รายงานวิจัยฉบับสมบูรณ์

โครงการ คาพาซิทีฟไบโอเซนเซอร์ที่มีความไวในการวิเคราะห์
Capacitive Biosensor for Sensitive Analysis

โดย ปณต ถาวรังกูร และคณะ

สิงหาคม 2552

สัญญาเลขที่ BRG4980023

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มหาวิทยาลัยสงขลานครินทร์

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

โครงการนี้ได้รับทุนอุดหนุนการวิจัยจากสำนักงานกองทุนสนับสนุนการวิจัย (สกว.) ทุนองค์ความรู้ใหม่ที่เป็นพื้นฐานต่อการพัฒนา สัญญาเลขที่ BRG4980023 ตั้งแต่วันที่ 1 เดือนกันยายน พ.ศ. 2549 ถึงวันที่ 31 เดือนสิงหาคม พ.ศ. 2552

ขอขอบคุณการสนับสนุนนักศึกษาจากโครงการปริญญาเอกกาญจนาภิเษก (คปก.) ศูนย์ความเป็นเลิศด้านนวัตกรรมทางเคมี (PERCH-CIC) และ สถานวิจัยการวิเคราะห์สารปริมาณน้อยและไบโอเซนเซอร์ (TAB-RC) มหาวิทยาลัยสงขลานครินทร์

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บทคัดย่อ

โครงการนี้ศึกษาคาพาซิทีฟไบโอเซนเซอร์โดยใช้เทคนิคโพเทนชิโอสเตติกในการวัดความจุไฟฟ้า ศึกษาไบโอเซนเซอร์ที่มีความไววิเคราะห์สูงสำหรับหาปริมาณสารต่างๆ โดยตรงจากปฏิกิริยาการจับกันของคู่แอฟฟินิตี ในขั้นต้นได้ศึกษาเซนเซอร์สองระบบโดยตรึงสารที่เป็นส่วนหนึ่งของคู่แอฟฟินิตีบนขั้วอิเล็กโทรดทอง ระบบหนึ่งสำหรับวัดปริมาณของกรดนิวคลิอิกโดยการจับกันของคู่แอฟฟินิตีระหว่างฮิสโทนที่ถูกตรึงบนผิวอิเล็กโทรดกับกรดนิวคลิอิกในสารตัวอย่าง อีกระบบหนึ่งตรึง 3-อมิโนฟีนิลโบโรนิคแอซิด ซึ่งเป็นซูโดแอฟฟินิตีลิแกนด์ สำหรับตรวจหาแบคทีเรียโดยอาศัยการจับกันระหว่างกรดโบโรนิคและสารประกอบไดออลบนผิวของแบคทีเรีย อีกส่วนหนึ่งของโครงการเป็นการศึกษาความเป็นไปได้ของการใช้อิเล็กโทรดทองแบบฟิล์มบางเป็นขั้วไฟฟ้าแบบใช้แล้วทิ้งเพื่อลดขั้นตอนในการเตรียมอิเล็กโทรด เตรียมอิเล็กโทรดโดยใช้เทคนิคการระเหยด้วยความร้อนและตรึงแอนติฮิวมันซีรัมอัลบูมินบนผิวทองเพื่อใช้หาปริมาณฮิวมันซีรัมอัลบูมิน ทุกระบบที่ได้ศึกษาให้ค่าต่ำสุดของการตรวจวัดดีมาก อยู่ในช่วง พิโกถึงเฟมโตโมลาร์ อิเล็กโทรดที่เตรียมขึ้นสามารถนำมาใช้วัดใหม่ได้อย่างน้อย 30 ครั้ง เมื่อทดสอบกับตัวอย่างจริงผลที่ได้สอดคล้องกับวิธีมาตรฐาน เพื่อให้การวัดความจุไฟฟ้าทำได้สะดวกยิ่งขึ้นจึงได้พัฒนาทั้งส่วนของอุปกรณ์และส่วนของชุดคำสั่งสำหรับการตรวจวัดตามเวลาจริง อุปกรณ์ที่พัฒนาขึ้นให้ผลตามเวลาจริงสอดคล้องกับผลที่ได้จากเครื่องมือทางไฟฟ้าเคมีเชิงพาณิชย์ การพัฒนาระบบที่ให้ผลตามเวลาจริงนี้ช่วยให้เห็นผลขณะทำการทดลองทำให้สามารถวิเคราะห์ผลได้รวดเร็วขึ้น ต่างจากการทดลองก่อนหน้านี้ซึ่งต้องคำนวณค่าความจุไฟฟ้าหลังจากการทดลอง ผลจากงานวิจัยนี้แสดงให้เห็นว่าการวัดความจุไฟฟ้าโดยเทคนิคโพเทนชิโอสเตติกเป็นวิธีที่ให้ความไวในการวิเคราะห์สูง และมีความเหมาะสมอย่างยิ่งในการวิเคราะห์สารปริมาณน้อย

คำสำคัญ คาพาซิทีฟไบโอเซนเซอร์ ความจุไฟฟ้า ความไววิเคราะห์สูง แอฟฟินิตี ไม่ติดฉลาก ตามเวลาจริง การวิเคราะห์สารปริมาณน้อย อิเล็กโทรดแบบใช้แล้วทิ้ง ซูโดแอฟฟินิตี

Abstract

This project focuses on capacitive biosensors using potentiostatic capacitance measurement. Highly sensitive biosensors were investigated for direct detection of trace amount of various compounds based on affinity binding interaction. The study was initially relied on the immobilization of sensing element on solid gold electrode where two different models were investigated. One is the detection of nucleic acids by its affinity binding to immobilized histone. The other is the use of a pseudoaffinity ligand, 3-Aminophenylboronic acid, to detect bacteria through the affinity binding between boronic acid moiety and diol-compound on the bacteria surface. Another part of the project was to investigate the use of disposable electrode to facilitate electrode preparation process. Anti-human serum albumin immobilized on thin gold film fabricated by thermal evaporation technique was used to detect human serum albumin. All investigated systems provided very low detection limit, pico to femto molar. The electrode, immobilized with sensing molecules, can be regenerated and reused at least 30 times. When applied to analyze real samples, the results agreed well with conventional methods. In addition, hardware and software of a real-time capacitive measuring system was developed. The results obtained with this system compared well with those from the commercial off-line system. The development of this real-time system enabled the results to be observed when the tests were being carrying out. This helped to speed up the analysis process which previously has to be done after the experiment. The results of these investigations demonstrated that the potentiostatic capacitance measurement is a highly sensitive technique that is well suited for trace level analysis.

Keywords: Capacitive biosensor; Capacitance; Highly sensitive; Affinity; Label-free; Real-time; Trace analysis; Disposable electrode; Pseudoaffinity

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Executive Summary

The objectives of this project were to develop a label-free capacitive affinity biosensor system for sensitive detection of analytes and to simplify the arrangement and handling of the sensor through the development of a disposable electrode and a real-time capacitive detection device. Sensing molecules were immobilized on gold working electrodes. Capacitance change due to the binding of analytes in the sample to the immobilized molecules was detected using potentiostatic step method. A commercially available potentiostat was initially employed in two investigations based on the immobilization of sensing elements on solid gold electrodes. One was the detection of nucleic acids using histone as biological sensing molecules. The system provided very good reproducibility of the signal with wide linear range and could detect DNA at an extremely low level of 10 fg ml^{-1}. The other was the use of 3-Aminophenylboronic acid (3-APBA) that is more stable than the biological component, to detect bacteria through the affinity binding between boronic acid moiety and diol-compounds on the bacteria surface. Good performances were also obtained. Its stability was proven by the higher number of reusability. One modified electrode could be reused up to 58 times, compared to about 43-45 times for electrodes based on biological components.

Another investigation was the use of disposable electrode to facilitate electrode preparation process. Electrodes coated with thin layer of gold by thermal evaporation technique was used as working electrode to measure the capacitance in a label free immunosensor system, using the detection of human serum albumin (HSA) by the immobilized anti-HSA as a model. Very low detection limit was obtained at 8.0 fM. Although this electrode was aimed to be used as “disposable”, it was found that each modified electrode can be reused up to 30 times.

To further improve this system, hardware and software of a real-time capacitive measuring system was constructed. Potential pulse was generated by electronic circuits and applied to the capacitance measuring unit. The current response was collected and sent to the capacitive analyser program where the capacitance was calculated, shown on the monitor as a function of time and saved on the computer for further analysis. The results obtained from this device, on the detection of affinity reaction between HSA and anti-HSA, agreed well with those obtained from the commercial off-line system.

The results from this project demonstrated the potential of this capacitive system to provide label-free sensitive detection of analytes through affinity binding reactions. Together with the developed real-time detection device, the operation of this system would be quite straight forward, leading to wider application.

1. Introduction

There is a growing need in many fields for analytical tools that can selectively detect substances at very low concentrations, *e.g.*, clinical analysis, process control and environmental monitoring. There will also be a situation when the samples to be analysed are mixture of compounds. If dilution can be applied, then the interfering effects of the matrix will be reduced. But at the same time the concentration of target analyte will also become lower. Therefore, sensitive assays are necessary and the use of affinity-based biosensors is promising since they can provide high sensitivity and selectivity.

Affinity-based biosensors use, for example, receptors, antibodies or nucleic acids to provide selective interaction with a given ligand to form a complex (Mello & Kubota, 2002) and detect the changes cause by the affinity reactions by appropriate transducers. The sensitivity obtained depends on the transducer and on the affinity of biological sensing molecules (Berggren *et al.*, 1998). Affinity biosensors can be divided into label-free and labeled. However, labeled affinity biosensors are expensive, time-consuming and real-time measurements are most often not possible. For label-free affinity detection, capacitive measuring system has been shown to be a highly sensitive technique (Berggren *et al.*, 2001). This technique has been used to detect the interaction of several affinity binding pairs (Berggren & Johansson, 1997; Berggren *et al.*, 1998; Bontidean *et al.*, 1998; Hu *et al.*, 2002; Bontidean *et al.*, 2003; Jiang *et al.*, 2003; Hedström *et al.*, 2005) with detection limits in the nano to femto molar range. Therefore, the low concentration detection of other affinity binding pairs such as nucleic acids or some pseudoaffinity ligands would also be possible.

The objectives of this project are the development of a label-free capacitive affinity biosensor flow-injection system for sensitive detection of analytes and to simplify the arrangement and handling of the sensor through the development of a disposable electrode and a real-time capacitive detection device. To reach these objectives four sub-projects were investigated. The study was initially based on the immobilization of the sensing element, on solid gold electrode, and the sensitive detection by the capacitive measuring system. The study investigated two different models. One is the detection of nucleic acids, which in some case the product and in some case the impurity in biotechnology processes (Levy *et al.*, 2000; Lokteff *et al.*, 2001), using a biological sensing element. The other is the use of pseudoaffinity ligand , 3-Aminophenylboronic acid (3-APBA) that is more stable than the biological component, to detect bacteria through the affinity binding between boronic acid moiety and diol-compounds (Gabai *et al.*, 2001) on bacteria surface. The other two sub-projects were the investigation of the possibility to develop this technique into a disposable device for a quick analysis and to develop a real-time capacitance measurement system.

2. Flow-Injection Capacitance Measurement

The capacitive biosensor is operated in a flow injection system. This gives a range of advantages including continuous detection, reproducibility and high sampling rate (Bosque-Sendra *et al.*, 2003; Hansen & Wang, 2004). The basic experimental set-up of the flow injection capacitive system is shown in Figure 2.1. A three-electrode system is connected to the potentiostat. The working electrode is the modified gold electrode where the sensing element is immobilized.

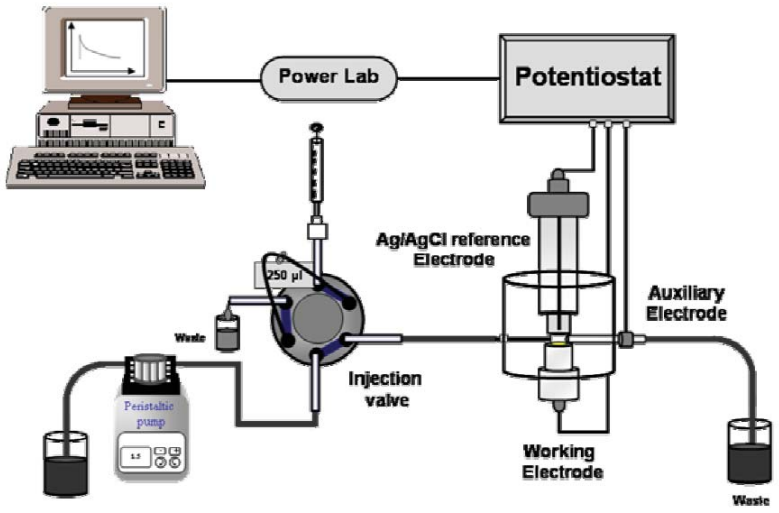

Figure 2.1 Schematic diagram showing the flow injection capacitive system

A potentiostatic step method was used to measure the capacitance. This method is based on the assumption that the system could be described as a simple RC model (Berggren & Johansson, 1997). When a potential pulse is applied to the modified gold electrode (working electrode) the current response signals can be described by

$$i(t) = \frac{u}{R_S}\exp\left(-\frac{t}{R_S C}\right) \qquad (1)$$

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* is the total capacitance measured at the working electrode/solution interface. The potential pulse was applied at a regular interval and the analog current responses from the potentiostat was sampled at a set frequency and recorded on a computer using the Powerlab data acquisition system system (ADInstrument, Australia). The current responses was monitored, first with the buffer solution, then during the injection of the analyte that will bind to the immobilized sensing elements on the gold electrode. The surface of the electrode will then be regenerated with regenerating solution to remove the analyte from the electrode to be ready for next analysis.

After the experiment the current responses was analysed to obtain the capacitance. This is done by taking the logarithm of the current, plotted it as a function of time where a linear relationship will be obtained follows the equation

$$\ln i(t) = \ln\left(\frac{u}{R_S}\right) - \frac{t}{R_S C} \qquad (2)$$

Then, C and R_s were obtained from the slope and intercept of the linear least-square fitting of ln $i(t)$ versus t. The capacitance was plotted as a function of time. When the solution containing the analyte was injected, it bound to the immobilized sensing elements on the gold electrode causing the capacitance to decrease until it reached a stable value. The change in capacitance, due to the binding, is the difference between C after the binding and C before the binding (Figure 2.2).

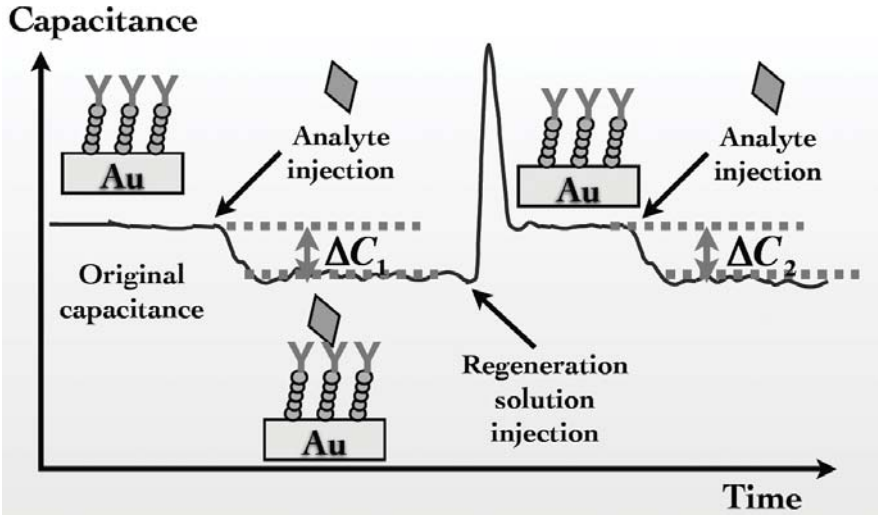

Figure 2.2 The decrease in capacitance (ΔC_1) resulting from the binding between immobilized biological sensing molecule and the analyte. After dissociation under regeneration condition the electrode can be reused.

3. Capacitive Affinity Biosensor to Detect DNA

3.1 Introduction

In biological and biopharmaceutical products, such as monoclonal antibodies, lymphokines and vaccines, quantification of residual cellular DNA from the host cell in the purification process is also important since they have to meet specific requirements regarding contaminating cellular DNA. Guidelines of The World Health Organization (WHO) recommends that the residual cellular DNA permitted in purified product contain less than 10 ng per dose (WHO, 1998). In this work a flow injection capacitive biosensor was applied for the rapid determination of trace amount of residual DNA based on the affinity binding of DNA to histone by immobilizing whole histone on gold electrode surface via self-assembled monolayer (SAM) of thioctic acid. This system was validated with real samples by determining genomic DNA contamination in crude shrimp protein preparation. Since this work has already been published in *Biosensors and Bioelectronics* (Numnuam *et al.*, 2009) (Impact factor 2008: 5.143) (see Appendix) only a short summary is presented.

3.2 Experimental

Histones from calf thymus and shrimp were immobilized on gold electrodes covered with SAM of thioctic acid. Each of these histones was used to detect DNA from calf thymus, shrimp and *Escherichia coli*. Operating conditions of the flow injection biosensor system were optimized for the affinity binding between DNA and histones from calf thymus. Parameters affecting the capacitive response were studied. These included regeneration solution (type, pH and concentration), sample volume, flow rate, and carrier buffer (type, pH and concentration). The optimum of each parameter was determined as a balance between the sensitivity (slope of the calibration curve) or capacitance change and analysis time. Using the obtained optimum conditions, the performances of the system i.e., linear range, lower detection limit, selectivity were investigated. Effects of histones and DNAs from different sources i.e. calf thymus and white shrimp were also tested.

3.3 Results

3.3.1 System performance

Assayed and optimized conditions of parameters affecting the capacitive response are shown in Tables 3.1-3.2.

Table 3.1. Assayed and optimized conditions of the type, pH and concentration of regeneration solution. The efficiency of DNA removal from the histone immobilized on the electrode was studied by injecting 1 ng l^{-1} of standard DNA solution.

Parameter of regeneration solution	Condition	Efficiency of DNA removal (%average residual activity)	Regeneration time (min)
Type	50 mM glycine – HCl, pH2.4	87± 2	13-16
	HCl pH 2.4	83 ± 2	12-14
	50 mM NaOH	70 ± 4	17-21
	50 mM KCl	54 ± 4	35-45
Concentration	10 mM Glycine-HCl, pH 2.4	81 ± 2	10-12
	25 mM Glycine-HCl, pH 2.4	93 ± 3	13-16
	50 mM Glycine-HCl, pH 2.4	85 ± 2	14-18
	75 mM Glycine-HCl, pH 2.4	74 ± 1	14-19
	100 mM Glycine-HCl, pH 2.4	58 ± 3	15-21
pH	25 mM Glycine- HCl, pH 2.2	86 ± 3	12-13
	25 mM Glycine- HCl, pH 2.4	97 ± 3	12-15
	25 mM Glycine- HCl, pH 2.6	88 ± 2	12-16
	25 mM Glycine- HCl, pH 2.8	81 ± 1	13-17

Table 3.2. Assayed parameters and optimized values of the capacitive system. Capacitive change is from the injection of 1 ng l^{-1} of calf thymus DNA.

Parameter	Investigated values	Capacitive change (-nF cm^{-2})	Analysis time (minute)	Optimized values
Concentration of buffer Tris-HCl, pH 7.2 (mM)	5	164 ± 25	12	10
	10	160 ± 5	14	
	25	103 ± 5	15	
	50	68 ± 4	17	
	100	18 ± 5	19	
pH of buffer 10 mM Tris-HCl	7.00	101 ± 1	14	7.00
	7.20	71 ± 6	14	
	7.40	37 ± 3	14	
	7.60	32 ± 3	14	
	7.80	26 ± 2	14	
	8.00	15 ± 2	14	
Flow rate (µl min^{-1})	50	169 ± 10	18	100
	100	165 ± 3	14	
	250	116 ± 3	12	
	500	65 ± 13	8	
Sample volume (µl)	50	29 ± 3	10	250
	100	65 ± 5	11	
	150	76 ± 5	12	
	200	99 ± 2	12	
	250	106 ± 4	13	
	300	104 ± 5	14	
	400	106 ± 2	17	

Under optimum conditions, histones from calf thymus and shrimp provided the same lower detection limit of 10^{-5} ng l^{-1} for DNA from different sources, *i.e.*, calf thymus, shrimp and *E. coli*. The standard curve for the affinity reaction between calf thymus histone and DNA shows sigmoidal behavior and two linear ranges, 10^{-5} to 10^{-2} ng l^{-1} and 10^{-1} to 10^{2} ng l^{-1}, could be obtained (Figure 3.1). The immobilized histones were stable and after regeneration good reproducibility of the signal could be obtained up to 43 times with a %R.S.D. of 3.1.

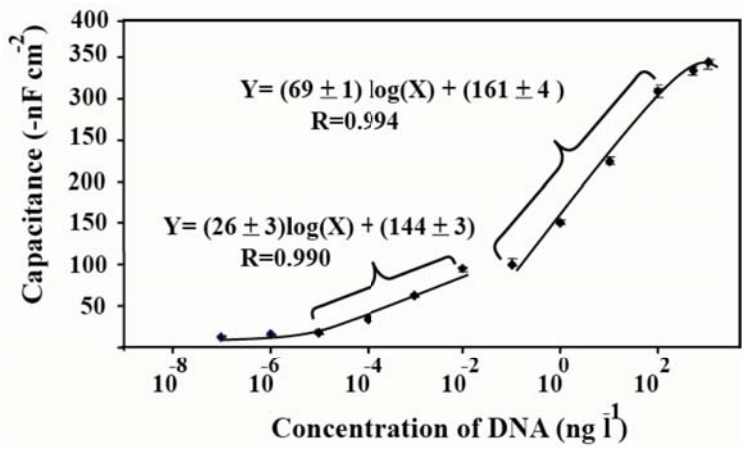

Figure 3.1 Capacitance change vs. the logarithm of calf thymus DNA concentration detected by immobilized calf thymus histone modified electrode under optimum conditions.

3.3.2 Selectivity

Since different sources of DNA or histones may affect the binding and, hence, the response of the capacitive system, the effect of DNA source was studied by using immobilized calf thymus histone and shrimp histone to detect DNA from calf thymus, white shrimp and *E. coli.* The studies indicated that histones can bind better with DNA from the same source and give higher sensitivity than the binding with DNA from different sources (Figure 3.2).

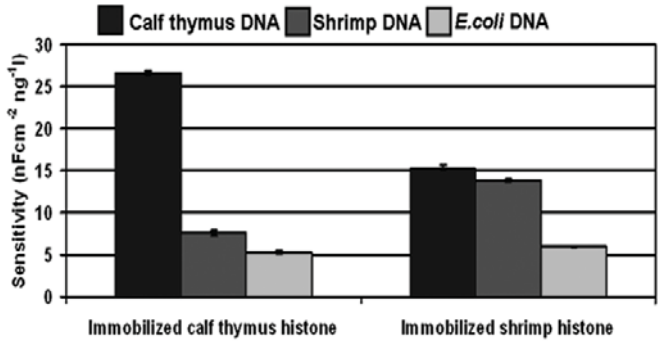

Figure 3.2 Sensitivities of immobilized calf thymus histone and shrimp histone for the detection of DNA from calf thymus, white shrimp and *E.coli*.

The effect of a mixture of DNA from different sources was also studied using immobilized calf thymus histones to detect the mixture of DNA from calf thymus, white shrimp and *E. coli* at different concentrations (Figure 3.3).

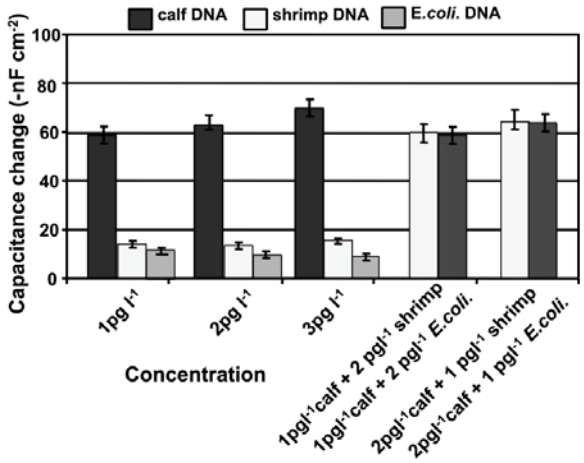

Figure 3.3 Effect of DNA from calf thymus, shrimp , *E. coli* and their mixture on the binding with calf thymus histone.

3.3.3 Recoveries

To validate the capacitive detection system with real samples, the recovery of spiked DNA (0.1-1.5 pg l^{-1}) in crude shrimp protein samples were tested. The analysis was carried out with immobilized shrimp histone. The responses obtained from spiked shrimp protein sample were used to calculate the concentration from the calibration curve. Recoveries were obtained between 80% and 116% (Table 3.3). From the results, percentage of recovery and relative standard deviation of all spiked DNA (in ng l^{-1} range) in crude shrimp protein are acceptable (Taverniers *et al.*, 2004) (acceptable recovery in the µg l^{-1} level is 40-120% and R.S.D. is 30-45.3%). These results show that the developed capacitive biosensor is suited for quantification of DNA.

Table 3.3 Recovery of shrimp DNA from spiked crude shrimp protein (n=3)

Spiked concentration	Immobilized shrimp histone				Immobilized calf thymus histone			
	Sample 1		Sample 2		Sample1		Sample 2	
	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)
Matrix matched calibration (pgl^{-1})								
0.10	80 ±18	22	-	-	-	-	-	-
0.50	116 ± 15	13	-	-	-	-	-	-
1.0	107 ± 7	7	-	-	-	-	-	-
1.5	100 ± 6	6	-	-	-	-	-	-
Dilution ($ng\ l^{-1}$)								
10	73 ± 2	3	88 ± 23	26	77 ± 6	8	95 ± 17	18
50	109 ± 5	5	98 ± 17	17	115 ± 15	13	117± 22	19
100	103 ± 4	4	113 ± 4	4	101 ± 6	6	116 ± 18	16
150	110 ± 1	1	108 ± 5	5	106 ± 7	7	101 ± 9	9

3.4 Conclusions

The results demonstrated the possibility of using the capacitive biosensor system for direct assay of affinity binding between DNA and histone protein. The lower detection limit of this system, 10^{-5} ng l^{-1}, is much lower than the 10 ng per dose guideline set by WHO (WHO, 1998). Since histone from one source can also detect DNA from other sources, this system is suitable for screening DNA contaminants independent of their origin. However, if the source of DNA is known a calibration curve can be constructed and this system can be used to quantify the amount of DNA which will be useful for a number of biotechnology process. This method can be used to investigate DNA in samples with different matrices. In case of trace DNA level, matrix matched calibration curve can be applied, for example, residual DNA in biological or biopharmaceutical products. For quantification of high concentrations of DNA, such as in samples after cell lysis, the matrix interference can be reduced by dilution where the standard curve could be used to determine the DNA in diluted real sample. Comparing to other commonly used methods for determination of DNA concentration, this system is more advantageous. For example, spectrophotometric method (absorbance at 260 nm, or the A260 method), although this method is accurate and reproducible, it is relatively insensitive and can only measure DNA concentrations that is greater than 5 μg ml^{-1} (Noites *et al.*, 1999) while the develop capacitive system can detect DNA as low as 10^{-8} ng ml^{-1}. In addition, analysis time of the caacitive technique (13–15 min) was much shorter than other DNA quantitation methods (30 min to 5 h) (Projan *et al.*, 1983; Schmidt *et al.*, 1996). Although, the preparation of electrode requires some time, one electrode can be reused up to 40 times by using the appropriate regeneration solution and this helps to reduce the cost of analysis.

4. Phenylboronic Acid Affinity Sensor

4.1 Introduction

The uses of compound with ligands that are more stable than the biological component are attractive when operating in crude solutions/suspensions. Such ligands are often chemically and physically stable and can stand sanitation steps. One interesting group of compounds is boronic acids. They form covalent bonds with cis-diols to generate five or six-membered cyclic esters in a basic aqueous media while the esters reversibly disassociate once upon the media pH is changed to an acidic condition and have been widly used to capture and isolation of cis-diol-containing molecules such as carbohydrates, RNA, nucleosides, glycoproteins and glycopeptides (Ren *et al.*, 2009). Boronic acids have been widely applied as sensing material in various sensors (Wang *et al.*, 2002; Schneider *et al.*, 2007) and as solid-phase in affinity chromatography extraction (Tsikas, 2001).

3-Aminophenylboronic acid (3-APBA) is a derivative that have a boronic acid moiety that can be used as recognition molecules for diol-compound (Gabai *et al.*, 2001). It has been applied as sensing material for the detection of various saccharides (Soh *et al.*, 2003; Sun *et al.*, 2004; Ma & Yang, 2005; Přibyl & Skládal, 2005; Torun *et al.*, 2009) and glycoproteins (Liu *et al.*, 2005; Přibyl & Skládal, 2006). Detection principles are based on electrical (Liu *et al.*, 2005; Ma & Yang, 2005), optical (Soh *et al.*, 2003; Sun *et al.*, 2004; Přibyl & Skládal, 2006) or mass changes (Přibyl & Skládal, 2005, 2006). Since the affinity binding causes change to the surface properties, therefore, it is possible to apply the capacitive system to detect the binding interaction.

One interesting application is the use of 3-APBA to detect bacteria. This can be done based on the binding between 3-APBA and glycoprotein and polysaccharide, diol compounds, on bacteria cell wall. Although the binding will not be selective to a particular species of bacteria since they all contain molecules with saccharides on their surface (Bower & Rosenthal, 2006) it will be useful as a rapid screening method. In this work we study the possibility of applying the capacitive system, with immobilized 3-APBA, for the detection of bacteria. To the best of our knowledge this is the first time that 3-APBA has been applied in a label-free affinity detection system for bacteria.

4.2 Experimental

4.2.1 Materials

11- mercaptoundecanoic acid was from Aldrich (MO, USA), 3-Aminophenylboronic acid, N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (N-hydroxy-2,5-pyrrolidinedione, NHS) were obtained from Sigma–Aldrich (Steinheim, Germany). All other chemicals were analytical grade. Buffers were prepared with 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 mm, with subsequent degassing.

4.2.2 Immobilization of 3-aminophenylboronic acid

Gold electrode was polished with alumina slurries (particle size 5μm, 1μm and 0.3μm) 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 with pure nitrogen gas (Limbut et al.,2006). The cleaned electrode was immersed in 11-mercaptoundacanoic acid solution, thoroughly rinsed with absolute ethanol and DI water then dried with pure nitrogen gas. In this step self-assembled 11-mercaptoundecanoic acid monolayer was formed on gold surface. The good formation of 11-mercaptoundecanoic acid is dependent on time and concentration, so concentration and incubation time were studied. The terminal carboxylic group of the SAM was activated by using 0.05 M of EDC with 0.03 M of NHS in phosphate buffer (pH 5.00). The obtained monolayer of N-hydroxysuccinimide-activated carboxylic groups was able to bind with primary amino group of 20 μl 3-aminophenylboronic acid (400 ppm) in phosphate buffer (pH 7.00). The electrode was kept at 4°C overnight. The immobilized layers were evaluated by cyclic voltammetry (Eco Chemie μ-autolab B.V., Netherland, and software package GPES 4.9). A reduction of redox peaks of ferrocyanide probe ($K_3[Fe(CN)_6]$) after each immobilized step confirmed that the layer was added on the electrode.

4.2.3 Preparation of bacteria standard

Pure cultures of *E. coli*, *Salmonella spp.* and *Staphylococcus aureus* were prepared in 250 ml conical flasks containing 100 ml of Difco™ Nutrient Broth at 37 °C in a shaker incubator (Heidolph UNIMAX 1010 and INKUBATOR 1000, Germany) at 150 rpm for 24 h. They were collected by centrifugation (10 min, 6,000 rpm) (DUPONT instruments, USA), washed three times by resuspending in 10 ml of 10 mM Tris-HCl buffer solution pH 7.5 and centrifuged again under the same conditions. The culture turbidity was adjusted to match a 0.5 McFarland standard, and serially diluted (10^8−10^2 CFU ml^{-1}) in 10mM Tris-HCl (pH 7.5).

4.2.4 Capacitance measurement

The modified electrode was used as the working electrode in a capacitive system and connected to the potentiostat (Model EA161, EDAQ, Australia). The transient current response obtained by applying a potentiostatic step (pulse every minute) of +50 mV was recorded and used to calculate the capacitance of the surface as described by Limbut et al. (2006).

4.2.5 Optimization of Flow Capacitive system

Primary conditions used in the flow injection capacitive system were carrier buffer 10mM Tris–HCl pH 7.6, 10 mM phosphate buffer pH 5.5 for regeneration solution, flow rate at 50 µl min^{-1} and a sample volume of 300 µl (1.5×10^4 CFU ml^{-1} of *E. coli).* The effect of type, pH and concentration of regeneration solution was firstly optimized followed by pH, concentration of carrier buffer, flow rate, and sample volume. Three replications were performed for each test value. The optimization was studied by changing one parameter and keeping other parameters constant. The optimum was considered by balancing between sensitivity and analysis time.

4.2.6 Real sample analysis

Thirteen real water samples were analyzed including three tap water, four bottled water, one raw water from a water reservoir, one well water, two from wastewater treatment ponds and three domestic wastewater samples. They were analyzed by the flow injection capacitive system and compared with the standard plate count method as described by Hobson et al. (Hobson *et al.*, 1996). In brief 10 µl of diluted water sample was spread onto agar plates (18 ml of Difco™ Nutrient Agar) and incubated at 37 ºC for 16-24 h, 5 replications for each sample. Total number of colonies was counted and multiplied by the dilution factor to obtain the concentration.

4.3 Results and discussion

4.3.1 Immobilization

The effect of concentration and incubation time of 11-mercaptoundecanoic acid on the formation of SAM on gold surface was tested by considering the reductive desorption of the monolayer in thiol solution via the reaction

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

At negative potential the thiol groups can be reduced with consequent thiol desorption from gold surface. The charge under desorption peak is used to estimate the surface concentrations of the thiol solutions in monolayer as follows:

$$\Gamma = Q/nFA$$

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 the electrode surface area (cm^2) (Mirsky, 2002). The degree of insulation was observed from the redox peaks using cyclic voltammetry with ferrocyanide as a redox probe (i.e. $K_3[Fe(CN)_6]$) in the electrolyte solution.

Incubation time was fixed at 20 min to study the effect of concentration in the range of 1-10 mM. The surface coverage increased from 1 to 4 mM. Between 4 and 10

mM nearly the same amount of surface coverage were obtained. All concentrations provided surface coverage of a monolayer, within the range of 10^{-9} to 10^{-10} mol cm^{-2} (Bard & R.Faulkner, 2000). However, only 10 mM showed good insulating surface, observed from the absence of the cyclic voltammogram redox peaks. Then, 10 mM was used to study the incubation time from 10 to 60 min. They all gave rise to monolayer, but the layer only showed good insulation from 50 min onwards. Therefore, 10 mM of 11-MUA at 50 min incubation time were used for the formation of SAM.

During the immobilization steps cyclic voltammogram was used to observed the degree of insulation. At the clean gold surface the redox couple could be oxidized and reduced. The amplitude of the peaks were very much reduced when 11-mercaptoundecanoic acid was self-assembled on the clean gold surface. When 3-APBA was placed on the electrode to coupling with the aldehyde groups, the insulating property of the electrode surface was further increased and could be seen as a flat cyclic voltammogram indicating that 3-APBA was covered on the electrode.4.3.2 Optimization

Regeneration solution

The regeneration solution was injected to remove *E.coli* from the binding with 3-APBA on the electrode surface, enabling the electrode to be reused. To evaluate the performance of the regeneration solution, residual activity of the 3-APBA electrode was calculated from the capacitance change (ΔC) due to the binding between *E.coli* and 3-APBA before and after regeneration using the equation

$$\%\text{Residual activity} = \frac{\Delta C_2}{\Delta C_2} \times 100$$

Where ΔC_1 and ΔC_2 are the capacitance change before and after regeneration, respectively. To break the 3-APBA-diol complex, acidic buffer was considered (Liu *et al.*, 2008; Zhang *et al.*, 2008). Three buffers, acetate pH 5.50, phosphate pH 5.50 and glycine-HCl pH 3.50 were studied. Acetate buffer gave high residual activity (86 ± 10 %) and shortest regeneration time (20 min). Therefore, further study on concentration of acetate buffer was studied between 5 mM and 25 mM. The highest percentage residual activities was obtained at 10 mM. The effect of pH between 3.50 to 5.50 was then studied and pH 4.50 provided the highest percentage residual activity. Therefore, 10 mM acetate buffer pH 4.50 was used as the regeneration solution.

Buffer solution

Tris-HCl buffer has often been used in capacitive systems because it provided a more steady baseline (Limbut *et al.*, 2006b; Loyprasert *et al.*, 2008; Numnuam *et al.*, 2009) and it was used in this work. Since the binding of anion boronate with diol compound depend on the component of matrices such as pH and electrolyte concentration (Lau *et al.*, 2000), the pH and concentration of Tris-HCl buffer were investigated. Concentration of Tris-HCl was studies at 5mM, 10mM, 15mM and 20mM. The highest

response was obtained at 10 mM, so this concentration was used to study the pH of Tris-HCl buffer at 7.00, 7.20, 7.40, 7.60, 7.80 and 8.0. The response was highest at pH 7.50.

Sample Volume

The effect of sample volume on capacitance change was investigated at 200, 250, 300, 350, 400, 450 and 500 µl. The signal of capacitance change increased with sample volume reaching a steady level between 350 µl and 400 µl. Therefore, 350 µl was chosen because the volume was lower and the analysis time was also shorter (20 min compared to 23 min).

Flow rate

The flow rate of solution through the measuring cell can affect the binding between *E. coli* and the immobilized 3-APBA on gold surface. Flow rate was test at 25 µl min^{-1}, 50 µl min^{-1}, 100 µl min^{-1}, 150 µl min^{-1} and 200 µl min^{-1}. When the flow rate increased the capacitance change decreased. There was not much difference between the responses of 25 µl min^{-1} and 50 µl min^{-1} (6%) but the analysis time of 50 µl min^{-1} was much shorter than 25 µl min^{-1} (25 min and 40 min, respectively). Therefore, 50 µl min^{-1} was chosen.

4.3.3 System performances

Linear dynamic range and detection limit

The gold electrode modified with 3-aminophenylboronic acid was applied in the flow capacitive system under optimum conditions. A linear relationship between the capacitance change and the logarithm of *E. coli* concentration was obtained between 1.5×10^2 to 1.5×10^6 CFU ml^{-1} (Figure 4.1). The limit of detection (LOD) was 100 CFU ml^{-1}, based on IUPAC Recommendation 1994 (Buck & Lindner, 1994). This LOD is much lower than the acceptable heterotrophic plate count (HPC) bacteria (also known as colony counts and previously known as standard plate count) in municipal drinking-water in most countries that have been set at less than 500 CFU ml^{-1} (Robertson & Brooks, 2003).

Selectivity

The effect of different bacteria (*E. coli*, *Salmonella spp.* and *Staphylococcus aureus*) was studied. The same concentrations (2×10^2 - 8×10^2 CFU ml^{-1}) of bacteria were inject to test the binding with 3-ABPA. The sensitivity (slope of calibration curve) obtained from *E. coli* (14.6 ± 1.3 -nF cm^{-2}/log CFU ml^{-1}) was slightly higher than the one from *Salmonella spp*. (12.7 ± 0.6 -nF cm^{-2}/log CFU ml^{-1}) and the lowest was from *Staphylococcus aureus* (6.8 ± 0.6 -nF cm^{-2}/log CFU ml^{-1}). This is probably due to the fact that gram-negative bacteria (*E. coli* and *Salmonella spp.*) have polysaccharide (O-antigen) on the outer membrane (Shen *et al.*, 2007), and are easier to bind to 3-APBA. On the other

hand *Staphylococcus aureus*, the gram-positive bacteria, has peptidoglycan in the cell wall (Manning, 2004), and is not as readily available to bind with 3-APBA as in gram-positive bacteria.

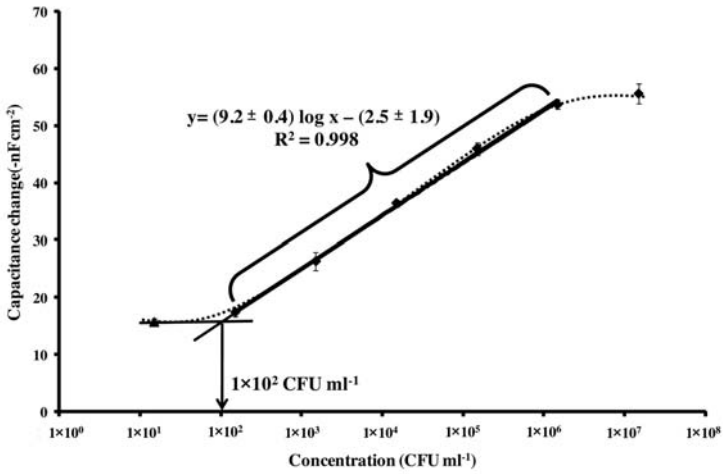

Figure 4.1 Capacitance change vs. the logarithm of *E.coli* concentration detected by electrodes with immobilized 3-APBA optimum conditions.

The mixture of different bacteria was also studied. First the response to each type of bacteria was test at 1×10^3, 2×10^3 and 3×10^3 CFU ml^{-1}. Then the mixture of 1×10^3 CFU ml^{-1} of *E. coli* + 2×10^3 CFU ml^{-1} of *Salmonella spp*. or *Staphylococcus aureus* and 2×10^3 CFU ml^{-1} of *E. coli* + 1×10^3 CFU ml^{-1} of *Salmonella spp*. or *Staphylococcus aureus* were tested. The results indicated that the mixture of bacteria gave lower response than when the the individual responses were combined (Figure 4.2). From the data it is clear that 3-APBA is not selective to a particular type of bacteria. This would be an advantage for the screening process where the purpose is to find out whether there is any bacteria but does not require to know the type of bacteria.

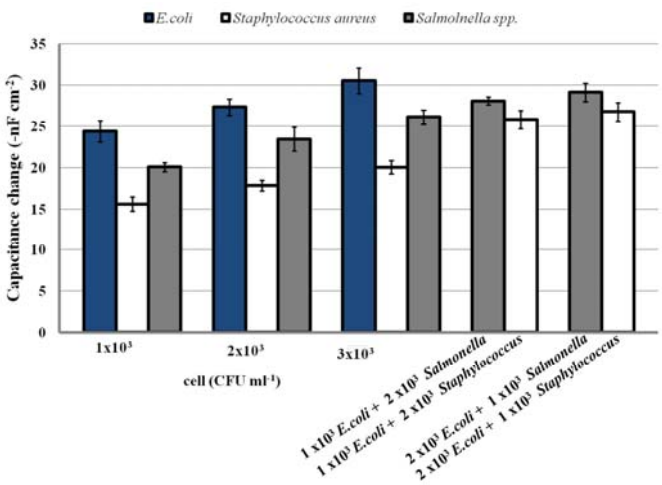

Figure 4.2 Capacitive responses from *E. coli, Staphylococcus aureus, Salmonella spp.* and their mixtures.

Stability

The reproducibility of 3-APBA modified electrode was investigated by monitoring capacitance change (-nF cm^{-2}) at the same concentration of standard *E. coli* (1.5×10^4 CFU ml^{-1}). After analysis, 10 mM acetate buffer pH 4.5 was injected to break the binding between *E.coli* and 3-APBA. For the first 58 cycles the binding activity of 3-APBA electrode retained an average of 98.6 ± 6.6% of the original capacitance change. After 58 times percentage residual activity of electrode decreased rapidly. This suggested that one preparation of the electrode can be reused with good reproducibility up to 58 times. This electrode was then tested by cyclic voltammetry to ensure that the self-assembled monolayer was not destroyed by the regeneration solution. Voltammograms of modified electrode before and after use were not different. That is, the decrease of responses is probably caused by the lost of 3-APBA activity.

The reusability of this electrode is better than when biological components were employed. For examples, capacitive affinity biosensors with immobilized antibodies can be reused between 43-45 times (Limbut *et al.*, 2006b; Loyprasert *et al.*, 2008). Similar reusability (43 times) has also been obtained with immobilized histone (Numnuam *et al.*, 2009). In addition the cost of 3-APBA is much lower than these biological molecules.

4.3.4 Real samples analysis

The effect of matrices in water sample was first studied. Known concentrations of *E. coli* were spiked into real water samples in the range of 2×10^2 to 8×10^2 CFU ml^{-1}. The plot of responses *versus* concentration was the spike curve. The running buffer was spiked with *E. coli* in the same range and used to construct the standard curve. The slopes of standard curve and spike curve were then compared by two-way ANOVA (R Development core Team, 2006). If there is no significant difference, it indicated that there is no matrix effect. The results found that, only the drinking and tap water samples showed no matrix effect, so these two types of samples can be analyzed directly. Other types of samples need a procedure to reduce the effect of matrix before analysis. One possibility is by dilution. The spike water samples were diluted by 10 mM Tris-HCl buffer pH 7.5 at several dilution factors. Depending on the types of samples, at 1,000-10,000 times dilution the matrix had no effect. Therefore, the standard curve could be use to determine *E. coli* by diluting the samples 1,000-10,000 times.

Table 4.1 shows the results obtained from real samples. The six “non detectable” results for the capacitive detection system indicated that the bacteria in the sample was lower than the limit of detection, 100 CFU ml^{-1}. However, they all corresponded well with the standard plate count where no bacteria was detected. The results from the capacitive sensor system were lower than the plate count technique between 3-10 %. This is probably because the calibration curve that used to determine the concentration of bacteria for the capacitive system was prepared using *E. coli* which normally provided the high response (see ***Selectivity*** in 4.3.3). Also the sample contained mixture of bacteria which normally provided lower responses than the combined responses of individuals (Figure 4.2).

However, this system would still be very useful since it can be applied as a rapid method (analysis time 25 min, regeneration time 20 min) for the estimation of number of bacteria compared to the plate count technique that requires 16-24 h.

It is interesting to see that in terms of bacterial level the well water and all of the tap waters are better than some bottled waters. It might seem surprising, however, a number of reports in several countries have also found high level of bacteria in bottled water, higher than the guideline of 500 CFU ml^{-1} (Armas & Sutherland, 1999; Lalumandier & Ayers, 2000; Venieri *et al.*, 2006; Zamberlan da Silva *et al.*, 2008).

4.4 Conclusions

This work demonstrated the potential of using 3-Aminophenylboronic acid in a label-free capacitive affinity sensor. The system was applied to detect bacteria based on the affinity binding reaction between 3-APBA and the diol compounds on bacterial surface using *E. coli* as a model. At optimum conditions a wide linear range was obtained with a low detection limit of 100 CFU ml^{-1}, much lower than the guideline for drinking water (500 CFU ml^{-1}) (Robertson & Brooks, 2003). This compound is more stable and less expensive than using biological components, such as antibodies. One preparation of electrode could be reused up to 58 times compared to about 43-45 times when using antibodies or histone (Limbut *et al.*, 2006b; Loyprasert *et al.*, 2008; Numnuam *et al.*, 2009). This helps to reduce analysis cost even further. Several types of real water samples were tested. There were no matrix effect from bottled drinking water and tap water. That is, these two types of samples can be inject directly into the detection system. For other types of samples, only a simple dilution is required. The number of bacteria obtained from the capacitive sensor is slightly lower than those from standard plate count method. However, it would still be very useful for rapid screening of bacteria, especially in drinking water, since the analysis time is about 25 min for each sample.

Table 4.1 Number of bacteria in water samples obtained from the plate count (n = 5) and capacitive sensor system (n =3) (nd: non detectable)

Samples	Standard Plate Count ($\times 10^4$ CFU ml^{-1})	Capacitive Sensor ($\times 10^4$ CFU ml^{-1})
Tap water 1	nd	nd
Tap water 2	nd	nd
Tap water 3	nd	nd
Bottled water 1	0.038 ± 0.008	0.035 ± 0.002
Bottled water 2	nd	nd
Bottled water 3	0.060 ± 0.007	0.058 ± 0.004
Bottled water 4	nd	nd
Well water	nd	nd
Reservoir water	47 ± 6	42 ± 3
Wastewater treatment pond 1	4.5 ± 0.5	4.16 ± 0.05
Wastewater treatment pond 2	2.6 ± 0.6	2.38 ± 0.08
Domestic wastewater 1	184 ± 7	175 ± 5
Domestic wastewater 2	191 ± 8	180 ± 3
Domestic wastewater 3	192 ± 9	186 ± 2

5. Development of Disposable Electrode

5.1 Introduction

The flow-injection capacitance measurement system described above uses solid gold electrode and is designed to be reusable. Therefore, the handling of the assay is rather time consuming since it will require a regeneration step. If the technology is going to become a useful analytical technique the arrangement and the handling of the sensor need to be simplified. One of the goals of this work is to design disposable electrodes to replace the solid gold electrode. This can be done using sputtering or thermal evaporator technique to place a thin layer of gold onto a glass slide. This will hold so small amounts of gold that it can be used as a disposable device. Electrodes were prepared by sputtering and thermal evaporator technology, in collaboration with laboratories at National Electronics and Computer Technology Center (NECTEC) and National Materials Technology Center (MTEC), respectively.

For a potentiostatic capacitance detection system the electrode surface has to be totally insulated and this can be done during the immobilization steps. A common method is to immobilized the biological sensing elements via SAMs of sulfur compounds on gold (Berggren & Johansson, 1997; Berggren *et al.*, 1998; Limbut *et al.*, 2006a; Limbut *et al.*, 2007). However, a good formation of SAM requires quite a long time and an alternative technique using electropolymerization of a non-conducting polymer was also investigated. Using the sputtered and thermal evaporated electrodes it was found that during electropolymerization process the sputtered gold layer was not as robust as the thermal evaporated one and the gold layer was normally peeled off. Therefore, detail study was carried out for the electrode prepared by thermal evaporation process and has been published in *Electroanalysis* (Teeparuksapun *et al.*, 2009) (Impact factor 2008: 5.143) (see Appendix), therefore, a short summary is presented.

5.2 Experimental

Disposable electrodes were fabricated by coating chromium (5 nm) and gold (200 nm) on glass strips (5.0 mm × 25.4 mm) and used in a label-free immunosensor system. Human serum albumin (HSA) and its antigen (anti-HSA) were used as a model system. Electropolymerization of *o*-phenylenediamine was used for the immobilization of anti-HSA by covalent binding.

Figure 5.1 shows the experiment set-up of the flow injection capacitive system. The operating conditions were optimized for the type and pH of regeneration solution, sample volume, flow rate, type, concentration and pH of carrier buffer. The operating conditions were considered by balancing between high capacitance signal and the time needed for one analysis. Using the optimum conditions, system performances were tested. The system was then applied to analyse HSA in human serum samples and the results

compared to albumin bromocresol green method (BCG) (the result obtained from Songklanagarind Hospital).

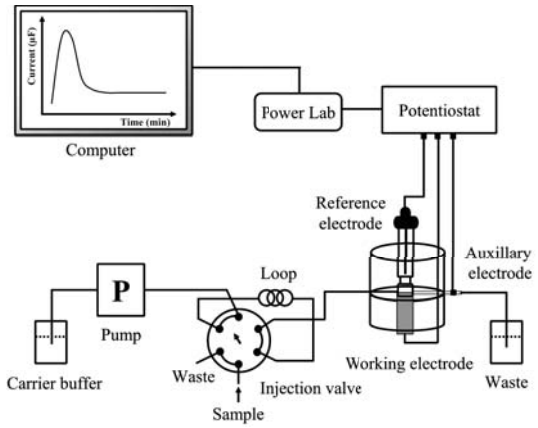

Figure 5.1 Schematic diagram showing the flow injection capacitive immunosensor system. The peristaltic pump carries the carrier buffer to the flow cell and passes to the outlet. Samples were injected to the system through injection valve and carried by carrier buffer through the flow cell. The results retrieved during the potentiostatic step due to the interaction of HSA to anti-HSA modified electrode are processed using a potentiostat measurement and control system powered by a computer.

5.3 Results

Figure 5.2 shows a typical two-dimensional perspective view of nanoscopic images of gold electrode fabricated by thermal evaporation method on glass slide. The gold grain and grain boundaries are clearly visible. The three-dimensional AFM image is also shown in Figure 5.2b. The gold electrode surface exhibits granular domain structures with a diameter of 20-28 nm. The surface of the gold electrode was obtained with the average roughness of 2.3 nm and the root-mean-square roughness of 2.9 nm.

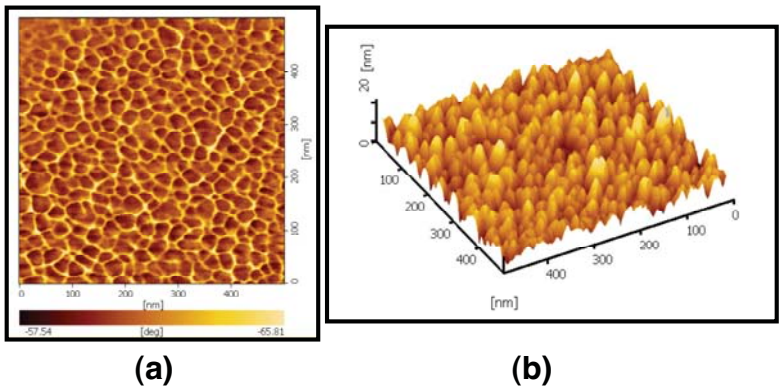

Figure 5.2 The AFM images of the gold electrode surface prepared by thermal evaporation technique (a) 2 dimensional and (b) 3 dimensional images.

Table 5.1 shows the optimum operating conditions. Using these conditions to analyse standard HSA a linear relationship was obtained in the range 1.0×10^{-14} to 1.0×10^{-9} M with the limit of detection of 8.0×10^{-15} M (Figure 5.3). The electrode can be reused up to 30 times.

Table 5.1 Optimum and investigated value of operating conditions (n=3).

Parameters	Investigated value	Optimum
Regeneration solution		
50 mM Glycine-HCl (pH)	2.2, 2.8	-
HCl (pH)	2.2, 2.4, 2.5, 2.6, 2.8	2.5
Sample volume (µL)	150, 200, 250, 300, 350	250
Flow rate (µL min^{-1})	25, 50, 100, 150, 200	100
Buffer solution		
type	tris-HCl, glycine-HCl	tris-HCl
concentration (mM)	5,10, 15, 20	10
pH	7.0, 7.2, 7.4, 7.6, 7.8	7.0

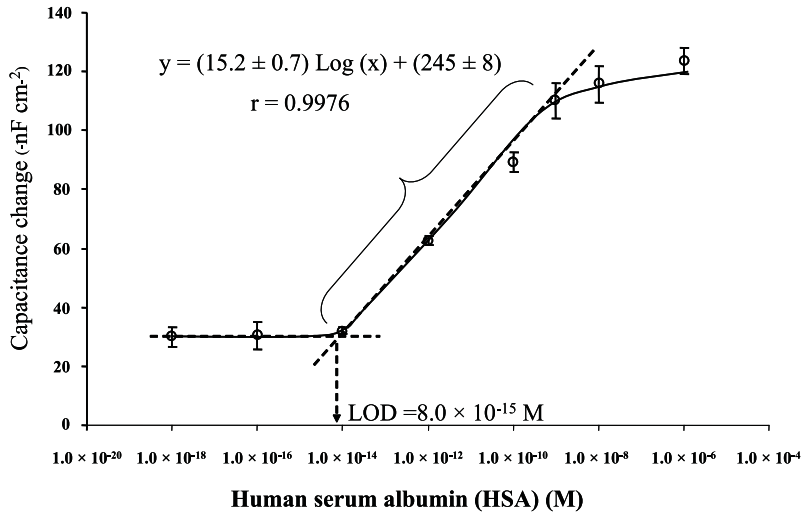

Figure 5.3 Capacitance change vs. logarithm of HSA concentration for an anti-HSA modified electrode under optimum conditions.

Selectivity to HSA was tested using bovine serum albumin (BSA), the protein that has molecular weight close to HSA. The maximum capacitance change obtained from the injection of BSA is much lower than the response obtained by the injection of HSA at the same concentration. This is also lower than the response of HSA at the limit of detection LOD indicating that the anti-HSA modified electrode has high selectivity to the detection of HSA.

The results of 16 human serum samples obtained by a capacitive immunosensor system were compared with albumin BCG method. Figure 5.4 shows the linear regression line plotted between the two methods. Concentrations of HSA determined by capacitive immunosensor system were lower than the results obtained from BCG method for all

samples. Several authors have reported that BCG method can overestimate albumin concentration. This is because the lack of specificity of Bromocresal Green (BCG) since other proteins, particulary α-and β globulin in human sample, also bind to the dye (Carfray *et al.*, 2000; Stokol *et al.*, 2001).

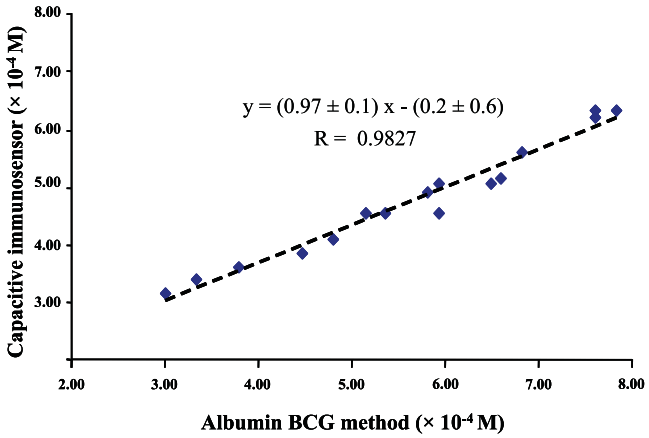

Figure 5.4 Linear regression line plotted between concentration of HSA obtained by capacitive immunosensor and Albumin BCG method.

5.4 Conclusions

The electrode fabricated by thermal evaporation method can be used as a working electrode in capacitive immunosensor system. AFM images show good characteristic of surface properties. This electrode can be used without any surface pretreatment such as mechanical polishing and electrochemical treatment. A large number of electrodes can be fabricated at one time with a very small amount of gold making it possible to use them as disposable electrode. The use of electropolymerization of *o*-PD instead of the formation of SAM on the electrode surface as an immobilization layer required much less preparation time, only 8 min while the formation of SAM of alkylthiol needed 12-24 hours (Limbut *et al.*, 2006a). The modified electrodes, which are simple and rapid to prepare, when incorporated in a capacitive immunosensor system could provide high sensitivity and low detection limit. The electrode can be reused up to 30 times and this helps to reduce the cost of analysis. The developed system showed good performance for the determination of albumin in human serum samples. With its low detection limit this technique could be used for the analysis of albumin in other type of sample such as urine in which the concentration of albumin is much lower than human serum sample. It is also possible to apply the proposed technique with other biological sensing elements to detect other analytes of interest.

6. Development of Real-time Measurement System

6.1 Introduction

In the above procedure the system does not have the ability to calculate and display the required capacitance while running the experiment. In this work we improve the measuring system by developing both the hardware to generate and control the potentiostatic pulses and a data acquisition program that will sample the current responses digitally, do the necessary transformation and show the calculated capacitance as a function of time on the computer screen as well as storing all data for further analysis.

The hardware and software of the real-time capacitive system were developed and the system was tested for the detection of an antigen based on antibody-antigen affinity interaction. Anti-human serum albumin (anti-HSA) and human serum albumin (HSA) is used as a model in this study.

6.2 Hardware

The real time measurement hardware (Figure 6.1) can be divided into two parts. One is the commercially available interface card. The other is the capacitance measuring unit composed of the electrochemical cell driver, the amplifier and the electrochemical cell containing 3 electrodes. The interface card receives the parameters, such as the frequency and the amplitude of the potentiostatic step perturbation from the software (capacitive analyzer, see 6.3) and then deliver them to the electrochemical cell driver. The amplifier maintains the potential difference between the working electrode (WE) and the reference electrode (RE). The electrical currents in the electrochemical cell is converted at the resistor R_{sense} and the obtained voltage is amplified with a suitable amplification controlled by the interface card. The amplification can be chosen from 100-1,000 and the amplification unit also attenuates and filters out undesired interferences. The amplified signal will then be digitized by the interface card and sent to the electrochemical analyzer (Figure 6.2) for calculating and displaying electrical signals, such as the resistance and capacitance in real time throughout the operational time.

6.3 Capacitive analyzer

"Capacitive Analyzer" is the program developed for data acquisition and analysis. Figure 6.2 shows the principle of the program.

To determine the capacitance the received voltage response is used to calculate the current response.

$$i(t) = \frac{u}{R_s} \exp\left(-\frac{t}{R_S C} \right) \qquad (1)$$

Then the program determines the logarithm of the current values to obtain

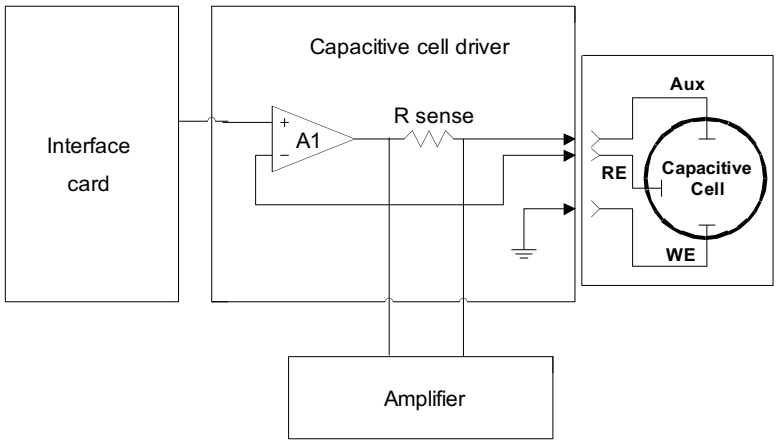

Figure 6.1 Block diagram of the hardware of the real-time capacitive measurement system.

$$\ln i(t) = \ln\left(\frac{u}{R_S}\right) - \frac{t}{R_S C} \qquad (2)$$

The linear least-square fitting of ***ln i(t)*** versus ***t*** is carried out. The values of capacitance ***C*** and resistance R_s are then calculated from the slope and Y intercept of the linear regression line. The capacitance is determined at the set time interval and the calculated capacitance is plotted on the computer monitor as a real-time response and stored. Figure 6.3 shows the computer monitor display of the capacitive analyzer showing the currents waveform, capacitance and resistance versus time in real time.

6.4 Detection of antigen

The system is tested for the detection of an antigen based on antibody-antigen affinity interaction. Anti-human serum albumin (anti-HSA) and human serum albumin (HSA) is used as a model in this study.

6.4.1 Immobilization of anti-HSA on gold surface

A gold electrode (Ø 3.0 mm, 99.99% purity) was used as the working electrode in the three electrodes electrochemical detection system. Gold surface was prepared as described earlier (4.2.2). The cleaned gold electrodes were immediately put in thiourea solution (250 mM in DI water pH 1.0). After 24 h the electrodes were thoroughly rinsed with DI water. SAM of thiourea on the gold surface was reacted with glutaraldehyde for 20 min. The electrodes were rinsed with DI water and dried with pure nitrogen gas. Then 20 µl of 0.5 g ml^{-1} anti-HSA in phosphate buffer, pH 7.0 was placed on the modified surface and left for the reaction to take place overnight at 4°C. The electrode was then immersed in 0.1 M ethanolamine pH 8.50 for 7 min, this step was to occupy all the aldehyde groups which did not couple to the anti-HSA. Finally, the electrode was reacted in a 10 mM 1-dodecanethiol ethanolic solution for 20 min to block the bare spots on the electrode surface (Figure 6.4).