CHAPTER III

EXPERIMENTAL

3.1 Materials

All reagents and materials are analytical grade and use without further purification

1. Acetonitrile : Aldrich
2. Bovine Serum Albumin (BSA) : Sigma
3. Cysteamine : Aldrich
4. 3,3-Dithiodipropionic acid : Aldrich
5. Ethanol : Merck
6. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide : Fluka
7. Glycine : Fluka
8. Hydrochloric acid : Fluka
9. Hydrogen peroxide : Merck
10. *N*-hydroxysuccinamide : Fluka
11. 2-mercaptoethanol : Aldrich
12. 3-mercaptopropionic acid : Aldrich
13. 11-mercaptoundecanoic acid : Aldrich
14. Milli-Q water : Mill-Q Lab system
15. Monoclonal antibody against *Vibrio harveyi* : Department of Biology, Faculty of Science, Srinakharinwirot University, Bangkok.
16. Phosphate buffer saline (PBS) : Aldrich
17. Sinapinic acid : Bruker
18. Trifluoroacetic acid : Aldrich
19. *Vibrio harveyi* : Department of Biology, Faculty of Science, Srinakharinwirot University, Bangkok.

15. *Vibrio vulnificus* : Department of Biology, Faculty of Science, Srinakharinwirot University, Bangkok.

16. *Vibrio paraheamolyticus* : Department of Biology, Faculty of Science, Srinakharinwirot University, Bangkok.

3.2 Equipments

3.2.1 Reflection-Absorption Infrared Spectroscopy (RAIRS)

All spectra were collected by a Nicolet Magna 750 FT-IR spectrometer equipped with a liquid nitrogen-cooled mercury-cadmium-telluride (MCT) detector. Spectra in the infrared region (4000-650 cm^{-1}) were collected with 512 co-addition scans at a spectra resolution of 4 cm^{-1}. A commercial accessory (the Seagull^{TM}, Harrick Scientific,USA) was employed for all spectral acquisition. A polarized radiation with an 86° angle of incidence was employed.

3.2.2 Contact Angle Measurements

Contact angle goniometer model 100-00 and a Gilmont syringe with a 24-gauge flat-tipped needle (Ramé-Hart, Inc., USA) was used for the determination of water contact angles. The measurements were carried out in air at room temperature. A droplet of Milli-Q water is placed on the tested surface by bringing the surface into contact with a droplet suspended from a needle of the syringe. A silhouette image of droplet was projected on the screen and the angle is measured. The reported angle is an average of 3 measurements on different area of each sample.

3.2.3 Quartz Crystal Microbalance (QCM)

Quartz Crystal Microbalance PM-710 plating monitor were used. The monitor uses a quartz crystal as the basic transducing element. The AT-cut quartz crystal itself is a flat circular plate approximately one inch (2.5 cm) in diameter and 0.011 to 0.0132 inches (0.028-0.033 cm) thick for 5 MHz. The crystal is excited into

mechanical motion by an external oscillator. The frequency at which the quartz crystal oscillates is lowered by the addition of material to its surface. All frequency readings were recorded at room temperature.

3.2.4 Matrix-assisted Laser Desorption Ionization (MALDI)

Molecular weight of monoclonal antibody was analyzed on Microflex MALDI-TOF mass spectrometry (Bruker Daltonics, Germany) using Sinapinic acid (SA) as a matrix. Trifluoroacetic acid (0.1%) in acetonitrile:water (1:2) was used as a diluent for the preparation of MALDI-TOF samples.

3.3 Methods

3.3.1 Self-assembly Monolayer (SAM) Formation of Carboxyl-terminated Thiol

A gold-coated quartz crystal was pretreated with Pirannha solution (1:3 (30% v/v) H_2O_2–concentrated H_2SO_4) for 5 min, After pretreatment, the crystals was rinsed thoroughly with distilled water and dried under stream of nitrogen. The pretreated quartz crystal was immersed in an ethanol solution of a desired end functionalized thiol (carboxyl-terminated thiol: 3-mercaptopropionic acid (MPA); 11-mercaptoundecanoic acid (MUA); 3,3-dithiodipropionic acid (DTDPA) or mixed selected carboxyl-terminated thiol with cysteamine (CE) or 2-mercaptoethanol (ME)) with its both sides in contact with the solution for 24 h to form an SAM. The SAM-modified crystal was then rinsed with ethanol and Milli-Q water successively and dried under stream of nitrogen. A resonant frequency at equilibrium of the crystal mounted on the QCM cell was measured under dried state before and after SAM formation. A frequency shift due to SAM formation was designated as $\triangle f_s$.

The same procedure for SAM formation of carboxyl-terminated thiol was also applied for the gold-coated glass slide. The gold coating was accomplished by vacuum deposition of 50 nm of chromium followed by 100 nm of gold onto a glass slide in Thermal vacuum evaporator model Auto 306 EDWARDS. The SAM-modified slide was then characterized by RAIRS and water contact angle measurement.

3.3.2 Activation of Carboxyl Groups of SAM-modified Substrates

The SAM-modified crystal obtained from 3.3.1 was treated with a 2.5 mL carbodiimide aqueous solution (45 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI) and 15 mM *N*-hydroxysuccinimide (NHS)) at room temperature for a certain period of time to convert the terminal carboxyl group to an active *N*-hydroxysuccinimidyl group (NHS). After rinsing with Milli-Q water, the activated SAM-modified crystal (NHS-modified crystal) was dried under stream of nitrogen. A resonant frequency at equilibrium of the crystal mounted on the QCM cell was measured under dried state before and after the activation. A frequency shift due to the activation was designated as $\triangle f_a$.

The same procedure for activation was also applied for SAM-modified gold-coated glass slide. After the activation, the slide was subjected to analysis by RAIRS and water contact angle measurement.

3.3.3 Immobilization of Monoclonal Antibody (MAb) against *Vibrio harveyi* on NHS-modified Substrate

The NHS-modified crystal obtained from 3.3.2 was incubated in 0.1 mg/mL phosphate buffer solution (pH = 7.4) of monoclonal antibody against *Vibrio harveyi* at 4°C for at least 15 h. The crystal was then rinsed with PBS to remove excess antibody before soaking in 50 μL of 1% BSA (or ethanolamine) in PBS to block unreacted sites from nonspecific adsorption. After rinsing with PBS and Milli-Q water, the crystal was dried under stream of nitrogen, yielding MAb-immobilized crystal. A resonant frequency at equilibrium of the crystal mounted on the QCM cell was measured under dried state before and after the immobilization. A frequency shift due to the immobilization was designated as $\triangle f_i$.

The same procedure for immobilization was also applied for NHS-modified gold-coated glass slide. After the immobilization, the slide was subjected to analysis by RAIRS.

3.3.4 Determination of Bacteria Binding

The phosphate buffer solution (40 μL) containing a selected bacteria (*Vibrio harveyi*, *Vibrio vulnificus* or *Vibrio paraheamolyticus*) having a predetermined

concentration was spread over the entire Au electrode on one side of the MAb-immobilized crystal obtained from 3.3.3. After 1 h incubation at 4°C, the crystal was rinsed with PBS and Milli-Q water, and dried under stream of nitrogen. A resonant frequency at equilibrium of the crystal mounted on the QCM cell was measured under dried state before and after the bacteria binding. A frequency shift due to the bacteria binding was designated as $\triangle f_b$.

The same procedure for bacteria binding was also applied for MAb-immobilized gold-coated glass slide. After the binding, the slide was subjected to analysis by RAIRS.

3.3.5 Regeneration of MAb-immobilized Quartz Crystal

The regeneration of the quartz crystal immobilized with MAb against *Vibrio harveyi* was investigated by immersing the used quartz crystal previously subjected to bacteria binding in 0.1 M glycine/HCl buffer solution (pH 2.3) for 1 h followed by thorough rinsing with Milli-Q water to desorb the bound bacteria. The crystal was then dried under stream of nitrogen. A resonant frequency at equilibrium of the crystal mounted on the QCM cell was measured under dried state before and after the next cycle of bacteria binding. A frequency shift due to the bacteria binding was recorded and compared with the shift of the previous cycle of bacteria binding.