Appendix A

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A reusable capacitive immunosensor for carcinoembryonic antigen (CEA) detection using thiourea modified gold electrode

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bstract

A capacitive immunosensor based on a self-assembled monolayer (SAM) of thiourea on gold electrode has been developed. Anti-rcinoembryonic antigen (anti-CEA) was immobilized on a self-assembled thiourea monolayer (SATUM) via covalent coupling. Under optimum nditions, the decrease in capacitive signal when carcinoembryonic antigen (CEA) standard was injected could be determined with a detection mit of 10 pg ml^{-1} and linearity in the range of 0.01–10 ng ml^{-1}. The immobilized anti-CEA on SATUM gold electrode was stable and after generation good reproducibility of the signal could be obtained up to 45 times with an RSD lower than 3.4%. Good agreement was obtained hen CEA concentrations of human serum samples determined by the flow injection capacitive immunosensor system were compared to those tained using an enzyme linked fluorescent assay (ELFA) method ($P < 0.05$).

ywords: Capacitance; Immunosensor; Carcinoembryonic antigen; Thiourea; Self-assembled monolayer

Introduction

Carcinoembryonic antigen (CEA), is a highly glycosylated l surface glycoprotein (180 kDa), belonging to a group of bstances known as the tumor-associated antigens (TAA) [1]. is an important tumor marker responsible for clinical diag-sis of over 95% of all colon tumors, 50% of breast tumors, well as tumors of the lung, pancreas, ovaries, and others of thelial tissue origin, especially of the gastrointestinal tract . Various commonly available methods were developed for EA, such as enzyme linked fluorescent assay (ELFA) [3] microparticle enzyme immunoassay (MEIA) [4]. However, se methods are expensive, time consuming procedures requir-potentially dangerous materials [5]. Therefore, development a new method with high sensitivity and specificity for the ect detection of CEA is highly desirable and an immunosensor registering the direct binding between antibody and antigen may be the answer.

Immunosensors are based on binding interactions between the immobilized biomolecules and the analyte of interest [6,7]. The sensitivity obtained depends on the properties of the transducer and the affinity of the biological sensing molecules and the analyte. Electrochemical immunosensors offer good possibilities for sensitive detection of unlabeled proteins (direct detection). Several direct immunosensors based on electrochemical transducers have been developed, such as potentiometric [8,9], amperometric [10], conductimetric [11] and impedimetric [12]. Also capacitive transducers have been investigated as a highly sensitive approach [13–19]. The principle of capacitance immunosensor is based on the electrical double-layer theory [20]. An electrode that is coated with the biorecognition element has a stable capacitance signal. When the analyte binds to the biorecognition element on the electrode it causes the capacitance to decrease. For a biosensor based on a capacitive transducer, the immobilization of the recognition element is of vital importance for the ability to detect the binding event.

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Self-assembled monolayers (SAMs) of thiols, sulfides, d disulfides by spontaneous adsorption of sulfur-containing olecules were frequently used to immobilize the biorecogni-n element (i.e. protein). The affinity between sulfur and gold oms is extremely high, resulting in the formation of SAMs at are highly stable in air, water, and organic solvents at room mperature [21]. The most frequently used building block for AMs is thioctic acid. A SAM is formed after treating a clean ld surface with the disulfide thioctic acid, and coupling of anti-dies is performed using a carbodiimide reaction [13,15,17]. nother way to graft antibody onto the sensor surface is by cova-ntly coupling the antibody on a SAM with amino groups that e derivatized using glutaraldehyde. Glutaraldehyde was intro-ced to react with the self-assembled 2-mercaptoethylamine nolayer on a gold electrode to covalently immobilize the tibody [22]. Since, thioctic acid and 2-mercaptoethylamine expensive, a cheaper thiol reagent, thiourea, was studied. hiourea (NH_2CSNH_2) was chosen because of its low envi-nmental impact, easier handling of reagent and its ability strongly adsorb on gold [23,24]. Furthermore, it has amino ups (R-NH_2) that can be used for covalently coupling of anti-dies.

This paper reports the development of a capacitive munosensor for the direct detection of carcinoembryonic igen (CEA) using anti-carcinoembryonic antigen (anti-CEA) mobilized on a self-assembled thiourea monolayer (SATUM) gold electrode. The system performance of the capacitive munosensor was tested for the determination of CEA level human serum samples and the results were compared with a mmercial method (enzyme linked fluorescent assay (ELFA), DAS® CEA).

Materials and methods

1. Materials

Monoclonal anti-human carcinoembryonic antigen (anti-EA) and carcinoembryonic antigen (CEA) from human fluids were obtained from Sigma (St. Louis, USA), thiourea was obtained from BDH laboratory reagents (Poole, England) and 1-dodecanethiol was obtained from Aldrich (Milwaukee, USA). 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.

2.2. Immobilization of anti-CEA

The immobilization of anti-CEA on gold surface is a three-step procedure. The initial step is to prepare the gold electrodes (Ø3 mm, 99.99% purity). They were polished by a polishing machine (Gripo® 2V, Metkon Instruments Ltd., Turkey) using alumina slurries with particle diameters 5, 1, and 0.30 μm, respectively, and subsequently cleaned through sonication in distilled water and ethanol, each for 15 min, followed by electrochemical etching in 0.5 M H_2SO_4 using cycling electrode potential from 0 to 1.5 V versus Ag/AgCl reference electrode with a scan rate of 0.1 V s^{-1} for 25 scans. The electrode was then dried in an atmosphere of pure nitrogen gas. The next step is to modify the gold electrode with thiourea. The cleaned electrode was immersed in a 250 mM thiourea solution at room temperature for 24 h, thoroughly rinsed with distilled water and dried with pure nitrogen gas. In this step self-assembled thiourea monolayer was formed on gold surface. The final step is the coupling of anti-CEA to the modified gold electrode. This was done by treating the modified gold electrode with 5% (v/v) glutaraldehyde in 10 mM sodium phosphate buffer pH 7.00 at room temperature for 20 min, thoroughly rinse the electrode with sodium phosphate buffer before drying, this step was to activate the aldehyde groups. Then 20 μl of 1.0 mg ml^{-1} of anti-CEA was placed on the surface of the electrode and reaction took place overnight at 4 °C. The electrode was immersed in 0.1 M ethanolamine pH 8.00 for 30 min; this step was to occupy all the aldehyde groups that did not couple to the anti-CEA. Before placing the biosensor in the flow cell, the electrodes were treated

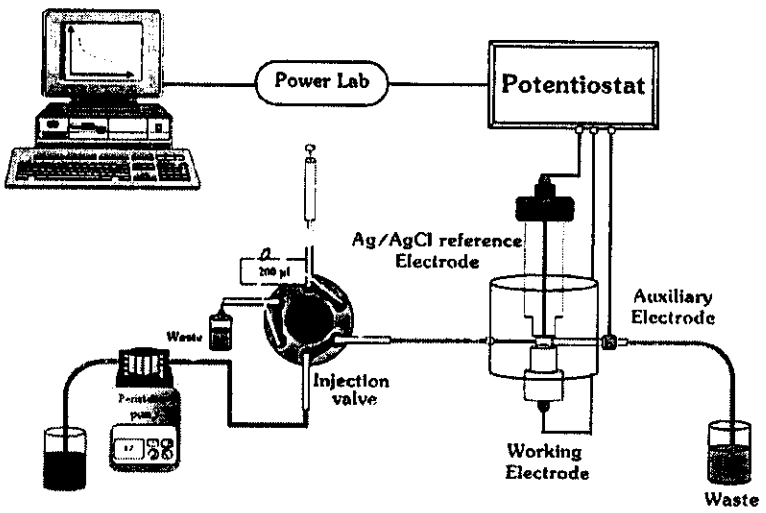


Fig. 1. Schematic diagram showing the flow injection capacitive immunosensor system.

in a 10 mM 1-dodecanethiol ethanolic solution for 20 min to block any pinholes or bare spots on the electrode surface.

2.3. Capacitance measurement

Fig. 1 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. The capacitance of the electrode surface was evaluated from the current response obtained from the application of a 50 mV potentiostatic step.

2.4. Optimization of the flow injection capacitive immunosensor

The operating conditions of the capacitive immunosensor in the flow injection system were optimized for the type and pH of regeneration solution, sample volume, flow rate and the type and pH of running buffer. These are summarized in Tables 1 and 2. The running buffer used throughout the experiments was 10 mM Tris–HCl, pH 7.00 (except when the effects of buffer types were tested). The effects of the type and pH of regenerating solution were tested with conditions; 100 μl min^{-1} flow rate of buffer, 250 μl sample volume. The effect of different types of buffer solutions was tested at a sample volume of 200 μl. The optimization of each parameter was performed by changing a single parameter while keeping the other parameters constant. The operating conditions were considered by balancing between the sensitivity and the time needed for one analysis.

Table 1
Efficiency of CEA removal from anti-CEA immobilized on the electrode studied by injecting 0.1 ng ml^{-1} CEA

Regeneration solution	Percentage of average residual activity
High ionic strength	
1 M NaCl	29 ± 13
4 M KCl	33 ± 11
2 M $MgCl_2$	42 ± 5
Low pH	
50 mM glycine–HCl, pH 2.50	59 ± 16
HCl, pH 2.50	67 ± 5
High pH	
5 mM NaOH	15 ± 3
50 mM NaOH	17 ± 6
Low pH	
HCl, pH 3.00	9 ± 6
HCl, pH 2.50	67 ± 5
HCl, pH 2.00	94 ± 2
HCl, pH 1.50	86 ± 9

The efficiency is given as a percentage residual activity, per cent of the sensor response to CEA after regeneration compared to the response before regenera-

Table 2
Assayed and optimized values used in the study of the flow injection capacitive immunosensor system

Parameters	Investigated values	Concentration of CEA (ng ml^{-1})	Optimum
Flow rate (μl ml^{-1})	25, 50, 100, 200, 400, 800	0.1	100
Sample volume (μl)	50, 75, 100, 150, 200, 250, 300	0.1	200
Buffer solutions			
Type	10 mM Tris–HCl buffer, pH 7.00 10 mM phosphate buffer, pH 7.00	10^{-3} to 10^{-1}	10 mM Tris–HCl Buffer, pH 7.00
pH	6.60, 6.80, 7.00, 7.20, 7.40, 7.60, 7.80, 8.00, 8.20	0.1	7.00

2.5. Determination of the amount of CEA in serum samples

CEA in serum samples were analysed by the ELFA technique, VIDAS® CEA method (the results obtained by Hat Yai Hospital) and the flow injection capacitive immunosensor system under optimum conditions; 100 μl min^{-1} flow rate of running buffer (10 mM Tris–HCl buffer pH 7.00) and 200 μl sample volume.

3. Results and discussion

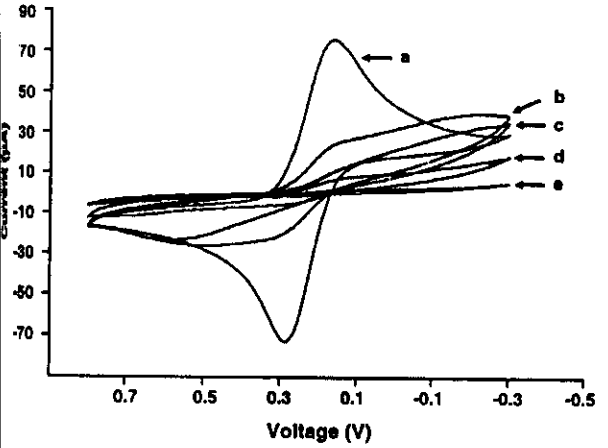
3.1. Electrochemical performance of the immobilization process

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 the electrolyte solution as shown in Fig. 2. At the clean gold surface the redox couple was oxidized and reduced (Fig. 2a). The redox peaks decreased when thiourea was self-assembled on the clean gold surface (Fig. 2b). When glutaraldehyde was reacted with the amine and then anti-CEA was linked covalently on the electrode via reaction with aldehyde groups, the insulating property of the electrode surface was further increased (Fig. 2c 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 of Fig. 2.

3.2. Capacitance evaluation

Continuously during the binding event between CEA and anti-CEA, potential pulses, 50 mV, are applied to the gold electrode yielding current response signals, which can be described by Eq. (1):

$$i(t) = \frac{u}{R_s}\exp\left(\frac{-t}{R_s C_{total}}\right) \quad (1)$$



g. 2. Cyclic voltammograms of a gold electrode obtained with 5 mM $[Fe(CN)_6]$ in 0.1 M KCl solution at a scan rate of 0.1 V s^{-1} 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-CEA–glutaraldehyde-amine SATUM, and (e) as in (d) after 1-dodecanethiol treatment.

here $i(t)$ is the current in the circuit as a function of time, u the ase potential applied, R_s the dynamic resistance of the recogni- n layer, t the time elapsed after the potential step was applied, d C_{total} is the total capacitance measured at the working elec- de/solution interface (Fig. 3). Taking the logarithm of Eq. (1) obtain:

$$\ln i(t) = \ln\frac{u}{R_s} - \frac{t}{R_s C_{total}} \quad (2)$$

Then, C_{total} and R_s were obtained from the slope and intercept the linear least-square fitting of $\ln i(t)$ versus t. The measure- nt of C_{total} was done every minute and the results were later tted as a function of time. Fig. 4 shows the change in capaci- ce due to affinity binding between CEA and anti-CEA on the rking electrode. When CEA was injected into the flow cell ound to the anti-CEA immobilized on the electrode causing al to decrease and the capacitance change (ΔC) could be rmined.

3.3. Optimization of the flow injection capacitive immunosensor

3.3.1. Regeneration solution

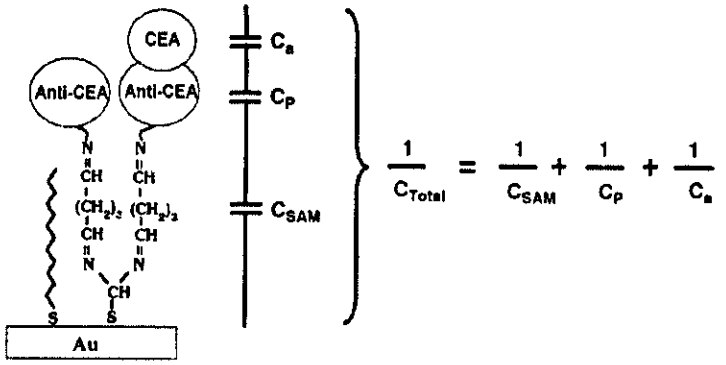
The interaction between CEA and the immobilized anti-CEA is via electrostatic force [25] and CEA can be removed from anti-CEA by using regeneration solution. Ideally, regeneration of the working electrode should remove any non-covalently bound CEA analyte without disrupting the activity of the anti-CEA molecules immobilized on the electrode. Regeneration allows surfaces to be reused many times, saving both time and money. To evaluate the performance of the regeneration solution, the residual activity of the anti-CEA electrode was calculated from the capacitance change (ΔC) as a consequence of the binding between CEA (i.e. 0.1 ng ml^{-1} of CEA standard) and anti-CEA before (ΔC_1) and after regeneration (ΔC_2) (Fig. 4) as follows:

$$\%\text{residual activity} = \frac{\Delta C_2 \times 100}{\Delta C_1} \quad (3)$$

The criteria for regenerating the electrode surface is "if post-regeneration binding remains above 90% compared to the binding efficiency before regeneration, the used conditions should be seen as adequate" [26]. A common approach for CEA–anti-CEA regeneration is to use different types of regenerating agents of three categories, that is, high ionic strength, low pH, and high pH and the results are shown in Table 1.

High ionic strength and high pH regeneration agents were shown to be ineffective with very low percent residual activity values (15 ± 3 to $42 \pm 5\%$), indicating that many anti-CEA molecules were still occupied by CEA after the regeneration step. However, at low pH, i.e. 50 mM glycine–HCl buffer, pH 2.50 and HCl, these were shown to be effective with a percent residual activity of 59 ± 16 and $67 \pm 5\%$, respectively. So, in this case HCl solution was chosen for further optimization.

The influence of pH of HCl solution, ranging from 3.00 down to 1.50 was studied (Table 1). The values of percent residual activity increased from 9 ± 6 to $94 \pm 2\%$ when pH decreased from 3.00 to 2.00. At pH 1.50 the percent residual activity was



Anti-CEA thiourea modified gold electrode

1. Capacitive layers on the gold electrode surface; (C_{SAM}) the capacitance change related to the self-assembled thiourea monolayer (SATUM), (C_P) the itance change of protein layer, (C_a) the capacitance change as a result of analyte interaction, (C_{total}) the total capacitance change measured at the working ode/solution interface.

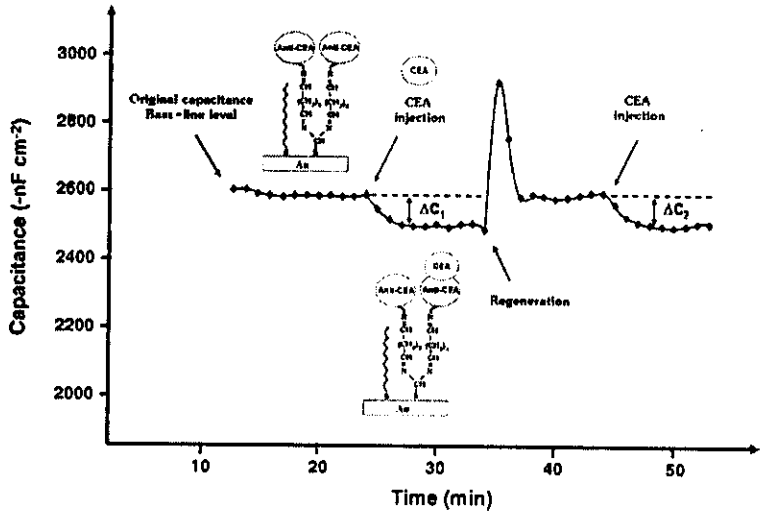


Fig. 4. Capacitance response of the anti-CEA modified electrode. The base-line signal in the carrier buffer was first recorded. After CEA was injected, the association of the immunocomplex between CEA and anti-CEA caused the capacitance to decrease (ΔC_1). Regeneration solution was then injected to remove CEA from anti-CEA. When the signal base-line was recovered a new analysis cycle was applied.

lower than at pH 2.00. This may be because at pH 1.50 some parts of SAM were destroyed by the very low pH [22]. Since at pH 2.00 residual activity values above 90% were achieved, HCl pH 2.00 should be used as the regeneration solution in the continued experiments.

To make sure that the SAM layer would not be destroyed during long term analysis, two modified electrodes were tested. One was prepared with only the SAM layer and 1-dodecanethiol, the other with anti-CEA immobilized on the SAM layer and 1-dodecanethiol. Fifty regeneration cycles were applied. Cyclic voltammograms were obtained before regeneration and after every 10 cycles. All cyclic voltammograms were the same for both electrodes (similar to Fig. 2e). This indicates that the SAM was still attached to the gold substrate. The stability of the SAM in this system was probably due to its short exposure time, only a few minutes at a time, to the regeneration solution. In most batch systems [12,18,22] the acid solution is usually used to regenerate modified electrode for 5–20 min. In this work a flow injection system was used (flow rate 100 μl min^{-1}, sample volume 200 μl), and the acid solution was retained on the modified electrode for only 2–3 min/cycle.

It should be noted that the capacitance of the system increased during regeneration (Fig. 4). This is because the capacitance also depends on the ionic strength of the solution. Higher ionic strength solution will give a higher value of capacitance [13,22]. When the regeneration solution was injected into the system to dissociate the binding between CEA and anti-CEA, the capacitance signal is higher than the base-line capacitance because the ionic strength of the regeneration solution (HCl, pH 2.00) is higher than the ionic strength of the carrier buffer (10 mM Tris–HCl).

3.2. Flow rate

In a flow injection capacitive immunosensor system, the flow rate of the buffer passing through the capacitive flow cell is the main factor affecting the yield of interaction between CEA and immobilized anti-CEA on the electrode surface. So optimization of flow rate is necessary. The changes in capacitance registered when varying the flow rates in the range of 25–800 μl min^{-1} increased when the flow rate decreased from 800 to 100 μl min^{-1}. However, for 25 and 50 μl min^{-1} the capacitance changes did not differ significantly from that observed at 100 μl min^{-1} ($P < 0.05$). So, 100 μl min^{-1} was chosen (Table 2).

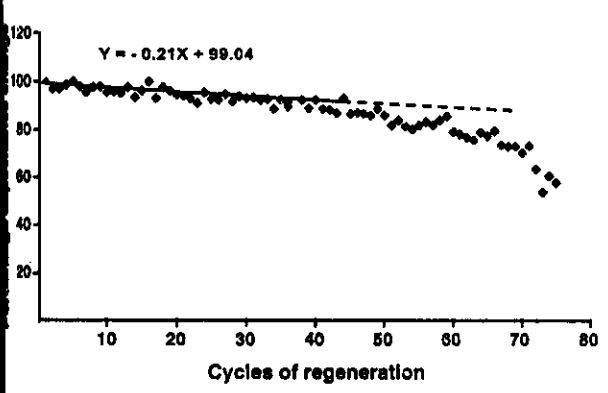
3.3.3. Sample volume

The change in capacitance signal ($-nF\,cm^{-2}$) increased as the sample volume increased from 50 to 200 μl. At sample volumes 200, 250 and 300 μl the capacitance change reached a maximum plateau. So, 200 μl was chosen because it has a lower analysis time than at 250 and 300 μl (Table 2).

3.3.4. Buffer solutions

3.3.4.1. Type. The influence of the type of buffer used in the flow injection capacitive immunosensor system (Table 2) was investigated and 10 mM Tris–HCl buffer pH 7.00 was chosen to be used in further analysis because it gave the highest sensitivity.

3.3.4.2. pH. The influence of pH during the binding reaction was studied between 6.60 and 8.20 for the same concentration of CEA (0.1 ng ml^{-1}) in 10 mM Tris–HCl buffer solution. (The buffering capacity of Tris–HCl is low at pH values below 7.00, but still the same buffer was used.) The change of capacitance increased with increasing pH from 6.60 to 7.00 and then decreased as the pH increased further. This result shows that the maximum change in capacitive signal occurs at pH 7.00 (Table 2). Therefore, pH 7.00 of Tris–HCl was used as the buffer in the binding reaction.



5. Reproducibility of the response from the anti-CEA modified electrode injections of a fixed volume of a standard solution of CEA (10 ng ml^{-1}) with generation and reconditioning steps between each individual assay.

4. Reproducibility

After regeneration, to remove CEA analyte from the anti-EA molecules immobilized on the electrode, CEA in the ndard solution was detected by the regenerated electrode eatedly to test the reproducibility. The performance of the i-CEA–thiourea modified electrode was evaluated intermit-ly over 5 days (15 times per day) by monitoring the change the capacitance signal at the same concentration of standard A (10 ng ml^{-1}).

Fig. 5 shows the percentage capacitance change (%) versus cycles of regeneration. The binding activity of anti-CEA mobilized by the self-assembled thiourea monolayer method ained about 91% of the original capacitance change signal r 3 days (45 times of regeneration). The result indicated that anti-CEA on SATUM electrode can be reused with good roducibility for up to 45 times with an RSD lower than 3.4%.

5. Linear dynamic range, detection limit, and selectivity

Discrete pulse injections of CEA standard solutions of con-trations ranging from 1×10^{-3} to 1×10^{2} ng ml^{-1} with mediate regeneration steps using HCl solution, pH 2.00 e performed. Fig. 6a shows the calibration curve for A under optimal conditions. A linear relationship between capacitance change and the logarithm of CEA concen-ion was obtained within a dynamic detection range of $^{-2}$ to 10 ng ml^{-1}. The linear regression equation was ΔC F cm^{-2}) = 31 log CEA (ng ml^{-1}) + 96, with a correlation fficient of 0.999. The detection limit was 10 pg ml^{-1} based IUPAC Recommendation 1994 [27]. This linear relationship itable for an "order of magnitude" test of CEA. For a good ntitative analysis of CEA in serum samples, a calibration e between the response and concentration of CEA standard ld provide a more accurate result. Since the concentration EA in blood of healthy humans is <2.5 ng ml^{-1} [28] and e analysis the serum will be diluted about 100 times to ce the matrix effect. The linear concentration calibration investigated in the range between 0.007 and 0.13 ng ml^{-1} 6b) that would cover the concentration in diluted serum.

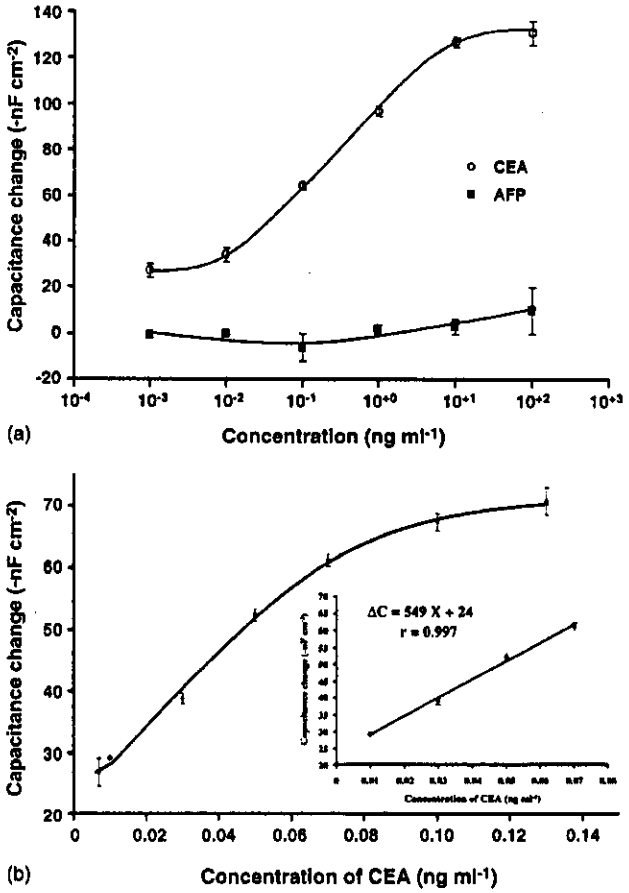


Fig. 6. (a) Capacitance change vs. the logarithm of CEA and AFP concentration for a transducer surface with immobilized anti-CEA under optimized conditions (100 μl min^{-1} flow rate, 200 μl sample volume, 10 mM Tris–HCl buffer, pH 7.00). (b) Capacitance change vs. concentration of CEA. The inset shows the relationship between the capacitance change and the concentration of CEA in the concentration range from 0.01 to 0.07 ng ml^{-1}.

In this case linearity was found between 0.01 and 0.07 ng ml^{-1}, i.e. ΔC (−nF cm^{-2}) = 549 × concentration (ng ml^{-1}) + 24.

The effect of substances that might interfere with the response of the CEA capacitive biosensor system was also studied. Alpha-fetoprotein (AFP) was used to test the selectivity of the capacitive biosensor system for CEA, because it is another tumor marker. The capacitance changes of AFP at the concentration range 1×10^{-3} to 1×10^{2} ng ml^{-1} on the anti-CEA immobilized on SATUM electrode was lower than the detection limit of CEA (Fig. 6a). These results suggested that the system was specific to CEA.

3.6. Comparison between the results obtained from the capacitive immunosensor system and ELFA technique (VIDAS® CEA)

The analysis of CEA using the capacitive immunosensor system and ELFA technique were done on the same serum samples. Ten samples were analysed and the concentrations were found in the range of 0.58–5.28 ng ml^{-1}. Comparisons between the

two analysis techniques was done by the regression line method [29] and the Wilcoxon signed rank test [30]. For the regression line method, the regression equation of the concentration of CEA obtained from the capacitive immunosensor system (y) and ELFA technique (x) is $y = 1.00 \pm 0.05x + 0.02 \pm 0.05$ with a correlation coefficient of 0.996. The results showed that the slope and the intercept did not differ significantly ($P < 0.05$) from the ideal value of 1 and 0, respectively, thus, there is no evidence for systematic differences between the methods. The Wilcoxon signed rank test was also used and there is no evidence for systematic differences between the results obtained from the capacitive immunosensor system and the ELFA technique ($P < 0.05$). That is, the concentrations determined by the capacitive immunosensor system are in good agreement to the ELFA technique.

4. Conclusions

Self-assembled thiourea monolayer is suitable for the immobilization of anti-CEA via covalent binding. The modified electrode, which is simple to prepare, when incorporated in a capacitive immunosensor system could provide high sensitivity, low detection limit, good selectivity and specificity for the assay of CEA. Using the appropriate regenerating solution, good reproducibility was obtained. The electrode can be reused up to 45 times and this helps to reduce the cost of analysis. The capacitive immunosensor system is potentially useful for direct assay of the interaction between CEA and anti-CEA on self-assembled thiourea monolayer without multiple washing and separation steps. This technique can be applied for the quantitative analysis of amount of CEA in human serum.

Acknowledgements

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Appendix B

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A comparative study of capacitive immunosensors based on self-assembled monolayers formed from thiourea, thioctic acid, and 3-mercaptopropionic acid

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Abstract

A procedure was developed for the covalent coupling of anti-alpha-fetoprotein antibody (anti-AFP) to a gold surface modified with a self-assembled monolayer (SAM) of thiourea (TU). The performance of the SAM-antibody layer was compared to those of similar layers based on thioctic acid (TA) and 3-mercaptopropionic acid (MPA) by using flow injection capacitive immunosensor system. Covalent coupling of anti-AFP on self-assembled thiourea monolayer (SATUM) modified gold electrode can be used to detect alpha-fetoprotein with high efficiency, similar sensitivity, the same linear range (0.01–10 $\mu g\, l^{-1}$) and detection limit (10 $ng\, l^{-1}$) as those obtained from sensors based on self-assembled thioctic acid monolayer (SATAM) and self-assembled 3-mercaptopropionic acid monolayer (SAMPAM). The system is specific for alpha-fetoprotein and can be regenerated and reused up to 48 times. Therefore, self-assembled monolayer using thiourea which is cheaper than thioctic acid and 3-mercaptopropionic acid is a good alternative for biosensor applications when SAMs are used.

Keywords: Thiourea; Capacitive immunosensor; Thioctic acid; 3-Mercaptopropionic acid; Self-assembled monolayer; Alpha-fetoprotein

1. Introduction

Immunosensors are based on binding interactions between immobilized biomolecules and the analyte of interest and their subsequent detection by appropriate detector (Mattiasson, 1984; Taylor, 1991). Several electrochemical detection principles have been used, such as potentiometric (Tang et al., 2004a; Taylor et al., 1991), amperometric (Ramanaviciene and Ramanavocous, 2004), conductimetric (Yagiuda et al., 1996), and impedemetric (Tang et al., 2004b). Capacitive measurement has also been investigated as a highly sensitive approach (Berggren et al., 1998, 2001; Berggren and Johansson, 1997; Bontidean et al., 1998; Hedström et al., 2005; Hu et al., 2002, 2005).

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), metal oxides surfaces (Gebbert et al., 1992, 1994), and self-assembled monolayers (SAMs) of sulfur compounds on gold (Berggren et al., 1998; Berggren and Johansson, 1997; Hedström et al., 2005).

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SAMs 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 and it is 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

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

or disulfide

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

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 versus standard 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-mercaptopropionic acid (MPA; $HSC_2H_4CO_2H$) (Disley et al., 1998; Sawaguchi et al., 2001; Vaughan et al., 1999). The carboxylic groups of the SAMs were 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 the protein solution where the activated electrophilic group attached 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; SC_2H_4-NH_2), was also an effective surface to which protein could be immobilized (Jiang et al., 2003). Glutaraldehyde 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 (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) that can be modified to covalently couple to the antibody. To our knowledge no one has applied it to immunosensors.

This paper reports the development of a procedure for the immobilization of antibody to a gold surfaces modified with a SAM of thiourea. The performance was compared with that of the commonly used thioctic acid and 3-mercaptopropionic acid. Alpha-fetoprotein (AFP) and anti-alpha-fetoprotein antibody (anti-AFP) were used as a model system. 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.

2. Materials and methods

2.1. 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), 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.

2.2. Methods

2.2.1. Preparation of the 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, 15 min each, in distilled water and absolute ethanol 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 versus 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.

2.2.2. Modification of 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 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 SAMs 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

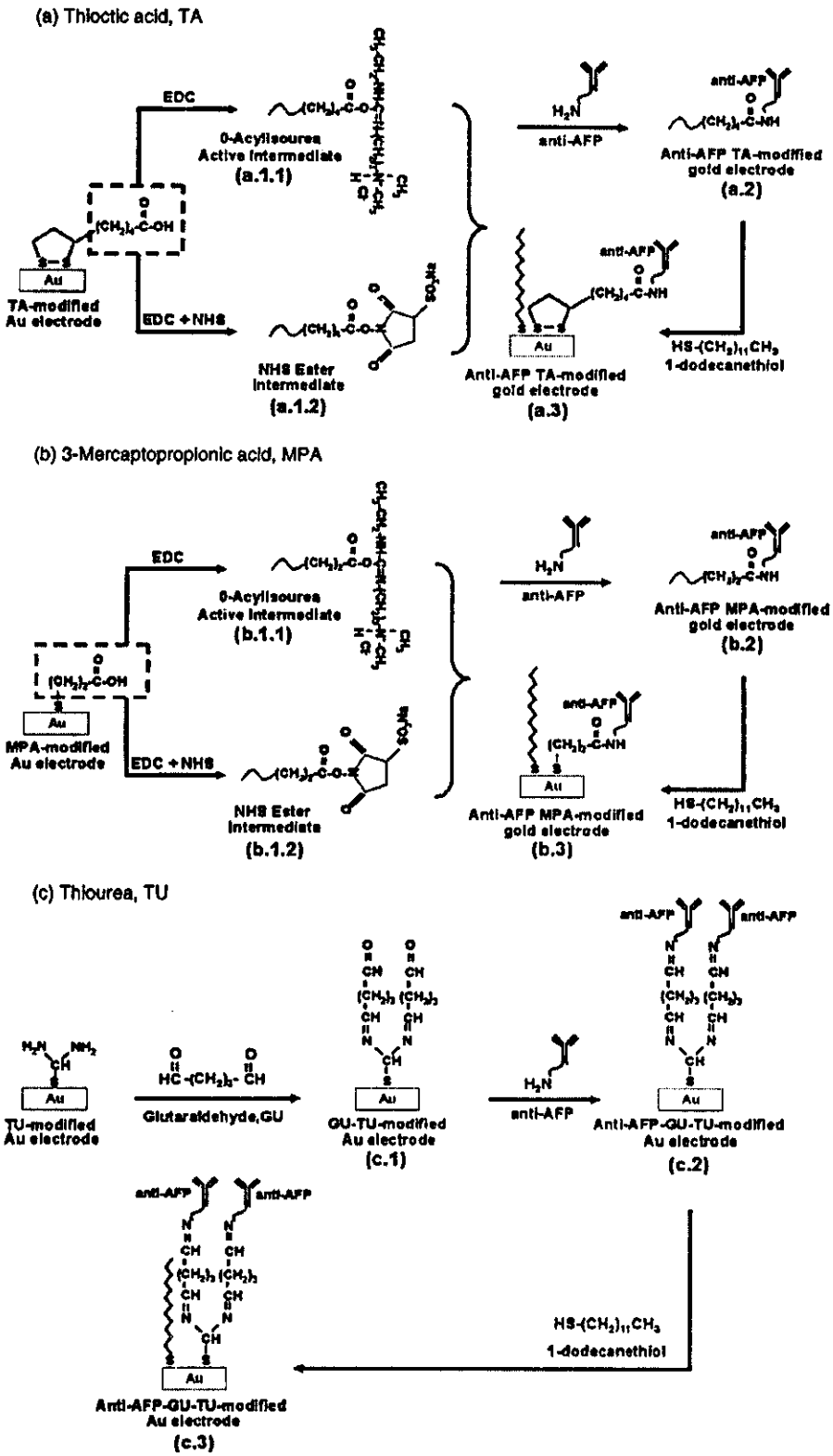

Fig. 1. Reaction mechanism for the anti-AFP immobilized on a self-assemble thiolate monolayer, (a) thioctic acid (TA), (b) 3-mercaptopropionic acid (MPA), and (c) thiourea (TU).

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.

2.2.3. Immobilization of anti-AFP

Anti-AFP, used as the sensing element, was immobilized on SATAM, SAMPAM, and SATUM through covalent binding. For SATAM and SAMPAM, their carboxylic groups were activated for an amine reaction by using 0.05 M of EDC in phosphate buffer (pH 5.00) (Fig. 1(a.1.1 and b.1.1)) or 0.05 M of EDC with 0.03 M of NHS in phosphate buffer (pH 5.00) (Fig. 1(a.1.2 and b.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 (Fig. 1(a.2 and b.2)). Finally, the electrode was reacted in a 10 mM 1-dodecanethiol ethanolic solution for 20 min (Fig. 1(a.3 and b.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 (Fig. 1(c.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 (Fig. 1(c.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 (Fig. 1(c.3)) to block the bare spots on the electrode surface.

2.3. Capacitance measurement

Fig. 2 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 laboratory built Ag/AgCl reference electrode was placed opposite to the working electrode.

Continuously during the binding event between AFP and anti-AFP, potential pulses, 50 mV, are applied to the gold electrode yielding current response signals, which can be described by Eq. (1).

$$i(t) = \frac{u}{R_s} \exp\left(\frac{-t}{R_s C_{total}}\right) \quad (1)$$

where $i(t)$ is the current in the circuit as a function of time, u the pulse potential applied, R_s the dynamic resistance of the recognition layer, t the time elapsed after the potential step was applied, and C_{total} is the total capacitance measured at the working electrode/solution interface. Taking the logarithm of Eq. (1) we obtain

$$\ln i(t) = \ln \frac{u}{R_s} - \frac{t}{R_s C_{total}} \quad (2)$$

Then, C_{total} and R_s were obtained from the slope and intercept of the linear least-square fitting of $\ln i(t)$ versus t. The measurement

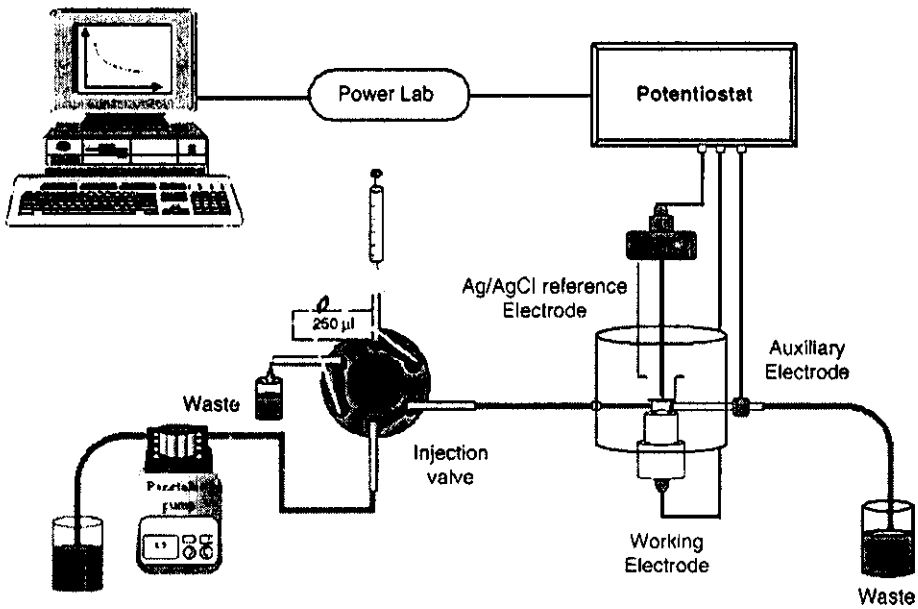

Fig. 2. Schematic diagram showing the flow injection capacitive immunosensor system.

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.

3. Results and discussion

3.1. 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 scan rate 100 mV s^{-1}. The efficiency of the formation of thiol SAMs on electrode surfaces can be described in term of surface coverage which can be estimated by comparing the area of the reduction peak of electroadsoption of oxygen atom on the modified and bare gold electrode (Sabatani et al., 1987). The percent surface coverage was calculated by using Eq. (3).

$$\text{surface coverage (\%)} = \frac{(Q_{MGE} - Q_{BGE}) \times 100}{Q_{BGE}} \quad (3)$$

where Q_{MGE} is the amount of electric charge exchanged during the electroadsorption of oxygen (C cm^{-2}) of the modified gold electrode and Q_{BGE} is the amount of electric charge exchanged during the electroadsorption of oxygen (C cm^{-2}) of the bare gold electrode. In cases of TA and MPA, the percent surface coverage was found to increase with immersion up to 12 h (Fig. 3(a)).

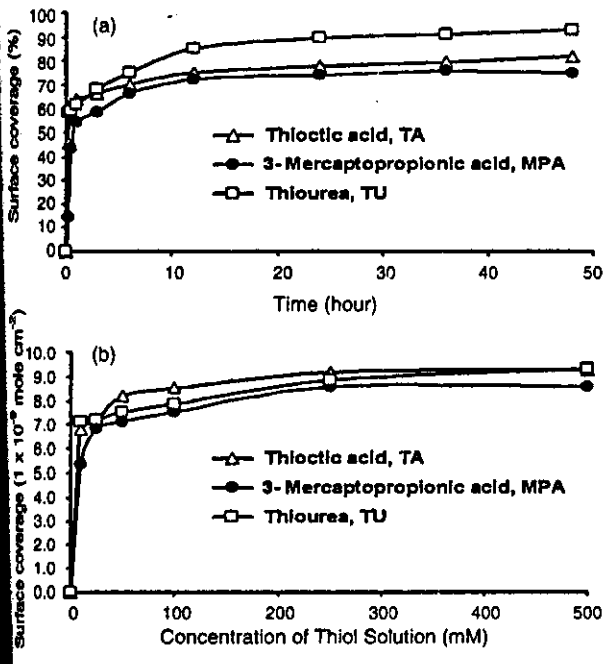

Fig. 3. The effect of (a) immersion times and (b) concentration of the thiol solutions for the formation of SAMs on gold electrode surface.

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.

3.2. Concentration of thiol solutions

The effect of concentrations 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 reaction (4).

$$RS\text{–}Au + e^- \rightarrow Au + RS^- \quad (4)$$

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. Cyclic voltammograms of reduction peak of thiolate monolayer in 0.5 M KOH, indicating the desorption of thiol bound to the gold electrode surface (Weisshaar et al., 1993). The charge under desorption peak can be used to estimate the surface concentrations of the thiol solutions in monolayer as follows:

$$\Gamma = \frac{Q}{nFA} \quad (5)$$

where Γ is the surface coverage (mol cm^{-2}), Q the total charge (C), n the number of electron transferred, F the Faraday's constant (96,485.4 C mol^{-1}), and A is the electrode surface area (cm^2). Fig. 3(b) 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 the gold electrode. At this concentration, the surface coverage for monolayers of TA, MPA, and TU were 9.2×10^{-9}, 8.6×10^{-9}, and 8.9×10^{-9} mol cm^{-2}, respectively.

3.3. Immobilization of anti-AFP

3.3.1. SATAM and SAMPAM

The immobilization of anti-AFP on SATAM, and SAMPAM using covalent method were done using EDC, and EDC/NHS solution for intermediate (Johnsson et al., 1991; Staros et al., 1986). The performances of these electrodes are shown in Table 1. The sensitivity of SATAM and SAMPAM activated with EDC/NHS were higher than SATAM and SAMPAM 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 1, determined by detecting the amount of protein in the solution before and after immobilization by the silver binding method (Krystal, 1987).

3.3.2. SATUM

The effect of glutaraldehyde was tested by determining the immobilization yield. The concentration was tested between

ble 1
rformances of anti-AFP covalently immobilized on self-assemble thioctic acid monolayer (SATAM), self-assemble 3-mercaptopropionic acid monolayer (SAM-AM), and self-assemble thiourea monolayer (SATUM)

rameters	Performances		
	SATAM	SAMPAM	SATUM
near range ($\mu g\,l^{-1}$)			
Activated with EDC	0.01–10	0.01–10	NA
Activated with EDC/NHS	0.01–10	0.01–10	NA
Activated with glutaraldehyde	NA	NA	0.01–10
mit of detection ($ng\,l^{-1}$)			
Activated with EDC	10	10	NA
Activated with EDC/NHS	10	10	NA
Activated with glutaraldehyde	NA	NA	10
nsitivity (Δc ($-nF\,cm^{-2}$)/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
nalysis 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
mobilization 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
rcentage capacitance change decreasing rate within 48 times of regeneration (%/time)	−0.20	−0.19	−0.18

A: not applicable.

05 and 10% (v/v). When the concentration of glutaraldehyde creased the immobilization yield also increased reaching a aximum at 5% (v/v) of glutaraldehyde and then leveled off. r the effect of incubation time (1–240 min), the highest immo-ilization yield is at 20 min. Therefore, 5% (v/v) glutaraldehyde d incubation time of 20 min were use for further experiments activate the SATUM electrode in 10 mM sodium phosphate ffer pH 7.00 at room temperature. Anti-AFP was then immo-ilized on the activated electrode.

4. Electrochemical performance of the process of ti-AFP immobilization

In the capacitive immunosensor system the insulating prop-y of the self-assembled monolayer on the electrode surface f vital importance. The degree of insulation was examined g cyclic voltammetry with a permeable redox couple (i.e. $[Fe(CN)_6]$) in the electrolyte solution. Fig. 4 shows an exam-of these voltammograms for SATUM. At the cleaned gold face the redox couple was oxidized and reduced according to ve a. When thiourea was self-assembled on the clean gold face the redox peaks decreased (curve b). Then aldehyde up of glutaraldehyde was reacted with the amine and the -AFP was linked covalently on the electrode via reaction the aldehyde group. The insulating property of the elec-e surface was further increased (curves c and d). A final ping of the electrode surface was achieved by the treatment 1-dodecanethiol, as can be seen from the disappearance of redox peaks in curve e. Similar cyclic voltammograms for SATAM and SAMPAM were also obtained indicating that all these modified surface were well insulated and were suitable for the capacitive measuring system.

3.5. Linear range, detection limit, and selectivity

When AFP was injected into the flow cell, it bound to the immobilized anti-AFP on the electrode causing the capacitance

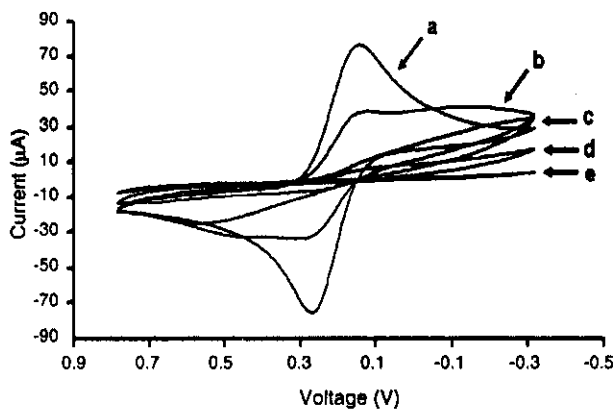

Fig. 4. Cyclic voltammograms of a gold electrode obtained in a 5 mM $K_3[Fe(CN)_6]$ containing 0.1 M KCl solution at scan rate of $0.1\,V\,s^{-1}$. All potentials are given vs. Ag/AgCl reference electrode. The voltage range was −0.3 to 0.8 V. (a) Clean gold electrode, (b) self-assembled thiourea monolayer (SATUM) electrode, (c) glutaraldehyde-amine SATUM, (d) anti-AFP-glutaraldehyde-amine SATUM, and (e) as in (d) but after 1-dodecanethiol treatment.

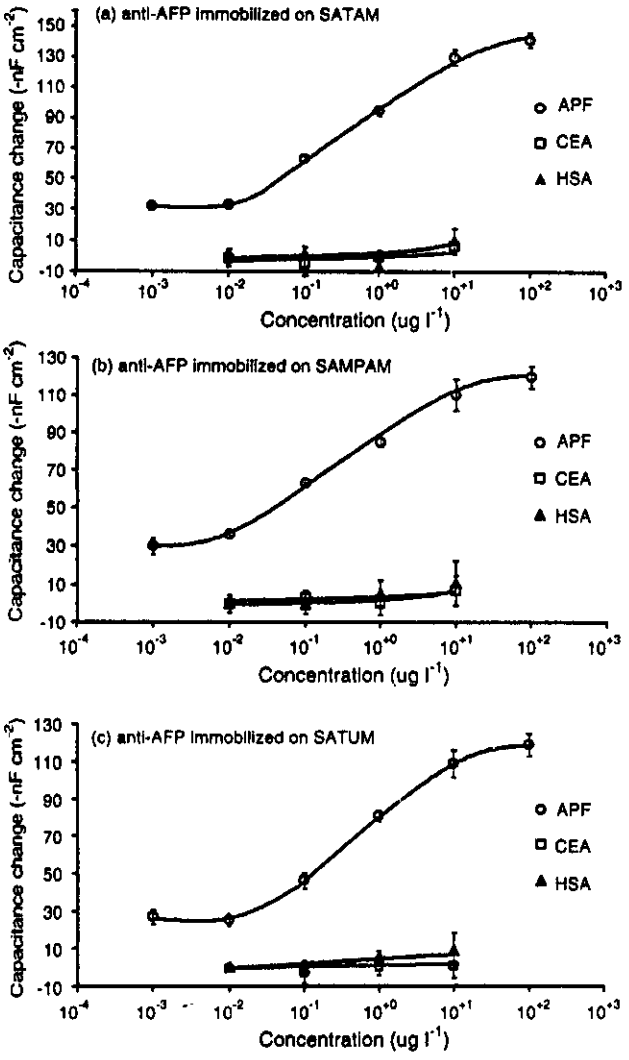

Fig. 5. Responses of the anti-AFP to alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), and human serum albumin (HSA) using (a) self-assembled thioctic acid monolayer (SATAM), (b) self-assembled 3-mercaptopropionic acid monolayer (SAMPAM), and (c) self-assembled thiourea monolayer (SATUM).

to decrease. Discrete pulse injections of AFP standard ranging from 0.001 to 100 $\mu g\,l^{-1}$ with intermediate regeneration steps using glycine–HCl buffer solution, pH 2.80, were performed. Fig. 5 shows 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}. 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 $\mu g\,l^{-1}$. The detection limits of SATAM, SAMPAM, and SATUM were 10 $ng\,l^{-1}$, based on IUPAC Recommendation 1994 (Buck and Lindner, 1994).

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 of HSA and CEA at the concentration range 0.01–10 $\mu g\,l^{-1}$ on the anti-AFP immobilized on SATAM, SAMPAM, and SATUM electrodes were much lower than those from AFP (Fig. 5) 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 specific to AFP.

3.6. Reproducibility

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

Percentage capacitance change decreasing rate after regeneration (%/time) of the three anti-AFP-modified electrodes are shown in Table 1. After 48 times of regeneration, the binding activity of anti-AFP immobilized on SATAM, SAMPAM, and SATUM retained more than 90% of the original capacitance change. That is, anti-AFP immobilization on SATAM, SAMPAM, and SATUM electrodes can be reused with good reproducibility up to about 48 times with the relative standard deviation (R.S.D.) of 4.2, 4.3, and 3.6%, respectively.

4. 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-mercaptopropionic acid, is certainly a good alternative to be applied for the immobilization of antibodies on gold surfaces.

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