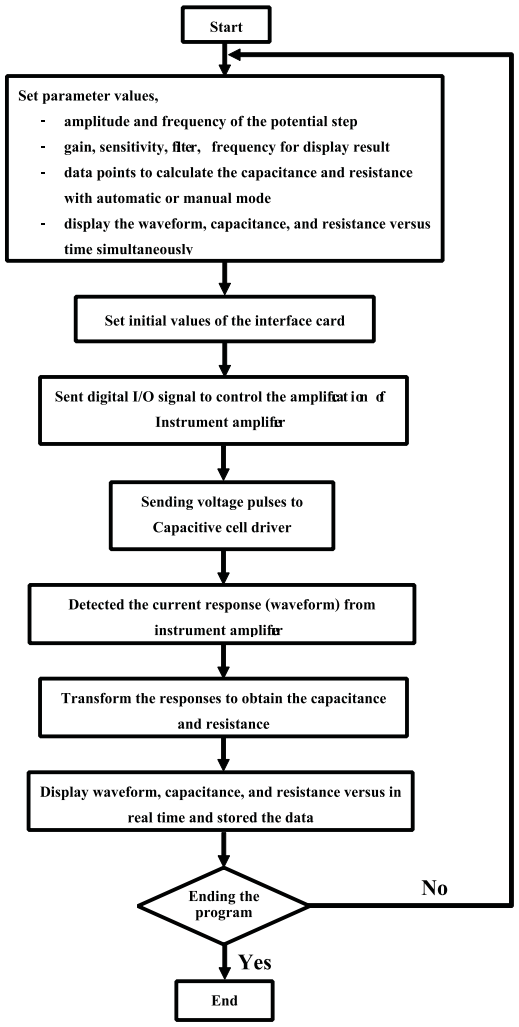

Figure 6.2 Flow chart of the capacitive analyzer program.

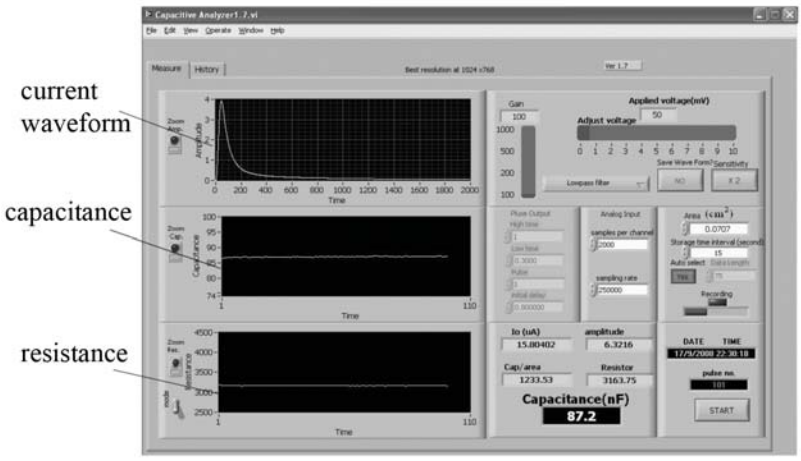

Figure 6.3 Capacitive analyzer displays the currents waveform, capacitance and resistance versus time in real time.

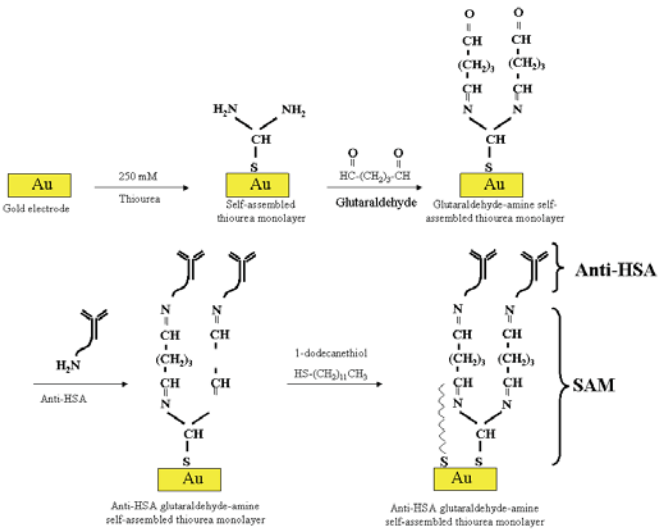

Figure 6.4 Immobilization steps of the anti-HSA immobilized on a self-assemble thiourea monolayer.

6.4.2 Capacitance measurement

A flow injection capacitive immunosensor system is employed (Figure 2.1). Three electrodes were placed in the immunosensor flow cell and connected to the capacitive online. The working electrode was the modified gold electrode. A custom made platinum electrode was used as the auxiliary. A custom made Ag/AgCl reference electrode was placed opposite to the working electrode.

During the binding event between human serum albumin (HSA) and anti-HSA, +50 mV potential pulses are applied to the gold electrode yielding current response signals where the total capacitance (C_{total}) at the working electrode/solution interface can be obtained. The measurement of C_{total} was done every minute and the results were plotted between capacitance *versus* time. When the solution containing HSA was injected into the flow cell, HSA bound to the immobilized anti-HSA 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 to remove HSA from anti-HSA immobilized electrode. Figure 6.5 shows an example of the real-time responses obtained during the injection of different HSA samples.

6.4.3 System performances

To investigate the linear range discrete pulse injection of HSA standard ranging from 1.0×10^{-18} to 1.0×10^{-8} M with intermediate regeneration steps using HCl solution pH 2.50 were performed. Figure 6.6 shows the calibration curve of 200 µl injections of HSA into 10 mM Tris-HCl buffer solution, pH 7.0 at a flow rate of 50 µl min^{-1}. A linear relationship between the capacitance change and logarithm of HSA concentration were

obtained between 1.0×10^{-16} to 1.0×10^{-8} M. The detection limit was 1.0×10^{-16} M, based on IUPAC Recommendation 1994 (Buck & Lindner, 1994).

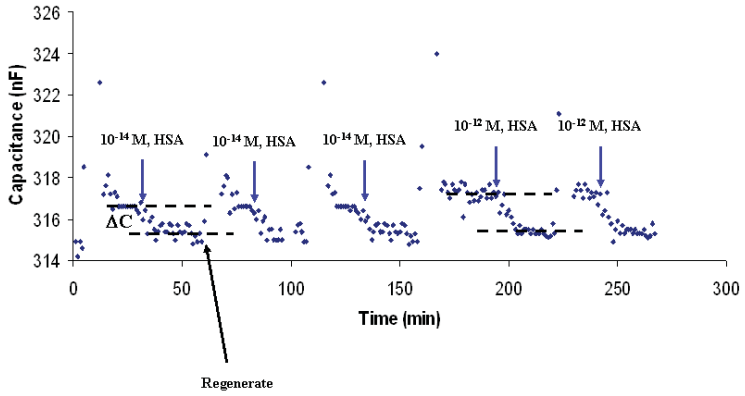

Figure 6.5 Real-time capacitive responses showing capacitance change (ΔC) as a function of time caused by binding of HSA and anti-HSA with subsequent regeneration solution.

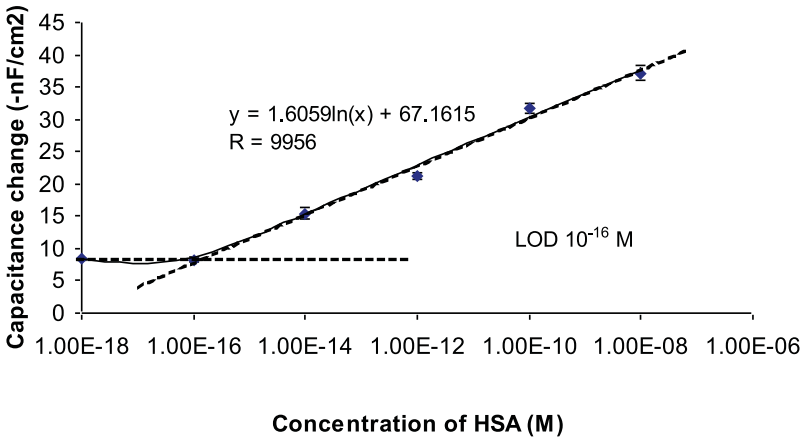

Figure 6.6 Response of a flow injection capacitive immunosensor system for anti-HSA and HSA binding.

Figure 6.7 shows the capacitive responses obtained using the normal capacitive system and the newly developed real-time (on-line) systems. As can be seen, the results of the two systems agreed well.

The real-time system was then applied to detect HSA in human urine samples obtained from Sonklanagarind Hospital, Hat Yai, Songkla. Figure 6.8 shows the regression line of the plot between concentrations of HSA analyzed by the real-time capacitive system and the values obtained from the hospital. The two sets of results were also statistically compared using Wilcoxon sign rank test and the results indicated that there was no significant difference between the two methods ($P > 0.05$).

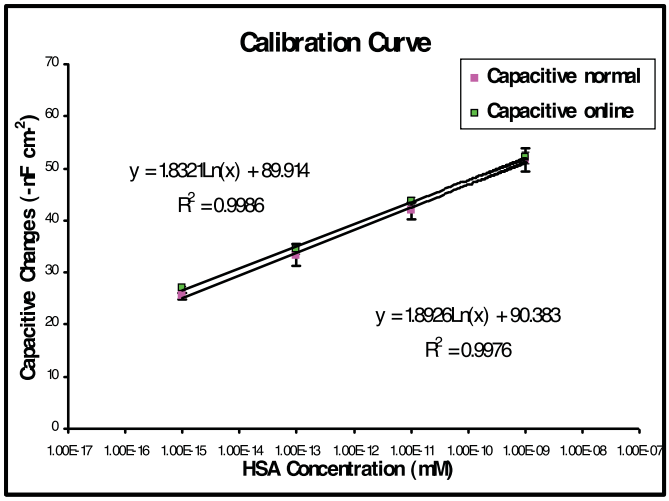

Figure 6.7 Comparison between responses obtained using the real-time (online) and normal capacitive systems.

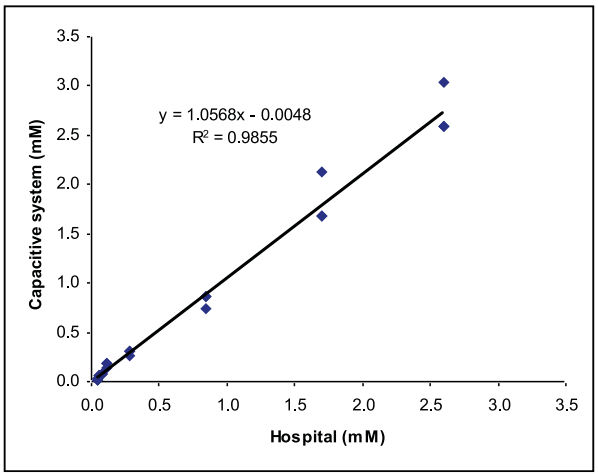

Figure 6.8 Plot of urine HSA concentrations analysed by real-time capacitive immunosensor compared to the results obtained by the hospital.

6.5 Conclusions

A real-time capacitive system was developed and successfully tested for the detection of an antigen based on antibody-antigen affinity interaction. Compared to other commonly used real-time system for affinity detection such as surface plasmon resonance (SPR), this system is less complicated with lower cost. Application for a patent (Thailand) is underway. A manuscript is also being prepared.

Output

Publications

Numnuam A, Kanatharana P, Mattiasson B, Asawatreratanakul P, Wongkittisuksa B, Limsakul C & Thavarungkul P. (2009). Capacitive biosensor for quantification of trace amounts of DNA. *Biosensors and Bioelectronics* **24**, 2559-2565.

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Wannapob R, Kanatharana P, Numnuam A, Limbut W, Asawatreratanakul P, Wongkittisuksa B, Limsakul C & Thavarungkul P. Phenylboronic acid capactive affinity sensor for bacteria detection. *In preparation*.

Wongkittisuksa B, Limsakul C, Kanatharana P, Asawatreratanakul P, Limbut W, Numnuam A & Thavarungkul P. Development of real-time capacitive measurement system for affinity biosensor. *In preparation*.

Patent

ระบบตรวจวัดอิเล็กโตรเคมิคอลเซนเซอร์ (Electrochemical sensor) แบบตรวจวัดตามเวลาจริง (real time) สำหรับวัดค่าทางไฟฟ้า เลขที่คำขอ 0901000618 วันรับคำขอ 13 กุมภาพันธ์ 2552

Conference presentations

Keynote/Invited speaker

Kanatharana P. (2009). Food Quality Control: Alternative Methods Based on Sensor. In NRCT-CPS Conference IV Drug Discovery: Drug Targeting and Drug Delivery. 21-23 August, โรงแรมวรบุรีอโยธยาคอนเวนชั่นรีสอร์ท พระนครศรีอยุธยา

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Thavarungkul P, Numnuam A, Kanatharana P, Asawatreratanakul P, Wongkittisuksa B & Limsakul C. (2008). Label-free Detection of Trace Amount of DNA. In *การประชุมนักวิจัยรุ่นใหม่ พบ เมธีวิจัยอาวุโส สกว. ครั้งที่ 8* 16-18 ตุลาคม 2551 โรงแรมฮอลิเดย์อินน์ รีสอร์ท รีเจนท์ บีช ชะอำ จังหวัดเพชรบุรี

Thavarungkul P, Kanatharana P, Asawatreratanakul P, Wongkittisuksa B, Limsakul C, Limbut W, Numnuam A, Thammakhet C, Teeparuksapun K & Wannapob R. (2009). Capacitive biosensor for sensitive analysis. In *การประชุมนักวิจัยรุ่นใหม่ พบ เมธีวิจัยอาวุโส สกว ครั้งที่ 9*. 15-17 ตุลาคม 2552 โรงแรมฮอลิเดย์อินน์ รีสอร์ท รีเจนท์ บีช ชะอำ จังหวัดเพชรบุรี

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Appendix

Publications

Submitted patent

Biosensors and Bioelectronics 24 (2009) 2559–2565

Contents lists available at ScienceDirect

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Capacitive biosensor for quantification of trace amounts of DNA

Apon Numnuam[a,b], Proespichaya Kanatharana[a,b], Bo Mattiasson[c], Punnee Asawatreratanakul[a,d], Booncharoen Wongkittisuksa[a,e], Chusak Limsakul[a,e], Panote Thavarungkul[a,f,*]

[a] Trace Analysis and Biosensor Research Center, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand
[b] Department of Chemistry and Center for Innovation in Chemistry, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand
[c] Department of Biotechnology, Lund University, Box 124, 221 00, Lund, Sweden
[d] Department of Biochemistry, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand
[e] Department of Electrical Engineering, Faculty of Engineering, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand
[f] Department of Physics, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand

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ABSTRACT

A flow injection capacitive biosensor system to detect trace amounts DNA has been developed based on the affinity binding between immobilized histone and DNA. Histones from calf thymus and shrimp were immobilized on gold electrodes covered with self-assembled monolayer (SAM) of thioctic acid. Each of these histones was used to detect DNA from calf thymus, shrimp and *Escherichia 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. Under optimum conditions, both 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. 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. When applied to analyze residual DNA in crude protein extracted from white shrimp recoveries were obtained between 80% and 116%.

1. Introduction

DNA quantification is critical for many biological studies since it is often used as a reference for measurements of other biologically active components in biological fluids and genetic diagnosis (Huang et al., 2002; Georgiou and Papapostolou, 2006; Prem Kumar et al., 2005). In biological and biopharmaceutical products, such as monoclonal antibodies, lymphokines and vaccines, quantification of residual cellular DNA from the host cells 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) published in 1986 recommend that the residual cellular DNA permitted in purified products contain less than 100 pg per dose (WHO, 1987). In 1997, this value was changed to 10 ng per dose (WHO, 1998).

The quantification method commonly used for residual DNA determination is real-time quantitative polymerase chain reaction (Q-PCR) (Lovatt, 2002). Other biochemical assay methods include spectrophotometry where DNA absorbs maximally around 260 nm (Samuel et al., 2003), densitometric scans of gels from agarose or polyacrylamide electrophoresis (Projan et al., 1983), chemiluminescence (Ma et al., 2004), spectrofluorimetric and resonance light scattering (RLS) methods (Li et al., 2002; Wang et al., 2005). Each method has its strengths and weaknesses in terms of sensitivity, specificity, running time, robustness, material safety/toxic waste, reagent stability and cost (Li et al., 2002; Ma et al., 2004). For example, in the case of the fluorescence method, many fluorescent reagents have been used to enhance fluorescence intensity for DNA determination, such as ethidium bromide, 4,6-diamidino-2-phenylindole, bis-benzimidazole dye Hoechst 33258. However, the preparation of reagents is inconvenient (Wang et al., 2005) and some reagents, such as ethidium bromide is a carcinogen (Link and Tempel, 1991). Therefore, development of alternative methods which are more convenient and have high sensitivity and specificity for the detection of DNA is desirable and use of affinity biosensors is an interesting approach.

Affinity biosensors are based on binding interaction between the immobilized biomolecule and the analyte of interest (Mattiasson, 1984; Wang, 2000). Affinity biosensors can be categorized as label-free (direct) and labeled. Between the two, label-free affinity biosensor is more attractive since it requires less steps. To detect the affinity binding reaction, capacitive transducers have been applied

* Corresponding author at: Trace Analysis and Biosensor Research Center, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand. Tel.: +66 74 288753; fax: +66 74 212817.
E-mail address: panote.t@psu.ac.th (P. Thavarungkul).

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and found to be very sensitive (Berggren et al., 2001, 1998; Berggren and Johansson, 1997; Bontidean et al., 2001, 1998; Hedström et al., 2005; Hu et al., 2002; Limbut et al., 2006) To detect DNA, it may be possible to apply histones as recognition element since histones are the basic proteins which are complexing with DNA *in vivo* to form the nucleosome (Helliger et al., 1988). The four core histones (H2A, H2B, H3and H4) form an octamer, which DNA is wrapped around to form the nucleosome particle and the histone H1 acts as the linker between the constituents of the nucleosome particle (Allan et al., 1981; Yoshikawa et al., 2001).

In this work we investigated the application of a flow-injection capacitive biosensor for the rapid determination of 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. The technique was tested using DNA preparations from various sources, i.e., calf thymus, shrimp and *Escherichia coli*. The signals of the binding reaction of DNA to white shrimp and calf thymus histones were also compared. This system was also validated with real samples by using it to determine genomic DNA contamination in crude shrimp protein preparation.

2. Materials and methods

2.1. Chemicals

Deoxyribonucleic acid (DNA) and histone from calf thymus, *N*-3-(dimethylamino-propyl-*N*′-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxy succinimide (*N*-hydroxy-2,5-pyrrodinedione, NHS) and white saponin were purchased from Sigma–Aldrich (Steinheim, Germany). Thioctic acid and 1-dodecanethiol were obtained from Aldrich (Milwaukee, USA). All other chemicals were of analytical grade. Solutions and buffers used in the capacitive biosensor system were prepared with deionized water. Before use, buffers were filtered through an Albet® nylon membrane filter with pore size 0.20 μm with subsequent degassing. DNA from white shrimp (*Penaeus merguiensis)* was provided by Center for Genomic and Bioinformatic Research, Faculty of Science, Prince of Songkla University, Songkhla, Thailand. *E. coli* DNA was obtained from Department of Biotechnology, Lund Universitiy, Sweden and purified by using Freez'N Squeeze DNA gel extraction spin column (Bio-Rad, USA). Histones from white shrimp (*Penaeus merguiensis*) erythrocyte nucleoprotein were prepared by using a slightly modified procedure from that reported by Murry et al. (1968). In brief, shrimp erythrocytes were lysed by vigorous stirring with white saponin solution (0.6% (w/v) in 0.14 M NaCl). Nuclei were collected by centrifugation at 500 × *g* for 1 h. Whole histone was then extracted from the nuclei with 0.2 M H_2SO_4 and centrifuged at 18,600 × *g* for 1 h. Ethanol was added to precipitate histones which were collected by centrifugation and washed 3 times with ethanol.

2.2. Immobilization of histone

The conditions used for immobilization of histones to gold surface modified with self-assembled monolayer of thioctic acid followed the process described by Limbut et al. (2006). In this case 20 μl of appropriate concentration of histone (see Section 3.2.1) was placed on each electrode and the reaction took place at 4 °C for 24 h. During the immobilization steps, the degree of insulation from different layers on the electrode surface is demonstrated by cyclic voltammetric measurements performed in a three electrode electrochemical batch cell containing 5 mM $K_3[Fe(CN)_6]$ and 0.1 M KCl, at a scan rate of 0.1 V s^{-1}. The modified gold electrode was used as the working, Ag/AgCl as a reference and a platinum rod was the auxiliary electrode. The electrodes were coupled to a potentiostat (ML 160, AD Instruments, Australia) connected to a computer.

2.3. Capacitance measurements

The measurements were performed in a three electrode flow cell with a dead volume of 10 μl by applying +50 mV potential pulses, one pulse per minute to the modified gold electrode through the Powerlab system (AD Instrument, Australia) (Fig. 1). The resulting current response from each pulse was sampled with a frequency of 40 kHz. The capacitance of the electrode surface was calculated from the current response as described by Limbut et al. (2006).

2.4. Optimization of the capacitive biosensor

Operating conditions of the flow injection capacitive biosensor system were optimized for the affinity binding between DNA and histones from calf thymus. Parameters affecting the capacitive response were studied, i.e., regeneration solution, sample volume, flow rate, and carrier buffer. The parameters were optimized one by one by comparing responses obtained after injections of standard DNA solution, three replications for each test value. The optimum of each parameter was determined as a compromise 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.

2.5. Real sample analysis

A crude protein extract from white shrimp was used as a representative of a real sample. This extract is used in the study of protein interaction with white spot syndrome virus in shrimp. In the preparation process, DNA was removed by DNaseI treatment and residual DNA is generally detected by UV spectrometry. To test the capacitive biosensor system, residual DNA in the extract was analyzed under optimum conditions, including the study of matrix effect and recovery.

3. Results and discussion

3.1. Insulating property of working electrode

Capacitive measurements require a proper insulation of the electrode surface in order to prevent disturbing redox reactions at the applied potential. The degree of insulation obtained by different layer in the electrode preparation was studied with cyclic voltammetry. Using the cleaned gold electrode, the reversible peaks for oxidation and reduction were clearly observed during cycling of potential. These peaks were significantly reduced with SAM modification followed by histone immobilization. After treating with 1-dodecanethiol to block any pinhole on the electrode surface, the redox peaks disappeared completely indicating that the electrode was totally insulated.

3.2. Optimization of the capacitive biosensor system

The capacitance of the binding event between histone and DNA is determined from the current response when a potentiostatic step of 50 mV is applied on the electrode (Limbut et al., 2006). The affinity binding between DNA and the immobilized histone molecules on the working electrode will result in an increase of the thickness of the layer and this would cause the total capacitance (C_{tot}) to decrease (Fig. 1). Fig. 2 shows an example of a plot of the calculated C_{tot} value with respect to time where the capacitance change (ΔC_1, ΔC_2) due to the binding can be determined. Since the interaction between DNA and histone are via non-covalent bonds, DNA could

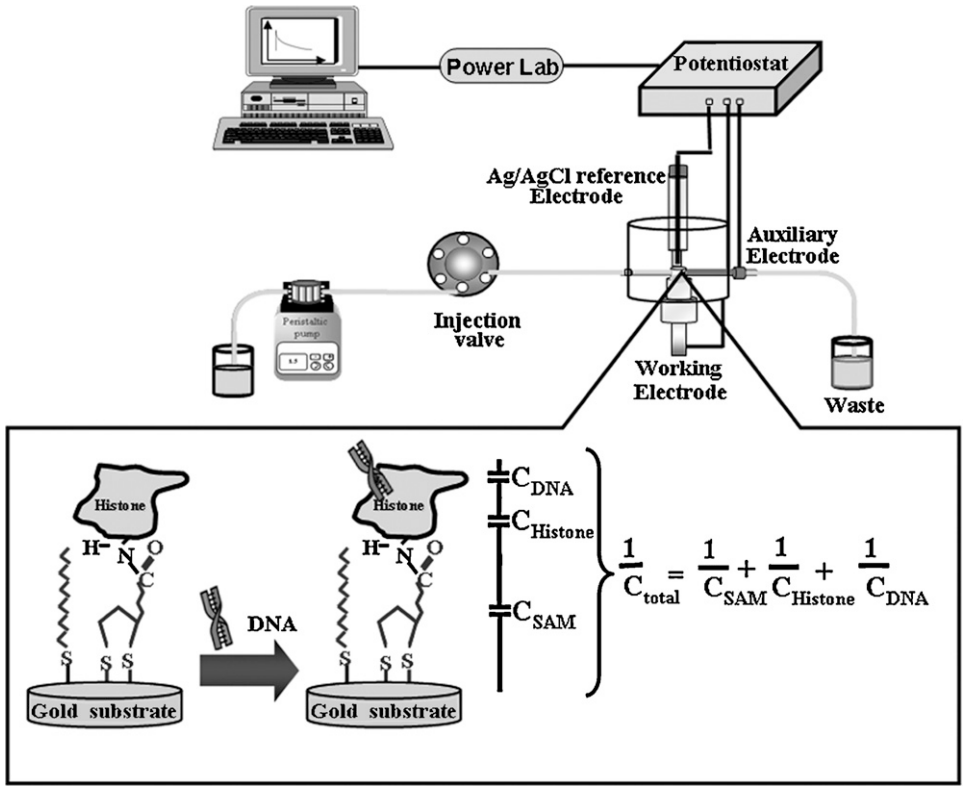

Fig. 1. Schematic diagram of the flow injection capacitive biosensor system. The total capacitance measured at the working electrode/solution interface (C_{tot}) comes from C_{SAM}; the capacitance of self-assembled thioctic acid monolayer, $C_{Histone}$; the capacitance of histone layer and C_{DNA}; the capacitance DNA analyte interaction (modified from Limbut et al., 2006).

be dissociated from the histone on the electrode surface by using regeneration solution. Optimizations were carried out as follows.

3.2.1. Concentration of histone

The effect of different concentrations of histone on the electrode response was studied. Solutions of histones (25 μg ml^{-1}, 50 μg ml^{-1}, 75 μg ml^{-1} and 100 μg ml^{-1}) were used for the immobilization process. Four electrodes were tested by injecting standard DNA solutions between 0.1 ng l^{-1} and 100 ng l^{-1}. The sensitivity (slope of calibration curve) increased with increasing histone concentration and stabilized for electrodes produced from 50 μg ml^{-1}

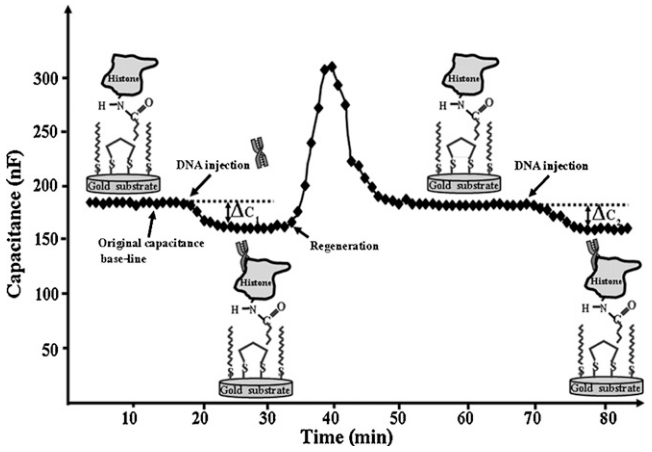

Fig. 2. An example of the capacitance (C_{tot}) plots as a function of time. The binding between histone and DNA causes the capacitance to decrease (ΔC_1) with subsequent signal increase due to dissociation under regeneration conditions. After regeneration, the system can be reused to detect DNA in a new injection (ΔC_2).

(87 ± 2 nF cm^{-2} ng^{-1} l) and upwards. To see the specific binding of DNA on the sensor surface, an electrode without immobilized histone was tested by injecting the same range of standard solutions of DNA. The responses did not change with concentrations and were lower than the response of the limit of detection (see Section 3.4). This result indicated that the immobilized histone is specific to DNA.

3.2.2. Regeneration solution

The biosensor needs regeneration of the sensor surface before measurement can be repeated. The goal is to break the non-covalently bonds between DNA (the analyte) and histones immobilized on the gold surface, while maintaining the activity of the histones.

To evaluate the performance of the regeneration solution, percentage of residual activity (%residual DNA-binding activity) of histone was calculated from capacitive change given by a consecutive binding between DNA (1 ng l^{-1} with sample volume 300 μl) and histone before (ΔC_1) and after ((C_2) regeneration (Fig. 2) according to the equation:

$$\%\,\text{Residual activity} = \frac{\Delta C_2 \times 100}{\Delta C_1} \quad (1)$$

Four different types of regenerating agents were compared. 50 mM KCl and high pH solution, 50 mM NaOH, were less effective than a low pH of 50 mM glycine–HCl, pH 2.4 and HCl pH 2.4. They gave low percentage residual activity, less than 70%, and required a longer regeneration time, more than 17 min, compared to 13 min for low pH. At low pH, glycine–HCl gave higher percentage residual activity (87 ± 2%) than HCl (83 ± 2%), therefore, glycine–HCl, pH 2.4 was further investigated to see the influence of its concentration (10–100 mM). At 25 mM the highest residual activity of 93 ± 3% was achieved. The effect of pH was then tested at this concentration

and 97 ± 3% residual activity was gained at pH 2.4. Therefore, 25 mM glycine–HCl, pH 2.4 was chosen for the continued experiments.

3.2.3. Buffer solution

Initially two widely used biochemical buffer types were tested, 10 mM of sodium phosphate and Tris–HCl buffer, pH 7.20, by analysing standard DNA between 0.1 ng l^{-1} and 100 ng l^{-1} in these buffers. When analysis was carried out in the two buffers, there was no difference between sensitivity (slope of calibration curve), i.e., 88 ± 2 and 89 ± 5 nF cm^{-2} ng^{-1} l for Tris–HCl and sodium phosphate buffer, respectively. However, the assays in Tris–HCl gave a higher response and a more steady baseline. Different concentrations of Tris–HCl buffer (5–100 mM) were then tested. The highest change in the capacitive signal, from injection of 1 ng l^{-1} DNA, were obtained at 5 mM (164 ± 25 nF cm^{-2}) and 10 mM (160 ± 5 nF cm^{-2}) but a more steady baseline was obtained at 10 mM so this concentration was selected. When optimizing the sensor response at different pH of Tris–HCl buffer (pH 7.00–8.00), the maximal capacitive change was found at pH 7.00. Therefore, 10 mM Tris–HCl, pH 7.00 was used in the continued experiments.

3.2.4. Flow rate

The effect of flow rate was investigated by injecting 300 μl of 1 ng l^{-1} of DNA solution at different flow-rates (50–500 μl min^{-1}). The capacitance change decreased as the flow rate increased due to the reduction of binding interaction time. The flow rate of 50 μl min^{-1} gave the highest response (169 ± 10 nF cm^{-2}), however, nearly the same response was also obtained at 100 μl min^{-1} (165 ± 3 nF cm^{-2}), but the analysis time was shorter, 14 min compared to 18 min at 50 ml min^{-1}, so 100 μl min^{-1} was chosen.

3.2.5. Sample volume

Generally, an increase in response can be achieved with an increase in sample volume. Therefore, the effect of sample volume on capacitance changes to 1 ng l^{-1} of DNA standard was studied from 50 μl to 400 μl. The change in capacitance signal increased with sample volume until 250 μl and become steady, therefore, 250 μl was chosen.

In summary the optimum conditions are: 25 mM glycine–HCl, pH 2.40 for regeneration solution, 10 mM of Tris–HCl, pH 7.00 for carrier and sample buffer solution, flow rate at 100 μl min^{-1} and 250 μl of sample volume, the analysis time was 13–15 min with another 13 min of regeneration time.

3.3. Reproducibility

To test whether the response of the histone modified electrode can be reproduced after the removal of DNA by regeneration solution, the same concentration of 1 ng l^{-1} of DNA was injected into the system with subsequent regeneration. The change in capacitance signal after regeneration was used to calculate the percentage of residual activity of the histone electrode by comparing the response to the initial capacitance change. The histone immobilized on the self-assembled monolayer method retained 95.2 ± 3.1% of its ability to bind DNA after 43 times of regeneration and the residual activity after this point dropped below 90%. The results indicate that the histone electrode can be reused with good reproducibility up to at least 40 times.

3.4. Linear dynamic range and detection limit

Standard calf thymus DNA solutions ranging from 1×10^{-7} ng l^{-1} to 1×10^{3} ng l^{-1} were injected with intermittent regeneration steps using 25 mM glycine–HCl, pH 2.4. Fig. 3 shows the calibration curve for DNA under optimal conditions. The plot between capacitance change and logarithm of DNA concentration showed a sigmoidal

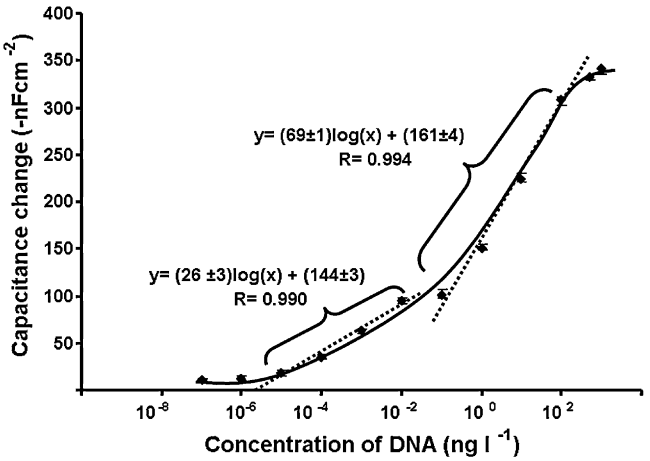

Fig. 3. Capacitance change vs. the logarithm of calf thymus DNA concentration detected by electrodes with immobilized calf thymus histone under optimum conditions (100 μl min^{-1} flow rate, 250 μl sample volume, 10 mM Tris–HCl buffer, pH 7.00).

behavior (within limit of the experimental error) two linear ranges with different sensitivities, i.e., from 10^{-5} ng l^{-1} to 10^{-2} ng l^{-1} and 10^{-1} ng l^{-1} to 10^{2} ng l^{-1} can be obtained. The lower concentration range (10^{-5} ng l^{-1} to 10^{-2} ng l^{-1}) was employed for further study (see Sections 3.5 and 3.6). This sigmoidal curve might be explained by the model of cooperative binding of ligands to multimeric protein (Nelson and Cox, 2000). In this work, whole histones of calf thymus were used (H1, H2A, H2B, H3 and H4) which bind to DNA with different affinity (Cui et al., 2005), with H1 having a higher affinity to DNA than the other four histones (Cui et al., 2005). In this case H1 would be the major histone that binds to DNA at low concentration. The binding between H1 and DNA may change the conformation of DNA which will then drive the cooperative binding of other lower affinity histone onto DNA molecule at higher concentration. At concentration higher than 10^{2} ng l^{-1} the binding of DNA by histones on the electrode becomes saturated, so the plateau curve was obtained. From the results, the detection limit was found to be 10^{-5} ng l^{-1} based on IUPAC Recommendation 1994 (Buck and Lindner, 1994).

3.5. Selectivity

Since different sources of DNA or histones may affect the binding and, hence, the response of the capacitive system, it is important to investigate this. First, the effect of DNA source was studied by using immobilized calf thymus histone to detect DNA from calf thymus, white shrimp and *E. coli*. The calibration curves were investigated between 10^{-5} ng l^{-1} and 10^{-2} ng l^{-1} and these are shown in Fig. 4a. The sensitivity (slope of calibration curve) obtained with calf thymus DNA was about fourfolds higher than the one obtained from white shrimp DNA, and the lowest sensitivity was obtained from *E. coli* DNA. This is probably due to the size of DNA, longest DNA is from calf thymus, followed by shrimp and *E. coli* (qualitative comparison of sizes was tested by using agarose gel electrophoresis). The layer on the electrode surface with a longer DNA should be thicker and this would cause C_{tot} to decrease more than when a shorter DNA was bound, so calf thymus DNA would give the highest response. Although the sensitivity of the system to each DNA is different, the lower limit of detection is the same. That is, when the system was used to detect the DNA of calf thymus, white shrimp, or *E. coli* the lower limit of detection is 10^{-5} ng l^{-1}.

Histones from a different source, i.e., white shrimp, were then immobilized on gold electrodes at the same concentration as that of calf thymus histones (50 μg ml^{-1}) and studied was car-

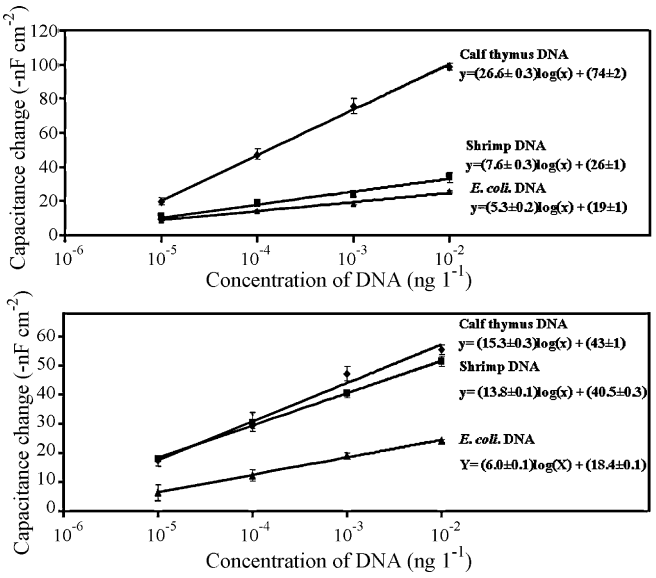

Fig. 4. Calibration curve for DNA from calf thymus, white shrimp and *E. coli* with (a) immobilized calf thymus histone and (b) white shrimp histone.

ried out by injecting DNA from calf thymus, white shrimp and *E. coli* (Fig. 4b). The results show that shrimp histone can also bind with DNA from all three sources with the same lower limit of detection of 10^{-5} ng l^{-1}. That is, neither calf nor shrimp histone is selective for DNA from its own species. Comparing between Fig. 4a and b, it can be seen that when using shrimp histone (Fig. 4b) the sensitivity to shrimp DNA can be increased from 7.6 ± 0.3 nF cm^{-2} ng l^{-1} to 13.8 ± 0.1 nF cm^{-2} ng l^{-1} while the sensitivity to calf thymus DNA decreased form 26.6 ± 0.3 nF cm^{-2} ng l^{-1} to 15.3 ± 0.3 nF cm^{-2} ng l^{-1}. That is, histone can bind better with DNA from the same source. The relatively high sensitivity to calf thymus DNA when using shrimp histone is probably due to the longer DNA of calf thymus as discussed in the previous paragraph.

The effect of a mixture of DNA from different sources was also studied by using immobilized calf thymus histones to detect the mixture of DNA from calf thymus, white shrimp and *E. coli* at different concentrations. The measurement was first done for each DNA at 1 pg l^{-1}, 2 pg l^{-1} and 3 pg l^{-1}. The response from calf thymus gave the highest response at every concentration (Fig. 5). Mixtures of DNA from different sources at different concentrations were then

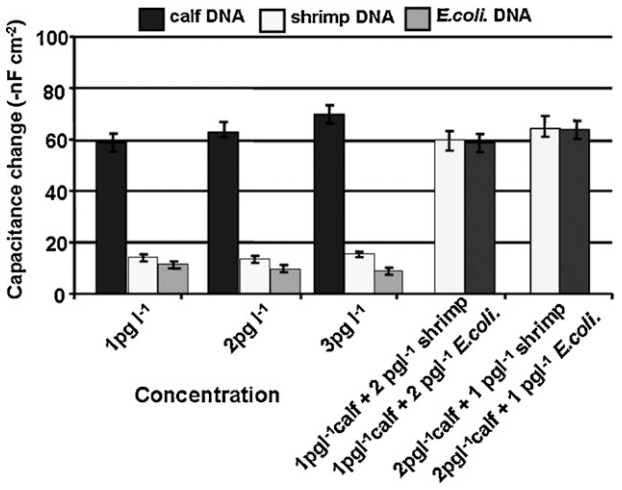

Fig. 5. Effect of DNA from calf thymus, shrimp, *E. coli* and a mixture of them on the binding with calf thymus histone.

studied, i.e., 1 pg l^{-1} of calf thymus DNA + 2 pg l^{-1} of shrimp or *E. coli* DNA, 2 pg l^{-1} of calf thymus DNA + 1 pg l^{-1} of shrimp or *E. coli* DNA. The results obtained from the mixture were less than the sum of individual but slightly higher than calf thymus DNA alone. That is, the data reflect the presence of calf thymus DNA and that calf histone can provide more selective binding to calf DNA than shrimp and *E. coli*.

3.6. Determination of residual shrimp DNA

Determination of residual DNA was carried out using a particle free homogenate of shrimp protein as real sample. This extract is normally used in the study of protein interaction with white spot syndrome virus. Since the protein in the extract may interfere with the detection of residual DNA via non-specific binding to the surface of the modified electrode, bovine serum albumin (BSA) was used to test this influence. Using the electrode with immobilized calf thymus histone, the responses due to BSA at the concentration range 0.01–100 ng l^{-1} were almost constant and were much lower than the responses from DNA. It can be suggested that non-specific binding from proteins do not play an important part in the response.

When analyzing real samples, the matrix may also cause some interference to the response. The influence of the matrix in real sample was studied by using immobilized shrimp histone. Crude shrimp protein sample without DNA (prepared by adding excess DNaseI to digest residual DNA) was spiked with known amount of shrimp DNA at 0.1–1.5 pg l^{-1} and these were used to perform the matrix matched calibration assays. A calibration curve was also obtained when analyzing standard solution in the same concentration ranges (standard calibration curve). The assay for each concentration was carried out in triplicate and the average sensor response and standard deviation were calculated. The slope of standard calibration curve and matrix matched calibration curve were compared by two-way ANOVA (analysis of variance) calculated by R solfware (R Development Core Team, 2006). The results showed that the slope of regression lines of standard and matrix matched calibration curves were significantly different ($P < 0.05$), indicating that there was effect of matrix interference on the sensor response (IUPAC Technical Report, 2002). One way of reducing the matrix effect is by dilution. Since the LOD of this system is very low (10^{-5} ng l^{-1}), it is possible to dilute the sample several times. This will be appropriate when the concentration of DNA is high, e.g., when DNA is product of process. However, when the detection is for residual DNA only trace amount will exist, in this case matrix matched calibration should be employed.

3.6.1. Matrix matched calibration

Matrix matched calibration was used for the detections of the trace amount of DNA. The response when injecting crude shrimp protein sample ($n = 3$) were used to calculate the concentration of residual DNA from the matrix matched calibration curve and no DNA was detected.

To further 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 histones. The responses obtained from spiked shrimp protein sample were used to calculate the concentration from the matrix matched calibration curve. Recovery percentage was evaluated by the following equation:

$$\%\,\text{Recovery} = \frac{C_1 - C_2}{C_3} \times 100 \quad (2)$$

where C_1 is the concentration of analyte measured in the spike sample, C_2 is the concentration of analyte measured in the blank and C_3 is the concentration of analyte spiked into the sample (Taverniers et al., 2004). From the results (Table 1), percentage of recovery and rel-

Table 1
Recovery of shrimp DNA from spiked solutions of crude shrimp protein ($n = 3$); using matrix matched calibration (Section 3.6.1) and dilution methods (Section 3.6.2).

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 ($pg\,l^{-1}$)								
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

ative standard deviation of all spiked DNA (in $pg\,l^{-1}$ range) in crude shrimp protein are acceptable. Since the acceptable recovery in the $\mu g\,l^{-1}$ level is 40–120% and R.S.D. is 30–45.3% (Taverniers et al., 2004).

3.6.2. Dilution

Crude shrimp protein samples were spiked with shrimp DNA at $10\,ng\,l^{-1}$ to $150\,ng\,l^{-1}$ and diluted 10,000 times (following Thavarungkul et al., 2007) with 10 mM Tris–HCl buffer, pH 7.0. These were analyzed ($n = 3$) and the calibration curve was compared to that of standard solution. The results showed that there was no matrix effect for the diluted samples, that is, the matrix effect can be reduced by dilution. Therefore, the standard curve could be used to determine the residual DNA contamination in diluted real sample.

The recovery of spiked shrimp DNA at $10\,ng\,l^{-1}$ to $150\,ng\,l^{-1}$ in crude shrimp protein sample was tested. The spiked samples were then diluted 10,000 times with Tris–HCl buffer, pH 7.0 to reduce the matrix effect. The analysis was carried out by two capacitive biosensor systems, one with immobilized shrimp histone and the other with calf thymus histone. The capacitance changes obtained from the capacitive systems were used to calculate the concentrations from the calibration curve of standard obtained prior to the test. From the results (Table 1) 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 $\mu g\,l^{-1}$ 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.

The spiked samples were also measured by UV spectrometry at λ_{260}. However, no response was obtained from any of spiked samples (DNA at 10–150 $ng\,l^{-1}$) since the detection limit of this method is $5\,\mu g\,ml^{-1}$ (Noites et al., 1999). That is, the developed capacitive system can detect DNA at a much lower concentration.

4. Conclusions

The results demonstrated the possibility of using the capacitive biosensor system for direct assay of affinity binding between DNA and histone protein on self-assembled thioctic acid monolayer modified electrode. For the system using calf thymus histone to detect calf thymus DNA, it provides a lower detection limit of $10^{-5}\,ng\,l^{-1}$ and wide linear ranges 10^{-5}–$10^{-2}\,ng\,l^{-1}$ and 10^{-1}–$10^{2}\,ng\,l^{-1}$. 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 A_{260} method), although this method is accurated and reproducible, it is relatively insensitive and can only measure DNA concentrations that is greater than $5\,\mu g\,ml^{-1}$ (Noites et al., 1999) while the develop capacitive system can detect DNA as low as $10^{-8}\,ng\,ml^{-1}$. In another method, densitometry scan of gel electrophoresis, DNA quantification between $15\,ng\,ml^{-1}$ and $700\,ng\,ml^{-1}$ can be determined (Projan et al., 1983). However, gel electrophoresis is not a very accurate quantification method, with an error range of around 10–20% (Levy et al., 2000; Projan et al., 1983). For spectrofluorometric method, although this technique is very sensitive and can detect DNA with concentration between $15\,ng\,ml^{-1}$ and $250\,ng\,ml^{-1}$ (Noites et al., 1999), it needs fluorescence dye to stain DNA such as ethidium bromide or other fluorescence stains that are powerful carcinogen. In addition, analysis time of this 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). A slight drawback of the capacitive system is the time required to modify the electrode surface, i.e., 2 days. Therefore, future investigation into a more rapid procedure would be interesting. 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.

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Full Paper

Disposable Electrodes for Capacitive Immunosensor

Kosin Teeparuksapun,[a,b,c] *Proespichaya Kanatharana*,[a,b,c] *Warakorn Limbut*,[a,b,d] *Chongdee Thammakhet*,[a,b,c] *Punnee Asawatreratanakul*,[a,e] *Bo Mattiasson*,[f] *Booncharoen Wongkittisuksa*,[a,g] *Chusak Limsakul*,[a,g] *Panote Thavarungkul*,[a,b,h]*

[a] Trace Analysis and Biosensor Research Center, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand
[b] Center for Innovation in Chemistry, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand
[c] Department of Chemistry, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand
[d] Department of General Science, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand
[e] Department of Biochemistry, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand
[f] Department of Biotechnology, Lund University, Box 124, 221 00 Lund, Sweden
[g] Department of Electrical Engineering, Faculty of Engineering, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand
[h] Department of Physics, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand
*e-mail: panote.t@psu.ac.th

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Abstract
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. 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. A linear relationship was obtained in the range from 1.0×10^{-14} to 1.0×10^{-9} M with a limit of detection of 8.0×10^{-15} M. Each modified electrode can be reused up to 30 times. The developed system was applied for human serum samples and compared to Albumin BCG method.

Keywords: Disposable electrode, Thermal evaporation, *o*-Phenylenediamine, Capacitive immunosensor, Human serum albumin

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1. Introduction

Solid gold electrodes are currently in widespread use in both electrochemical analysis and bioanalysis [1–4], primarily because of a broad potential window of gold for a redox reaction, rich surface chemistry and suitability for various sensing and detection applications [5]. It is also generally used as working electrode in the development of electrochemical biosensors [4, 6, 7, 8] where the electrode surfaces must be carefully cleaned and pretreated for the immobilization coupling chemistries. The properties of the gold electrode substrate, especially surface topography and roughness, play an important role in the properties of the resulting immobilization [5].

Cleaning of solid gold electrode surface is frequently accomplished by repeated polishing with different particle sizes of alumina slurry and subsequently cleaned through sonication to remove any physisorbed multilayer [3, 7, 9], sometime subjected to electrochemical treatment with H_2SO_4 and mounted in a Teflon electrode holder which restricted a certain exposed area [3, 4, 7]. Once the electrode with immobilized biomolecules has lost its activity, these electrodes need to be oxidized with Piranha solution prior to reuse, followed by the pretreatment steps as described previously. This sequence of treatments is cumbersome. Although alumina can be used successfully for the polishing of gold surface, there are problems created by additives in the alumina preparations [10]. Inhalation of alumina powder may causes irritation to respiratory tract, coughing and shortness of breath [11]. Therefore, the use of alumina powder should be avoided. Due to these drawbacks, an alternative inexpensive strategy to obtain a smooth surface without polishing is of interest. Thermal evaporation is one of the techniques that can be used to fabricate thin gold film which can be used in biosensor design. These include a sensor for surface plasmon resonance (SPR) [12, 13], electrodes for conductivity measurement [14], and quartz crystal microbalance (QCM) [15].

Among label-free electrochemical immunosensors, capacitive measurement based on potentiostatic step has proven to be a sensitive detection system [3, 6, 7, 16, 17]. In this approach, biomolecules (e.g., antibodies) are immobilized on the electrode surface. The change in the dielectric properties is measured when an analyte (e.g., antigen) binds to the biomolecule on the electrode, causing capacitance to decrease [3, 6, 7, 17]. The most important step for the construction of a capacitive immunosensor is the immobilization of biomolecules on an insulating layer of the electrode surface. A self-assembled monolayer (SAM) of alkylthiol is generally employed to make such a layer on

gold rod electrodes [3, 6, 7, 16, 17]. However, this technique needs several hours [3, 6, 7] and the use of gold rod is suffering from the difficulties of polishing steps. Therefore, another strategy to obtain an insulating layer such as electropolymerization of nonconducting polymer on a smooth thin film surface with no pretreatment step is of interest.

Non-conducting polymers are emerging as a novel support matrix for the immobilization of biomolecules [18]. Polymer of *ortho*-phenylenediamine (*o*-PD) is one of the nonconducting polymers which has been widely used [19, 20]. Electropolymerization of *o*-PD will form a 1,4-substituted benzonoid-quinoid with free amino groups exposed on the backbone of the poly *o*-PD [21]. Cross-linking of these free amino groups with biomolecules using a bifunctional reagent such as glutaraldehyde can provide a biosensor with stable performance [22–24]. This method was selected as an immobilization technique in this study.

In this report is presented the use of thin gold film electrodes fabricated by thermal evaporation technique as disposable working electrodes for capacitive measurement of samples without any pretreatment steps. Anti human serum albumin (anti-HSA) and human serum albumin (HSA) were used as a model affinity binding pair. Great reduction of electrode modification time was achieved through the immobilization of anti-HSA on the surface of the electrode modified with a nonconducting layer of *o*-PD by electropolymerization instead of SAM. Performances of the modified gold thin-film electrodes were tested in a capacitive immunosensor system.

2. Experimental

2.1. Materials

Glass microscope slide (25.4 mm × 76.2 mm) (Sailbrand 23, China) was used as the base for electrodes fabrication. *Ortho*-phenylenediamine (*o*-PD) was obtained from BDH laboratory reagent (Poole, England). Glutaraldehyde 25% (w/v) was obtained from Fluka (Buchs, Switzerland). Polyclonal antibody raised against human serum albumin (HSA) was obtained from Dako (Glostrup, Denmark). Bovine serum albumin (BSA) was obtained from Sigma (USA). Pure HSA from ICN Biomedicals (Ohio, USA) was used as antigen. All other chemicals used were analytical grade. All buffers were prepared with water treated with a reverse osmosis-deionizing 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. Fabrication of Gold Electrodes

Glass slides were cut into strips (5.0 mm × 25.4 mm), cleaned for 5 min with Piranha solution while exposed to sonication and rinsed with deionized water several times. They were dried in an oven at 100 °C for 1 hour. Since a thin gold film which is directly coated on a substrate can easily come off during electrochemical measurement, a chromium layer is required to enhance the adhesion [25–27]. Chromium was first coated on the glass surface using a thermal evaporator (Edwards Auto 306, UK) by applying a current of 60 mA at 5×10^{-6} mbar. In this step chromium was deposited in a layer of ca. 5 nm. Then gold was coated on the chromium layer by applying a current of 40 mA at the same pressure until a thickness of ca. 200 nm was obtained. Surface properties of the fabricated electrodes were characterized using Atomic Force Microscopy (AFM) (Seiko instrument, Japan). The scans were obtained by the computer controlled device SPI4000 in a contacting mode using a cantilever of the type CSG10 (Molecular Device and Tools for Technology (NT-MDT)).

2.3. Antibody Immobilization

Immobilization procedure was modified from Karalemas [24], Cheng et al. [28], and Limbut et al. [7]. Electropolymerization of *o*-PD was performed by cyclic voltammetry in the potential range of 0–0.8 V (vs. Ag/AgCl) at a scan rate of 50 mV s^{-1} from a 5 mM [28] solution of *o*-PD in 10 mM sodium acetate buffer pH 5.18. In this step, polymer was coated on the electrode surface and free amino groups are provided on the backbone of a poly *o*-PD chain [21].

The electrode was rinsed with distilled water to remove undeposited polymer before being dipped in 5.0% (v/v) glutaraldehyde in 10 mM sodium phosphate buffer pH 7.0 for 20 min at room temperature, thoroughly rinsed with 10 mM sodium phosphate buffer pH 7.0. In this step the amino group of the polymer is modified by coupling to one of the aldehyde groups of glutaraldehyde leaving one free aldehyde group. The modified electrode was then dried with nitrogen gas and 20 μL of anti-HSA (0.5 g/L) prepared in 10 mM sodium phosphate buffer pH 7.0 was placed on the surface and coupling reaction took place overnight at 4 °C. Finally, the electrode was immersed in 0.1 M ethanolamine pH 8.0 for 20 min to occupy all aldehyde groups that did not couple to anti-HSA. When not in use electrodes were kept at 4 °C in a closed Petri dish filled with nitrogen gas.

The electropolymerization efficiency of *o*-PD was studied at 5, 10, 15 and 20 cycles, respectively. To evaluate the results of the electropolymerization procedures the sensitivities of the electrodes to standard HSA from 1.0×10^{-14} to 1.0×10^{-10} M were determined and compared.

2.4. Capacitive Measurement

Figure 1 shows the basic experimental set-up of the flow injection capacitive immunosensor system. The flow cell with a dead volume of 10 μL contained a Ag/AgCl reference electrode at the top, a modified thin-film gold working electrode at the bottom and a stainless steel tube (i.d. 0.4 mm, o.d. 1.1 mm, length 20 mm) acting as an auxiliary electrode and solution outlet. Capacitive measurements

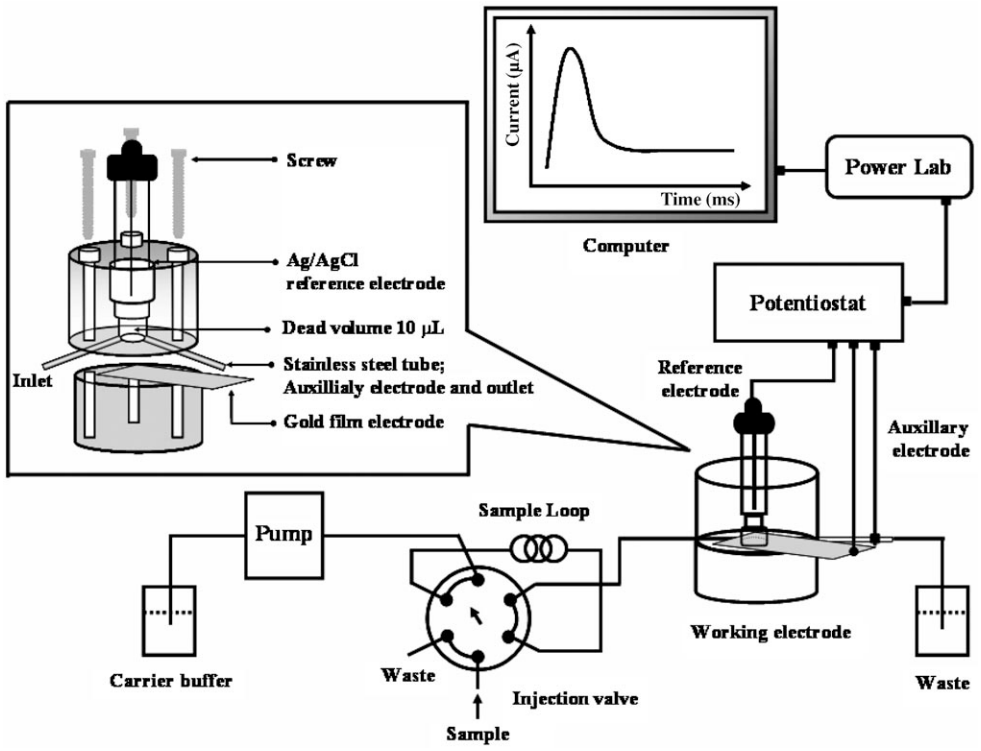

Fig. 1. Schematic diagram showing the flow injection capacitive immunosensor system.

were done through a potentiostatic step method. Potential pulses of 50 mV were applied to the gold electrode yielding current response signals. The total capacitance was calculated from the current response as described by Limbut et al. [3]. The binding interaction between HSA and the immobilized anti-HSA on the electrode causes the capacitance to decrease and this is proportional to the concentration of HSA. To recondition the sensor for analysis of a new sample, HSA was removed from the surface of the electrode using regeneration solution.

The operating conditions of the flow injection capacitive immunosensor system were optimized for the type and pH of regeneration solution, sample volume, flow rate, type, concentration and pH of carrier buffer. These are summarized in Table 1. All optimizations were carried out using standard HSA 1.0×10^{-14} M except the part for the type of carrier buffer, standard HSA in the range from 1.0×10^{-14} to 1.0×10^{-10} M were investigated.

2.5. Real Sample Analysis

Human serum samples were obtained from Songklanagarind Hospital, Hat Yai, Thailand. Dilution was used to reduce the matrix effect. The calibration curve of standard HSA prepared in 10 mM Tris-HCl pH 7.0 was first performed. Then standard samples of HSA at various concentrations were spiked into the human serum samples. The spiked samples were then diluted and analyzed in the capacitive immunosensor system at optimum conditions. At appropriate dilution (see Sec. 3.7.) the responses of the spiked samples were plotted against the known concentration of HSA [29]. The slopes of the standard curves obtained by standard and matrix-based curve were compared to evaluate the matrix effect using two-way analysis of variance (two-way ANOVA) calculated by R software [30].

The dilution factor that can reduce the effect of matrix interferences was then used for the analysis of albumin in

Table 1. Optimized parameters and values used in the study of flow injection capacitive immunosensor system.

Parameters	Investigated values	Optimum
Regeneration		HCl pH 2.5
Glycine-HCl (pH)	2.2, 2.8	
HCl (pH)	2.2, 2.4, 2.5, 2.6, 2.8	
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 buffer, Sodium phosphate buffer	Tris-HCl
Concentration	5, 10, 15, 20	10
pH	7.0, 7.2, 7.4, 7.6, 7.8	7.0

human serum samples. The results obtained by capacitive immunosensors method were compared with Albumin BCG method. In brief, human serum sample was added with bromocresal green (BCG), albumin in human serum binds quantitatively with BCG resulting in the formation of a green-blue color which can be measured by spectrophotometer (the results obtained from Songklanagarind Hospital).

3. Results and Discussion

3.1. Electrode Surface Characterization

Figure 2a shows a two-dimensional AFM perspective view of nanoscopic images of gold surface thermally evaporated on glass slide. The gold grain and grain boundaries are clearly visible. The three-dimensional AFM image is also shown in Figure 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.

3.2. Electropolymerization of *o*-PD

Cyclic voltammograms of gold electrode during electropolymerization in the presence of 5 mM *o*-PD in 10 mM sodium acetate buffer pH 5.18 were investigated. Along with the increase in the number of potential cycles, the anodic current decreased significantly. This decrease in oxidation current was due to the loss of activity of the electrode surface when covered with newly formed polymer film [31].

In the capacitive immunosensor system the insulating property of polymeric layer on the surface of electrode is of vital importance [32]. In this study the concentration of *o*-PD was fixed at 5 mM. Therefore, the number of cycles of *o*-PD electropolymerization is the main parameter affecting the insulating property. The degree of insulation was examined using cyclic voltammetry with $K_3[Fe(CN)_6]$ in electrolyte solution. The degree of insulation increased with increasing number of cycles. However, when these electrodes, coated through different numbers of electropolymerization cycles of *o*-PD, were activated with 5.0% (v/v) glutaraldehyde, with subsequent immobilization of anti-HSA and then treatment with 0.1 M ethanolamine at pH 8.0, all electrodes were completely insulated, indicated by the absence of redox peaks of the voltammograms. Therefore, the responses of these electrodes to standard HSA (1.0×10^{-14} to 1.0×10^{-12} M) were then used to determine the best condition for preparing the electrodes. The results showed that the sensitivity (slope of calibration curve) increased when the number of cycles increased from 5 to 15 cycles. This is probably due to the increase of density of polymer layer with increasing number of electropolymerization cycles. At 20 cycles the sensitivity (38.2 ± 0.1 (– nF cm^{-2}) (log M)$^{-1}$) of the electrode was nearly the same as after 15 cycles of treatment (38.3 ± 3.8 (– nF cm^{-2}) (log M)$^{-1}$). It is possible that at 15 cycles the electrode was almost completely covered with the polymeric layer, therefore higher number of cycle will not increase the amount of polymer exposed to the surface. Thus, 15 cycles was chosen as an optimum number of cycles for electropolymerization of*o*-PD for the coating procedure and this was used throughout the experiment.

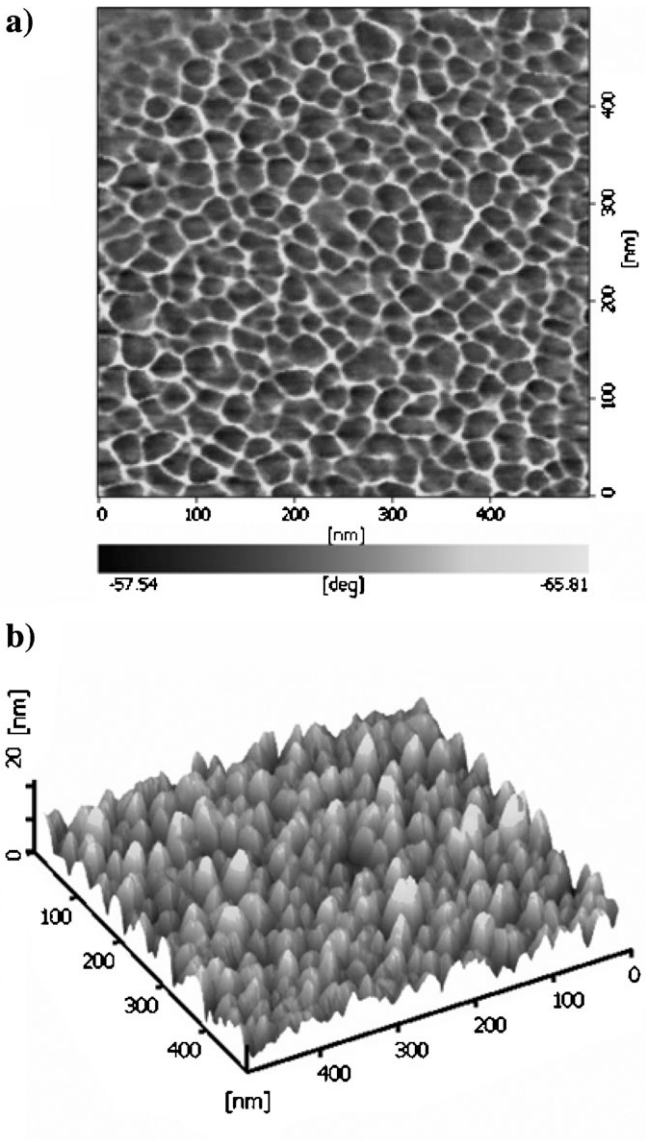

Fig. 2. AFM images of bare gold surface fabricated by thermal evaporation technique a) 2-dimensional and b) 3-dimensional.

3.3. Optimization

3.3.1. Regeneration Solution

Ideally, regeneration of the working electrode should remove any noncovalently bound HSA without destroying

the activity of the anti-HSA immobilized on the electrode. Initially glycine-HCl and HCl were tested. However, for glycine-HCl after the regeneration the signal took very long time before returning to the baseline. Therefore, only HCl solution was further tested. To test the performance of the regeneration solution, the percentage residual activity was evaluated by Equation 1 [3].

$$\%\,\text{residual activity} = \Delta C_2 \times 100/\Delta C_1 \quad (1)$$

Where ΔC_1 and ΔC_2 are the capacitance change before and after regeneration, respectively. The influence of pH of HCl solution, ranging from 2.8 to 2.2 was studied (Table 1). The highest percentage residual activity were obtained at pH 2.5 ($99 \pm 2\%$) which was equal to data obtained at pH 2.4 ($99 \pm 5\%$) but at pH 2.5, the standard deviation is lower, therefore, pH 2.5 was used as regeneration solution for further experiments.

3.3.2. Sample Volume and Flow Rate

The flow rate and volume of the sample passing through capacitive flow cell are two main factors affecting the yield of binding between HSA and anti-HSA in the flow injection capacitive immunosensor system. These two parameters were optimized at the same time. The sample volume was investigated at 150, 200, 250, 300, and 350 μL. For each sample volume flow rate was studied at 25, 50, 100, 150 to 200 μL min^{-1}. Balancing between high response and short analysis time, a sample volume of 250 μL at 100 μL min^{-1} provided the best performance, and these were used for further experiments.

3.3.3. Buffer Solutions

The concentration and pH of running buffers, Tris-HCl and sodium phosphate buffer were optimized (Table 1). At every concentration of the two buffers, pH 7.0 provided the highest responses and the concentration of 10 mM gave the highest capacitance signal. When the two buffers at their optimum concentration and pH were compared, 10 mM Tris-HCl pH 7.0 gave better sensitivity (7.9 ± 0.4 (– nF cm^{-2}) $(\log M)^{-1}$) than 10 mM sodium phosphate buffer pH 7.0 (6.98 ± 0.04 (– nF cm^{-2}) $(\log M)^{-1}$). Therefore, 10 mM Tris-HCl pH 7.0 was chosen as the carrier buffer.

3.4. Linear Dynamic Range and Detection Limit

Figure 3 shows the result for discrete pulse injections of standard HSA solutions ranging from 1.0×10^{-18} to 1.0×10^{-6} M with intermediate regeneration steps using HCl, pH 2.5. A linear relationship between the capacitance change and the logarithm of HSA concentration was obtained in the range of 1.0×10^{-14} to 1.0×10^{-9} M. The linear regression equation was Y (– nF cm^{-2}) $= (15.2 \pm 0.7) \log [\text{HSA (M)}] + (245 \pm 8)$. The detection limit was 8.0×10^{-15} M based on IUPAC recommendation 1994 [33]. The performance of the capacitive immunosensor system presented in this work provided wider linear range than the one reported by Hedström et al. [6] (2.5×10^{-11} to 2.5×10^{-9} M). In their work, SAM of alkanethiol was used for the immobilization of anti-HSA on gold rod electrode, indicating that electropolymerization of *o*-PD on thermally evaporated gold electrode can be used successfully in the capacitive immunosensor system.

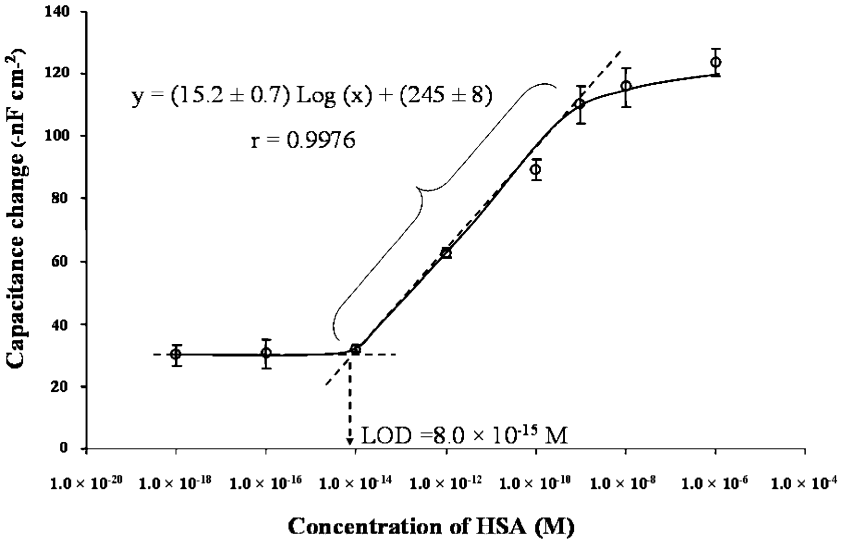

Fig. 3. Capacitance change vs. logarithm of HSA concentration for an anti-HSA modified electrode under optimum conditions (flow rate 0.1 mL min^{-1}, sample volume 250 μL, 10 mM Sodium phosphate buffer pH 7.0).

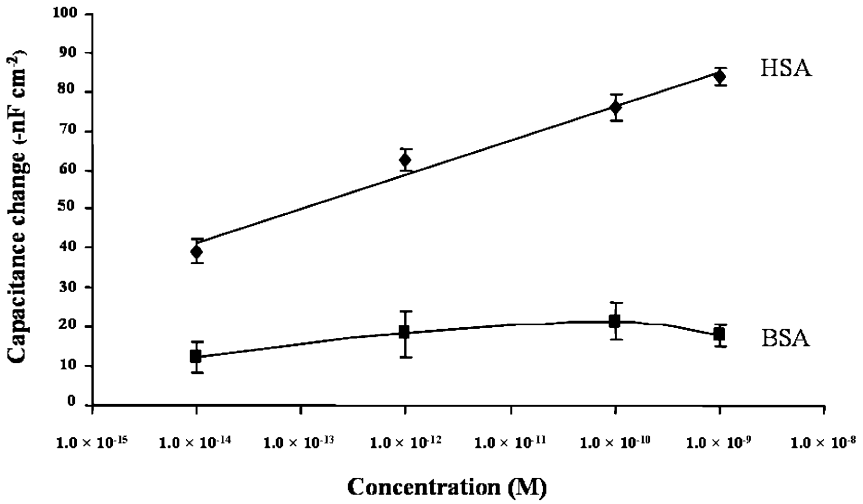

Fig. 4. Selectivity of anti-HSA modified electrode to BSA at a concentration range from 1.0×10^{-14} to 1.0×10^{-9} M.

3.5. Selectivity

To demonstrate that the developed capacitive immunosensor is specific to HSA, other substances which might interfere with the response of the system was studied. Bovine serum albumin (BSA), the analogous protein from bovine with a molecular weight close to that of HSA was selected for the test of selectivity. It is furthermore often reported that cross-reactivity is observed between anti-HSA and the two serum albumins [34]. The capacitance changes from the binding of BSA to the anti-HSA modified electrode at the linear range of HSA, 1.0×10^{-14} to 1.0×10^{-9} M, were studied. The results are shown in Figure 4. The maximum capacitance change obtained from the injection of 1.0×10^{-10} M BSA is 21 ± 5 ($-$ nF cm^{-2}), almost 4 times lower than the response obtained by the injection of HSA at the same concentration. This is also lower than the response of HSA at 8.0×10^{-15} M ($-$ 41 nF cm^{-2}), the limit of detection, indicating that the anti-HSA modified electrode has high selectivity to the detection of HSA.

3.6. Reproducibility

To study the reproducibility of responses, standard HSA 1.0×10^{-14} M was injected to the capacitive immunosensor system with subsequent regenerating with HCl pH 2.5. The performance of the electrode was evaluated intermittently over 3 days (12 times/day). For the first 30 cycles of regeneration the average percentage capacitance change was $94 \pm 3\%$. Then, the response decreased rapidly to about $78 \pm 2\%$. To confirm that the insulating layer of the polymer was not destroyed after long term of regeneration, cyclic voltammograms of the electrode were obtained in ($K_3[Fe(CN)_6]$) solution before and after the reproducibility study. Both cyclic voltammograms were similar which indicated that the insulating layer of the polymer was not destroyed. Therefore, the decrease in percentage capacitance change after 30 times of injections may be due to the loss of activity of anti-HSA after long period of analysis.

The reproducibility between electrodes preparation was also tested by comparing the responses of five electrodes to standard HSA 1.0×10^{-14} M. The average response of each electrode when compared to the signal without HSA (blank) was higher by about 10 folds with $\%RSD = 25$. Sensitivities of two sets of five electrodes were also studied and found that the sensitivity varied from one electrode to another. For the two sets, the *RSD* of sensitivities were 18 and 23%. That is, the responses of different electrodes were not reproducible. This is as expected since the voltammograms obtained after the electrode surfaces were electropolymerized with *o*-PD were not the same, hence, different surface area. This would certainly affect the immobilization yield and cause the different response signal. This means that when a new electrode is used, new calibration is needed. However, since one electrode can be reused up to 30 times most of the required analysis can probably be performed within one electrode.

3.7. Real Samples Analysis

To determine the concentration of albumin in real samples, their concentrations have to fall within the linear range of the response. The concentration of albumin in human serum for hypoalbuminemia and hyperalbuminemia patients are in the range of 7.3×10^{-5} to 1.0×10^{-3} M [35] and in this work, a linear relationship between the capacitance change ($-$ nF cm^{-2}) and the logarithm of HSA concentration was obtained in the range of 1.0×10^{-14} to 1.0×10^{-9} M. Therefore, the samples were diluted starting from 10^6 times so that their albumin concentration would fall in the linear range.

Serum samples were also spiked with different concentration of standard HSA and then diluted for 10^6 times. The concentrations after dilution were 3.0×10^{-10}, 5.0×10^{-10}, 7.0×10^{-10} and 1.0×10^{-9} M. Five serum samples were tested

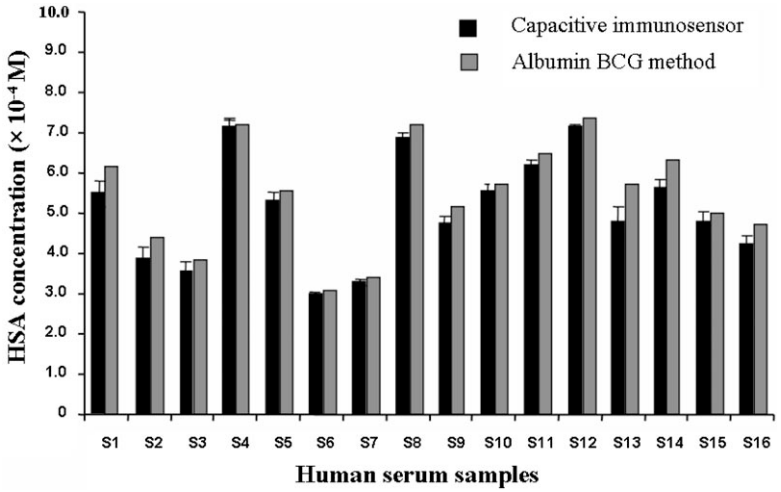

Fig. 5. Comparison of albumin concentration analyzed by capacitive immunosensor and Albumin BCG method.

for matrix interferences. The slope of regression line of the standard HSA prepared in 10 mM Tris-HCl buffer pH 7.0 was not significantly different from the slope of the diluted real samples ($P < 0.05$), indicating that there is no effect of matrix at 10^6 times dilution. The dilution factor of 10^7 times was also tested for the same five samples. As expected, the result also showed no effect of matrix interference.

The analysis of albumin in human serum samples was performed by diluting the samples at 10^6 or 10^7 times to be in the linear range of the calibration curves. Sixteen samples were analyzed. The obtained response ($-$ nF cm^{-2}) was used to calculate the concentration of HSA in the sample from the calibration curve of standard HSA.

Figure 5 shows the concentrations of albumin in human serum samples analyzed by capacitive immunosensor and BCG method (results obtained from Songklanagarind Hospital). Comparison between the two methods was done by regression line method. The regression equation of albumin concentration obtained from capacitive immunosensor system (y) and BCG method (x) is $y = (0.97 \pm 0.1)x - (0.2 \pm 0.6)$ with a correlation coefficient (r) of 0.9827. Concentrations of HSA determined by capacitive immunosensor system were lower than the results obtained from BCG method by about 3%. Differences between the two methods for the 16 tested samples are in the range of -0.03 to -0.9×10^{-4} M. Several authors have reported that BCG method can overestimate albumin concentration up to $1.0 - 1.4 \times 10^{-4}$ M compared to the immunoassay methods [36, 37]. This is due to the lack of specificity of Bromocresal Green (BCG) since other proteins, particulary α- and β globulin in human sample, also bind to the dye [36–38].

To further validate the capacitive immunosensor, percentage recovery of the added HSA was evaluated. It was calculated by Equation 2.

$$\text{Recovery } (\%) = (C_1 - C_2) \times 100/C_3 \quad (2)$$

where C_1 = concentration determined in fortified sample, C_2 = concentration determined in unfortified sample and C_3 = concentration of fortification [30]. Recoveries of the five human serum samples are summarized in Table 2. Since the acceptable recovery in analytical analysis in the 1.0×10^{-4} M level is 90–107% and *RSD* is 5.3–8% [39]. Therefore, recoveries for all samples are acceptable.

Table 2. Percentage recovery of albumin in five human serum samples at concentration of fortification 3.0×10^{-4}, 5.0×10^{-4}, 7.0×10^{-4}, and 10.0×10^{-4} M with dilution 10^6 and 10^7 times.

Spiked concentration (M)	Percentage recovery				
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Dilution 10^6 times					
3.0×10^{-4}	96 ± 6	91 ± 6	91 ± 2	91 ± 7	90 ± 7
5.0×10^{-4}	94 ± 4	92 ± 1	92 ± 4	95 ± 1	96 ± 4
7.0×10^{-4}	90 ± 1	90 ± 2	91 ± 3	92 ± 3	90 ± 2
10.0×10^{-4}	98 ± 3	96 ± 4	96 ± 3	97 ± 4	98 ± 3
Dilution 10^7 times					
3.0×10^{-4}	91 ± 6	91 ± 7	93 ± 3	91 ± 3	93 ± 3
5.0×10^{-4}	91 ± 4	93 ± 6	94 ± 4	97 ± 5	100 ± 3
7.0×10^{-4}	92 ± 6	97 ± 5	103 ± 5	102 ± 6	101 ± 1
10.0×10^{-4}	95 ± 3	96 ± 4	100 ± 2	99 ± 5	95 ± 7

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. By using thermal evaporation, the fabrication of each electrode is inexpensive since 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. Different sizes and patterns can also be fabricated to meet the application requirement and mass production of this type of electrode is also a real possibility.

Electropolymerization of *o*-PD can be used to fabricate an insulated thin film on a disposable gold electrode. Compared to SAM technique, this proposed method is very rapid since the electropolymerization step took only 8 min while the SAM formation of alkylthiol needed 12 – 24 hours [7]. The electrode coated with *o*-PD film can be used for anti-HSA immobilization via covalent coupling using glutaraldehyde. 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. Moreover 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. On this basis, we suggest that the proposed technique will provide an easy and low cost biosensor that can be applied to many classes of biological substances. Future work may include the use of other polymers to enhance immobilization yields and the fabrication of a disposable component where all three electrodes, i.e., working, auxiliary and references electrodes, are placed on the same substrate would be interesting for sensor chip development.

5. Acknowledgements

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6. References

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คำขอรับสิทธิบัตร/อนุสิทธิบัตร

☑ การประดิษฐ์

☐ การออกแบบผลิตภัณฑ์

☐ อนุสิทธิบัตร

ข้าพเจ้าผู้ลงลายมือชื่อในคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ ขอรับสิทธิบัตร/อนุสิทธิบัตร ตามพระราชบัญญัติสิทธิบัตร พ.ศ.2522 แก้ไขเพิ่มเติมโดยพระราชบัญญัติสิทธิบัตร (ฉบับที่ 2) พ.ศ.2535 และพระราชบัญญัติสิทธิบัตร (ฉบับที่ 3) พ.ศ.2542

สำหรับเจ้าหน้าที่	
วันรับคำขอ 13 ก.พ. 2552	เลขที่คำขอ 0901000618
วันยื่นคำขอ 4 กพ 52	
สัญลักษณ์จำแนกการประดิษฐ์ระหว่างประเทศ	
ใช้กับแบบผลิตภัณฑ์ ประเภทผลิตภัณฑ์	
วันประกาศโฆษณา	เลขที่ประกาศโฆษณา
วันออกสิทธิบัตร/อนุสิทธิบัตร	เลขที่สิทธิบัตร/อนุสิทธิบัตร
ลายมือชื่อเจ้าหน้าที่	

1. ชื่อที่แสดงถึงการประดิษฐ์/การออกแบบผลิตภัณฑ์
ระบบตรวจวัดอิเล็กโตรเคมิคอลเซนเซอร์ (Electrochemical sensor) แบบตรวจวัดตามเวลาจริง (real time) สำหรับวัดค่าทางไฟฟ้า

2. คำขอรับสิทธิบัตร/การออกแบบผลิตภัณฑ์นี้เป็นคำขอสำหรับแบบผลิตภัณฑ์อย่างเดียวกันและเป็นคำขอลำดับที่
ในจำนวน คำขอ ที่ยื่นในคราวเดียวกัน

3. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร และที่อยู่ (เลขที่ ถนน ประเทศ)
มหาวิทยาลัยสงขลานครินทร์ ที่อยู่ 15 ถนนกาญจนวณิชย์ อ.หาดใหญ่ จ.สงขลา 90110 และสำนักงานกองทุนสนับสนุนการวิจัย ที่อยู่ ชั้น 14 อาคาร เอส เอ็ม ทาวเวอร์ 979/17-21 ถนนพหลโยธิน แขวงสามเสนใน เขตพญาไท กรุงเทพฯ 10400

3.1 สัญชาติ	ไทย
3.2 โทรศัพท์	0-7428-7409
3.3 โทรสาร	0-7428-7412
3.4 อีเมล์	jittiyut.y@psu.ac.th

4. สิทธิในการขอรับสิทธิบัตร/อนุสิทธิบัตร
☐ ผู้ประดิษฐ์/ผู้ออกแบบ ☑ รับโอน ☐ ผู้ขอรับสิทธิโดยเหตุอื่น

5. ตัวแทน (ถ้ามี) ที่อยู่ (เลขที่ ถนน จังหวัด ประเทศ รหัสไปรษณีย์)

5.1 ตัวแทนเลขที่	
5.2 โทรศัพท์	
5.3 โทรสาร	
5.4 อีเมล์	

6. ผู้ประดิษฐ์/ผู้ออกแบบผลิตภัณฑ์ และที่อยู่ (เลขที่ ถนน ประเทศ)
รองศาสตราจารย์บุญเจริญ วงศ์กิตติศึกษา รองศาสตราจารย์ปณต ถาวรังกูร รองศาสตราจารย์ชูศักดิ์ ลิ่มสกุล รองศาสตราจารย์เพริศพิชญ์ คณาธารณา นายวรากร ลิ่มบุตร นายนัฐพร พงศ์พนธุ์วิทูร นายด้าย บัณฑิศักดิ์ นายพุทธิพล กัลป์จารุ ผู้ช่วยศาสตราจารย์พรรณี อัศวตรีรัตนกุล นางสาวจงดี ธรรมเขต นายชิตนนท์ บูรณชัย นางสาวสุชีรา ลอยประเสริฐ นายอาภรณ์ นุ่มน่วม นางสาวสุภาพร คาวัลย์ ที่อยู่ 15 ถนนกาญจนวณิชย์ อ.หาดใหญ่ จ.สงขลา 90110

7. คำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้แยกจากหรือเกี่ยวข้องกับคำขอเดิม
ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรขอให้ถือว่าได้ยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ในวันเดียวกับคำขอรับสิทธิบัตรเลขที่
วันยื่น เพราะคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้แยกจากหรือเกี่ยวข้องกับคำขอเดิมเพราะ
☐ คำขอเดิมมีการประดิษฐ์หลายอย่าง ☐ ถูกคัดค้านเนื่องจากผู้ขอไม่มีสิทธิ ☐ ขอเปลี่ยนแปลงประเภทของสิทธิ

หมายเหตุ ในกรณีที่ไม่อาจระบุรายละเอียดได้ครบถ้วน ให้จัดทำเป็นเอกสารแนบท้ายแบบพิมพ์นี้ โดยระบุหมายเลขกำกับข้อและหัวข้อที่แสดงรายละเอียดเพิ่มเติมดังกล่าวด้วย

8. การยื่นคำขอนอกราชอาณาจักร

วันยื่นคำขอ	เลขที่คำขอ	ประเทศ	สัญลักษณ์จำแนกการประดิษฐ์ระหว่างประเทศ	สถานะคำขอ
8.1				
8.2				
8.3				

8.4 ☐ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรขอสิทธิให้ถือว่าได้ยื่นคำขอนี้ในวันที่ได้ยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรในต่างประเทศเป็นครั้งแรกโดย
☐ ได้ยื่นเอกสารหลักฐานพร้อมคำขอนี้ ☐ ขอยื่นเอกสารหลักฐานหลังจากวันยื่นคำขอนี้

9. การแสดงการประดิษฐ์ หรือการออกแบบผลิตภัณฑ์ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรได้แสดงการประดิษฐ์ที่หน่วยงานของรัฐเป็นผู้จัด
วันแสดง วันเปิดงานแสดง ผู้จัด

10. การประดิษฐ์เกี่ยวกับจุลชีพ

10.1 เลขทะเบียนฝากเก็บ	10.2 วันที่ฝากเก็บ	10.3 สถาบันฝากเก็บ/ประเทศ

11. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอยื่นเอกสารภาษาต่างประเทศก่อนในวันยื่นคำขอนี้ และจะจัดยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ที่จัดทำเป็นภาษาไทยภายใน 90 วัน นับจากวันยื่นคำขอนี้ โดยขอยื่นเป็นภาษา
☐ อังกฤษ ☐ ฝรั่งเศส ☐ เยอรมัน ☐ ญี่ปุ่น ☐ อื่น ๆ

12. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอให้อธิบดีประกาศโฆษณาคำขอรับสิทธิบัตร หรือรับจดทะเบียน และประกาศโฆษณาอนุสิทธิบัตรนี้ หลังจากวันที่ เดือน พ.ศ.
☐ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรขอให้ใช้รูปเขียนหมายเลข ในการประกาศโฆษณา

13. คำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ประกอบด้วย
ก. แบบพิมพ์คำขอ 2 หน้า
ข. รายละเอียดการประดิษฐ์
หรือคำพรรณนาแบบผลิตภัณฑ์ 5 หน้า
ค. ข้อถือสิทธิ 2 หน้า
ง. รูปเขียน 8 รูป 5 หน้า
จ. ภาพแสดงแบบผลิตภัณฑ์
☐ รูปเขียน รูป หน้า
☐ ภาพถ่าย รูป หน้า
ฉ. บทสรุปการประดิษฐ์ 1 หน้า

14. เอกสารประกอบคำขอ
☑ เอกสารแสดงสิทธิในการขอรับสิทธิบัตร/อนุสิทธิบัตร
☐ หนังสือรับรองการแสดงการประดิษฐ์/การออกแบบผลิตภัณฑ์
☐ หนังสือมอบอำนาจ
☐ เอกสารรายละเอียดเกี่ยวกับจุลชีพ
☐ เอกสารการขอนับวันยื่นคำขอในต่างประเทศเป็นวันยื่นคำขอในประเทศไทย
☐ เอกสารขอเปลี่ยนแปลงประเภทของสิทธิ
☐ เอกสารอื่น ๆ

15. ข้าพเจ้าขอรับรองว่า
☑ การประดิษฐ์นี้ไม่เคยยื่นขอรับสิทธิบัตร/อนุสิทธิบัตรมาก่อน
☐ การประดิษฐ์นี้ได้พัฒนาปรับปรุงมาจาก.....

16. ลายมือชื่อ (☑ ผู้ขอรับสิทธิบัตร / อนุสิทธิบัตร; ☐ ตัวแทน)

.....
(รองศาสตราจารย์สุรพล อารีย์กุล)
รองอธิการบดีฝ่ายวิจัยและบัณฑิตศึกษา ปฏิบัติราชการแทน
อธิการบดีมหาวิทยาลัยสงขลานครินทร์

.....
(ศาสตราจารย์ปิยะวัติ บุญ-หลง)
ผู้อำนวยการสำนักงานกองทุนสนับสนุนการวิจัย

หมายเหตุ บุคคลใดยื่นคำขอรับสิทธิบัตรการประดิษฐ์หรือการออกแบบผลิตภัณฑ์ หรืออนุสิทธิบัตร โดยการแสดงข้อความอันเป็นเท็จแก่พนักงานเจ้าหน้าที่ เพื่อให้ได้ไปซึ่งสิทธิบัตรหรืออนุสิทธิบัตร ต้องระวางโทษจำคุกไม่เกินหกเดือน หรือปรับไม่เกินห้าพันบาท หรือทั้งจำทั้งปรับ