Chapter 7

A Comparative Study of Capacitive Immunosensors Based on Self-Assembled Monolayers Formed From Thiourea, Thioctic Acid and 3-Mercaptopropionic Acid

7.1 Introduction

Capacitive immunosensor is based on the principle that for an electrolytic capacitor the capacitance depends on the thickness and dielectric behavior of a dielectric layer on the surface of a metal (Gebbert *et al.*, 1992). It can be constructed by immobilizing biorecognition elements in a thin layer on an electrode and measuring changes in the dielectric properties when an analyte binds to the biorecognition elements on the electrode, causing capacitance to decrease.

Immobilization is an important part in capacitive immunosensor since the electrode surface has to be electrically insulated. Different immobilization techniques have been developed and biorecognition elements can be immobilized on capacitive sensors via modified semiconductor surfaces (Barraud *et al.*, 1993; Bataillard *et al.*, 1988; 1991), metal oxides surfaces (DeSilva *et al.*, 1995; Gebbert *et al.*, 1992; 1994) and self-assembled monolayers (SAMs) of sulfur compounds on gold (Bain *et al.*, 1989; 1998; Berggren and Johansson, 1997; Bontidean *et al.*, 1998; 2000; Bontidean *et al.*, 2003; Hedström *et al.*, 2005; Jiang *et al.*, 2003; Mirsky *et al.*, 1997).

SAM is a particularly suitable immobilization technique for capacitive biosensor (Riepl *et al.*, 1999) since it allows electrochemical insulation of the surface of a working gold electrode. It is also an excellent immobilization technique for protein, it shields proteins from direct contact with solid surface, thus, reduces the risk of the sensing element denaturation (Wadu-Mesthrige *et al.*, 2000). Furthermore, the proteins, use as the sensing element, are immobilized through covalent binding and they can be exposed to a high or low pH, often uses in regeneration, leading to a reusable system (Frey and Corn, 1996). SAMs can be formed at room temperature by spontaneous adsorption of alkanethiol on gold surfaces (Nuzzo and Allara, 1983; Porter *et al.*, 1987) by the reaction of sulfide

$$RSH + Au \longrightarrow RS^{-}Au^{+}\cdot Au + \tfrac{1}{2} H_2$$

or disulfide

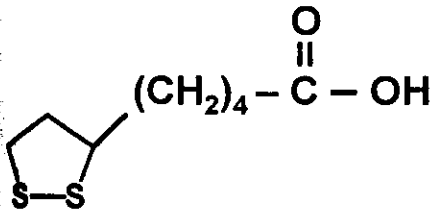
$$RS\text{-}SR + Au \longrightarrow RS^{-}Au^{+}\cdot Au$$

The affinity between sulfur and gold atoms is extremely high, resulting in the formation of SAMs that are highly stable in air, water, and organic solvents at room temperature (Bain *et al.*, 1989; Chaki and Vijayamohanan, 2002). They are also stable for a wide range of potential, from -400 to +1400 mV *vs* saturated calomel electrode in diluted sulphuric acid solution, which is especially significant for electrochemical sensing (Finklea *et al.*, 1987).

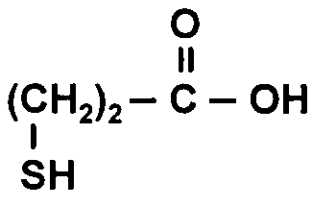
Capacitive biosensors have often been based on SAMs of thioctic acid (TA; $S_2C_7H_{13}$-CO_2H) (Berggren *et al.*, 1998; Berggren and Johansson, 1997; Disley *et al.*, 1998; Hedström *et al.*, 2005; Liu *et al.*, 1999) and 3-mercaptopropioic acid (MPA; $HSC_2H_4CO_2H$) (Disley *et al.*, 1998; Sawaguchi *et al.*, 2001; Vaughan, *et al.*, 1999). To immobilize proteins, the carboxylic groups of the SAMs were first activated with 1-ethyl-3-(3-diamino)propyl-carbodiimide (EDC) (Akram *et al.*, 2004; Berggren *et al.*, 1998; Berggren and Johansson, 1997; Hedström *et al.*, 2005), and sometimes together with a succinimide, *i.e.*, *N*-hydroxysulfosuccinimide (NHS) (Gooding and Hibbert, 1999; Staros *et al.*, 1986; Vaughan, *et al.*, 1999). Then the activated groups were exposed to protein solution where the activated electrophilic group attached to the primary amino group of amino acid residues, forming a new peptide bond between SAM and protein. SAM containing amine modified entities, such as 2-mercaptoethylamine (MEA; HSC_2H_4-NH_2), was also an effective surface to which protein could be immobilized (Jiang *et al.*, 2003). Glutaradehyde can also be used as it was introduced to react with the self-assembled MEA monolayer on the gold electrode to covalently immobilize the protein. However, only a few studies on capacitive immunosensors were reported based on the amine-modified SAM (Mirsky *et al.*, 1997).

Since, TA, MPA and MEA are rather expensive, an alternative cheaper thiol reagent, thiourea was investigated. Thiourea (TU; NH_2CSNH_2) was chosen because of its low environmental impact, easier handling of reagent and the fact that it is strongly adsorbed on gold (Holze and Schomaker, 1990; Ubaldini *et al.*, 1998). It has amino groups (R-NH_2) (Figure 7.1) that can be modified to covalently couple to the antibody. To our knowledge no one has applied it to immunosensors.

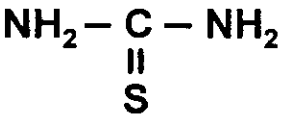
This project reports the development of a procedure for the immobilization of antibody to a gold surface modified with a SAM of thiourea. The performance was compared with that of the commonly used thioctic acid and 3-mercaptopropioic acid. Alpha-fetoprotein (AFP) and Anti-alpha-fetoprotein antibody (anti-AFP) were used as a model affinity pair. The evaluation of each method for immobilization was done using a flow injection capacitive immunosensor system. The comparison was done by observing several analytical parameters, such as sensitivity, linear range, limit of detection, specificity and reproducibility.



Thioctic acid, TA



3-Mercaptopropionic acid, MPA



Thiourea, TU

Figure 7.1. Structure of thiol compounds.

7.2 Materials

Anti-AFP and AFP from human fluids were obtained from Dako (Denmark). 3-mercaptopropionic acid, *N*-(3-Dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC) and *N*-Hydroxysuccinimide (*N*-Hydroxy-2,5-pyrrolidinedione, NHS) were obtained from Sigma-Aldrich (Steinheim, Germany), thioctic acid 98% and 1-dodecanethiol were obtained from Aldrich (Milwaukee, USA), and thiourea was obtained from BDH laboratory reagents (Poole, England). All other chemicals used were of analytical grade. All buffers were prepared with distilled water treated with a reverse osmosis-deionized system. Before use, the buffers were filtered through an Albet® nylon membrane filter (Albet, Spain), pore size 0.20 μm, with subsequent degassing.

7.3 Methods

7.3.1 Preparation of gold surface

Gold electrodes (Ø 3 mm, 99.99% purity) were polished (Gripo® 2V polishing machine, Metkon Instruments Ltd., Turkey) with alumina slurries (particle diameters 5, 1, and 0.30 μm) and then cleaned through sonication subsequentially in distilled water and absolute ethanol, 15 min each, to remove any physisorbed multilayer (Yang *et al.*, 1995). They were then washed in distilled water and dried with pure nitrogen gas. Each electrode was pretreated by electrochemical etching in 0.5 M H_2SO_4 solution by cycling potential from 0 to +1500 mV *vs.* Ag/AgCl reference electrode with a scan rate of 0.1 V s^{-1} for 25 scans. Finally they were dried with pure nitrogen gas.

7.3.2 SAMs formation

A cleaned gold electrode was immediately immersed in a thiol solution (thioctic acid, 3-mercaptopropionic acid or thiourea) at room temperature for a fixed period of time (see later) before being thoroughly rinsed with distilled water and dried with pure nitrogen gas. In this step self-assembled thioctic acid monolayer (SATAM), self-assembled 3-mercaptopropionic acid monolayer (SAMPAM), or self-assembled thiourea monolayer (SATUM) was formed on the gold surface.

A good formation of SAM on gold surface depends on both the time (Dubois and Nuzzo, 1992; Kim *et al.*, 1993; Wink *et al.*, 1997) and concentration of thiol solutions (Kim *et al.*, 1993; Liu *et al.*, 1999; Wink *et al.*, 1997). The effects of these factors were investigated. The optimization of the immersion time was studied by immersing cleaned gold electrodes in 100 mM of thiol reagents for 0, 0.25, 0.50, 1, 3, 6, 12, 24, 36 and 48 h. The concentrations of thiol solutions were then optimized by immersing cleaned gold electrodes in thioctic acid and 3-mercaptopropionic acid for 12 h (optimum time) and in thiourea solution for 24 h (optimum time) at concentrations of thiol solutions, 0, 10, 25, 50, 100, 250, and 500 mM.

7.3.3 Immobilization of anti-AFP

Anti-AFP, used as the sensing element, was immobilized on SATAM and SAMPAM through both non-covalent (*i.e.*, electrostatic) and covalent bindings. For SATUM the anti-AFP was only immobilized through covalent binding.

For non-covalent immobilization of anti-AFP on SATAM and SAMPAM cleaned gold electrode surface was modified with 250 mM of TA and MPA for 12 h. Then 20 µl of 0.5 mg ml^{-1} of anti-AFP in phosphate buffer, pH 4.50 was placed on the modified surface and left for the reaction to take place overnight at 4°C (Figure 7.2(1.1) and Figure 7.3(1.1)). Finally, the electrode was reacted in a 10 mM 1-dodecanethiol ethanolic solution for 20 min to block any pinholes or bare spots on the electrode surface (Figure 7.2(1.2) and Figure 7.3(1.2)).

For covalent method, the carboxylic group of SATAM and SAMPAM were activated for an amine reaction for 5 h by using 0.05 M of EDC in phosphate buffer (pH 5.00) (Figure 7.2(2.1.1) and Figure 7.3(2.1.1)) or 0.05 M of EDC with 0.03 M of NHS in phosphate buffer (pH 5.00) (Figure 7.2(2.1.2) and Figure 7.3(2.1.2)) for 5 h (Johnsson *et al.*, 1991; Staros *et al.*, 1986) and then rinsed with 10 mM sodium phosphate buffer, pH 7.00 and dried. Then 20 µl of 0.5 mg ml^{-1} of anti-AFP was placed on the electrode and reaction took place overnight at 4°C (Figure 7.2(2.2) and Figure 7.3(2.2)). Finally, the electrode was reacted in a 10 mM 1-dodecanethiol ethanolic solution for 20 min (Figure 7.2(2.3) and Figure 7.3(2.3)) to block the bare spots on the electrode surface.

In the case of SATUM, glutaraldehyde was introduced to react with the SATUM on the gold surface. The amine groups will be modified and free aldehyde groups will be exposed to which protein can couple. The time and amount of glutaraldehyde that were suitable for the reaction were optimized. The optimum conditions were then used to treat the surface of gold electrode (Figure 7.4(1)), before being thoroughly rinsed with sodium phosphate buffer, pH 7.00 and dried. Then 20 µl of 0.5 mg ml^{-1} anti-AFP was placed on the electrode and reaction took place overnight at 4°C (Figure 7.4(2)). The electrode was then immersed in 0.1 M ethanolamine pH 8.00 for 30 min, this step was to occupy all the aldehyde groups which did not couple to the anti-CEA. Finally, the electrode was reacted in a 10 mM 1-dodecanethiol ethanolic solution for 20 min (Figure 7.4(3)) to block the bare spots on the electrode surface.

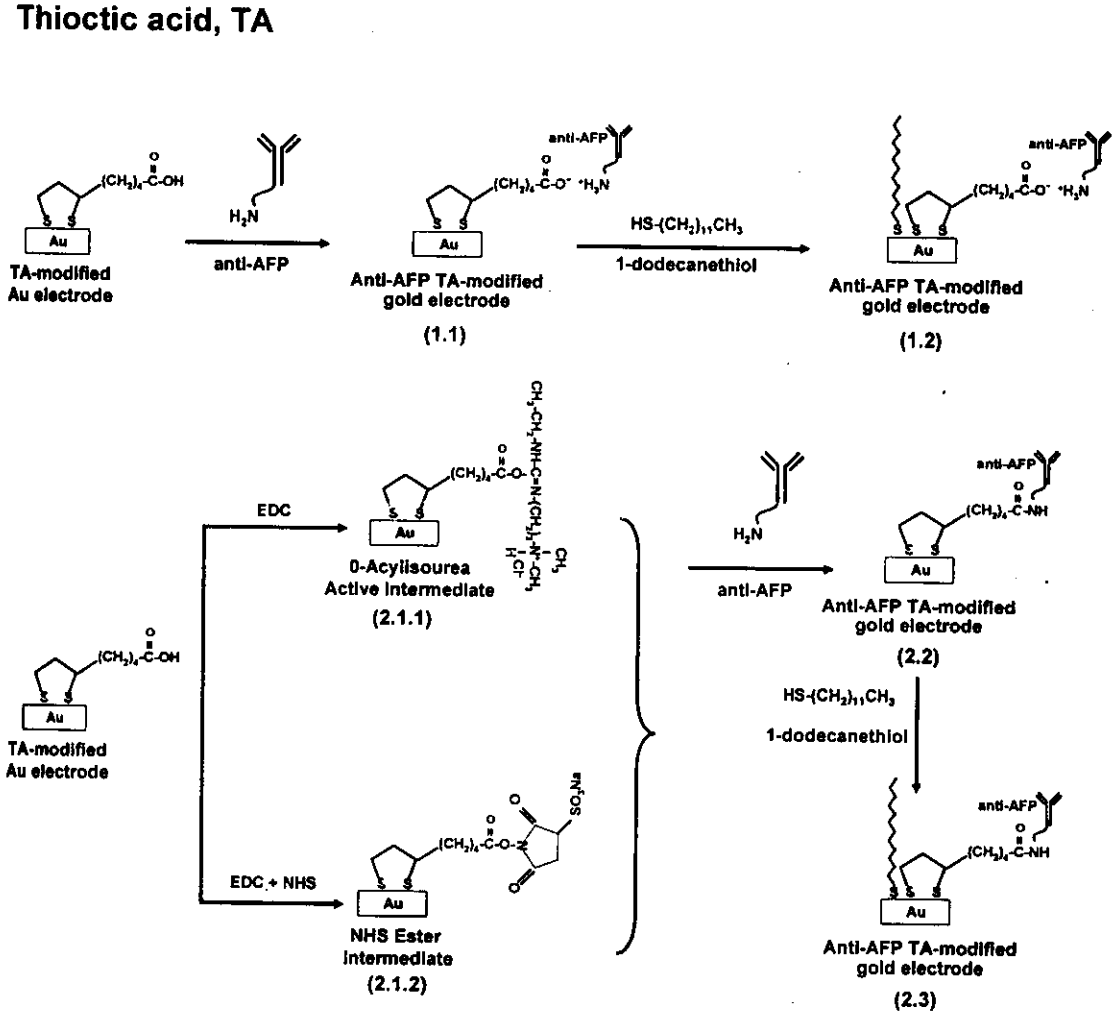


Figure 7.2 Reaction mechanism for the anti-AFP immobilized on a self-assemble thioctic acid monolayer.

3-Mercaptopropionic acid, MPA

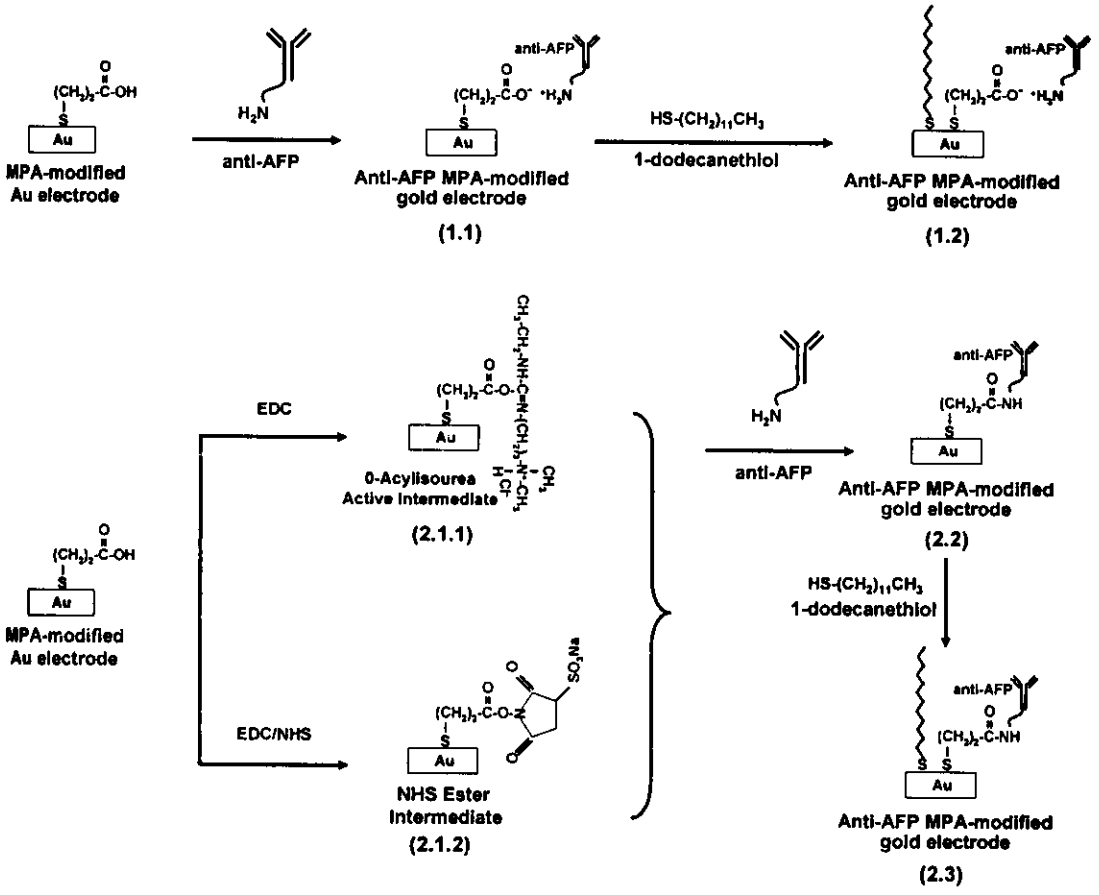


Figure 7.3 Reaction mechanism for the anti-AFP immobilized on a self-assemble 3-mercaptopropionic acid monolayer.

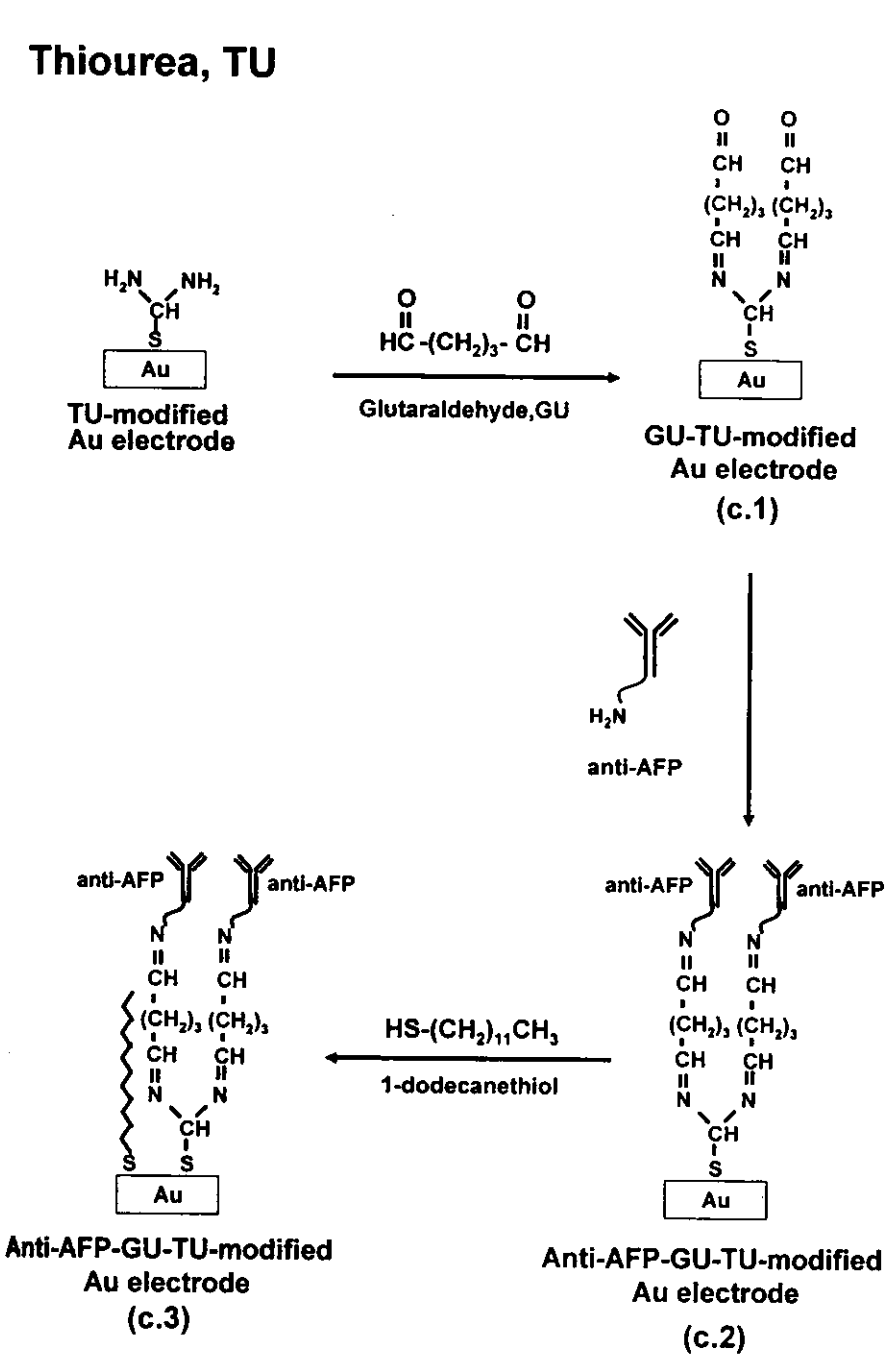


Figure 7.4 Reaction mechanism for the anti-AFP immobilized on a self-assemble thiourea monolayer.

7.3.4 Determination of the immobilization yield

The quantity of anti-AFP immobilized on SATAM, SAMPAM, and SATUM was determined by the difference between the concentration of the anti-AFP in the solution before and after immobilization. The amount of anti-AFP was determined by the silver binding method (Krystal, G., 1987; Krystal *et al.*, 1985; 1989). The advantages of this method over other methods are its sensitivity with lower limit of detection (ng of amounts of protein in solution), linear range of the assay for the majority of standard proteins tested between 15 and 2000 ng, and there is little or no interference from carbohydrates, nonionic detergents, or ethanol, and pretreatment of protein samples with Bio-Gel P-6 chromatography (BIO-RAD Laboratories, U.S.A.) for removing salt and other small interfering substances from protein samples.

For the measurement of anti-AFP sample, 5 µl of sample was diluted to 500 µl with distilled water containing sodium dodecyl solfate (SDS) (0.02%) and Tween 20 (0.4%) to prevent passive adsorption of silver onto glass and plastic surfaces. This solution was applied to Bio-Gel P-6 column, mounted on 12×75 mm polystyrene tube, was then centrifuged at 1,500 rpm for 5 min. Fifty µl of eluted sample was taken for assay, diluted to 1 ml with distilled water in microtubes. Then 20 µl of 2.5% glutaraldehyde was added to each sample and vortex-mixed for 2 seconds. This mixture was added with 200 µl of the ammoniacal silver solution (adding 1 ml of 4% (w/v) sodium hydroxide and 0.2 ml of concentrate ammonium hydroxide to 18.6 ml of distilled water, and then followed by the dropwise addition of 0.2% (w/v) silver nitrate) and vortex-mixed for another 2 seconds. The reaction tubes were allowed to sit at room temperature for 10 min then stopped the color development by adding 40 µl of the 30 mg/l sodium thiosulfate solution. Absorbances at 399 nm were measured in 1-mL glass cuvettes *vs.* reagent blank.

7.3.5 Capacitance measurement

Figure 7.5 shows the basic experimental set-up of the flow-injection capacitive immunosensor system. Three electrodes were placed in the immunosensor flow cell (10 µl) and connected to the potentiostat (ML 160, AD Instruments, Australia). The working electrode was the modified gold electrode. A stainless steel tube (I.D. 0.4 mm, O.D. 1.1 mm, length 25 mm) was used as the auxiliary electrode and outlet. A lab built Ag/AgCl reference electrode was placed opposite to the working electrode.

Continuously during the binding event between AFP and anti-AFP, 50 mV potential pulses are applied to the gold electrode yielding current response signals as described in section 3.6.3.4 (equation 14) where the total capacitance (C_{total}) at the working electrode/solution interface can be obtained. The measurement of C_{total} was done every minute and the results were later plotted as a function of time. When the solution containing AFP was injected into the flow cell, AFP bound to the immobilized anti-AFP on the electrode causing the capacitance to decrease until it reached a stable value. The change in capacitance due to the binding was obtained by subtracting C_{total} after the binding from the C_{total} before the binding. The surface of the electrode was then regenerated with 10 mM glycine-HCl, pH 2.80 (Maupas *et al.*, 1997) to remove AFP from anti-AFP immobilized electrode.

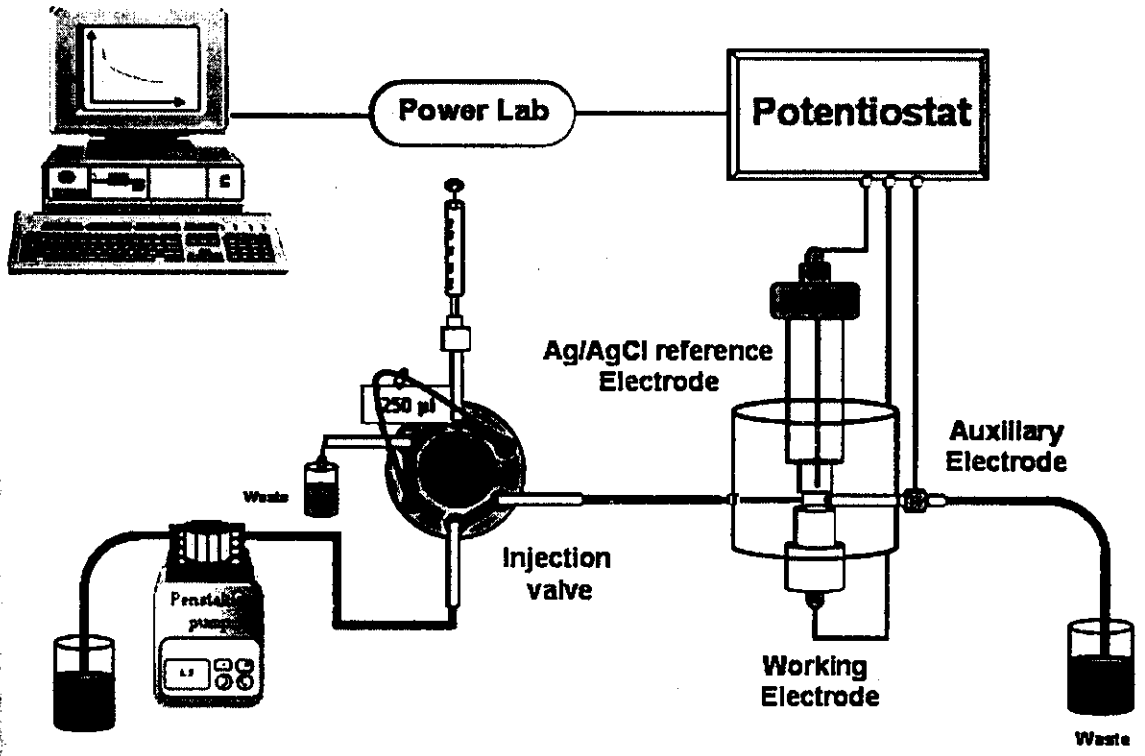


Figure 7.5 Schematic diagram showing the flow injection capacitive immunosensor system.

7.4 Results and discussion

7.4.1 Immersion times

The effect of immersion times of thiol solutions for the formation of SAMs on gold electrode surfaces was tested by using cyclic voltammetry technique in 0.1 H_2SO_4 at a scan rate of 100 mV s^{-1}. Figures 7.6 - 7.8 show examples of cyclic voltammograns for thioctic acid modified electrode after 0, 6 and 24 h, respectively. The efficiency of the formation of thiol SAM on a electrode surface can be described in term of surface coverage which can be estimated by comparing the area of the reduction peak of electro-adsoption of oxygen atom on the modified and bare gold electrode (Sabatani *et al.*, 1987). The percent surface coverage was calculated by using equation 1.

$$\% \text{ surface coverage} = \frac{(Q_{MGE} - Q_{BGE}) \times 100}{Q_{BGE}} \qquad (1)$$

Where Q_{MGE} is the amount of electric charge exchanged during the electroadsorption of oxygen (coulombs cm^{-2}) of the modified gold electrode, and Q_{BGE} is the amount of electric charge exchanged during the electroadsorption of oxygen (coulombs cm^{-2}) of the bare gold electrode. For TA and MPA, the percent surface coverage was found to increase with immersion up to 12 h (Figure 7.9). For longer immersion times, the percent coverage did not differ significantly ($P < 0.05$). For the TU modified gold electrode, the insignificant difference of percent surface coverage was obtained after 24 h. Therefore, 12 h is the optimum immersion time for TA and MPA while 24 h is used for TU.

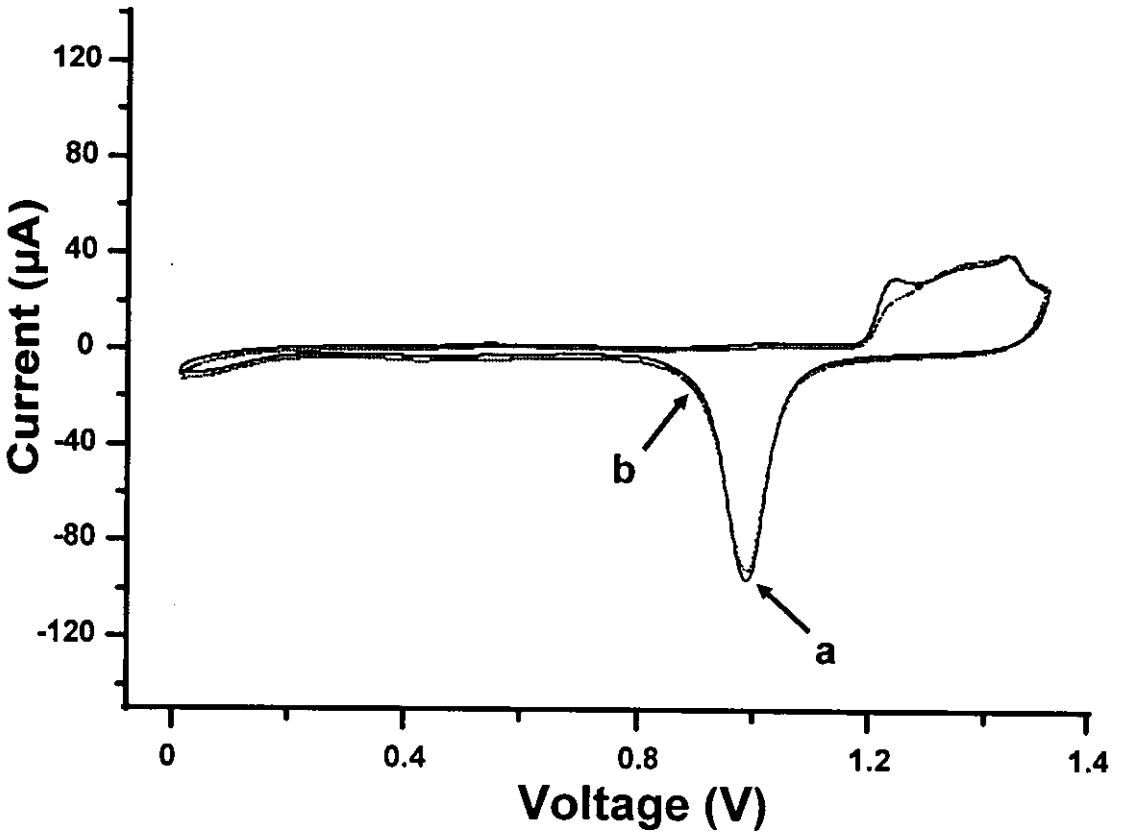


Figure 7.6 Cyclic voltammograms for bare gold electrode (a), thioctic acid modified electrode after 0 h (b). All scans were performed in 0.1 M H_2SO_4, with a scan rate of 100 mV s^{-1}.

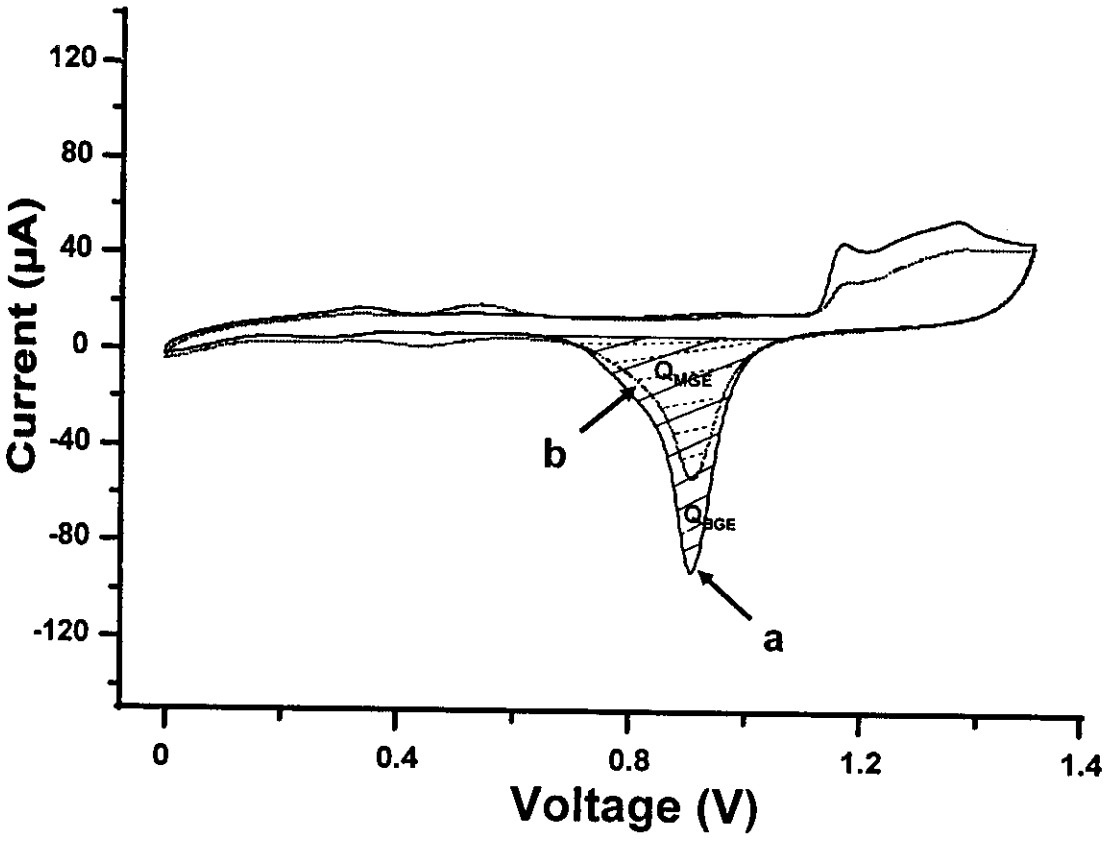


Figure 7.7 Cyclic voltammograms for bare gold electrode (a), thioctic acid modified electrode after 6 h (b). All scans were performed in 0.1 M H_2SO_4, with a scan rate of 100 mV s^{-1}. Q_{MGE} is the amount of electric charge exchanged during the electroadsorption of oxygen of the modified gold electrode, Q_{BGE} is the amount of electric charge exchanged during the electroadsorption of oxygen of the bare gold electrode.

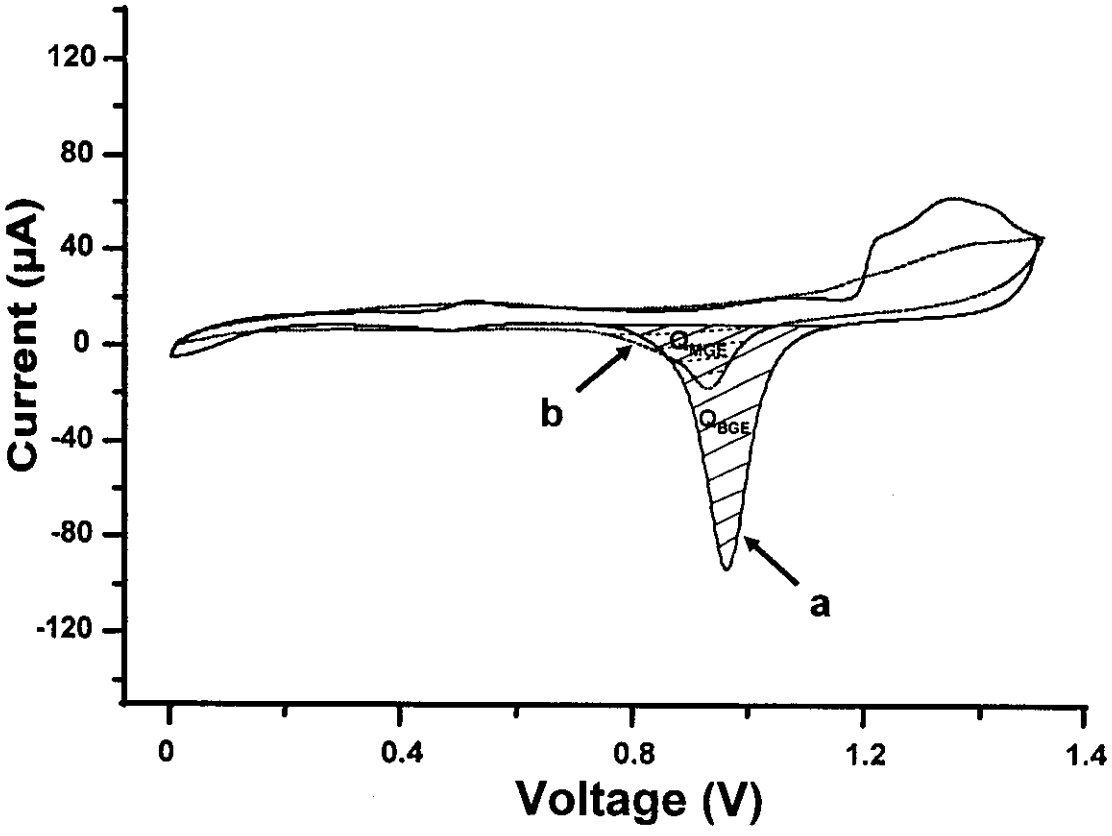


Figure 7.8 Cyclic voltammograms for bare gold electrode (a), thioctic acid modified electrode after 24 h (b). All scans were performed in 0.1 M H_2SO_4, with a scan rate of 100 mV s^{-1}. Q_{MGE} is the amount of electric charge exchanged during the electroadsorption of oxygen of the modified gold electrode, Q_{BGE} is the amount of electric charge exchanged during the electroadsorption of oxygen of the bare gold electrode.

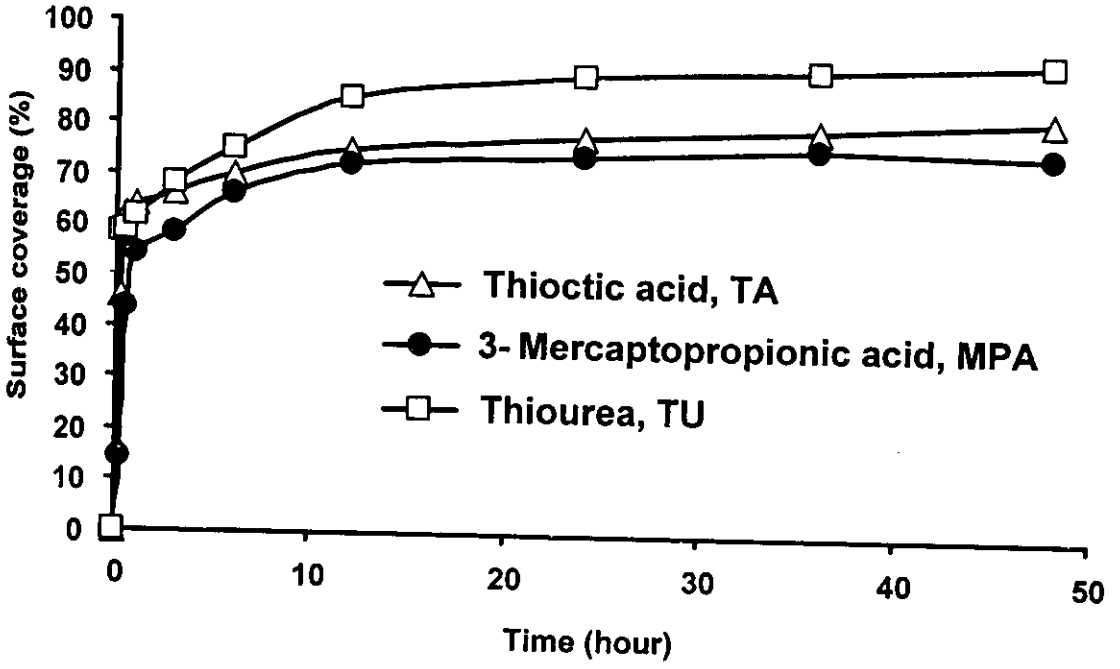


Figure 7.9 The effect of incubation times of thiol solutions for the formation of SAMs on gold electrode surfaces.

7.4.2 Concentration of thiol solutions

The effect of concentration of thiol solutions for the formation of SAMs on gold electrode surfaces were tested by considering the reductive desorption of these monolayers in thiol solutions via the reaction

$$RS\text{-}Au + e^- \longrightarrow Au + RS^-$$

At negative potentials the bond of thiol groups to gold can be reduced with consequent thiol desorption (Weisshaar *et al.*, 1992). Thiol solutions (TA, MPA and TU) were characterized making use of this property. A cyclic voltammogram of the reduction peak of thiolate monolayer in 0.5 M KOH indicated the desorption of thiol

bound to the gold electrode surface (Weisshaar *et al.*, 1993). Figure 7.10 shows an example of the cyclic voltammograms for the reduction of the Au-S bond of thioctic acid modified electrode. The charge under desorption peak can be used to estimate the surface concentration of the thiol solution in monolayer as follows (Bard and Faulkner, 2001; Wang, 2000):

$$\Gamma = \frac{Q}{nFA} \qquad (2)$$

Where Γ is surface coverage (mol cm^{-2}), Q is the total charge (coulombs), n is number of electron transferred, F is Faraday's constant (96,485.4 coulombs mol^{-1}), and A is the electrode surface area (cm^2). Figure 7.11 shows the effect of the concentration of thiol solutions for the formation of SAMs. The increase in surface coverage (mol cm^{-2}) started to level off close to 250 mM of all thiol solutions. Therefore, 250 mM of TA, MPA, and TU were chosen for the modification of the surface of gold electrodes. At this concentration, the surface coverage for monolayers of TA, MPA, and TU were 9.2×10^{-9} mol cm^{-2}, 8.6×10^{-9} mol cm^{-2}, and 8.9×10^{-9} mol cm^{-2}, respectively. These results indicated that the TA, MPA, and TU modified gold electrodes were monolayer because the values of surface coverage were in the range of 10^{-10} to 10^{-9} mol cm^{-2} (Bard and Faulkner, 2001).

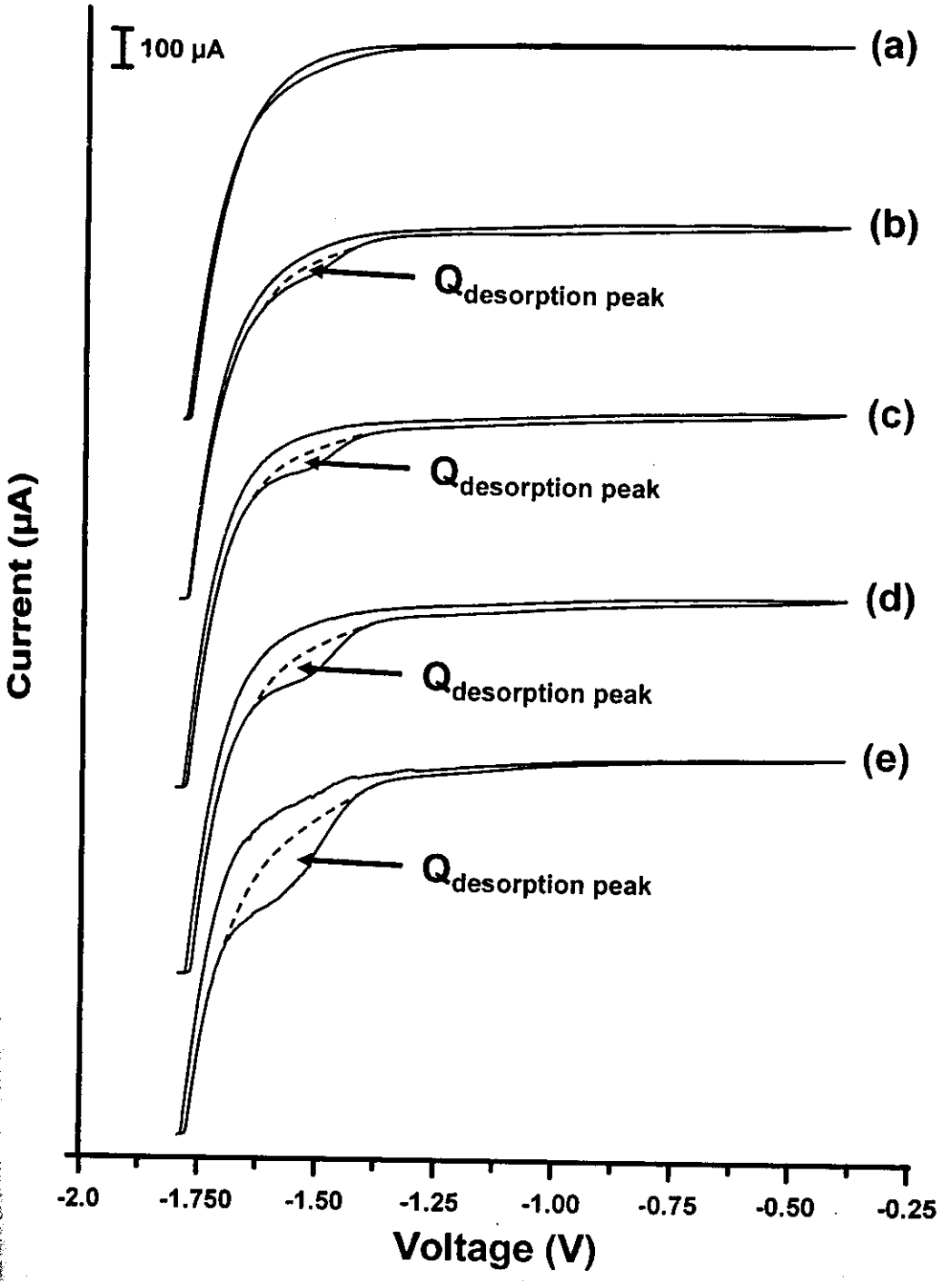


Figure 7.10 Cyclic voltammograms for the reduction of the Au-S bond, (a) bare gold electrode, (b) 10 mM thioctic acid modified electrode, (c) 50 mM thioctic acid modified electrode, (d) 100 mM thioctic acid modified electrode, (e) 250 mM thioctic acid modified electrode. All scans were performed in 0.1 M KOH with a scan rate of 100 mV s^{-1}.

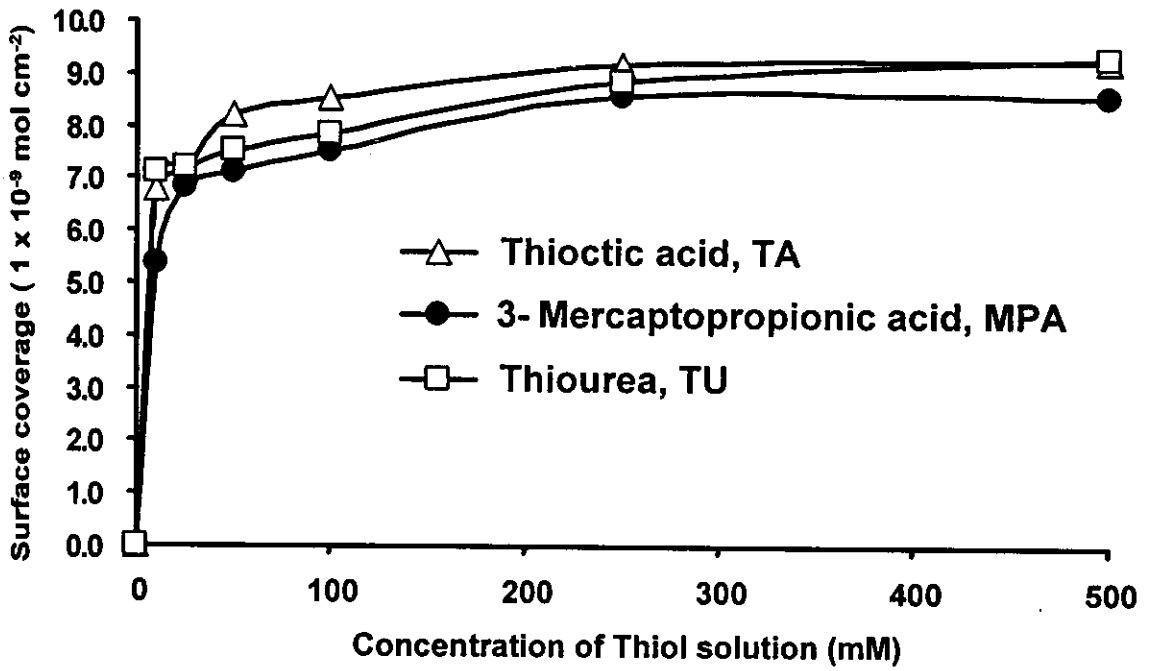


Figure 7.11 The effect of concentration of thiol solutions for the formation of SAMs on gold electrode surfaces.

7.4.3 Immobilization of anti-AFP

7.4.3.1 SATAM and SAMPAM

7.4.3.1.1 Electrostatic binding

For electrostatic binding of anti-AFP on SATAM and SAMPAM, standard AFP (0.01 μg ml^{-1}) was injected into the system, it bound to anti-AFP immobilized on the electrode causing the capacitance to decrease about 12 and 20 nF cm^{-2} for SATAM and SAMPAM, respectively. The electrode surface was then regenerated by 10 mM glycine-HCl, pH 2.80 to remove AFP from anti-AFP. However, after three times of regeneration, the response decreased by about 75% and 60% for SATAM and SAMPAM, respectively. This is because the force of the electrostatic binding between anti-AFP and SATAM, and SAMPAM is not strong

enough and the regeneration solution removed both the AFP and immobilized anti-AFP from the electrode surface. So, non-covalent method is not suitable for this system.

Although this method can not be reused, it gave good immobilized yield (tested by silver binding method (section 7.3.4)) of 76.7±0.4 % and 72.6±1.5 % for SATAM and SAMPAM, respectively. Therefore, if an analytical system does not require a reusable electrode, non-covalent method is one technique that can be applied.

7.4.3.1.2 Covalent binding

The immobilization of anti-AFP on SATAM, and SAMPAM using covalent method were done using EDC, and EDC/NHS solution as intermediate (Johnsson *et al.*, 1991; Staros *et al.*, 1986). The performances of these electrodes are shown in Table 7.1. The sensitivity of SATAM and SAMPAM activated with EDC/NHS were higher than those activated with EDC by about 1.5 times. This is correlated to the higher amount of the immobilized anti-AFP, which is shown as immobilization yield in Table 7.1, determined by detecting the amount of protein in the solution before and after immobilization by the silver binding method (section 7.3.4).

7.4.3.2 SATUM

The effect of glutaraldehyde was tested by determining the immobilization yield. The concentration was tested between 0.05 and 10 % (v/v). When the concentration of glutaraldehyde increased the immobilization yield also increased reaching a maximum at 5% (v/v) of glutaraldehyde and then leveled off (Figure 7.12(a)). For the effect of incubation time (1 to 240 min), the highest immobilization yield is at 20 min (Figure 7.12(b)). Therefore, 5% (v/v) glutaraldehyde and incubation time of 20 min were use for further experiments to activate the SATUM electrode in 10 mM sodium phosphate buffer pH 7.0 at room temperature. Anti-AFP was then immobilized on the activated electrode.

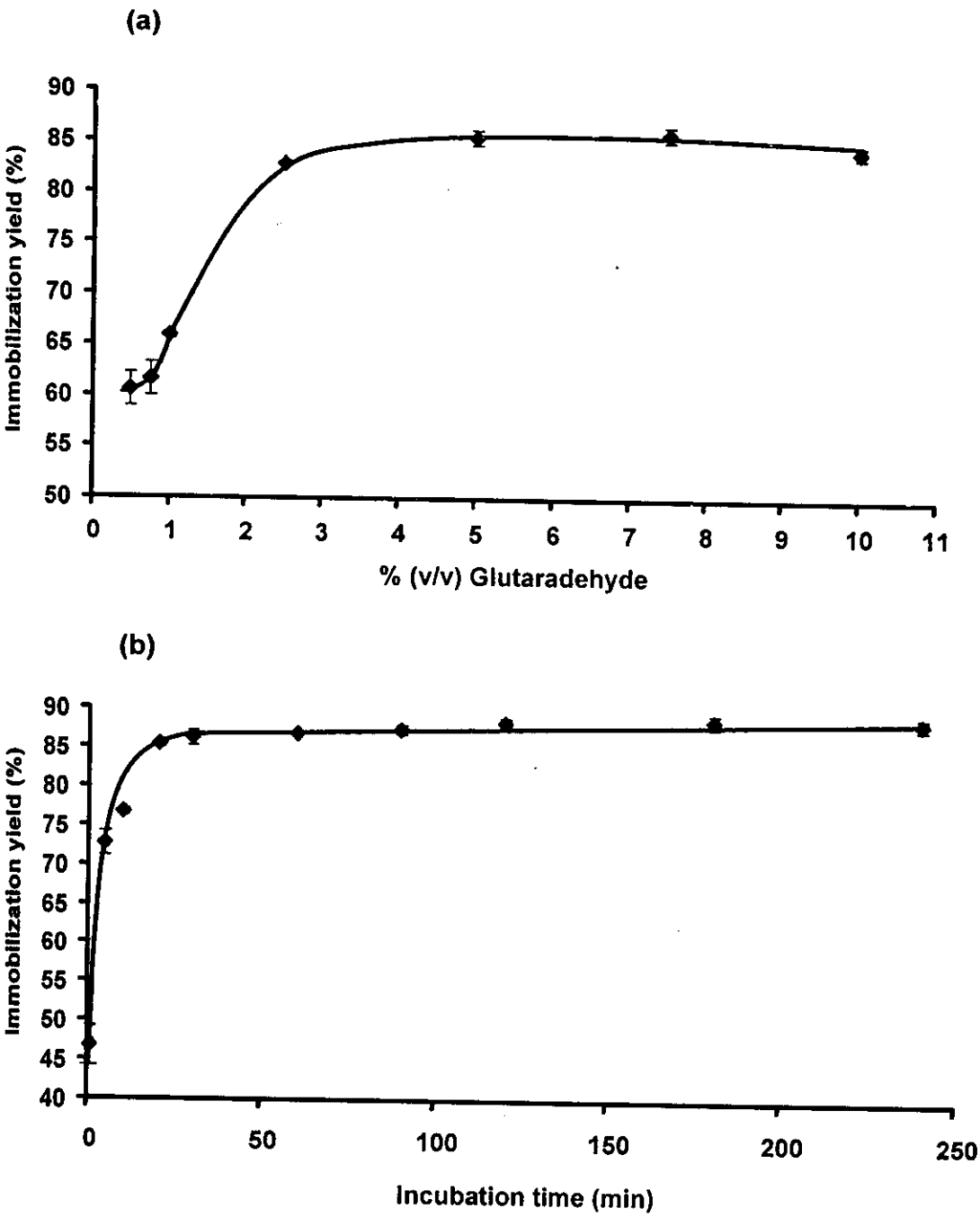


Figure 7.12 The effect of the concentration (a) and incubation times (b) of glutaraldehyde to activated self-assemble thiourea monolayer for anti-AFP immobilization.

Table 7.1 Performances of anti-AFP covalently immobilized on self-assembled thioctic acid monolayer (SATAM), self-assembled 3-mercaptopropionic acid monolayer (SAMPAM), and self-assembled thiourea monolayer (SATUM). (NA: not applicable)

Parameters	Performances		
	SATAM	SAMPAM	SATUM
Immobilization yield (%), (n=3)			
- activated with EDC	65.8 ± 0.2	60.5 ± 1.6	NA
- activated with EDC/NHS	88.6 ± 0.8	88.3 ± 0.6	NA
- activated with glutaraldehyde	NA	NA	88.5 ± 0.8
Linear range ($\mu g\ l^{-1}$)			
- activated with EDC	0.01 to 10	0.01 to 10	NA
- activated with EDC/NHS	0.01 to 10	0.01 to 10	NA
- activated with glutaraldehyde	NA	NA	0.01 to 10
Limit of detection ($ng\ l^{-1}$)			
- activated with EDC	10	10	NA
- activated with EDC/NHS	10	10	NA
- activated with glutaraldehyde	NA	NA	10
Sensitivity ($\Delta c(-nF\ cm^{-2})/\log AFP(\mu g\ l^{-1})$)			
- activated with EDC	21.3	16.9	NA
- activated with EDC/NHS	32.2	24.5	NA
- activated with glutaraldehyde	NA	NA	28.7
Analysis time (min)			
- activated with EDC	13-15	13-15	NA
- activated with EDC/NHS	13-15	13-15	NA
- activated with glutaraldehyde	NA	NA	13-15

7.4.4 Electrochemical performance of the process of Anti-AFP immobilization

In the capacitive immunosensor system the insulating property of the self-assembled monolayer on the electrode surface is of vital importance. The degree of insulation was examined using cyclic voltammetry with a permeable redox couple (*i.e.*, $K_3[Fe(CN)_6]$) in an electrolyte solution. Figure 7.13 shows the cyclic voltamograms for SATAM, and SAMPAM, respectively. At the cleaned gold surface the redox couple was oxidized and reduced according to curve a. (Figure 7.13(A), and (B)). Then, the redox peaks decreased due to steps of modified SAMs and immobilization of anti-AFP on electrode surface, the insulating property of the electrode surface was further increased. A final capping of the electrode surface was achieved by the treatment with 1-dodecanethiol, as can be seen from the disappearance of the redox peaks (curve d Figure 7.13(A) and (B)).

For SATUM (Figure 7.14), at the cleaned gold surface the redox couple was oxidized and reduced according to curve a. When thiourea was self-assembled on the clean gold surface the redox peaks decreased (curve b.). Then aldehyde group of glutaraldehyde was reacted with the amine and the anti-AFP was linked covalently on the electrode via reaction with the aldehyde group. The insulating property of the electrode surface was further increased (curve c and d). A final capping of the electrode surface was achieved by the treatment with 1-dodecanethiol, as can be seen from the disappearance of the redox peaks in curve e.

All cyclic voltammograms for SATAM, SAMPAM, and SAMPAM (Figure 7.13 and 7.14) indicated that all these modified surface were well insulated and were suitable for the capacitive measuring system.

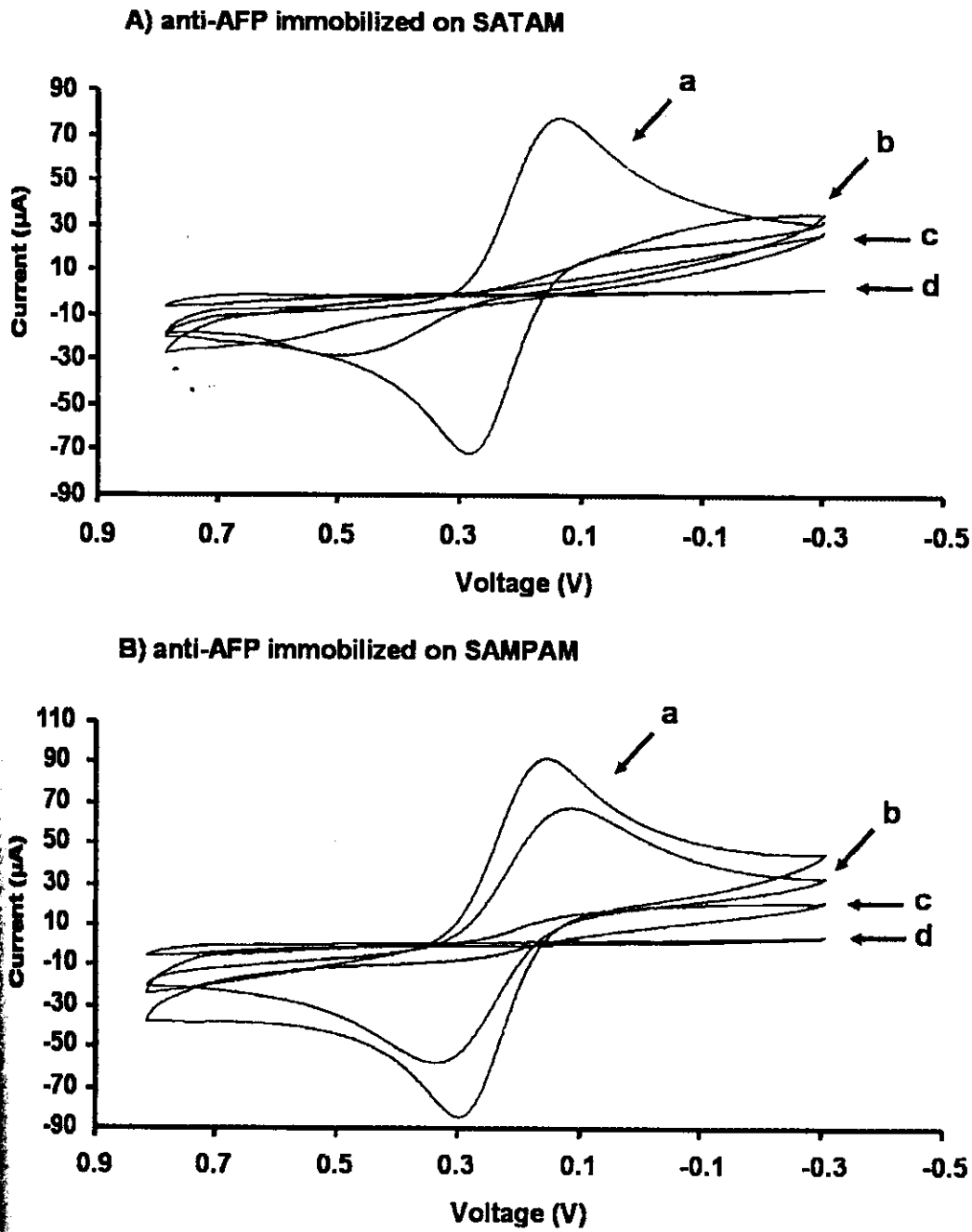


Figure 7.13 Cyclic voltammograms of a gold electrode obtained in a 5 mM $K_3[Fe(CN)_6]$ containing 0.1 M KCl solution at scan rate of 0.1 V s^{-1}. All potentials are given *vs.* Ag/AgCl reference electrode. The voltage range was -0.3 to 0.8 V. (a) clean gold electrode, (b) self-assembled thiol monolayer electrode, (c) anti-AFP self-assembled thiol monolayer electrode, and (d) as in (c) but after 1 dodecanethiol treatment.

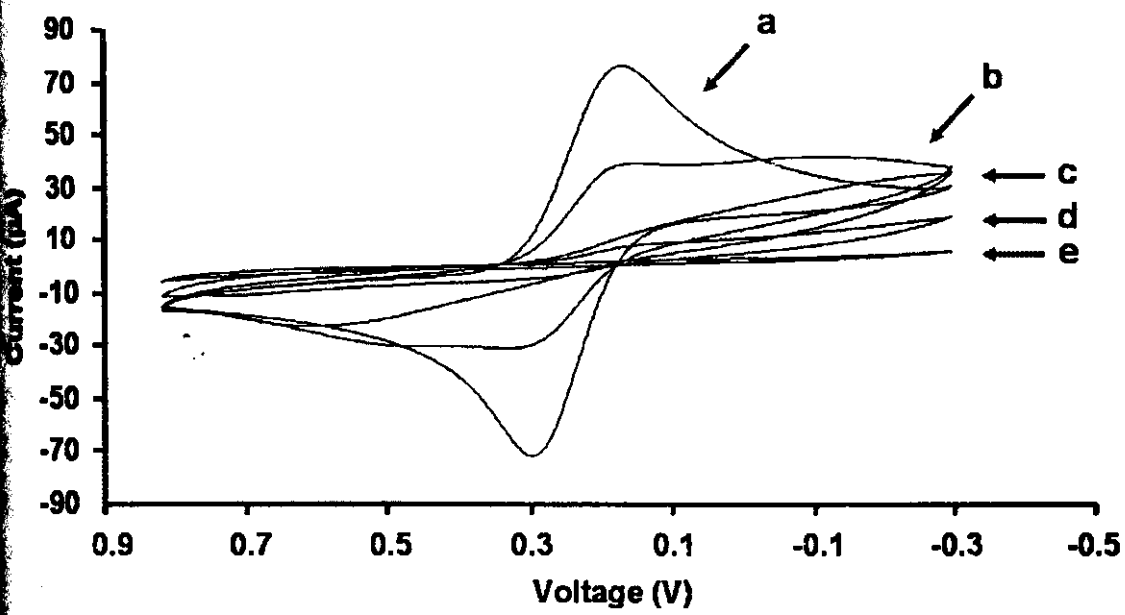


Figure 7.14 Cyclic voltammograms of a gold electrode obtained in a 5 mM $K_3[Fe(CN)_6]$ containing 0.1 M KCl solution at scan rate of 0.1 V s^{-1}. All potentials are given *vs.* Ag/AgCl reference electrode. The voltage range was -0.3 to 0.8 V. (a) Clean gold electrode, (b) self-assembled thiourea monolayer (SATUM) electrode, (c) glutaraldehyde-amine SATUM, (d) anti-AFP glutaraldehyde-amine SATUM, and (e) as in (d) but after 1-dodecanethiol treatment.

7.4.5 Linear range and detection limit

When AFP was injected into the flow cell, it bound to the immobilized anti-AFP on the electrode causing the capacitance to decrease. Discrete pulse injections of AFP standard ranging from 0.001-100 μg l^{-1} with intermediate regeneration steps using glycine-HCl buffer solution, pH 2.80 were performed. Figures 7.15 - 7.17 show the calibration curves for 250 μl injection into 10 mM Tris-HCl buffer solution, pH 7.60 at a flow rate of 0.10 ml min^{-1} for SATAM, SAMPAM, and SATUM, respectively. A linear relationship between the capacitance change and logarithm of AFP concentration of SATAM (r = 0.999), SAMPAM (r = 0.999), and SATUM (r = 0.996) were obtained between 0.01 and 10 μg l^{-1}. The detection limits of SATAM, SAMPAM and SATUM were 10 ng l^{-1}, based on IUPAC Recommendation 1994 (section 4.2) (Buck and Lindner, 1994).

7.4.6 Selectivity

The effect of substances that might interfere with the response of the AFP capacitive biosensor system was also studied. Human serum albumin (HSA) and carcinoembryonic antigen (CEA) were used to test the selectivity of the capacitive biosensor system for AFP, because the physical and chemical characteristics of albumin are similar to AFP (Bader *et al.*, 2004) and CEA, like AFP, is one type of the tumor markers. The capacitance changes due to HSA and CEA at the concentration range 0.01-10 μg l^{-1} were much lower than those from AFP (Figures 7.15 - 7.17) and lower than the detection limit of AFP. A much higher concentration of HSA, 68 mg l^{-1}, was also tested and the capacitance change was also much lower than the value at the detection limit for AFP. From these results, we suggested that these systems were selective to AFP.

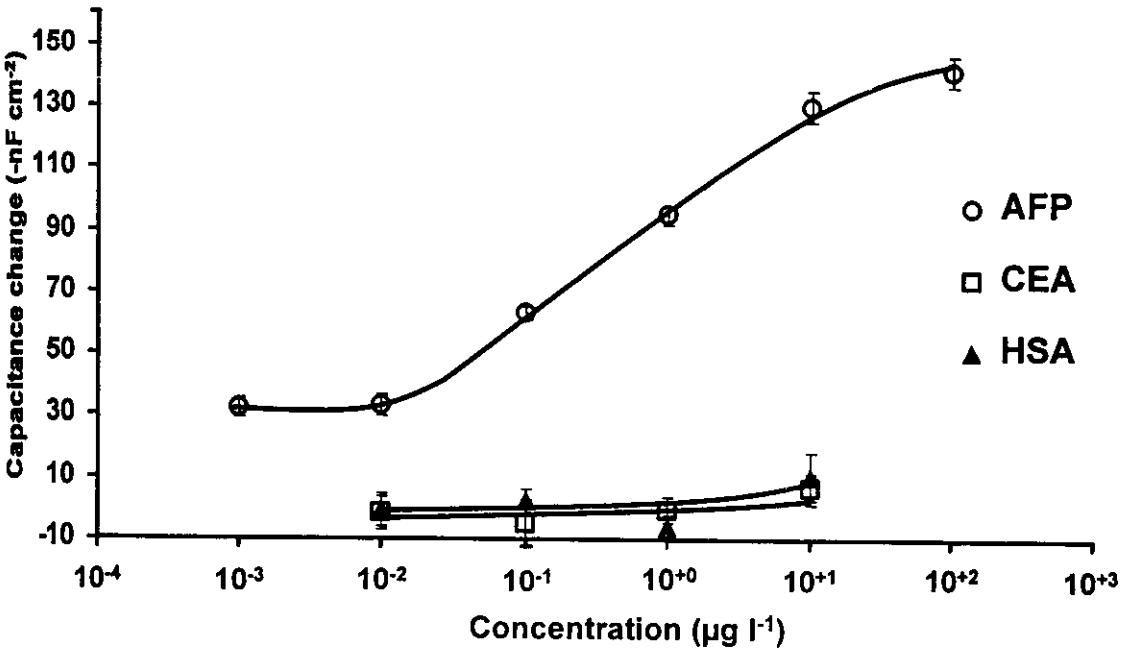


Figure 7.15 Responses of the anti-AFP to Alpha-fetoprotein (AFP), Carcinoembryonic antigen (CEA) and Human serum albumin (HSA) using self-assemble thioctic acid monolayer (SATAM).

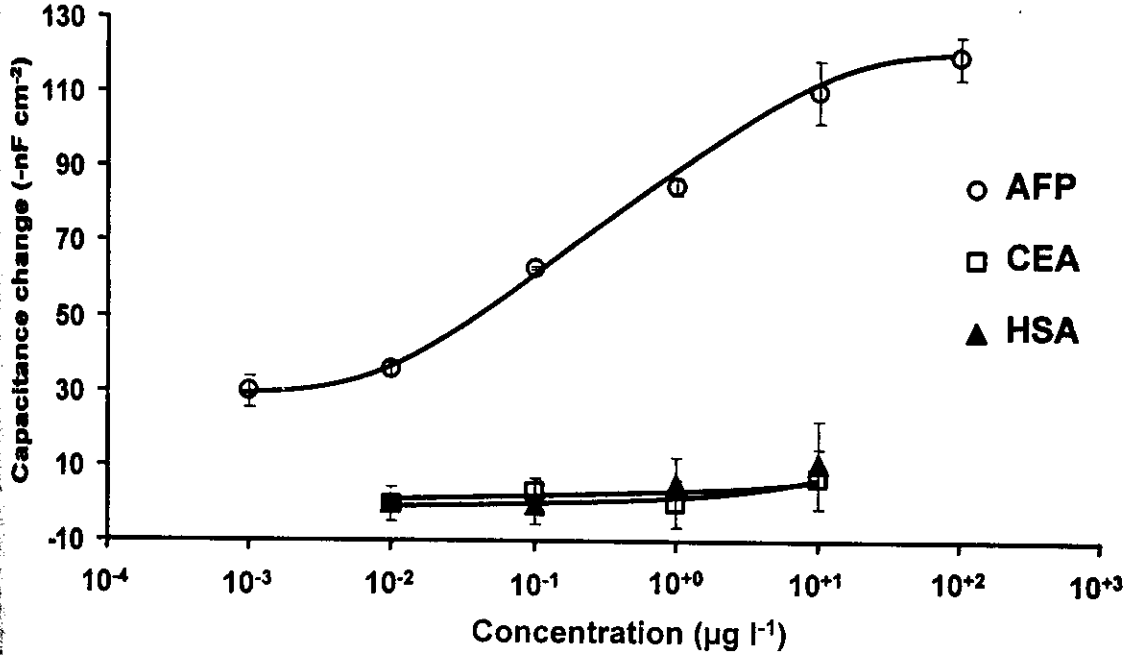


Figure 7.16 Responses of the anti-AFP to Alpha-fetoprotein (AFP), Carcinoembryonic antigen (CEA) and Human serum albumin (HSA) using self-assemble 3-mercaptopropionic acid monolayer (SAMPAM).

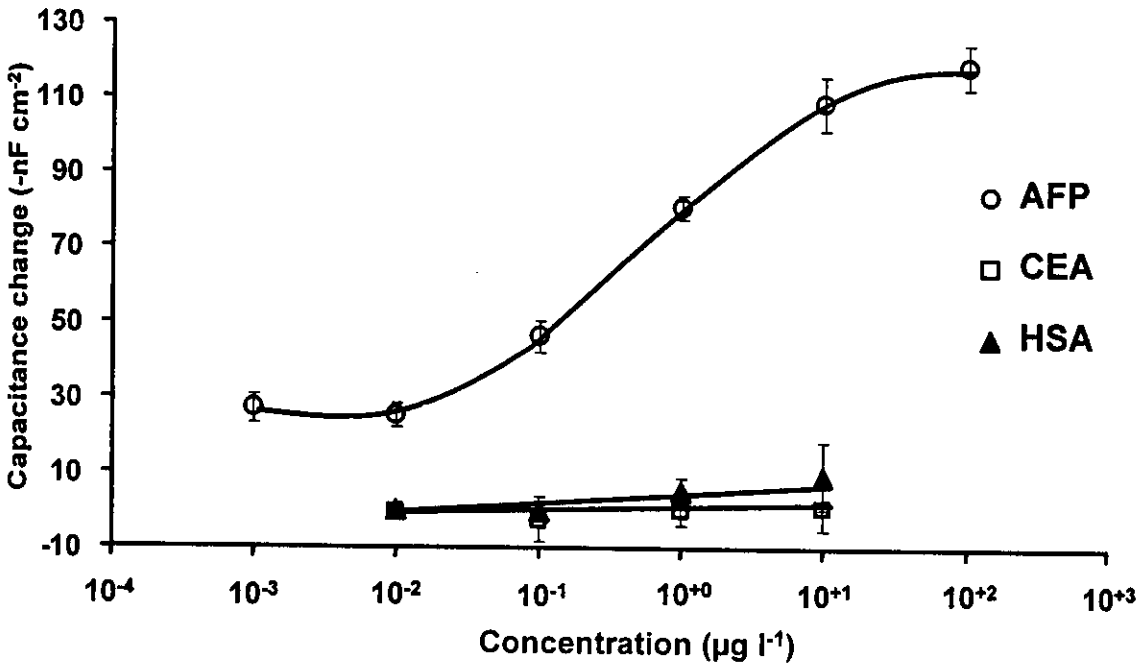


Figure 7.17 Responses of the anti-AFP to Alpha-fetoprotein (AFP), Carcinoembryonic antigen (CEA) and Human serum albumin (HSA) using self-assemble thiourea monolayer (SATUM).

7.4.7 Reproducibility

In this work, 10 mM glycine-HCl buffer solution, pH 2.80 (Maupas *et al.*, 1997), was used as regeneration solution to break the affinity binding between AFP and anti-AFP. AFP was detected by regenerating the electrode intermittently over 6 days (12 times per day). The reproducibility performance of anti-AFP-modified electrodes were evaluated by monitoring the change of capacitance signal at the same concentration of standard AFP (1 $\mu g\ l^{-1}$) at a flow rate of 0.10 ml min^{-1} Tris-HCl buffer solution, pH 7.60 and a sample volume of 250 μl.

Figures 7.18 - 7.20 show the percentage of capacitance change (-nF cm^{-2}) versus the cycles of regeneration. After 4 days or 48 times of regeneration the binding activity of anti-AFP immobilized on SATAM (Figure 7.18), SAMPAM (Figure 7.19), and SATUM (Figure 7.20) retained about 92%, 93%, and 91%, respectively, of the original capacitance change signal. That is, anti-AFP immobilization on SATAM, SAMPAM, and SATUM electrodes can be reused with good reproducibility upto 48 times with the relative standard deviation (RSD) of 4.2 %, 4.3 %, and 3.6 %, respectively.

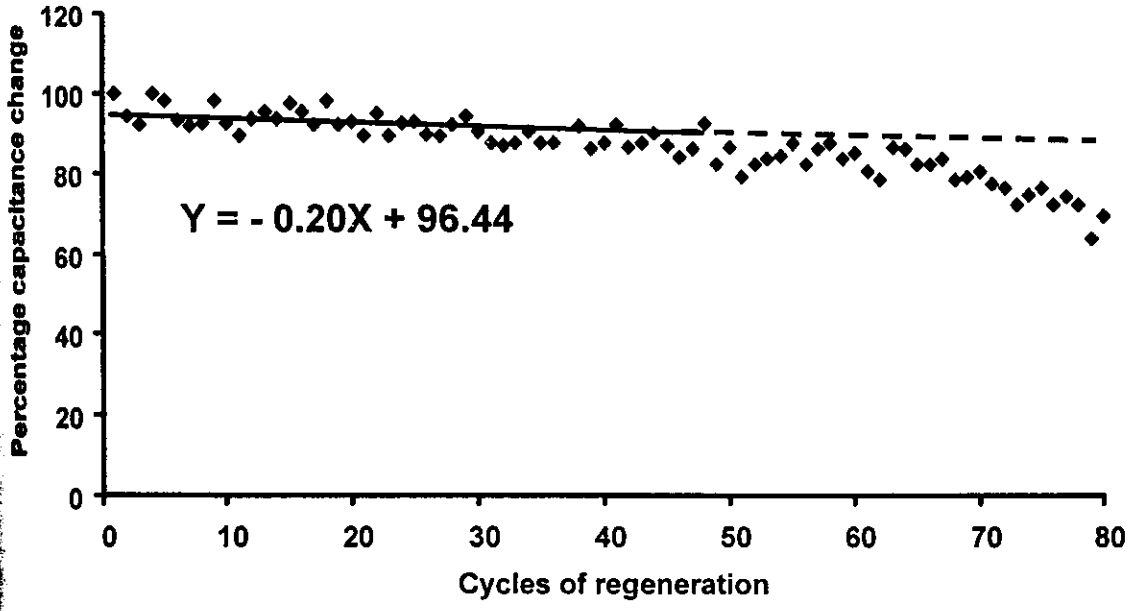
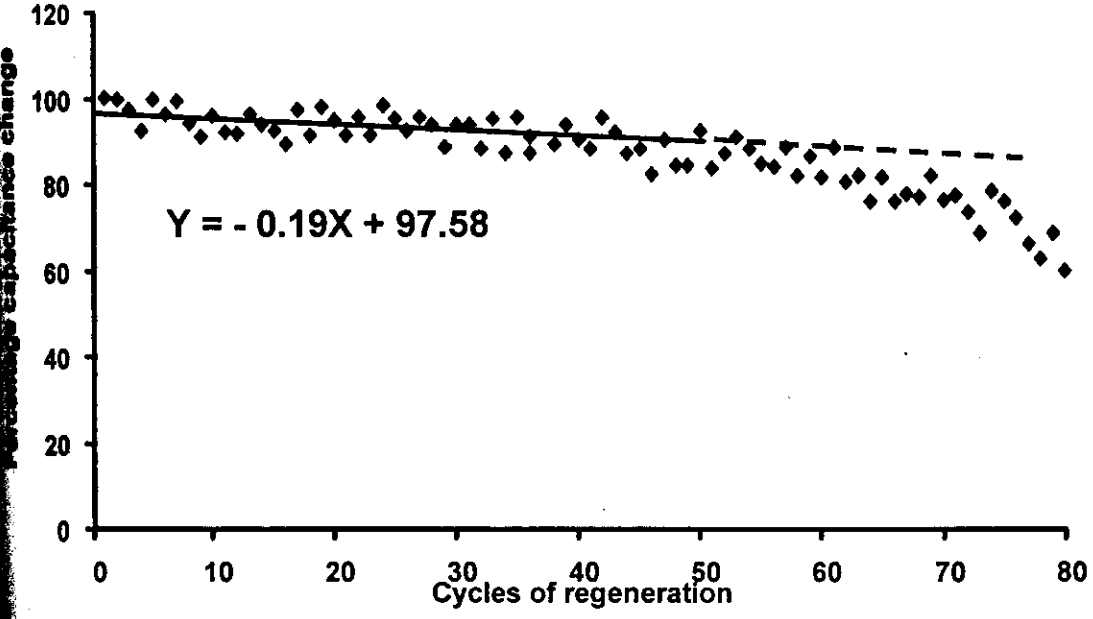


Figure 7.18 The reproducibility of the anti-AFP on self-assemble thioctic acid monolayer (SATAM).



gure 7.19 The reproducibility of the anti-AFP on self-assemble 3-mercaptopropionic acid monolayer (SAMPAM).

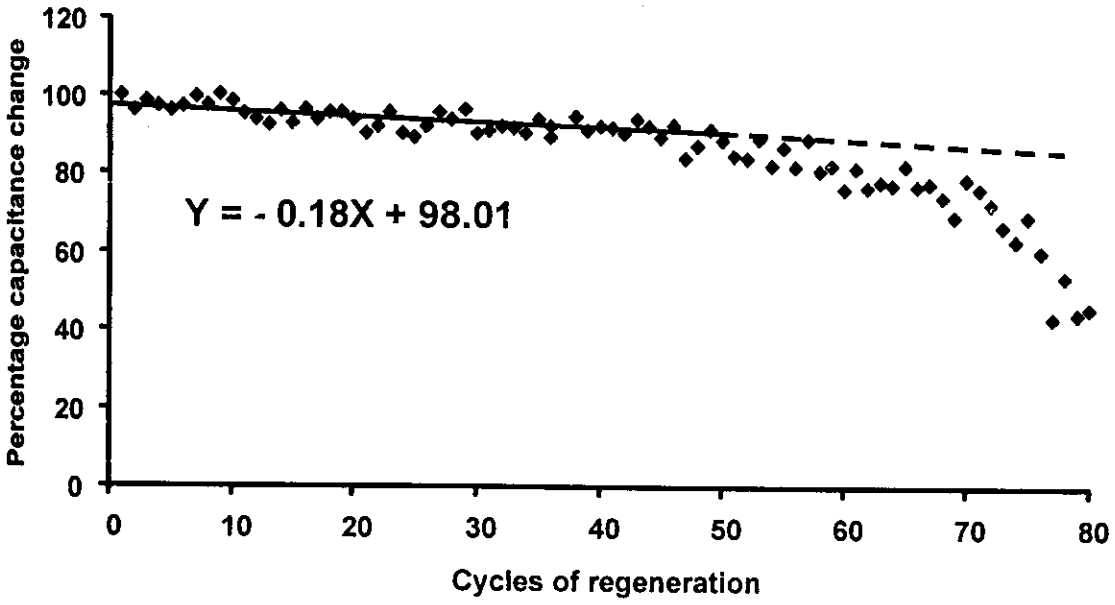


Figure 7.20 The reproducibility of the anti-AFP on self-assemble thiourea monolayer (SATUM).

7.5. Conclusions

Covalent coupling of anti-AFP on self-assembled thiourea monolayer (SATUM) modified gold surface with glutaraldehyde had been proven to be a good and reliable immobilization technique for a flow injection capacitive immunosensor system. The modified electrode was sensitive and selective to the presence of AFP. The electrode can also be regenerated and reused. Comparing with the covalent coupling of anti-AFP on self-assembled thioctic acid monolayer (SATAM) and self-assembled 3-mercaptopropionic acid monolayer (SAMPAM) activated by EDC/NHS, the proposed SATUM can certainly match their performances. That is, all the systems gave the same linear range with nearly the same sensitivity and detection limits. Good stability was obtained for all the systems, *i.e.*, they can be regenerated and reused up to 48 times with good reproducibility. Therefore, thiourea, which is cheaper than thioctic acid and 3-mercaptopropioic acid, is certainly a good alternative to be applied for the immobilization of antibodies on gold surfaces.