CHAPTER IV

RESULTS AND DISCUSSION

The aim of this work is to immobilize monoclonal antibody against *Vibrio harveyi* (*V. harveyi*) onto gold electrode of quartz crystals to be used for QCM analysis, employing a three-step procedure. The first step involves a formation of self-assemble monolayer of carboxyl-terminated alkanethiol. The second step is an activation of the monolayer, using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) and *N*-hydroxysuccinamide (NHS) to convert the terminal carboxyl group on the surface to an active NHS ester. The third step is a coupling of monoclonal antibody (MAb) against *V. harveyi*. The stepwise procedure of the SAM formation, the activation of carboxyl groups followed by the coupling reaction with MAb against *V. harveyi* is shown in Figure 4.1.

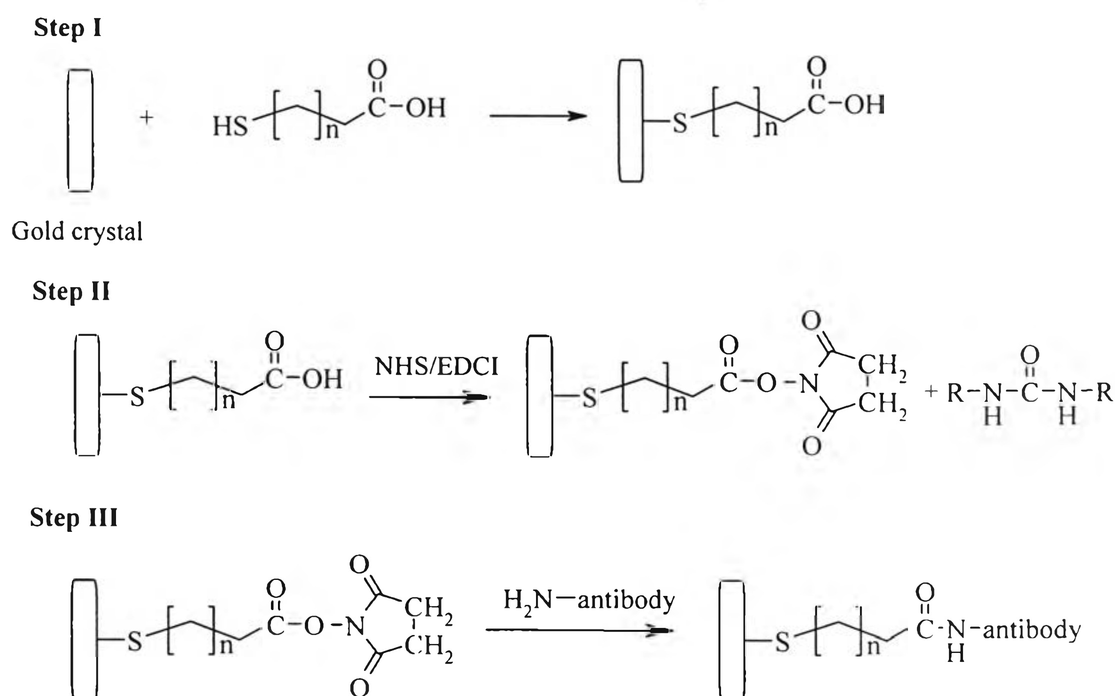

Figure 4.1 Schematic diagram showing the procedure for the preparation of a *Vibrio harveyi* immunosensor.

4.1 Self-assembly Monolayer (SAM) Formation of Carboxyl-terminated Alkanethiol onto Gold Surface

The SAMs formed by alkanethiols on a gold surface were commonly used as the functionized monolayer to immobilize biological compounds because of the formation of a strong metal-thiolate bond with no oxide formation at the electrode surface and the availability of free carboxyl group for linkage with the *N*-terminal of bioactive molecules.

Three thiol compounds including 11-mercaptoundecanoic acid (MUA), 3,3-dithiodipropionic acid (DTDPA), and 3-mercaptopropionic acid (MPA) were used for the SAM formation. Their structures are shown in Figure 4.2. The self assembly process was monitored by QCM and water contact angle analyses. The frequency shift due to SAM formation ($\triangle f_s$) together with the water contact angle of the self assembled monolayer obtained from MPA on gold surface as a function of immersion time are plotted in Figure 4.3. The QCM data suggests that the SAM process was complete after 24h while the contact angle reached the lowest value of 35.2 ± 1.9^o indicating the enhanced hydrophilicity of the surface by the terminal carboxyl groups at the other end of MPA.

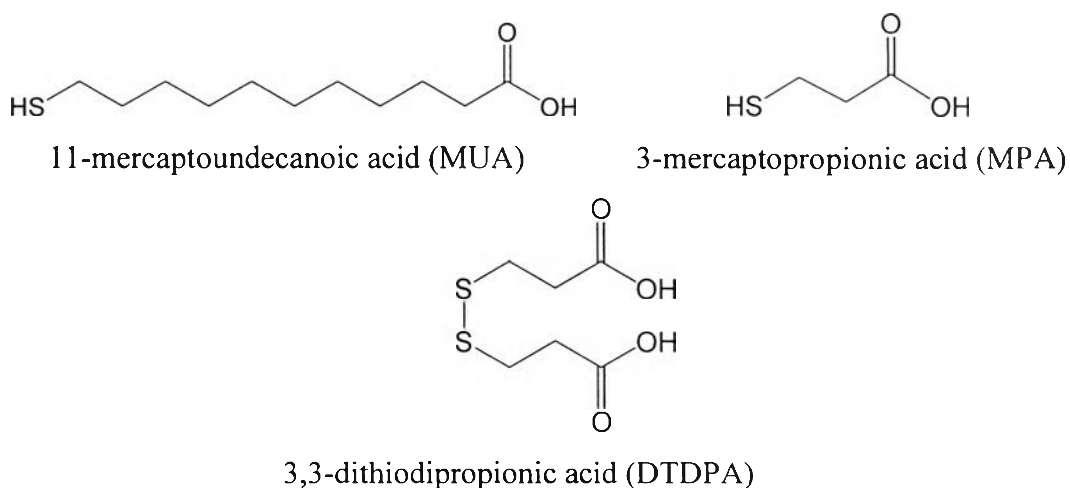

Figure 4.2 Structures of three thiol compounds.

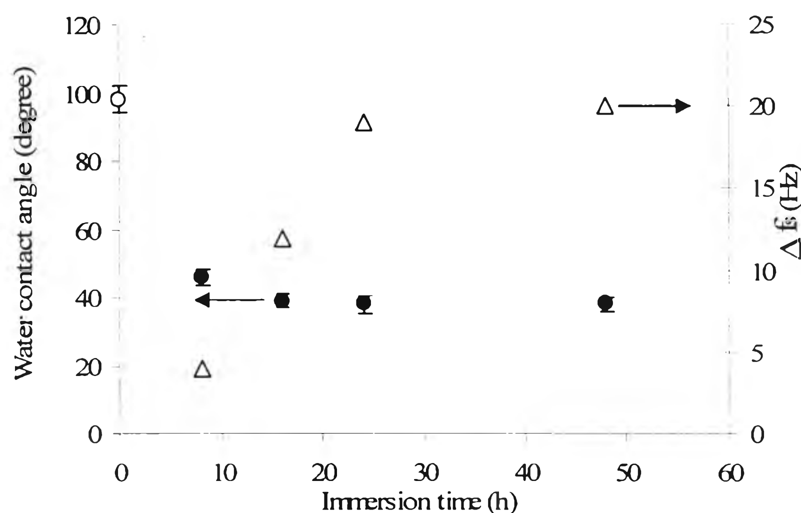

Figure 4.3 Frequency shift due to SAM formation ($\triangle f_s$) and water contact angle of SAM obtained from 10 mM MPA as a function of time.

Using the immersion time of 24h, effects of alkanethiol concentration on SAM formation of all three alkanethiols were investigated. The results are shown in Figure 4.4. The frequency change was in the following order: MUA>MPA>DTDPA at all concentration. This may stem from the fact that MUA possesses the highest molecular weight in comparison with other two alkanethiols so the highest $\triangle f_s$ was noticed for the self-assembly of MUA. Also, it has been reported that the monolayer assembled from the dithiol, DTDPA is inferior to that of the monothiol, MPA.[18] Thus the monolayer of DTDPA should be less dense than the one from MPA. That is why its $\triangle f_s$ exhibited the lowest value among three alkanethiols. The concentration of 10 mM seems to be the optimal concentration for the self assembly of MPA and DTDPA. On the other hand, the concentration in such a range did not have a significant impact on the SAM formation from MUA.

The gold-coated substrates bearing the monolayers assembled from the immersion time of 24h were subjected to contact angle analysis. The water contact angle data plotted in Figure 4.5 are in good agreement with the speculation previously discussed based on QCM data. The concentration of 10 mM is high enough to achieve the maximum packing of alkanethiols and the highest hydrophilicity. The higher hydrophobicity of the MUA monolayer in comparison with the monolayers of other

two alkanethiols may result from a disruption in the ordered SAM by underlying defaults of the substrate. Areas of increased surface roughness may expose the hydrophobic methylene groups of the alkanethiol to the monolayer/air interface causing the contact angle to be higher than the expected polar layer of carboxyl groups. The same speculation is also valid in the case of the monolayer assembled from DTDPA. The alkanethiol molecule tends to fold and flat down on the surface when the packing is not dense. The hydrophobic methylene groups of the alkanethiol thereby dominate at the monolayer/air interface, making the surface hydrophobic despite the presence of the hydrophilic terminal. For this reason, MPA was chosen for the preparation of the carboxyl-terminated monolayer for a subsequent immobilization of monoclonal antibody.

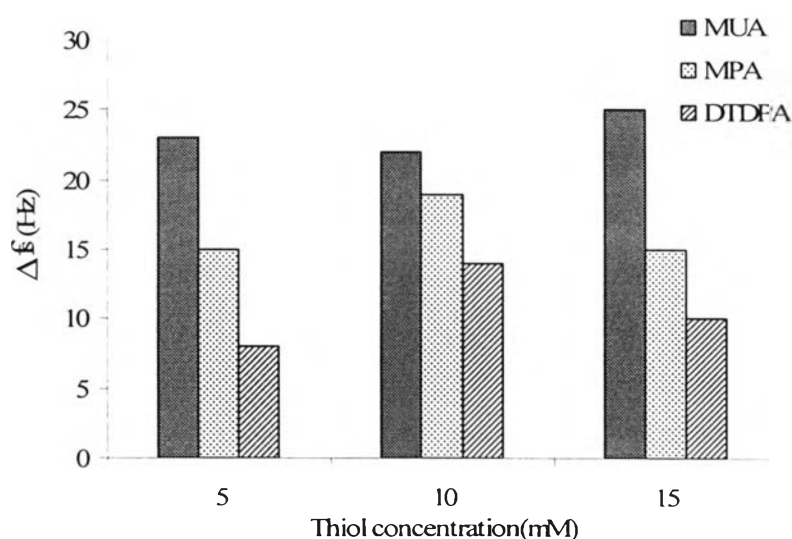

Figure 4.4 Frequency shift due to SAM formation (Δf_s) of alkanethiols as a function of concentration.

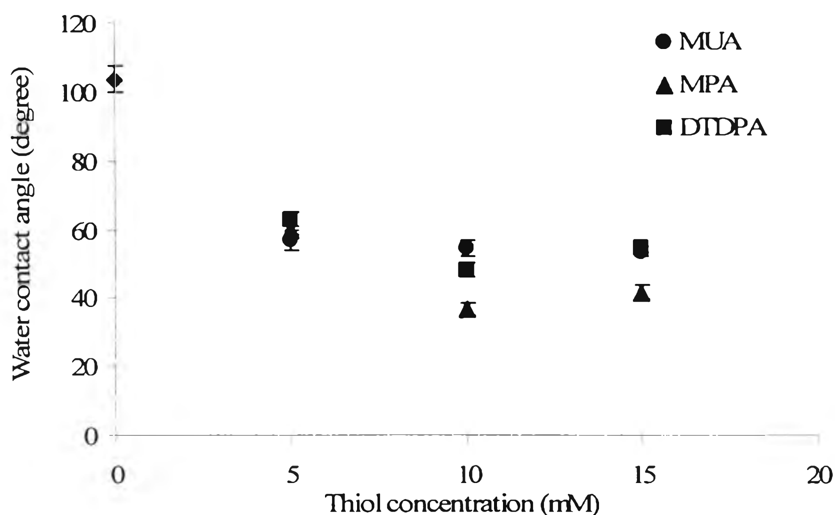

Figure 4.5 Water contact angle of SAM of alkanethiol as a function of thiol concentration.

Reflection Absorption Infrared Spectroscopy (RAIRS) was used to confirm the monolayer formation of carboxyl-terminated alkanethiol on gold substrate. The technique can provide information regarding the chemical structure of the assembly. The spectra shown in Figure 4.6 verify that the carboxyl-terminated monolayers were formed. A characteristic feature of IR vibration was observed: C-H stretching in the range of 2800-3000 cm^{-1} and C=O stretching of carboxyl group at 1740 cm^{-1}.

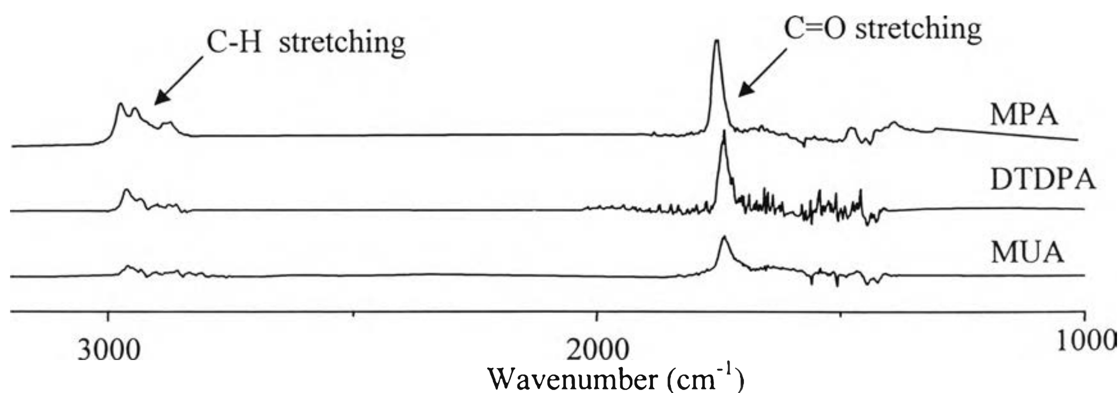

Figure 4.6 RAIRS spectra of carboxyl-terminated monolayers on gold-coated substrates.

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4.2 Activation of Carboxyl Groups of SAM-modified Substrates

In this research, the carboxyl groups of the MPA monolayer were activated by an heterogeneous reaction of MPA-modified substrates with EDCI and NHS to convert the terminal carboxyl group to an active NHS ester. NHS/EDCI is known as an effective system to generate active NHS ester groups in aqueous system that are quite stable for a reasonably long period of time in neutral condition and less prone to hydrolysis. The mechanism of the activation of carboxyl groups on the surface is shown in Figure 4.7.

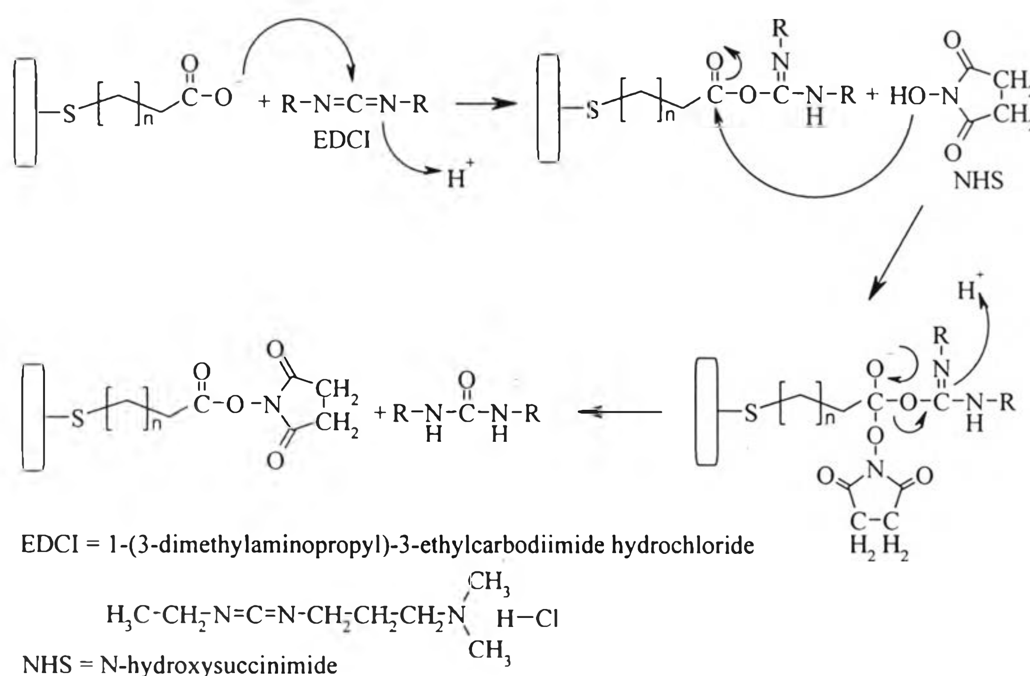

Figure 4.7 Mechanism of the activation of surface carboxyl groups by NHS/EDCI system.

In order to achieve the optimal condition for activation, two parameters namely immersion time, NHS/EDCI concentration were investigated. The extent of activation was followed by QCM and water contact angle analyses. Figure 4.8 depicts a frequency shift due to the activation (Δf_a) and its corresponding water contact angle as a function of immersion time. The surface obviously became more hydrophobic after the conversion of carboxyl groups to NHS groups. A continuous decrease of both water contact angle and Δf_a if the activation was longer than 45 min may be explained as a result of the unwanted hydrolysis that removes the existing NHS

groups from the surface. Using 30 min for the activation, an effect of NHS/EDCI on activation was determined. Results from Figure 4.9 suggested that the most suitable concentration of NHS/EDCI was 15:45 mM.

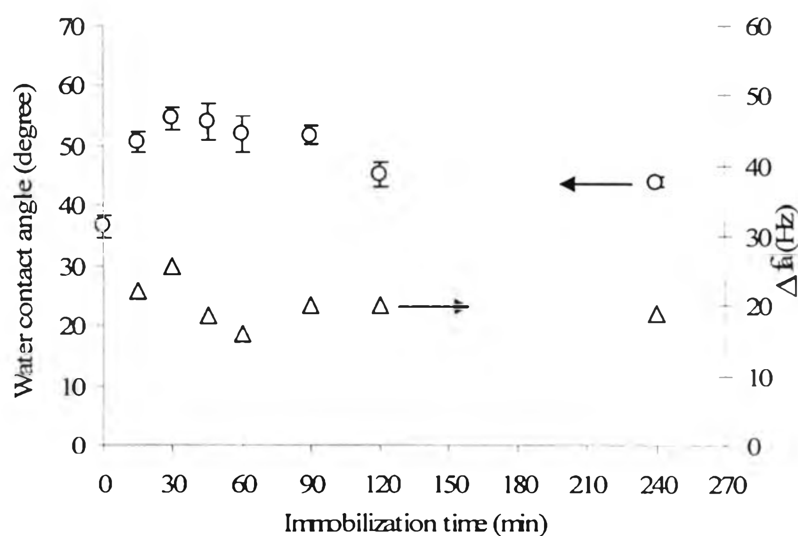

Figure 4.8 Water contact angle and frequency shift due to the activation ($\triangle f_a$) of the MPA-modified substrate as a function of immersion time using 15:45 mM of NHS/EDCI.

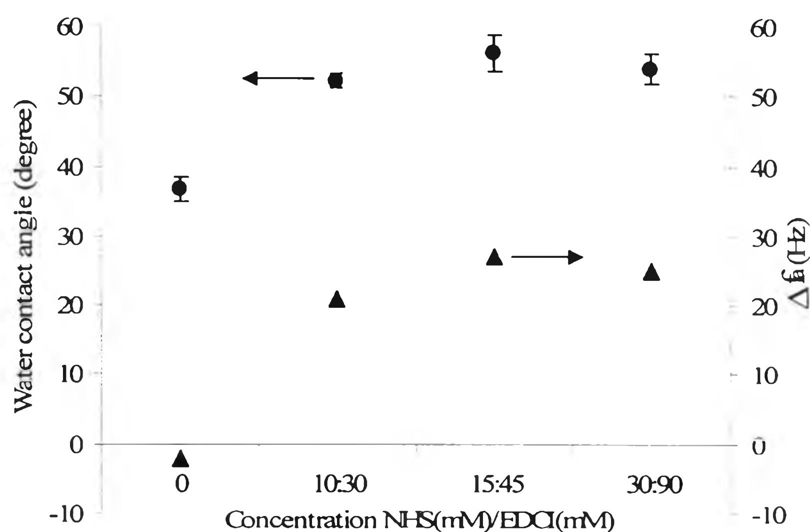

Figure 4.9 Water contact angle and frequency shift due to the activation ($\triangle f_a$) of the MPA-modified substrate as a function of NHS/EDCI concentration using 30 min immersion time.

4.3 Immobilization of Monoclonal Antibody against *Vibrio harveyi*

The immobilization of the antibody to the NHS-modified substrate was done by the formation of covalent amide bonds between the NHS groups and the amino groups of the *N*-terminal and/or the amino side groups of lysine residues of the antibody.

Monoclonal antibody (MAb) was characterized by matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF). The MALDI-TOF spectrum of the monoclonal antibody is shown in Figure 4.10. The obtained mass of the monoclonal antibody in spectrum was 152170 *m/z*.

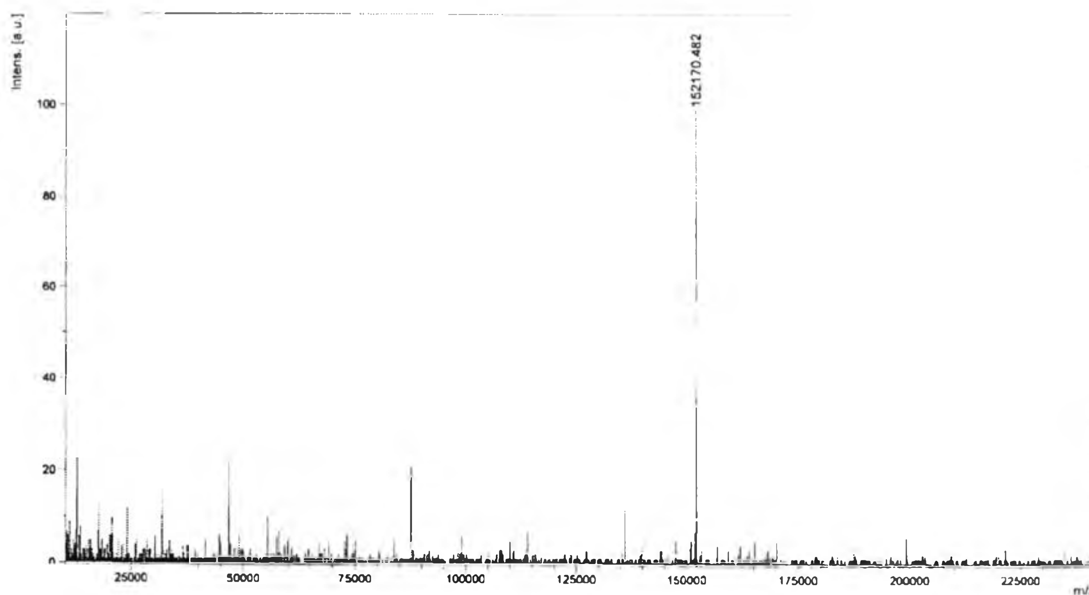

Figure 4.10 MALDI-TOF spectrum of monoclonal antibody against *Vibrio harveyi*.

Since it is the amount of MAb immobilized on electrode that largely determines the sensitivity of the immunosensor, it is quite necessary to seek for an optimal condition for MAb immobilization. To investigate an influence of the immobilization time, the concentration of MAb was fixed at 0.1 mg/mL and the immobilization time was varied from 8 to 15, and 24 h. The extent of MAb immobilization as a function of time is expressed in term of a frequency shift due to the MAb immobilization ($\triangle f_i$) as illustrated in Figure 4.11. Apparently, 15 h is sufficiently long for the MAb immobilization to reach the maximum extent.

An effect of MAb concentration on the antibody immobilization on the NHS-modified substrate was determined by varying the MAb concentration in the range of 0.05-1.0 mg/mL using the immobilization time of 15h. Figure 4.12 shows the frequency shift due to the MAb ($\triangle f_i$) as a function of the MAb concentration. The $\triangle f_i$ has a tendency to increase when the concentration was higher than 0.1 mg/mL. It is believed that the high concentration cannot only enhance the density of the MAb, but it should also promote non-specific adsorption. In order to obtain an appropriate concentration for MAb immobilization, this set of MAb-modified substrates has to be subjected to a treatment with a blocking reagent prior to the binding with the target bacteria. The result will be discussed in the next section.

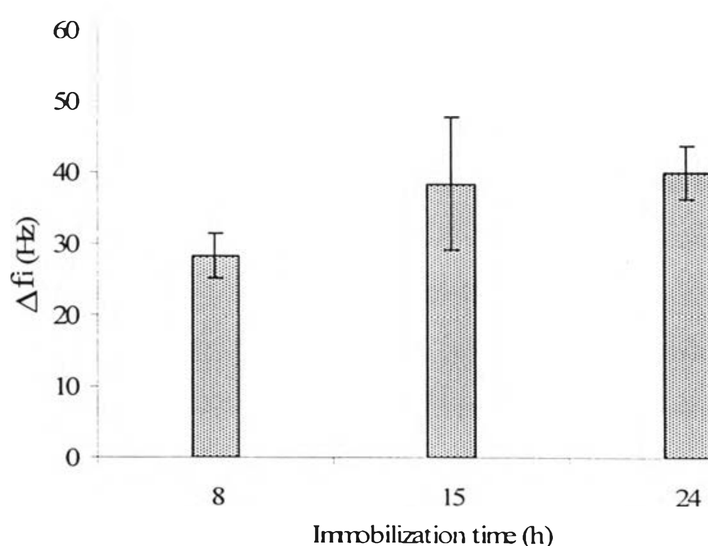

Figure 4.11 Frequency shift due to the MAb immobilization ($\triangle f_i$) on the NHS-modified substrate as a function of immobilization time using 0.1 mg/mL MAb.

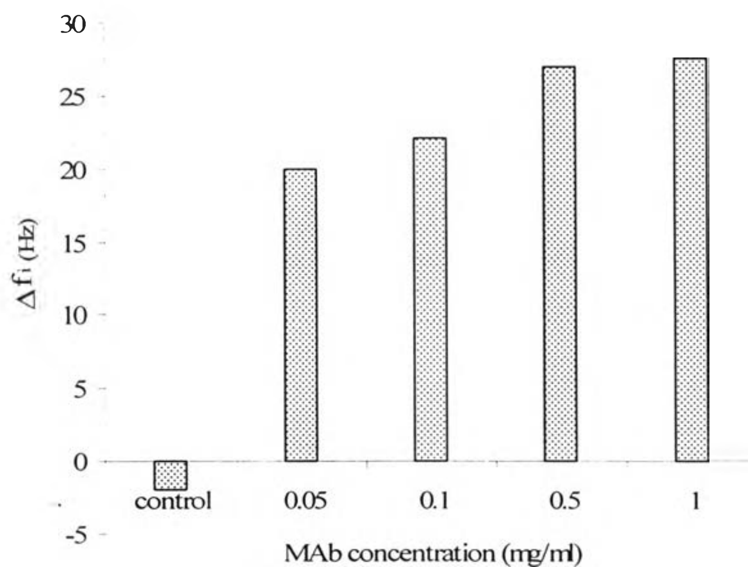

Figure 4.12 Frequency shift due to the MAb immobilization ($\triangle f_i$) as a function of MAb concentration using the immobilization time of 15h.

4.4 Determination of Bacteria Binding

4.4.1 Variables Affecting Bacteria Binding

(a) Blocking Reagent

It has been reported that the characteristic of the immobilized antibody is critical to the ability of the sensor to bind with the target bacteria. The immobilized antibody layer having a good characteristic should not have unreacted or non-specific sites which generally cause detrimental effect on the bacteria detection. To block the unreacted or non-specific sites, two blocking reagents namely bovine serum albumin (BSA) and ethanolamine were tested. According to Figure 4.13, the binding of *V. harveyi* was significantly improved after the treatment with both blocking reagents. In this particular case, BSA seems to be more effective in blocking than ethanolamine. This investigation truly confirms the necessity of the blocking step.

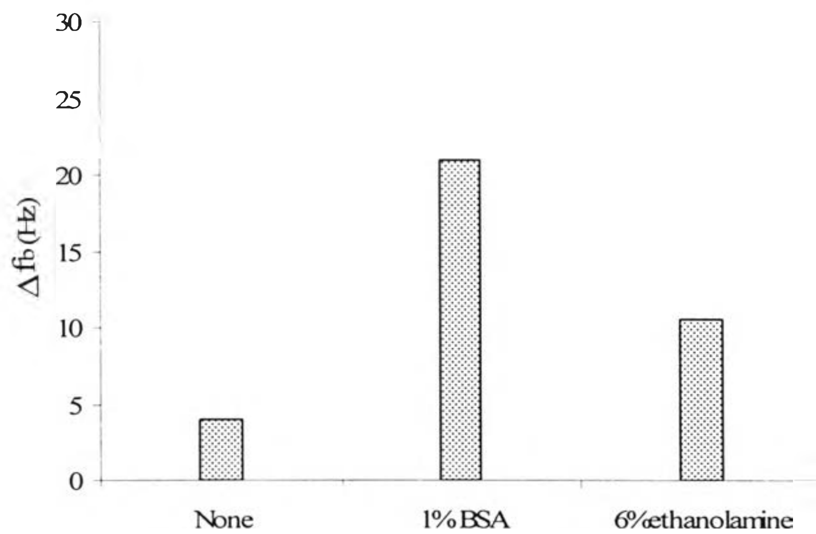

Figure 4.13 Frequency shift due to the *V. harveyi* binding ($\triangle f_b$) with the MAb-immobilized substrate after the treatment with blocking reagents. The concentration of *V. harveyi* used was 10^5 CFU/mL.

(b) Concentration of Monoclonal Antibody

The set of MAb-immobilized substrates prepared by using different concentrations of the MAb was tested for binding with *V. harveyi* having a concentration of 10^5 CFU/mL after the treatment with 1%BSA. Results displayed in Figure 4.14 suggested that the MAb concentration of 0.1 mg/mL is the optimal concentration for MAb immobilization. The data also implies that the density of MAb-modified substrates prepared from the concentration of 0.5 and 1 mg/mL may be so high that the binding of *V. harveyi* is no longer favorable. This leads to the next level of investigation on how to tailor the density of immobilized MAb more efficiently in order to improve the binding efficiency of the immunosensor. This research plans to do so by using self-assembly of mixed alkanethiols. The results are described in (c)

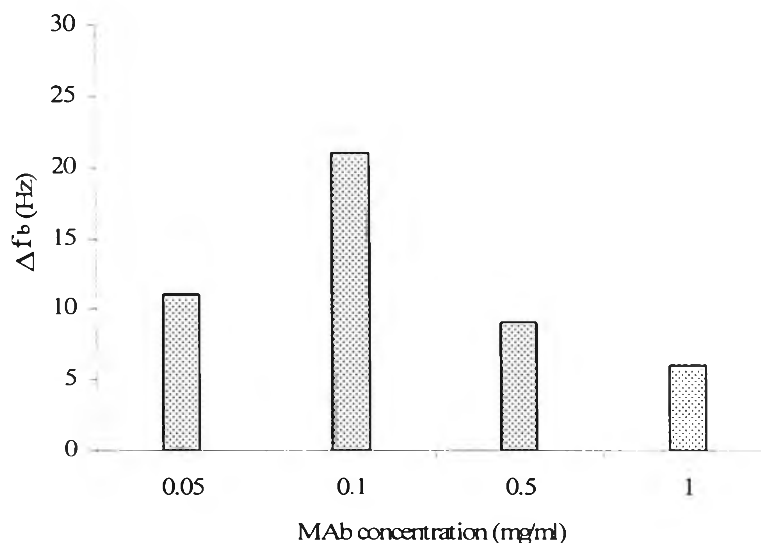

Figure 4.14 Frequency shift due to the *V. harveyi* binding ($\triangle f_b$) with the MAb-immobilized substrate as a function of MAb concentration used in the immobilization step after the treatment with 1%BSA.

(c) Mixed Self-assembled Monolayers

There are usually two alkanethiols used for the preparation of mixed SAMs. One carries a functional group to attach the probe molecule (MAb against *V. harveyi* in this case), while the other is used as 'dilution reagent' or 'blocking reagent' to control the density of reactive groups on the surface and/or to prevent non-specific adsorption of interfering species. This research used MPA as the thiol that carries the carboxyl group for MAb immobilization. Two functionalized alkanethiols were selected as the dilution agents. One is a hydroxyalkanethiol, 3-mercaptoethanol (ME) and the other is an aminoalkanethiol, cystamine (CE). The same stepwise reaction (shown in Figure 4.1) was used for the immobilization of MAb except that the mixed SAM (MPA+ME or MPA+CE) was used in stead of the single SAM (MPA). QCM was used for monitoring the MAb immobilization and *V. harveyi* binding.

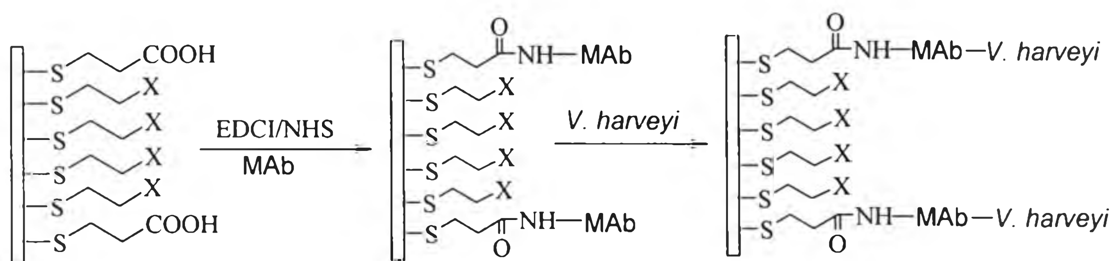

Figure 4.15 Schematic diagram showing the MAb immobilization and *V. harveyi* binding of the mixed SAMs; x = NH_2 for CE and x = OH for ME.

Figure 4.16 shows the frequency shift due to the MAb immobilization ($\triangle f_i$) together with the frequency shift due to *V. harveyi* binding ($\triangle f_b$) of the mixed MPA-CE SAM as a function of dilution ratio between MPA and CE. Interestingly, the dispersion of the carboxyl group of MPA in the mixed SAMs makes carboxylic group more accessible, and as a result mixed SAM can bind more MAb than the homogeneous SAM of MPA of which the carboxyl group is imbedded in a two-dimensional structure and therefore less accessible for activation and/or for immobilization of MAb. The amino group of CE not only performs as 'dilution reagent' to reduce the steric hindrance, but also is partly activated by NHS/EDCI and therefore it can supply additional binding sites for MAb. This helps explaining the continuous elevation of $\triangle f_i$ as the dilution ratio decreases. The subsequent binding of *V. harveyi* corresponds well with the MAb immobilization. The greater the $\triangle f_i$, the higher the $\triangle f_b$.

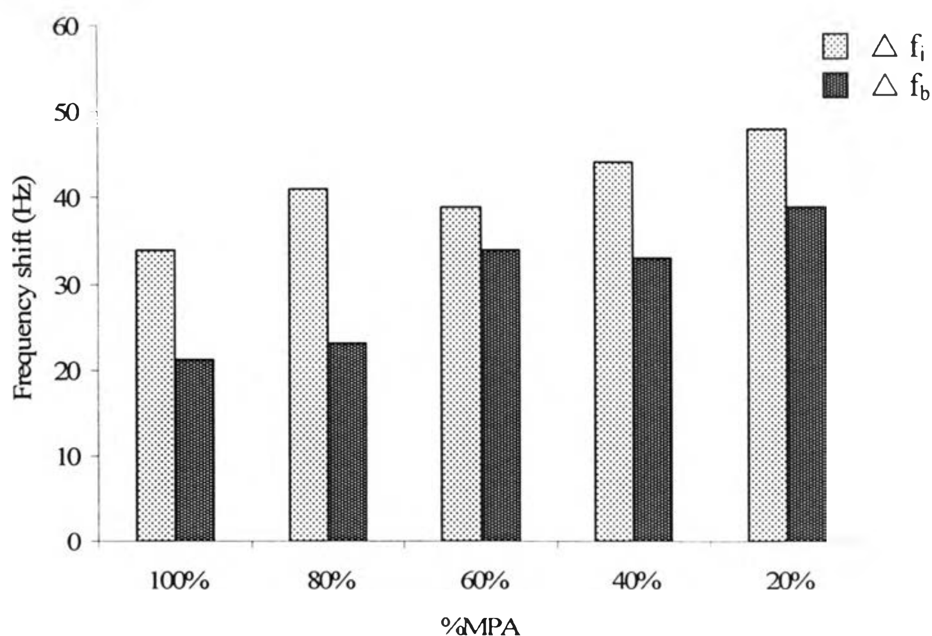

Figure 4.16 Frequency shifts due to the MAb immobilization ($\triangle f_i$) and the *V. harveyi* binding ($\triangle f_b$) of the MPA-CE mixed SAM as a function of dilution ratio (%MPA).

Figure 4.17 shows the frequency shift due to the MAb immobilization ($\triangle f_i$) together with the frequency shift due to *V. harveyi* binding ($\triangle f_b$) of the mixed MPA-ME SAM as a function of dilution ratio between MPA and ME. Unlike CE, the hydroxyl group of ME can only act as 'dilution reagent' and 'blocking reagent' but was not capable of providing additional sites for MAb immobilization. That is why only a certain dilution ratio (80%MPA:20%ME) can improve the MAb immobilization and the subsequent *V. harveyi* binding. Too much dilution led to the too low density of active binding sites for MAb immobilization. As a result, the immunosensor's sensitivity was decayed.

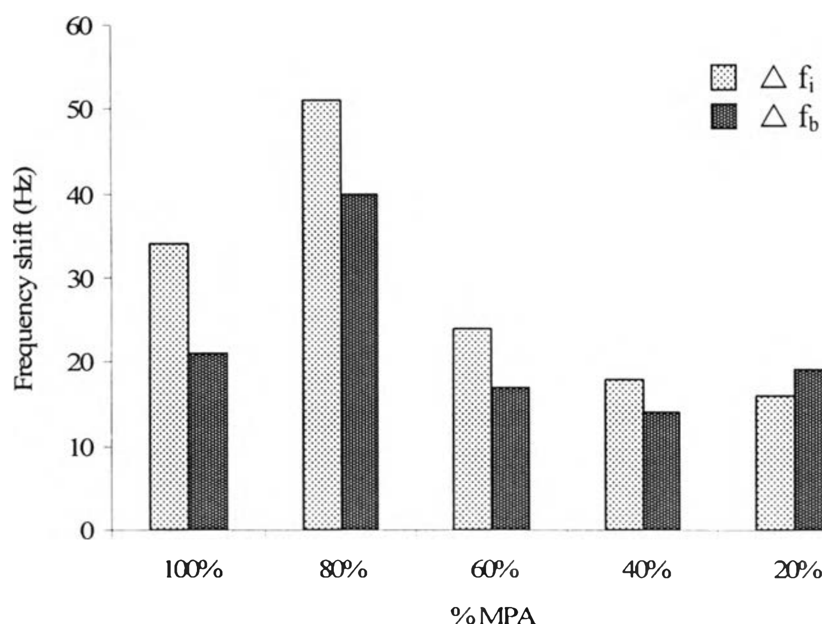

Figure 4.17 Frequency shifts due to the MAb immobilization ($\triangle f_i$) and the *V. harveyi* binding ($\triangle f_b$) of the MPA-ME mixed SAM as a function of dilution ratio (%MPA).

4.4.2 Confirmation of Monoclonal Antibody Immobilization and Bacteria Binding

Figure 4.18 displays RAIRS spectra of the gold-coated substrates obtained after the MAb immobilization and the *V. harveyi* binding in comparison with that bearing SAM of MPA. Evidently, the carbonyl stretching at ~1740 $cm^{-1}$of the MPA monolayer disappeared after the MAb immobilization and the *V. harveyi* binding. New signals from MAb and *V. harveyi* of C=O stretching (amide I) and N–H bending (amide II) emerged at ~1650 cm^{-1} and ~1540 cm^{-1}, respectively. Moreover, there was a weak signal of a broad peak corresponding to N-H and/or O-H stretching appearing in the range of 3000-3600 cm^{-1}. These data verified that MAb and *V. harveyi* have been successfully immobilized on the gold-coated substrates.

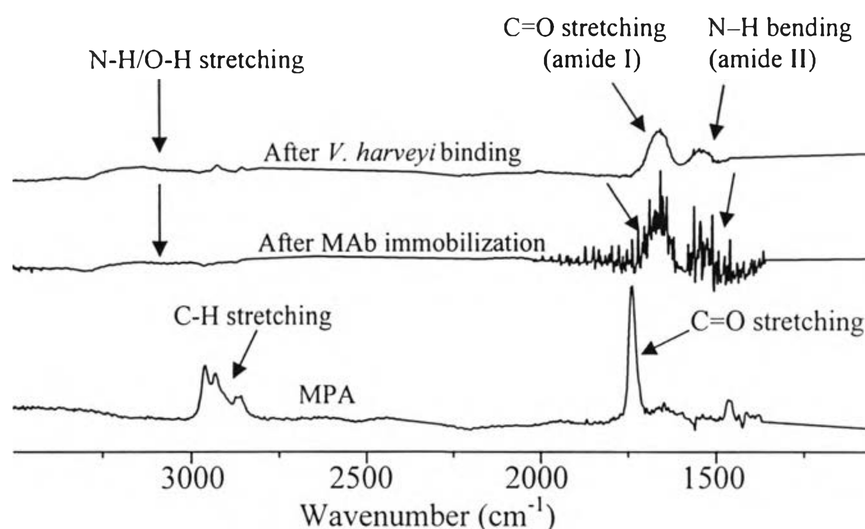

Figure 4.18 RAIRS spectra of the gold-coated substrates before and after MAb immobilization and *V. harveyi* binding.

4.4.3 Efficiency of the Immunosensor

The efficiency of immunosensor is generally expressed in terms of sensitivity, specificity, and regeneration. The results illustrated in Figure 4.19 suggested that this QCM-based immunosensor was quite sensitive and gave a working range of 10^3 to 10^7 CFU/mL. This value is comparable with the conventional technique like ELISA whose detection limit is 10^4 CFU/mL. The fact that the frequency shift is varied as a function of the *V. harveyi* concentration indicates that the quantification of *V. harveyi* in the working range is possible.

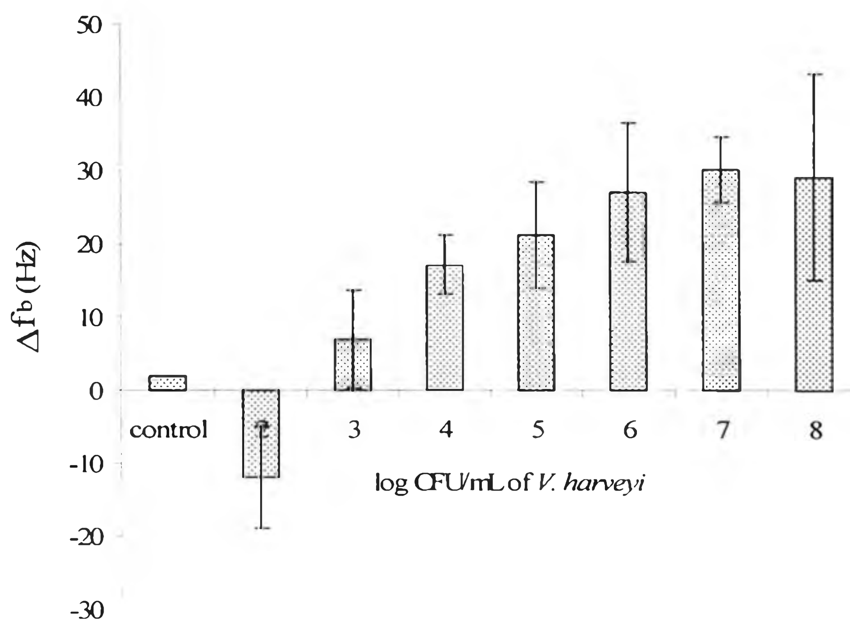

Figure 4.19 Frequency shift due to *V. harveyi* binding ($\triangle f_b$) of the MAb-immobilized substrate prepared from MPA monolayer as a function of *V. harveyi* concentration.

A major concern in the development of immunosensors is their specificity. Because quite often, related microorganisms with widely differing health effects were found together. To detect the specificity of the developed immunosensor, two kinds of non-specific species, *Vibrio vulnificus* (*V. vulnificus*) and *Vibrio paraheamolyticus* (*V. paraheamolyticus*) were used. Results from Figure 4.20 suggested that the sensor was specific to *V. harveyi* with a slight cross reactivity to *V. vulnificus*, but not specific to *V. paraheamolyticus*.

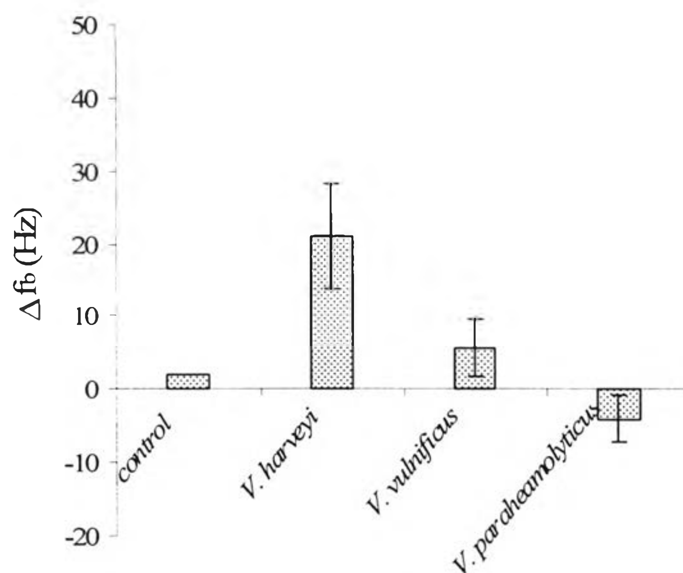

Figure 4.20 Frequency shift due to bacteria binding ($\triangle f_b$) on the MAb-immobilized substrate prepared from MPA monolayer.

The regeneration properties of the developed QCM-based immunosensor were investigated by rinsing the used probes in 0.1 M glycine/HCl buffer solution (pH 2.3) followed by washing with distilled water several times to desorb the bound bacteria. As demonstrated in Figure 4.21, the immunosensor can be partially regenerated using 60 min soaking time. The prolonged treatment in the acidic solution cannot only desorb the bacteria, but also perhaps further remove some of the immobilized MAb as can be seen from the increase of $\triangle f_b$ when 120 min was applied for the regeneration. In order to achieve a better regeneration efficiency of the sensor, others parameters (concentration, type of reagent) affecting the regeneration has to be thoroughly investigated.

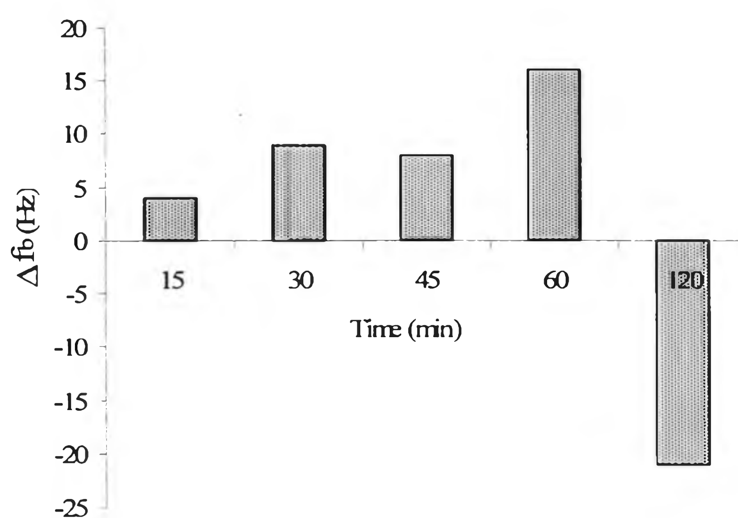

Figure 4.21 Frequency shift due to *V. harveyi* binding ($\triangle f_b$) of the MAb-immobilized substrate prepared from MPA monolayer after 1 cycle of regeneration in 0.1 M glycine/HCl buffer solution (pH 2.3) as a function of regeneration time.