). The chemicals used are listed below:

1. Human Immunoglobulin G antibody produced in goat: Anti-HIgG (Sigma, USA)
2. Immunoglobulin G from human serum: HIgG (Sigma, USA)
3. Bovine serum albumin: BSA (Merck, Germany)
4. N-Hydroxysuccinimide: NHS (Merck, Germany)
5. N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride: EDC (Merck, Germany)
6. Potassium phosphate monobasic: KH_2PO_4 (Fisher Scientific, USA)
7. di-Sodium hydrogen orthophosphate dodecahydrate: $Na_2HPO_4.12H_2O$ (Ajax Finechem, Australia)
8. Potassium chloride: KCl (Ajax Finechem, Australia)
9. Sodium chloride: NaCl (Loba Chemie, India)
10. Sodium hydroxide: NaOH (Merck, Germany)
11. Potassium hexacyanoferrate (II) trihydrate: $K_4[Fe(CN)_6].3H_2O$ (Scharlab, Spain)
12. Potassium hexacyanoferrate (III): $K_3[Fe(CN)_6]$ (Scharlab, Spain)

2.2 Materials and instruments

Materials and instruments used in this work are listed as follow:

1. Graphene oxide: GO (AIE Lab, CMU, Thailand)
2. Screen-printed carbon electrode: SPCE, WE (AIE Lab, CMU, Thailand)

3. Silver/silver chloride electrode: Ag/AgCl, RE (Metrohm, Switzerland)

4. Stainless steel electrode: AE

5. Syringe pump 5.0 mL syringe barrel, 6 cm moving plunger, 48000 steps motor, Model XLP-6000 (Cavro, USA)

6. 10-Position selection valve (Valco instrument, USA)

7. PTFE tubing (1.59 mm O.D., 0.5 mm I.D.) (Upchurch Scientific, USA)

8. Flow through electrochemical cell (by Miss Preeyaporn Reanpang)

9. Digital multimeter (ProsKit, USA)

10. Home-made amperometric detector (by Assoc. Prof. Dr. Jaroon Jakmunee)

11. E-corder 210 (eDAQ Pty Ltd., Australia)

12. Autolab potentiostat (Metrohm, Netherland)

13. Plasma cleaner (PDC-32G, Harrick, USA)

14. pH meter model 744 (Metrohm, Switzerland)

15. Micropopette 20, 100, 1000 µL (Eppendorf, Germany)

16. Personal computer

2.3 Software

1. Autolab (Metrohm, Switzerland)

2. eDAQ Chart (eDAQ, Australia)

3. Microsoft Excel 2003 (Microsoft, USA)

2.4 Preparation of solution

2.4.1 Phosphate buffer saline solution (10 mM PBS, pH 7.4)

8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 were dissolved in DI water and the volume was made up to 1000 mL to obtain 10 mM phosphate buffer saline solution.

2.4.2 Ferri/ferrocyanide solution (10 mM $[Fe(CN)_6]^{4-/3-}$)

0.3293 g of $K_3[Fe(CN)_6]$ and 0.4224 g of $K_4[Fe(CN)_6]$ were dissolved in PBS and the volume was made up to 100 mL to obtain 10 mM $[Fe(CN)_6]^{4-/3-}$.

2.4.3 Sodium hydroxide solution (5 mM NaOH)

0.05 g of NaOH was dissolved in DI water and the volume was made up to 250 mL to obtain 5 mM NaOH.

2.4.4 Anti-HIgG solution (0.1 mg mL^{-1} Anti-HIgG)

7.5 µL of Anti-IgG was pipetted into the cup and the volume was made up to 500 µL by PBS.

2.4.5 HIgG solution (0.1 mg mL^{-1} HIgG)

9.75 µL of IgG human serum was pipetted into the cup and the volume was made up to 500 µL by PBS.

2.4.6 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide solution (0.4 M EDC)

0.0621 g of EDC was dissolved in DI water and the volume was made up to 1 mL to obtain 0.4 M EDC.

2.4.7 N-Hydroxysuccinimide solution (0.1 M NHS)

0.0115 g of NHS was dissolved in DI water and the volume was made up to 1 mL to obtain 0.1 M NHS.

2.4.8 Bovine serum albumin solution (0.5% w/v BSA)

0.01 g of BSA was dissolved in DI water and the volume was made up to 2 mL to obtain 0.5% w/v BSA.

2.4.9 Graphene oxide (1 mg mL^{-1} GO)

0.0010 g of graphene oxide particle was dispersed in 1 mL of DI water.

2.5 Electrochemical immunosensor preparation

The preparation of immunosensor was carried out following the previous work [Jumpathomg, 2016]. SPCE was treated in a plasma cleaner chamber for 1 minute [Reanpang, 2015]. The treated screen-printed carbon electrodes (SPCEs) were modified two times with 2 µL of GO and dried at ambient temperature for 30 min. The electrodes were droped with 20 µL of EDC/NHS (1:1) solution and dried for 30 min in order to activate the electrode. Then incubated with 20 µL of anti-HIgG solution for 30 min at

4°C, blocked unreacted site with 20 µL of BSA and evaluated the current signal by SIA-amperometry. After that, the immunosensor electrode was disassembled from the EC and was incubated offline in a standard HIgG or sample solution at room temperature for 10 min to allow binding between anti-HIgG and HIgG and evaluated the current signal again. The procedures of the immunosensor preparation and detection signal are presented in Figure 2.1.

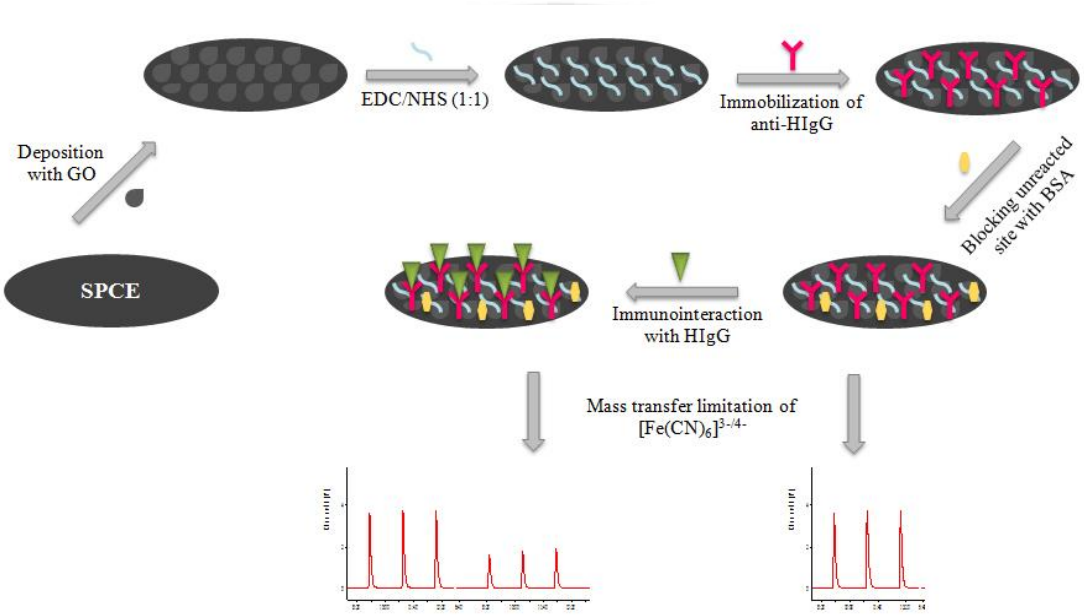

Figure 2.1 The preparation of electrochemical immunosensor for HIgG determination.

2.6 Characterization of the electrode

2.6.1 Effect of material on electrode

All electrodes (bare SPCE, GO/SPCE, anti-HIgG/GO/SPCE and HIgG/ anti-HIgG/GO/SPCE) were characterized by CV using the 10 mM $[Fe(CN)_6]^{3-/4-}$ as a redox probe. The electrochemical immunosensing process was evaluated based on the current of $[Fe(CN)_6]^{3-/4-}$ redox probe in PBS electrolyte solution by applying a fixed potential of +350 mV vs Ag/AgCl to the WE.

2.6.2 Morphology of the electrode

Surface of all electrodes were characterized by SEM. The electrode surface samples were dried, mounted over SEM stubs and measured using the following

condition: 30,000X magnification, 15 kV accelerating voltage and ~15 mm working distance.

2.7 SIA system and operational procedure for determination of HIgG

The immunosensor electrode as described in section 2.5 was utilized in SIA-amperometric system for HIgG determination. The SIA system consists of PBS carrier solution which is used as the electrolyte of the system, a syringe pump (SP), a holding coil (HC), a selection valve which the ports are connected to $[Fe(CN)_6]^{3-/4-}$ vessel, and also to electrochemical cell (EC), a home-made amperometer (D) [Reanpang, 2015; Upan, 2016] that was connected to the signal recording unit (eDAQ), and a computer (PC) as shown in Figure 2.2.

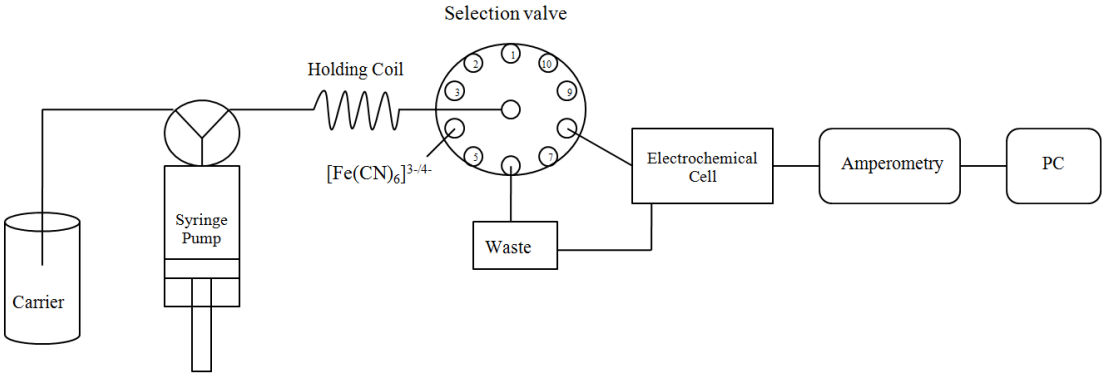

Figure 2.2 SIA system for determination of HIgG: carrier = 0.1 M PBS pH 7.4, SP = syringe pump, HC = holding coil, selection valve, EC = electrochemical cell, D = amperometer, PC = personal computer, and W = waste.

The EC has three electrodes, including a Ag/AgCl electrode as a reference electrode, stainless steel electrode as a auxiliary electrode and anti-HIgG/GO/SPCE as a working electrode. To carry out the immunointeraction, the immunosensor was incubated out of EC at room temperature for 10 min with different concentration of HIgG.

The operational sequences for the determination of HIgG by SIA-amperometric system are as follow. Firstly, PBS carrier solution was filled in the HC, $[Fe(CN)_6]^{3-/4-}$ redox probe was filled the tubing that connected to the port 4 of selection valve and the electrochemical cell and the tubing connecting to the port 8 of the selection valve, respectively. Then operational sequences were started by aspirating, the PBS carrier

solution and then pushing through port 8 to the electrochemical cell. Afterward, the selection valve was switched to port 4, 100 µL of $[Fe(CN)_6]^{3-/4-}$ was aspirated and then set through port 8 to the electrochemical cell as described in Table 2.1.

Table 2.1 The operational sequences for the determination of HIgG.

Step	Selection valve	Flow rate (mL min^{-1})	Volume (mL)	Position	Description
1	Syringe pump	6	Full	Aspirate	PBS was filled in the HC.
2	Port-8	6	5.0	Inject	PBS was pushed through EC.
3	Port-4	6	0.1	Aspirate	$[Fe(CN)_6^{3-/4-}]$ (redox probe) was filled the tubing.
4	Syringe pump	6	2.5	Aspirate	PBS was filled in the HC.
5	Port-8	2	Empty	Inject	Reagent was pushed through EC for recording the peak current.

The amperometric peak current was recorded, one peak each for triplicate injection. The analytical responses was obtained from the current before (I_0) and after (I) the occurance of immunointeraction between anti-HIgG and HIgG. The relative decreasing of electrical current was evaluated as:

$$\% \text{ decreasing current} = \frac{I_0 - I}{I_0} \times 100 \qquad 2.1$$

Finally, the electrochemical cell and the electrodes were cleaned by deionized water.

2.8 Optimization of SIA system

Effect of some parameters in SIA system which affected to the performance of the analytical response, i.e., flow rate, applied potential, concentration of $[Fe(CN)_6]^{3-/4-}$, and time for immunointeraction were studied, respectively. The results were compared in the term of percentage of decreasing current after binding of HIgG on the electrode. The optimum condition of each parameter was selected for the next experiment.

2.8.1 Effect of flow rate

The effect of flow rate was studied in the range of 1.0 - 5.0 mL min^{-1}. The other conditions were kept constant as summarized in Table 2.2.

Table 2.2 The conditions for the study of flow rate.

Parameters	Condition
Applied potential	350 mV
Phosphate buffer saline	10 mM pH 7.4
Concentration of ferri/ferrocyanide	10 mM
Immunointeraction time	4˚C, 30 min

2.8.2 Effect of applied potential

The effect of applied potential was studied in the range of 250-500 mV. The other conditions were established in Table 2.3.

Table 2.3 The conditions for the study of applied potential.

Parameters	Condition
Flow rate	2 mL min^{-1}
Phosphate buffer saline	10 mM pH 7.4
Concentration of ferri/ferrocyanide	10 mM
Immunointeraction time	4˚C, 30 min

2.8.3 Effect of concentration of ferri/ferrocyanide

The effect of concentration of ferri/ferrocyanide was studied in the range of 5-15 mM. The other conditions were established in Table 2.4.

Table 2.4 The conditions for the study of concentration of ferri/ferrocyanide.

Parameters	Condition
Flow rate	2 mL min^{-1}
Applied potential	350 mV
Phosphate buffer saline	10 mM pH 7.4
Immunointeraction time	4˚C, 30 min

2.8.4 Effect of immunointeraction time

The effect of immunointeraction time between HIgG and anti-HIgG was studied at room temperature in the range of 5-20 min. The other conditions were summarized in Table 2.5.

Table 2.5 The conditions for the study immunointeraction time.

Parameters	Condition
Flow rate	2 mL min^{-1}
Applied potential	350 mV
Phosphate buffer saline	10 mM pH 7.4
Concentration of ferri/ferrocyanide	10 mM

2.9 Analytical performances

2.9.1 Calibration graph and detection limit

The calibration graph was studied for concentration of IgG in the range of 2 to 500 ng mL^{-1} under the optimum conditions of SIA system. Percentage of decreasing current was plotted against HIgG concentration. The limit of detection (LOD) was calculated in term LOD = 3 signal/noise ratio of the calibration graph.

2.9.2 Precision study

2.9.2.1 Repeatability

The repeatability of immunosensor was examined by incubating 50 ng mL^{-1} HIgG on electrode surface, injecting 11 replicates of redox probe and analyzing decrease current percentage between before and after immunointeraction of anti-HIgG and HIgG. The precision was evaluated in term of percentage of relative standard deviation (%RSD) value as the equation:

$$\%RSD = \frac{S.D.}{\bar{X}} \times 100 \qquad 2.2$$

When; S.D. = Standard deviation

$\bar{X}$ = Mean value

2.9.2.2 Reproducibility

The reproducibility of immunosensor was examined by incubating 50 ng mL^{-1} HIgG on 7 electrodes and analyzing decrease current percentage between before and after immunointeraction of anti-HIgG and HIgG. The precision was evaluated in term of percentage of relative standard deviation (%RSD) value as the equation 2.2.

2.9.2.3 Stability

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. Then, they were analyzed for decrease current percentage between before and after immunointeraction of anti-HIgG and HIgG compared daily for 1 month.

2.10 Determination of HIgG in real samples

The proposed electrochemical immunosensor was investigated for its applicability to determine HIgG in real sample such as urine samples. Four urine samples that collected from AIE lab members were diluted with PBS (pH 7.4) (sample:PBS, 1:1) and spiked with certain concentration of HIgG because HIgG concentrations in all the samples were below the detection limit of the method. The samples were subjected to immunointeraction on electrode surface instead of standard HIgG (Figure 2.1) and evaluate the current signal by SIA-amperometric system three times. Calibration graph was used for quantitative analysis of HIgG recoveries. The sensitivity and influence of urine matrix are evaluated from standard addition method. The samples were spiked with standard HIgG concentration in the range of 10 - 100 ng mL^{-1}. The results were evaluated from standard addition graph.