CHAPTER IV

RESULTS AND DISCUSSION

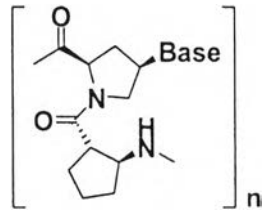
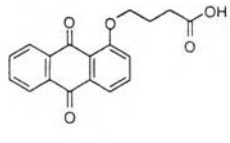


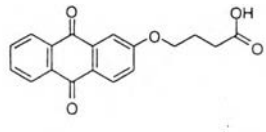
Figure 4.1 The structure of acpcPNA

In this work, pyrrolidinyl peptide nucleic acids (acpcPNA, **Figure 4.1**) carrying a redox-active label or reporter were synthesized and employed as sensor probes for electrochemical DNA sequence detection on a screen printed carbon paste electrode (SPCE) in an immobilization-free fashion. It is expected that this format should require low cost of instrumentation, simple sample preparation and low volume and concentration of sample. Details of which will be illustrated in this chapter.

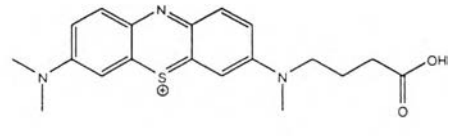
4.1 Synthesis and characterization of electrochemically-active labeled acpcPNA



4-(Anthraquinone-1-yloxy)butyric acid (1AQ)



4-(Anthraquinone-2-yloxy)butyric acid (2AQ)



Methylene blue butyric acid (MB)

Synthesis of pyrrolidinyl peptide nucleic acids carrying a redox-active label requires modification of the PNA via a suitable chemistry such as amide bond formation. Three organic redox-active labels were chosen for the modification. All of which requires functionalization with a carboxyl group prior to the coupling to the PNA. These carboxy-functionalized labels including 4-(anthraquinone-1-yloxy)butyric acid (1AQ), 4-(anthraquinone-2-yloxy)butyric acid (2AQ) and methylene blue butyric acid (MB) were synthesized and characterized by NMR and mass spectrometry. All carboxyl-functionalized electrochemically-active labels were activated as pentafluorophenyl (Pfp) ester prior to the coupling to the acpcPNA via acylation of the free amino group, either at the *N*-terminal or internal position (via APC-linker).

The successful attachment of the redox-active label onto the acpcPNA was verified by MALDI-TOF MS analysis. In all cases, the labeled PNAs were purified to more than 90% purity by reverse-phase HPLC (**Figure A7-A20**). Retention time and yield of all labeled PNAs used in this study are summarized in **Table 4.1**.

Table 4.1 Retention time, m/z (MALDI-TOF), and yield of redox-active labeled acpcPNA

PNA	Sequence (N→C)	t_R (min)	m/z (calcd)	m/z (found)	%yield*
1AQ-T2	1AQ-TT-Lys	31.2	991.9	991.3	22.8
2AQ-T2	2AQ-TT-Lys	24.2	991.9	991.3	27.2
MB-T2	MB-TT-Lys	21.9	1038.8	1037.4	12.8
1AQ-T9	1AQ-TTT TTT TTT-Lys	34.4	3429.1	3432.0	25.0
2AQ-T9	2AQ-TTT TTT TTT-Lys	24.7	3429.1	3430.2	20.3
MB-T9	MB-TTT TTT TTT-Lys	23.6	3476.6	3477.5	14.2
T5-1AQ-T4	Ac-TTT T(1AQ)TT TTT-Lys	30.7	3429.3	3431.9	7.9
T5-2AQ-T4	Ac-TTT T(2AQ)TT TTT-Lys	24.0	3429.3	3436.4	16.2
T5-MB-T4	Ac-TTT T(MB)TT TTT-Lys	33.6	3519.6	3521.8	9.5
2AQ-WSSV-Lys	2AQ- TCA AAT TCA GA-Lys	31.6	4134.3	4137.6	16.8
2AQ-WSSV-Ser	2AQ- TCA AAT TCA GA-Ser	31.3	4093.2	4091.9	1.6
2AQ-WSSV-Glu	2AQ- TCA AAT TCA GA-Glu	31.8	4135.2	4133.8	0.6
2AQ-B1502-Lys	2AQ-CGC GCA GGT TCC-Lys	30.2	4348.8	4351.1	6.4
2AQ-B1513-Lys	2AQ-GGA GCG CGA TCC-Lys	30.2	4418.2	4415.6	6.8

* Yields of purified labeled PNA that are calculated based on original loading of the resin used for the PNA synthesis.

4.2 PNA-DNA binding properties

The PNA-DNA binding properties of redox-active labeled acpcPNAs were studied by thermal denaturation. This was performed spectrophotometrically by

monitoring the absorbance at 260 nm at various temperatures ranging from 20 to 95 °C. Heating disrupts of the hydrogen bonds and the base pairings, which results in separation of the duplex into single stranded components. When the absorbance at 260 nm was plotted against with temperature, a sigmoidal curve is obtained. The temperature at the midpoint of the S-curve which correspond to 50% denaturation is called melting temperature (T_m), which can be taken as an indication of the stability of the duplex. In practice, this value can be obtained from the maximum of the first derivative plot between normalized absorbance at 260 nm as a function of temperature. Data in **Table 4.2** reveal that the labeling at the *N*-terminus of acpcPNA resulted in a more stable hybrid as shown by higher melting temperatures than the corresponding unlabeled acpcPNA. The T_m increase was observed for both complementary and single base mismatched hybrids. The increase in stability can be explained by π-π interaction between the redox-active label with the terminal base pair of the PNA-DNA duplex. In contrast, hybrids of internally labeled acpcPNA with DNA are less stable than the corresponding unlabeled acpcPNA. This suggests that the redox-active label at the internal position does not stack well between the base pairs or in the grooves of the duplex. Accordingly, the bulky labeling group interferes with the base pairing of the PNA-DNA duplex, resulting in the observed destabilization of the duplexes. In all cases, the melting temperatures of hybrids with complementary DNA are 20-30 °C higher than hybrids with single base mismatched DNA. It can therefore be concluded that all labeled acpcPNA exhibit high DNA affinity as well as specificity as observed in unlabeled acpcPNA.

Table 4.2 Melting temperature of labeled acpcPNA

PNA	DNA Sequence (5'→3')	T_m (°C)	ΔT_m[a] (°C)	ΔT_m[b] (°C)
T9	AAA AAA AAA	80.0	-	-
	AAA AIA AAA	50.0	-30.0	-
1AQ-T9	AAA AAA AAA	80.0	-	0.0
	AAA AIA AAA	48.1	-31.9	31.9
2AQ-T9	AAA AAA AAA	85.6	-	-5.6
	AAA AIA AAA	58.8	-26.8	21.2
MB-T9	AAA AAA AAA	84.3	-	-4.3
	AAA AIA AAA	58.4	-25.9	21.6
Ac-T4(NH)T5	AAA AAA AAA	76.5	-	-
	AAA AIA AAA	48.9	-27.6	-

PNA	DNA Sequence (5'→3')	T_m (°C)	ΔT_m [a] (°C)	ΔT_m [b] (°C)
T5-1AQ-T4	AAA AAA AAA	60.9	-	15.6
	AAA ATA AAA	30.9	-30.0	45.6
T5-2AQ-T4	AAA AAA AAA	60.3	-	16.2
	AAA ATA AAA	27.9	-32.4	48.6
T5-MB-T4	AAA AAA AAA	69.1	-	7.4
	AAA ATA AAA	37.5	-31.6	39.0
2AQ-WSSV-Lys	TCT GAA TTT GA (Dcomp)	82.7	-	-
	TCT GAC TTT GA (DsmC)	60.5	-22.2	-
	TCT GAG TTT GA (DsmG)	54.5	-28.2	-
	TCT GAT TTT GA (DsmT)	63.4	-19.3	-
	CTA AGT CTG AA T TTA GGG G (Dcomp19mer)	>90.0	-	-
	CTA AGT CTG AA T TTA GGG G (with 100 mM NaCl)	83.7	-	-
	CTA AG TCT GCA TTT AG GG G (DsmC19mer)	70.5	(-19.5)	-
	CTA AG TCT GCA TTT AG GG G (with 100 mM NaCl)	61.0	-22.7[b]	-
2AQ-B1502-Lys	GGA ACC TGC GCG (DcompB1502)	81.0	-	-
	GGA TCG CGC TCC (DcompB1513)	<20.0	-	-
	AGCCTGCGGA ACCTGCGCGG CTACTACAAC (DcompB1502-30mer)	>90	-	-
	AGCCTGCGGA ACCTGCGCGG CTACTACAAC (with 100 mM NaCl)	84.7	-	-
2AQ-B1513-Lys	GGA TCG CGC TCC (DcompB1513)	81.1	-	-
	GGA ACC TGC GCG (DcompB1502)	<20.0	-	-
	AACCTGCGGA TCGCGCTCCG CTACTACAAC (DcompB1513-30mer)	>90	-	-
	AACCTGCGGA TCGCGCTCCG CTACTACAAC (with 100 mM NaCl)	81.8	-	-

[a] T_m duplex (mismatched) – T_m duplex (complementary)

[b] T_m duplex (labeled) – T_m duplex (unlabeled)

4.3 Preparation and characterization of screen-printed carbon electrode (SPCE)

4.3.1 Preparation of the unmodified, positively and negatively charged polymer modified SPCE

The SPCE was prepared as previously described [26]. Three major complements in the SPCE are working electrode (screened with carbon ink mixture), counter electrode (screened with carbon ink mixture) and reference electrode (screened with Ag/AgCl ink). An insulator is screened to limit the area of the electrode so as to minimize the volume of the sample required. As the analyte is prepared in aqueous solution, the insulator used in this work must be hydrophobic such as nail polish. The unmodified working electrode consisted of carbon ink and graphite in the optimized amounts (1 g carbon ink: 0.2 g graphite) obtained from an earlier study [26]. The Ag/AgCl was first screened as the reference electrode. After that, the carbon ink mixture was screened as the working and counter electrodes. Lastly, the insulator was screened. The pattern of the SPCE used in this work is shown as **Figure 4.2**.

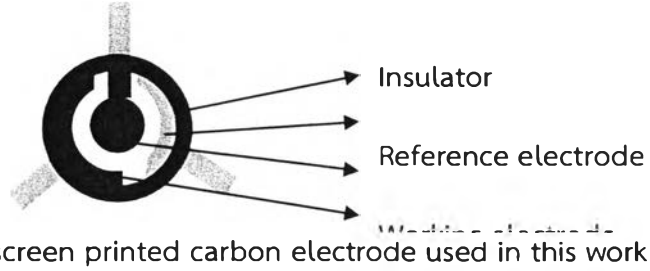


Figure 4.2 The pattern of the screen printed carbon electrode used in this work [26]

The polymer-modified SPCE was prepared similarly to the unmodified SPCE electrode, but with a positively charged or negatively charged polymer mixed in the carbon ink mixture. There are several possible ways to modify an electrode with a charged species (usually a polymer). Firstly, the polymer is added to the carbon ink solution before the electrode screening [26]. Secondly, a solution of the polymer or its monomer may be dropped or coated onto the unmodified electrode [21]. Finally, carbon-paste electrodes can be electrochemically pre-treated (with strong base solution such as sodium hydroxide) to oxidize its surface into carboxyl groups [35]. In this work, the first way is preferred over the other because no additional coating step is required, and it is easier to control the amount of the polymer to be added to the electrode. The modified carbon ink was prepared by mixing the commercial carbon ink with graphite powder in the proportion that had been determined earlier (1 g carbon ink: 0.2 g graphite) [26]. Next, the polymer solution (1% w/v) was added to

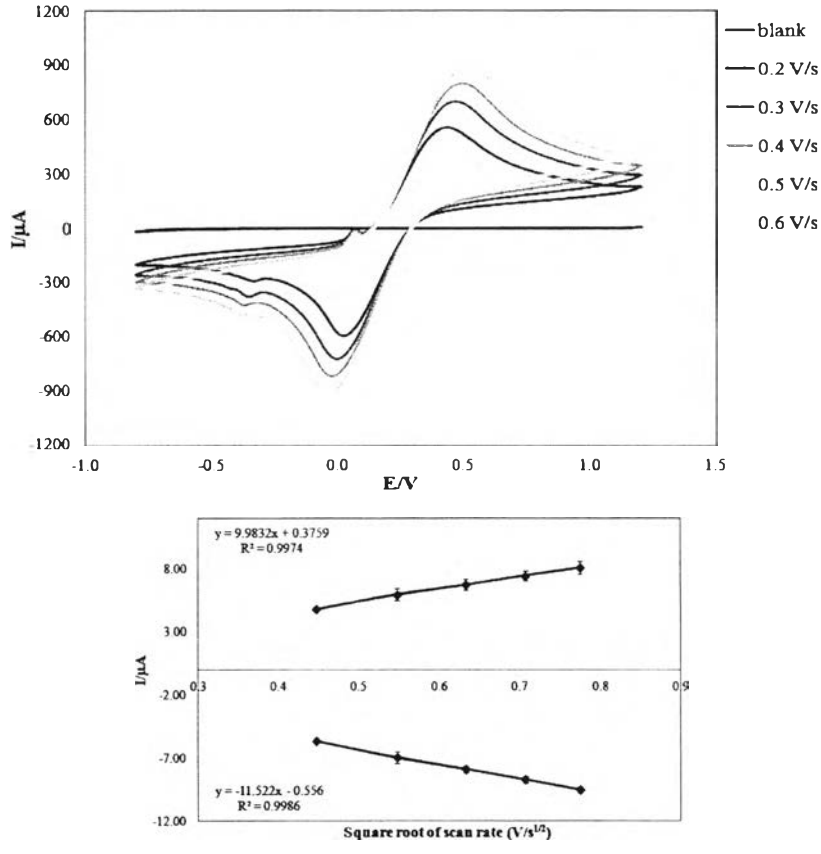
the solution. Finally, the binding solution containing diethylene glycol monobutyl ether and ethylene glycol monobutyl ether acetate was added in order to form a well dispersed paste with sufficient stickiness to allow efficient screening.

Various types of positively charged polymer including chitosan (CHT), *N*-[(2-hydroxyl-3-trimethylammonium)propyl])chitosan chloride (HTACC-CHT) and poly(quarternized(dimethylamino)ethyl methacrylate) (PQDMAEMA), as well as polyacrylic acid (PAA) as a representative negatively charged polymer were investigated to maximize the difference between the signals of the label on the PNA probe before and after hybridization with the complementary DNA target.

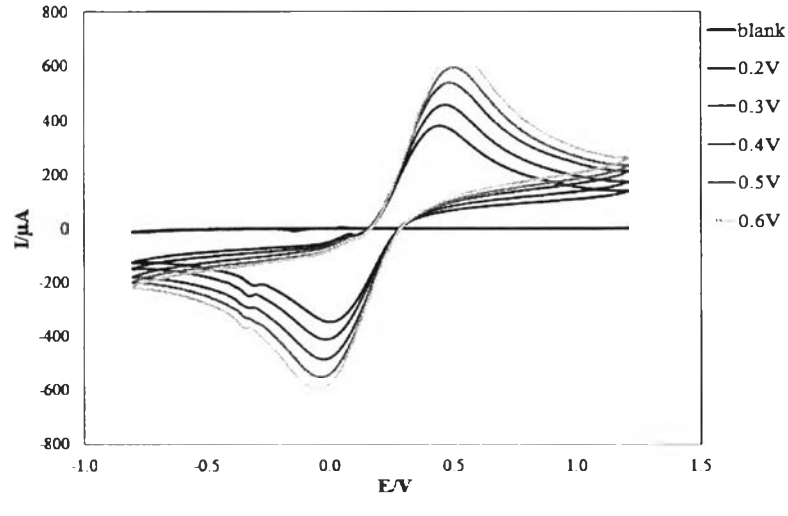
4.3.2 Performance of the unmodified, positively charged and negatively charged SPCE

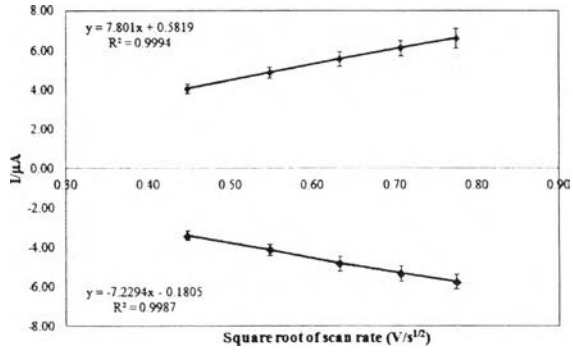
To assess their suitability of the modified SPCE as a platform for DNA sequence determination, the electrochemical behavior of the unmodified, PQDMAEMA (positively charged) and PAA (negatively charged) modified SPCE was studied. The experiments were carried out by determining the current of $[Fe(CN)_6]^{3-/4-}$ at different scan rates by cyclic voltammetric (CV) method. The results showed that the ferri/ferro cyanide gave reversible peaks on all electrodes. Moreover, the anodic peak currents of $[Fe(CN)_6]^{3-/4-}$ increases linearly as a function of square root of the scan rates on all kinds of SPCE (see **Figures 4.3**).

The linearity of the anodic peak currents against the square root of the scan rates on the PQDMAEMA, unmodified and PAA modified SPCE is in the range of 200-600 mV s^{-1} with a correlation coefficient (R^2) = 0.9974, 0.9994 and 0.9987 for reduction peak and R^2 = 0.9986, 0.9987 and 0.9997 for oxidation peak respectively. The results imply that the oxidation and reduction of the redox-active reporter on these modified electrodes is diffusion controlled [72].

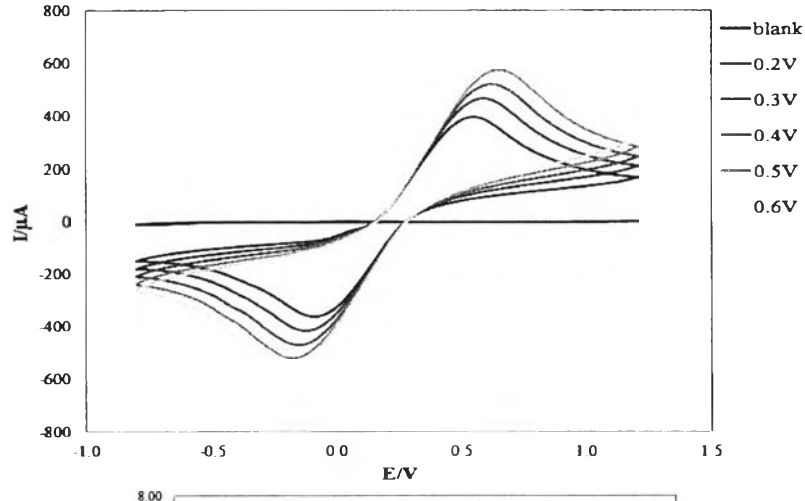


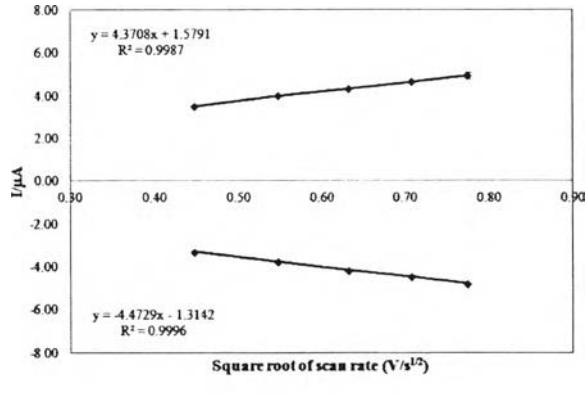
(A)





(B)



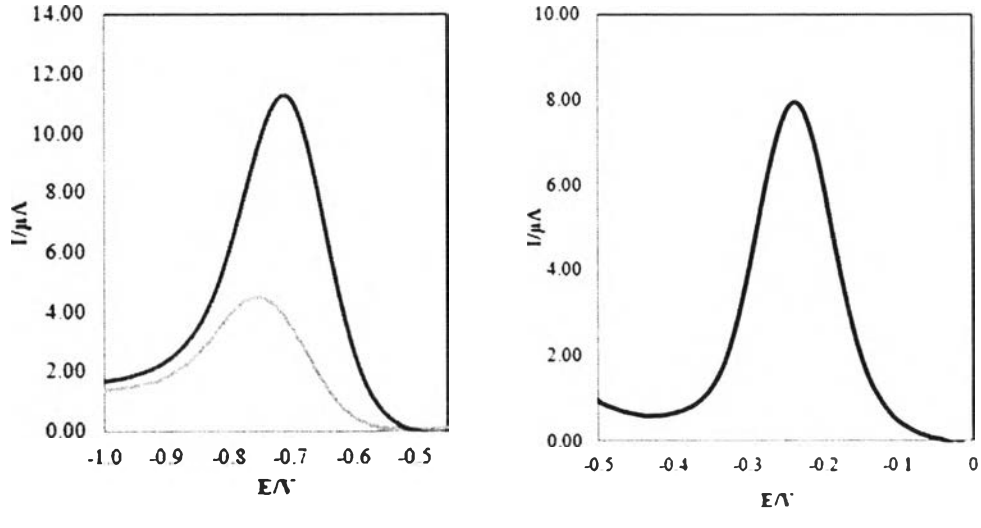


(C)

Figure 4.3 Cyclic voltammograms of 1 mM [Fe(CN)6]3-/4- in 0.5 M KCl (20 μL) and the linear relationship between anodic peak currents and scan rates on (A), 1% PQDMAEMA (positively charged) (B) unmodified and (C) 5% PAA (negatively charged) modified SPCE

4.4 Characteristic of electrochemical signal of labeled acpcPNA

Before the labeled acpcPNA can be used as an electrochemical probe, the electrochemical signals of the synthesized redox-labeled acpcPNA were first measured by square wave voltammetry (SWV) on the unmodified SPCE. Different redox-active reporter gave different redox peaks depending on the nature of the label and types of electrode. Electrochemical signals of the 1AQ-, 2AQ- and MB-labeled acpcPNA T2 were measured on unmodified electrodes in order to find the most suitable label that provides strong signal and sharp peak. In order to measure the signal, the labeled acpcPNA samples (50 μM in 10 mM phosphate buffer pH 7.4) were dropped onto the unmodified SPCE electrode which was pre-treated in 0.5 M NaOH at a potential of 1.3 V for 30 s. The labels 1AQ, 2AQ and MB gave signals at -0.75, -0.71 and -0.24 V respectively. The highest signals were obtained from 2AQ and MB labeled PNA, suggesting more efficient electron transfer processes than the 1AQ label. Therefore, 2AQ- and MB-labeled acpcPNA were selected for further studies.



(A) (B)

Figure 4.4 SWV signals of labeled acpcPNA (50 μM, 20 μL) (A) 1AQ-T2 (yellow) 2AQ-T2 (orange) and (B) MB-T2 in 10 mM phosphate buffer (pH 7.4)

4.5 Proof of principle for immobilization-free DNA sequence detection employing labeled acpcPNA

It is known for some times that the electrochemical signal of redox-active DNA probes can change in response to the hybridization with the correct DNA targets. The signal may increase (**Figure 4.5(A)**) or decrease (**Figure 4.5(B)**) after the hybridization, depending on the relative efficiency of the electron transfer process between the label and the electrode. This may be controlled by choosing a right combination of the probe and the label [73].

Figure 4.5 DNA Biosensors based on redox labeled probe immobilization with (A) a classical probe and (B) a hairpin probe [73].

In this work, an immobilization-free method for PNA-based DNA sequence detection is proposed. Although PNA is normally uncharged, it is anticipated that by suitable modification of the PNA and the electrode by some charged species it should be possible to immobilize the PNA by electrostatic attraction. Upon binding to the DNA target, the electrochemical signal should change according to the mechanisms shown in **Figure 4.5** analogously to DNA [21].

To prove the principle of immobilization-free method for electrochemical DNA detection proposed in this work, the electrochemical signal of the labeled acpcPNA probes before and after hybridized with complementary DNA on the pre-treated unmodified SPCE were compared. The free terminally-labeled AQ- and MB-labeled acpcPNA probes (2AQ-T9 and MB-T9) (50 μM in 10 mM phosphate buffer pH 7) were dropped onto the unmodified SPCE electrodes and the signal was measured by SWV. The signal of MB-labeled PNA appeared at -0.24 V (**Figure 4.6**), which was in good agreement with the MB-T2 (**Figure 4.6**). Importantly, when the complementary was added to the solution, the signal completely disappeared (**Figure 4.6**). When the DNA contained a single mismatched base, the signal was suppressed to a smaller

extent when compared to complementary DNA. Similar results were obtained with 2AQ-T9. The disappearance of the signal can be explained by the differential ability of PNA and DNA (or PNA-DNA hybrids) to electrostatically interact with the SPCE electrodes. Since the PNAs contain a positively charged lysine as a solubility enhancer, it carries a net positive charge which can electrostatically attract the negatively charged pre-treated electrode. This results in efficient electron transfer process. Upon binding with the complementary DNA target, the negatively charged PNA-DNA duplex and the negative charge on the surface of the electrode will repulse each other. This will separate the labeled probe from the electrode, and therefore the electron transfer process should be less efficient. On the other hand, if sequence of DNA is non-complementary, some PNA strand can still electrostatically attract to the electrode and thus the signal is visible. These preliminary results had proven the concept and were the basis for designing of the immobilization-free PNA-based DNA sensor in this work (**Scheme 4.1**).

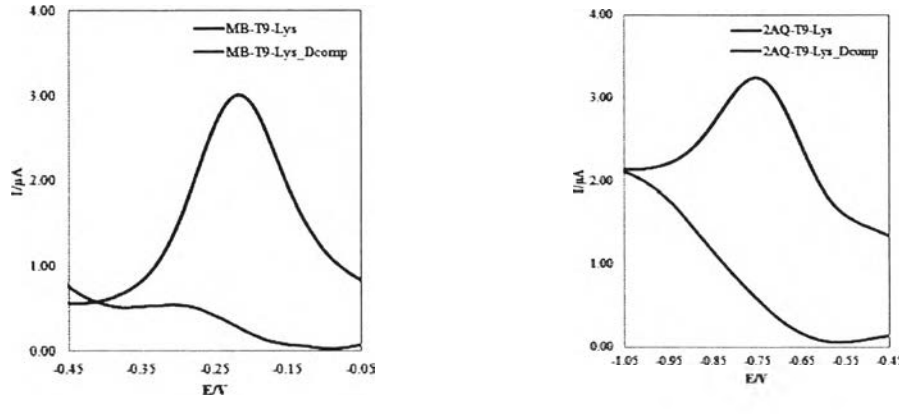
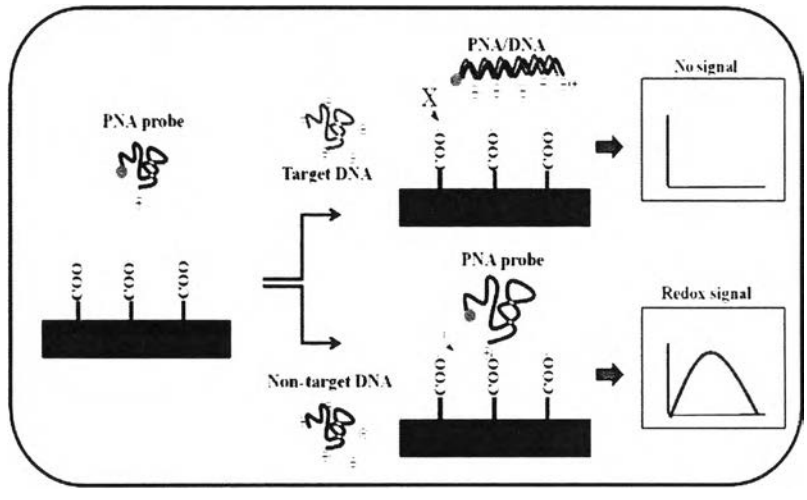


Figure 4.6 SWVs of (left) MB-T9-Lys and (right) 2AQ-T9-Lys (50 μM, 20 μL) before and after hybridized with complementary DNA in 10 mM phosphate buffer (pH 7.4)



Scheme 4.1 Working hypothesis of the present immobilization-free electrochemical DNA sensor

4.6 Comparison of performance of terminally and internally-labeled acpcPNA probes

The performances of two types of 2AQ- and MB-labeled acpcPNA, i.e. at internal and terminal positions, with the same T9 sequence were compared by measuring the electrochemical signal of the labeled PNA before and after hybridized with complementary and mismatched DNA. The PNA and PNA-DNA solution (50 μM in 10 mM phosphate buffer pH 7.4) was dropped onto the unmodified SPCE electrode and the signal was measured by SWV. The results are illustrated in **Figure 4.7.** In all systems, the complementary and multiple mismatched DNA targets were clearly distinguished. However, in three systems including MB-labeled (both internal and terminal labeling) and 2AQ-labeled (internal labeling only) PNA, discrimination of single mismatched from fully complementary DNA targets were not possible since both types of DNA completely suppressed the probe signal. Nevertheless, clear difference between the signal of complementary and single mismatched DNA targets could be observed with the terminally 2AQ-labeled PNA. The different performance between the four systems does not correlate with the T_m difference between the complementary and mismatched PNA-DNA hybrids (**Table 4.2**). Although the reason is still unclear, 2AQ-labeled PNA was chosen for the next studies.

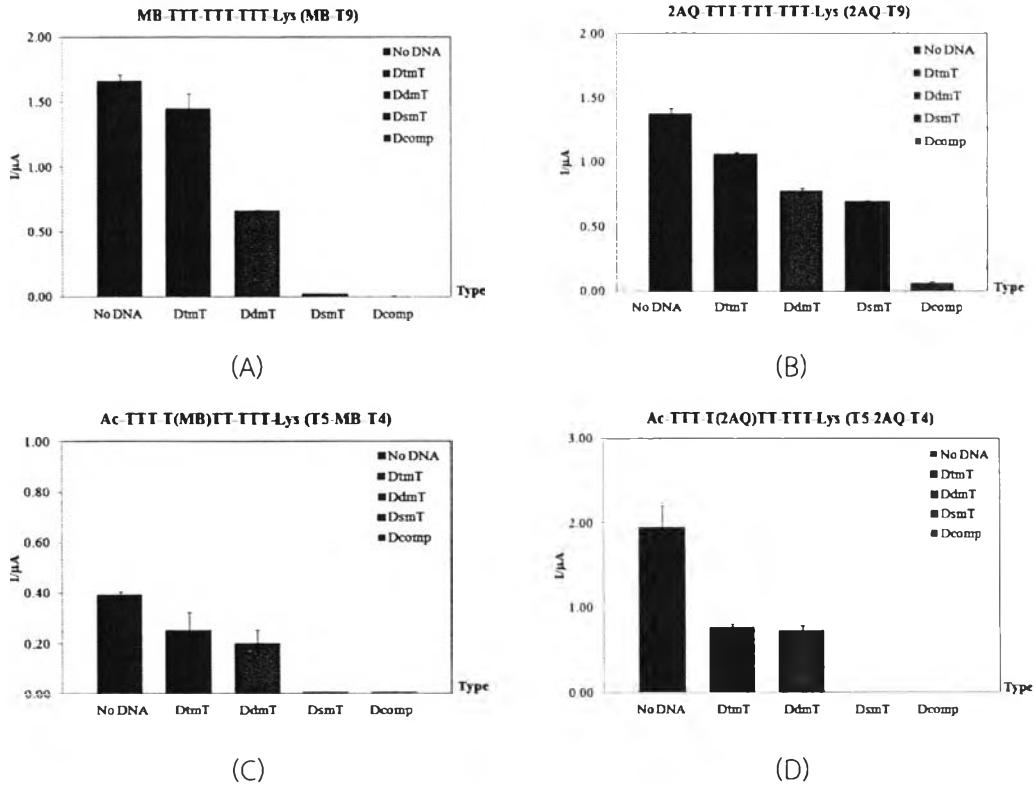


Figure 4.7 The selectivity of labeled PNA probe (50 μM, 20 μL) (A) MB-T9-Lys (terminal) (B) 2AQ-T9-Lys (terminal) (C) T5-MB-T4-Lys (internal) (D) T5-2AQ-T4-Lys (internal) on unmodified, pretreated SPCE in 10 mM phosphate buffer (pH 7.4)

4.7 Effects of buffer

To study the effect of buffer, various types of buffer including acetate buffer pH 4.6, citrate buffer pH 4.6, MES buffer pH 7.0, phosphate pH 7.4, Tris-HCl pH 8.0 and Tris-Borate pH 9.0 were investigated. The PNA 2AQ-T2 (50 μM in 30 mM acetate buffer or 10 mM for other buffers) were dropped onto the pre-treated unmodified SPCE electrode the signal measured by SWV. From **Figure 4.8**, the following observation can be made. At low pH (acetate and citrate buffers, both at pH 4.6), the signals were weak and the potential of the anthraquinone label shifted to -0.60 V, while still maintaing a good shape. At neutral pH (MES buffer pH 7.0 and phosphate buffer pH 7.4), the signal was stronger and the potential of anthraquinone label shifted to -0.90 V, with severe broadening of the peaks. The best signal strength and shape were observed in high pH buffers (Tris-HCl buffer pH 8.0 and Tris-Borate buffer pH 9.0) whereby the potential of the anthraquinone label was -0.70 V. It appears that

in basic buffers, the carboxyl groups on the electrode surface exist in the deprotonated form and can attract more strongly to the lysine-modified PNA. However, the effects of counter ions cannot be completely ruled out.

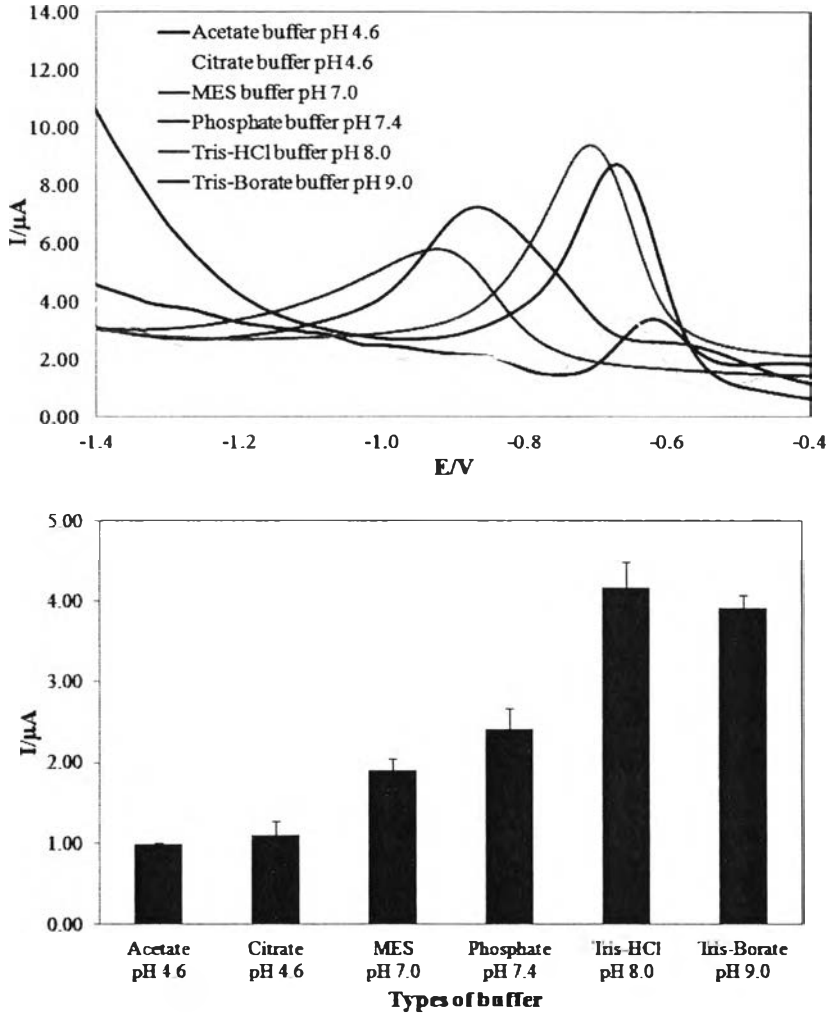


Figure 4.8 (Top) SWVs of 2AQ-T2 (50 μM, 20 μL) on unmodified, pretreated SPCE in various buffers and (Bottom) bar graph of the signal received

Table 4.3 Redox potential and peak width at half height of 2AQ-T2 in various buffers

Type of buffer	Redox potential (V)	I (μA)	Peak width at half height (V)
30 mM acetate pH 4.6	-0.624	0.98	0.101
10 mM citrate pH 4.6	-0.664	1.11	0.091
10 mM MES pH 7.0	-0.916	1.91	0.262
10 mM Phosphate pH 7.4	-0.845	2.41	0.211
10 mM Tris-HCl pH 8.0	-0.704	4.17	0.151
10 mM Tris-Borate pH 9.0	-0.664	3.91	0.161

Next, the specificity of the mixed sequence PNA probe 2AQ-WSSV-Lys was measured in various buffers. No discrimination between complementary and single mismatched DNA targets were observed in citrate (B) and MES (C) buffers because both complementary and mismatched DNA targets completely suppressed the 2AQ signal. On the other hand, the signals resulting from complementary and single mismatched DNA can be clearly distinguished in the cases of acetate (A), phosphate (D), Tris-HCl (E) and Tris-Borate (F) buffers. According to **Figure 4.9**, the Tris-HCl (E) and Tris-Borate (F) buffers gave much better results than the other two systems because the signal from single mismatched DNA target was almost the same as that of single stranded 2AQ-PNA. Incidentally, the two Tris buffers provided the best signal strength and shape as shown in the aforementioned study. However, only Tris-HCl buffer was chosen for subsequent studies because it can be prepared to have a wider pH range covering neutral and basic pH.

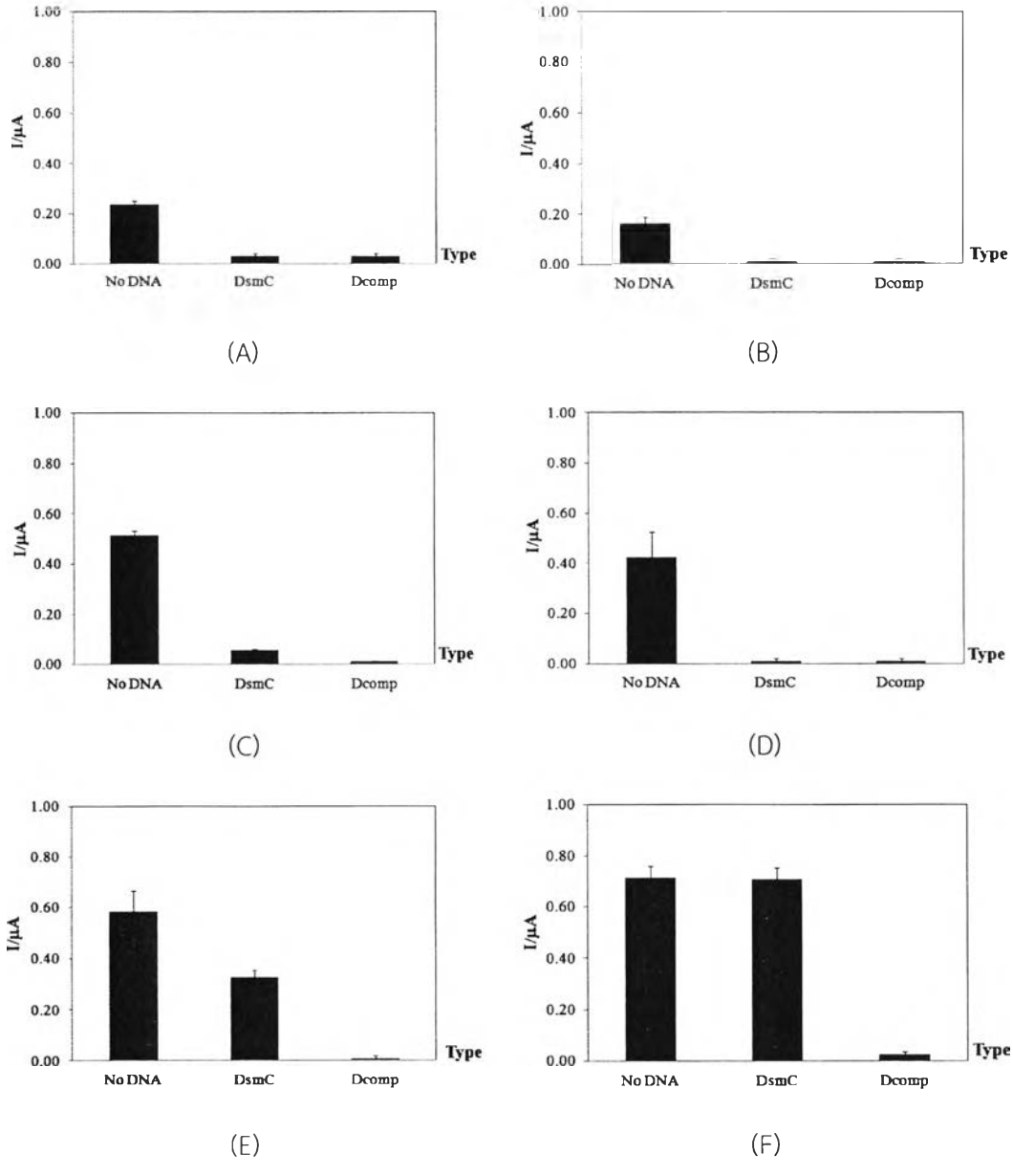


Figure 4.9 Selectivity of 2AQ-WSSV-Lys (50 μM, 20 μL) on unmodified, pretreated SPCE in various buffers (A) 30 mM acetate buffer (pH 4.6) (B) 10 mM citrate buffer (pH 4.6) (C) 10 mM MES buffer (pH 7.0) (D) 10 mM phosphate buffer (pH 7.4) (E) 10 mM Tris-Cl buffer (pH 8.0) (F) 10 mM Tris-Borate buffer (pH 9.0)

4.8 Reusability of the unmodified SPCE

Next, the reusability of the unmodified SPCE was studied. The used SPCE electrode was washed with water followed by 1.0 M NaOH solution and 20%

aqueous MeCN (washing with water is required after each step. and then dried at room temperature). The NaOH washing alone could not remove all the MB-T9 PNA, thus subsequent washing with aqueous acetonitrile was required. [16]. After this treatment, no signal due to the MB label was observed when the blank buffer was measured indicating that the PNA was completely removed. The SPCE can be reused for at least 5 times without noticeable degraded performance (**Figure 4.10**).

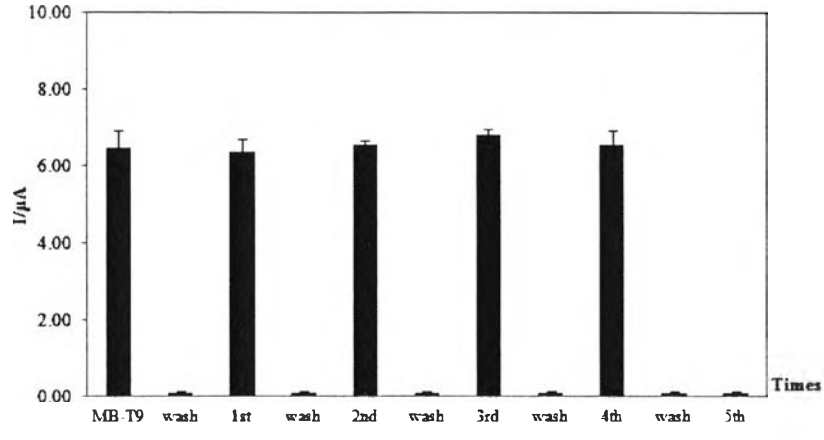
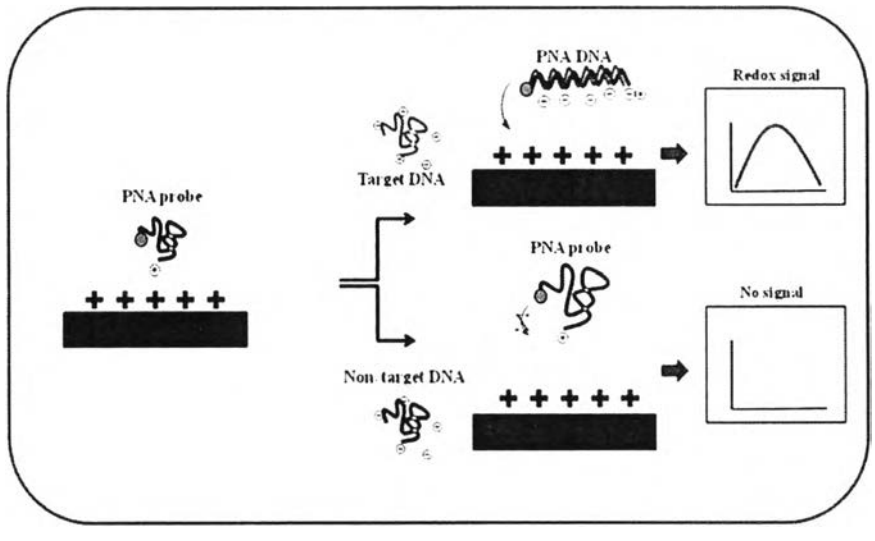


Figure 4.10 Reusability of the unmodified SPCE. The SWV signal of MB-T9 PNA (50 μM, 20 μL) was measured on unmodified, pretreated SPCE in 10 mM phosphate buffer pH 7.4.

4.9 Positively-charged modified SPCE

Although the proof-of-principle experiments work well, there are two limitations. Firstly, the concentration of the PNA probe/DNA target required to get a reasonable signal was high (in the micromolar levels). Secondly, the detection was in a signal-off mode, whereby the signal was decreased in the presence of the correct DNA target. It is more preferable to develop a signal-on detection since the signal change can be detected more readily. It was proposed that both aspects might be controlled by modulation of the charge on the surface of the electrode. Since the electrode is made of a carbon paste, the charge modulation could be easily achieved by adding some charged polymers into the carbon paste mixture used for the screening of the electrode (section 4.3). First of all, CHT was initially chosen as the polymer for modification of the SPCE since this had been done before [Jampasa et al., 2014], although the original purpose in that work was to use the amino group of the CHT as a handle for covalent immobilization of the probe.

To test whether the positively-charged electrode can switch the detection to signal-on mode, the electrode prepared from carbon paste mixture consisting of 1% w/v CHT was tested. The 2AQ-WSSV-Lys PNA and PNA-DNA solutions (50 μM in 10 mM phosphate buffer pH 7.4) were dropped onto the CHT-modified SPCE electrodes and the signals obtained were compared. As shown in **Figure 4.11**, the positively-charged electrode can indeed switch the detection mode to signal on as shown by the absence of signal in the case of PNA probe alone, and a signal was regained in the presence of DNA. The absence of signal from single stranded PNA probe can be explained by the repulsion between the positively-charged lysine-modified PNA and the also positively-charged electrode. Upon hybridization with the DNA, the PNA-DNA hybrid became negatively-charged and thus attracted to the electrode, allowing an efficient electron transfer from the anthraquinone label as shown in **Scheme 4.2.**



Scheme 4.2 Principle of immobilization-free DNA detection on the positively charged modified SPCE

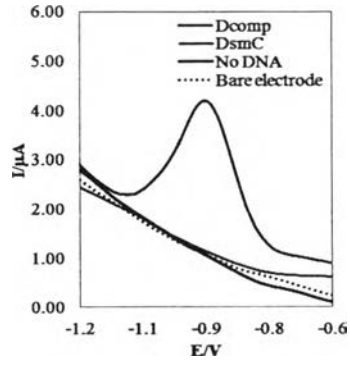


Figure 4.11 The signal of 2AQ-WSSV-Lys (50 μM, 20 μL) in 10 mM phosphate buffer pH 7.0 on the CHT modified SPCE

However, CHT has a limited solubility in water and can generate positive charge only at a pH well below neutral (< 5.5) [74-76]. To overcome these limitations, other positively-charged polymers including HTACC-CHT and PQDMAEMA were also compared with CHT. Structures and properties of the polymers used in this study are summarized in **Figure 4.12** and **Table 4.4** respectively.

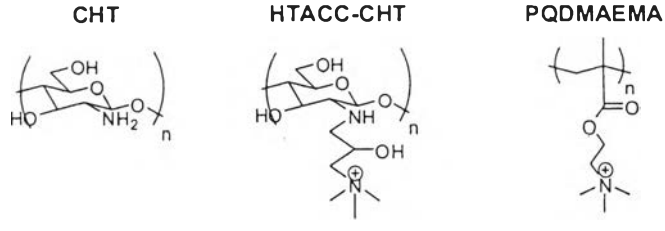


Figure 4.12 Structures of CHT, HTCAA-CHT and PQDMAEMA

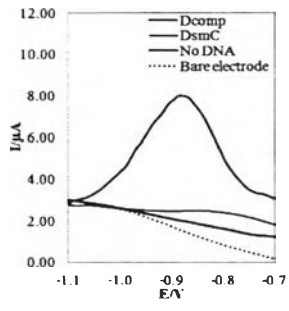
Table 4.4 Characteristics of the three positively-charged polymers used in this study

Polymer	Working pH range	Source	Water solubility
CHT	below 5.5	semi-synthetic	poor (only in acidic solution)
HTACC-CHT	all pH	semi-synthetic	good
PQDMAEMA	all pH	synthetic	excellent

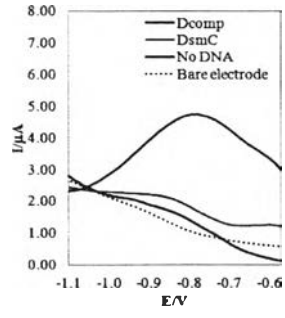
Firstly, three different electrodes modified with each of the three polymers at 1% w/v in the carbon ink paste before the screening were prepared. The signals obtained from 2AQ-WSSV-Lys PNA as well as its hybrids with complementary and single-mismatched DNA (50 μM in 10 mM phosphate buffer, 20 μL) were compared for each electrode. The effect of pH of the buffer was also studied at the same time by varying the pH of the phosphate buffer in the range of 5.5, 7.0 and 8.0. The results summarized in **Figure 4.13** show that the highest signal of complementary PNA-DNA duplex was obtained on PQDMAEMA-modified SPCE at pH 8.0. In addition, the signal from single base mismatched PNA-DNA duplex completely disappeared. Under the same conditions, CHT and HTACC-CHT gave lower signals for complementary duplex, and higher signals for single base mismatched duplex. Accordingly, PQDMAEMA-modified SPCE at pH 8.0 was chosen for the next studies.

The signal on CHT modified SPCE was quite high in acidic condition. It can be explained by protonation of the amino group on CHT in acidic solution (pH < 5.5). HTACC-CHT and PQDMAEMA modified SPCE gave signals with poor shapes suggesting inefficient electron transfer process. Under neutral and basic conditions, the signal

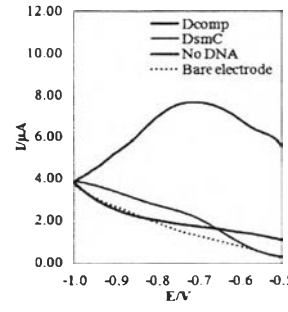
shapes and strengths were good for HTACC-CHT and PQDMAEMA, but not CHT because of the deprotonation.



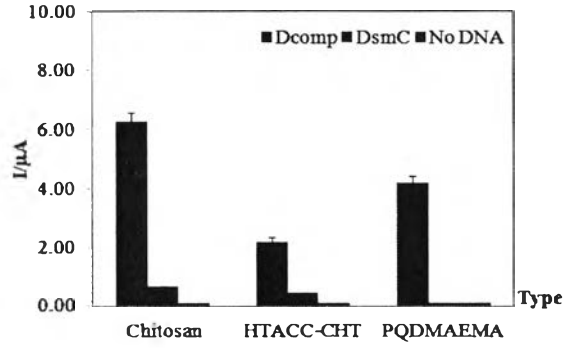
1% CHT (pH 5.5)



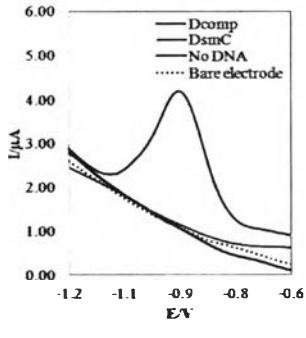
1% HTACC-CHT (pH 5.5)



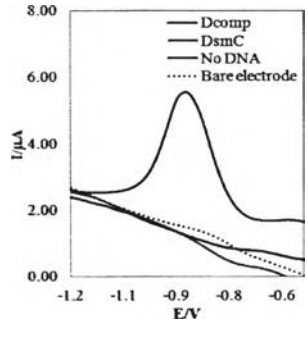
1% PQDMAEMA (pH 5.5)



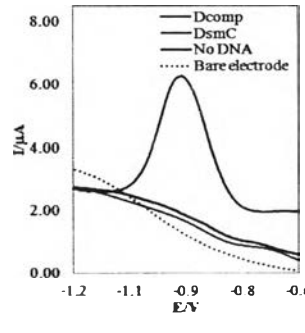
(A)



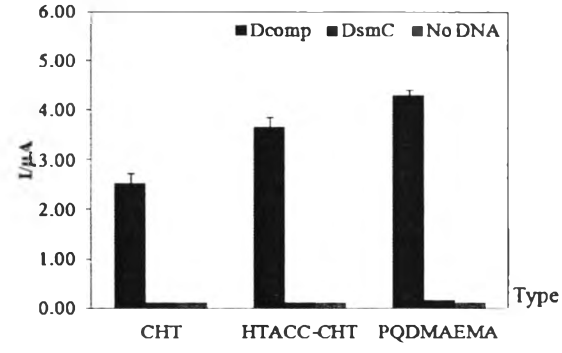
1% CHT (pH 7.0)



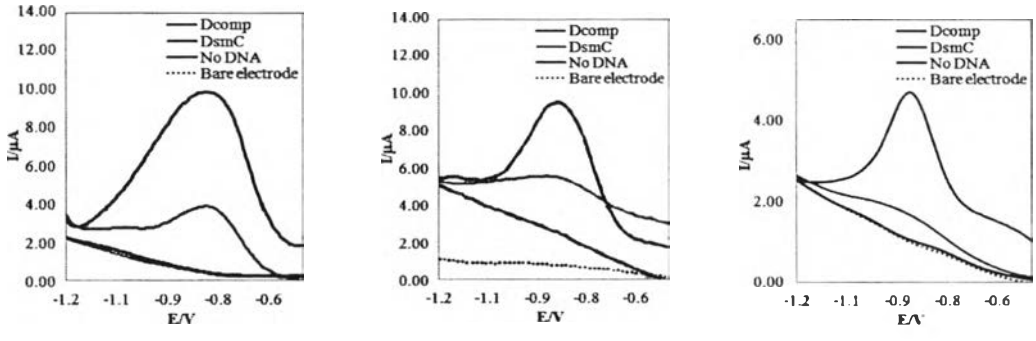
1% HTACC-CHT (pH 7.0)



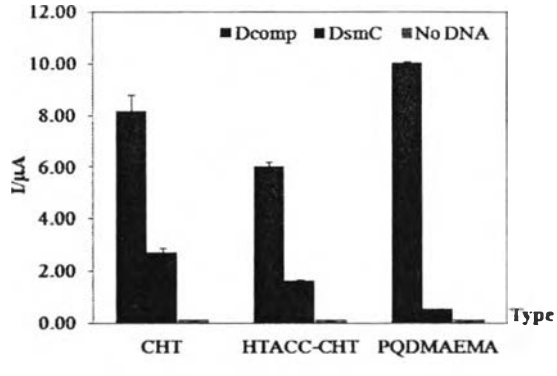
1% PQDMAEMA (pH 7.0)



(B)



1% CHT (pH 8.0) 1% HTACC-CHT (pH 8.0) 1% PQDMAEMA (pH 8.0)



(C)

Figure 4.13 Comparison of the 2AQ-WSSV-Lys (50 μM, 20 μL) before and after hybridized with single base mismatched and complementary DNA in 10 mM phosphate buffer (A) pH 5.5, (B) pH 7.4 and (C) pH 8.0 on various positively charged modified SPCE the (left)

4.10 Effect of type and pH of buffer

From above results, the pH of the buffer seemed to have significant influences to the electrochemical signal. As previously shown in section 4.6 on the unmodified electrode, the nature of the buffer itself can also have dramatic effects on the signal strength and shape, the effect of the buffer type was also re-investigated on the PQDMAEMA-modified positively-charged electrode at pH 7 and 8. In agreement with the previous study, Tris-HCl buffer consistently showed a better performance than phosphate buffer. Importantly, the clear differentiation between complementary and single mismatched DNA targets could be obtained both at pH 7 and 8, even when the concentration of the PNA/DNA was decreased from 50 μM to 5 μM. Tris-HCl buffer pH 8 was chosen for the next experiments because it gave higher signal for complementary duplex and lower signal for single mismatched duplex compared to pH 7.

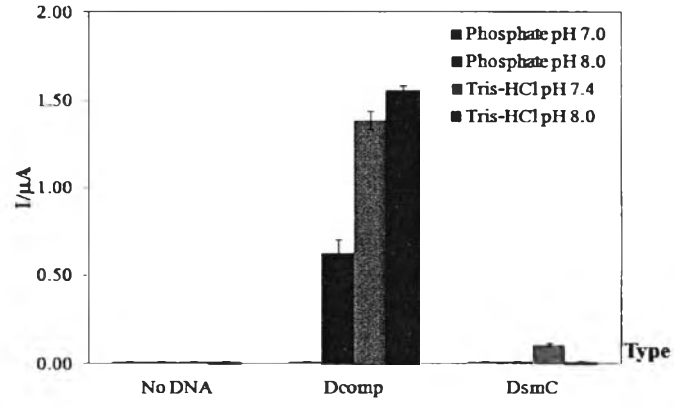


Figure 4.14 Effect of buffer to the 2AQ-WSSV-Lys (5 μM, 20 μL) signal (left) before and after hybridized with (center) complementary and (right) single base mismatched DNA in 10 mM phosphate buffer pH (purple) 7.0 and (blue) 8.0 and Tris-HCl buffer pH (green) 7.4 and (red) 8.0.

4.11 Effect of accumulation potential

All results above suggest that it was possible to use PQDMAEMA-modified SPCE for a highly specific signal-on detection of DNA sequence using 2AQ-labeled acpcPNA probes. However, another major problem of low sensitivity still remained. In all previous experiments, the concentration of PNA and DNA required to give acceptable signals was in micromolar range (5 to 50 μM). This is far too high to be useful for detection of real DNA samples. Subsequent experiments were designed with the attempt to improve the sensitivity of the detection.

Accumulation of applied potential is another attractive option to increase the signal. The potential accumulation at positive value is commonly used to increase positive charge of the working electrode [77, 78]. This can increase the amount of the negatively charged PNA-DNA duplexes onto electrode surface by electrostatic adsorption and may result in a more efficient electron transfer.

To test this hypothesis, a 2AQ-WSSV-Lys PNA-DNA sample (5 μM in 10 mM Tris-HCl buffer pH 8.0) was dropped onto the PQDMAEMA modified SPCE electrode. The AQ signal was measured at varying accumulation potential of 0.1, 0.3, 0.5, 0.7 and 0.9. As expected, the more accumulation potential applied, the higher signal was obtained. From **Figure 4.15**, the maximum signal was observed at the accumulation potential of 0.7 V by increasing from 1.0 μA to 3.1 μA. At higher accumulation potentials, the signal was decreased because these potential is over the potential working range of carbon electrode. Therefore, the accumulation potential at 0.7 V was enhanced the signal 3 times to the original signal and chosen for the next experiments. From accumulation effect, the concentration of PNA/DNA can be decreased to 500 nM

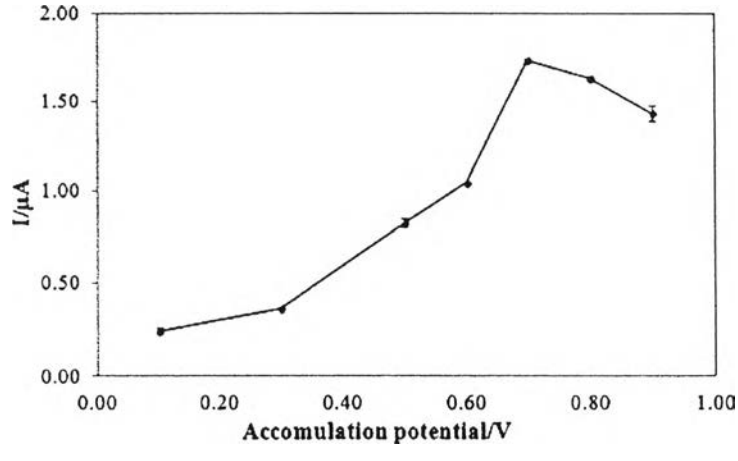


Figure 4.15 Effect of accumulation potential to the signal of hybrid of 2AQ-WSSV-Lys with complementary DNA (5 μM in 10 mM Tris-HCl buffer pH 8.0, 20 μL) on 1% PQDMAEMA modified SPCE.

4.12 The effect of amounts of polymer

The amounts of the polymer in the SPCE, which may be controlled by the amount of the polymer added in the carbon ink paste mixture before screening of the working and counter electrodes, should have significant effects on the sensitivity because it determines the "positiveness" of the electrodes. This was the next

parameter to be investigated. PQDMAEMA was prepared as aqueous solution (0.5, 1.0, 5.0, 10.0, 15.0 and 20.0% w/v) before mixing with the carbon ink mixture to give the final concentration of the polymer at 0.13, 0.26, 1.3, 2.6, 3.9 and 5.2% (before screening). The electrode prepared from each carbon ink mixture with different concentration of PQDMAEMA was used to detect the signals of 2AQ-WSSV-Lys and its hybrids with complementary and single mismatched DNA to select the best system providing optimized result in signal. The optimal electrode should give low signal before hybridization and high after hybridization with complementary DNA. Ideally, the signal after hybridization with single mismatched DNA should be the same as before hybridization. The PNA and PNA-DNA samples (500 and 50 nM in 10 mM Tris-HCl buffer pH 8.0, 20 µL) was dropped onto the SPCE electrodes prepared from carbon ink mixtures with 0.13, 0.26, 1.3, 2.6, 3.9 and 5.2% w/v PQDMAEMA. The signals were measured and compared. The results are shown in **Figure 4.16**. The data revealed that increasing the amounts of the polymer resulted in stronger electrochemical signals. Unfortunately, at the concentration of 5% or above, the background signal (PNA without DNA) became so high that it outweighed the increased signal of the PNA-DNA hybrid. Nevertheless, the in all cases, the single mismatched hybrid gave signals that were not significantly different from the background signal of single stranded PNA indicating the high specificity. From **Figure 4.16**, 0.26% PQDMAEMA modified SPCE exhibits the best compromise between the signal and background. Therefore, 0.26% PQDMAEMA modified SPCE was chosen for further studies.

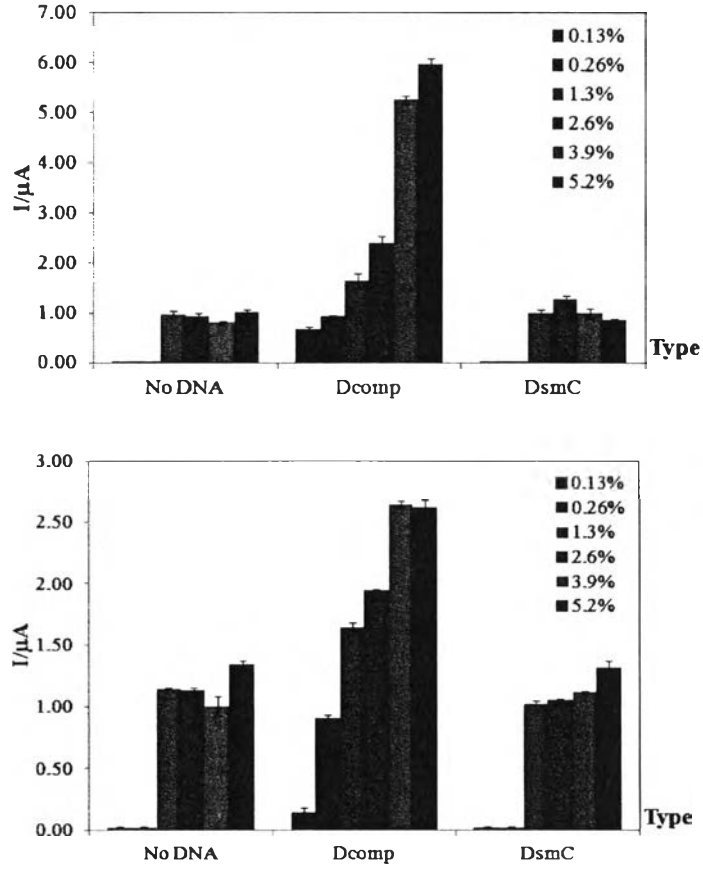


Figure 4.16 Effect of the amounts of PQDMAEMA in the modified SPCE to the 2AQ-WSSV-Lys signal ((Top) 500 and (Bottom) 50 nM in 10 mM Tris-HCl buffer pH 8.0, 20 μL). The % polymer is expressed as the amount of the polymer (w/v) in the carbon ink mixture used for the electrode screening.

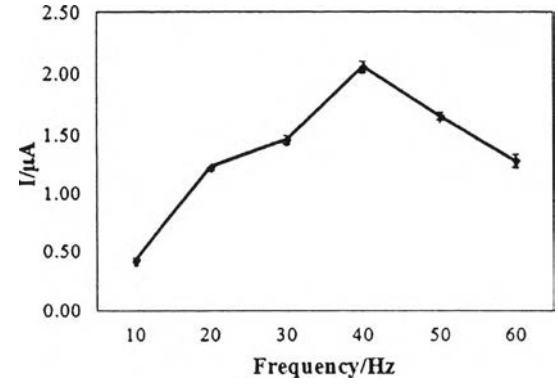
4.13 Optimization of SWV parameters

The square-wave voltammetric technique (SWV) used in the detection of the electrochemical signal of anthraquinone-labeled PNA has a few parameters to optimize including frequency, step potential and amplitude. These parameters can affect the kinetic rate of redox-active species on the redox reaction and hence should have significant effects to the signal strength. The 2AQ-WSSV-Lys PNA-DNA samples (50 nM in 10 mM Tris-HCl buffer pH 8.0, 20 μL) and dropped onto the 0.26% PQDMAEMA modified SPCE. The frequency of the applied potential was varied in the range of 10, 20, 30, 40, 50 and 60 Hz, the step potential was varied in the range of 10, 26, 50, 75, 100 and 125 mV and the amplitude was varied in the range of 100, 250, 500, 750, 1000, 1250 and 1500 mV. The order of optimization is frequency, step potential and amplitude respectively. Firstly, step potential of 0.100 V and amplitude

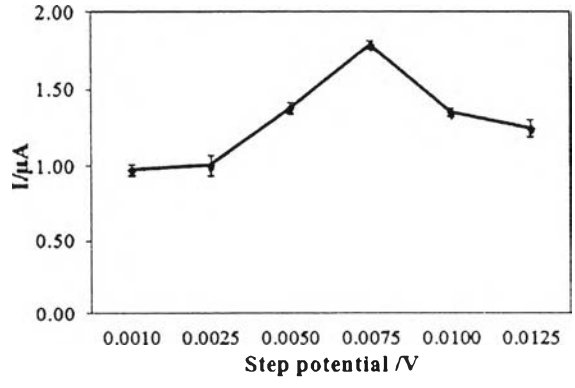
of 0.050 V were used in the frequency optimization. The optimized frequency was then fixed and used for optimization of the step potential. After the frequency and step potential were optimized, the amplitude was finally optimized to yield all optimized SWV parameters as shown in **Table 4.5**. Consequently, the SWV signal of the complementary duplex at 50 nM in 10 mM Tris-HCl buffer pH 8.0 on 0.26% PQDMAEMA modified SPCE is increased from 0.9 μA (before SWV parameters optimization) to 2.4 μA (after SWV parameters optimization).

Table 4.5 Optimization of SWV parameters

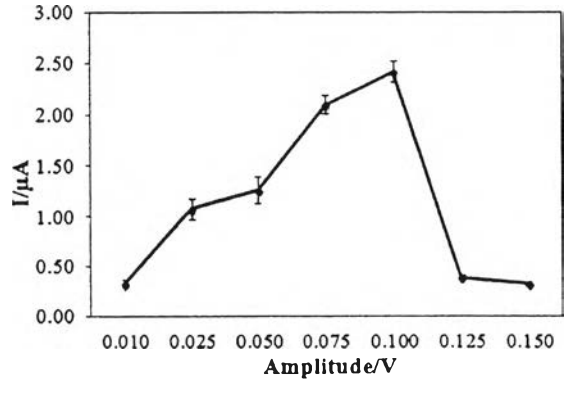
Parameters	Optimized range	Optimized value
Frequency (Hz)	10-60	40
Step potential (V)	0.001-0.0125	0.0075
Amplitude (V)	0.010-0.150	0.100



(A)



(B)



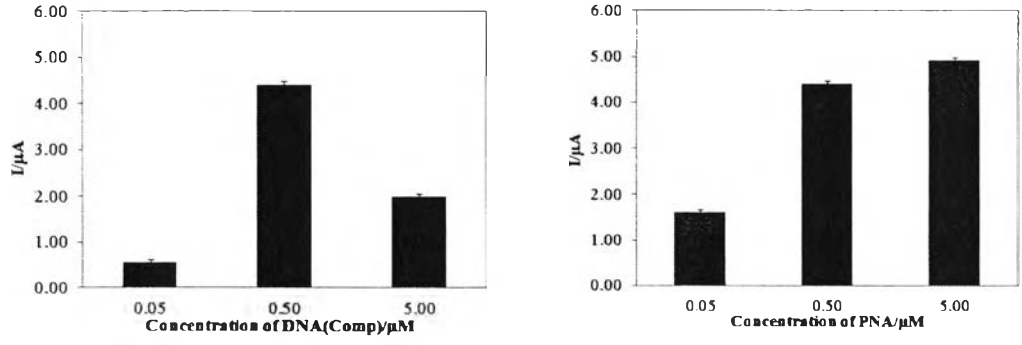
(C)

Figure 4.17 Optimization of SWV parameters for the detection of the signal from 2AQ-WSSV-Lys PNA-DNA sample on 0.26% PQDMAEMA modified SPCE (50 nM, 10 mM Tris-HCl buffer pH 8.0, 20 μL)

4.14 Effect of analyte composition

After the quantity of polymer in the modified SPCE was optimized, the composition of the analyte (PNA-DNA duplex) was investigated. In previous experiments, the PNA and DNA amounts were kept equal. In this experiment, the amount of DNA was varied to higher than, equal to and lower than the amount of the PNA. In the first experiment, the PNA solution was prepared to give the final concentration of 0.5 μM in 10 mM Tris-HCl (pH 8.0) and the DNA was varied to give the final concentration of 0.05, 0.5 and 5 μM. In the other experiment, the concentration of the PNA was varied (0.05, 0.5 and 5 μM) but the DNA concentration was fixed at 0.5 μM. The samples were dropped onto the 0.26% PQDMAEMA modified SPCE and the signals were recorded and compared. The results from the first experiment (varied concentration of DNA) in **Figure 4.18(A)** showed that the maximum signal was obtained at equimolar amounts of PNA and DNA. This is not surprising as lower amount of DNA means the formation lower amounts of PNA-DNA hybrid - which is responsible for the detected signal. On the other hand, when the concentration of DNA was higher than PNA, the excess DNA which is also negatively charged can competitively adsorb onto the electrode and thus reducing the amount of the adsorbed PNA-DNA hybrid thereby decreasing the electrochemical signal. This phenomenon had been reported before on positively charged modified ITO electrodes [21].

In another experiment, the concentration of DNA was fixed at 0.5 μM and the concentration of PNA was varied from 0.05 to 5 μM as shown in **Figure 4.18(B)**. The same results as in the previous experiment were obtained at low amount of PNA. At high amount of PNA, the signal did not change from when the PNA and DNA were present in equal amounts. This means that the excess PNA does not increase the signal and is therefore unnecessary. Accordingly, the suitable ratio of PNA to DNA is 1:1.



(A) (B)

Figure 4.18 Effect of analyte composition (A) DNA (B) PNA on the 0.26% PQDMAEMA modified SPCE (0.5 μM in 10 mM Tris-HCl buffer pH 8.0, 20 μL)

4.15 Calibration curve

To investigate the analytical performance of this system, the relationship between the concentration of the sample (PNA-complementary DNA duplex) and the electrochemical signal was studied. In this experiment, the concentration of the 2AQ-WSSV-Lys PNA was fixed at 50 nM, and the concentration of DNA was varied in the range of 0.1, 0.35, 1, 3.5, 5.0, 10, 35, 50, 100 and 350 nM. The PNA or PNA-DNA samples were dropped onto the 0.26% PQDMAEMA modified SPCE and the signal was measured and compared. The calibration curve was obtained by plotting the current (I) of the PNA probe signal against DNA concentrations. From **Figure 4.19** (top), the current increased linearly (R^2 = 0.997) as a function of concentration of the PNA-DNA duplex in the range of 3.5-50 nM. At DNA concentrations > 50 nM, the signal decreased because the excess DNA prevented the PNA to access to the electrode (section 4.14). At low DNA concentrations (<3.5 nM), the signal appeared to be decreased rapidly and down to zero at 0.1 nM or less. Thus, limit of detection (LOD) should be 3.67 nM as calculated from 3.3SD/m, where SD = residual standard deviation of the linear regression and m = slope of the calibration curve [79]. Limit of quantitation (LOQ) can be calculated similarly, using the equation LOQ = 10SD/m. As a result, this system shows LOQ of 11.12 nM. The LOD and LOQ in this range should be sufficient for analysis of PCR-amplified DNA samples. Consequently, this immobilization-free electrochemical DNA detection by acpcPNA should be suitable be for using as a DNA sensor.

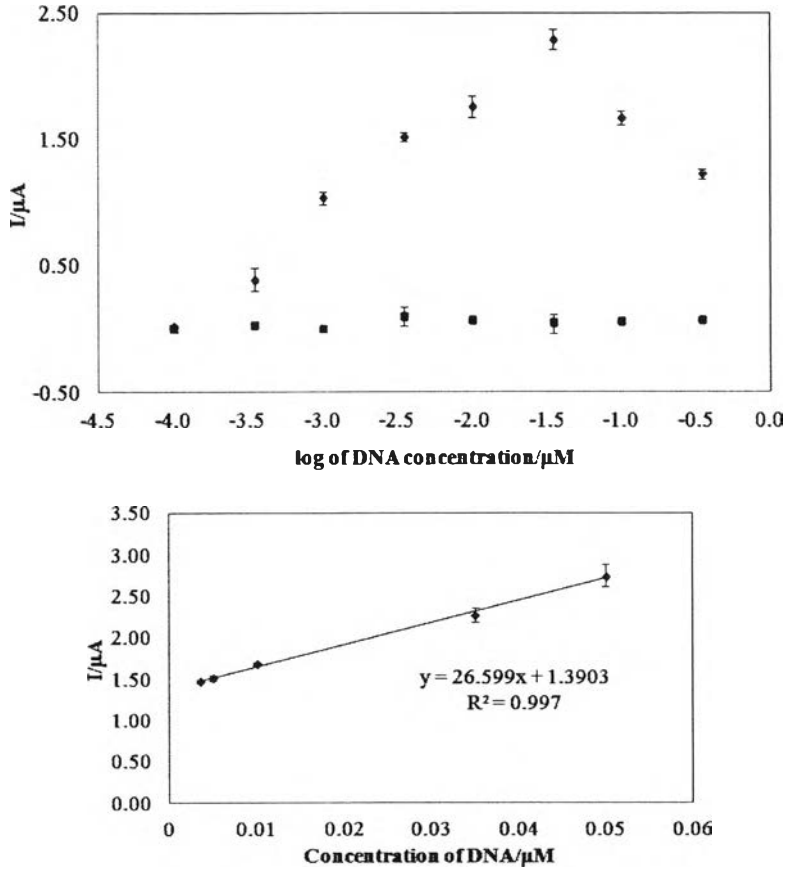


Figure 4.19 (Top) Scan of linear range and (Bottom) calibration curve of this system on 0.26% PQDMAEMA modified SPCE in 10 mM Tris-HCl buffer pH 8.0 (20 μL)

4.16 Selectivity of the PNA probe

Selectivity of the 2AQ-WSSV-Lys probe was studied by comparing the signal of PNA before and after hybridization with complementary and various single mismatched DNA with the following sequences.

DsmC6 5'-TCT GAC TTT AG-3'	DsmA4 5'-TCT AAA TTT AG-3'
DsmG6 5'-TCT GAG TTT AG-3'	DsmT4 5'-TCT IAA TTT AG-3'
DsmT6 5'-TCT GAI TTT AG-3'	DsmA7 5'-TCT GAA ATT AG-3'
DsmC5 5'-TCT GCA TTT AG-3'	DsmC7 5'-TCT GAA CTT AG-3'
DsmG5 5'-TCT GGA TTT AG-3'	DsmG7 5'-TCT GAA GTT AG-3'
DsmT5 5'-TCT GIA TTT AG-3'	DsmA11 5'-TCT GAA TTT AA-3'

The PNA and PNA-DNA samples (50 nM in 10 mM Tris-HCl buffer pH 8.0, 20 µL) were dropped onto the 0.26% PQDMAEMA modified SPCE and the signal measured and compared. As expected, the signal of the anthraquinone was very small when the PNA was hybridized with single base mismatched DNA. In most cases the signal was almost the same as single stranded PNA. The only exception is the DNA DsmA11 carrying the mismatched base at the terminal position, which gave similar signal to the complementary hybrid. This is not unexpected due to the similarity of the mismatched and the complementary DNA sequences. This experiment suggests that the 2AQ-WSSV-Lys PNA probe exhibits excellent selectivity in distinguishing between complementary and mismatched DNA.

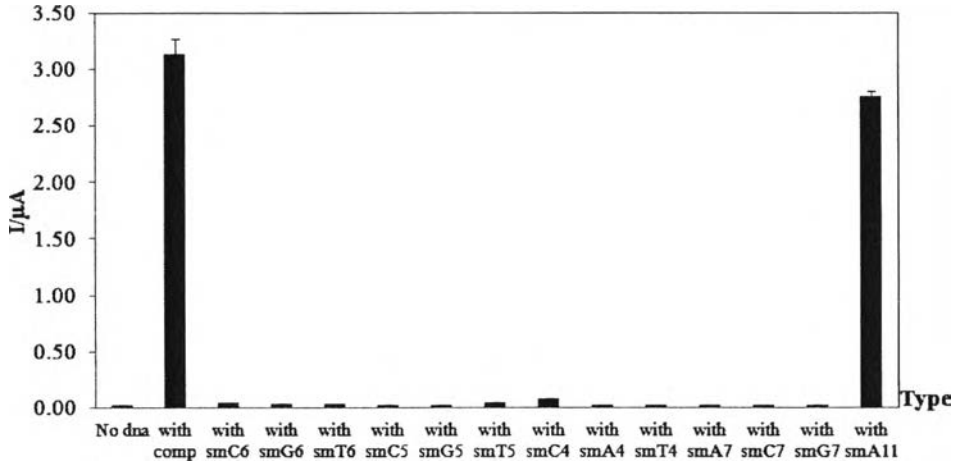


Figure 4.20 Selectivity of 2AQ-WSSV-Lys (50 nM, 20 µL) on 0.26% PQDMAEMA modified SPCE in Tris-HCl buffer at pH 8.0

4.17 Effect of non-complementary DNA

Although the PNA probe exhibits very high selectivity between complementary and mismatched DNA, it is interesting to challenge this by increasing the concentration of the mismatched DNA relative to the complementary DNA. The 2AQ-WSSV-Lys and its hybrid with complementary DNA were prepared to give the final concentration of PNA and DNA = 50 nM in 10 mM Tris-HCl buffer (pH 8.0), and the amount of non-complementary DNA was varied in the range of 50 nM, 0.5 and 5 µM which are 1, 10 and 100 times of the complementary DNA. The samples were dropped onto the 0.26% PQDMAEMA modified SPCE and compared the signal. As shown in **Figure 4.21**, the signal decreased according to the amount of non-complementary DNA added. This can be explained by a competitive binding of the complementary PNA-DNA hybrid by the excess non-complementary DNA, which

resulted in less amounts of adsorbed PNA-DNA hybrid and thus lower signal. Nevertheless, a clear signal can still be observed even when the single mismatched DNA was present at 100 times higher than the complementary DNA. In the absence of the complementary DNA, the signal due to the mismatched DNA is virtually indistinguishable from the background at all concentrations, confirming the high specificity of the PNA probe.

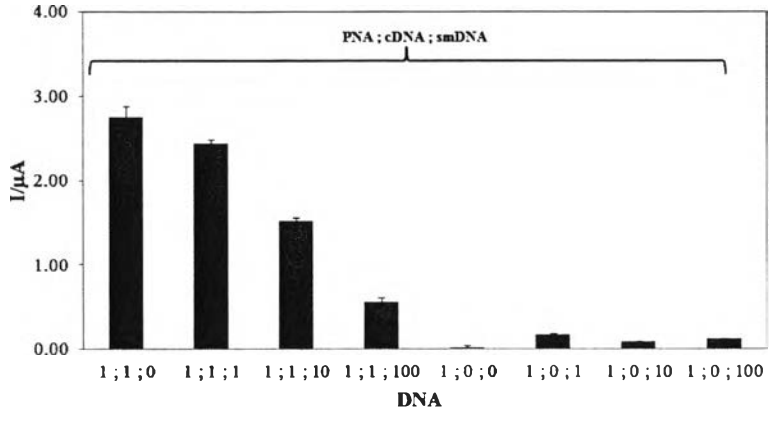


Figure 4.21 Effect of non-target DNA (single base mismatched DNA) to the signal of PNA-DNA hybrid (50 nM, 20 μL) on 0.26% PQDMAEMA modified SPCE in 10 mM Tris-HCl buffer pH 8.0

4.18 Effect of long and double-stranded DNA targets

So far the DNA detection experiments were carried out with single stranded synthetic DNA which has exactly the same length as the probe. This is very different from the real situation whereby DNA samples are present as duplexes and are usually much longer than the probe. To assess the possibility of detection of long and double-stranded DNA targets, two 19 base pairs DNA targets (Dcomp19mer, DsmC19mer) were designed to have complementary or non-complementary sequence to the 2AQ-WSSV-Lys PNA probe (see **Table 4.2**). The sequence were chosen to be similar to the real WSSV DNA and the region of PNA binding was placed at the central part of the DNA duplex so that there were 4 bases pairs on each side of the PNA-DNA duplex.

To compare the signal and specificity with long, single stranded DNA, the PNA and PNA-DNA samples (50 nM in 10 mM Tris-HCl buffer pH 8.0, 20 μL) were dropped onto 0.26% PQDMAEMA modified SPCE and the signals were measured every 10 min until the signal was stable. The results in **Figure 4.22** showed that the signal was almost the same as that obtained from the DNA target with identical length to the

probe. It indicated that the length of target DNA has no effects to the electron transfer process.

Next, the experiment was repeated with complementary and mismatched long, double-stranded DNA targets. The DNA duplexes were formed by mixing two complementary DNA strands together. The formation of the desired DNA duplexes was verified by melting temperature analysis (66.8 °C for B1502 duplex and 65.4 °C for B1513 duplex with or without salt) The PNA and PNA-dsDNA samples (50 nM in 10 mM Tris-HCl buffer pH 8.0, 20 µL) were dropped onto the 0.2% PQDMAEMA modified SPCE electrode. The signal was detected every 10 min until the signal was stable. The time required for the signal to reach maximum in this experiment was significantly longer (up to 40 min) than in the case of single strand DNA (**Figure 4.23(A)**). The appearance of the signal, albeit rather slowly, in the case of complementary DNA duplex suggested that binding of the PNA to the dsDNA, presumably by duplex invasion, could occur. After 40 min, the signal was stable and was similar to the signal obtained from single stranded DNA target with equal length to the probe. On the other hand, no signal was observed with the non-complementary DNA duplex even after 40 min (**Figure 4.23(B)**), suggesting the high selectivity between complementary and mismatched long, duplex DNA targets.

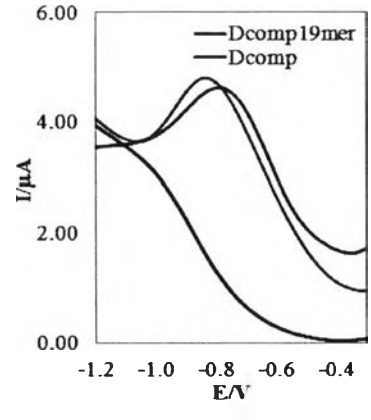


Figure 4.22 Effect of long length target DNA to the signal of PNA-DNA hybrid (50 nM, 20 µL) on 0.26% PQDMAEMA modified SPCE in 10 mM Tris-HCl buffer pH 8.0

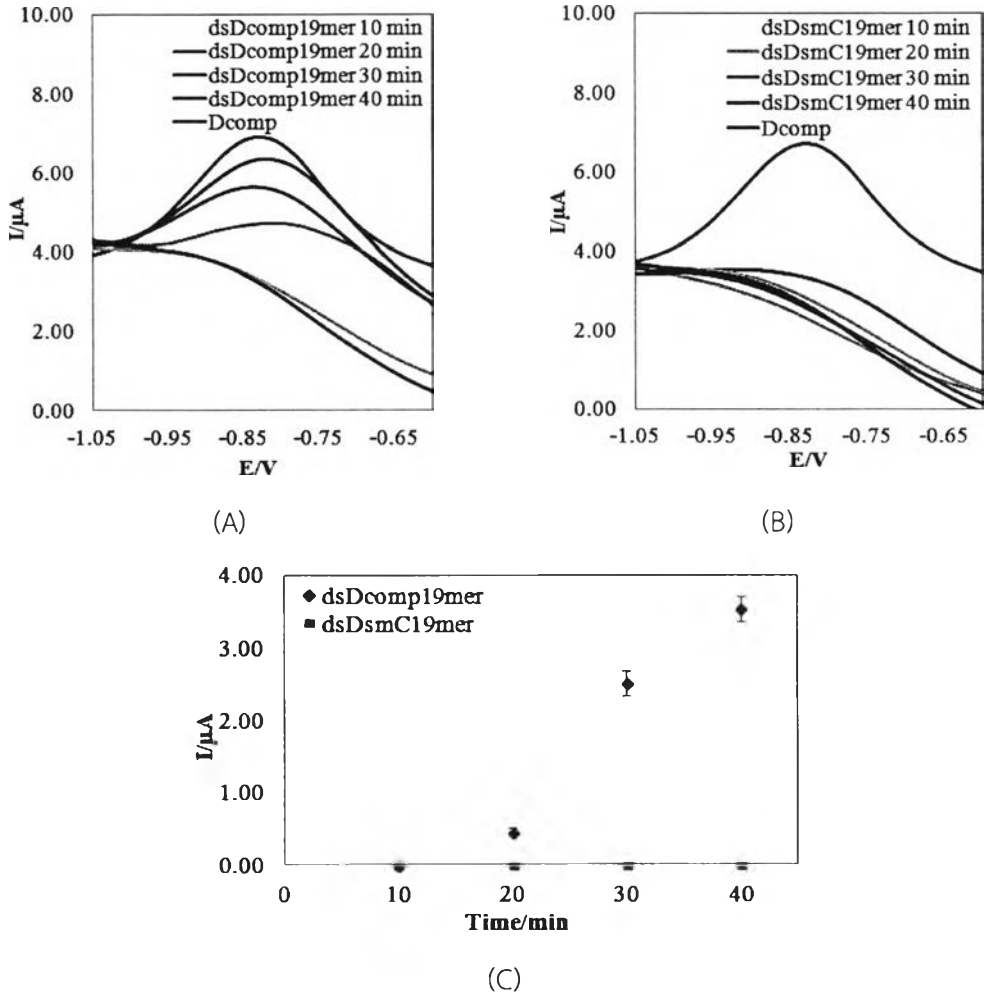


Figure 4.23 Invasion of AQ-WSSV-Lys PNA (50 nM) to the 19bp double-stranded (A) complementary and (B) single base mismatch DNA (50 nM) on 0.26% PQDMAEMA modified SPCE in 10 mM Tris-HCl buffer pH 8.0 (total volume = 20 µL)

Furthermore, the presence of salt such as NaCl is an important factor that can stabilize dsDNA by decreasing the electrostatic repulsion of the negatively-charged phosphate of the DNA backbones. Therefore, the effect of NaCl to the detection of double strand DNA was studied. The 2AQ-WSSV-Lys PNA and PNA-dsDNA samples (50 nM in 10 mM Tris-HCl buffer pH 8.0 with or without 100 mM NaCl, 20 µL) were dropped onto the 0.26% PQDMAEMA modified SPCE electrode and the signal was detected every 10 min until it was stable. As expected, the time required to obtain the maximum signal was longer in the presence of NaCl. In the case of dsDcomp10mer, 50 min was required to obtain the maximum signal. This can be explained by the increased stability of DNA duplexes at high salt concentrations.

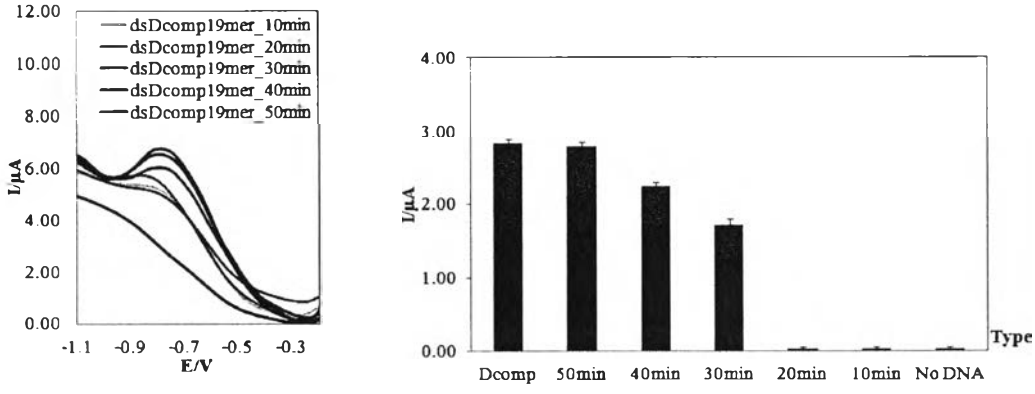


Figure 4.24 Invasion of PNA (50 nM) on the long double strand target DNA (50 nM) in the presence of 100 mM NaCl on 0.26% PQDMAEMA modified SPCE in 10 mM Tris-HCl buffer pH 8.0 (total volume = 20 μL)

To improve the invasion kinetics, the double-stranded DNA was separated into their single stranded components. This could be achieved by denaturation of the DNA duplex by heating to 90°C to break the hydrogen bonds. In the case of denatured dsDcomp19mer, the signal appeared and reached maximum immediately after addition of the PNA under both conditions with and without NaCl. No signals could be observed with the denatured dsDsmC19mer.

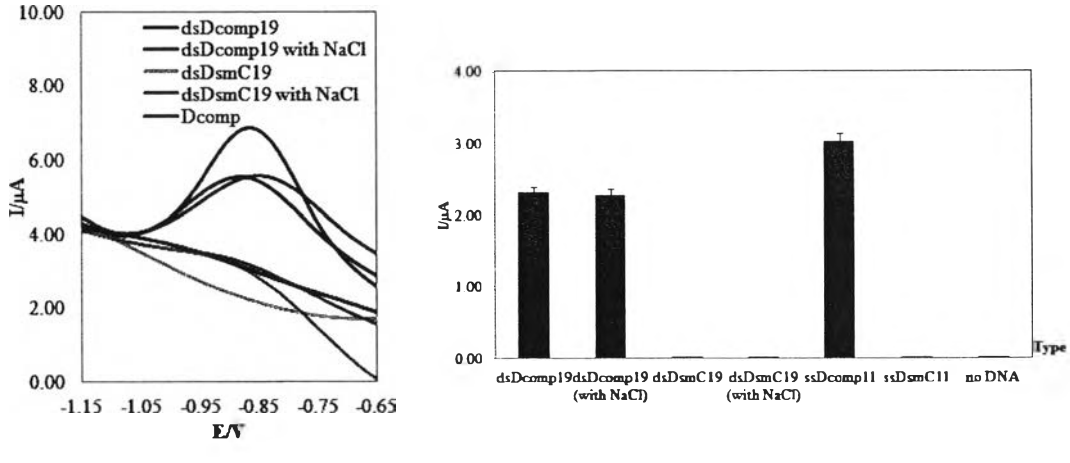


Figure 4.25 Invasion of PNA (50 nM) on the long double strand target DNA (50 nM) by using thermal denaturation method on 0.26% PQDMAEMA modified SPCE in 10 mM Tris-HCl buffer at pH 8.0 (total volume = 20 μL)

4.19 Detection of blind samples

To test the reliability of the new technique for DNA sequence detection, the detection of blind samples were conducted. Six blind samples, which consisted of a synthetic WSSV DNA (Dcomp or DsmC) were prepared. These samples were diluted to give the final concentration of 50 nM in 10 mM Tris-HCl buffer (pH 8.0) and dropped onto the 0.26% PQDMAEMA modified SPCE electrode before the signal was measured. After that, the concentrated PNA stock solution was added to the sample solution on the electrode to give the final PNA concentration of 50 nM without causing significant volume change and the signal was measured again. If a signal was observed, then the sample is positive. In case of no signal, there were still possibilities of negative samples, or malfunctioned electrodes. To distinguish between the two possibilities, the complementary DNA (Dcomp) was added to this solution at 50 nM without changing the volume. If the signal appeared, the electrode was working properly. In the first measurement (without PNA), no signal was observed for all samples, which means that there was no false positive results. After adding the PNA probe, only in samples #2 and #4 that the signal was observed similar to the positive control experiment, indicating that they were WSSV positive samples. For the remaining samples, the signal was observed after adding WSSV positive DNA (Dcomp), indicating that they are WSSV negative samples (DsmC) and that the electrodes and the probe were functioning properly. The results illustrated in **Figure 4.26** are fully consistent with the key provided by Prof. Dr. Tirayut Vilaivan who prepared the blind samples (positive: #2, #4; negative: #1, #3, #5, #6).

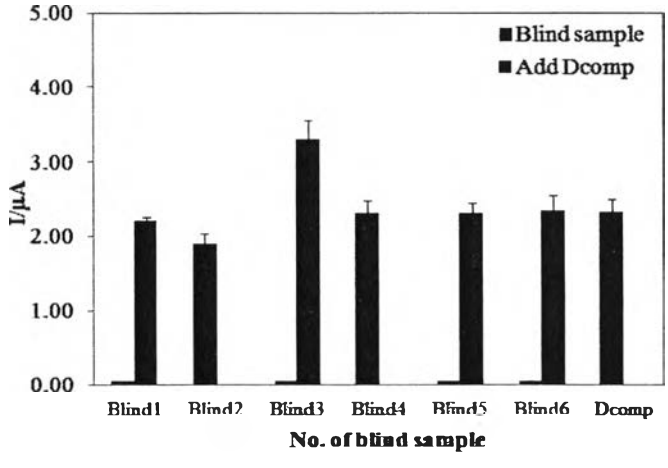
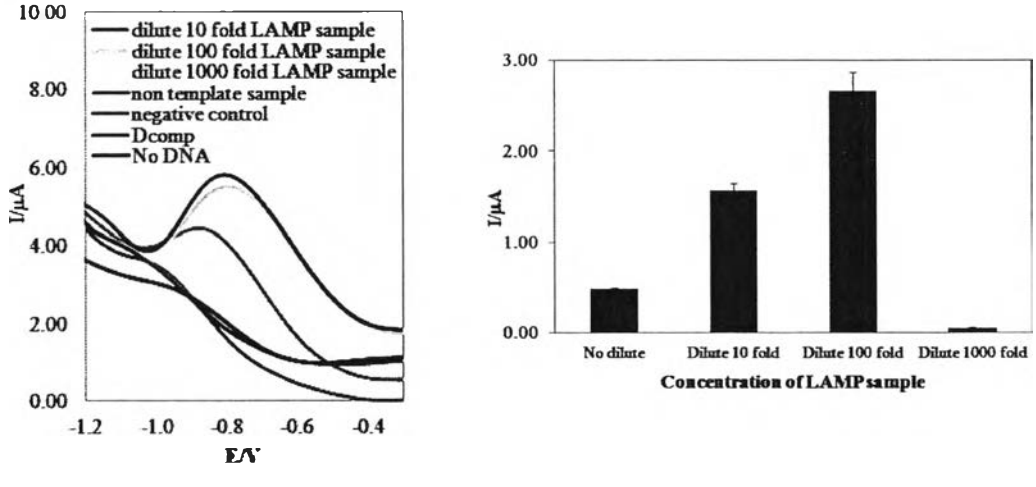


Figure 4.26 Detection of blind DNA samples using 2AQ-WSSV-Lys probe (50 nM) on the 0.26% PQDMAEMA modified SPCE in 10 mM Tris-HCl buffer pH 8.0 (20 μL)

4.20 Detection of LAMP-amplified DNA samples

To test the applicability of the technique for detection of real DNA samples, DNA samples were extracted from infected shrimps and amplified by LAMP technique [80] using a set of primers that were specific for amplification of the WSSV DNA sequence (section 3.3.5). Since the concentration of the LAMP samples is unknown, the samples were also diluted to 10, 100 and 1,000 times in order to avoid having excessive amounts of DNA that would interfere with the detection (section 4.14). The mixture of PNA with heat-denatured DNA sample (50 nM PNA, various dilution of DNA in 10 mM Tris-HCl buffer pH 8.0, 20 µL) was dropped onto 0.26% PQDMAEMA modified SPCE electrode and the signal was measured. As shown in **Figure 4.27**, the signal was observed at both 10-fold and 100-fold dilutions, but not at 1,000-fold dilution. Only weak signal was observed in the undiluted sample. Interestingly, the maximum signal was observed at 100-fold dilution rather than in more concentrated samples, which suggest that the 100-fold diluted LAMP sample contains approximately the same amount of DNA samples to the PNA probe used in the experiment (50 nM). The results confirmed that it is possible to use the developed immobilization-free technique for electrochemical detection of real DNA samples that was amplified by LAMP technique.



(A) (B)

Figure 4.27 (A) Detection of heat-denatured LAMP WSSV samples at no, 10-fold, 100-fold and 1,000-fold dilution using 2AQ-WSSV-Lys (50 nM) compared with non-template sample and negative control (100 dilution) on 0.26% PQDMAEMA modified SPCE in 10 mM Tris-HCl buffer pH 8.0 (20 µL) (B) comparison of the signal at various dilution of LAMP samples

4.21 Specificity test with real DNA samples

After the successful detection of LAMP-amplified WSSV DNA, DNA samples from other common of shrimp viruses including IHNNVSV, YHV and TSV were used to study the selectivity of this probe in real samples. As shown in **Figure 4.28**, this system exhibits an excellent specificity. No signals could be detected in all samples except WSSV.

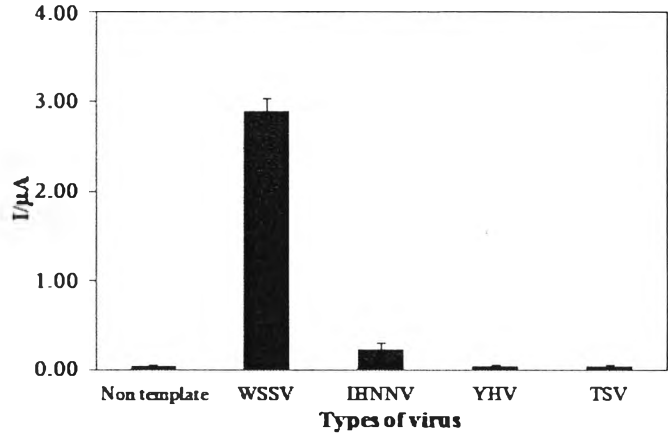
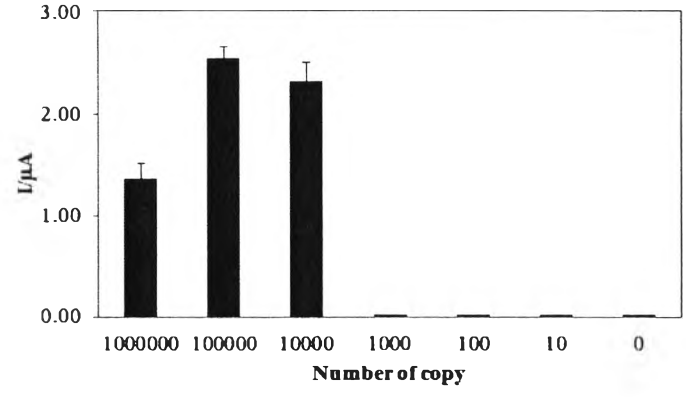


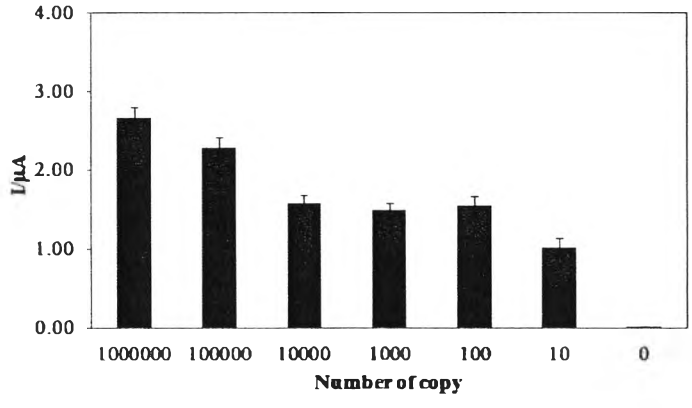
Figure 4.28 Specificity of 2AQ-WSSV-Lys (50 nM) in detection of heat-denatured LAMP samples on 0.26% PQDMAEMA modified SPCE in 10 mM Tris-HCl buffer pH 8.0 (20 μL)

4.22 Sensitivity of LAMP samples

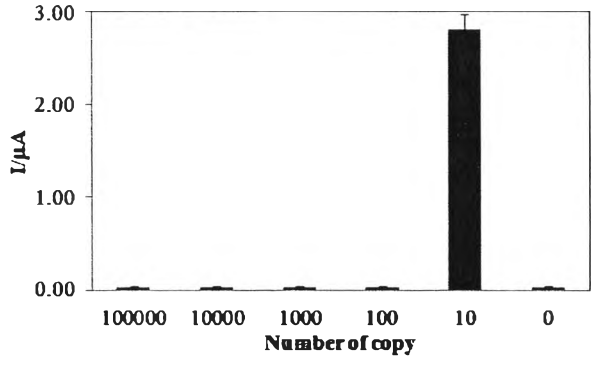
To test the sensitivity of the technique, the samples were amplified with starting with different number of DNA copies (10^6, 10^5, 10^4, 10^3, 10^2, 10 and 0). The undiluted LAMP samples were compared with the 10 and 100 times undiluted samples. The samples were prepared and measured as described in 4.20. According to **Figure 4.29**, the signal was observed at 10^6, 10^5 and 10^4 copies for 100-fold dilution and at 10^6, 10^5, 10^4, 10^3, 10^2 and 10 copies for 10-fold dilution. On the other hand, the signal was present only at 10 copies in undiluted samples. This can be explained by the presence of too much DNA in the undiluted samples that interfered with the detection (section 4.14). It confirmed that the LAMP sample is powerful technique to amplified DNA sample and also confirmed the low limit of detection in this method.



(A)



(B)

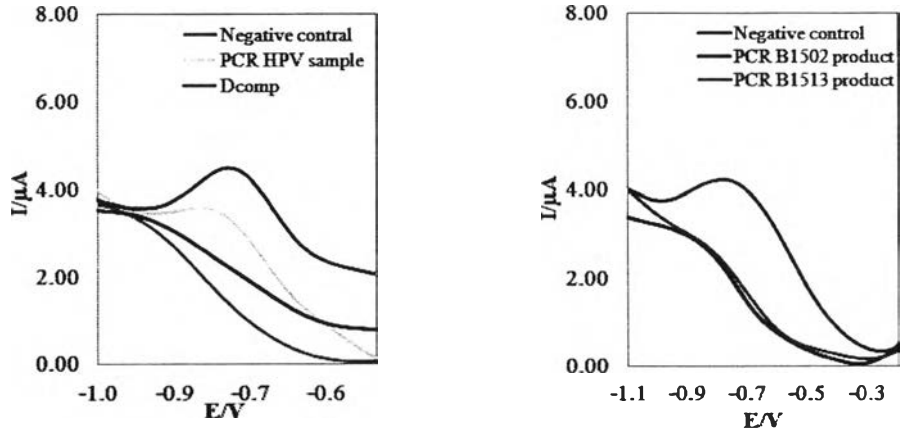


(C)

Figure 4.29 Sensitivity in detection of heat-denatured LAMP samples at different dilutions (A) 100-fold, (B) 10-fold and (C) no dilution with 2AQ-WSSV-Lys (50 nM) on 0.26% PQDMAEMA modified SPCE in 10 mM Tris-HCl buffer pH 8.0 (20 μM)

4.23 Detection of PCR-amplified DNA samples

DNA samples prepared by PCR are different from LAMP in such a way that PCR sample is a faithful copy of a particular region of the entire DNA sequence of the subject of interest that is usually several hundred base pairs in length. On the other hand, LAMP sample consists of much shorter DNA region that is repeating many times in the same DNA strand. This difference makes effective concentration of the DNA region of interest in the LAMP sample much higher than the PCR sample. To further demonstrate the application of the present technique in detection of PCR-amplified samples, two sets of clinically relevant human DNA samples (HPV and HLA B1502/1513) were prepared by PCR. As heat-denaturation was found to be inefficient, the PCR sample was denatured by chemical denaturation method instead (section 3.3.5). The PNA (1AQ-HPV for detection of HPV and 2AQ-B1502 for detection of B1502) and PNA-DNA samples (50 nM PNA, 10-fold dilution of DNA in 10 mM Tris-HCl buffer pH 8.0, 20 µL) were dropped onto 0.26% PQDMAEMA modified SPCE and the signal was measured. The results shown in **Figure 4.30** clearly demonstrate that the technique is also applicable for detection of PCR-amplified DNA samples.



(A) (B)

Figure 4.30 Detection of PCR (A) HPV type 16 (HPV-negative C33 was used as negative control sample) and (B) B1502 samples (HLA B1513 was used as negative control sample) on 0.26% PQDMAEMA modified SPCE in 10 mM Tris-HCl buffer pH 8.0 (20 µL)

4.24 Understanding the interaction of charges on the SPCE and PNA

The charge on the surface of the SPCE is one of the key elements of immobilization-free method presented in this work. However, another important factor that had not been considered in details is the charge on the PNA probe. To understand more on the interaction of charges on the SPCE and PNA, electrochemical behaviors of differently charged PNA (neutral, positively-charged and negatively-charged) on differently charged SPCE electrodes (unmodified, positively-charged and negatively-charged) were investigated. The differently charged PNA were synthesized by replacing the positively charged lysine in 2AQ-WSSV-Lys with serine (neutral) or glutamic acid (negatively charged) to form 2AQ-WSSV-Ser and 2AQ-WSSV-Glu, respectively. The preparation of the neutral and positively-charged (PQDMAEMA, 0.26%) SPCE was the same as previously described (section 4.3.1), but no pre-treatment was performed on the neutral electrode to avoid formation of the negatively-charged carboxyl groups on the electrode surface. The negatively-charged electrode was prepared by mixing polyacrylic acid (PAA, 1.3%) to the carbon ink mixture used for the electrode screening. The PNA samples (50 nM in 10 mM Tris-HCl pH 8.0, 20 µL) were dropped onto the electrodes and the electrochemical signals were measured and compared.

As shown in **Figure 4.31**, the charges on both PNA and SPCE critically affect the electrochemical signal of the 2AQ-WSSV PNA probes obtained before and after hybridization with DNA. The results are fully consistent with the working hypothesis previously shown in **Schemes 4.1** and **4.2**. The positively-charged single stranded PNA 2AQ-WSSV-Lys yielded the signal only on the negatively-charged PAA-modified electrode. The negatively-charged single stranded PNA 2AQ-WSSV-Glu yielded the signal only on the positively-charged PQDMAEMA-modified electrode. Unexpectedly, the supposedly neutral PNA 2AQ-WSSV-Ser gave the signal on both positively-charged and negatively-charged electrodes. This suggests additional binding modes of PNA to the electrode that cannot yet be identified. The non-pre-treated unmodified (neutral) electrode gave no signal with all three PNA systems, confirming that the charge modification is essential for the present immobilization-free DNA detection method.

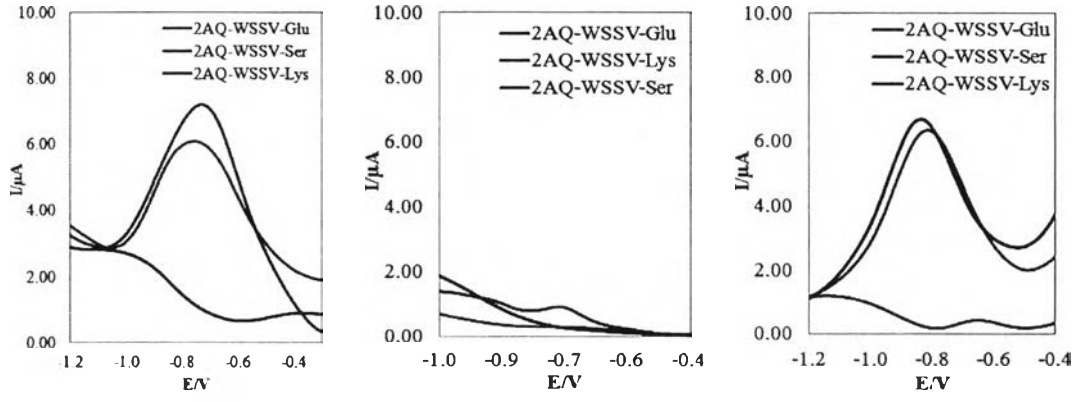
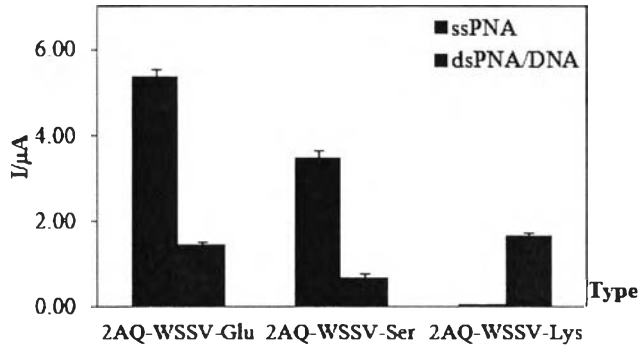


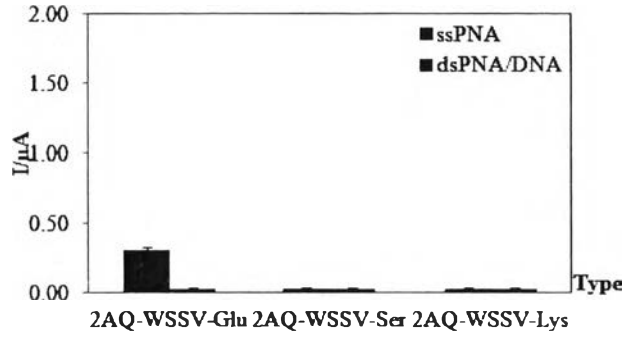
Figure 4.31 Comparison of the 2AQ-WSSV-Lys, 2AQ-WSSV-Ser and 2AQ-WSSV-Glu (all at 50 nM) on the (A) positively-charged (B) unmodified (C) negatively-charged modified SPCE in 10 mM Tris-HCl buffer pH 8.0 (20 μL)

The signal change in the presence of DNA was also explored with all three types of PNA and three types of electrode. The PNA and PNA-DNA samples (50 nM in 10 mM Tris-HCl pH 8.0, 20 μL) were dropped onto the electrodes and the electrochemical signals were measured and compared. On the positively-charged modified SPCE (**Figure 4.32A**), the signal was suppressed when the DNA was present in the case of negatively-charged (2AQ-WSSV-Glu) and neutral PNA (2AQ-WSSV-Ser). Since the negatively-charged PNA-DNA hybrids are supposed to be present on the electrode due to the favorable electrostatic interaction, the lower signal in these two cases may reflect the poorer electron transfer from the PNA-DNA hybrid compared to the free PNA. In the case of positively-charged PNA (2AQ-WSSV-Lys), the same level of signal was observed. However, since the single stranded PNA gave no signal, this result in the net signal increase in the presence of DNA, which is in agreement with our previous results (sections 4.9-4.23). The results were completely opposite on the negatively-charged modified SPCE. The addition of the DNA caused disappearance of the signal in all cases. However, since the single-stranded negatively-charged PNA (2AQ-WSSV-Glu) gave no signal originally, no net signal change could be detected. On the other hand, a clear reduction of the signal was observed in both the positively-charged (2AQ-WSSV-Lys) and neutral PNA (2AQ-WSSV-Ser). In all cases, no signal/signal change on the neutral SPCE could be observed. Again, all of these results emphasize that the charge modification is essential for the present immobilization-free DNA detection method. Furthermore, it also suggests the possibility of using a combination of neutral or positively-charged PNA with negatively

charged electrodes for detection of DNA in a signal-off mode. This possibility will be explored in the future.



(A)



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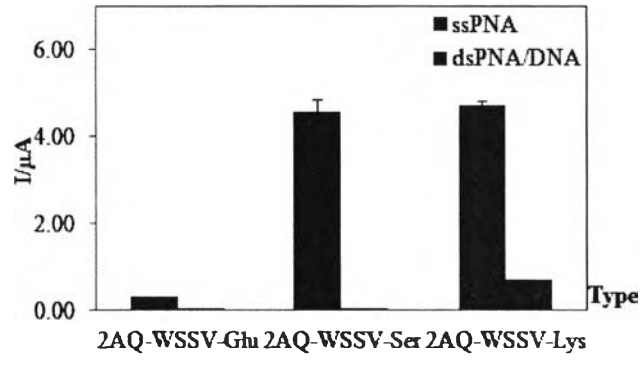

(C)

Figure 4.32 Comparison of the 2AQ-WSSV-Lys, 2AQ-WSSV-Ser and 2AQ-WSSV-Glu before and after hybridized with complementary DNA on the (A) positively-charged (B) unmodified (C) negatively-charged modified SPCE in 10 mM Tris-HCl buffer pH 8.0 (20 μL)