พอลิเอไมด์นิวคลีอิกแอซิดโพรบสำหรับการตรวจหาดีเอ็นเอของไวรัสฮิวแมนแพปพิลโลมาชนิด 16 ด้วยวิธีทางเคมีไฟฟ้า

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POLYAMIDE NUCLEIC ACID PROBE FOR DETECTION OF HUMAN PAPILLOMA VIRUS DNA TYPE 16 BY ELECTROCHEMICAL METHOD

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Petrochemistry and Polymer Science
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Thesis Title	POLYAMIDE NUCLEIC ACID PROBE FOR DETECTION OF HUMAN PAPILLOMA VIRUS DNA TYPE 16 BY ELECTROCHEMICAL METHOD
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ศักดิ์ดา จำปาสา: พอลิเอไมด์นิวคลีอิกแอซิดโพรบสำหรับการตรวจหาดีเอ็นเอของไวรัสฮิวแมนแพปพิลโลมาชนิด 16 ด้วยวิธีทางเคมีไฟฟ้า (POLYAMIDE NUCLEIC ACID PROBE FOR DETECTION OF HUMAN PAPILLOMA VIRUS DNA TYPE 16 BY ELECTROCHEMICAL METHOD) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.ดร. อรวรรณ ชัยลภากุล, 106 หน้า.

งานวิจัยนี้ ได้พัฒนาวิธีการตรวจหาดีเอ็นเอของไวรัสฮิวแมนแพปพิลโลมา(HPV) ความเสี่ยงสูงชนิด 16 โดยใช้โพรบ acpcPNA ร่วมกับการตรวจวัดทางเคมีไฟฟ้า โพรบได้ออกแบบให้มีความจำเพาะเจาะจงต่อดีเอ็นเอของไวรัส HPV ชนิด16 การตรวจวัดอาศัยหลักการเปลี่ยนแปลงสัญญาณทางเคมีไฟฟ้าของตัวติดฉลากที่สามารถเกิดปฏิกิริยารีดอกซ์ได้ ตัวติดฉลากที่ใช้ คือ แอนทราควิโนน ซึ่งถูกติดไว้บริเวณปลายสายโซ่ของโพรบ โพรบที่ถูกติดฉลากสามารถตรวจสอบได้ด้วยเทคนิค MALDI-TOF MS และด้วยจากการศึกษาทางความร้อนมีค่าอุณหภูมิการแยกสายอยู่ที่ 69.9 องศาเซลเซียส ขั้วไฟฟ้าที่ใช้ในการศึกษาครั้งนี้ คือขั้วไฟฟ้าพิมพ์สกรีนคาร์บอนที่ดัดแปรด้วยไคโตซาน โพรบถูกตรึงลงบนผิวหน้าขั้วด้วยพันธะโควาเลนต์โดยใช้กลูตารอลดีไฮด์เป็นตัวครอสลิงค์ การตรวจวัดทางเคมีไฟฟ้าศึกษาด้วยเทคนิคสแควร์เวฟโวลแทมเมททรีภายใต้สภาวะที่เหมาะสม จากผลการทดลองพบว่า สัญญาณของโพรบมีค่าลดลงเมื่อไฮบริไดเซชันกับดีเอ็นเอเป้าหมาย และมีค่าความสัมพันธ์ที่เป็นเส้นตรงกับความเข้มข้นของดีเอ็นเอในช่วง 0.02 – 12 μM ที่ค่าสัมประสิทธิ์สหสัมพันธ์เท่ากับ 0.996 ขีดจำกัดค่าความเข้มข้น (LOD) และค่าปริมาณต่ำสุด (LOQ) ของการวิเคราะห์ อยู่ที่ 0.004 μM และ 0.014 μM ตามลำดับ วิธีที่พัฒนาขึ้นนี้ได้ประสบความสำเร็จในการตรวจวัด HPV DNA ชนิด 16 ในตัวอย่างเซลล์ไลด์ (SiHa) พบว่า ค่าความเข้มข้นที่ได้ คือ 1.25 ± 0.08 μM ซึ่งมีค่าสอดคล้องกับค่าที่ได้การเปรียบเทียบจากตัวมาตรฐาน นอกจากนี้โพรบที่ออกแบบยังมีความจำเพาะเจาะจงสูงเมื่อศึกษาเทียบกับ HPV DNA ชนิดอื่นๆได้แก่ ชนิด 18, 31 และ 33 ซึ่งหวังว่าวิธีนี้จะเป็นอีกวิธีหนึ่งทางเลือกที่ใช้ในการคัดกรองมะเร็งปากมดลูกในระยะเริ่มแรก ที่มีราคาไม่แพง ซึ่งเหมาะสำหรับประเทศที่ยากจนหรือมีทรัพยากรอยู่อย่างจำกัด

สาขาปิโตรเคมีและวิทยาศาสตร์พอลิเมอร์ ลายมือชื่อนิสิต……

ปีการศึกษา……2555…… ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก……..

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม……..

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม…….....

5372470823: MAJOR PETROCHEMISTRY AND POLYMER SCIENCE
KEYWORDS: REDOX-ACTIVE LABEL / HUMAN PAPILLOMA VIRUS / POLYAMIDE NUCLEIC CID / ANTHRAQUINONE

SAKDA JAMPASA: POLYAMIDE NUCLEIC ACID PROBE FOR DETECTION OF HUMAN PAPILLOMA VIRUS DNA TYPE 16 BY ELECTROCHEMICAL METHOD: PROF. ORAWON CHAILAPAKUL, Ph.D., 106 pp.

In this work, a novel method for detection of high-risk human papillomavirus (HPV) DNA type 16 employing pyrrolidinyl polyamide nucleic acid (acpcPNA) probe coupled with electrochemical detection was developed. A capture probe was designed to bind specifically to HPV DNA type 16. The change of electrochemical signal of redox-active acpcPNA probe was observed. Anthraquinone was used as a redox-active label, which was attached at the N-terminus of PNA probe. The AQ-modified acpcPNA (PNA-AQ) was characterized by MALDI-TOF MS and thermal denaturation study with a complementary synthetic DNA target corresponding to the HPV DNA sequence (T_m = 69.9 ^oC). Chitosan-modified screen-printed carbon electrode was used in this study. The PNA-AQ probe was covalently immobilized onto the electrode surface using glutaraldehyde as a cross linking agent. Electrochemical detection was performed under optimal parameters using a square-wave voltammetric method. As a result, the probe signal decreased when exposed to the target DNA. The developed method showed a linear range between 0.02 –12 μM of DNA target with a correlation coefficient of 0.996. The limit of detection (LOD) and limit of quantitation (LOQ) was found to be 0.004 μM and 0.014 μM, respectively. Finally, this novel method was successfully applied to detect the HPV DNA type 16 in cell-lines sample (SiHa). The concentration of HPV DNA that this method can detect was found to be 1.25 ± 0.08 μM. This developed method produced results in good agreement with the standard marker. Moreover, the sensor probe has very high selectivity over non-complementary oligonucleotides, including HPV type 18, 31 and 33 DNA. This detection method can serve as a novel and inexpensive tool for early stage detection of cervical cancer, which is in demand in developing countries.

Field of Study : Petrochemistry and Polymer Science Student's Signature…….....
Academic Year :……2012..... Advisor's Signature……
Co-advisor's Signature.……
Co-advisor's Signature.……

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LIST OF ABBREVIATIONS

A	Adenine
acpc	Aminocyclopentane carboxylic acid
Ag/AgCl	Silver/silver chloride
AQ	Anthraquinone
C	Cytosine
^{o}C	Degree Celsius
CE	Counter electrode
CHT-SPCE	Chitosan modified screen-printed electrode
CIN	Cervical intraepithelial neoplasia
CV	Cyclic voltammetry
DA	Dopamine
DBU	1, 8-diazabicyclo-[5, 4, 0]-undec-7-ene
DIEA	N-ethyldiisopropylamine
DMF	N, N-dimethylformamide
DNA	Deoxyribose nucleic acid
dNTPs	Deoxynucleotide Triphosphates
E	Potential
E^0	Formal reduction potential
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide)
$E_{p,a}$	Anodic peak potential
$E_{p,c}$	Cathodic peak potential
Fmoc	Fluorenylmethyloxycarbonyl chloride

G	Guanine
g	Gram
HATU	O-(7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate
HDME	Hanging mercury drop electrode
HOAt	Hydroxyl-7-azabenzotriazole
HPLC	High performance liquid chromatography
HPV	Human papillomavirus
i	Current
i_f	Forward half-cycle
$i_{p, a}$	Anodic peak current
$i_{p, c}$	Cathodic peak current
i_r	Reverse half-cycle
LOD	Limit of detection
LOQ	Limit of quantitation
min	Minute
mL	Milliter
mM	Millimolar
mV	Millivolt
m/z	Mass per charge
n	Number of electron
ng/μL	Nanogram per microliter
nm	Nanometers

NMR	Nuclear magnetic resonance
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEI	Polyethylenemine
PNA	Peptide nucleic acid
PNA-AQ	Peptide nucleic acid modified with anthraquinone
ppm	Part per million
RE	Reference electrode
R_f	Retention factor
RNA	Ribonucleic acid
SD	Standard deviation
SPCE	Screen-printed carbon electrode
SWV	Square-wave voltammetry
T	Thymine
TLC	Thin layer chromatography
T_m	Melting temperature
WE	Working electrode
w/v	Weight / Volume
X	Spacer
µA	Microampere
µL	Microliter
µM	Micromolar

CHAPTER I

INTRODUCTION

1.1 Introduction

Cervical cancer is one of the leading types of cancer responsible for deaths in females around the world. Human papillomavirus (HPV) has been proven to be the major cause of cervical cancer. HPV infections can be transmitted by sexual, skin and mucous membranes and are especially contagious in developing countries where there are limited resources allocated for public healthcare. There are many types of HPV that can infect the genital areas of men and women or affect other parts of the body. Some types of HPV can cause genital warts or cancer [1].

The HPV virus can distribute in our bodies for a long period time without showing any symptoms of an infection. Hence, when the symptoms become apparent and the diagnosis reveals the cervical cancer positive status, the person is already approaching the last stage of the infection which it is difficult to treat. The symptoms are different depending on the type of HPV infection. HPV can sub-classify into two groups: High risk and Low risk HPV; where only high risk HPV infection, such as HPV types 16 and 18, can lead to cervical cancer [1,2].

Nowadays the most popular methods for the screening and diagnosis of the HPV infection are Pap smear and Hybrid captures test (developed by Digene Corporation). Although the two techniques are widely used, they have disadvantages. Pap smear provides a low sensitivity, requirement for an expert to analyze the data and to some extent false negative results are received. Likewise, Hybrid captures test is time consuming and expensive [3]. So, two mentioned techniques are unsuitable for limited resource countries.

As previously reported techniques for detection of HPV DNA, leaky surface acoustic wave, piezoelectric and fluorescence spectroscopy have been applied [4,5]. In addition electrochemical detection for the diagnosis of HPV-related sequences has been also developed. The electrochemical detection is an attractive method due to its advantages including high sensitivity, small volume of sample requirement, low cost,

simplicity and portability, making it an excellent candidate for point-of-care DNA diagnostic method [6].

The general working principle of DNA electrochemical biosensor involves immobilization of a DNA or PNA probe onto the electrode surface. Subsequent hybridization with the correct target DNA causes a change in the electrochemical signal [7]. Different types of electrode can act as electrochemical transducers for DNA detection including gold electrode, hanging mercury drop electrode (HMDE). However, using these types of electrode is expensive and harmful. Carbon-based electrode is an alternative choice to use as electrochemical transducers for DNA detection due to its inexpensive, easy to modify and environmentally friendly [8].

Polyamide Nucleic Acid or peptide nucleic acid (PNA) is an artificially synthesized DNA analogue that was discovered by Neilsen's group. The PNA structure is composed of repeating N-(-2-aminoethyl)-glycine units linked with peptide bond as the backbone chain replacing of deoxyribose sugars and phosphate groups in the DNA backbone. PNA bind with the complementary DNA or RNA under Watson & Crick rules like DNA-DNA and DNA-RNA duplexes. The special features of PNA are greater specificity, sensitivity, physical & chemical stability and high binding affinity to the natural complementary DNA or RNA more than DNA [9]. Because of these excellent properties, the PNA was therefore promisingly used as biomolecular sensor probe for disease diagnostic. Recently, a new pyrrolidinyl PNA system was developed by Vilaivan's group [10]. The newly developed PNA system possesses an α,β-peptide backbone deriving from D-proline/2 aminocyclopentanecarboxylic acid (known as acpcPNA). The acpcPNA showed a stronger binding affinity and higher specificity toward complementary DNA target than Nielsen's PNA. Consequently, the acpcPNA was also applied as an excellent probe to detect the DNA target by combination with suitable detection techniques such as MALDI-TOF mass spectrometry, fluorescence microscopy, surface plasmon resonance (SPR) [11, 12, 13, 14, 15, 16], but these techniques involve complicated instrument with high cost of operation.

Many immobilization methods of PNA/DNA probe onto the electrode surface have been reported. Physically or chemically modified electrode with a sensor probe including electrostatic adsorption and covalent attachment is widely performed [7, 8,

17]. The immobilization of sensor probe is an important step to the detection signal. The covalent immobilization of the PNA/DNA probe is an attractive method because it provides reusable of probe-modified electrode. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) (EDC) coupling agent is a commonly used to immobilize PNA/DNA probe onto the functionalized-electrode [18]. However due to the decomposition of EDC in aqueous solution, using EDC is not preferable. In this work, the covalent attachment of PNA-AQ probe using a biocompatible, environmentally friendly chitosan polymer was proposed.

For electrochemical monitoring of HPV DNA various types of electroactive species for PNA/DNA labels/indicators in electrochemical detection of the PNA/DNA hybridization such as methylene-blue, ferrocene and anthraquinone are widely used. These labels may be covalently attached to the probe or with other methods [19, 21, 21].

Because we realized in cervical cancer crisis that caused by high-risk HPV, especially in developing countries where there are limited resources allocated for public healthcare, cervical cancer is always present. These countries are in need of a well-developed technology to solve this problem. It is our inspiration to develop a screening method for the detection of HPV DNA in the primary stage, which can save the life or reduce the death rate of these people. In this work, we will focus on high-risk HPV type 16 that has been proven to be the major cause of cervical cancer.

A novel electrochemical biosensor based upon acpcPNA probe coupled with the electrochemical detection that provides low cost, high sensitivity and fast analysis for the detection of human papillomavirus (HPV) DNA type 16 was proposed. This developed method presented the possibility for the detection of HPV DNA in cell-line sample. We hoped that the detection platform would be applicable as inexpensive tool in screening for the HPV-DNA type 16 in the primary stage of cervical cancer.

1.2 Objective of the research

The two main goals of this work are as follows:

1. To develop a screening method for the detection of human papillomavirus (HPV) DNA type 16 coupled with electrochemical detection.
2. To apply the developed method that provides rapidity, high sensitivity and low cost for the determination of HPV DNA in cell-line sample.

1.3 Scope of the research

Electrochemical biosensor for the detection of human papilloma virus **(**HPV**)** DNA type 16 employing pyrrolidinyl peptide nucleic acid **(**acpcPNA**)** as a sensor probe was developed. AcpcPNA capture probe use in this work is a conformationally restricted acpcPNA with a sequence of (N)-AQ-CGACCTCCACATACLysNH$_2$-(C) which was designed to be complementary to the HPV Type 16 DNA. The PNA probe was synthesized on a Tentagel resin equipped with Rink amide linker by standard Fmoc solid-phase peptide synthesis procedure. The redox-active label anthraquinone (AQ) was covalently attached to the N-terminus of the acpcPNA probe through N-acylation. The successful AQ-modified acpcPNA probe (PNA-AQ) was verified by MALDI-TOF MS and by thermal denaturation study with a complementary synthetic DNA target corresponding to the HPV DNA. Chitosan was used for introduction of amino group to the electrode surface. This functional group was utilized for the immobilization of PNA-AQ onto the electrode surface by covalent attachment. The electrode was modified by PNA-AQ probe via cross-linking of the amino group on electrode surface and the amino group of lysine side chain on the PNA probe using the glutaraldehyde. Hybridization with the target DNA was studied by measuring the peak current of AQ using a square-wave voltammetric (SWV) method. The analytical parameters such as sensitivity, specificity and reproducibility have been studied. Influences of chitosan, glutaraldehyde and electrochemical parameter were examined to obtain the optimal conditions. This developed method was also applied to detect the HPV DNA type 16 in cell-line.

CHAPTER II

THEORY AND LITERATURE SURVEY

2.1 Cervical cancer

2.1.1 What is cancer? [2]

Cancer begins in cells, the building blocks that make up tissues. Tissues made up the cervix and other organs of the body. Normal cervical cells grow and divide to form new cells as the body needs them. When normal cells grow old or get damaged, they die, and new cells take their place. Sometimes, this process goes wrong. New cells form when the body does not need them, and old or damaged cells do not die as they should. The buildup of extra cells often forms a mass of tissue called a growth or tumor.

Cervical cancer begins in cells on the surface of the cervix (Figure 2.1). The cervix is the lower part of the uterus (womb). It is sometimes called the uterine cervix. The fetus grows in the body of the uterus (the upper part). The cervix connects the body of the uterus to the vagina (birth canal). The part of the cervix closest to the body of the uterus is called the endocervix. The part next to the vagina is the exocervix (or ectocervix). The two main types of cells covering the cervix are squamous cells (on the exocervix) and glandular cells (on the endocervix). These two cell types meet at a place called the transformation zone. Most cervical cancers start in the transformation zone.

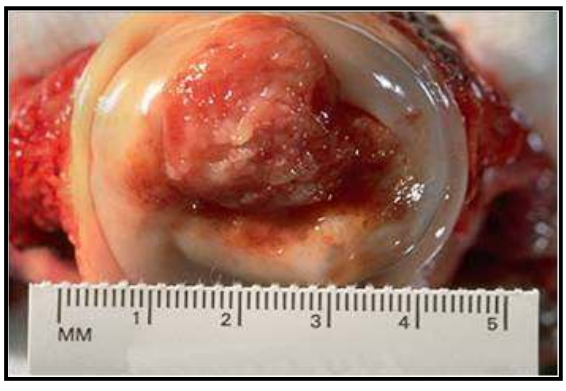


Figure 2.1 The cervical cancer [22].

2.1.2 Cervical cancer definition

Cervical cancer, or cancer of the cervix, is cancer of the entrance to the uterus (womb). The cervix is the narrow part of the lower uterus, often referred to as the neck of the womb. Cervical cancer occurs most commonly in women over the age of 30. Various strains of the human papillomavirus (HPV), a sexually transmitted infection, play a role in causing most cases of cancer of the cervix.

2.1.3 Cause of cervical cancer

Cervical cancer is one of the leading types of cancer relating to responsible for deaths in females around the world. It is almost always caused by HPV. HPV infection is always transferring through sex, skin and mucous membranes. Especially, who have multiple partners. This cancer is the major cause of death of people in developing countries that there are really need the well-technology and cost-effective to solve and treat it in early state of cancer. Although most people have no symptoms some people with HPV infection will have visible genital warts. They are raised, flesh-colored soft growths that may occur singly or in clusters. The HPV virus can cause warts on the penis, vulva, urethra, or around the anus. In women, HPV can invade the vagina and cervix. Vaginal and cervical warts are flat and not easily visible without special procedures. However, both men and women can be infected with the HPV virus and not have any visible warts on the genitalia. This is because this virus lives in the cells.

But these diseases can be naturally treated unlike high-risk HPV infection that can lead to cancer. When a high-risk HPV type is appeared, the risk of cancerous lesions increases. High-risk HPV types are detected in more than 90% of patients who were diagnosed as cervical cancer positive. The two types of high risk HPV will always be presented which are 16 and 18. High-risk HPV can initiate abnormal cell changes in the cervix. If these changes are not treated over a long period, they can lead to dysplasia, a precancerous condition, and then to cancer.

2.1.4 Cervical cancer symptoms

The HPV virus can distribute in our bodies for a long period time without showing any symptoms of an infection. Hence, when the symptoms become apparent and the diagnosis reveals the cervical cancer positive status, the person is already approaching the last stage of the infection when it is difficult to treat. The symptoms are different depending on the type of HPV infection.

Early cervical cancers usually don't cause symptoms. When the cancer grows larger, women may notice abnormal vaginal bleeding:

- Bleeding that occurs between regular menstrual periods
- Bleeding after sexual intercourse, douching, or a pelvic exam
- Menstrual periods that last longer and are heavier than before
- Bleeding after going through menopause

This stage is call cervical intraepithelial neoplasia (CIN) or dysplasia (precancerous condition) [23, 24]. Dysplasia can divide into three categories as shown in the Figure 2.2.

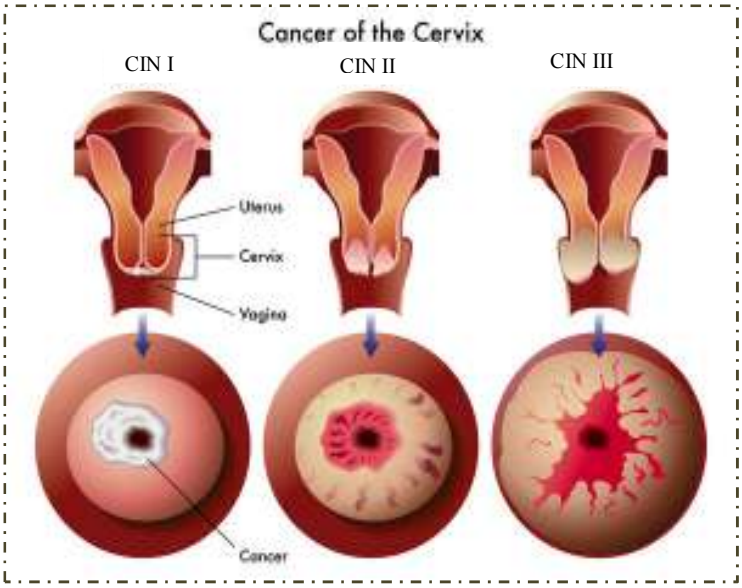


Figure 2.2 Dysplasia or precancerous condition of cervical cancer [22].

CIN I

The least risky types, represents only mild dysplasia, or abnormal cell growth. It is confined to the basal 1/3 of the epithelium. This corresponds to infection with HPV, and typically will be cleared by immune response in a year, though can take several years to clear.

CIN II

Moderate dysplasia confined to the basal 2/3 of the epithelium

CIN III

Severe dysplasia that spans more than 2/3 of the epithelium, and may involves the full thickness. This lesion may sometime also be referred to as cervical carcinoma in situ.

CIN is the potentially premalignant transformation and abnormal growth (dysplasia) of squamous cells on the surface of the cervix. CIN is not cancer, and is usually curable. Most cases of CIN remain stable, or are eliminated by the host's immune system without intervention. However a small percentage of cases progress to cervical cancer,

2.1.5 Risk factors

When patients who were diagnosed as cervical cancer, it is natural to wonder what may have caused the disease. Doctors usually can't explain why one woman develops cervical cancer. However, we do know that a woman with certain risk factors may be more likely than other women to develop cervical cancer. A risk factor may increase the chance of developing a disease. Other risk factors, such as age, socioeconomic and ethnic factors, high sexual activity, family history, use of oral contraceptives, having many children, immunossupression and smoking, can act to increase the risk of cervical cancer among infected women with HPV and more.

A woman's risk of cervical cancer can be reduced by getting screening test of regular cervical cancer screening tests. If abnormal cervical cell changes are found early, cancer can be prevented by removing or killing the changed cells before they

become cancer cells. Moreover, a woman can reduce risk of cervical cancer is by getting an HPV vaccine before becoming sexually active (between the ages of 9 and 26). Therefore, even women who get an HPV vaccine need regular cervical cancer screening tests.

2.1.6 Detection of cervical cancer

Many techniques were applied to detect or screen the cervical cancer, but the most popular methods which are commonly used in general hospitals are Pap smear and Hybrid capture test.

Pap smear

Pap smear or knows as the cytological test. It is a routine screening test for the cervical cancer. The surface of the cervix is scraped with a small brush and the scraped-off cells from the cervix are fixed in a preservative solution (Figure 2.3). The slide is called a Pap smear and is sent to a special laboratory where it is performed and evaluated by highly trained technicians and doctors. The principle of this method is observed the color changed between normal and abnormal cell after dying [25].

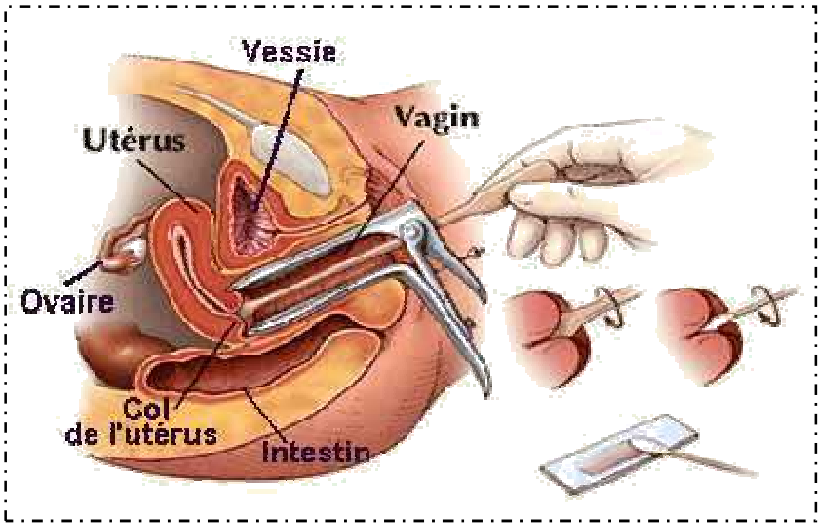


Figure 2.3 Schematic illustration of Pap smear test [25].

Hybrid capture test

The hybrid capture HPV (HC2) test manufactured by Digene is a microtitration plate hybridization test intended for detection of low-risk (LR) and high-risk (HR) types of HPV DNA in cervical swab specimens. The test is able to detect 5 LR HPV types (6, 11, 42, 43 and 44) and 13 HR HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). Specimens potentially containing HPV DNA hybridize in solution with several specific RNA probes. Resulting RNA/DNA hybrid molecules are fixed to RNA/DNA hybrid specific antibodies on the surface of the plate wells. The specific antibodies subsequently react with monoclonal antibodies to RNA/DNA hybrid molecules conjugated to alkaline phosphatase to be detected by a chemoluminiscent substrate. Light produced as a result of the substrate split is measured by a luminometer and expressed in relative light units (RLU). The RLU value allows for semiquantitative determination of viral DNA quantity in the specimen. The method also uses signal amplification and thus the determination sensitivity becomes comparable with that provided by PCR methods. The presence of some HPV DNA types can be detected with a sensitivity of 1pg viral DNA/ml of specimen as shown in Figure 2.4 [26].

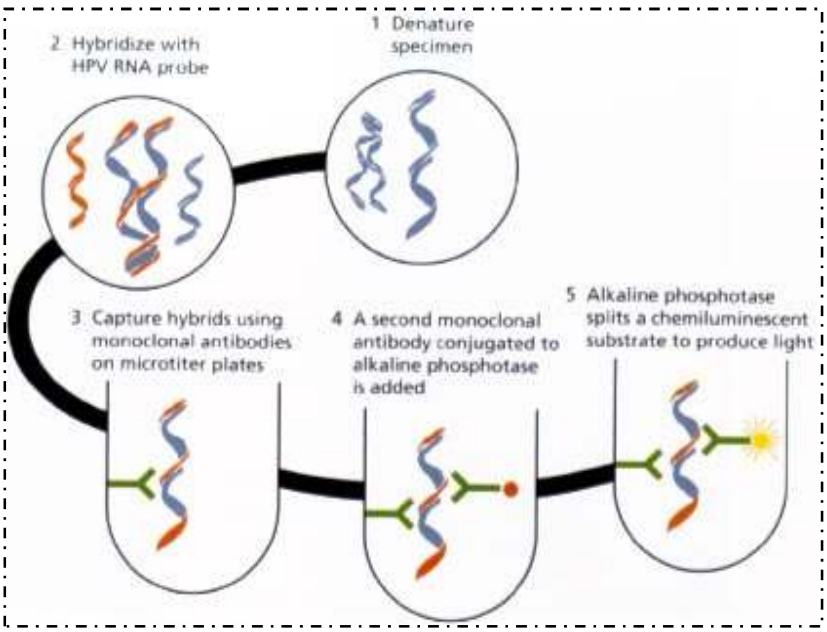


Figure 2.4 Hybrid Capture test principle [26].

2.1.7 Treatment of Cervical cancer

The choice of treatment depends mainly on the size of the tumor and whether the cancer has spread. The treatment choice may also depend on whether you would like to become pregnant someday. Treatment options for women with cervical cancer are follows [2].

- Surgery
- Radiation therapy
- Chemotherapy
- A combination of these methods

2.1.8 Cervical cancer situation

As the statistic information, cervical cancer is one of the leading types of cancer responsible for deaths in females around the world. Cervical cancer mostly appears in women who are age in range of 30-50 years old. The number of cervical cancer patients has increased continuously for 500,000 people every year and 200,000 of them died which is 80 percent of all patients.

For Thailand, the cervical cancer is the main cause of death of Thai females. As the statistic information records that from 1998 to 2010, a number of died people in Thailand have been reached to the maximal in the central part following with northern, eastern, and southern respectively. Moreover, the trend of the death rate of patients caused by cervical cancer has been increasing continuously every year. From the information, it can conclude that about 5,000 people every year or 14 people per day have been died. The result is shown below in the Figure 2.5

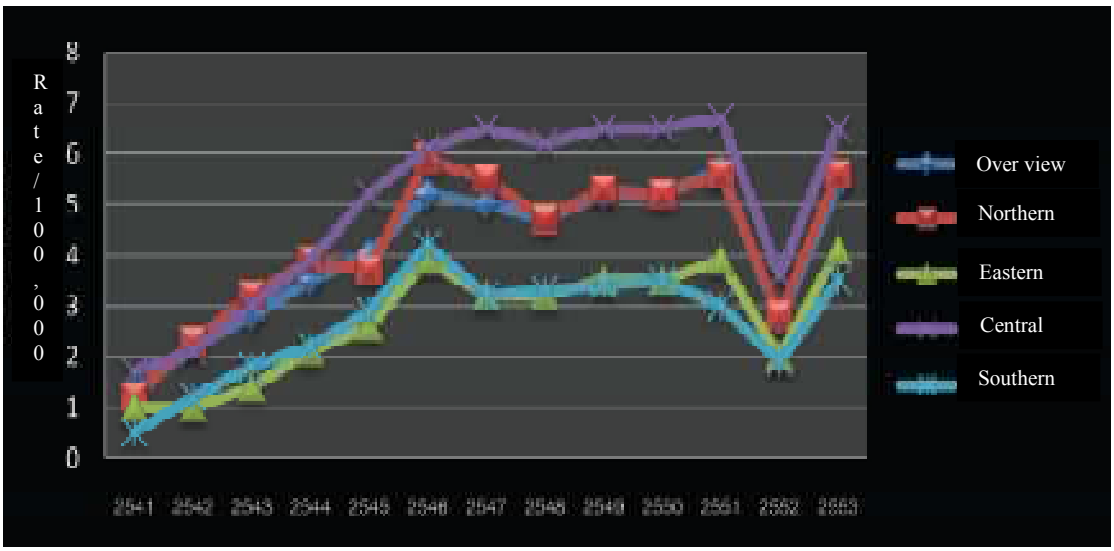


Figure 2.5 A number of died people caused by cervical cancer [28].

For new cervical cancer patients in Thailand, the researcher s report that from 2006 to 2009, the northern, eastern and central the new cervical cancer patients are increased around 4,000 people per year. But for the southern, the new patients around 1,400 people per year are increased. From the Figure 2.6 the new patient is slightly decreased. In every year, the new patients around 10,000 people will be increased or average 27 people per day [27, 28, 29].

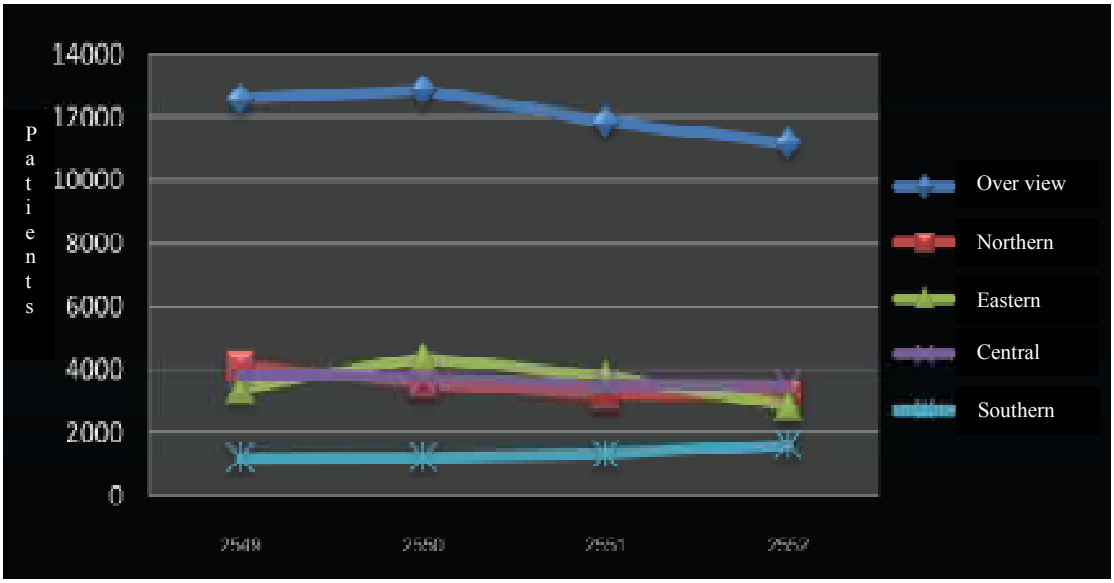


Figure 2.6 A number of new patients in each year [29].

2.2 Polyamide or peptide nucleic acid (PNA)

2.2.1 Peptide nucleic acid background

Peptide nucleic acid or Polyamide Nucleic Acid (PNA) is an artificially synthesized DNA analogue which was discovered by Neilsen's group. The PNA structure is composed of repeating N-(-2-aminoethyl)-glycine units linked together with peptide bonds (orange circle) as the backbone chain instead of the deoxyribose sugars and phosphate backbone DNA (red circle) as shown in Figure 2.7 [9, 30, 31, 32].

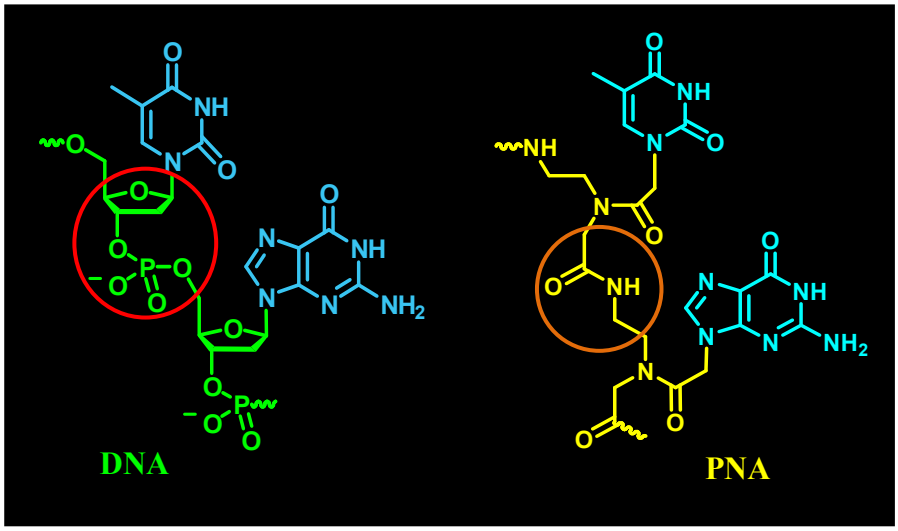


Figure 2.7 Comparison of PNA and DNA structure.

PNA binds with the complementary DNA or RNA following Watson & Crick analogous to like DNA-DNA and DNA-RNA duplexes [68] (Figure 2.8). The special features of PNA are great specificity, sensitivity, physical & chemical stability and high binding affinity to the natural complementary DNA or RNA. The higher binging affinity of PNA than DNA is due to the electrochemically neutral nature of PNA. Thus, the electrostatic repulsion was eliminated when the PNA-DNA duplex was formed. The hybridization between PNA and DNA is independent on the ionic strength concentration [33]. Therefore, stable hybrids can occur in low salt concentration. Moreover, PNA is also resistant to nuclease and protease enzyme.

Because of these excellent properties, PNA has become an attractive tool for the researchers, and it was therefore extensively used as biomolecular probe for diagnostic applications [34, 35]. After the discovery of PNA, many research groups have tried to develop the new PNA systems that provide improved properties such as binding affinity or specificity.

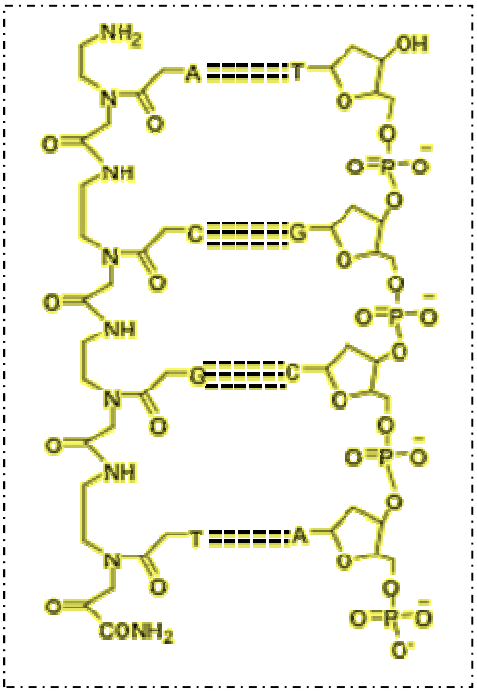


Figure 2.8 Illustration of PNA binding with DNA under Watson & Crick rules [68].

2.2.2 Development of peptide nucleic acid

Recently, a new pyrrolidinyl PNA system was developed by Vilaivan's group. The newly developed PNA system (known as acpcPNA) possesses an α,β-peptide backbone deriving from D-proline/2-aminocyclopentanecarboxylic acid [10]. The structure of acpcPNA-DNA hybrid and repeating units of DNA, Nielsen's PNA and acpcPNA are illustrated in Figures 2.9 and 2.10, respectively. The acpcPNA shows a stronger binding affinity and higher specificity toward complementary DNA target than Nielsen's PNA. AcpcPNA has been used as a probe to detect the target DNA by

dealing with a suitable detection technique such as MALDI-TOF mass spectrometry, fluorescence microscopy, surface plasmon resonance (SPR), and electrochemical detection [11, 12, 13, 14, 15, 16, 69, 70].

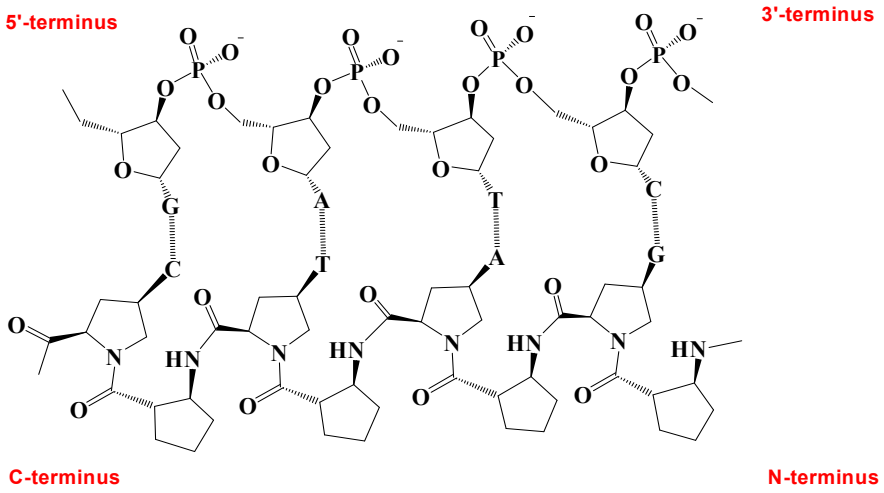
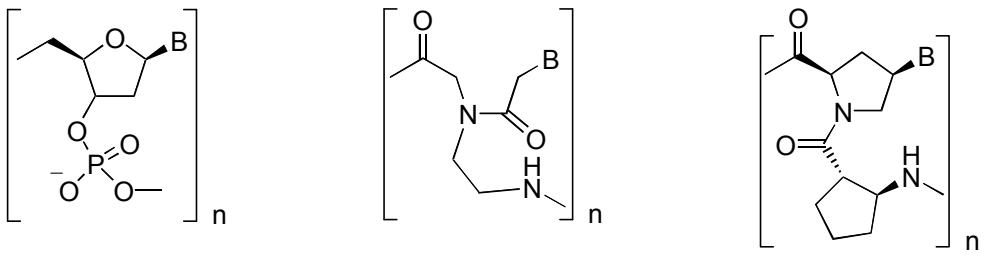


Figure 2.9 Illustration of binding form between acpcPNA and natural DNA [10].



DNA | Nielsen's PNA B = A, T, C, G | Vilaivan's *SS*-ACPC PNA

Figure 2.10 Contrast of the repeating units between DNA, Nielsen's PNA and acpcPNA [10].

2.3 Electrochemical detection [37, 38]

Electrochemistry is a branch of chemistry concerned with the interrelation of electrical and chemical effects. A large part of this field deals with the study of chemical changes caused by the passage of an electric current and the production of electrical energy by chemical reactions. In fact, the field of electrochemistry encompasses a huge array of different phenomena (e.g., electrophoresis and corrosion), devices (electrochromic displays, electroanalytical sensors, batteries, and fuel cells), and technologies (the electroplating of metals and the large-scale production of aluminum and chlorine).

Scientists make electrochemical measurements on chemical systems for a variety of reasons. They may be interested in obtaining thermodynamic data about a reaction. They may want to generate an unstable intermediate such as a radical ion and study its rate of decay or its spectroscopic properties. They may seek to analyze a solution for trace amounts of metal ions or organic species. In these examples, electrochemical methods are employed as tools in the study of chemical systems in just the way that spectroscopic methods are frequently applied. There are also investigations in which the electrochemical properties of the systems themselves are of primary interest, for example, in the design of a new power source or for the electrosynthesis of some product. Many electrochemical methods have been devised. Their application requires an understanding of the fundamental principles of electrode reactions and the electrical properties of electrode-solution interfaces.

2.3.1 Cyclic voltammetry [39]

Cyclic voltammetry (CV) is a variety of potentiodynamic electrochemical measurement. It is the most widely used technique for obtaining qualitative information about electrochemical reactions. It is generally used to study the electrochemical properties of an analyte in solution. In a cyclic voltammetry experiment, the working electrode potential is ramped linearly versus time like linear sweep voltammetry. This ramping is known as the experiment's scan rate (V/s).

However, cyclic voltammetry takes the experiment a step further than linear sweep voltammetry.

Cyclic voltammetry consists of scanning linearly the potential of a stationary working electrode, using a triangular potential waveform, as shown in Figure 2.11. The voltage is swept between two values at a fixed rate, however now when the voltage reaches V_2 the scan is reversed, and the voltage is swept back to V_1. Depending on the information sought, single or multiple cycles can be used. During the potential sweep, the potentiostat measures the current resulting from the applied potential. The resulting current-potential plot is termed a cyclic voltammogram. The cyclic voltamogram is a complicated, time-dependent function of a large number of physical and chemical parameters.

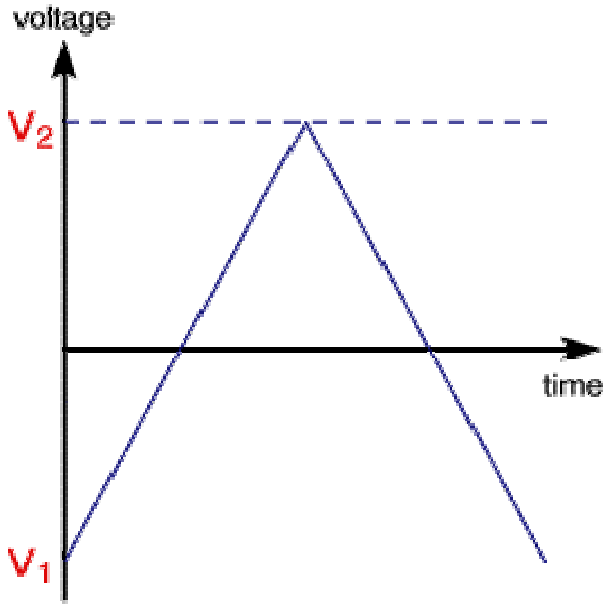


Figure 2.11 Illustrates a triangular potential waveform of CV [39].

The potential is applied between the reference electrode and the working electrode and the current is measured between the working electrode and the counter electrode. This data is then plotted as current (i) vs. potential (E). The forward potential scanned produces a current peak for any analyses that can be reduced or oxidized depending on the initial scan direction. As the potential approaches the characteristic E^0 for the redox process, the current will increase until a peak is

reached. After traversing the potential region in which the reduction process takes place, the direction of the potential sweep is reversed. During the reverse scan, it will reach the potential that will reoxidize the product formed and generate a current of reverse polarity from the forward scan, resulting in the oxidation peak that will usually have a similar shape to the reduction peak. As a result, information about the redox potential and electrochemical reaction rates of the compounds is obtained.

Cyclic voltammetric measurements were characterized by a peak potential. E_p is the potential at which the current reaches its maximum value; i_p is the value that is called the peak current. The $i_{p,a}$ and $E_{p,a}$ are the anodic peak current and anodic peak potential, respectively. The $i_{p,c}$ and $E_{p,c}$ are the cathodic peak current and cathodic peak potential, respectively (Figure 2.12).

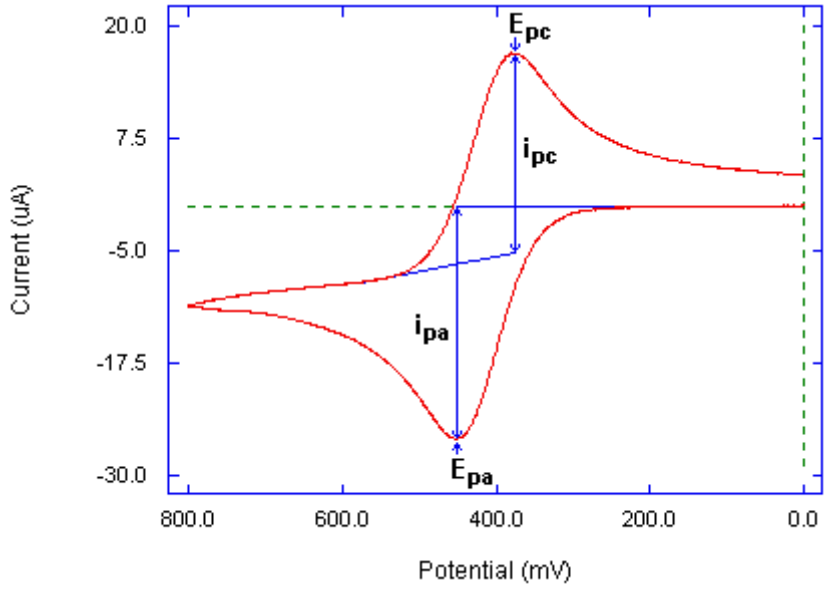


Figure 2.12 Typical reversible cyclic voltammetry with the initial sweep direction towards more positive potential [40].

The forward sweep produces an identical response to that seen for the LSV experiment. When the scan is reversed we simply move back through the equilibrium positions gradually converting electrolysis product. The current flow is now from the solution species back to the electrode and so occurs in the opposite sense to the forward seep but otherwise the behavior can be explained in an identical manner. For

a reversible electrochemical reaction the CV recorded has certain well defined characteristics.

I) The voltage separation between the current peaks is

$$\Delta E = E_p^a - E_p^c = \frac{59}{n}\text{mV} \qquad \text{(Equation 2.1)}$$

II) The positions of peak voltage do not alter as a function of voltage scan rate
III) The ratio of the peak currents is equal to one

$$\left|\frac{i_p^a}{i_p^c}\right| = 1 \qquad \text{(Equation 2.2)}$$

IV) The peak currents are proportional to the square root of the scan rate

$$i_p^a \text{ and } i_p^c \propto \sqrt{v} \qquad \text{(Equation 2.3)}$$

For instance if the electronic transfer at the surface is fast, and the current is limited by the diffusion of species to the electrode surface, then the current peak will be proportional to the square root of the scan rate.

2.3.2 Reversible system

The shape of a reversible cyclic voltammogram with an electrode of fixed area is shown in Figure 2.19. The peak current for a reversible couple is given by the Randles-Sevcik equation below:

$$i_p = 2.69\times10^5 n^{3/2} A D^{1/2} C v^{1/2} \qquad \text{at } 25°\text{C} \qquad \text{(Equation 2.4)}$$

Where n is the number of electron, A is the electrode area (in cm^2), D is diffusion coefficient (in $cm^2 \cdot s^{-1}$), C is the concentration of electroactive species (in $mol \cdot cm^3$) and v is the potential scan rate (in $V \cdot s^{-1}$).

Accordingly, the current is directly proportional to the concentration and increases with the square root of the scan rate. Such dependence on the scan rate is indicative of the electrode reaction controlled by mass transport. The reverse-to-forward peak current ratio is in unity for a simple reversible couple. This peak ratio can be strongly affected by chemical reactions coupled to the redox process. The current peaks are commonly measured by extrapolating the proceeding baseline current.

2.3.3 Irreversible system

For irreversible processes, the individual peaks are reduced in size and widely separated. The peak current given by

$$i_p = (2.99\times10^5)n\,(\infty n_a)^{1/2} A C D^{1/2} \nu^{1/2} \qquad \text{at } 25°C \qquad \text{(Equation 2.5)}$$

Where n is the number of electrons, A is the electrode area (in cm^2), D is diffusion coefficient (in $cm^2 \cdot s^{-1}$), C is the concentration of electroactive species (in $mol \cdot cm^3$) and ν is the potential scan rate (in $V \cdot s^{-1}$).

The peak current is still proportional to the bulk concentration but will be lower in height. The peak current of the irreversible processes is about 80% of the peak for a reversible. The shape of an irreversible cyclic voltammogram with an electrode of fixed area is shown in Figure 2.13.

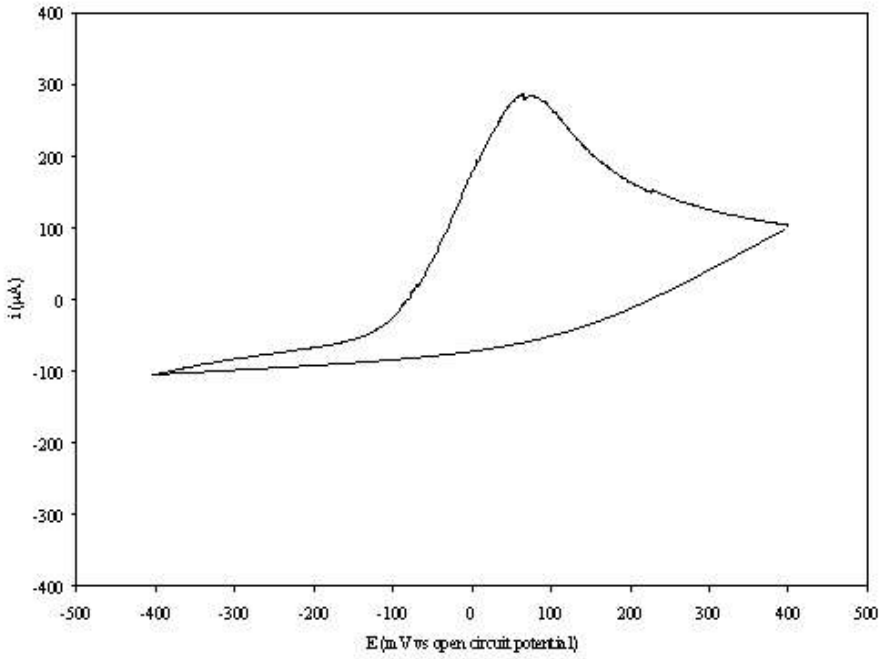


Figure 2.13 Cyclic voltammogram for irreversible redox process [41].

2.3.4 Square Wave Voltammetry [42]

Square Wave Voltammetry (SWV) is an extremely useful technique for measuring trace levels of organic and inorganic species. The sensitivities of SWV are better than those of normal pulse voltammetry. The potential wave form for SWV is shown in Figure 2.14. The potential wave form consists of a square wave of constant amplitude superimposed on a staircase wave form. The current is measured at the end of each half-cycle, and the current measured on the reverse half-cycle (i_r) is subtracted from the current measured on the forward half-cycle (i_f). This difference current ($i_f - i_r$) is displayed as a function of the applied potential.

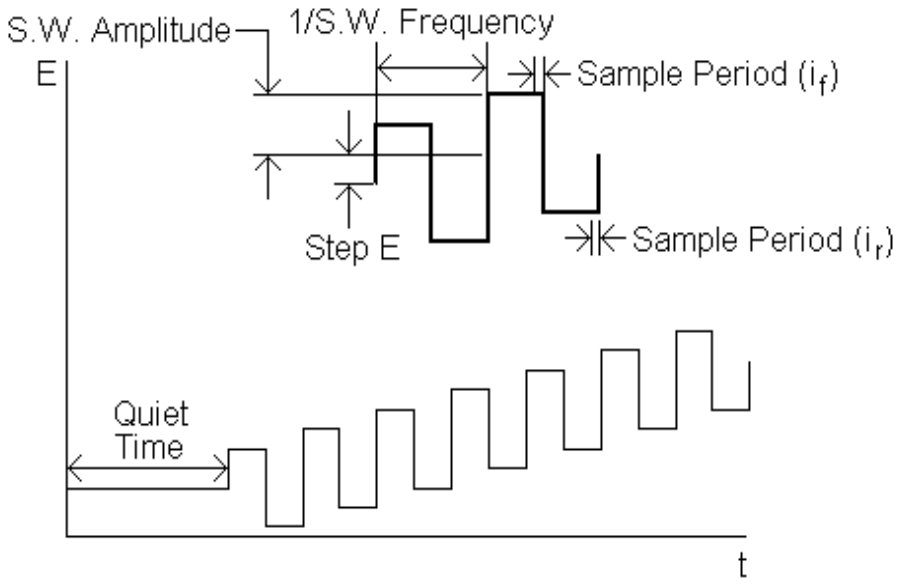


Figure 2.14 Potential wave form for square wave voltammetry [42].

There are two advantages to measuring the different current. First, it increases the discrimination against the charging current, since any residual charging current is subtracted out. Second, the shape of the current response is a symmetric peak, rather than the sigmoidal curve typically found for normal pulse voltammetry. At potentials well negative of the redox potential, the current is diffusion-controlled, and the potential pulse has no effect; hence, the forward and reverse currents are equal, and the different current is again zero. The largest difference between the forward and reverse currents (and hence the largest current response) is at the redox potential (Figure 2.15).

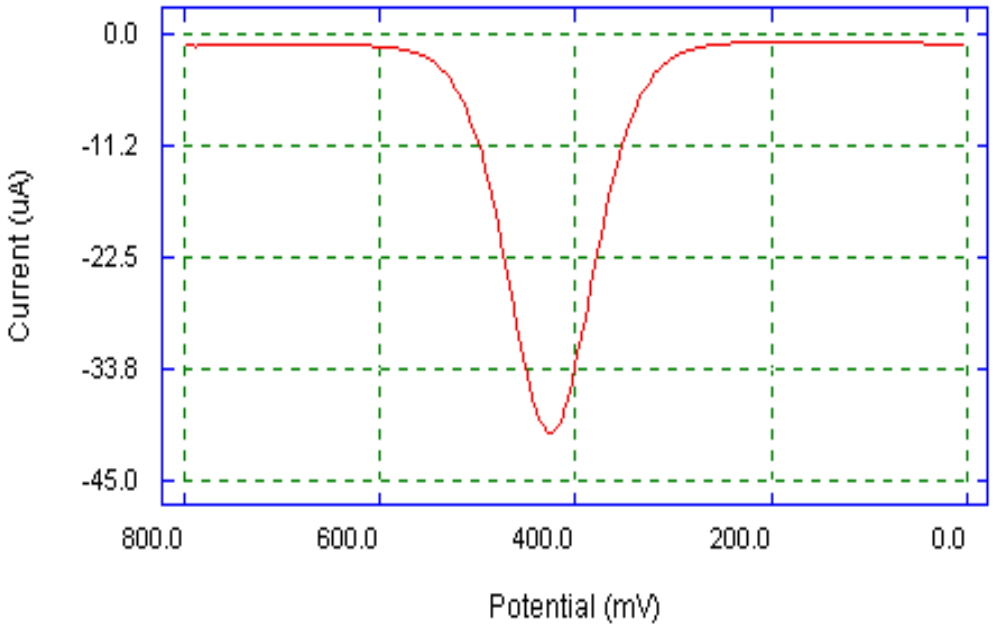


Figure 2.15 A typical square wave voltammogram [42].

2.4 Anthraquinone (AQ) redox-active species

2.4.1 Anthraquinone background

Anthraquinone (AQ) occurs naturally in certain plants, fungi and insects and it contributes to the coloring pigment of such organisms. Due to this property, the compound is used commercially to manufacture dyes. In powdered form, anthraquinone exhibits a color that ranges from gray to yellow and green. However, it produces a variety of different colored dyes, including alizarin (red), oil blue A and oil blue 35, quinizarine green SS and solvent violet 13. Anthraquinone (Figure 2.16) is a derivative of anthracene, a coal-tar by product characterized by a chemical structure consisting of a polycyclic aromatic hydrocarbon and three fused rings of benzene.

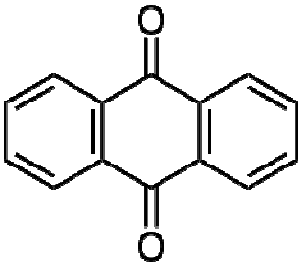


Figure 2.16 Anthraquinone structure [44].

AQ also called anthracenedione or dioxoanthracene that is an aromatic (a hydrocarbon characterized by general alternating double and single bonds between carbons) organic compound. This compound is an important member of the quinone family. Quinone is a class of organic compounds that are formally derived from aromatic compounds .The term is also used in the more general sense of any compound that can be viewed as an anthraquinone with some hydrogen atoms replaced by other atoms or functional groups. These derivatives include many substances that are useful or play important roles in living beings. Anthraquinone is identified by many other names, such as anthrachinon, dioxoanthracene, and several different trade names, including Hoelite and Corbit. AQ also presents certain safety challenges to the manufacturing industry. For instance, the substance is highly combustible and cannot be used near an open flame or extreme heat since the compound produces toxic fumes when burned. Environmentally, the compound is toxic to fish and does not readily biodegrade.

2.4.2 Anthraquinone application

There are other applications for AQ in addition to producing dyes. For example, it is used as a digester additive in the production of wood pulp and paper. A derivative called 2-ethylanthraquinone is used in producing hydrogen peroxide. In the field of medicine, it is used as a laxative, as an antimalarial medication to prevent or cure malaria and also used in the treatment of cancer. AQ has a medieval history of long term use as a reptile repellent and is used to determine the presence of geese in particular. This action may be due to the laxative properties that the compound

possesses when introduced as treated birdseed or grass. In fact, its presence is what lends laxative qualities to several well-known herbs used to treat constipation, such as senna pods, aloe, rhubarb, buckthorn and cascara sagrada.

Not only those benefits mentioned above but AQ was also broadly used as a redox active species for indicator/labeling of PNA-DNA hybridization [53]. Because its structure consists of two carbonyl groups that can provide the redox peaks when an appropriate potential was applied and due to its thermal stability and high sensitivity thereby, AQ has become an attractive compound to many researchers. AQ was applied as a redox active species for many purposes in electrochemical detection.

The redox mechanism of AQ involves two electrons transfer. Thus, two redox peaks can be achieved. AQ mechanism is presented in the Figure 2.17.

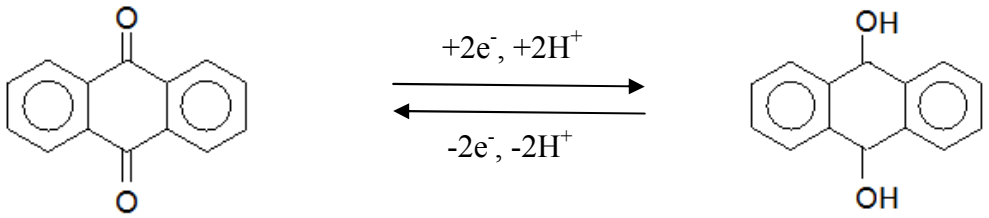


Figure 2.17 AQ mechanisms with two electrons transfer [45].

2.5 Literature surveys

In 1996, Wang and co-worker [46] studied the specificity and sensitivity between PNA and DNA probe to detect the target DNA and also other parameters which affected the hybridization. Both PNA and DNA probe were immobilized onto the electrode surface by electrostatic adsorption. $Co(phen)_3^{3+}$ was used as the redox-active label to offer the electrochemical signal after the hybridization. The electrochemical response of redox-active label was observed. The result found that the PNA probe provide more sensitivity and specificity than the DNA probe.

In 2008, Siriwong et al. [47] reported the using of acpcPNA as a sensor probe to detect the target DNA. The result revealed that the acpcPNA is more stable than the DNA probe. This was due to PNA backbone has a neutral charge whereas DNA

backbone is a negative charge. Therefore, the electrostatic repulsion is eliminated when the PNA-DNA duplexes were formed which affected the specificity and the stability of hybridization.

In 2010, Ananthanawat et al. [14] compared the bind of different probe when they were exposed to target DNA including acpcPNA, aegPNA and DNA probes. This study aim was to study the specificity of various types of probe. The results indicated that the acpcPNA shows more specificity, sensitivity and only antiparallell toward the complementary DNA than another probe.

In 2011, Farjami et al. [48] developed a simple and robust “off-on” signaling genosensor platform to improve selectivity for single-nucleotide detection. The off-on genosensor design used for detection of a cancer biomarker TP53 gene sequence favored discrimination between the healthy and SNP-containing DNA sequences, which was particularly pronounced at short hybridization times. The DNA beacons were immobilized onto gold electrodes. MB was used as a redox active label at the end of DNA beacons to allow electrochemical detection. The result displays that a typical “on-off” change of the electrochemical signal was observed upon hybridization of the 27-33 nucleotide long hairpin DNA to the target DNA. The DNA hairpin beacons down to 20 nts provided improved genosensor selectivity for SNP and allowed switching of the electrochemical genosensor response from the on-off to the off-on mode. This due to the electron transfer mechanism between MB and electrode surface.

In 2006, Lai et al. [49] reported an electrochemical method for the sequence-specific and rapid detection of unpurified amplification products of the gyrB gene of Salmonella typhimurium. In this work MB was used as indicator/label of hybridization by attachment at the end of probe. Probe-modified MB was covalently immobilized onto electrode surface. The results exhibit that the sensor does not respond when challenged with control oligonucleotides. This sensor is fully electronic and requires neither cumbersome, expensive optics nor high voltage power supplies.

This developed method well suited for implementationin portable PCR microdevices directed at, for example, the rapid detection of pathogens

In 2001, Xu et al. [50] proposed the characterization of ssDNA on chitosan-modified electrode. Platinum was used as the working electrode. Chitosan was accumulated onto electrode as a film form. Single-strand DNA probe could bind with chitosan on a platinum electrode via forming a tight DNA-chitosan complex. FCA-modified DNA probe can hybridize efficiently with the complementary sequences and be successfully used for the sequence-specific DNA detection. The amounts of immobilized plasmid DNA are in range from 0.01 to 5.0 μmol/L. The regressing equation ranging from 0.01 to 0.20 μmol/L was $y = 2.073x + 0.238$ (x is the concentration of denatured plasmid DNA, $\times 10^{-7}$) and the regressing coefficient (r^2) of the linear curve was 0.9972. A detection limit to detection of the plasmid DNA was found to be 5.0 nmol/L.

In 2010, Khaled et al. [8] modified the screen-printed carbon electrode using chitosan mixed into carbon ink for the determination of heavy metals including Pb, Cu, Cd and Hg. These elements form ion-associates with the protonated amino group in the chitosan molecule at the electrode surface, and then the differential pulse anodic stripping voltammetry was performed. The screen printed electrodes exhibited adequate shelflife (6 months)**.** The developed screen-printed carbon electrodes modified with chitosan were also successfully applied for the determination of Pb^{2+} real water sample.

In 2002, Yi. et al [51]. In this work their used chitosan for the covalent binding of single stranded DNA oligonucleotide probes in fluorescence based nucleic acid hybridization assay. Chitosan was deposited onto 96-well microtiter plate format and leading to hybridization experiments. Glutaraldehyde was used as a cross-linking between chitosan and the probe agent due to it quite robust. The results demonstrate the portability of a DNA hybridization assay based on covalent coupling to chitosan, which, in turn, can be deposited onto various surfaces. More arduous surface preparation techniques involving silanizing agents and hazardous washing reagents are eliminated using this technique.

In 2011, Tran et al. [52] reported the developed electrode using the polymerization of polyaniline-multiwalled carbon nanotube film (PANi–MWCNT) on platinum electrode arrays (IDA) for the detection of human papillomavirus (HPV) infection. Peptide aptamers was used as capture reagent. Label free, electrochemical detection of the specific immune reaction between antigen peptide aptamer HPV- 16-L1. The advantage of this technique consists of reagentless and multiple detection of antigen–antibody complex formation on well conducting IDA interface of PANi–MWCNT, without intermediate steps or any labeling reagents, as normally required in the previous works.

In 2011, Ozkan et al. [17] studied the electrochemical parameters for MB on binding to DNA at HMDE, GCE and CPE in the solution and at the electrode surface. MB was used as the label/hybridization of DNA/PNA probe. Adsorptive transfer stripping voltammetry (AdTSV), differential pulse voltammetry (DPV), and alternating current voltammetry (ACV) techniques were applied in this work. The changes in the voltammetric peak of MB were observed. These results showed that MB could be used as an effective electroactive hybridization indicator for DNA biosensors.

In 2012, Kang et al. [53] fabricated multiplex encoders and decoders test using for detection of DNA target couple with electrochemical method that uses electronic (electrochemical) signals as its readout. These devices use two or more sequence-specific DNA probes, with each being modified with a distinct redox reporter. MB, AQ and FCA were used as the redox-active label by covalent attachment. These probes, when interrogated together, serve as encoders and decoders, converting patterns that are encoded and decoded by the presence or absence of specific DNA sequences into specific electronic outputs. This work demonstrated the multifunctional, bio-electrochemical devices.

In 2010, Civit et al. [54] proposed high sensitivity and selectivity method of an electrochemical genosensor array for the individual and simultaneous detection of two high-risk human papillomavirus DNA sequences. Two types high-risk HPV are

16E7p and HPV45E6. This technique provided LOD in the range of pM which is sufficient to detect real RNA/DNA samples obtained from PCR amplification. For multiplexed detection format, high selectivity was observed over the non-specific sequence. The proposed method can be used as platform for the development of an electrochemical high throughput screening assay of multiple high-risk DNA sequences.

In 2005, Li et al. [55] fabricated the electrode based on chitosan doped with carbon nanotube (CNT). This electrode was applied to detect salmon sperm DNA using methylene blue (MB) as a DNA indicator. The result reveal that CNTs can enhance the sensitivity and accelerate the rate of electron transfer electroactive species. The detection limit was found to be 0.252 nM fish sperm DNA and no interference was found in the presence of 5 lg/ml human serum albumin. The differential pulse voltammetry signal of MB was linear over the fish sperm DNA concentration range of 0.5–20 nM.

In 2008, Luo et al. [56] reported an electrochemical method for sequence-specific detection of DNA without solid-phase probe immobilization. TIO was used as the electrode and it was cleaned to obtain the negative charge on electrode surface. In this work, PAH positive charge polymer was coated onto electrode surface. When PNA probe hybridized with DNA target the meant the positive charge of PAH can trap the hybridized form on electrode surface. When it gets close to the electrode, the electrochemical response of electro active species can be detected. This method can also detect the target DNA without PCR.

In 2010, Luo et al. [57] introduced a novel electrochemical method for detecting sequence-specific DNA based on competitive hybridization. This method method utilizes the competition between the target DNA (t-DNA) and a ferrocene-labeled peptide nucleic acid probe (Fc-PNA) to hybridize with a probe DNA (p-DNA). The neutral PNA backbone and the electrostatic repulsion between the negatively-charged DNA backbone and the negatively-charged electrode surface are then exploited to determine the result of the competition through measurement of the

electrochemical signal of Fc. Upon the introduction of the t-DNA, the stronger hybridization affinity between the t-DNA and p-DNA releases the Fc-PNA from the Fc-PNA/p-DNA hybrid, allowing it to freely diffuse to the negatively charged electrode to produce a significantly enhanced electrochemical signal.

In 2007, Wei et al. [58] reported the new method for the pretreatment of screen-printed carbon electrodes (SPCEs) by NaOH and pre-anodized in low concentration of NaOH. This electrode was applied to detect the dopamine (DA) and ascorbic acid (AA). The result revealed that the pretreated electrodes (after pretreated two steps) exhibited excellent electrocatalytic behavior for the redox of DA. Interference from AA in the detection of DA could be effectively eliminated due to which resulted from the functionalization of the electrode surface in the pretreatment step.

CHAPTER III

EXPERIMENTAL

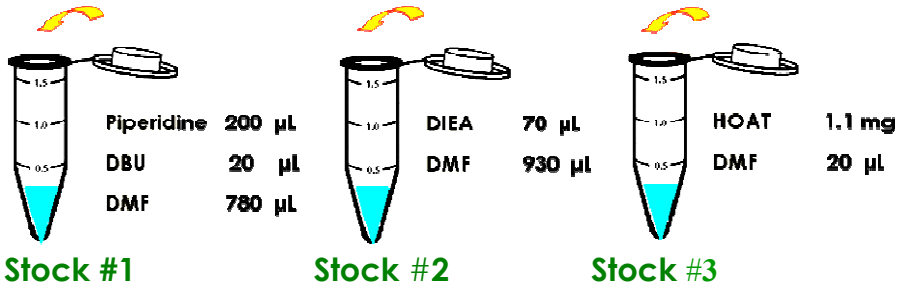
This chapter will provide information regarding instruments, equipment, chemicals, electrode preparation, sample preparations and methodology, which were used in this presented work. Not only the information is presented but the performance of each step will also be described.

3.1 Synthesis of the acpcPNA probe [36]

In this section, the synthesis of acpcPNA probes is described. The PNA probe used in this work is a conformationally restricted acpcPNA with a sequence of (N)-AQ-CGACCTCCACATACLysNH$_2$-(C). This sequence was designed to be complementary with the DNA of HPV Type 16. Lysinamide was included at the C-terminus for two reasons; first as a PNA solubility enhancer and second as a handle for immobilization of the PNA (via the amino group on the lysine side chain) on the electrode.

3.1.1 Preparation of stock solution

The stock solutions to use in each step were prepared. The stock solutions consist of three solutions which were called stock solution #1, stock solution #2 and stock solution #3. The compositions of each solution are shown below.



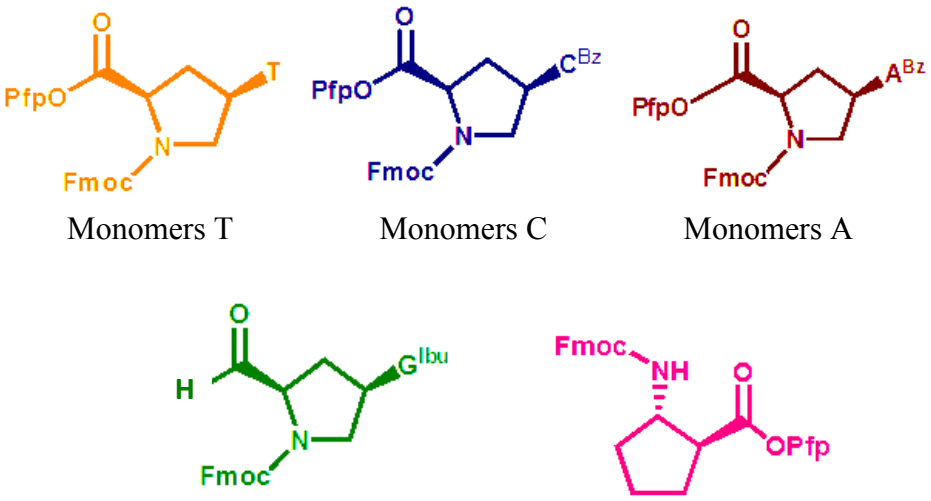
DBU (1, 8-diazabicyclo-[5, 4, 0]-undec-7-ene)

DMF (N, N-dimethylformamide)

DIEA (N, N diisopropylethylamine)

HOAt (hydroxyl-7-azabenzotriazole)

There are five base monomers that were used to synthesize the acpcPNA probe (Figure 3.1). The monomers are **T**, **C**, **A, G** and **X**, as illustrated below. These monomers were supplied by Dr. Tirayut Vilaivan.



Monomers T Monomers C Monomers A

Monomers G Monomers Spacer

Figure 3.1 The structure of monomers **T**, **C**, **A, G** and **X** (Spacer)

The monomer weights of each base which were used to synthesize acpcPNA are presented as follows:

Monomer T pfp 3.77 mg (Thymine)

Monomer C pfp 4.30 mg (Cytosine)

Monomer A pfp 4.44 mg (Adenine)

Monomer G OH 3.50 mg + HATU 2.2 mg + Stock 2 30 µL, 40 min. (Guanine)

Monomer X 3.10 mg (spacer)

3.1.2 Synthesis procedure

The PNA was synthesized on a Tentagel resin equipped with Rink amide linker by standard Fmoc solid-phase peptide synthesis following our previously described protocol [36]. The synthesis procedure consisted of deprotection, coupling and capping step. After the last step, which was the capping step, the synthesis cycle was repeated until the desired probe sequence was obtained. The synthesis procedures are illustrated in Figure 3.2.

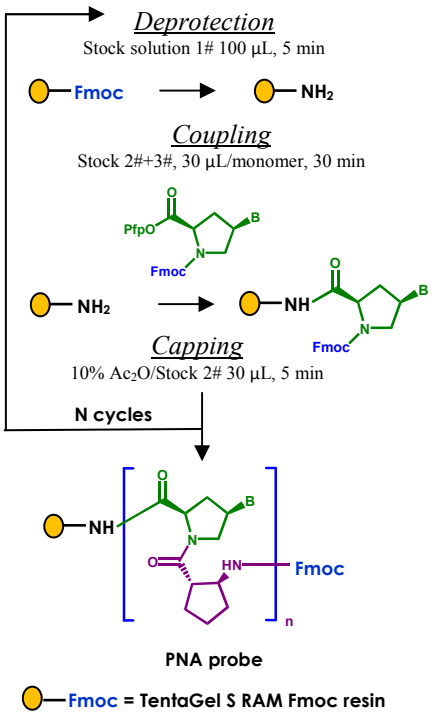


Figure 3.2 Schematic illustration of the synthetic procedures of acpcPNA probe

3.2 Labeling of the PNA probe

After receiving the PNA probe, next step was the labeling of PNA probe. In this work, an anthraquinone (AQ) was used as a redox-active label of the PNA probe by acylation reaction.

3.2.1 Modification of starting reagent

The commercially available 1-hydroxy-9,10-anthraquinone was used as a starting reagent, which was converted to 1-carboxymethoxyanthraquinone by alkylation with tert-butyl bromoacetate followed by subsequent deprotection. 1-Hydroxyanthra-9,10-quinone (1 eq) (1 mmol), tert-butyl bromoacetate (2 eq) (1 momol) and K_2CO_3 (2 eq) (1 mmol) were mixed together in a round bottom flask in DMF. The reaction was stirred under the temperature of 60 ^oC, and it was monitored by thin layer chromatography (TLC) technique. The tert-butyl ester intermediate was isolated and hydrolyzed with trifluoroacetic acid (TFA). The procedure is illustrated below (Figure 3.3).

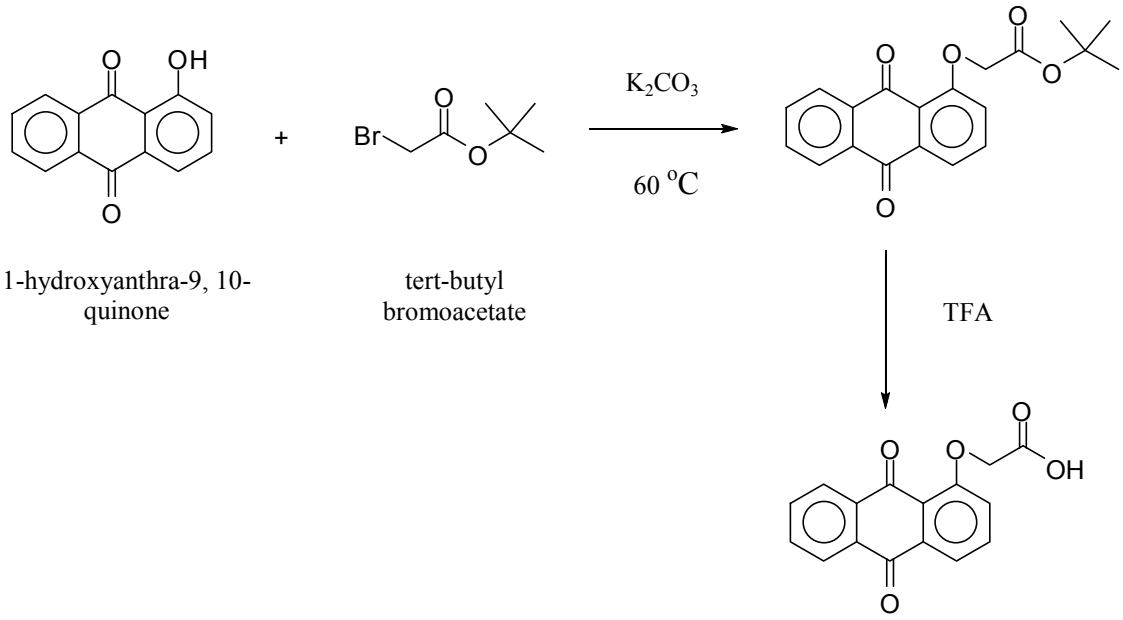


Figure 3.3 Schematic illustration of the modification of 1-hydroxyanthra-9,10-quinone.

3.2.2 Labeling of the acpcPNA with modified anthraquinone [59]

The N-Fmoc-deperotected (0.5 µmol) PNA on the solid support was treated with 1-carboxymethoxyanthraquinone (4 eq), HATU (4 eq), and N, N diisopropylethylamine (DIEA) (6 eq) in dimethylformamide (DMF) to attach the AQ, label at the N-terminus as shown in Figure 3.4. The reaction was left overnight at room temperature. The progress of the reaction was monitored by MALDI-TOF mass spectrometry (MS) on a Microflex MALDI-TOF mass spectrometer (Bruker Daltonics). After the reaction was completed, the modified PNA on the solid support was treated with 1:1 v/v aqueous ammonia:dioxane at 60 ºC overnight in a sealed tube to remove the nucleobase protecting groups. The AQ-labeled PNA probe (PNA-AQ) was next cleaved from the solid support with trifluoroacetic acid and purified by reverse phase HPLC. The identity of the PNA-AQ was verified by MALDI-TOF MS. The purity was confirmed to be >90% by reverse phase HPLC.

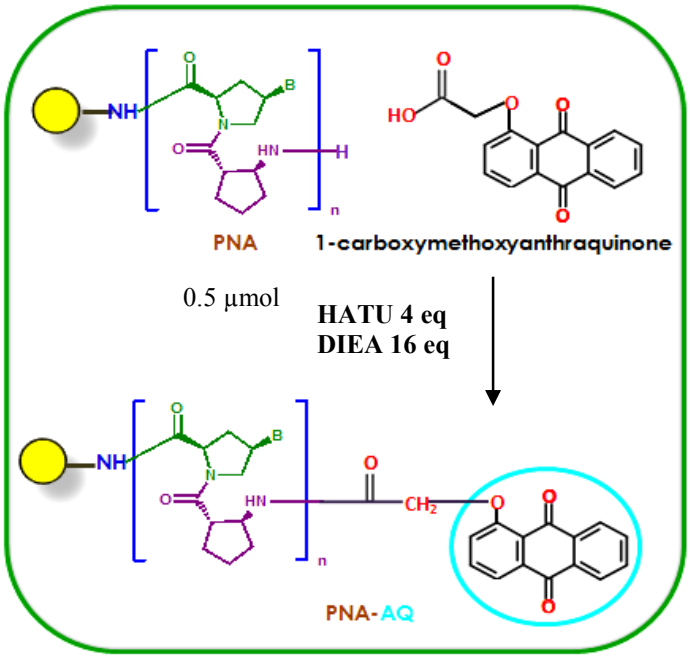


Figure 3.4 Schematic illustration of the labeling of the acpcPNA probe with 1-1-carboxymethoxyanthraquinone by acylation reaction.

3.3 Thermal denaturation study

This experiment was performed to measure the stability of PNA-DNA duplexes. The hybridization carried out by mixing together 1 μM PNA probe and 1 μM targets DNA, in PBS pH 7.4.

The absorbance of the PNA-DNA mixture was determined as a function of temperature using a UV-spectrophotometer at 260 nm. The temperature was applied with the rate of 1 ^{o}C/min.

3.4 Immobilization of PNA-AQ probe

In this work, two methods for immobilization of PNA-AQ probe onto the electrode surface were proposed.

For the first method, the PNA-AQ probe was immobilized onto the electrode surface through carboxy-functionalized screen-printed carbon electrode (SPCE) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) as a coupling agent.

The second method, the PNA-AQ probe was immobilized onto the electrode surface via the amino group on the electrode surface (using chitosan as the introduction of amino group) and the amino group of the lysine side chain on the PNA probe using the glutaraldehyde as a cross linking agent.

3.4.1 Immobilization of PNA-AQ probe onto the electrode surface through carboxy-functionalized SPCE

3.4.1.1 Preparation of screen-printed carbon electrode (SPCE)

3.4.1.1.1 Instruments and equipment

The instruments and equipments involved in the preparation of electrode are listed in Table 3.1.

Table 3.1 List of instruments and equipment for the preparation of electrode.

Instruments	Suppliers
Screen-printed blocks	Chaiyaboon Co., Bangkok, Thailand
Hot air oven	Memmert, USA
Ultrasonic Steri-Cleaner	Sterdy Industrial Company Ltd.
Polyvinylchloride (PVC) substrate	Yingyong plastic glass Company Ltd.

3.4.1.1.2 Chemicals

The chemicals for the preparation of electrode are listed in Table 3.2.

Table 3.2 List of chemicals for the preparation electrode and all solutions.

Instruments	Suppliers
Carbon ink	Electrodag PF-407C, Acheson, California, USA
Silver/silver chloride ink	Electrodag 7019, Acheson, California, USA
Ultrasonic Steri-Cleaner	Sterdy Industrial Company Ltd.
Polyvinylchloride (PVC) substrate	Yingyong plastic glass Company Ltd.
Acetone**,** AR grade	Merck, Germany
Graphite powder particle < 100 μm	Sigma Aldrich
Paint nail (Insulator)	A glossary shop
Diethylene glycol monobutyl ether	Merck
Ethylene glycol monobutyl ether acetate	Merck

Note: Diethylene glycol monobutyl ether and ethylene glycol monobutyl ether acetate were mixed together with the ratio of 1:1 v/v, which was used as binder solution.

3.4.1.1.3 Methodology

For the electrochemical detection, there were various kinds of electrodes that were applied to detect the electrochemical signal of the analytes. In this work, a low

cost screen-printed carbon electrode (SPCE) was used which the pattern was designed by Adobe Illustrator program (Figure 3.5).

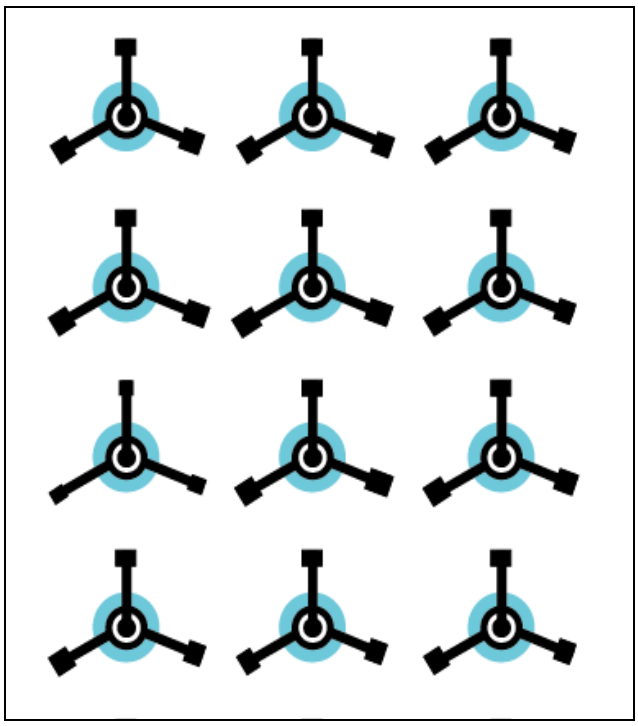


Figure 3.5 Presents the electrode pattern that was designed by Adobe Illustrator program.

3.4.1.1.3.1 The preparation of carbon ink

Ink compositions consisted of the three components as shown below. All components were mixed together with a suitable ratio of carbon ink, graphite powder and binder solution (1g: 0.2 g: 1 mL).

Graphite powder

Carbon ink

Binder solution

3.4.1.1.3.2 The screen-printed blocks

The screen-printed blocks are composed of silver block, carbon block and insulator block as displayed below.

Silver block

Carbon block

Insulator block

3.4.1.1.3.3 Inks for the preparation of electrode

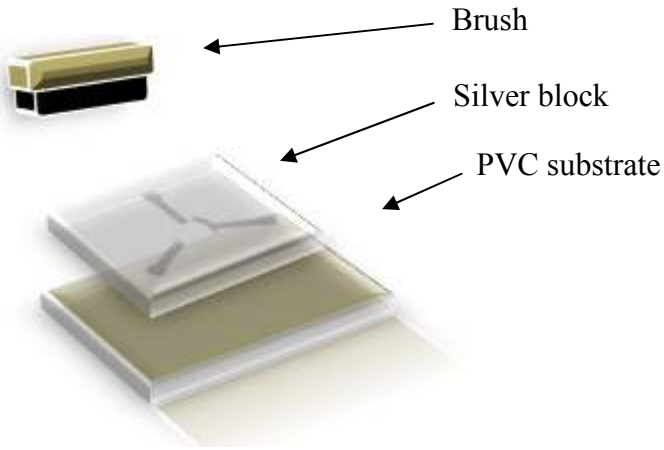
The preparation of SPCE consists of three-inks as exhibited below.

Silver ink Carbon ink (from section 3.4.1.1.3.1) Insulator

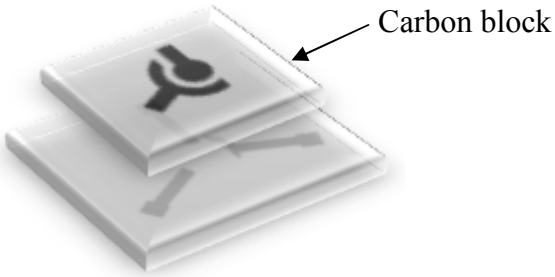
3.4.1.1.3.4 The screening procedure [60]

Carbon inks were mixed with a suitable ratio of carbon ink, graphite powder and binder solution (1 g: 0.2 g: 1 mL). The preparation of the electrode consisted of three steps. First step, the silver ink was printed on PVC substrate as the base layer that was used for the connecting pads and the reference electrode (RE). The next step was the printing of carbon ink on the same substrate as the second layer which was used for the working (WE) and counter electrode (CE). Next, the insulator was printed as the last layer. After each step was performed, the electrode was dried in the oven at 55 ^{o}C for 1 hour. When the completed electrodes were achieved (Figure 3.7), prior to using the electrode, it was tested with the ferri/ferro cyanide solution to confirm the good characteristic of electrode. If the electrode was ready to use, the reversible peak of iron should be obtained. The screening procedure is illustrated in Figure 3.6.

Step I.



Step II.



Step III.

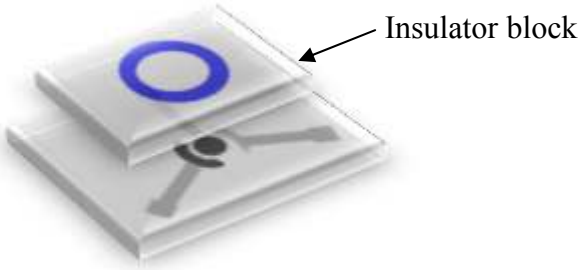


Figure 3.6 The schematic illustration of the screening procedure of the SPCE.

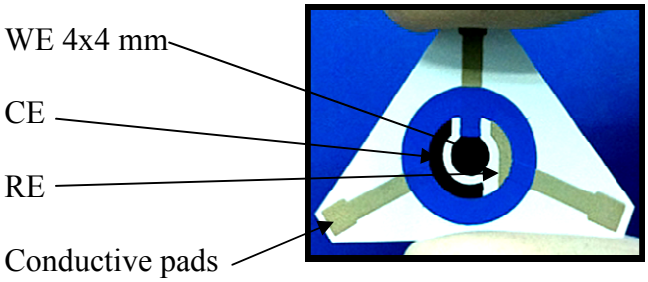


Figure 3.7 The finished SPCE.

3.4.1.2 Characterization of SPCE

When the finished electrodes were obtained, prior to using the electrode, it was tested with the ferri/ferro cyanide solution to confirm the acceptable characteristic of electrode. If the electrode is ready to use, the reversible peak of iron should be obtained.

3.4.1.2.1 Instruments and equipments

The instruments and equipments for the characterization of electrode are listed in Table 3.3.

Table 3.3 List of instruments and equipments for the characterization of electrode.

Instruments	Suppliers
Potentiostat (Autolab PGSTAT 30)	Metrohm, Switzerland
Faraday cage	Copperplate, custom made

3.4.1.2.2 Chemicals

Table 3.4 List of chemicals for the preparation of $[Fe(CN)_6]^{3-}$ solution.

Chemicals	Suppliers
Hexacyanoferrate (III) $[Fe(CN)_6]^{3-}$	Merck
Hexacyanoferous (II) $[Fe(CN)_6]^{4-}.3H_2O$	Merck
Potassium chloride	Merck
Mill-Q water	-

3.4.1.2.3 Preparation of 0.5 M potassium chloride solution

1.86 g of potassium chloride was dissolved in Milli-Q water 50 mL.

3.4.1.2.4 Preparation of 1 mM ferri/ferro cyanide solution

8.2 mg of $[Fe(CN)_6]^{3-}$ and 0.010 mg $[Fe(CN)_6]^{4-}$. $3H_2O$ were dissolved in 50 mL potassium chloride (0.5 M).

3.4.1.2.5 Methodology

A potentiostat (Autolab PGSTAT 30) (Figure 3.8) was used for cyclic voltammetric detection. The CV was performed at a scan rate of 100 mV s^{-1}, and the potential was scanned from –0.6 V to 1.2 V vs Ag/AgCl.

Figure 3.8 A potentiostat (Autolab PGSTAT 30)

Note: All electrochemical measurements were performed with a PGSTAT 30 potentiostat (Metrohm Siam Company Ltd.), and controlled with the General Purpose Electrochemical System (GPES) software.

3.4.1.3 The immobilization and hybridization of PNA-AQ probe

3.4.1.3.1 Chemicals

The chemicals for the immobilization and hybridization of PNA-AQ probe are listed in Table 3.5.

Table 3.5 List of chemicals for the preparation of all solutions.

Chemicals	Suppliers
EDC, AR grade	Merck, Germany
Sodium hydroxide, AR grade	Sigma Aldrich
Hexacyanoferrate (III) $[Fe(CN)_6]^{3-}$	Merck
Dopamine chloride, AR grade	Merck
Hydrochloric acid, AR grade	Merck
Sodium chloride (NaCl), AR grade	Sigma

Potassium dihydrogen phosphate ($KH_2P\ O_4$), AR grade	Sigma Merck
Disodium hydrogen phosphate ($Na_2HP\ O_4$), AR grade	Merck
Potassium chloride (KCl), AR grade	Merck
Milli-Q water	-

3.4.1.3.2 Preparation of phosphate buffer solutions (PBS) pH 7.4

8.15 g NaCl, 0.27 g KH_2PO_4, 1.41 g Na_2HPO_4 and 0.154 KCl were dissolved in 1000 mL Milli-Q water. The pH was adjusted with phosphoric acid or sodium hydroxide. PBS buffer of pH 7.4 contained 140 mM NaCl, 2 mM KH_2PO_4, 10 mM Na_2HPO_4, and 2 mM KCl. For PBS of pH 6, it was adjusted with conc. hydrochloric acid. PBS pH 7.4 in all experiments was prepared with the same protocol.

3.4.1.3.3 Preparation of PNA and DNA solution

PNA and DNA stock solutions were prepared with daily fresh Milli-Q water and were kept frozen prior to use. More diluted PNA and DNA solutions were prepared in PBS pH 7.4. The concentration of each stock solution was determined spectrophotometrically from the calculated molar extinction coefficients at 260 nm (ε_{260}).

3.4.1.3.4 Preparation of dopamine stock solutions (1 mM)

Dopamine solution was prepared in PBS buffer pH 6. 10 mg of dopamine chloride was dissolved in 50 mL of PBS pH 6. Other concentrations of dopamine solution were prepared by dilution of the dopamine stock solution.

3.4.1.3.5 Preparation of sodium hydroxide solution (1 M)

Sodium hydroxide solution was prepared in Milli-Q water. 2 g sodium hydroxide was dissolved in 50 mL Milli-Q water. Other concentrations of sodium hydroxide solution were prepared by dilution of the 1 M sodium hydroxide stock solution.

3.4.1.3.6 Preparation of EDC solution (10 mM)

0.19 g EDC was dissolved in 100 mL Milli-Q water.

3.4.1.3.7 The immobilization and hybridization procedure

This step is an important that is the immobilization and hybridization of the PNA probe onto the electrode surface. First, the electrode was pretreated with 0.5 M NaOH solution at 1.4 V for 180 s to generate the carboxylate groups on the electrode surface, which was used to immobilize the PNA probe. This was followed by the EDC (10 mM) coupling step and was left for 30 minutes. Then, PNA-AQ (20 µM) probe solution was dropped onto the electrode surface and the reaction was left more than 8 hours at room temperature. Finally, target DNA (20 µM) solution was dropped onto the modified electrode, and left 10 minutes for hybridization. After each step, the electrode was rinsed twice with PBS buffer. The immobilization and hybridization procedure are summarized in Figure 3.9.

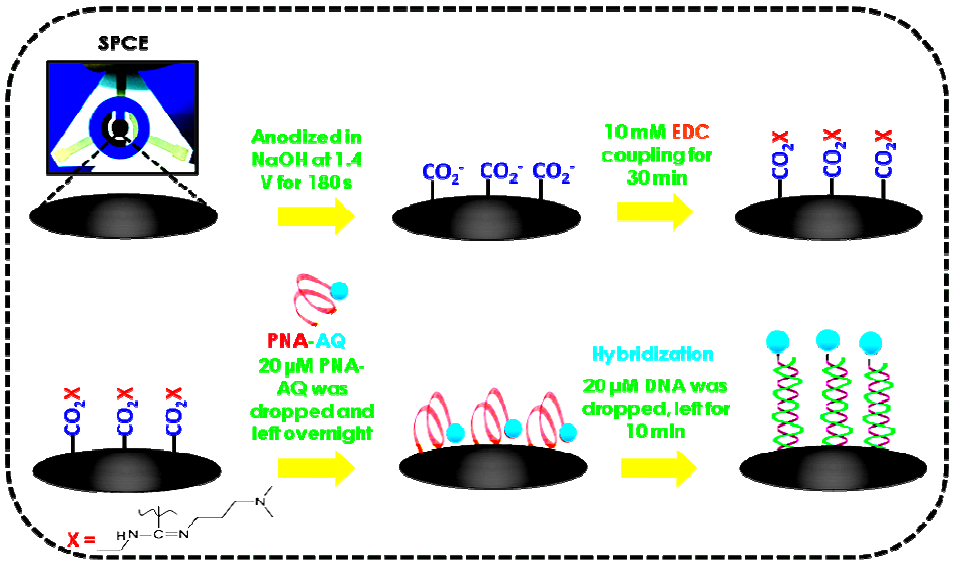


Figure 3.9 Schematic illustration of the immobilization and hybridization procedure of PNA-AQ probe

3.4.1.3.8 Methodology

A potentiostat (Autolab PGSTAT 30) was used to perform square-wave voltammetric (SWV) experiments. SWV was scanned in the potential range from -1.6 V to 0 V vs. Ag/AgCl. Parameters: pulse amplitude of 50 mV, frequency of 30 and a step potential of 30 mV.

All parameters which affect the immobilization of PNA-AQ onto the electrode surface were investigated. The parameters are pretreatment time, pretreatment potential, and sodium hydroxide concentration. The presence of carboxylate group on the electrode surface influences the immobilization of PNA-AQ probe which is consequently affected by these parameters. The dopamine current was used to confirm the presence of caboxylate group on the electrode surface.

3.4.2 Immobilization of PNA-AQ probe onto the electrode surface using chitosan polymer

3.4.2.1 Preparation of chitosan-modified screen-printed carbon electrode (CHT-SPCE)

3.4.2.1.1 Instruments and equipments

The instruments and equipments involved in the preparation of electrode are listed in Table 3.6.

Table 3.6 List of instruments and equipments for the preparation of CHT-SPCE.

Instruments	Suppliers
Screen-printed blocks	Chaiyaboon Co., Bangkok, Thailand
Hot air oven	Memmert, USA
Ultrasonic Steri-Cleaner	Sterdy Industrial Company Ltd.
Polyvinylchloride (PVC) substrate	Yingyong plastic glass Company Ltd.

3.4.2.1.2 Chemicals

The chemicals for the preparation of CHT-SPCE are listed in Table 3.7.

Table 3.7 List of chemicals for the preparation of CHT-SPCE.

Chemicals	Suppliers
Carbon ink	Electrodag PF-407C, Acheson, California, USA
Silver/silver chloride ink	Electrodag 7019, Acheson, California, USA
Graphite powder particle < 100 μm	Sigma Aldrich
Acetone	Merck, Germany
Chitosan flake with 90%DD (Mw = 15 kDa)	Seafresh Chitosan (Thailand)
Glacial acetic acid 100%, AR grade	Merck
Paint nail (insulator)	A glossary shop

3.4.2.1.3 Preparation of 4%w/v Chitosan solution

The preparation of chitosan solution is described; 0.2 g of chitosan flake was dissolved in 5 mL of 1% acetic acid. The solution was stirred over night at room temperature to ensure that the chitosan was completely dissolved.

3.4.2.1.4 Methodology

3.4.2.1.4.1Ink compositions

Ink compositions consisted of four components as shown below.

Graphite powder

Carbon ink

Chitosan solution 4% w/v **Binder solution**

4% w/v chitosan solution, carbon ink and binder solution were mixed together with the ratio of 0.35 g (0.4%): 0.2 g: 1 g: 1.5 mL, respectively.

3.4.2.1.4.2 The screen-printed blocks

The screen-printed blocks were composed of silver block, carbon block and insulator block as displayed below.

Silver block Carbon block

Insulator block

3.4.2.1.4.3 The screening procedures

The preparation of CHT-SPCE was performed with the same procedure as described before (see 3.4.1.1.3.4)

3.4.2.2 Immobilization and hybridization of PNA probe

3.4.2.2.1 Chemicals

Table 3.8 List of chemicals for the preparation of all solutions in the immobilization and hybridization step.

Chemicals	Suppliers
Synthetic DNA oligonucleotides, related to HPV DNA type 16	Science (Bangkok, Thailand).
Glutaraldehyde (70% in water. Biochemica)	Fluka
Sodium chloride (NaCl), AR grade	Merck
Potassium dihydrogen phosphate ($KH_2P\ O_4$), AR grade	Merck
Disodium hydrogen phosphate (Na_2HPO_4), AR grade	Merck

Potassium chloride (KCl), AR grade	Merck
Mill-Q water	-

3.4.2.2.2 Preparation of phosphate buffer solutions (PBS)

PBS buffer of pH 7.4 contained 140 mM NaCl, 2 mM KH_2PO_4, 10 mM Na_2HPO_4, and 2 mM KCl.

8.15 g NaCl, 0.27 g KH_2PO_4, 1.41 g Na_2HPO_4 and 0.154 KCl were dissolved in 1000 mL Milli-Q water. The pH was adjusted with phosphoric acid or sodium hydroxide.

3.4.2.2.3 Preparation of PNA solutions

PNA and DNA stock solution were prepared with daily fresh Milli-Q water and was kept frozen prior to use. More diluted PNA and DNA solutions were prepared in phosphate buffer (PBS) pH 7.4.

3.4.2.2.4 The immobilization and hybridization procedures

PNA-AQ probe was covalently immobilized onto the electrode surface using the following procedure. A 10 μL of 5 % aqueous solution glutaraldehyde (as cross-linking agent) was dropped onto the electrode surface and incubated at 40 ^{o}C for three hours and the electrode was washed with PBS pH 8 for three times. 5 μL of PNA-AQ probe solution (15 μM) was pipetted onto the electrode surface and was left at room temperature overnight. The electrode was kept in a small box to prevent the evaporation of solution. Before the hybridization of PNA probe, the washing step was performed with PBS pH 7.4 twice to remove the excess and nonspecifically adsorbed PNA-AQ probe on the electrode surface. The modified electrode was hybridized with the target DNA for 10 minutes by dropping 5 μL of the target DNA (15 μM) solution onto the electrode surface and the electrode was rinsed with PBS buffer pH 7.4. The immobilization and hybridization procedures are illustrated as shown in Figure 3.10.

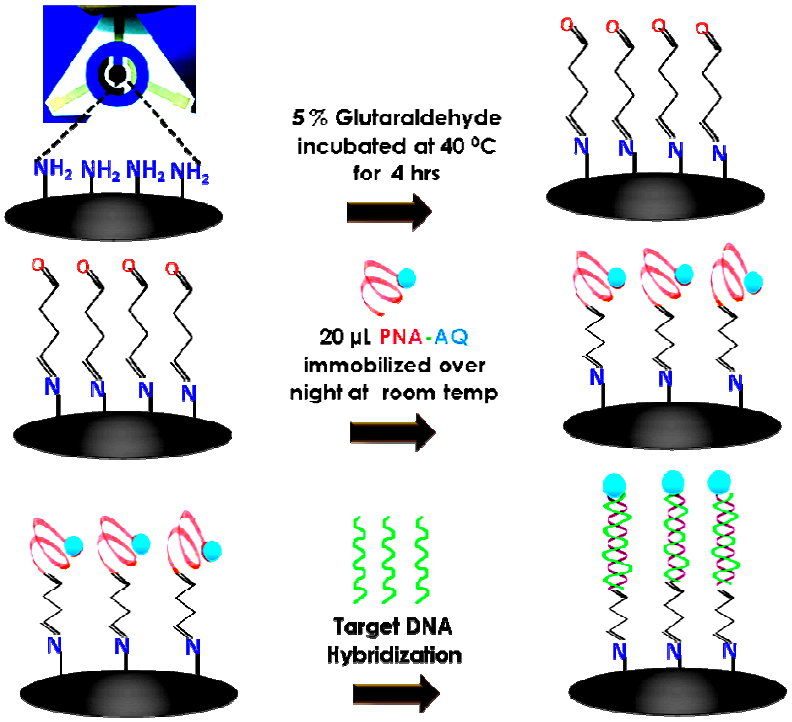


Figure 3.10 The schematic illustration of the immobilization and hybridization of PNA-AQ probe onto CHT-SPCE.

3.4.2.2.5 Methodology

A potentiostat (Autolab PGSTAT 30) was used to perform square-wave voltammetric (SWV) experiments. SWV was scanned in the potential range from -1.6 V to 0 V vs. Ag/AgCl. Parameters: pulse amplitude of 50 mV, frequency of 30 and a step potential of 30 mV.

3.5 Optimization of the experimental conditions

3.5.1 Effect of the graphite amounts

The graphite amounts affect the sensitivity of electrode. Thus, this factor was investigated. The ink preparation step was prepared with the same procedure. Various amounts of graphite powder (0.5, 0.3, 0.2, 0.1 and 0.05 g.) were mixed into the ink in the preparation step.

3.5.2 The immobilization and hybridization of PNA-AQ probe onto electrode surface

3.5.2.1 Variation of the chitosan amounts

The preparation of chitosan solution and the carbon ink were prepared with the same procedure as described before (see 3.4.2.1.3, 3.4.2.1.4.). The chitosan amounts of 1%, 2%, 3%, 4% and 5% w/v were varied to modify the carbon ink. The final concentrations of chitosan in carbon are 0.1, 0.2, 0.3, 0.4 and 0.5% respectively. We expected that when the CHT amount was increased, the $-NH_2$ groups on the electrode surface would also increase which will affect the immobilization of probe onto electrode surface. The modified-SPCE was also examined with 1 mM $[Fe(CN)_6]^{3-/4-}$ solution. Two types of polymer including CHT and polyethylenemine (PEI) (Mw = 2 kDa), were used to compare its role in the modification of SPCE.

3.5.2.2 Variation of the percent of glutaraldehyde cross-linking agent

Effects of different ratios of glutaraldehyde were examined. The stock solution of glutaraldehyde is 70% w/v in water. The 4, 5, 6, 7 and 8% w/v of glutaraldehyde solutions were prepared by dilution from the stock solution.

3.5.2.3 Variation of the hybridization time

Hybridization time affected the probe signal after it was hybridized with the DNA target. Hence, the hybridization time was one of parameter that needs to study. The hybridization times of 5, 10, 15, 20, 25 and 30 minutes were varied. Electrochemical detection was performed at frequency of 30 Hz, amplitude of 50 mV and step potential of 30 mV in PBS pH 7.4.

3.5.2.4 Methodology

The electrochemical measurement obtained with a square-wave voltammetric (SWV) method. SWV was scanned in the potential range from -1.6 V to 0 V VS. Ag/AgCl.

3.6 Optimization of the electrochemical detection

After the immobilization steps, the electrochemical parameter was then studied to obtain the optimal conditions.

The effects of frequency, amplitude, and step potential on electrochemical detection were investigated. The electrochemical parameters affected the kinetic rate of electroactive species on the redox reaction. It meant that we have to optimize a suitable condition for each parameter. The sensitivity and peak shape of a redox-active label were dependent on these parameters.

The various frequencies, amplitudes and step potentials were studied. This study was carried with a potentiostat (Autolab PGSTAT 30) as well as electrochemical detection was performed by a square-wave voltammetry.

3.7 Calibration curve

After the optimal parameters were achieved, next step was the creation of the calibration curve.

The calibration curve was plotted between the changed current (ΔI) of PNA probe signal and various DNA concentrations. The 15 μM probe concentration was selected to immobilize onto the electrode surface because it was the lowest concentration that the modified-electrode could provide the signal.

3.8 Analytical performance

3.8.1 Linearity

The hybridization signal between PNA-AQ probe and the different concentrations of DNA target (0.02-12 μM) was analyzed by SWV under the optimal condition. The average of triplicate measurements was used to plot calibration curve which linear range can be obtained.

3.8.2 Limit of detection (LOD)

The limit of detection (LOD) was determined statistically from the calibration curve in the range of 0.02-12 μM and calculated from $3S_{blank}$ /S where S_{blank} was the standard deviation of blank measurement (n=3) and S was the slope of the linearity or the sensitivity of the method.

3.8.3 Limit of quantitation (LOQ)

The limit of quantitation (LOQ) was determined statistically from the calibration curve in the range of 0.02-12 μM and calculated from $10S_{blank}$ /S where S_{blank} was the standard deviation of blank measurement (n=10) and S was the slope of the linearity or the sensitivity of the method.

3.8.4 Repeatability

The repeatability was studied by ten replicate measurements of standard DNA solution (3, 6 and 9 μM). The repeatability was assessed in terms of the relative standard deviation (% RSD), using the following formula:

$$\% \text{ RSD} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

3.9 The selectivity of the acpcPNA probe

In this proposed method, the selectivity of acpcPNA-AQ sensor probe was also studied. The chemicals for this study are listed in the Table 3.9.

3.9.1 Chemicals

Table 3.9 List of chemicals for the preparation of DNA solution.

Chemicals	Suppliers
Type 16 : 5'-GCTGGAGGTGTATG-3',	Pacific Science (Bangkok, Thailand)
Type 18 : 5'- GGATGCTGCACCGG-3'	Pacific Science (Bangkok, Thailand)
Type 31 : 5'-CCAAAAGCCCAAGG-3'	Pacific Science (Bangkok, Thailand)
Type 33 : 5'-CACACAAGTAACTA-3'	Pacific Science (Bangkok, Thailand)
Hydrochloric acid, AR grade	Merck
Sodium chloride (NaCl), AR grade	Sigma
Potassium dihydrogen phosphate ($KH_2P\ O_4$), AR grade	Sigma
Disodium hydrogen phosphate ($Na_2HP\ O_4$), AR grade	Merck
Potassium chloride (KCl), AR grade	Merck
Mill-Q water	-

DNA type 16 is a complementary DNA to the PNA probe and therefore used as target DNA. Other types of DNA were used as non-complementary DNA to study the selectivity of the PNA probe including type 18, 31 and 33.

3.9.2 Preparation of various DNA concentrations

All DNA solutions were prepared in PBS pH 7.4 by dilution from the stock solution. The concentration of each sample was 15 μM.

3.9.3 Preparation of PBS pH 7.4

(See in 3.4.2.2.2)

3.9.4 Methodology

All electrochemical measurements were obtained with a SWV. SWV was scanned in the potential range from -1.6 V to 0 V VS. Ag/AgCl.

3.10 Detection of HPV DNA type 16 in cell-lines sample

3.10.1 Instruments and equipments

The instruments and equipments for detection of HPV DNA type 16 in cell-lines sample are listed in table 3.10.

Table 3.10 List of instruments and equipments for the detection of HPV DNA type 16 in cell-lines.

Instruments/equipments	Suppliers
Nanodrop 2000c Spectrophotometer	Thermo Sciencetific
PCR machine, eppendof vapo.protect	Eppendof, Ltd. Thailand
EnDURO power Supplies (Agarose Gel)	Labnet Internation. Ltd.
Syngene bio imaging	In Genius, Thailand

3.10.2 Chemicals

Table 3.11 List of chemicals for the detection of HPV DNA type 16 in cell-lines.

Chemicals	Suppliers
Primer forward 5' cactattttggaggactgga 3' T_m 50 ^oC	Pacific Science (Bangkok, Thailand)
Primer reverse	Pacific Science (Bangkok, Thailand)

5' gccttaaatcctgcttgtag 3' T_m 50 ^oC	
Cell-line SiHa HPV type 16	Supplied by my Co-advisor
Cell-line cervical cancer C33a	Supplied by my Co-advisor
dNTP	Jena Bioscience
Taq polymerase enzyme	Jena Bioscience

3.10.3 Sample preparation

In this present work, cell-line DNA sample (SiHa) was amplified by polymerase chain reaction (PCR) to increase the amounts of DNA. For performing the PCR, to obtain the optimal condition we need to prior test the condition of PCR procedure. The target range of PCR product is in L1 gene that composed of 243 base pairs (bp). The PCR conditions are listed in the Table 3.12

Table 3.12 The test conditions for PCR

Chemicals	Stock	[conc.]	use/µl
Primer forward	20 µM	0.4 µM	0.2
Primer reverse	20 µM	0.4 µM	0.2
Cell-line (SiHa) HPV type 16	-	50 ng/µL	1
dNTPs	10 mM	0.2 mM	0.2
buffer 10x + 15 mM $MgCl_2$	-	-	1
Taq polymerase Enzyme	5 unit/µL	0.5 U	0.1
H_2O	-	-	7.2

Annealing temperatures (T_m) of 48, 50 and 52 ^oC were selected as the test conditions. The amplification was performed with 35 cycles of PCR. The procedure consists of three steps as following [61].

Denaturation step: 95 ^oC, 10 min

Annealing step: 95 ^oC, 30 sec $\longrightarrow$ (48, 50, 52 sec), 30 sec

Extension/elongation step: 72 ^oC, 30 sec

Final elongation: 72 ^oC, 7 min

The obtained PCR products were analyzed with 2% agarose gel electrophoresis which was prepared as previous protocol [62]. This technique is employed for size separation. The sizes of PCR products were determined by comparison with a DNA ladder (0.5 μL) (a molecular weight marker), which contains DNA fragments of known size, run on the gel alongside.

Before the electrochemical detection, PCR product was denatured in 0.5 M NaOH (50-100 μL) with 1.5 M NaCl and incubates at room temperature for 5 minutes [63, 64, 67]. Finally, denatured DNA was neutralized with sodium acetate and pipetted to hybridize with probe-modified electrode. The cervical cancer cell-line C33a was used as the negative control to compare with the positive control (SiHa) for electrochemical detection.

CHAPTER IV

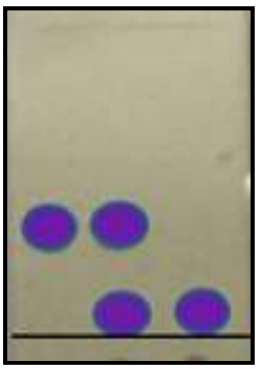
RESULTS AND DISCUSSION

In this work, the newly developed method based upon pyrrolidinyl peptide nucleic acid (acpcPNA) as a sensor probe for detection of HPV DNA type 16 was proposed. The results and discussion of all experiments were illustrated in this chapter. The proposed method was successfully applied to detect HPV DNA type 16 in cell-line samples. It is hoped that this detection platform will be applicable as an efficient and inexpensive tool in screening for the HPV-DNA type 16 in the primary stage of cervical cancer.

4.1 Synthesis of a carboxy functionalized 1-Hydroxyanthra-9,10-quinone

The commercially available 1-hydroxyanthra-9,10-quinone was used as the starting material. Before the labeling of PNA probe, it was converted to 1-carboxymethoxyanthraquinone by alkylation with tert-butyl bromoacetate. This will install a carboxyl group that would later form an amide bond with the PNA probe. The reaction was monitored by thin layer chromatography (TLC) as shown in Figure 4.1. The successful modification was confirmed by proton nuclear magnetic resonance (NMR) in Figure 4.2.

According to Figure 4.1, the result shows the desired product has formed after the reaction was set under a suitable condition. The product showed R_f of 0.093 (1:4 EtoAc: Hexane), which was very different from the starting reagent. The product can be used for the next step without further purification because the reaction was completed.



S M P

Figure 4.1 Comparison of TLC results with different R_f of starting reagent (S) and product (P).

S; represent starting reagent

M; represent mixture of starting material and product

P; represent the product

Figure 4.2 displays ^{1}H NMR spectra of the obtained product. Proton signal of the methylene group appeared at the chemical shift of 5 ppm, OH group showed chemical shift spectra at 13 ppm and ^{1}H-NMR of aromatic rings presented at the chemical shift from 7.5 to 8.2 ppm. This data can confirm the expected product structure.

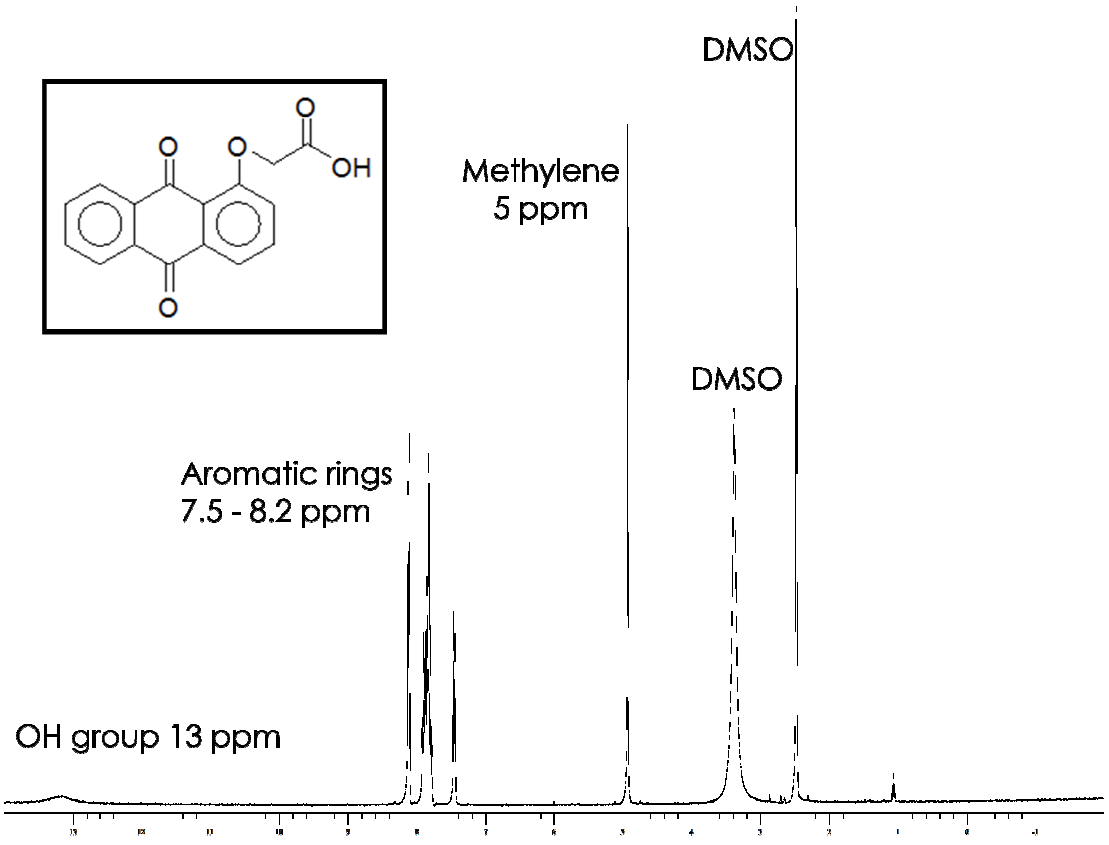


Figure 4.2 ^{1}H-NMR of the modified structure [53].

4.2 The characterization of the unlabeled and labeled PNA probe

In this section, confirmation of the successful synthesis of the unlabeled and labeled PNA probes was described. The unlabeled and labeled PNA probes were characterized by MALDI-TOF mass spectrometry. This technique not only confirmed the successful synthesis of PNA probe but also verified the stability of AQ moiety after the labeling. As shown in Figure 4.3, a 14-mer PNA probe of the unlabeled probe with a sequence of CGACCTCCACATAC showed a mass peak at m/z 4644.60 (calcd ± 2 m/z). After labeling, the mass increased to m/z 4909.76 (calcd ± 2 m/z). The increment of PNA mass coincides with the AQ-label. Therefore, successful labeling of the PNA probe with the electroactive label had been achieved.

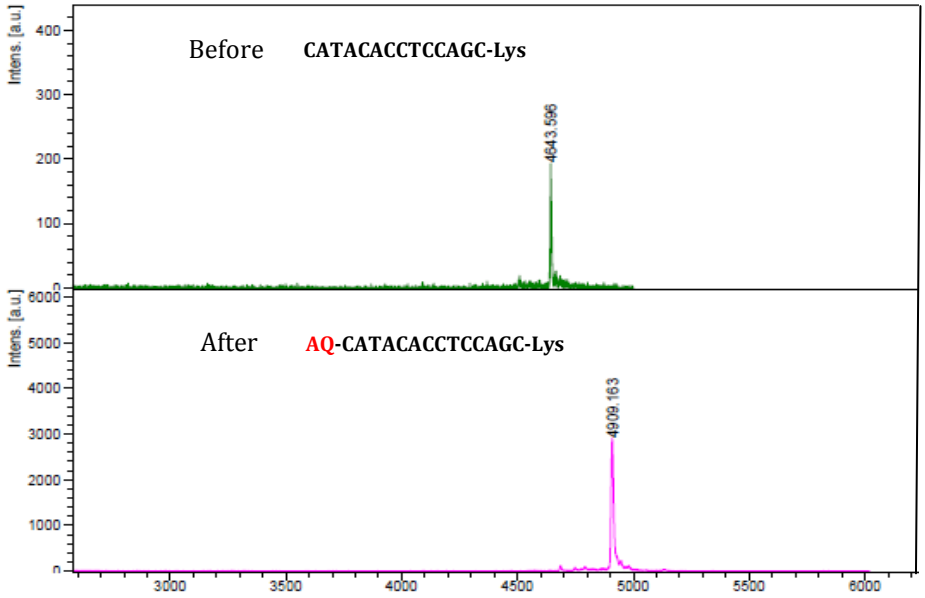


Figure 4.3 MALDI-TOF mass spectra of PNA probe before and after labeling with AQ.

4.3 Thermal denaturation study of PNA-DNA duplexes

The stability of PNA hybridization was studied by thermal denaturation with a complementary synthetic DNA corresponding to the HPV DNA type 16. The results revealed that the PNA and DNA formed a stable hybrid with a melting temperature (T_m) of 69.9 ^{o}C as illustrated in Figure 4.4.

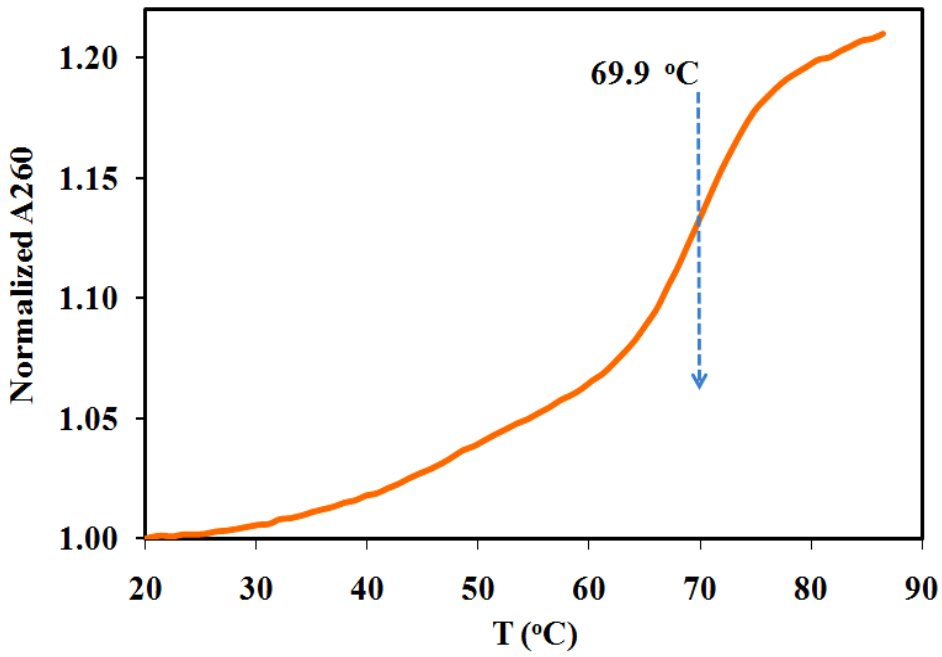


Figure 4.4 Displays the melting temperature (T_m) of PNA-DNA duplexes.

4.4 Characterization of electrode

Prior to using the electrode, it was tested with the ferri/ferro cyanide solution to confirm the acceptable characteristic. If the electrode is ready to use, a reversible peak of iron should be obtained. This meant the electrode has an acceptable characteristic which is ready to use as displayed in Figure 4.5.

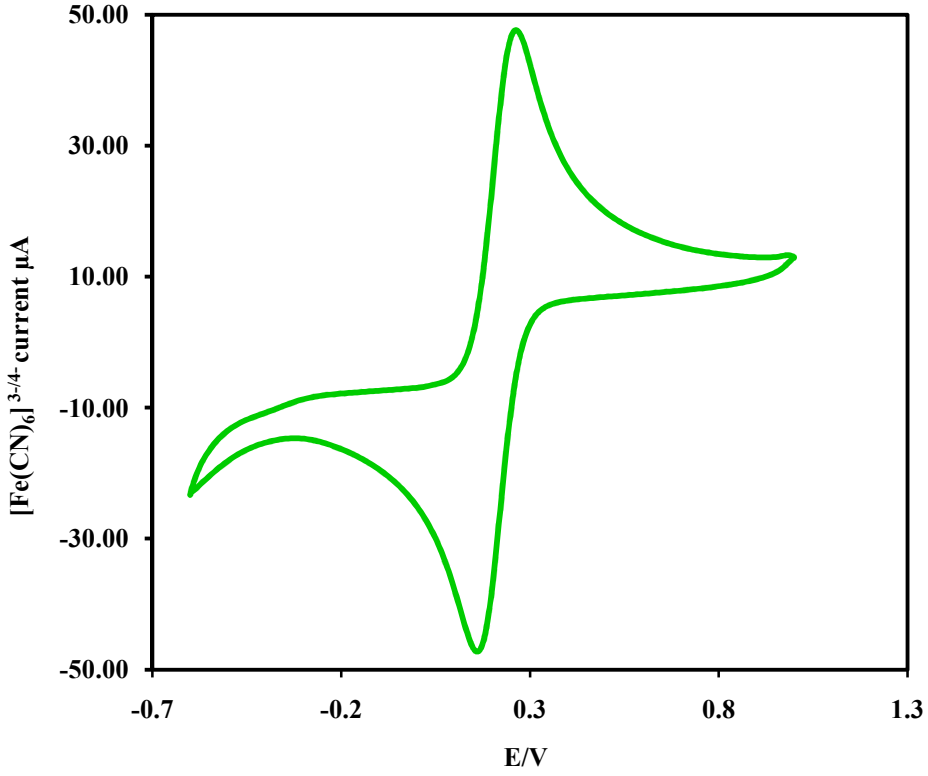


Figure 4.5 Cyclic voltammogram of 1 mM $[Fe(CN)_6]^{3-/4-}$ in 0.5 M KCl at scan rate of 100 mV/s

4.5 The effect of graphite amounts on the sensitivity of SPCE

A low cost screen-printed carbon electrode (SPCE) was used in this study. Three-electrode system was designed by Adobe Illustrator program. The designed pattern is shown in Figure 4.6. The composition of ink can affect the sensitivity of the electrochemical measurement. Hence, we have to optimize a suitable ratio of ink composition. Ink compositions are composed of graphite powder (most effect), binder solution and carbon ink. The propose of this study is to optimize the appropriate amount of graphite powder that yield the highest sensitivity (determined from the $[Fe(CN)_6]^{3-/4-}$ current).

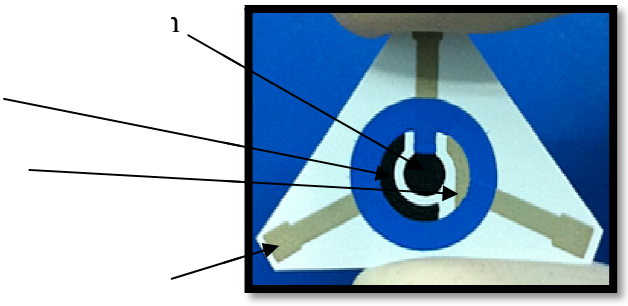


Figure 4.6 The design of three-electrode system of SPCE.

Figure 4.7 displays cyclic voltammograms obtained from 1mM $[Fe(CN)_6]^{3-/4-}$ in 0.5 M KCl using different amounts of graphite powder as the modifier. The result revealed that the current of $[Fe(CN)_6]^{3-/4-}$ is dependent on the amount of graphite. When the amount of graphite powder was increased, the current of $[Fe(CN)_6]^{3-/4-}$ also increased. It reached maximum at 0.2 g of graphite powder (Figure 4.8 plot between current against graphite amounts). When we added 0.3 g and 0.5 g of graphite powder to modify the screen-printed carbon electrode, the currents were slightly decreased. Therefore, the appropriate amount of graphite powder to modify a screen-printed carbon electrode was found to be 0.2 g. The result explained by the maximum distribution of graphite particles and highest surface area on electrode.

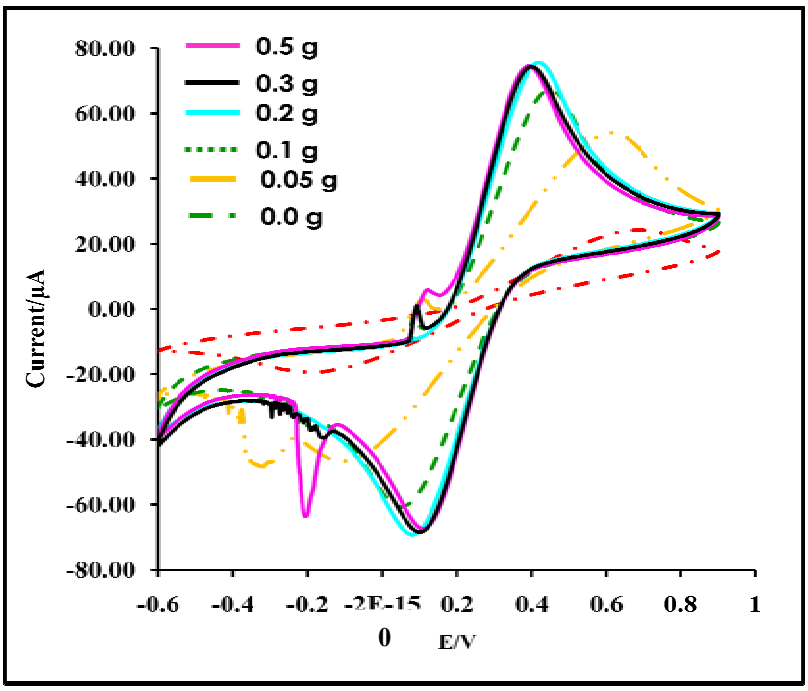


Figure 4.7 Cyclic voltammograms of 1mM $[Fe(CN)_6]^{3-/4-}$ in 0.5 M KCl with different amounts of graphite powder. Scan rate: 100 mV/s.

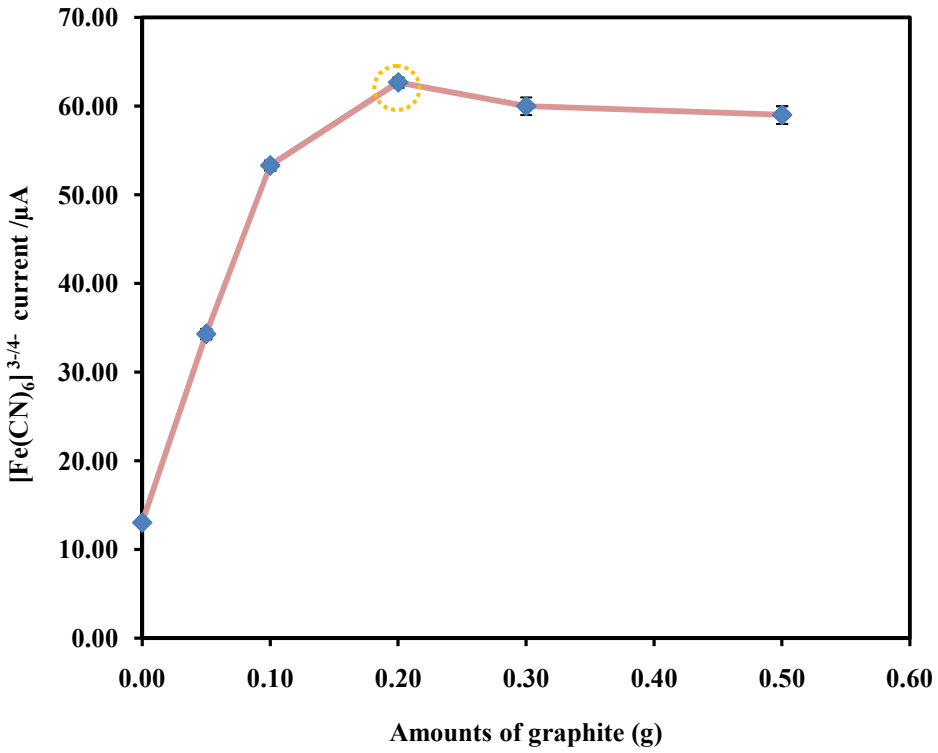


Figure 4.8 The influence of graphite powder amounts on $[Fe(CN)_6]^{3-/4-}$ currents.

4.6 Study of the electrochemical behavior of PNA-AQ probe before the immobilization

The electrochemical behavior of the unimmobilized PNA-AQ probe was studied by square-wave voltammetry. The result was found as provided in Figure 4.9 that a redox-active label peak appears at around -0.6 V. A redox-active label peak was utilized as the analyte peak to confirm the successful immobilization of PNA-AQ sensor probe onto electrode surface and to observe the change or electrochemical respond after hybridization with the DNA target.

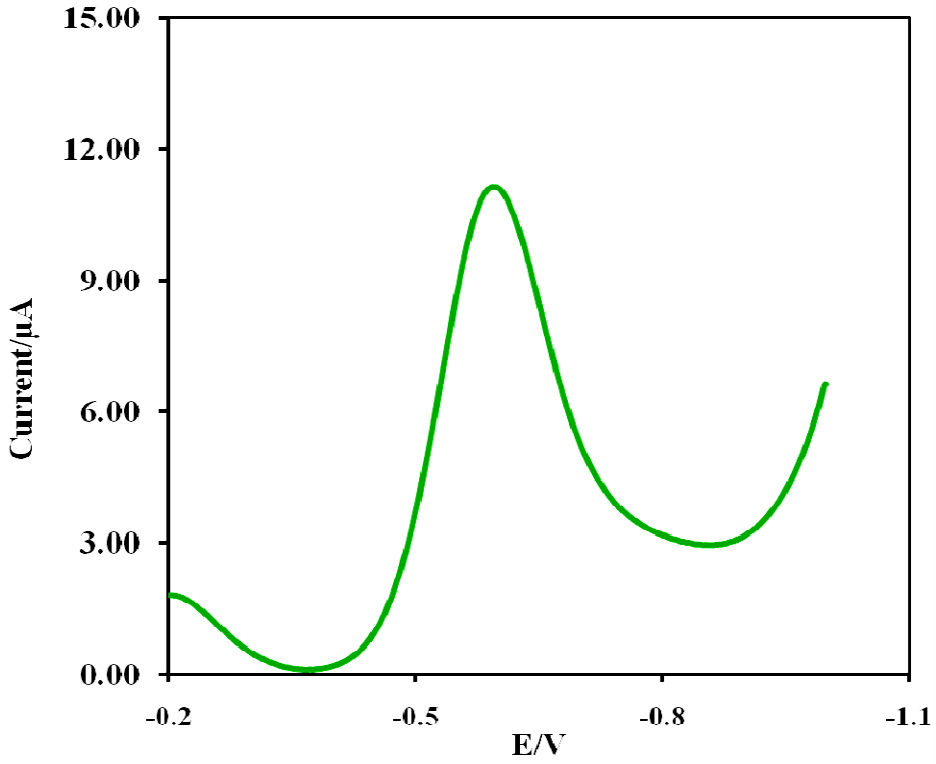


Figure 4.9 A square-wave voltammogram of PNA-AQ probe (50 µM) before the immobilization onto the electrode surface. Parameters: Frequency 25 Hz, amplitude 50 mV and step potential 30 mV, in PBS pH 7.4.

4.7 Immobilization of PNA-AQ probe

As proposed in the chapter III, two immobilization methods of PNA-AQ probe onto the electrode surface was performed and the results are described in this section.

4.7.1 Immobilization of PNA-AQ probe onto the electrode surface through carboxy-functionalized SPCE

In this immobilization method, the PNA-AQ probe was immobilized onto the electrode surface through carboxylate groups on the electrode surface.

4.7.1.1 Confirmation of the presence of carboxylate groups on the electrode surface

The carboxylate electrode was tested with the dopamine solution, which was prepared in acidic condition. The different dopamine (DA) currents between pretreated and non-pretreated electrode was used as the indicator of the presence of carboxylate groups on the surface. When the carboxylate groups were presented, the dopamine current should be increased. The result revealed that, the DA current was increased in the pretreated electrode compared to the non-pretreated electrode (Figure 4.10). The increase of DA currents were explained by attraction of the positive charge of DA molecules being attracted to the electrode surface with the negative charged carboxylate groups on the electrode surface[58], as shown in Figure 4.11.

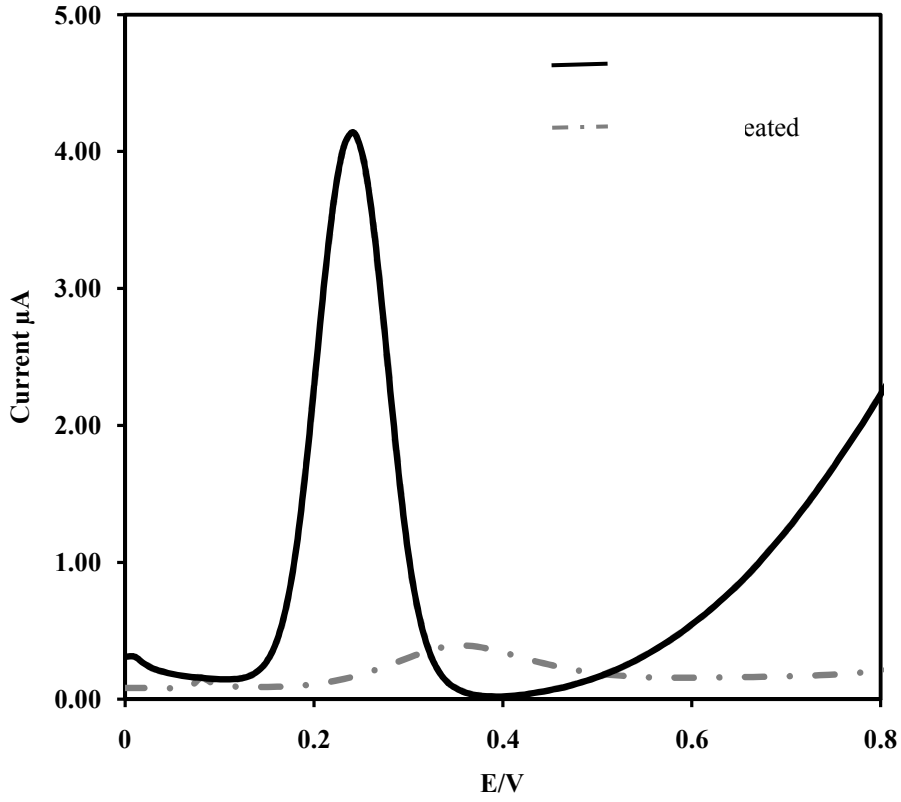


Figure 4.10 Comparison of square-wave voltammogram of 5 µM DA in PBS pH 6.0 between pretreated and non-pretreated electrode. Parameter: scan rate 100 mV, amplitude 40 mV, frequency 10 Hz.

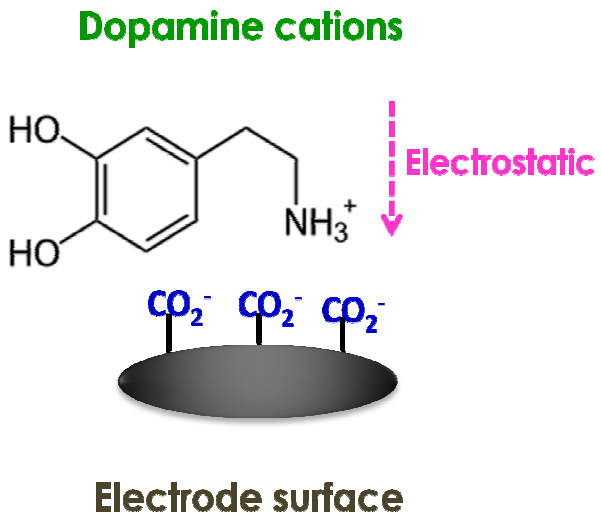


Figure 4.11 Schematic illustration of the interaction between carboxylate anions and positive charge of DA molecules on the electrode surface by electrostatic interaction.

4.7.1.2 Optimization of parameters for the immobilization process

All parameters that may affect the formation of carboxylate group on the electrode surface were studied. Because the amounts of presented carboxylate group on the surface affect the immobilization efficiency.

4.7.1.2.1 Pretreatment potential

The first parameter investigated was the applied potentials. The results showed that the DA currents were increased with increasing applied potentials. The maximum current was obtained at 1.8 V. As displayed in the Figure 4.12, when the applied potential was higher than 1.8 V, the DA currents were slightly decreased. This is due to the overloaded potential and the formation of bubbles on the electrode surface which could cause damage to electrode. Therefore, the applied potential of 1.8 was selected as the pretreatment potential.

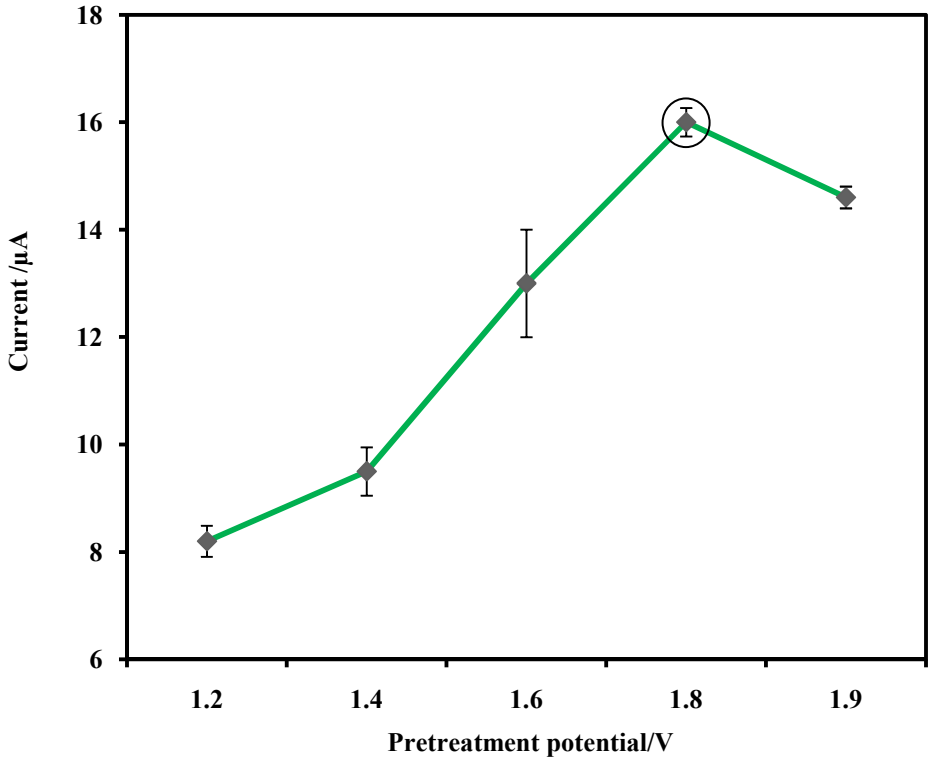


Figure 4.12 Effects of the pretreatment potentials on the DA currents

4.7.1.2.2 Pretreatment time

The second parameter investigated was the treatment time. The pretreatment times of 10, 20, 30, 40 and 50 s were designated to pre-treat the electrode in the pretreatment steps. The result is shown in the Figure 4.13.

According to Figure 4.13, a similar trend of the DA currents to the applied potential effect was obtained. The DA currents were increased with increasing the treatment time. It meant that a lot of carboxylate groups were presented on the electrode surface. The DA currents were upsurged with the intensifying electrostatic attraction interaction between dopamine and carboxylate anions on the surface. Due to this reason, the DA currents were upsurged with increasing the treatment time. At 40 s of pretreatment time causes a highest current of DA. Hence, it was selected as the pretreatment time.

In contrast, at pretreatment times of higher than 50 s, the presence of carboxylic group on the electrode surface was reduced which causes the reducing DA current and the error values reach to maximum. The reasoning for this case is that when the electrode was applied with a long pretreatment time, the electrode surface not only changed into a carboxylate group but other functional groups could also present on the electrode surface. Thus, at pretreatment time higher than 50 s was not suitable for the pretreatment of electrode.

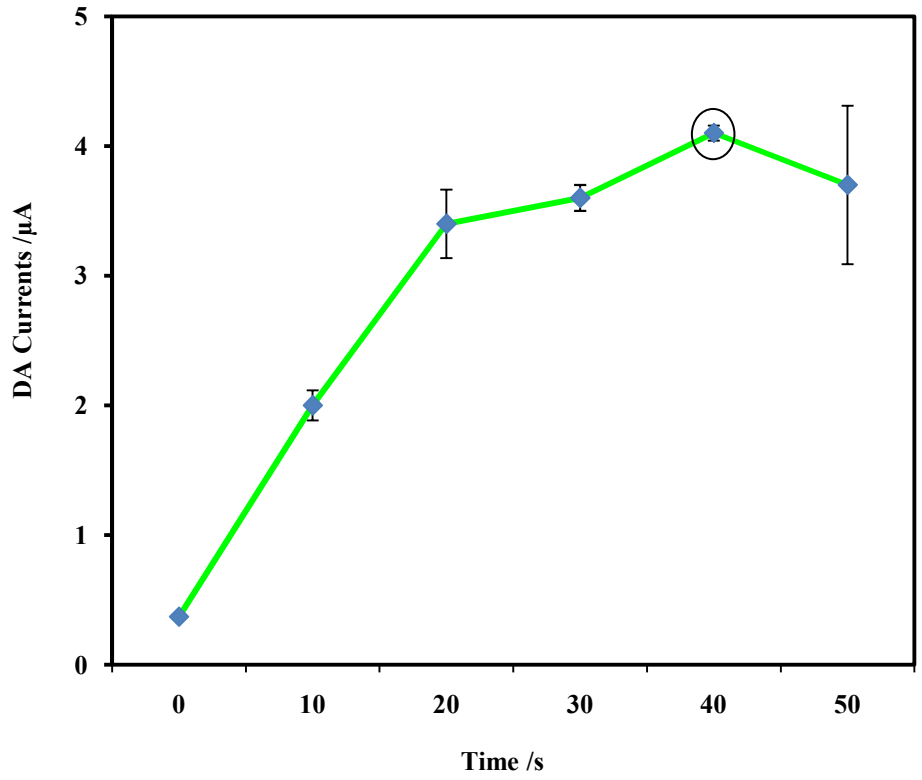


Figure 4.13 Effects of pretreatment times on DA currents, pretreated in 0.5 M NaOH at 1.6 V

4.7.1.2.3 Sodium hydroxide concentration

Sodium hydroxide concentration is one of the factors which may influence the presence of carboxylate groups on the electrode surface.

Figure 4.14 shows that the DA currents were improved with increasing concentration of NaOH. The DA current reached to a maximum at 0.3 M NaOH and became stable at concentrations higher than 0.3 M NaOH. Thus, 0.3 NaOH was chosen for pretreatment of electrode.

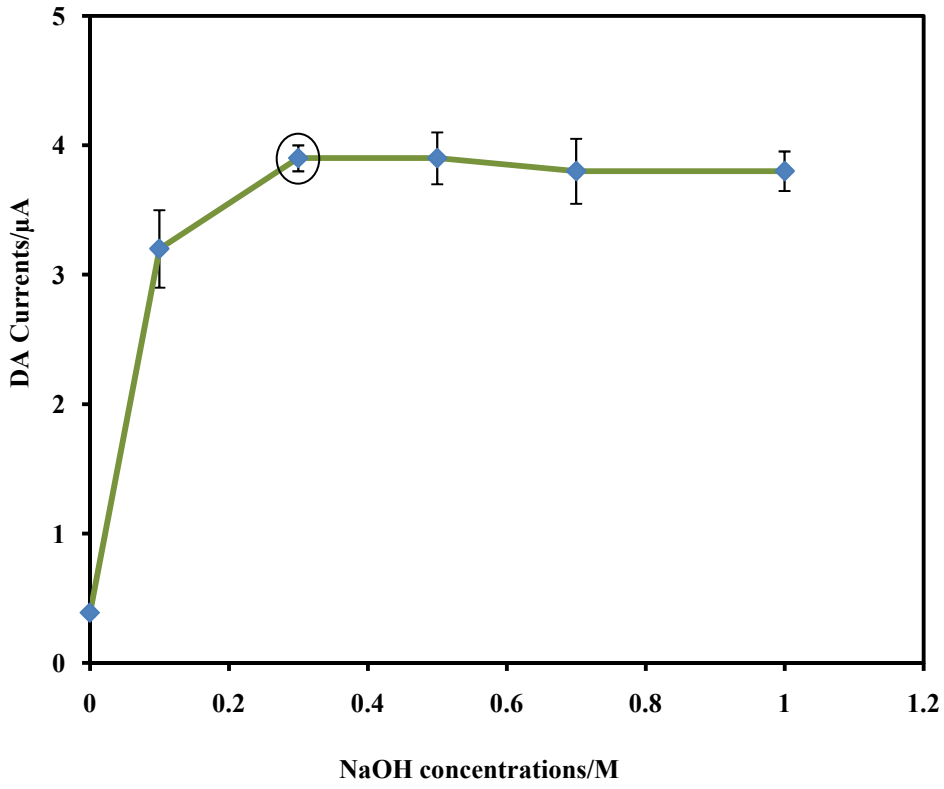


Figure 4.14 Effects of NaOH concentrations on DA currents

The optimum pretreatment condition for SPCEs were found as anodized at 1.8 V in 0.3 M NaOH for 40 s.

4.7.1.3 Immobilization and hybridization of PNA-AQ probe

The successful immobilization of PNA-AQ probe was followed by the redox-label peak of AQ after the immobilizing steps were performed. The hybridization occurred between the 20 µM PNA-AQ probes and the 20 µM synthetic DNA target which was studied by measuring the peak current of AQ using a square-wave voltammetric (SWV) method. The results in Figure 4.15 showed the redox signal response decreased by three folds after addition of the DNA. This is explained by the electron transfer space between redox-active label was increased after hybridization with the target DNA.

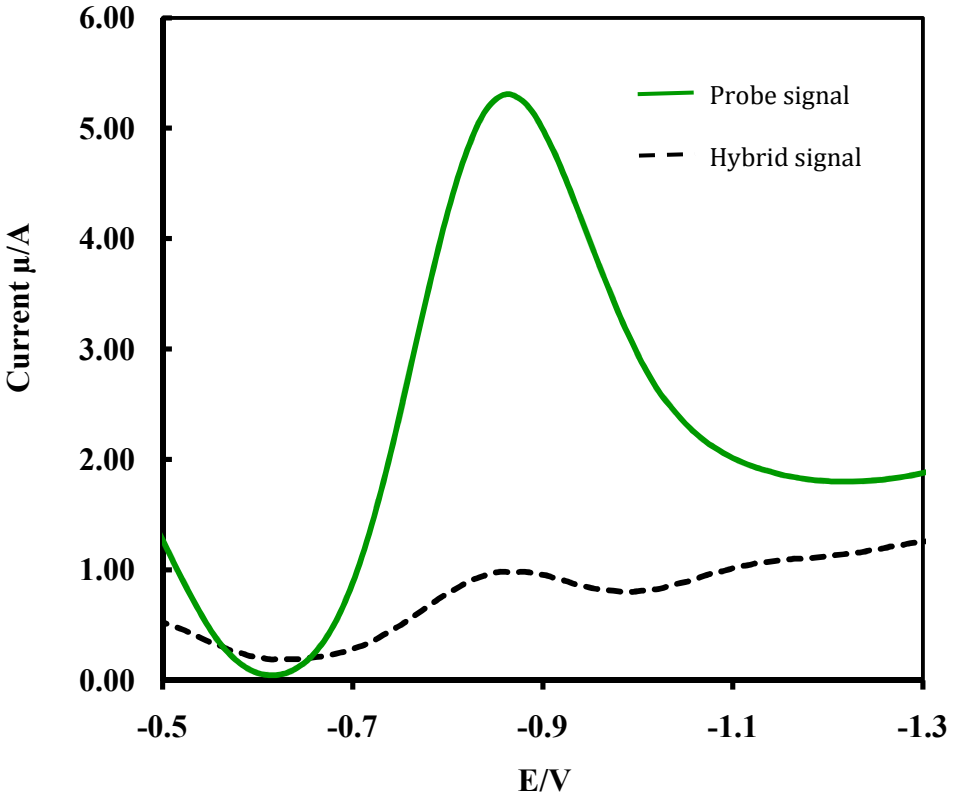


Figure 4.15 Square-wave voltammograms of PNA-AQ probe before and after hybridization with 20 µM target DNA. Parameters are frequency of 5 Hz, amplitude of 50 mV and step potential of 30 mV in PBS pH 7.4.

However, even in this immobilization method the optimum conditions for the immobilization of PNA-AQ onto the electrode were already achieved, but there was a problem about the reproducibility of the experiment, because we cannot control the amount of carboxylate groups on the electrode surface which influences the immobilization of PNA-AQ probe. Consequently, a new immobilization method was proposed and used in future study.

4.7.2 Immobilization of PNA-AQ probe onto chitosan-modified SPCE

4.7.2.1 The optimization of parameters for immobilization of PNA-AQ probe.

4.7.2.1.1 Effect of chitosan amount on the PNA-AQ probe signal

After identifying a suitable amount of graphite powder to modify the electrode, the next procedure is the immobilization of probe onto the electrode surface. The SPCE was modified with chitosan (CHT) which was used for the introduction of $–NH_2$ group on the electrode surface.

The CHT amounts of 0%, 1%, 2%, 3% and 4% w/v were designated to modify the carbon ink. We expected that the increasing of CHT amount would increasingly added the $-NH_2$ groups on the electrode surface, which would affect the immobilization of probe onto electrode surface. The CHT amount of 4% w/v provided the highest current of probe signal as illustrated in the Figure 4.16, 4% w/v CHT was selected even though the probe signal did not plateau because when CHT was higher than 4% w/v, the CHT solution became a gel, which resulted in a very poor dispersion in carbon ink. Therefore, 4% w/v CHT was chosen as the optimal amount to modify the carbon ink.

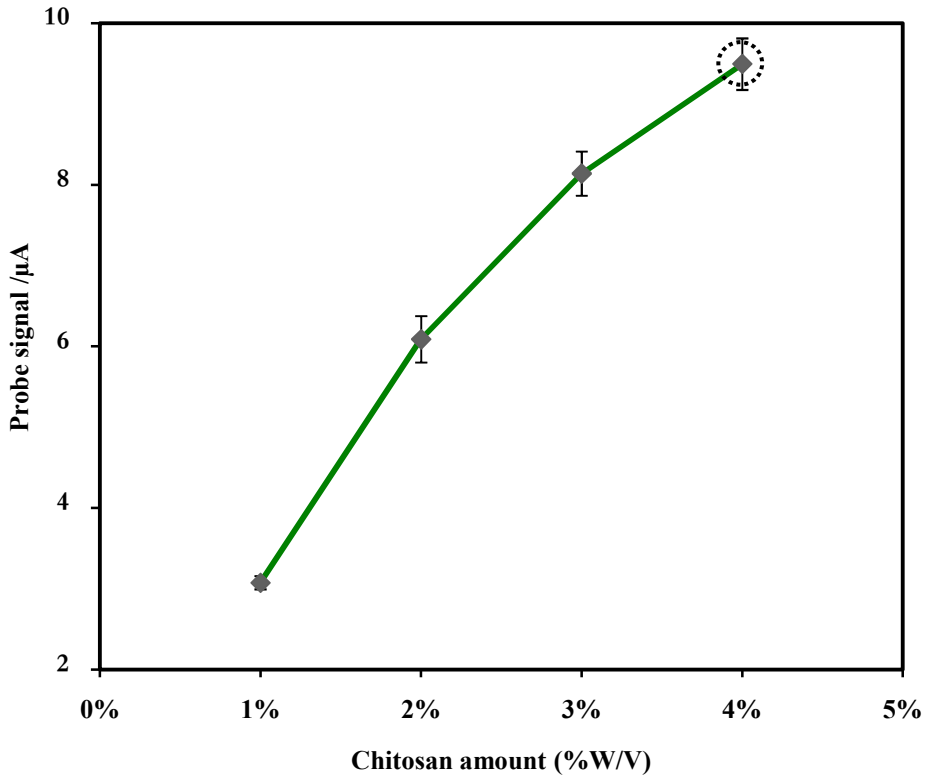


Figure 4.16 The effect of CHT amount on probe signal.

After obtaining the suitable amount of CHT to modify the carbon ink to give the best PNA probe signal, the sensitivity of modified electrode was performed by $[Fe(CN)_6]^{3-/4-}$. From Figure 4.17, the result shows that the increasing of CHT amount affected the $[Fe(CN)_6]^{3-/4-}$ current. The $[Fe(CN)_6]^{3-/4-}$ current is altered proportionally to the amount of CHT due to its naturally nonconductive polymer. The current was only slightly decreased when the CHT ratio was increased. The result shows that it was suitable to use 4% CHT to modify the SPCE. Therefore, 4% w/v of CHT was chosen.

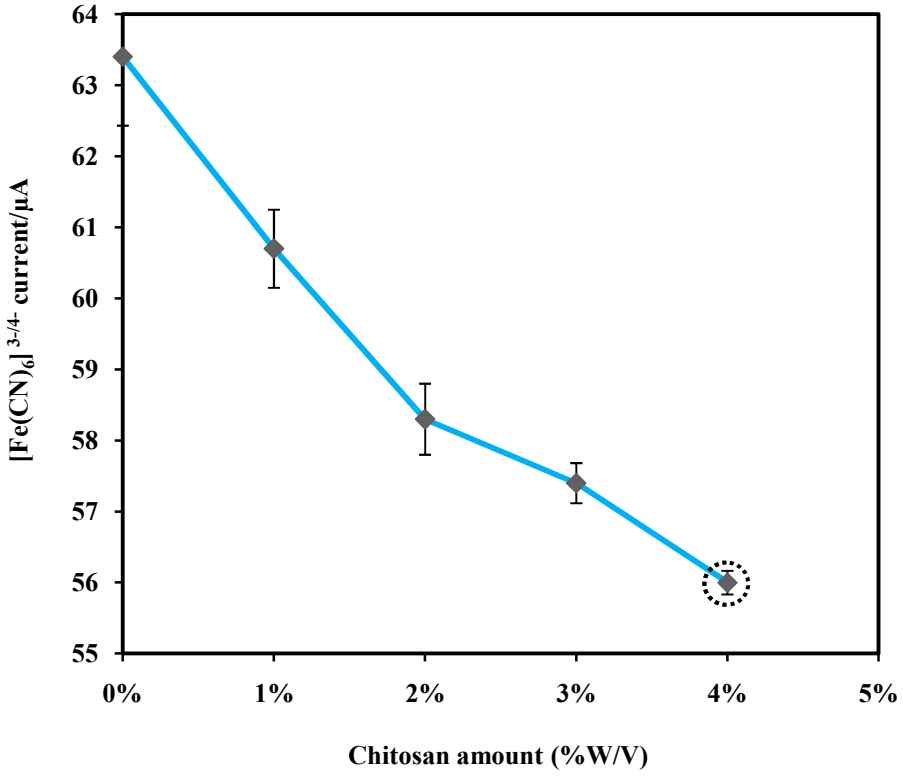


Figure 4.17 The effect of CHT amount on $[Fe(CN)_6]^{3-/4-}$ current.

In addition to chitosan, other polymers were also studied in the context of its function towards the introduction of $-NH_2$ group for the immobilization of PNA probe. Polyethylenemine (PEI) (Mw = 2 kDa) was compared to CHT. The structures of the two polymers are shown in Figure 4.18.

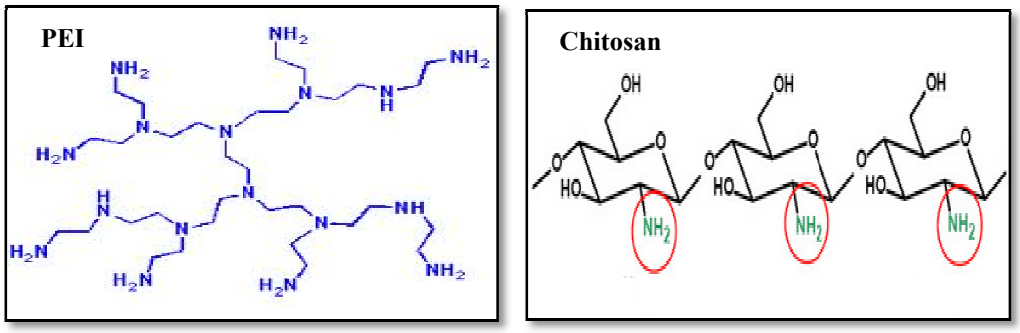


Figure 4.18 Comparison of the structures of two polymers between CHT and PEI.

Before the immobilization of PNA onto the electrode surface, we used 1 mM $[Fe(CN)_6]^{3-/4-}$ solution to test the sensitivity of two types of electrode. From the result (data not shown) the PEI modified-SPCE gave about 10 times lower sensitivity or lower current of 1 mM $[Fe(CN)_6]^{3-/4-}$ when compared to that of the CHT modified-SPCE. Therefore, CHT was the suitable polymer to modify the SPCE for the introduction of amino groups on the electrode surface.

4.7.2.2.2 Effect of % glutaraldehyde on the PNA-AQ probe signal

In this work, glutaraldehyde was used as a cross-linking agent in the immobilizing step. Thereby, we have to study its effect on the immobilization signal.

Figure 4.19 exhibits the probe signal when using different percentages of glutaraldehyde. From the results, we found that the signal was increased with increasing percent of glutaraldehyde up until 5% of glutaraldehyde where the signal plateaued thereafter. This was due to the limitation of the electrode surface area. The plateaued signal can be explained by the full coverage of the probe on the electrode surface. Hence, 5% of glutaraldehyde was selected as the maximum concentration of the cross-linking agent for the immobilizing the PNA probe.

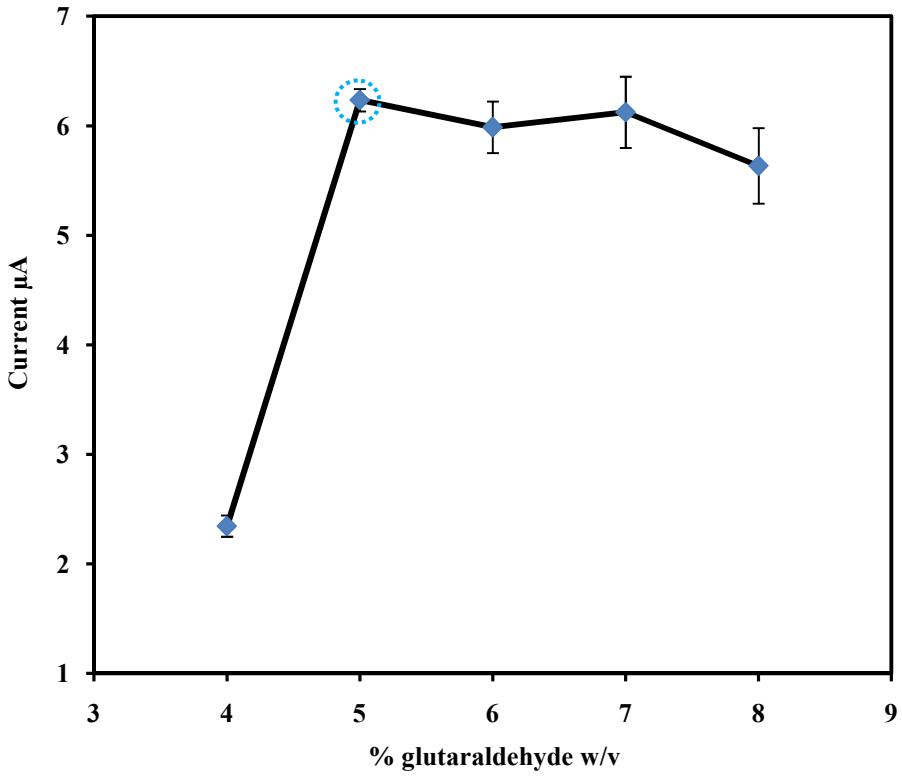


Figure 4.19 The effect of glutaraldehyde on probe signal.

In the immobilizing step, we need to apply the temperature to activate the reaction between amino groups and glutaraldehyde cross-linking agent. The temperature of 40 ^{o}C was selected as the activation temperature for 3 hours to force the reaction. When the temperature was higher than 40 ^{o}C, the electrode surface peeled off from the PVC substrate and got damaged. Therefore, this temperature was picked as the maximal temperature to accelerate the reaction.

4.7.2.2 Optimization of parameters for the electrochemical detection

In this part, the aim was to optimize the parameters for an electrochemical measurement. The frequency, amplitude, and step potential were investigated. We found that the optimal parameters for an electrochemical detection of the PNA probe after it was immobilized onto the electrode surface were at frequency of 35 Hz,

amplitude of 50 mV, and step potential of 30 mV. The results of optimizing each parameter are described below.

4.7.2.2.1 Effect of frequency

Electrochemical parameters affected the kinetic rate of electroactive species. It meant that we have to optimize a suitable condition for each parameter to obtain the best condition. The sensitivity and peak shape of a redox-active label are dependent on these parameters. Frequency was the first parameter studied. The result is displayed in Figure 4.20.

Figure 4.20 shows the effect of frequency on probe signal. From the result it was found that probe signal was slightly raised with increasing frequency value and the highest current was obtained at the frequency of 30 Hz. When frequency value was higher than 30 Hz, the probe signal was decreased. So it can be concluded that frequency values higher than 30 Hz was not suitable for the electrochemical detection. Not only the current was decreased but the error values were also maximized. Thus, the frequency of 30 was selected for the optimal frequency.

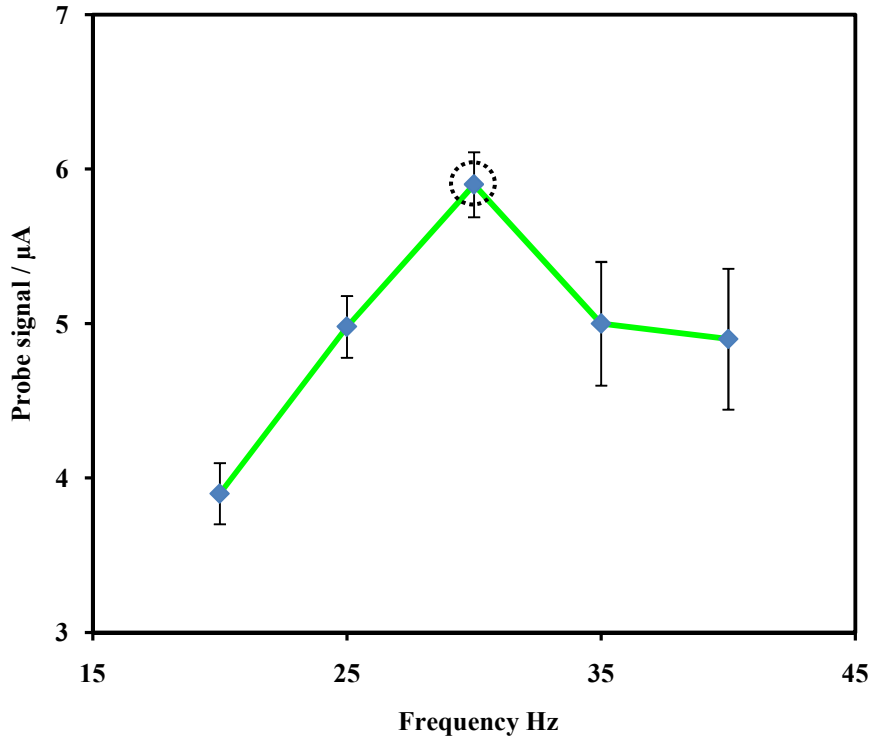


Figure 4.20 The effect of frequency on probe signal.

4.7.2.2.2 Effect of Amplitude

Amplitude is also one of the parameters that affected the kinetic rate and peak shape of a redox-active label. According to Figure 4.21, the amplitude of 50 mV was the optimal condition to apply and to obtain the highest current for electrochemical detection. The result is shown below.

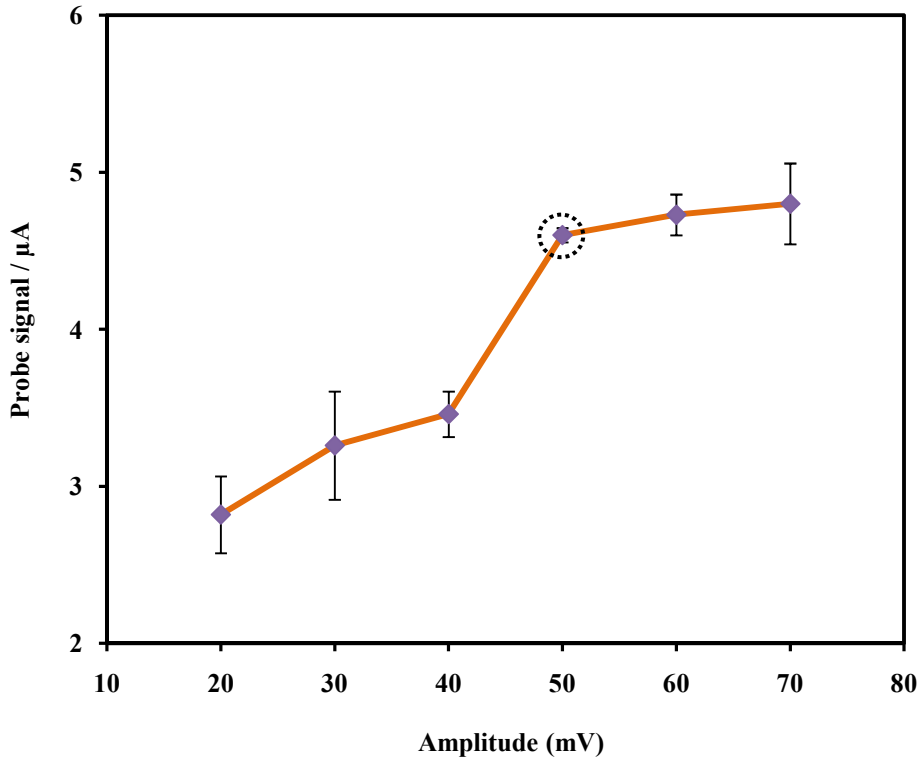


Figure 4.21 The effect of amplitude on probe signal.

4.7.2.2.3 Effect of step potential

Figure 4.22 illustrates the effect of step potential on probe signal. The result revealed that at the step potential of 30 mV, the current was maximized above all other step potentials. The result is shown below.

All electrochemical parameters that affected the kinetic rate of a redox active label have been studied. These parameters include the frequency, amplitude and step potential. The optimal condition for each parameter is summarized in table 4.1

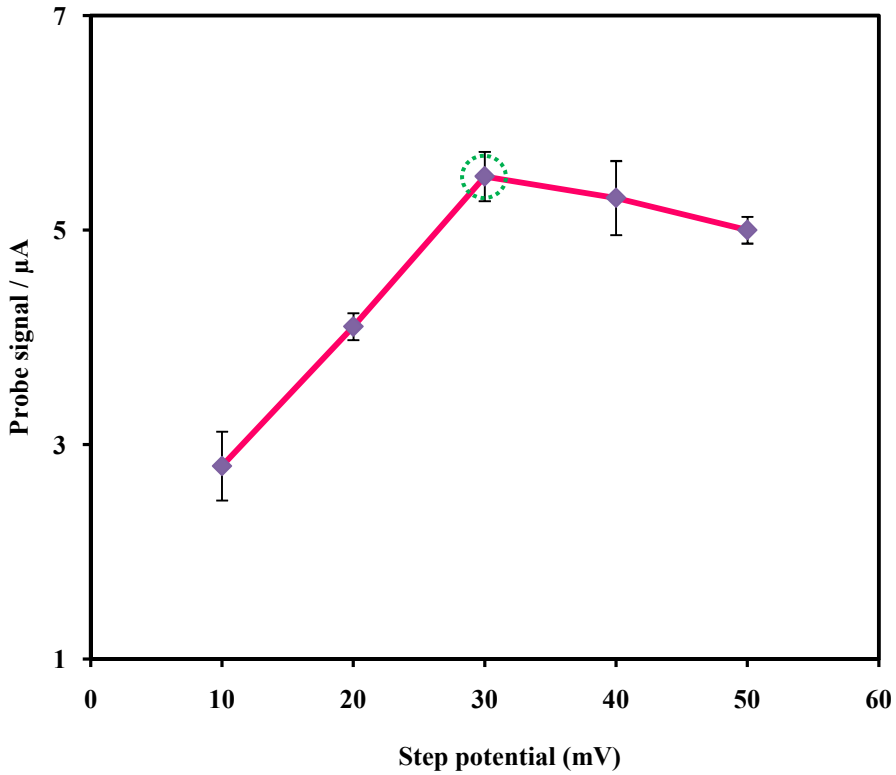


Figure 4.22 The effect of step potential on probe signal.

Table 4.1 The optimal conditions of each parameter for electrochemical measurement

Parameters	Optimal parameter
Frequency	30 Hz
Amplitude	50 mV
Step potential	30 mV

4.7.2.3 Immobilization and hybridization of PNA-AQ probe

The immobilization and hybridization of PNA-AQ probe with the complementary DNA are important steps. We need to perform carefully because they will influence the current obtained. The aim of this experiment was to determine the electrochemical response before and after the hybridization of the PNA probe.

4.7.2.3.1 Immobilization of PNA probe

The PNA probe was covalently attached onto the electrode surface through a glutaraldehyde cross-linking agent. If the PNA probe was truly immobilized onto the electrode, the redox-active label peak will appear at around -0.8 V.

The result exhibits a redox-active label peak at around -0.8 V after PNA probe was immobilized onto electrode surface as we expected. This result can confirm that the PNA probe has been successfully immobilized onto electrode as displayed in Figure 4.23.

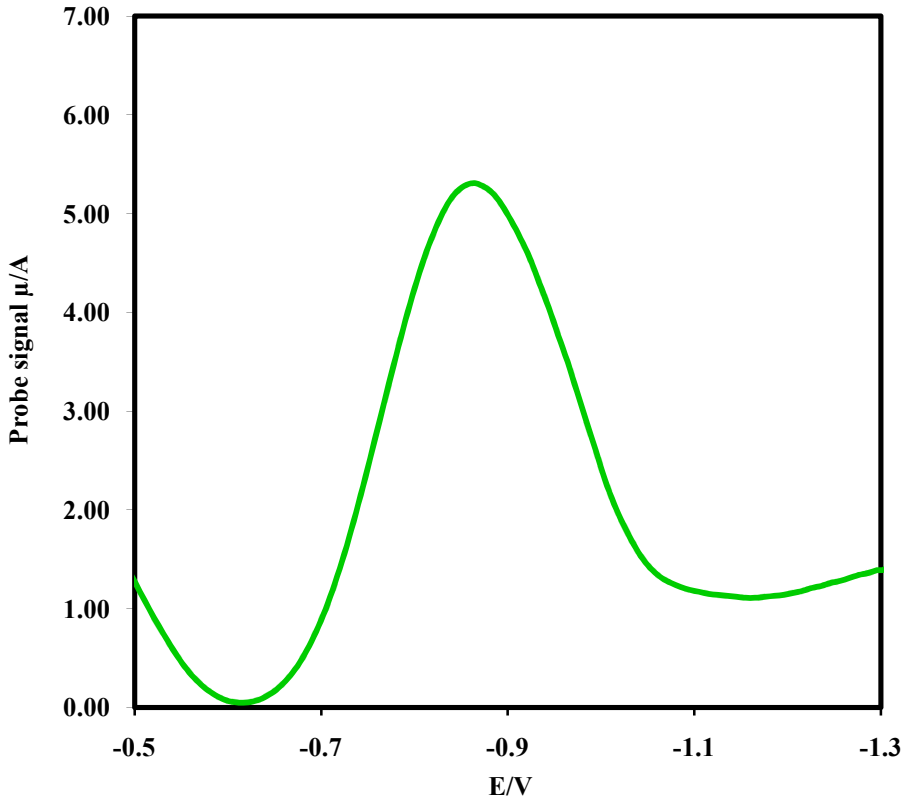


Figure 4.23 Square-wave voltammogram of PNA-AQ probe after immobilized onto the electrode surface. Frequency 30 Hz, amplitude 50 mV and step potential 30 mV in PBS pH 7.4.

4.7.2.3.2 Hybridization of PNA probe with the target DNA

After the immobilization step, hybridization was subsequently performed. Hybridization occurred between the 15 µM PNA-AQ probes and the 15 µM synthetic

DNA target which was studied by measuring the peak current of AQ using a square-wave voltammetric (SWV) method.

From the result, the signal was very different for the electrochemical response of PNA probe after hybridization has occurred. The redox signal response decreased by three folds after the addition of the complementary DNA as shown in the Figure 4.24. This result is explained by the rigidity of PNA-DNA duplexes, which affected the accessibility and electron transfer between the AQ label and the electrode surface [66]. The electron transfer mechanism is illustrated in Figure 4.25.

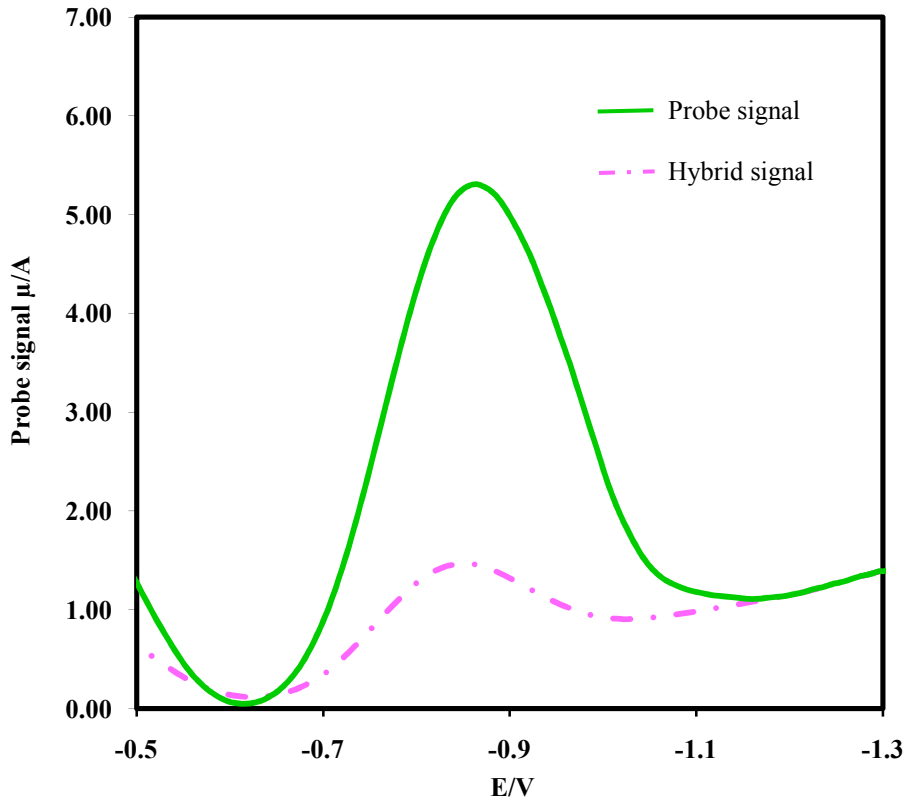


Figure 4.24 Square-wave voltammogram comparison of PNA-AQ probe before and after hybridization with 15 µM target DNA. Parameters are frequency of 25 Hz, amplitude of 50 mV and step potential of 30 mV in PBS pH 7.4. Hybridization time is 10 minutes.

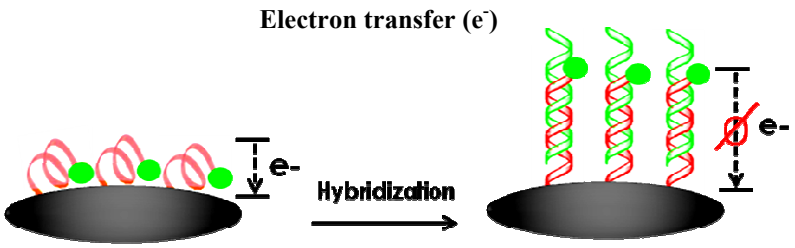


Figure 4.25 The schematic illustration of the electron transfer mechanism between the AQ label and the electrode surface.

4.7.2.3.3 Study of the hybridization times

In this section, the effect of hybridization times was studied. The hybridization times of 5, 10, 15, 20, 25 and 30 minutes were experimented. The concentration of target DNA was fixed at 15 µM.

Figure 4.26 illustrates the SWV comparison of the hybridization time effect on the electrochemical response of the PNA probes after hybridization with the target DNA. The results show that the PNA probe signals decreased with increasing hybridization time. The PNA probe signal plateaued after 15 minutes of hybridization time (Figure 4.27). This phenomenon was due to the completion of binding between target DNA and PNA probe at 15 minutes. Hence, 15 minutes was chosen as the optimal hybridization time

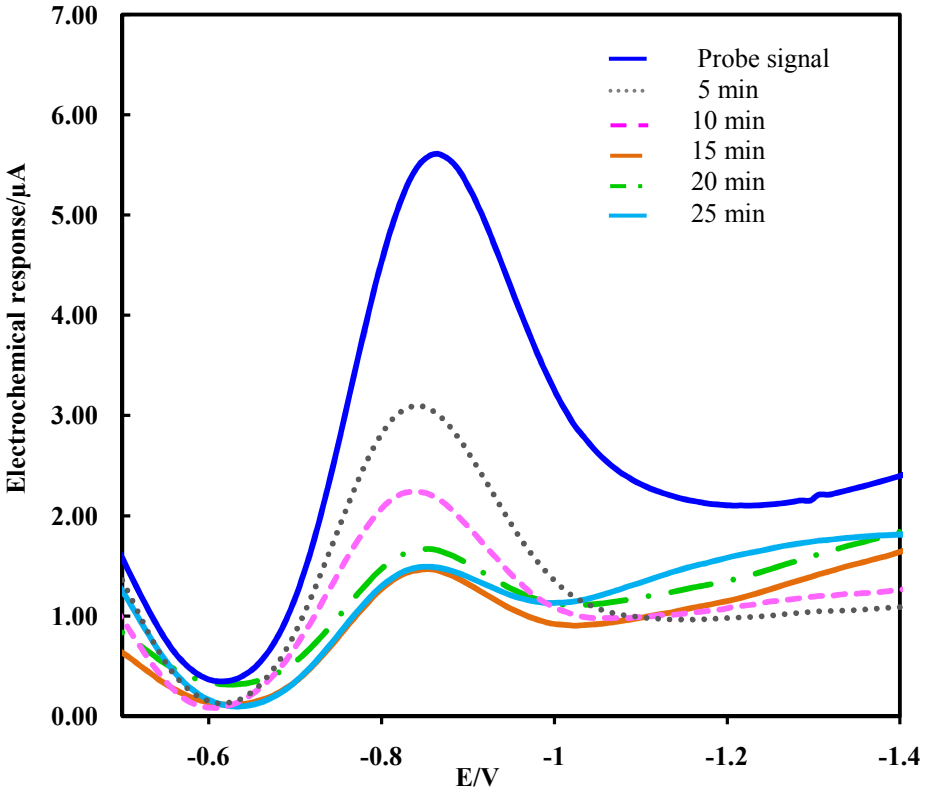


Figure 4.26 Comparison of square-wave voltammograms with various hybridization times on the electrochemical response. Parameters: Amplitude 50 mV, Frequency 30 Hz, Step potential of 30 mV in PBS pH 7.4.

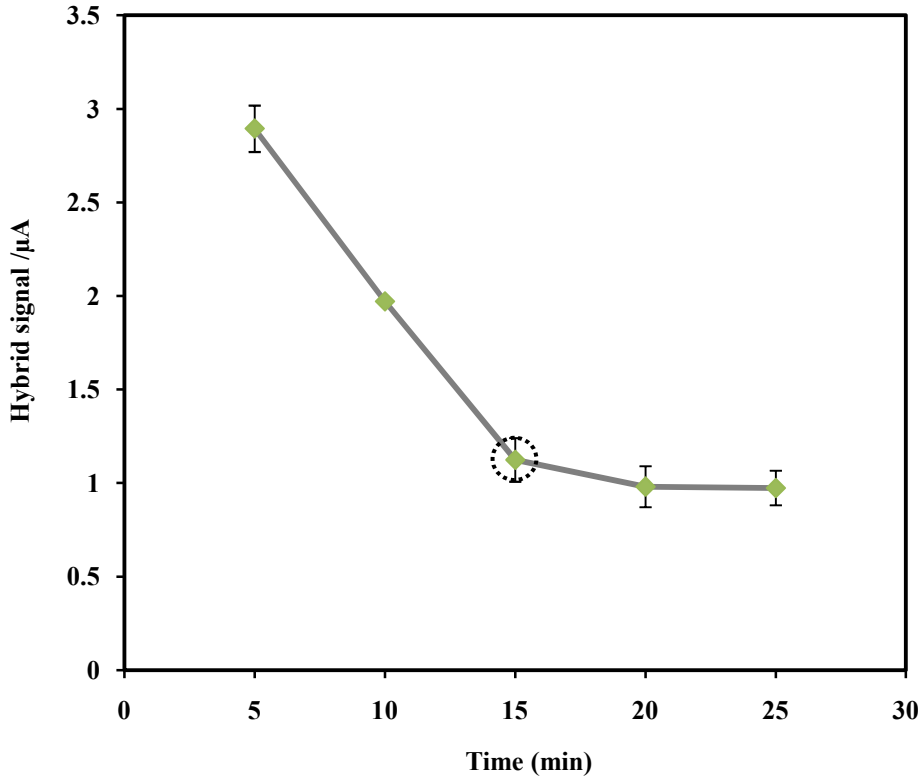


Figure 4.27 Hybridization signal against plot the hybridization time (minutes) Parameters: Amplitude 50 mV, Frequency 30 Hz, Step potential of 30 mV.

4.8 Calibration curve

After the optimal parameters were achieved, the next step is the creation of the standard curve. The standard or calibration curve was prepared by using oligonucleotide synthetic corresponding to HPV DNA at various concentrations. The calibration curve was obtained from a plot between the changed current (ΔI) of PNA probe signal and various DNA concentrations.

Figure 4.28 shows square-wave voltammograms of the electrochemical response of PNA-AQ probe after it was hybridized with target DNA, which was measured under the optimal experimental conditions.

The calibration curve in Figure 4.29 was plotted between ΔI of PNA probe signal and various DNA concentrations, which was obtained from the SWV analysis.

The 15 µM probe concentration was selected to immobilize onto the electrode surface because it was the lowest concentration that the modified electrode that can provide measured signal.

According to Figure 4.28, the lowest target DNA concentration that this method can be detected was found to be 0.02 µM. The lowest AQ signal was observed when the probe-modified electrode was exposed to 12.0 µM target DNA concentration. Calibration curve provided linear range between 0.02 to 12.0 µM unit with high coefficients of 0.996 (Figure 4.29). LOD and LOQ were found to be 0.004 µM and 0.014 µM, respectively.

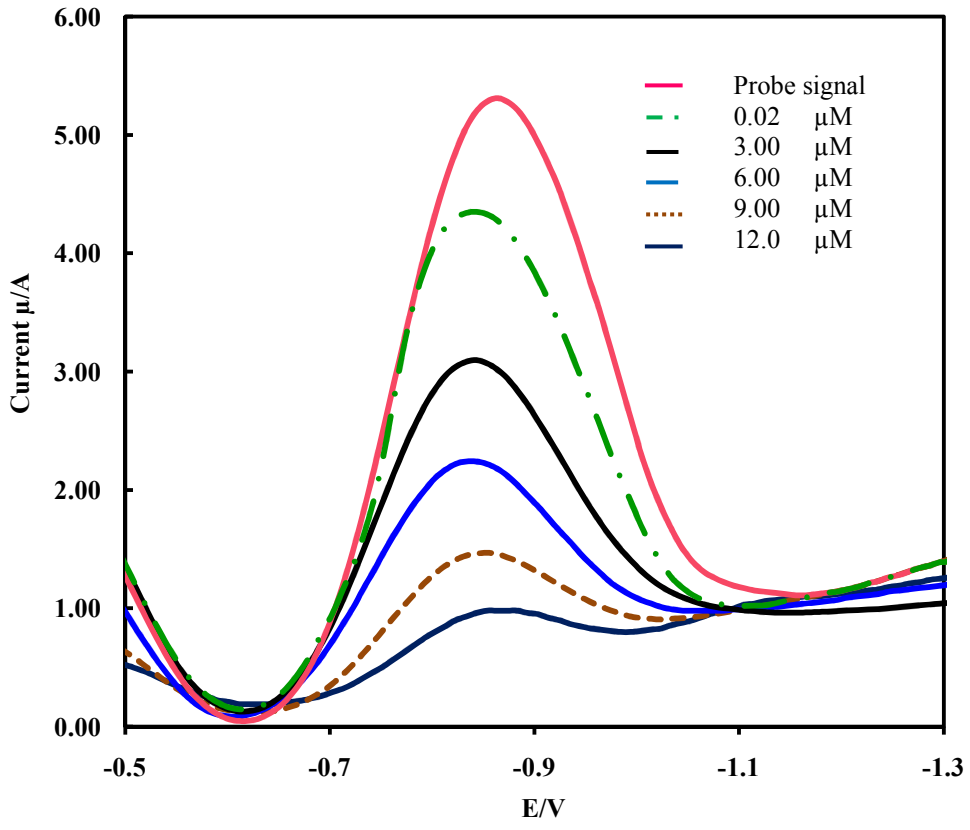


Figure 4.28 Square-wave voltammograms of electrochemical response of PNA-AQ probe after hybridization with target DNA (0.02, 3, 6, 9 and 12 µM), measured under the optimal experimental conditions.

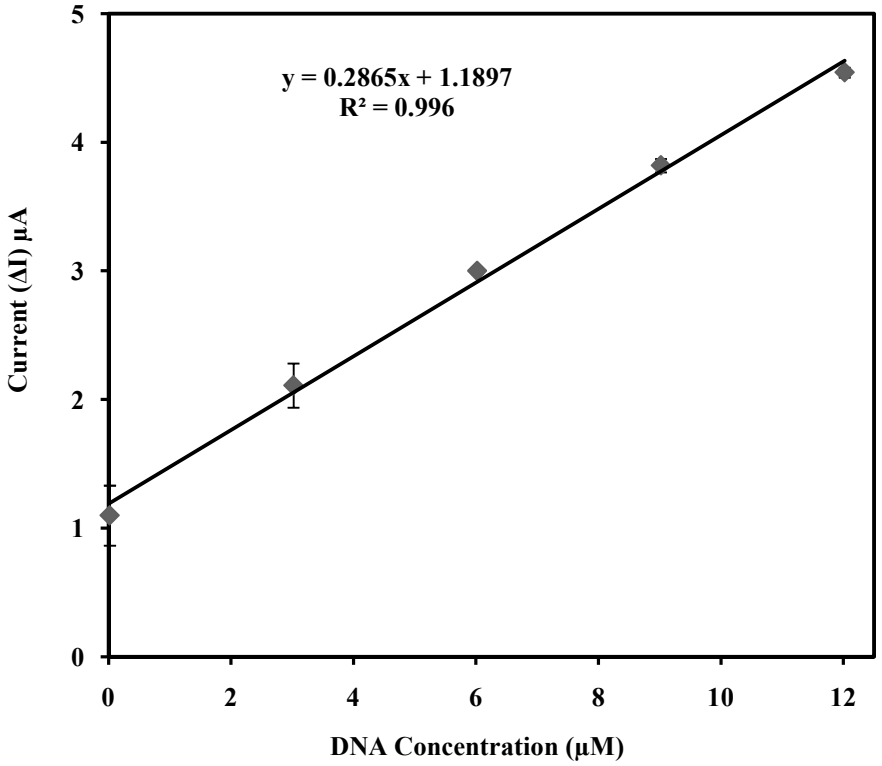


Figure 4.29 Calibration plots of probe signal against DNA concentration (0.02, 3, 6, 9 and 12 µM), obtained with SWV, measured under the optimal experimental conditions.

4.9 Repeatability

In this experiment, the relative standard deviations for ten repeating measurements of each sample at different concentrations are shown in Table 4.2. This indicated that the developed method exhibited the high reproducibility.

Table 4.2 The relative standard deviations of electrochemical detection (n=10)

Concentration (μM)	SD	%RSD
3	0.06	1.75
6	0.05	1.48
9	0.07	2.05

4.10 The selectivity of the acpcPNA probe

In this work, the selectivity of the sensor probe was examined by exposure of the acpcPNA-AQ probe to 14-mer noncomplementary oligonucleotides, including HPV DNA type 18, 31 and 33. All types are high risk HPV that have the potential to cause cervical cancer. These HPV strains are always present in women who are diagnosed as cervical cancer positive. The results revealed that the probe signal was slightly decreased when PNA probe was exposed to noncomplementary DNA target. It meant that PNA probes did not form any hybrid with the noncomplementary DNA. Therefore, it can be concluded that the PNA sensor probe selectively bind to the HPV DNA type 16 target sequences. The results are as shown in Figures 4.30 and 4.31.

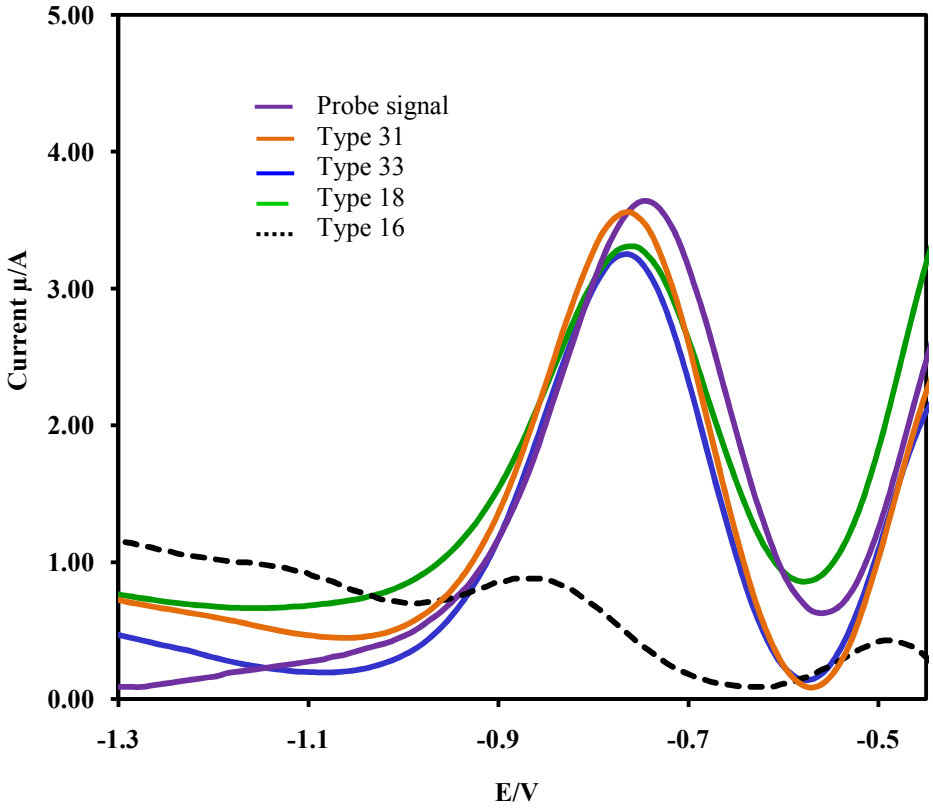


Figure 4.30 Square-wave voltammograms of the PNA probe signal after it was exposed to the different kinds of interference under the optimal condition. Hybridization occurred between PNA-AQ probes (15 µM) 1:1 DNA targets (15 µM)

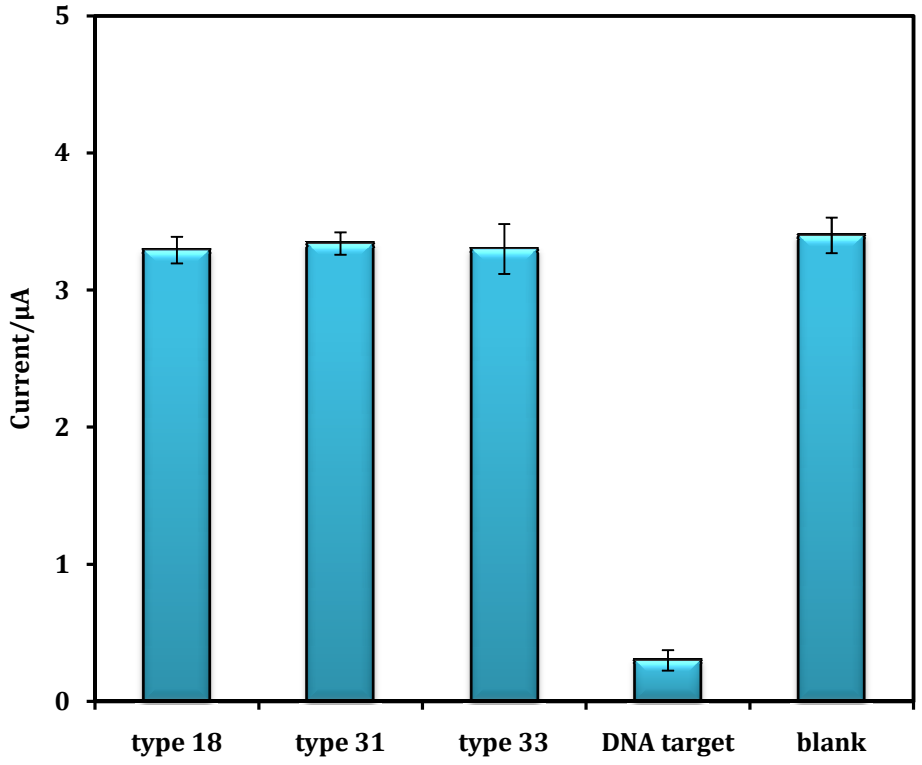


Figure 4.31 The comparison between PNA-AQ signal and the different types of interferences.

4.11 Analytical application in a real sample

4.11.1 Optimal condition of PCR

The DNA sample was first amplified by PCR. After achieved the PCR product, it was then characterized of PCR size by agarose gel electrophoresis. A suitable condition of PCR for DNA amplification is at annealing temperature of 52 ^{o}C. The result is illustrated in Figure 4.32, The band obtained from agarose gel electrophoresis that at annealing temperature of 52 ^{o}C only one bane was observed without non-specific DNA fragment when comparing to at 48 and 50 ^{o}C. Therefore, at annealing temperature of 52 ^{o}C was selected as the optimal temperature for PCR.

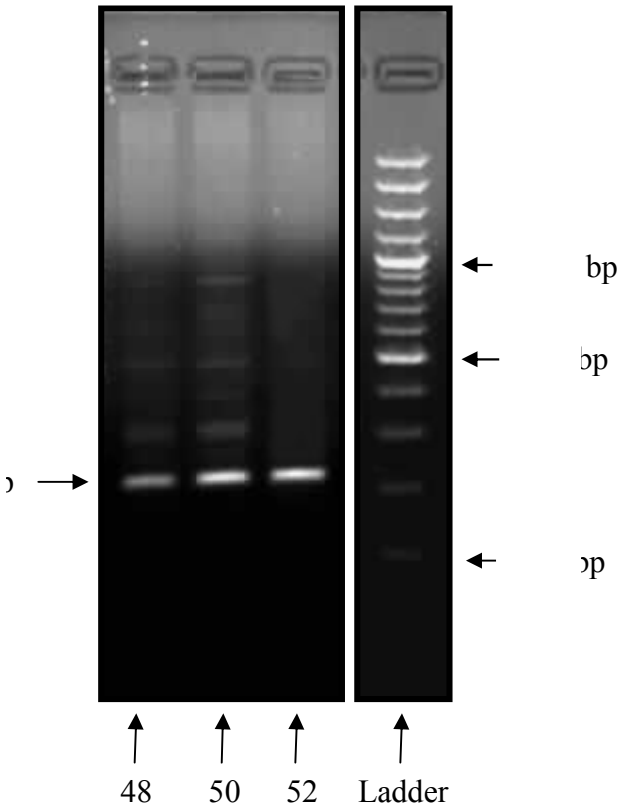


Figure 4.32 Comparison of DNA size between PCR product and DNA ladder obtain from agarose gel electrophoresis.

4.11.2 Sequence-specific DNA detection

For the detection of PCR product obtained from positive sample (SiHa Cell-line), the electrochemical detection was performed with the same procedure as standard DNA. Cell-line C33a was used as the negative control compare to positive control (SiHa Cell-line). The result shows that when the addition of negative control, probe signal does not decreased. It meant that PNA probes did not form any hybrid with the noncomplementary DNA. In the case of positive control, probe signal was decreased after it was exposed to positive control (SiHa Cell-line) as shows in Figure 4.33. Therefore, it can be concluded that the PNA sensor probe selectively bind to the HPV DNA type 16 target sequences. From the result, the potential was shift to the positive potential this phenomenon might be due to the interaction of DNA chain to both electrode surface and AQ molecule. DNA'chain has the long chain (243 bp) that

can affect the electron transfer of electroactive species. Hence, electron transfer can be enveloped by the bulky DNA chain and was not available for redox activity [65].

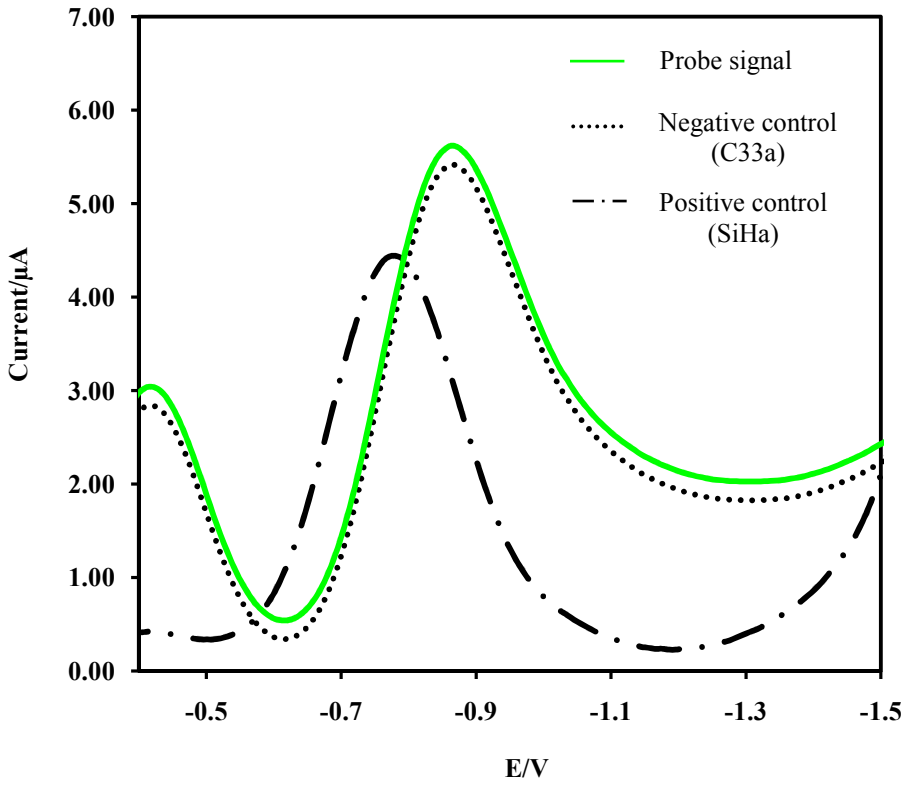


Figure 4.33 Square-wave voltammograms comparison between probe signal and Negative control (C33a), Positive control (SiHa) after hybridization.

4.11.3 Comparison the proposed method with real PCR concentration

The concentration of real sample or PCR product of cell-line (SiHa) was received by comparison to the calibration curve. The obtained concentration from this proposed method were illustrated in table (n=3).

Table 4.3 The concentrations of real sample obtains from this method (n=3)

No.	Concentration (μM)
1	1.25
2	1.24
3	1.28
Mean	1.25 ± 0.08 μM

In addition, the results were validated with the real concentration of PCR product. The real concentration of sample was received by comparison to standard marker which was received form agarose gel electrophoresis. The certain concentration of sample is 1.19 μM. This developed method produced results in good agreement. Therefore, we can say that this method has high accuracy for detection of HPV DNA in real sample.

CHAPTER V

CONCLUSIONS AND FUTURE PERSPECTIVE

5.1 Conclusions

A novel electrochemical biosensor based upon acpcPNA as sensor probe was successfully applied to detect HPV DNA type 16. All parameters that affect the electrochemical signal were investigated. The lowest target DNA concentration that this method can detect was found to be 0.02 μM. The lowest AQ signal was observed when the probe-modified electrode was exposed to 12.0 μM target DNA concentration. Calibration curve provided linear range between 0.02 to 12.00 μM with high coefficients of 0.996. The limit of detection (LOD) and limit of quantitation (LOQ) were found to be 0.004 and 0.014 μM, respectively. The results show that acpcPNA sensor probe had very high selectivity for HPV type 16 DNA over noncomplementary oligonucleotides, including HPV types 18, 31 and 33 DNA.

Furthermore, this method was successfully applied for the detection of HPV-DNA type 16 in cell-line sample (SiHa). The result obtained from this method was provides high accuracy when was compared to real PCR concentration. In conclusion, a newly developed platform using acpcPNA sensor probe couple electrochemical provides an attractive method for detection of HPV-DNA type 16. This sensor offers a promise to be an inexpensive tool for high-throughput screening for the cervical cancer in the primary stage which can save the life or reduce the death rate of these people.

5.2 Future perspective

In the future, this developed method can be applied for the simultaneous detection of multiple types of HPV-DNA. In addition, this detection platform could improve its sensitivity by using other types of electrode and/or modifying the electrode with some materials, such as a conductive polymer or nanomaterial, to obtain high sensitivity. If this technique can detect the HPV-DNA directly (usually in

sub-nanomolar concentration) without prior PCR amplification, it would become an even more attractive technique for this disease diagnosis. This would promise a more convenient method to screen for HPV-DNA.

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