Chapter 9
Conclusions

In this thesis, the development and evaluation of the performance of capacitive affinity biosensor systems have been described. This affinity biosensor is based on the immobilization of biorecognition element on a self-assembled monolayer (SAM) of sulfur-containing molecules on working gold electrode (WE). Binding of the target analyte to the biorecognition elements on the electrode causes the capacitance to decrease. The capacitance change due to the direct affinity reaction could then be determined using a potential step method.

To investigate the use of the capacitive measuring system for the direct detection (label-free) of affinity reaction. Initial study was carried out by immobilizing biorecognition elements on a gold working electrode (WE) surface via self-assembled thioctic acid monolayer (SATAM). Two affinity binding pairs, human serum albumin (HSA) and anti human serum albumin antibody (anti-HSA), and crystallizable fragment (Fc-fragments) from IgG (anti-HSA) and protein A, were investigated. The capacitive affinity biosensor system is clearly suitable for direct assay with a very low detection limit of 1 fM and 10 fM for HSA and protein A, respectively (Table 9.1). It requires short analysis time (12-17 min), is selective and sensitive to target analyte, and can be developed for other affinity binding pairs. Furthermore, the regeneration of the immunologically active surface on the electrode is also possible.

After this initial study the system was then applied for the direct detection of bacterial endotoxin in fermentation liquid from E.*coli*. Lectin (endotoxin neutralizing protein (ENP)) derived from American horseshoe crab was immobilized to a self-assembled thiol layer on the biosensor transducer (Au). The observed decrease in capacitive signal as a result of the injected endotoxin standard could be determined with a detection limit as low as 0.1 pM and linearity ranging 1.0×10^{-13} to 1.0×10^{-10} M (Table 9.1). When analyzing real samples, applying endotoxin

preparations purified from an *E.coli* cultivation, good agreement was obtained when comparing the results obtained from capacitive biosensor system to those utilizing the conventional method, *Limulus ambeocyt lysate* (LAL) test ($P < 0.05$). This proved that the system is acceptable for quantitative analysis of trace amount of bacterial endotoxin.

In addition to the use of the system to detect selected analytes a new self-assembled monolayer for capacitive immonosensor using thiourea (TU), a cheaper compound, was studied and compared to thioctic acid (TA) and 3-mercaptopropioic acid (MPA). Anti-alpha-fetoprotein and alpha-fetoprotein was used as the affinity pair. The performance of the self-assembled thiourea monolayer (SATUM) antibody layer was compared to those of similar layers based on TA and MPA by using a flow injection capacitive immunosensor system. Covalent coupling of anti-AFP on self-assembled thiourea monolayer 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) (Table 9.1). 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.

This new self-assembled monolayer using thiourea was then studied for the use with real samples. A reuseable capacitive immunosensor for the direct detection of carcinoembryonic antigen (CEA) using anti-carcinoembryonic antigen (anti-CEA) immobilized on a self-assembed thiourea monolayer via covalent coupling on gold electrode was then developed. Under optimum conditions, the decrease in capacitive signal when carcinoembryonic antigen (CEA) standard was injected could be determined with a detection limit of 10 $pg\ ml^{-1}$ and linearity in the range of 0.01 to 10 $ng\ ml^{-1}$. The immobilized anti-CEA on SATUM gold electrode was stable and after regeneration good reproducibility of the signal could be obtained up to 45 times with an RSD lower than 3.4%. Good agreement was obtained when CEA

concentrations of human serum samples determined by the flow injection capacitive immunosensor system were compared to those obtained using an enzyme linked fluorescent assay (ELFA) method ($P < 0.05$). The use of SAM and a flow injection system may also help to eliminate non-specific binding, a main problem found in a number of biosensor systems.

The performances of all flow injection capacitive biosensor systems are summarized in Table 9.1. Tables 9.2 - 9.5 show the comparison between the analytical features of the flow injection capacitive affinity biosensor system, standard and other biosensor methods for protein A, bacterial endotoxin, AFP, and CEA, respectively. It can be seen that these flow injection capacitive biosensor systems provide much better performance than other methods in almost all categories.

These results show that the flow injection capacitive affinity biosensor system can be applied for the direct detection of several target analytes. This technique is highly selective, can detect analyte with accuracy, using short analysis time (12-18 min). The preparation of the modified electrode, although requires several steps, is quite simple and is, most likely, not affected by non-specific binding protein. Furthermore, the regeneration of the activity of the working electrode can be performed by acid buffer solution with good reproducibility (%RSD < 4.3) enable the electrode to be reused for more than 40 times. Different preparations of the modified electrode provided similar sensitivity (slope), therefore, new calibration may not be necessary when changing the electrode. The detection limit of the system is generally very low, in the range of pico to femto molar, making this technique a good alternative approach to detect trace amount of affinity binding analytes. The use of this technique can definitely be applied to other affinity binding pairs. To further develop the system, one may try to eliminate the pretreatment procedures of gold surface prior to SAM which includes several polishing and cleaning steps until a very smooth surface is obtained. This can be achieved by fabricating a smooth surface gold electrode using, for example sputtering technique. This would require a very small amount of gold enable it to be used as a disposable device. Further development on miniaturization and multi analyte device are also possible.

Table 9.1 Performance of the flow injection capacitive biosensor systems for different analytes studied in this work.

Analytes	Type of SAM	Coupling biorecognition via	Limit of detection	Linear range	Linear regression equation	Regeneration solution	Analysis time
HSA	TA	EDC	1 fM	1.0×10^{-15} to 1.0×10^{-9} M and 1.0×10^{-7} to 1.0×10^{-5} M	ΔC (-nF cm^{-2}) = 12 log (M) + 34 and ΔC (-nF cm^{-2}) = 154 log (M) – 1134	100 mM glycine HCl, pH 2.20	12-17 min
Protein A	TA	EDC	10 fM	1.0×10^{-14} to 1.0×10^{-10} M	ΔC (-nF cm^{-2}) = 10 log (M) + 177	50 mM glycine HCl, pH 2.50	12-17 min
Endotoxin	TA	EDC	0.1 pM and 0.01 fM	1.0×10^{-13} to 1.0×10^{-10} M and 1.0×10^{-17} to 1.0×10^{-13} M	ΔC (-nF cm^{-2}) = 18 log (M) + 253 and ΔC (-nF cm^{-2}) = 3 log (M) + 66	25 mM glycine HCl, pH 2.40	13-18 min
AFP	TA	EDC	10 ng l^{-1}	0.01 to 10 μg l^{-1}	ΔC(-nF cm^{-2}) = 21 log (μg l^{-1}) + 62	10 mM glycine HCl, pH 2.80	13-15 min
AFP	TA	EDC/NHS	10 ng l^{-1}	0.01 to 10 μg l^{-1}	ΔC(-nF cm^{-2}) = 32 log (μg l^{-1}) + 96	10 mM glycine HCl, pH 2.80	13-15 min
AFP	3-MPA	EDC	10 ng l^{-1}	0.01 to 10 μg l^{-1}	ΔC(-nF cm^{-2}) = 17 log (μg l^{-1}) + 61	10 mM glycine HCl, pH 2.80	13-15 min
AFP	3-MPA	EDC/NHS	10 ng l^{-1}	0.01 to 10 μg l^{-1}	ΔC(-nF cm^{-2}) = 25 log (μg l^{-1}) + 86	10 mM glycine HCl, pH 2.80	13-15 min
AFP	TU	Glutaraldehyde	10 ng l^{-1}	0.01 to 10 μg l^{-1}	ΔC(-nF cm^{-2}) = 29 log (μg l^{-1}) + 80	10 mM glycine HCl, pH 2.80	13-15 min
CEA	TU	Glutaraldehyde	10 pg ml^{-1}	0.01 to 10 ng ml^{-1}	ΔC(-nF cm^{-2}) = 31 log (μg l^{-1}) + 96	HCl. pH 2.00	13-16 min

HSA; Human serum albumin
AFP; Alpha-fetoprotein
CEA; Carcinoembryonic antigen
TA; Thioctic acid
3-MPA; 3-mercaptopropionic acid
TU; Thiourea
EDC; N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride
NHS; N-Hydroxysuccinimide (N-Hydroxy-2,5-pyrrolidinedione)

Table 9.2 Comparison of the analytical feature for protein A detection.

Analytical feature	Standard method[1]	Immunoassay, ELISA[2]	Capacitive biosensor
Limit of detection (fM)	1.0×10^{4}	700	10
Linear range (M)	1.0×10^{-11} to 1.0×10^{-10}	1.4×10^{-12} to 0.2×10^{-10}	1.0×10^{-14} to 1.0×10^{-10}
Analytical time (min)	>210	>180	12-17
Complexity of the procedure	Yes	Yes	No
Label-free	No	No	Yes
Reusable	No	No	Yes

[1]Protein A Enzyme-linked immunosorbent assay (ELISA) for samples containing IgG (Immusystem AB)

[2]An ELISA for protein A (Steindl *el al.*, 2000)

Table 9.3 Comparison of the analytical feature for bacterial endotoxin detection.

Analytical feature	Standard method[1]	Luminescence biosensor[2]	Piezoelectric biosensor[3]	Capacitive biosensor
Limit of detection (pM)	0.1	2.0×10^{4}	0.25	0.1
Linear range (M)	1.0×10^{-13} to 1.0×10^{-11}	2.0×10^{-8} to 2.0×10^{-6}	2.5×10^{-13} to 2.0×10^{-12}	1.0×10^{-13} to 1.0×10^{-10}
Analytical time (min)	>48	-	20	13-18
Complexity of the procedure	Yes	Yes	No	No
Label-free	No	No	Yes	Yes
Reusable	No	No	No	Yes

[1]Chromogenic Limulus Amebocyte Lysate Assay (Cambrex)

[2]A luminescence endotoxin biosensor (Hreniak, 2004)

[3]Quartz crystal microbalance biosensor for the detection of endotoxins (Yang and Chen, 2002)

Table 9.4 Comparison of the analytical feature for alpha-fetoprotein (AFP) detection.

Analytical feature	Standard method[1]	Amperometric biosensor[2]	Piezoelectric biosensor[3]	Capacitive biosensor
Limit of detection (ng l^{-1})	600	10,000	50,000	10
Linear range (μg l^{-1})	600 to 100,000	10 to 10,000	50 to 800	0.01 to 10
Analytical time (min)	>60	>20	>60	13-15
Complexity of the procedure	Yes	Yes	No	No
Label-free	No	No	Yes	Yes
Reusable	No	No	No	Yes

[1]Enzyme Linked Fluorescent Assay (VIDAS® AFP)

[2]An amperometric biosensor for AFP (Kim *et al.*, 2001)

[3]A piezoelectric biosensor for the detection of AFP (Tsai and Lin, 2005)

Table 9.5 Comparison of the analytical feature for carcinoembryonic antigen (CEA) detection.

Analytical feature	Standard method[1]	Amperometric biosensor[2]	Piezoelectric biosensor[3]	Capacitive biosensor
Limit of detection (pg ml^{-1})	500	200	66,700	10
Linear range (ng ml^{-1})	0.5 to 200	0.5 to 120	66.7 to 466.7	0.01 to 10
Analytical time (min)	>90	>20	>20	13-16
Complexity of the procedure	Yes	Yes	No	No
Label-free	No	No	Yes	Yes
Reusable	No	No	No	Yes

[1]Enzyme Linked Fluorescent Assay (VIDAS® CEA)

[2]An amperometric biosensor for CEA (Li *et al.*, 2006)

[3]A piezoelectric biosensor for the detection of CEA (Shen *et al.*, 2005)