Electrochemical detection study of DNA

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**NANYANG
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**ELECTROCHEMICAL DETECTION STUDY
OF DNA**

**SCHOOL OF MECHANICAL AND AEROSPACE
ENGINEERING**

2007

ELECTROCHEMICAL DETECTION STUDY OF DNA

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School of Mechanical and Aerospace Engineering

A thesis submitted to the Nanyang Technological University

in fulfilment of the requirement for the degree of

Doctor of Philosophy

2007

Declaration

"This dissertation represents my own work except where otherwise indicated or acknowledged. No part of the dissertation has been concurrently submitted for any other qualification at any other university."

.....

Teh Huey Fang

May, 2007

Abstract

In molecular genetics, DNA amplification is one of the essential steps that a biologist performs routinely. The most common and established method used for this purpose is the polymerase chain reaction (PCR). Detection in PCR via fluorescence detection method is sensitive and routinely used in real-time quantitative PCR applications, but its bulky and expensive optical detection system cannot be easily miniaturized and integrated into a biochip. Electrochemistry-based detection is an ideal technique for miniaturized analytical systems, and its sensitivity and selectivity are comparable to the laser-induced fluorescence. The distinctive advantages of the electrochemical detection over optical detection techniques include the ease of miniaturization and portability, sample turbidity tolerance, low cost, low power consumption, and compatibility with biochip microfabrication technology.

In this thesis, we first carry out three studies of macro electrochemical detection for DNA analysis. Then, a portable microfluidic flow-through microdevice with an integrated electrochemical detection system for PCR products quantification is developed.

The first macro electrochemical detection study is based on the study of DNA purine bases - adenine and guanine, on a sol-gel carbon composite electrode (CCE). It is shown that adenine and guanine can be measured simultaneously by the differential pulse stripping voltammetry on a sol-gel CCE. For guanine and adenine, under optimum stripping conditions a linear calibration graph in the range of $2 \times 10^{-8} \sim 1 \times 10^{-6}$ M is obtained, with its limit of detection (LOD) of the stripping method calculated to be 0.06 μ M and 0.09 μ M for guanine and adenine, respectively. This CCE is useful for the simultaneous analysis of DNA purine bases from denatured DNA.

The second macro electrochemical detection study is based on covalent immobilization of a synthesized 21mer capture probe onto carboxylate-terminated 4-aminobenzoic acid (4-ABA) monolayers modified on a glassy carbon electrode (GCE)

surface for the square wave voltammetric (SWV) detection of sequence-specific synthetic target DNA. The decrease of the peak current of the methylene blue (MB), an electroactive indicator, is observed upon the hybridization of the probe with the target DNA. The reduction signal of MB is able to discriminate the target and non-complementary synthetic DNA sequence. The reproducibility for the three independently made electrodes is 90.3%, measured at 10 ng/ μ L of synthetic target DNA, with the LOD of 0.5 ng/ μ L. We also apply this approach to detect polymerase chain reaction (PCR) product instead of the synthetic DNA. The identification and quantification of the asymmetric PCR amplified samples at the end-point detection are demonstrated by the above method. This electrochemical detection method is sequence specific, and is able to detect minimum amount of PCR product after the 5th PCR cycle with the initial Lambda DNA template at concentration of 1 ng/ μ L.

The third electrochemical detection study, which is based on an *in-situ* EC detection method, is developed for the stationary electrochemical detection quantitative polymerase chain reaction (EC-qPCR) without the need for immobilization of the capture probe. Unlike the first and second studies, which are only applicable for the end-point detection, the method in the third study is based on the real-time cycle-by-cycle monitoring of the PCR. We have implemented this method in a stationary EC-qPCR reactor for the *in-situ* quantification of the PCR product during the amplification. A 3-electrode detector module is incorporated into the PCR tube in order to perform the electrochemical detection cycle-by-cycle during PCR. Since the PCR mixture remains in the PCR tube and experiences the PCR thermocycling, this device functions as a stationary EC-qPCR reactor. The 3-electrode detector module consists of three platinum metal wires of 0.1 mm in diameter and 2 mm in length submerged in the PCR mixture, respectively functioned as the working electrode, the counter electrode and the reference electrode, for the EC measurement of the PCR product inside the PCR tube. The reactor is then mounted in a thermal cycler for performing PCR while 3-electrode detector module in the reactor is connected to the EC workstation to achieve the PCR amplification and the *in-situ* EC measurements

concurrently. The electrochemically active DNA intercalator, which is methylene blue (MB), is incorporated into the PCR mixture to generate an electrochemical signal at every PCR cycle that is related to the quantities of the PCR product during PCR cycling.

In the stationary EC-qPCR reactor, since the platinum wires and DNA intercalators are in contact with the PCR mixture, a study of inhibition on the PCR is performed on the platinum wires with different radiuses and DNA intercalators with different concentrations. The gel electrophoresis is used to indicate the rough amount of the PCR product, while microcapillary electrophoresis is used to characterize in details about the yield of the PCR product. In this work, three platinum electrodes with a diameter of 0.1 mm are used in the 3-electrode detector module while 10 μM of the MB is incorporated into the PCR mixture for performing the stationary EC-qPCR study. The SWV method is utilized to measure the peak current I_p of the DNA intercalator at the extension phase of a PCR cycle where the dsDNA is formed. I_p at a PCR cycle versus the PCR cycle numbers is plotted, and the threshold line is used to obtain the threshold cycle number, C_T . The peak current I_p of the MB is observed to decrease further with the increase of the PCR cycle numbers, and then forms a plateau at the end of PCR thermal cycling. The C_T values obtained from the EC detection are 11, 17 and 34 for the initial template DNA concentrations of 1 $\text{ng}/\mu\text{L}$, 0.1 $\text{ng}/\mu\text{L}$ and 0.01 $\text{ng}/\mu\text{L}$, respectively.

In the fourth electrochemical detection study of this thesis, we implement the concept of the *in-situ* EC detection for the real-time quantification of the PCR product in a microfluidic flow-through EC-qPCR microdevice, where both the amplification of the target DNA sequence and subsequent EC detection of the PCR product are realized simultaneously at every PCR cycle in the same microdevice. An array of platinum microelectrodes fabricated on a glass slide is integrated into the microchannel made of polydimethylsiloxane (PDMS) to form a chip. Each microelectrode in the array for EC detection is located at the extension phase of a PCR cycle. The MB is incorporated in the PCR mixture to generate the electrochemical signal that is

proportional to the concentration of the PCR product. By using the SWV method of EC technique, the EC measurement for the peak current of the MB is taken when the flow front of the PCR mix segment in the microchannel fully submerges a particular 3-electrode EC detection station. The EC measurement circuit is subsequently switched to the next EC detection station for measuring the corresponding peak current of the MB when the flow front of the PCR mix segment submerges the next detection station. This EC measurement process proceeds until all the detection stations are used sequentially. During this *in-situ* EC detection process, the current signal is observed to decrease significantly at around 11, 15, and 18 cycles for the initial template DNA concentrations of 20 ng/ μ L, 2 ng/ μ L, and 0.2 ng/ μ L, respectively. A larger amount of the initial template DNA corresponds to an earlier onset of the exponential phase of the PCR, or an earlier detectable decrease in the current signal. The LOD for our microfluidic flow through EC-qPCR microdevice is around 0.2 ng/ μ L. This sensitivity is comparable to that obtained by using an optical fluorescence detection system. Considering the small size of the entire system and the potentials for further design improvements, this microfluidic flow-through EC-qPCR microdevice exhibits a new breakthrough for its concept and implementation for performing a portable, inexpensive, and robust real-time qPCR.

The sensitivity of the microfluidic flow-through EC-qPCR microdevice can be further increased by selecting electrochemically active DNA intercalators with higher duplex-specific affinity and reducing the size of microelectrodes. The design of the microdevice can be further functionalized as well, for example, the heaters and temperature sensors can be incorporated into the microfluidic flow-through EC-qPCR microdevice for more efficient heat-transfer and rapid thermal cycling, which will increase the precision and efficiency of the PCR process. Ideally, with these improvements, an automated and a truly portable EC detection system can be developed for the *in-situ* quantification of the PCR product in the future with commercial potential.

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List of Abbreviations

4-ABA	aminobenzoic acid
bp	base pairs
BSA	bovine serum albumin
C	cytosine
CCE	carbon composite electrode
CE	counter electrode
CV	cyclic voltammetry
Co(bpy) ₃ ³⁺	tris(2,2'-bipyridyl)cobalt (III)
Co(phen) ₃ ³⁺	tris(1,10-phenanthroline)cobalt(III)
C _T	threshold cycle
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphate
DPV	differential pulse voltammetry
DRIE	deep reactive ion etching
dsDNA	double stranded DNA
EB	ethidium bromide
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EIS	electrochemical impedance spectroscopy
E _{acc}	peak accumulation potential
E _p	peak potential
EC	electrochemical
EC-qPCR	electrochemical quantitative PCR
Fe(phen) ₃ ²⁺	tris(1,10-phenanthroline)iron(II)

$[\text{Fe}(\text{CN})_6]^{3-}$	ferricyanide ion
GCE	glassy carbon electrode
G	guanine
I_p	peak current
LSV	linear sweep voltammetry
μTAS	micro-total analytical systems
MB	methylene blue
MEMS	Micro-Electro-Mechanical-Systems
NAs	nucleic acids
NHS	N-hydrosulfosuccinimide
NTC	no-template control
ν	scan rate
PBS	phosphate buffere solution
PCR	polymerase chain reaction
PDMS	poly(dimethylsiloxane)
PEG	polyethylene glyco
RE	reference electrode
SAM	self-assembly monolayer
ssDNA	single stranded DNA
SAM	self-assembled monolayer
SCE	saturated calomel electrode
SWV	square wave voltammetry
T	tymine
t_{acc}	accumulation time
T_a	annealing temperature
T_g	glass temperature

List of Abbreviations

TAE	tris-acetate-EDTA
TBR	tris(2,2'-bipyridine)ruthenium (II)
WE	working electrode

List of Publications

Journal Papers

- [1] H.F. Teh, X. Yang, H. Gong, and S.N. Tan, Voltammetric and differential pulse stripping study of adenine on a sol-gel derived carbon composite electrode. *Electroanalysis*, 2004, 16, 769-773.
- [2] H.F. Teh, H. Gong, X. Dong, X. Zeng, L.K. Annie Tan, X. Yang, and S.N. Tan, Electrochemical biosensing of DNA with capture probe covalently immobilized onto glassy carbon surface. *Anal. Chim. Acta*, 2005, 551, 23-29.

Conference Paper

- [1] H.F. Teh, H. Gong, X. Dong, X. Zeng, L.K. Annie Tan, X. Yang, and S.N. Tan, Electrochemical biosensing of DNA with capture probe covalently immobilized onto glassy carbon surface, First International Conference on Bioengineering and Nanotechnology, Sept. 2004 (Singapore).

Chapter 1

Introduction

1.1 Background

One of the most important advances in biology area in the twentieth century is the discovery of the deoxyribonucleic acid (DNA), which is the carrier of genetic information. However, before the 1940's, most biologists believed that proteins were the chief carriers of hereditary material until the primary experiments carried out by Avery *et al.* in 1944, showed that DNA was the “transforming factor that conferred virulence upon non-virulent pneumococci [1].” The landmark paper in 1953 by Watson and Crick which correctly proposed the double-stranded helix structure of DNA led to an understanding of gene function in molecular terms and marked the beginning of a molecular revolution [2]. To date, advanced nucleic acid-based technologies, including nucleic acid hybridization, DNA sequencing reactions, the polymerase chain reaction (PCR) and automated fluorescent DNA sequencers, have streamlined and automated most of the nucleic acid analysis processes. DNA analysis has become an important tool for diagnostics of genetic diseases, identifications of disease-causing microorganisms, assessments of medical treatment, as well as forensic applications.

To meet the tremendous demand of DNA detections, it is essential to develop the fast, inexpensive, and automated analytical systems with high throughput. The most attractive approach of such a system is the analytical microdevices realized by the state-of-the-art microfabrication technologies. These microdevices such as lab-on-a-chip or micro-total analytical systems (μ TAS) can potentially replace the conventional bench-top instrumentations. The optical systems commonly employed in the conventional assay are sensitive and specific for DNA analysis. Optical detection has typically dominated DNA analysis due to its extremely low (pico-molar) detection limit. However, optical detection systems are difficult to be miniaturized due to the

complex and bulky optical components involved. Electrochemical systems with high compatibility to advanced microfabrication technology and high sensitivity similar to that of the fluorescence-based detection methods holds great promise for the realization of portable DNA analyzers. Furthermore, electrochemical reactions give an instant and direct electrical signal, which avoids the expensive signal transduction equipment as required in optical detection system.

1.2 Motivation and objectives

The main objective of this thesis is to develop electrochemical detection techniques both in macro- and micro-sizes for the DNA analysis. In this thesis, we carry out four studies with the electrochemical detection method for DNA analysis. The objective of the first macro electrochemical detection study is to study the electrochemical detection of the DNA purine bases - adenine and guanine, on a sol-gel carbon composite electrode (CCE). Cyclic voltammetry (CV) and differential pulse stripping voltammetry detection are applied for the individual nucleic acid bases study. Simultaneous determination of the guanine and adenine in the denatured DNA study by using the sol-gel CCEs are also carried out in this research. The objective of the second macro electrochemical detection study is based on the DNA hybridization study where a synthesized 21-mer capture probe is immobilized onto a carboxylate-terminated 4-aminobenzoic acid (4-ABA) monolayers modified on a glassy carbon electrode (GCE) surface for monitoring the DNA hybridization with the complementary target ssDNA in the solution by using square wave voltammetry (SWV) measurement. The capability of the sequence specific electrochemical detection for the identification and quantification of an asymmetric PCR amplified real samples at the end-point detection is also demonstrated in this study. The objective of the third electrochemical detection study, which is based on an PCR *in-situ* electrochemical (EC) detection method, is developed for conducting a quantitative real-time PCR with real time EC measurement (EC-qPCR) without the need for immobilization of the capture probe. The PCR amplification and the

electrochemical measurements are carried out concurrently in real-time during a PCR process. A 3-electrode module and methylene blue (MB) have been incorporated into the PCR mixture in the stationary EC-qPCR reactor to perform the SWV measurements for cycle-by-cycle monitoring, which enables the quantification of PCR products. The objective of the fourth study is to develop a microfluidic flow-through integrated PCR microdevice with the function of concurrent DNA amplification and EC detection to quantify the PCR products in real-time on the same integrated device. The EC results are compared with the fluorescence detection method.

1.3 Scope of the thesis

Chapter 1 provides a brief introduction and a description of our motivation, objectives, and study scopes. The rest of this thesis comprises the following portions:

Chapter 2 describes the working principles of PCR. This is followed by a review of existing methods available for the DNA analysis and detection of PCR products in macro-scale. In addition, a review of the state-of-the-art technologies in the development of microfabricated DNA analytical devices is also provided.

Chapter 3 demonstrates the voltammetric detection of individual nucleic acid bases by using the sol-gel CCEs. Graphite particles are incorporated into the sol-gel matrix to obtain the required electrical properties.

Chapter 4 describes our work towards the development of a DNA immobilization strategy on the GCE surface for the DNA hybridization study. This work deals with the covalent immobilization of a synthesized 21-mer capture probe onto a carboxylate-terminated 4-aminobenzoic acid (4-ABA) monolayer modified on a GCE surface via 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hydrosulfosuccinimide (NHS) cross-linking reaction for the voltammetric detection of the sequence-specific DNA. This work also involves PCR products instead of synthetic DNA.

Chapter 5 presents a PCR *in-situ* electrochemical (EC) detection method for conducting a quantitative real-time PCR with real time EC measurement (EC-qPCR). The PCR amplification and the electrochemical measurements are carried out concurrently in real-time during a PCR process. A macro 3-electrode module is incorporated into the PCR tube in order to perform SWV electrochemical detection during the PCR cycles. Electrochemically active DNA intercalators are incorporated into PCR mixture to generate electrochemical signals during the PCR thermal cyclings to obtain quantities of PCR products. The SWV method of the electrochemistry technique is utilized to measure the peak current I_p of the DNA intercalators at the extension phase of every PCR cycle where the dsDNA is formed.

In Chapter 6, the design and fabrication of a microfluidic flow-through EC-qPCR microdevice are described in detail. The experimental verification the electrochemical detection of the PCR products on the microdevice are carried out and the experimental results are discussed, which successfully demonstrated the capability of the EC electrode integrated microfluidic flow-through EC-qPCR microdevice for the simultaneous PCR product detection and amplification during the PCR cycles.

Chapter 7 is a summary of the work that has been done in this thesis. The suggestions of further improvements for the microfluidic flow-through EC-qPCR microdevice and future work are also presented.

Chapter 2

A Review of Existing Methods in DNA Analysis

2.1 DNA Chemistry and Biology

2.1.1 The structure of DNA

DNA is a linear polymer comprising of a large number of nucleotide units. The four units of DNA are nitrogenous bases or nucleotides: adenine (A), thymine (T), cytosine (C) and guanine (G), as shown in Figure 2-1. These monomers are connected in a polymeric single stranded DNA (ssDNA) along a sugar-phosphate backbone. All DNA bases are attached to deoxyribose, a five-carbon sugar molecule. A base connected to a sugar is referred to as a deoxyribonucleoside. A nucleoside connected to one, two or three phosphate groups is referred to as a deoxynucleoside monophosphate (dNMP), diphosphate (dNDP) or triphosphate (dNTP), respectively, as shown in Figure 2-2. Phosphodiester bonds connect each deoxyribonucleotide in the DNA chain. The phosphate attached to the last carbon of the deoxyribose sugar of the one nucleotide is attached to the third carbon of the sugar of the next nucleotide, thus forming one strand of nucleotides connecting 5' to 3'.

Figure 2-1: DNA bases. The four units of DNA bases are adenine (A), thymine (T), cytosine (C) and guanine (G).

Figure 2-2: Nucleotide structure consisting of a base, sugar molecule and phosphate groups.

From the analysis of the X-ray diffraction patterns obtained from crystals of DNA, Watson and Crick proposed a model for the structure of DNA [3]. This model predicted that double stranded DNA (dsDNA) molecule comprises two single strands of DNA running tightly-wound around each other to form a ladder in the shape of a helix as shown in Figure 2-3. Hydrogen bonds are formed between the complementary bases adenine (A) with thymine (T) and guanine (G) with cytosine (C). From any fixed position in the helix, one strand is oriented in the 5' → 3' direction and the other in the 3' → 5' direction. On its exterior surface, the double helix of DNA contains two deep grooves between the ribose-phosphate chains. These two grooves are termed as major and minor grooves.

Figure 2-3: DNA bases arranged in a double helix.

2.1.2 *In vitro* amplification of nucleic acids

The ability to specifically amplify very small quantities of nucleic acid has been invaluable in areas such as disease diagnostics, genetic analysis, forensics and

environmental testing. The amplification process constitutes only part of the complete analysis, as a sample preparation step must usually be performed prior to amplification, which is followed by a detection step.

2.1.3 Target amplification

The most sensitive molecular technique uses an enzymatic step to amplify the target nucleic acid before analysis of the specific sequence is performed. A variety of target amplification techniques have been developed, such as the PCR.

2.1.4 Polymerase Chain Reaction (PCR)

It should be noted that the amount of DNA in purified samples is typically insufficient for direct detection, thus a target amplification step is needed. In 1983, Kary Mullis at Cetus Corporation developed the molecular biology technique that revolutionized genetic research, earning him the Nobel Prize in 1993 [4, 5]. This technique, known as the PCR, transformed molecular biology into a multidisciplinary research tool. PCR has rapidly become one of the most widely-used techniques in molecular biology and for good reasons: it is a rapid, inexpensive and simple mean of producing copies of DNA molecules in large quantities, from minute quantities of source DNA material – even when the source DNA is of relatively poor quality. Researchers in the Human Genome Project are using PCR to look for markers in cloned DNA segments and to order DNA fragments in libraries. PCR involves preparation of the sample, the master mixture and the primers, followed by detection and analysis of the reaction products. The various steps of PCR are schematically shown in Figure 2-4.

Figure 2-4: Principle of the Polymerase Chain Reaction [6, 7].

PCR is an *in vitro* technique which allows the amplification of a specific deoxyribonucleic acid region that lies between two regions of known DNA sequences [8]. PCR involves DNA synthesis from two oligonucleotide primers that act as sites for initiation by the DNA polymerase and therefore defined region of the template DNA that will be copied. The primers are complementary to regions of known sequence on opposite strands of the template DNA and the 3'(OH)-end points towards the other primer. DNA polymerases require a primer to start the DNA synthesis. The primer is extended in the presence of the four deoxyribonucleotides (dATP, dGTP, dCTP and dTTP) and specific buffer conditions so that the DNA between the two primer binding sites will become amplified during each cycle of the PCR. The reaction tube is placed in a thermal cycler and subjected to a series of heating and cooling cycles as outlined in Figure 2-5. The reaction cycle involves template denaturation, primer annealing, and the extension of the annealed primers by DNA polymerase until enough copies are made for further analysis.

a. Temperature of a PCR cycle

In each PCR cycle, the reaction mixture is subjected to three different temperatures (Figure 2-5).

Figure 2-5: A typical temperature profile of a PCR [5].

Step 1: Denaturation

First the template DNA is denatured to separate the complementary strands (double-stranded DNA) into two single strands at the denaturation temperature, usually about 90 to 96°C (Figure 2-6). Since the hydrogen bonds linking the bases to one another are weak, they break at high temperatures, whereas the bonds between deoxyribose and phosphates, which are stronger covalent bonds, remain intact.

Figure 2-6: Denaturation by heat [9].

Step 2: Annealing

Next the reaction mixture is cooled to an annealing temperature to allow the oligonucleotide primers to hybridize on the template (Figure 2-7). The hybridisation or annealing temperature is usually about 40 to 60°C. During this annealing step, the thermostable DNA polymerase will be active and begin to extend the primers as soon as they anneal to the template. Annealing temperature (T_a) and time strongly influence the efficiency and specificity of the PCR reaction. Hence, even though there are some rules for deriving annealing temperature, it is recommended that this parameter be assessed empirically.

Figure 2-7: Annealing-primer binding to target [9].

Step 3: Extension

The extension temperature causes DNA synthesis to occur (Figure 2-8). This is usually set at about 70 to 75°C, just below the optimum for *Taq* DNA polymerase (enzyme). Once the primers anneal to the complementary DNA sequences, the temperature is raised to approximately 72°C and the enzyme *Taq* DNA polymerase is used to replicate the DNA strands. Extension always begins at the 3' end of the primer

making a double strand out of each of the two single strands. *Taq* DNA polymerase synthesizes exclusively in the 5' to 3' direction. Therefore, free nucleotides in the solution are only added to the 3' end of the primers constructing the complementary strands of the targeted DNA sequences.

Figure 2-8: Extension [9].

At the end of the first PCR cycle, there are now two new DNA strands identical to the original target. The PCR process repeats the first three steps for 25-40 times, as necessary for the specific application. Finally, reaction is allowed to cool to either room temperature or 4°C depending on the application and type of thermal cycler used.

The theoretical DNA amplification factor is 2^n , where n defines the number of PCR cycles performed. Ideally, after 20 cycles of PCR there will be a 2^{20} -fold amplification, assuming 100% efficiency during each cycle. The efficiency of a PCR will vary from template to template and according to the degree of optimization that has been carried out. The target sequence product (also known as an amplicon) obtained as a result of the process contains the oligonucleotide primer sequences at its ends. Although

extremely efficient, amplification of target sequences in an exponential manner is not an unlimited process. A number of factors act against the process being 100% efficient at each cycle. Their effects are more pronounced in the later cycles of PCR. Normally, the amount of enzyme becomes limiting after 25-30 cycles of PCR, which corresponds to a 10^6 -fold amplification, due to molar target excess. The enzyme activity also becomes limiting due to thermal denaturation of the enzyme during the process. Another factor that will reduce the efficiency is the reannealing of target strands as their concentration increases. The reannealing of target strands then competes with primer annealing. It is important to minimize any variance in the PCR procedure and strive to obtain cycle efficiencies of 100%. Thus, the final number of copies of the target sequence after amplification, X_{final} , is expressed by the formula below [10]:

$$X_{final} = (2^n - 2n)x \quad (2.1)$$

where:

n is the number of cycles;

$2n$ is the first product obtained after cycle 1 and second products obtained after cycle 2 with undefined lengths;

x is the number of copies of the original template.

b. The PCR mixture

The basic PCR mixture has four main ingredients: template dsDNA, a set of two primers to determine the amplified region, abundant *Taq* polymerase enzyme and free oligonucleotides (dNTPs) of each type (ATP, GTP, TTP and CTP) to be used as building bricks by the polymerase; but, clearly, there is more to the picture than it shows. The following is a brief description of the standard PCR mixture:

Template DNA

Template DNA is a basic prerequisite for conducting any PCR amplification. PCR amplification requires the presence of at least one DNA template strand. However, a minimum of 10^4 copies of template strands is recommended for easy operation.

Typical initial concentrations are between 0.01-0.1 ng per reaction for plasmid DNA and 10-500 ng per reaction for genomic DNA.

Taq Polymerase

A heat-resistance polymerase is required to perform the extension process of PCR without excessive contamination. Although *Taq* polymerase is the most widely used enzyme, there exist alternative polymerases that can improve yields in certain cases. The typical concentration for *Taq* polymerase is between 0.25-2 U, where U is the unit enzymatic activity for *Taq* polymerase, defined as follows: 1U = the amount of enzyme required to convert (in 30 min at 72°C) 10 nmol dNTP into acid insoluble materials.

Primers

Primes also play an essential role in the PCR, since they provide selectivity to the amplification process and are required by *Taq* polymerase to initiate extension. Typical primers have lengths between 17-18 bp long and a (G+C) content of between 40 and 60%. Optimum primer concentrations are 0.1-1 μ M.

dNTPs

Free oligonucleotides in solution are required by the *Taq* polymerase to replicate the template molecule during the extension step. It is important to ensure an equal concentration of each type of oligonucleotide (A, T, G and C), which should range between 50-250 μ M at pH7.5.

Buffers

The PCR buffer is a composite solution that adds indispensable elements to the PCR mixture. The buffer consists of three separate items:

Potassium chloride (KCl)

Salt is required to stabilize the DNA molecules, facilitating primer annealing and polymerase activity. Typical concentration for KCl is about 30-50 mM.

Magnesium chloride (MgCl₂)

Magnesium chloride is necessary for PCR since, in solution, this divalent cation provides the free Mg²⁺ ion required by the *Taq* polymerase to conduct its enzymatic activity. Optimal concentration for MgCl₂ is 1-1.5 mM. Conversely, excess free magnesium may increase the level of non-specific amplification. Magnesium ion stabilizes DNA duplex; hence high concentration of MgCl₂ decreases the efficiency of PCR.

Tris-HCl [Tris(Hydroxymethyl) aminomethane.HCl] buffer

The Tris-HCl buffer is required to set the global pH of the reaction, which affects both polymerase activity and specificity. The usual pH is 8.3 at 25°C (which will drop to 7.2 at 72°C), provided by a concentration of 10mM Tris-HCl buffer.

Ultra-pure water

To achieve the desired proportions and concentrations of all reactants, they are typically dissolved in sterile 18.2 MΩ ultra-pure water. Ultra-pure water is rendered free of organic material by double-distillation and then deionized in ionic (cationic and anionic) exchange columns. To achieve further sterility, milliQ water can be sterilized in autoclave prior to use.

c. Application of PCR

One of the great advantages of PCR is its ability to amplify a defined region of DNA from a very complex starting template such as genomic DNA. PCR has a strong impact on four main areas of biotechnology: gene mapping, cloning, DNA sequencing, and gene detection [11]. PCR is now used as a medical diagnostic tool to detect

specific mutations that may cause genetic disease, in criminal investigations and courts of law to identify suspects on a molecular level, and in sequencing of the human genome. Prior to PCR discovery, the use of molecular biology techniques for therapeutic, forensic, pharmaceutical, or medical diagnostic purposes was not practical or cost-effective. The development of PCR technology changes these aspects of molecular biology from a difficult science to one of the most accessible and widely used tools in genetic and medical research.

2.2 Overview of the Detection and Analyses of PCR Products

PCR products, or amplicons, consist of fragments of DNA which are normally of a length defined by the boundaries of the PCR primers. PCR products are often less than 10 kbp in length. Generally the following information can be derived from the analysis of specific PCR products:

- Detection of the presence of the target DNA sequence and variations within it;
- Quantization of the yield of a PCR amplification to determine the relative or absolute amounts of the initial DNA or RNA target;
- Sequence analysis, either by differential probe hybridization or by direct sequencing of the product.

Nucleic acid based analysis can be carried out in either heterogeneous or homogenous manner. Heterogeneous assays require further manipulation once the amplification reaction is complete. This may involve aliquoting a sample for a number of analytical procedures or adding certain components of a signal detection system. Homogenous assays require no further manipulation once the reaction is set up as signal-generation and detection are carried out in a single closed-tube when the reaction is complete, or continuously during the amplification (real-time detection). The quantity of samples and number of analyses required from one sample usually dictate the choice between homogeneous and heterogeneous systems.

The amplification product is studied in various ways in order to gain information about the DNA molecule that previously serves as the original template. The following are among the most important techniques for studying PCR:

- Direct detection by ethidium bromide
- Detection by gel electrophoresis
- Blotting and hybridisation with specific probes
- Detection by a combination of capture and detection probes
- Sequencing of the PCR product
- Immunological detection of PCR products
- Scintillation proximity assay (SPA)
- Electrochemiluminescent detection
- Surface plasmon resonance (SPR)
- Real-time quantitative PCR

2.2.1 Direct detection by ethidium bromide

The simplest method for detecting a PCR product is the addition of a small quantity of ethidium bromide (EB), a dye that becomes fluorescent after intercalating with strands of DNA in the vessel after the amplification procedure. When the reaction vessels are examined under an ultraviolet lamp, a positive reaction will emit light whereas negative reactions remain dark [12]. A single DNA molecule can contain hundreds of fluorophores and emit a strong signal. These light emitting DNA fragments can be observed using an epi-fluorescence microscope.

2.2.2 Detection by gel electrophoresis

Electrophoresis of an aliquot of the PCR sample on an agarose or polyacrylamide gel followed by visualization after staining with EB, is one of the simplest methods of amplicon detection [13]. After staining, ultraviolet transillumination allows visualization of the DNA in the gel. The gel may be photographed to provide a permanent record of the experimental result. Size markers or previously amplified

control PCR products can be electrophoresed in adjacent wells of the gel to allow accurate size determination of the amplicons.

The PCR product should be a fragment or fragments of DNA of defined length. The simplest way to check for the presence of these fragments is to load a sample taken from the reaction product, along with appropriate molecular-weight markers, onto an agarose gel which contains 0.8-4.0% EB. DNA bands on the gel can then be visualized under ultraviolet trans-illumination. By comparing product bands with bands from the known molecular-weight markers, one can visually identify any product fragments which are of the appropriate molecular weight.

The fundamental theory behind electrophoresis is to place the sample in a gel-like material (usually agarose or acrylamide) and expose the gel to an electric voltage. The negatively charged phosphate groups on the nucleic acids confer an overall negative charge, forcing the DNA molecules to migrate to the positive electrode (anode) during electrophoresis. As the sample migrates to the positive electrode, it has to pass through the pores of the gel. Molecules will be separated on the basis of size, in which smaller molecules easily migrate through the gel and will therefore run faster than larger molecules. The mobility of a DNA molecule is inversely proportional to the logarithm of its molecular weight. It is therefore possible to find out how big a fragment of DNA is by analyzing its mobility in a gel.

Gels can be poured in a variety of shapes, sizes, and pore sizes. The choice of gel size and type primarily depends on the type and size of the molecules that are being separated. Agarose gels are run horizontally and submerged in a tank of buffer, whereas acrylamide gels are run vertically with the gel sandwiched between two glass plates. A gel with a larger pore size (lower percentage agarose or acrylamide) will efficiently separate (resolve) molecules of higher molecular weight (long DNA fragments). Unfortunately such gels with larger pore sizes are not good for resolving smaller molecular weight DNAs because the DNA fragments separate too much and appear as diffuse bands. High percentage gels, with small pore sizes, are more

appropriate for resolving DNA fragments with smaller molecular weight into sharper bands. Agarose gels have lower resolving power than acrylamide but have a greater range of separation. Agarose gels are good for separating DNA fragments in the range of 200 bp to 50 kb in length. Acrylamide gels are better for high resolution of short DNA fragments.

Agarose gel results are obtained from the end point of the reaction. End-point detection is very time consuming. Results may not be obtained for days. Results are based on size discrimination, which may not be very precise. The end point result is variable from sample to sample. While gels may not be able to resolve these variations in yield, real-time PCR is sensitive enough to detect these changes. Some of the problems with end-point detection are:

- Poor Precision
- Low sensitivity
- Short dynamic range < 2 logs
- Low resolution
- Non - Automated
- Size-based discrimination only
- Results are not expressed as numbers
- Ethidium bromide for staining is not very quantitative
- Post PCR processing

2.2.3 Blotting and hybridisation with specific probes

When screening for known point mutations in the sequence amplified by PCR, amplicons can be transferred (blotted) to nitrocellulose or nylon membranes and analysed by hybridisation with a labelled oligonucleotide probe specific for the mutation. Washing the membrane at a critical temperature after hybridisation removes probes bound to sequences that were not a perfect match [14]. However, this is a time-consuming process, as it requires multiple PCR product handling steps, thus further

increases the risk of contamination.

2.2.4 Detection by a combination of capture and detection probes

The increased automation of PCR and the detection of PCR products have led to the development of more sophisticated techniques to detect the presence of specific amplification during the reaction. As an alternative to analysis by electrophoresis and blotting procedures, the PCR product can be detected by a combination of capture and detection probes. In addition to the two primers used for amplification, two oligomers are designed to hybridise as probes within the amplicon. One probe is used for capture (that is, for the binding of the PCR product to a solid phase such as micro plate, membrane or paramagnetic particle) and the other is labelled and used for detecting the immobilized PCR product.

2.2.5 Sequencing of the PCR products

The nucleotide sequence of the PCR product can be determined by sequencing either directly or after cloning into an appropriate vector. Sequencing the amplified stretch of DNA can be particularly important in the case of infection with HIV or hepatitis C virus (HCV) in order to determine the extent of sequence variability in regions within the envelope genes. The sequencing method requires single-stranded DNA as the starting material. The single-stranded DNA undergoes a procedure known as Sanger-Coulson method, in which a DNA polymerase synthesizes families of chain-terminated molecules. This reaction thus generates complementary strand fragments terminated at all possible positions of the matching dideoxide. These fragments can be separated in a gel and their relative positions indicate the location in the sequence where a specific base is present, hence the sequence could be read out directly.

2.2.6 Immunological detection of PCR products

A PCR-enzyme-linked immunosorbent assay (PCR-ELISA) was developed by P. Kurupati *et al.* for the detection of the PCR products from blood specimens [15]. Antibody digoxigenin peroxidase conjugate labeled amplified PCR products were hybridized to the biotin labeled probe on the streptavidin-coated microwell plates.

ABTS (2,2-azino-di-3-ethylbenzothiazoline sulfonate) was added for colour development. The absorbance at 405 nm was then measured using a plate reader. If more PCR products were hybridized to the probe, then more antibody digoxigenin peroxidase in the well would catalyze the colour reaction of ABTS, thus the higher is the optical density value.

2.2.7 Scintillation proximity assay

The scintillation proximity assay provides a means of analyzing PCR products without physical separation [16] and can be easily adapted to a microplate format. Molecules that are labelled with a low-energy β -emitter, such as ^3H , stimulate scintillation by being brought into proximity with a fluorophor-containing bead. The energy from unbound ^3H -labelled molecules remaining in solution is absorbed as heat and not detected. The QuantAmp kit of Amersham International plc contains streptavidin-coated fluomicrospheres (scintillation proximity assay beads), for the detection of biotinylated, ^3H -labelled DNA molecules. QuantAmp has been used for the quantification of baculovirus DNA by detecting biotinylated ^3H -labelled products in the exponential phase of amplification [17] and in semi-quantitative analysis of mRNA levels by using biotinylated internal probes for the PCR product [18].

2.2.8 Electrochemiluminescent detection

Electrochemiluminescent detection and quantification of PCR-amplified DNA is possible by using a mixture of biotinylated and tris(2,2'-bipyridine)ruthenium (II) (TBR) end-labelled reverse primers that permits fast post-PCR product detection [19, 20]. The probe-based detection system exploits a chemiluminescent reaction that takes place on the electrode surface in an electrochemical cell. The amplification products are then captured on streptavidin coated paramagnetic beads and quantified by measuring the electrochemiluminescent signal of the TBR label. Quantitative results can be obtained using a standard curve, which is generated with a synthetic external standard.

2.2.9 Surface plasmon resonance (SPR)

Asymmetric PCR products were detected using a flow injection-type sensor based on surface plasmon resonance [21]. A sequence-specific DNA probe is immobilized on the transducer to capture single-stranded PCR products from the solution. The detection is dependent on the formation of a double-stranded complex upon hybridization of the immobilized probe with the target DNA, which corresponds to the amplicons from asymmetric PCR.

2.2.10 Real-Time quantitative PCR

Real-time detection in the simplest form is achieved by the simultaneous amplification and detection of specific DNA sequences achieved through the addition of a DNA intercalating dye, EB to a PCR. Theoretically, as stated in Section 2.1.4, the theoretical DNA amplification factor is 2^n at any given cycle. However, in practice, the molecular amplification process is not as straightforward. A basic PCR run can be categorized into three phase (Figure 2-9) [22]:

Figure 2-9: PCR phases in Log scale.

Exponential: Exact doubling of product is accumulating at every cycle (assuming 100 % reaction efficiency). The reaction is very specific and precise.

Linear (High Variability): The reaction components are being consumed, the reaction is slowing, and products are starting to degrade.

Plateau (End-Point: Gel detection for traditional methods): The reaction has stopped, no more products are being made and if left long enough, the PCR products will begin to degrade.

Initially, exponential phase of PCR products occurs because all of the reagents are fresh and available, and the kinetics of PCR pushes the reaction to favour doubling of amplified products. As the reaction progresses, some of the reagents are being consumed as a result of the amplification. This depletion will occur at different rates for each replicate. The reaction would slow down and the PCR product would not be doubled at each cycle. This linear amplification can be seen in the linear phase of the reaction. The amplification eventually slows down and reaches a plateau. This plateau phase corresponds to the stage in traditional PCR where measurements for end-point detection are performed. In addition, the amplification may not be exponential to begin with, given variations in reaction conditions or the presence of inhibitors. Hence, end-point detection may cause the number of copies of molecular amplification to be inaccurate due to variable yield at the plateau phase (*Figure 2-9*).

2.2.11 Electrochemical detection of PCR products

In the identification and quantification of the PCR amplicons after the post-PCR processes, several authors have reported successful detections of the hybridization events by using PCR-amplified DNA from the real samples with electrochemical DNA biosensors designed for reliable measurements of clinical interest [23-28]. Ozsoz *et al.* used an electrochemical DNA biosensor (by immobilizing the molecules of oligonucleotide on the surface of a carbon paste electrodes and adding the MB as a hybridization indicator) coupled with the PCR amplification of DNA extracted from human blood for the electrochemical detection of Hepatitis B viral sequences [25]. A

disposable indicator-free electrochemical DNA biosensor which was applied in the detection of apolipoprotein E sequences in PCR samples was reported by Mascini *et al.* [23]. The configuration of the biosensor comprised the immobilisation of an inosine-modified (guanine-free) probe onto a screen-printed electrode transducer and the detection of the duplex formation in connection with the SWV measurement of the oxidation peak of guanine in the target sequence. Lai *et al.* reported on a novel method for the sequence-specific detection of unpurified amplification products based on self-assembly of a MB-labelled DNA probe on a gold electrode surface [26]. In the absence of a target, the stem-loop structure held the MB tag in proximity to the electrode surface and thus enabled an efficient electron transfer. Upon hybridization with the target PCR amplicons, a large change in the reduction peak current of MB was observed. Li *et al.* had demonstrated the utilization of an oligonucleotide-functionalized electro-conducting polymer, coupled with a nano particle based hybridization indicator for the detection of multi-labelled PCR amplicons [28]. Lee *et al.* demonstrated an electrochemistry-based approach for the detection of the asymmetric PCR amplification products of Chinese herbal genes [27]. In their work, a 5'-thiol-derivatized ssDNA probe was chemisorbed on a gold electrode surface for the hybridization-based detection of unpurified PCR amplicons. Subsequent signal transduction of the hybridization event was achieved by the linear sweep voltammetric scan of a commercially available redox-active DNA intercalator (Hoechst 33258) bound to the hybrid between the DNA probe and the PCR amplicons. The current signal obtained could be used to identify the sequence of the PCR amplicons and to quantify the amount of DNA template existed in the sample. The enzyme-based impedimetric detection of PCR products by oligonucleotide-modified screen-printed gold electrodes had been studied [24]. The detection of 195 bp amplicons was also achieved via its sandwich hybridization with a biotinylated signalling probe, in a flexible format that eliminated the need of biotinylated primers during the PCR amplification [24].

Kobayashi *et al.* [29] has developed a similar EC detection system for DNA

quantification based on an aggregation induced by Hoechst 33258. The PCR product was amplified every 5 cycles and mixed with redox-active Hoechst 33258 molecules in a 1:10 ratio. The mixture was then electrochemically measured individually. The results suggested that the electrochemical detection depend on DNA concentrations. However, this could not be achieved while the PCR process was in progress, that is, the authors, had to halt the PCR process every 5 cycles in order to perform their measurements. Consequently, this method is not suitable for real-time quantification of PCR products. This is partially due to the decrease in anodic peak current of Hoesch 33258 with the increase in the number of scans at the electrode even at the absence of PCR products.

2.3 Overview of DNA Analyses in Microdevices

There are some common restrictions associated with conventional PCR devices. These include:

(i) Slow thermal cycling and poor thermal uniformity

The basis of most commercial products for heating a sample is through intimate contact between a PCR reaction chamber holding the sample and a heating block that is rapidly heated and cooled (e.g. by using a Peltier element that can be heated and cooled). This heating/cooling block has a large heat capacity, which limits the rate at which the samples are heated and cooled [30]. The conventional PCR devices are capable of achieving up to $4^{\circ}\text{C min}^{-1}$ in the temperature range of $50\text{--}94^{\circ}\text{C}$ and require more than an hour to run a complete PCR reaction. Thus, with pre-PCR processing and PCR detection, the whole process can take several hours and at a cost of several hundred dollars [31]. In addition, conventional systems require relatively high reaction volume ($>25\text{ }\mu\text{L}$), thus contributing to a slower thermal cycle [32, 33]. Moreover, in practice, overheating and sub-cooling cannot be entirely avoided. Furthermore, one of the greatest problems with PCR carried out in plastic PCR plates (e.g. 96 well-plates) are that the temperature gradients within the well plate maybe as

high as 10°C. Therefore, there is a need to improve the performance of these PCR devices.

(ii) High power consumption and large size

The high power consumption and size of the conventional PCR thermocyclers are two other restrictions of preventing these devices from becoming portable for field use.

(iii) High risk of contamination

The products of the PCR is to be delivered with pipettes into the detection devices, thus there is a lot of sample handling activities. This increases the risk of contamination of PCR products by the surrounding environment, and subsequently affecting the accuracy of results of the DNA assays.

A typical PCR involving 25-35 cycles usually takes between two to three hours and is often the rate-limiting step in DNA analysis. In addition, the conventional DNA analysis techniques are time and labour-intensive, thus it cannot cope with the ever-growing demand for genetic testing in the 21st century. Miniaturization of the analytical systems offers significant advantages in terms of analytical performance (separation efficiency and detection sensitivity), speed, high throughput, cost, and sample/reagent consumption. Most importantly, all the analytical steps (sample preparation, target amplification and product detection) can be integrated into single self-contained microdevices for point-of-care DNA analysis applications [34, 35]. In recent years, many devices for PCR have been constructed to demonstrate the benefits of miniaturizing. These devices are commonly referred to as 'PCR chips' [36, 37]. This micro-PCR device, based on micro-electro-mechanical systems (MEMS) technology, provides a platform for PCR amplification and *in-situ* fluorescence detection, while others try to accommodate all the necessary fluid, thermal and detection functions under a common substrate to realize the conception of the miniaturized total chemical analysis system (μ TAS) [38, 39]. This leads to the development of high performance and reusable μ PCR devices. With the possibility of low cost fabrication, disposability of these devices is also investigated. The most straightforward approach in the

miniaturization of bioanalytical systems is to take advantage of the developed microfabrication technologies, and to shrink the conventional DNA assay platforms onto silicon, glass and plastic microdevices.

The advent of miniaturization has facilitated improvements in PCR instrumentation, allowing a reduction in sample volume and reagent cost, shorter cycling times and higher-throughput, with automation offering the possibility of “hands-free” analysis. Reaction times have been reduced to 90 s by adapting PCR to run in continuous flow system on a chip, while real-time PCR has been performed in a rapid air thermal cycler in 10-20 min. Affymetrix has developed a miniature device that integrates sample extraction, nested PCR and mutation detection of HIV-1 by array hybridization.

2.3.1 PCR microchip

The different approaches of performing PCR on a microchip have been classified into two main configurations: a time domain (well-based amplification) and a space domain approach (continuous flow-through-based amplification).

(i) Well-based amplification (Time domain PCR)

In the first configuration, the reaction mixture is kept stationary and the temperature of the surrounding reaction chamber is cycled between the different temperatures. The first on-chip PCR devices were presented by Northrup *et al.* [40] in 1993 and Wilding *et al.* in 1994 [41]. Both devices consisted of a silicon chip with a micro-well in which the sample was loaded. The entire chip was heated and cooled to provide the required thermocycling conditions. Northrup’s chip (Figure 2-10) was fabricated onto 0.5 mm silicon wafers that were chemically etched to provide $10 \times 10 \text{ mm}^2$ reservoirs. These pools of approximately 50 μL in capacity were then sealed with a glass cover, the surface of which having been bound with silicon. Polyethylene tubing was used to provide the system with inlets/outlets and the thermal cycling required for PCR was made available by a patterned polysilicon layer deposited by CVD (chemical vapour deposition) onto a heat-region (a silicon nitride (Si_3N_4) membrane) at the backside

of the chip. The polysilicon resistance was electrically accessed through two aluminium contact pads for heating, and cooling was attained by passive air convection. Temperature was monitored using a chrome-aluminium thermocouple. With this basic scheme, Northrup's team was able to conduct PCR amplifications of a 142 bp region of the GAG gene of HIV. Starting with 10^8 initial copies, a successful amplification in twenty 1 min cycles (20 min) was achieved, although the PCR yield was lower than the positive control run on a conventional thermocycler.

Figure 2-10: Microchip-based PCR developed by Northrup *et al.* [40].

(ii) Continuous flow-through based amplification (Space domain PCR)

Kopp *et al.* was first demonstrated a second configuration of the chip-based PCR device that used the continuous flow methodology, which consisted of a single channel winding through different temperature regions (Figure 2-11) [42]. In this format the reaction mixture was moved between different fixed temperature zones. The channels were 90 μm wide by 40 μm deep and covered the entire surface of a two square 0.55 mm thick Corning glass plate. The channels were silanized with dichlorodimethylsilane to prevent enzymatic binding. The sample was initially placed in a well, and pumped through the channel at a flow rate of 5.8nl/s using a Kloehn 50300 syringe pump. The length of the channel and flow rate determined the time spent in each temperature region. Kopp *et al.* reported on the best results with channel length ratios of 4:4:9 for denaturing : annealing : elongation respectively. The different temperature regions were maintained by resting the glass plate on three

copper blocks. The device did not need heating and cooling, and this permits faster cycling. A variant of this general design incorporates outlets at different distances along the serpentine channel, facilitating product collection after 20, 25, 30, 35, and 40 cycles [43-46]. Successive samples can be analyzed simultaneously by isolating the individual samples by segments of the diluent. The key disadvantage of the continuous flow methodology is evident from the geometry – increasing the number of channels on a chip requires significantly more real estate, thus the design cannot be truly micro-scale.

Figure 2-11: Microfluidic PCR chip layout developed by Kopp et al. [42].

2.3.2 Advantages of PCR miniaturization

As seen from the various PCR microchips that have been developed, PCR miniaturization can bring about the following advantages:

- *More precise temperature control.* Microsystems are more suited to precise temperature control than conventional systems due to the short paths for heat transport and its high surface area to volume ratio. Heaters and sensors for local temperature measurement can be located in very close proximity or connected to the chamber containing the sample (for example, thin film platinum temperature sensor). This can provide a more accurate reading on the sample temperature. The choice of geometries and materials allows configurations for well-controlled local

heat flow during denaturing, annealing and extension process [47].

- *Faster thermal cycle.* Since the heating element and cooling elements possess small volumes and small heat capacities, achieving high cooling and heating rates of about $15\text{--}40\text{ }^{\circ}\text{C s}^{-1}$ is possible [30]. Ultimately, the minimum reaction time is limited by the speed of the enzyme itself. In addition, since the sample volumes have shrunk significantly to the range of micro-litre or less, sample thermal cycling becomes faster [48, 49]
- *Cost reduction.* The cost of reagent is reduced due to miniscule reaction volumes. System costs are also much lower due to the introduction of batch fabrication technologies [50]. This allows single-use systems to be produced as well.
- *High throughput.* Coupled with the reduced analysis time, reduced reagent and fabrication costs, miniaturization can result in an increase of throughput [51, 52]. Appropriate microchip design could allow multiple samples to be tested.
- *Less waste production.* The coupling of traditional sample preparation with microchip electrophoresis is wasteful. For example, 20-50 μL of PCR product is generated with conventional thermal cycling. However, microchip analysis requires as little as 1 μL of sample with only a few pico-litres actually consumed. In the case of PCR chip, only a small volume of sample is needed for thermal cycling [53].
- *Ability to integrate with various platforms.* Sample preparation, PCR thermal cycling, PCR product detection and post-processing can be integrated onto a micro-fluidic chip. This means that the risk of cross-contamination due to sample handling and the risk of infection will be reduced and sample analysis will be faster through reduced manual manipulation [35, 54-58].

2.3.3 Integrated DNA analytical microdevices

The combination of selective amplification and separation techniques is the key to

generic DNA analysis systems. Since the early work of deoxyribonucleic acid (DNA) manipulations in microchips by Northrup [39] and Woolley *et al.* [59], tremendous research activities have been carried out to miniaturized conventional DNA analytical procedures in microchip platforms. These microdevices enjoy the miniaturization advantages of small size, low sample, reagent and power consumption, enhanced analytical performance (shorten the assay time), and high level of integration. There are three essential components in a complete DNA assay protocol, which include sample preparation, target amplification, and product detection. The implementation of these functionalities on-chip has been individually optimized prior to their final integration.

In the last few years, two teams succeeded in creating fully integrated PCR-plus-electrophoresis systems using similar approaches [55, 60]. Making combined use of micro-fluidic valves, hydrophobic vents, electro-kinetic injection and integrated or off-chip thermal elements, both groups produced functional monolithic chips for PCR-plus-electrophoresis analysis. Burns *et al.* [55] developed a device, which used microfabricated fluidic channels, heaters, temperature sensors and fluorescence detectors to analyze nanoliter-sized DNA samples (Figure 2-12). It incorporated sample injectors, mixturer, heating chamber, and separation channel with detectors.

Figure 2-12: Schematic of integrated device with two liquid samples and electrophoresis gel [55].

Lagally *et al.* [61, 62] developed a fully integrated system in glass for the manipulation, amplification and capillary electrophoresis separation of submicroliter volumes of DNA. Figure 2-13 shows the microfabricated PCR-capillary electrophoresis device. The PCR chambers are connected to a common bus through a set of valves. Hydrophobic vents at the other end of the PCR chambers are used to locate the sample and to eliminate gas. The PCR chambers are directly connected to the cross channel of the capillary electrophoresis for product injection and analysis [62]. It features the microfluidic loading and positioning of sample in eight 280 nL PCR chambers with the use of active valves and hydrophobic vents, rapid PCR amplification using thin film heaters, followed by direct injection and rapid separation on a microfabricated capillary electrophoresis channel.

Figure 2-13: Microfluidic PCR-capillary electrophoresis device [62].

Although many issues still need to be addressed in order to produce functional generic analysis systems for DNA, the goal of creating a sort of DNA μ -TAS is getting closer and, combined with advances in closed-kit analysis systems, its implications for research and clinical biology could certainly be as revolutionary as the introduction of integrated circuits, for computer science [63].

2.3.4 DNA amplicons detection

DNA detection in a microscale setting has been achieved through optical, microgravimetric (mass-sensitive) and electrochemical techniques. In the case of DNA analysis, recognition can be achieved with an oligonucleotide probe that is complementary to the target sequence, and signal transduction can be based on optical, microgravimetric or electrochemical methods.

a. Capillary electrophoresis - separation and EC detection

Capillary electrophoresis coupled with laser-excited confocal fluorescence detection, a miniaturized version of gel electrophoresis, is by far one of the most widely studied optical DNA detection methods. This method is usually used for PCR product sizing, DNA fragment analysis and sequencing. State-of-the-art capillary electrophoresis devices are capable of separating and detecting DNA from tens to hundreds of base pairs in less than 10 mins, with a resolution of a few base pairs.

The first generation of capillary electrophoresis systems with integrated electrochemical detection were developed by Woolley *et al.* [64]. Using indirect electrochemical detection, high-sensitivity DNA fragment restriction and PCR product-sizing were performed. The initial measurements are based on indirect DNA detection using the redox-active intercalator dye iron phenanthroline ($\text{Fe}(\text{phen})_3^{2+}$), which has been successfully applied to the detection of PCR products and DNA fragment sizing. With this technique, initial high background currents are obtained from free $\text{Fe}(\text{phen})_3^{2+}$ in the separation and sheath-flow buffers (Figure 2-14). When complexed, DNA- $\text{Fe}(\text{phen})_3^{2+}$ migrates past the detector and transient reductions in the background current are observed, indicating the presence of double-stranded DNA [60, 64-66].

Figure 2-14: DNA analysis using a CE chip with integrated electrochemical detection [65].

However, the capillary electrophoresis analysis of PCR products does not provide sequence information, which is essential in routine genetic assays of disease and pathogen diagnostics.

b. PCR - Electrochemical device for DNA amplification and detection

The first demonstration of a microfabricated silicon/glass-based device with functionalities of PCR target amplification and end point sequence-specific electrochemical detection was developed by Lee *et al.* [67]. In their work, gold electrodes within the PCR chamber were used for DNA probes immobilization and either electrochemical intercalators or silver-coated gold nanoparticles were used to detect the concentration of the PCR product of interest. Figure 2-15 shows the diagram of the integrated PCR-EC end-point detection microdevice. The integrated PCR-EC microdevice had a reaction chamber volume of approximately 8 μ L. First, the PCR-mixture containing the DNA analyte was loaded into the reaction chamber (Figure 2-15 (A)); second, asymmetric PCR was then carried out to produce single-stranded rich target amplicon (Figure 2-15 (B)). Third, the hybridization of the target amplicon to the probe immobilized on the working electrode would occur if the amplified sequence was complementary to the probe (Figure 2-15 (C)). Finally, the electrochemically-active hybridization indicator was bound to the hybridized amplicon, and the amount of PCR amplicons was determined by electrochemical

transduction (Figure 2-15 (D)).

Figure 2-15: A diagram depicting the assay principle of the integrated PCR-EC microdevice [67].

A plot of the peak current of the intercalator (Hoechst 33258) during electrochemical detection voltammetric against log of number of copies of the DNA template in the PCR reaction chamber is showed in Figure 2-16. The DNA template is from the gene of traditional Chinese medicinal herb *Fritillaria thunbergii* (~ 600 bp). Asymmetric PCR was carried out inside the reaction chamber of PCR-EC microdevice for 40 cycles. After the asymmetric PCR, the reaction chamber was cooled back to ambient temperature for 1 min. The reaction mixture was incubated for 1 hour, allowing the PCR product to hybridize with the probe immobilized onto the working electrode. The signal transduction of the hybridization event was done by introducing a 100 μM Hoechst 33258 solution into the reaction chamber for 5 min, followed by DPV scan of the gold electrode from +0.20 V to +0.80 V at a scan rate of 100 mV s^{-1} . The detection limit of this system was 0.4 $\text{fg}/\mu\text{L}$ of DNA template concentration.

Figure 2-16: A calibration plot of the differential pulse voltammetric peak current of the gold working electrode in the PCR-EC microdevice against the number of template molecules in the PCR-mixture [67].

Such EC detection devices can be significantly miniaturized and integrated onto a chip, where the detected target DNA need not to be labeled and only the presence of electrochemically active substances is necessary. Thus, this EC detection system is superior to other detection methods in terms of low-consumption, portability and miniaturization. However, the immobilization of sequence specific DNA probes onto the gold electrode is tedious and unreliable in differentiating similar DNA sequences.

2.4 Electrochemical DNA Biosensors

Aiming at lab-on-a-chip microdevices for DNA analysis, the entire detection system should ideally be an integrated on-chip. The optical and microgravimetric systems are difficult to be miniaturized due to the complex and bulky instrumentations involved. The electrochemical system, with its inherent advantages of miniaturization and compatibility with advanced microfabrication technology, holds great promise for the realization of portable DNA analyzers. The high sensitivity of electrochemical microdevices, approaching that of the fluorescence-based methods, makes them excellent candidates for trace DNA detection. Moreover, low cost and minimal power

requirement are desirable characteristics of the electrochemical transducers for routine and disposable device applications. Furthermore, electrochemical reactions give an electronic signal directly; there is no need for expensive signal transduction equipment. Moreover, because immobilized probe sequences can be readily confined to a variety of electrode substrates, detection can be accomplished with an inexpensive analyzer.

Electrochemical (EC) detection offers several advantages over conventional fluorescence detection in different areas such as the following [68-70]:

Power Requirements

EC detection measures small currents or voltages, so a large, expensive, and heavy power supply is not needed to power a light source.

Components

EC detection does not require optical components such as a light source, mirrors, filters, detectors, support mechanics, or movement mechanics for chip scanning. Consequently, EC detection systems are simpler and less expensive, and the fewer component requirements result in smaller footprints and less weight.

Portability

Since the instrument is small and light and power can be supplied by batteries, EC detection systems are potentially portable. This characteristic will enable significant applications of EC detection in the market in which small, inexpensive, and portable systems are necessary.

Performance

In optical detection schemes, the signal-to-noise ratio is limited by the amount of stray light from the incident beam that gets into the detector channel. EC detection has no incident background. The only existing background comes from the inherent background currents in the measurement systems and the capacitive charging currents at the chip. Since these currents are small, a higher signal-to-noise ratio and greater

sensitivity can be achieved with the EC detection mode.

In principle, sequence-specific electrochemical DNA detection has the following three steps: probe (or target) immobilization, hybridization, and electrochemical transduction, as shown in Figure 2-17.

Figure 2-17: Figure representation of the 3 typical steps involved in the sequence-specific electrochemical DNA detection.

2.4.1 Probe immobilization

Appropriate and careful choice of the method of immobilization of the selected probe is crucial to the success of the assay. Several workers reported on the importance of good design of the recognition layer [71-76]. An ideal method of immobilisation should [77] :

- Enable accurate control of the density of packing and orientation of probes in the immobilised layer to facilitate the recognition event. It has been found that layers, which are too densely packed, reduce the accessibility of the target sequence and thus the efficiency of hybridization.
- Furnish a stable layer under experimental and storage conditions.
- Result in minimum loss of the binding capacity. In general, interaction of the probe with the electrode surface results in reduced recognition efficiency and this should be minimized. Since hybridization involves coiling of the probe

around the target, some conformational mobility is needed.

- Result in simplicity and ease of operation.

Many strategies have been developed for anchoring DNA on surfaces of different probes:

a. Adsorption

Physical adsorption is the simplest method for the immobilization of nucleic acids on surfaces because it does not require reagents or special oligonucleotide modification. Either dipping the electrode in the probe solution or dropping a small volume of the probe solution onto the electrode surface and evaporating dry achieves physical adsorption of ssDNA probe onto an electrode surface.

Electrochemical adsorption is frequently employed for probe immobilization onto carbon electrodes [78-80]. This immobilization strategy was pioneered by Cai *et al.* in 1996 for highly sensitive measurements of ssDNA at carbon paste electrodes with adsorptive stripping potentiometry [81]. The positive potential favours adsorption of these polyanions through the sugar-phosphate skeleton, with the bases being oriented towards the solution [81, 82]. Electrostatic binding of DNA to the positively charged carbon electrode was sufficiently strong and bases were accessible for the hybridization with target DNA (Figure 2-18). Briefly, a fresh carbon paste electrode was first pre-treated by applying a potential of +1.70 V (versus Ag/AgCl reference electrode) for 1 min to increase its roughness and hydrophilicity. After that, the electrochemical adsorptive accumulation of ssDNA probe was preceded from a stirred solution at a potential of +0.50 V for 2 min. This accumulation potential enhances the stability of the immobilized probe through the electrostatic attraction between the positively charged carbon surface and the negatively charged sugar phosphate backbone. The hybridization between the probe and complementary target DNA will happen as depicted in Figure 2-18.

Figure 2-18: DNA immobilization by electrochemical adsorption [83]. (A) DNA probe absorption onto an electrochemical transducer by applying a positive potential. (B) Hybridization between the probe and the target at the same positive potential as the accumulation step.

Disadvantages

The probe surfaces prepared by the adsorption method suffer from poor hybridization efficiency and reusability (Figure 2-19). In principle, adsorption method is the simplest and cheapest means of immobilization because neither surface nor nucleic acid derivatisation is required. The binding capacity of the recognition layer is, however, limited, because of lack of control over the orientation of the adsorbed probes and their reduced flexibility. The resulting multiple-point linkage as depicted in Figure 2-19 usually leads to poor hybridization efficiency. Moreover, the release of nucleic acids from the surface during the hybridization is a potential problem that is hard to avoid. Reusability is difficult to achieve [77].

Figure 2-19: Poor hybridization efficiency caused by multiple sites of the adsorbed DNA probes.

b. Entrapment

This method enables more stable immobilization than adsorption does. Dialysis

membranes, cationic layers, polymers including conducting and cationic polymers, and sol-gel, have been employed for nucleic acid entrapment. One example of this is the bulk modification of carbon paste electrode with DNA probe. The carbon paste electrode was prepared by mixing probe with graphite powder, sol-gel and mineral oil [76, 84-88]. The immobilized probe retained its hybridization activity on confinement in the interior of the carbon paste matrix. Most importantly, the surface of these biocomposite hybridization biosensors can be renewed for subsequent reuse.

Disadvantages

Entrapment of nucleic acid in the bulk of a polymer results in high loading levels but the conformational mobility of the nucleic acid is relatively restricted, thus leading to kinetic and steric barriers during hybridization. Entrapment is effective in immobilizing the DNA but the efficient binding of the probe DNA with the target is reduced as the probe DNA is less accessible for the target DNA.

c. Affinity binding

The DNA probe immobilization can be done through avidin-biotin complexation [89, 90]. Each avidin or streptavidin molecule has four binding sites for biotin [91]. The strong binding affinity, close to that of a covalent bond, can be used to form a stable probe monolayer on an avidin/streptavidin-modified electrode surface. In this case, DNA probe has to be modified with a biotin group at its 5' or 3' terminus. Compared with the adsorptive immobilization, probe immobilized by avidin-biotin interaction has higher hybridization efficiency. This is because single point attachment at the end of the probe strand (standing upright) permits a higher degrees of freedom for hybrid formation than that of the flatten strand in the adsorptive immobilization strategy. In addition, the strong avidin-biotin binding allows regeneration of the probe surface for repetitive assays.

d. Covalent immobilization

The most widely used method to covalently immobilize DNA probe onto gold electrodes is based on thiol chemisorption. This originates from the extensively

investigated self-assembled monolayer (SAM) of alkanethiol on gold surface [92]. Initial study on the use of thiol-modified probe for the hybridization detection was based on microgravimetric method. Since then, a large number of electrochemical DNA biosensors have utilized gold electrodes chemisorbed with thiol-modified probe as the hybridization sensing platform [71, 93]. Chemisorption enables single-point attachment of the probe at the end-point thiol group. It combines a relatively strong and simple means of immobilisation with conformational mobility of the probe. Alkane thiol-terminated oligonucleotides are the most used and are already commercially available. Oligonucleotides with several successive phosphorothioate units on the 5'-terminus have also been used [94]. Covalent binding of the DNA probe via one (both) of its ends is advantageous as it can be well controlled and the non-specific molecules weakly bound to the surface can easily be removed.

2.4.2 Electrochemical transduction

The hybridization event can be detected via the change of current signal of a redox indicator (that associates with the newly formed surface hybrid), or from changes in other electrochemical parameters (such as capacitance or conductivity) or in the redox activity of the nucleic acid resulting from the duplex formation. Electrochemical transducers of the hybridization event can be divided into indicator-based and indicator-free approaches. The indicator-based approach is composed of three subclasses:

(i) Electrochemically active intercalators

Major processes involved in electrochemical DNA biosensors based on the use of redox indicators (Figure 2-20). The hybridization event is detected via the changes of current signal of an electroactive indicator. The first electrochemical DNA biosensor was reported by Millan *et al.* in 1993, which was based on the hybridization indicators tris(2,2'-bipyridyl)cobalt (III) ($\text{Co}(\text{bpy})_3^{3+}$) or tris(1,10-phenanthroline)cobalt(III) ($\text{Co}(\text{phen})_3^{3+}$) [95]. Mikkelsen *et al.* attached single stranded DNA probe sequences to a GCE and then hybridize test DNA samples to this DNA-modified electrode. These

groove binders or intercalators have higher affinities towards dsDNA (probe-target hybrid) than single-stranded (ss) ones (unhybridized probe). This leads to the accumulation of redox indicator at the hybrid-formed electrode surface and thus an increased current signal during voltammetric scan.

Figure 2-20: DNA biosensors based on the use of redox indicators [73].

Electroactive intercalators and groove binders other than metal complexes have been extensively studied since Hashimoto *et al.* published their work on the voltammetric characterization of some commercially available indicators [96]. Hoechst 33258 [97-99] and daunomycin [100] behave in a similar behaviour as $\text{Co}(\text{bpy})_3^{3+}$ and $\text{Co}(\text{phen})_3^{3+}$, were successfully applied for PCR product detection using microfabricated electrodes. Figure 2-21 shows the chemical structures of some electrochemically active DNA-binding molecules.

The desired properties of an ideal electrochemical hybridization indicator can be summarised as follows:

- Duplex-specific affinity – ssDNA affinity should be negligible or, at least, as low as possible.

- Redox potential should be inside those for the oxidation and reduction processes of the nucleobases. Low redox potential is preferred.
- Fast and reversible electron-transfer between indicator and electrode.
- Stable reduced and oxidised forms.
- Low toxicity and cost.

Figure 2-21: Chemical structures of electrochemically-active DNA-binding molecules [101].

A special redox threading intercalator (ferrocenyl naphthalene diimide), which binds four times more strongly to dsDNA than to ssDNA, was synthesized by Takenaka *et al.* to lower the detection limit for DNA detection [102]. Figure 2-22 shows the differential pulse voltammograms for the ferrocenyl naphthalene diimide indicator at the electrode before and after hybridization.

Figure 2-22: (A) Chemical structure of redox threading intercalator (ferrocenylnaphthalene diimide) (B) Differential pulse voltammograms for the ferrocenyl naphthalene diimide indicator at the dT20-modified electrode before (a) and after (b) hybridization on the curve [102].

Methylene blue was first studied by Kelly *et al.* [103]. Figure 2-23 shows the schematic of the electrocatalytic detection of DNA hybridization by Kelly *et al.* [103, 104]. Alkanethiol-modified probes were attached to gold electrodes and hybridized to target DNA. In presence of redox-active intercalator, reduction of ferricyanide in solution was observed. In this assay, electron flows from the electrode surface to the intercalated MB^+ to reduce it to leucomethylene blue (LB^+) in a reaction mediated by the surface-attached DNA. On the reducing ferricyanide ($\text{Fe}(\text{CN})_6^{4-}$) in solution, LB^+ is oxidized back to MB^+ , which can then participate in more electron transfer in an electrocatalytic cycle, involving repeated interrogation of the surface bound DNA. Longer interrogation times yield greater absolute signals and better discrimination between mismatched and fully matched DNA. All of the possible DNA single-base mismatches (including the thermodynamically stable GA mismatch) were detected by this assay [104]. Ozsoz *et al.* later investigated the interaction of methylene blue with exposed guanine base for hybridization study [25, 105-107]. There was a decrease in peak current upon hybrid formation.

Figure 2-23: Schematic of the electrocatalytic detection of DNA hybridization based on long-range electron transfer used by Kelly et al. [104].

One way to ensure that the hybridization indicator binds exclusively to either ssDNA or dsDNA is to label the probe or the target with a redox marker. Motorola's Clinical Micro Sensors Division is a biochip company employing bioelectronics, which is to form electronic circuits using biological molecules. They developed a novel sandwich hybridization assay with a redox ferrocene-modified reporter probe as shows in Figure 2-24 [108]. An insulated gold electrode was coated with a SAM containing DNA capture probe. Unlabeled complementary target was bound to the surface of the SAM through hybridization with the capture probe as well as a single-stranded ferrocene-modified reporter probe in the solution. If hybridization of all three oligonucleotides occurs, the signalling probe brings a ferrocene molecule to an electronic pad imbedded within the otherwise insulated electrode surface, which completes the circuits. The ferrocene-labeled reported probe, being complementary to the target in the region next to the capture probe-binding site, was then held in close proximity to the SAM. The core feature of this detection platform was the ability of the SAM to have electron transfer between the immobilized ferrocene and the gold, while insulating the electrode from the unbound reporter probes.

Figure 2-24: Schematic of the DNA hybridization biosensor developed by Clinical Microsensors Motorola which uses a sandwich type assay to transducer the hybridization event [108].

(ii) Redox enzyme

Another important class of hybridization indicators that has intrinsic signal amplification capability is redox enzymes [109]. Enzyme labels have been widely used in bioaffinity sensors, particularly in immunosensors. The used of enzyme labels to generate electrical signals also offers great promise for ultrasensitive EC detection of DNA hybridization. The majority of enzyme-amplified electrochemical DNA hybridization assays utilized horseradish peroxidase (HRP) (Figure 2-25). Willner *et al.* developed a novel method based on the enzyme-catalyzed precipitation of an insoluble product coupled with Faradaic impedance measurement [110-113]. In their approach, a capture probe-modified gold electrode was hybridized with a target-biotinylated reported probe, followed by the attachment of avidin-HRP. The enzyme catalyzed the oxidation of 4-chloro-1-naphthol by H_2O_2 , forming an insoluble precipitate on the conductive support and blocking the interfacial electron transfer.

Figure 2-25: Amplified electrical detection of DNA hybridization using HRP-functionalized liposomes and biocatalytic precipitation of the product of the enzymatic reaction [112].

(iii) Metal nanoparticles

Metal nanoparticles, particularly gold; have attracted substantial interest in electrochemical hybridization transduction owing to their extraordinarily high selectivity and sensitivity (Figure 2-26). In addition, greater signal amplification is achievable with the silver enhancement technique. Procedure-wise, the gold nanoparticle label is specifically bound to the formed hybrid, followed by a silver-staining step, in which silver-enhancing solutions are used to improve the signal of the gold particles by forming shells of silver surrounding the gold core. Since Taton *et al.* published their work on the nanoparticle probe-based scanometric DNA detection with extraordinary high selectivity in 2000 [114], there has been considerable interest in utilizing the gold nanoparticle as an electrochemical hybridization indicator. Authier *et al.* [115], and Wang *et al.* [116] demonstrated the electrochemical sequence-selective detection of DNA molecules with gold nanoparticle labels.

Figure 2-26: Schematic diagram of the silver-enhanced colloidal gold electrochemical detection of DNA hybridization by Cai *et al.* [117].

Indicator-free approaches are emerging technologies in the electrochemical DNA biosensors for simple and fast transduction of the hybridization events. Such protocols greatly simplify the sensing protocol as they eliminate the need for the indicator addition, association, and detection step and offer an instantaneous detection of the duplex formation.

One approach of label free electrochemical biosensing of DNA hybridization is based upon the intrinsic electroactivity of guanine base. Among four nucleic acids bases, the guanine moiety is most easily oxidised and is most suitable for such indicator-free hybridization detection [117, 118]. Wang *et al.* developed an electrochemical biosensor with the immobilization of an inosine-substituted (guanine free) capture probe onto a solid support carbon paste electrode and the hybridization was detected through the appearance of the guanine oxidation peak [119].

Another scheme for indicator-free electrochemical transduction of the hybridization event is based on changes in interfacial properties such as electrical transfer measurement [120]. The first electrical transport measurements on DNA molecules at least 600 nm long were reported by Fink and Schönenberger [121]. Direct measurements of electrical current as a function of the potential applied across DNA

indicated efficient conduction through the DNA ‘ropes’. Braun *et al.* [122] hybridized target DNA with surface bound oligonucleotides on two Au electrodes, thus stretching the DNA molecule in the microgap between these electrodes. DNA was then used as the template for the vectoral growth of a conductive silver wire.

It is also possible to exploit different rates of electron transfer through ss- and ds-DNA for probing hybridization (including mutation detection) via the perturbation in charge migration through DNA. Boon *et al.* [104, 123] demonstrated that such charge transport is disrupted by the presence of a single-base mismatch. Such disruption and point mutation were detected, using a gold electrode modified with thiolated DNA, by monitoring changes in the charge transport between an electroactive methylene blue intercalator and a ferricyanide redox species.

A new label-free electrochemical DNA hybridization detection method has recently been reported by Gooding *et al.* [124]. The change in flexibility of an ss-probe before and after hybridization caused an ion-gating effect, where the rigid rod like structure of the duplex opened up the SAM-modified Au electrode to the access of ions.

Microfabricated silicon field effect sensors were used to directly monitor the increase in surface charge when DNA was hybridized on the sensor surface [125]. The electrostatic immobilization of probe DNA on a positively charged poly-L-lysine layer allowed hybridization at low ionic strength, where field-effect sensing was the most sensitive case. Nanomolar DNA concentration could be detected within minutes, and a single-base mismatch within 12 mer oligonucleotides could be distinguished by using a differential detection technique with two sensors in parallel.

Chapter 3

Electrochemical Detection of Nucleic Acid Bases

3.1 Introduction

The analyses of purine bases, e.g. adenine and guanine, are involved in cellular energy transduction and signalling mediated by enzymatic oxidation reactions. Purine and pyrimidine bases, the building blocks in both DNA and RNA, play an essential role in various biological processes, such as involvement in cell metabolism. Because they are metabolized by liver, caffeine and some of its metabolites; xanthine, uric acid, theobromine and theophylline have been used to study functional impairment in liver disease. Therefore, quick, inexpensive and accurate methods for quantitative analysis of purines and pyrimidines are needed.

Guanine and adenine are easily oxidizable nucleobases [126]. The oxidation of nucleic acids has been the focus of considerable research in toxicology, because the resulting lesions are suspected to lead to ageing and cancer. Figure 3-1 shows the oxidation and reduction sites of DNA. Guanine certainly undergoes more reactions than the other physiological bases, its impressive repertoire including reactions with one-electron oxidants, alkylating agents, hydroxyl radicals, singlet oxygen and transition-metal complexes. The enhanced reactivity of guanine over the other bases is apparent in the one-electron oxidation potentials, which are +1.34 V (against the standard hydrogen electrode) for guanosine, +1.79 V for adenosine and much higher for the pyrimidines. Ever since the early days of radiation chemistry and biology, it has been realized that the purine and pyrimidine bases are the most sensitive to radiation-induced modification or destruction of the components of DNA, itself the most critical of the cellular targets.

Figure 3-1: Scheme of the electroactive sites in nucleic acid bases. Adenine (A) and cytosine (C) are reducible at mercury electrodes. Guanine (G) undergoes a chemically reversible reduction at the mercury electrodes, yielding an anodic peak G due to oxidation of its reduction product. A and G can be oxidized at carbon electrodes. Watson-Crick base pairs TA and CG are shown without the reduction sites involved in hydrogen bonding.

Guanine (2-amino-6-hydroxypurine) is a purine base consisting of two condensed heterocyclic pyrimidine and imadazole rings. Guanine bases play many important roles in the life processes, e.g. they are used as the information molecules in genes and used in the process of converting genes to proteins. Guanine is one of the components of nucleic acids, as well as vitamins and coenzymes. As the electron transfer properties of guanine and its derivatives *in vitro* provide useful information about *in vivo* behaviour, thus the electrochemical study of such compounds has been the subject of investigation for a long time [126]. Electro-oxidation of guanine is also useful for the detection of DNA and the design of DNA biosensor based on the oxidation of the structure. The structure of guanine is shown below:

Guanine

Adenine, an organic base of the purine family is one of the most important organic molecules for life as we known today. Purines are six-membered rings attached to five membered rings. When adenine is attached to DNA, it forms a bond with another molecule called thymine, a pyrimidine, on the other side of the DNA strand. The sequence of DNA in each organism allows for the diversity among all living organisms. Adenine combines with the sugar ribose to form adenosine, which in turn can be bonded with from one to three phosphoric acid units, yielding the three nucleotides adenosine monophosphate (AMP), adenosine diphosphate (ADP), and adenosine triphosphate (ATP). These adenine derivatives perform important functions in oxidative phosphorylation and trans-membrane signalling processes, and as secondary substrate or modulator for other enzymes processes. Thus, it is clear that adenine and its derivatives play very important role in various biochemical pathways. Investigation of the redox electrochemical behaviour of adenine and its derivatives can provide useful insights into their biological role [127]. The structure of adenine is shown below:

Adenine

Hence, electrochemical study of guanine, adenine or their residues from DNA samples has been undertaken on various electrode materials, e.g. pyrolytic graphite

electrode [128, 129], glassy carbon electrode [130-136], carbon paste electrode [137], mercury electrode [138], etc. Certain chemically modified electrodes have also been used for the determination of adenine content in DNA or RNA [139]. From the literature cited above, it is clear that carbon based electrode material is a suitable electrode matrix for the electrochemical analysis of guanine and adenine.

Our research group had been working with the applications of sol-gel based electrodes for biosensing and other applications [140-144]. Sol-gel technique offers the flexibility for the fabrication of electrodes with different configurations to suit different applications [84, 142]. A new class of sol-gel based CCEs can be formed by homogeneous dispersion of graphite powder in a suitable sol-gel precursor/monomer. Such CCEs had been reported to have various desirable properties for electroanalysis, such as low background current, good chemical and mechanical stability, easy preparation and wide potential window [145-147]. To our best knowledge, the electrochemical investigation of adenine and guanine on a sol-gel carbon composite electrode (CCE) had not been reported in the literature. This work described the electrochemical oxidation of guanine and adenine at a CCE. Cyclic voltammetry and differential pulse stripping voltammetry for the individual or simultaneous determination of guanine and adenine in DNA at low levels were carried out in this research.

3.2 Experimental Procedure

3.2.1 Chemicals

Methyltrimethoxysilane (MTMOS) was obtained from Merck-Schuchardt. Extra pure graphite powder was purchased from Merck. Guanine, adenine and calf thymus DNA were purchased from Sigma-Aldrich and used without further purification. All other chemicals are analytical reagent grade and used without further purification. All aqueous solutions were prepared with ultra pure water (Milipore Mili-Q water).

3.2.2 Instrumentation

Cyclic voltammetric and differential pulse stripping experiments were performed with CH Instruments Electrochemical Workstation (Model 660). The three-electrode system used in this study consisted of a sol-gel derived carbon composite working electrode; a platinum coil counter electrode and a saturated calomel reference electrode. Since dissolved oxygen did not interfere with the electrochemical study, no deaeration was performed. All experiments were performed in air-conditioned laboratory with temperature set at 25 ± 0.5 °C.

3.2.3 Preparation of typical MTMOS stock sol-gel solution

The stock sol-gel solution was prepared by mixing 0.5 ml of methanol, 0.8 ml of MTMOS, 1.0 ml of water and 0.1 ml of 1mM HCl [146]. The mixture was sonicated for 10 min. The stock sol-gel solutions were prepared freshly and stored for 1 day at room temperature before the fabrication of the electrodes.

3.2.4 Construction of the silica sol-gel derived carbon composite electrodes

The construction of the silica sol-gel derived CCE was according to the method from [146], as shown in Figure 3-2. A CCE was prepared as follows: 200 mg of carbon powder was mixed with 0.3 mL stock sol-gel in mortar and made into a homogeneous sol-gel carbon paste. Then the carbon sol-gel paste was packed into one end of a glass capillary with i.d. of 3 mm and o.d. of 5 mm and the electrode area was about 7.1 mm^2 . A copper wire was inserted through the opposite end to establish an electrical contact. The electrode was allowed to polymerize and left to dry for 3 days at room temperature to complete the polymerization. After the complete polymerization process, the electrodes were polished with fine emery paper and thoroughly rinse with water before use.

Figure 3-2: Construction of the silica sol-gel derived carbon composite electrodes.

3.2.5 Preparation of purine bases

Stock solutions of guanine and adenine of 1 mM were made in 0.1 M NaOH. Working solutions of guanine and adenine were prepared by adding small volumes of stock solution to the phosphate buffer solutions. The solutions were then sonicated to ensure homogenization.

3.2.6 Preparation of DNA samples

A gentle treatment of calf thymus DNA with 1 mM HCl resulted in the selective removal of its purine bases by cleavage of purine glycoside bounds. The calf thymus dsDNA was hydrolyzed for the quantification of guanine and adenine. Denaturation of DNA was performed by the following procedure: an accurately weighed sample of approximately 3 mg of DNA was treated with 0.5 ml of pure perchloric acid and stirred to dissolve for 10 min; then 0.5 ml of 9 M NaOH was then added to neutralize the acidic solution and the volume was made up to 10 ml with 0.5 M phosphate buffer (pH=3.0).

3.2.7 Detection procedures

The electrochemical experiments were performed in a 3 electrode electrochemical cell holding 10 mL air-saturated 0.5 M phosphate buffer (pH=3.0). For the cyclic voltammetric (CV) experiments, the $E_i = -0.20$ and $E_f = +1.20$ V and the scan rate (v) was set at 25 mV s^{-1} . The CVs were obtained in quiescent buffer solutions. For differential pulse adsorption stripping experiments, the CCE was cycled between -0.20 V and $+1.20$ V vs. SCE for 3 cycles at a scan rate of 25 mV s^{-1} in between the experiments. The accumulation potential (E_{acc}) applied onto CCE was -0.20 V vs. SCE, and accumulation time (t_{acc}) was 60 s. During the accumulation step prior to the stripping step, the buffer solution containing adenine was stirred at 250 rpm. After 5 s quiescent period, the anodic differential pulse stripping voltammograms were recorded from -0.20 V to $+1.20$ V under the following conditions: pulse amplitude = 50 mV , pulse width = 25 ms , scan rate = 10 mV s^{-1} .

3.3 Results and Discussion for Adenine Base Analysis

3.3.1 Characterization using cyclic voltammetry

The CV behavior of adenine on the sol-gel CCE was investigated at a scan rate of 25 mV s^{-1} in an aerated unstirred 0.5 M phosphate (pH=3.0). Figure 3-3 shows the cyclic voltammograms of a (a) blank solution and (b) $10 \text{ }\mu\text{M}$ adenine in the above buffer solution. The oxidation peak of adenine on the sol-gel CCE was well defined with a peak at $+0.98$ V. No reduction peak was observed in the cathodic sweep in the potential and scan range studied. Thus the electrochemical oxidation of adenine on sol-gel CCE could be considered as irreversible on the sol-gel CCE. We found that E_p shifted to positive value as the scan rate increases. This evidence supported that the reaction was not reversible, as reversible reaction should have E_p independent of v or other chemical step was involved in the adenine oxidation process. This irreversible redox behavior of adenine was similar to other previous work done on glassy carbon electrode [133]. Typically, the oxidation peak current increased when accumulation time was allowed for guanine on the sol-gel CCE.

Figure 3-3: Cyclic voltammograms of (a) a blank solution and (b) 10 μM of adenine in 0.5 M phosphate buffer (pH=3.0) on a sol-gel CCE electrode with a scan rate of 25 mV s^{-1} .

It had been widely reported in the literature that adenine or other purine bases have the tendency to adsorb on carbon electrodes [133, 148]. In order to investigate whether adenine adsorption was presented on CCE electrode surface, the following current function ($I_p/v^{1/2}$) versus $\log v$ was plotted in Figure 3-4 (A). Figure 3-4 (A) shows that there was an increase of ($I_p/v^{1/2}$) function versus $\log v$. This phenomenon demonstrated that there is adsorption of adenine on the sol-gel CCE. This is not surprising since the sol-gel CCE is porous [87, 149-151]. The oxidation peak current of adenine was plotted against $v^{1/2}$, and no linear relationship was found which implies that it is not a totally diffusion-controlled process. A plot of $\log I_p$ versus $\log v$ in the range of 0.01~1.00 V s^{-1} in 0.5 M phosphate buffer (pH=3.0) was shown in Figure 3-4 (B), the relationship obeyed to the following equation: $\log I_p = 1.362 + 0.736 \log v$ ($R^2 = 0.998$) where v is in V s^{-1} and I_p is in μA . A slope of 0.736 indicated that the electrode process was controlled simultaneously by heterogeneous electron transfer rate and adsorption. The process of adenine adsorption at the surface of sol-gel CCE was dependent on the potential applied to the electrode.

(A)

(B)

Figure 3-4: (A) Plot of $(I_p/v^{1/2})$ versus $\log v$; and (B) plot of $\log I_p$ versus $\log v$ for an adsorption test for 10 μM adenine on a sol-gel CCE in 0.5 M phosphate buffer ($\text{pH}=3.0$), scan rate= 25 mV s^{-1} .

3.3.2 Effect of pH

The solution pH affects the ionization of adenine and therefore the peak current as well as the peak potential. Both the oxidation peak current (I_p) and potential (E_p) were dependent on pH of the buffer solution. The effect of pH on the oxidation peak current was shown in Figure 3-5. When the pH was 2.0, the oxidation peak current was low

and it reached a maximum value at pH 3.0, while beyond this pH, the oxidation peak current decreased steadily. The decreasing portion of I_p with pH below pH 3.0 cannot be explained as the characteristic of adenine. Because adenine exists as a cation ($pK_a=3.8$) in this region, very good accumulation in the sol-gel carbon composite was expected leading to the opposite trend. It seemed that the effect internally associated with the sol-gel CCE in more acidic buffer solutions can cause this result. The E_p for adenine oxidation peak changed linearly with pH described by this equation: $E_p=1.3187-0.0581pH$ in the pH range of 2.0~9.0 ($R^2=0.991$). The slope of 58 mV / pH showed that two protons took part in the rate-determining step. The oxidation of adenine had been suggested to follow a 3 steps mechanism involving the total loss of 6 electrons and 6 protons [126].

Figure 3-5: Effect of pH on the oxidation peak current (I_p) for 10 μM adenine; other conditions are the same as in Figure 3-3.

3.3.3 Differential pulse stripping study: E_{acc} and t_{acc}

One of the aims of the current work was to develop sensitive voltammetric method for the detection of adenine content in DNA digest samples, thus differential pulse adsorption stripping voltammetric technique was employed for the remaining work. To obtain the optimum conditions for the detection of adenine, important parameters

to be investigated were the accumulation time (t_{acc}) and accumulation potential (E_{acc}) as these parameters affect the stripping current significantly. Figure 3-6 shows that I_p increased with t_{acc} initially for the first 60 s for adenine concentration of 10 μM and I_p attained electrode surface saturation beyond 60 s where it reached steady-state. This makes no significant changes in the current-accumulation potential profile. The effect of t_{acc} was investigated by cycling the potential of the sol-gel CCE +1.20 V and -0.40 V at a scan rate of 20 mV s^{-1} for 3 full cycles in between the stripping experiments.

Figure 3-6: Effect of accumulation time (t_{acc}) on the anodic peak currents obtained at a sol-gel CCE for 10 μM adenine solution in 0.5 M phosphate buffer (pH=3.0) using differential pulse stripping adsorption voltammetry with $E_{acc} = -0.20$ V vs. SCE and other conditions are as follows: pulse amplitude=50 mV, pulse width=25 ms, scan rate=5 mV s^{-1} .

The effect of E_{acc} on the stripping peak current was shown in Figure 3-7. I_p increased as the E_{acc} was changed from +1.00 V to -0.10 V, at which a maximum peak current was achieved. As the potential was further lowered to -0.40 V, the peak current decreased. This trend on the effect of E_{acc} on I_p is similar to other reported works on stripping experiments for the determination of adenine on denatured DNA on a glassy carbon electrode [139]. Thus, the optimum E_{acc} of -0.10 V was selected for the remaining work.

Figure 3-7: Effect of accumulation potential (E_{acc}) on the anodic peak currents obtained at a sol-gel CCE for 10 μM adenine solution in 0.5 M phosphate buffer (pH=3.0); t_{acc} =60 s and other conditions are as in Figure 3-6.

3.3.4 Response characteristics and analytical application

Figure 3-8 shows the calibration curve of the DNA in the range of 1 μM ~400 μM for the determination of adenine. Electroanalytical quantification of adenine was done in 0.5 M phosphate buffer (pH=3.0) electrolyte using differential pulse adsorption stripping mode under the optimum conditions: E_{acc} = -0.10 V, t_{acc} = 60 s, a linear calibration curve in the range of 2×10^{-8} M – 1×10^{-6} M was obtained (insert of Figure 3-8). The limit of detection was 0.09 μM for adenine. The calibration curve relating to the I_p and concentration of adenine was as follows: I_p (μA) = 0.854 [adenine/ μM] + 0.166 (R^2 =0.999). The peak current I_p of the oxidation was found to increase with concentration of adenine. It was clear that the calibration curve levels off when the concentration of adenine increased as this is typical of adsorption stripping analysis.

Figure 3-8: Calibration graph of adenine (0.1 μM to 400 μM) under optimum stripping conditions: $E_{acc} = -0.20\text{ V}$ vs. SCE and $t_{acc} = 60\text{ s}$ and other experimental conditions were as in Figure 3-6. The insert shows the calibration graph for the range of $2 \times 10^{-8} \sim 1 \times 10^{-6}\text{ M}$ adenine.

Figure 3-9 shows the calibration graph of adenine in acid denatured DNA. The detection limit of DNA calculated was 0.72 $\mu\text{g/mL}$ for adenine. The acid-denatured DNA gives two well-defined peaks at the pretreated sol-gel carbon electrode due to the oxidation of guanine (I) and adenine (II) residues (Figure 3-10). The unknown concentration of adenine in DNA was calculated from peak current using the calibration graph from Figure 3-9 for the simultaneous determination of adenine in acid denatured DNA.

Figure 3-9: Calibration graph for simultaneous determination of adenine in acid-denatured DNA; other experimental conditions are as in Figure 3-6, $t_{acc} = 60\text{ s}$, $E_{acc} = -0.20\text{ V}$.

Figure 3-10: Differential pulse stripping voltammogram of 10 μg of acid digest DNA, $t_{acc}=60$ s and other conditions are as in Figure 3-6. Peak (i) adenine and Peak (ii) guanine, $E_{acc}=-0.20$ V.

3.4 Results and Discussion for Guanine Bases Analysis

3.4.1 Characterization using cyclic voltammetry

The CV behavior of guanine on the sol-gel CCE was investigated at a scan rate of 25 $mV s^{-1}$ in an aerated unstirred 0.5 M phosphate (pH=7.0). Figure 3-11 shows the cyclic voltammograms of (a) a blank solution and (b) 10 μM guanine in the above buffer solution. The oxidation peak of guanine on the CCE was well defined with a peak at +0.70 V. No reduction peak was observed in the cathodic sweep in the potential and scan range studied and thus the electrochemical oxidation of guanine on sol-gel CCE is considered as irreversible on the sol-gel CCE.

Figure 3-11: Cyclic voltammograms of (a) a blank solution and (b) 10 μM of guanine in 0.5 M phosphate buffer (pH=7.0) on a sol-gel CCE electrode with a scan rate of 25 $mV s^{-1}$.

A plot of oxidation peak potential (E_p) versus scan rate (v) showed that E_p was a function of v (Figure 3-12), i.e. E_p shifted to positive value as the scan rate increased. This supported the fact that the reaction was not reversible as reversible reaction should have E_p independent of v or other chemical step was involved in the guanine oxidation process. This irreversible redox behavior of guanine was similar to other previous work done on carbon based electrodes, even though Brett *et al.* had reported that the oxidation of guanine was quasi-reversible [126]. Typically, the oxidation peak current increased when accumulation time was allowed for guanine on the sol-gel CCE.

Figure 3-12: Effect of scan rate (v) on the oxidation peak potential (E_p); other experimental conditions are as in Figure 3-11.

To investigate whether guanine adsorption was present on CCE surface, the following current function ($I_p/v^{1/2}$) versus $\log v$ was plotted (Figure 3-13). Figure 3-13 shows that there was an increase of ($I_p/v^{1/2}$) function versus $\log v$. This phenomenon demonstrated that there was adsorption of guanine on the sol-gel CCE, which is not surprising since the sol-gel carbon composite is porous. The oxidation peak current of guanine was plotted against $v^{1/2}$, and no linear relationship was found which implied that it was not a totally diffusion-controlled process. A plot of $\log I_p$ versus $\log v$ in the range of $0.01 \sim 1.00 \text{ V s}^{-1}$ in 0.5 M phosphate buffer ($\text{pH}=7.0$) was shown in Figure 3-14, the relationship obeyed to the following equation: $\log I_p = 1.333 + 0.720 \log v$ ($R^2=0.996$) where v is in V s^{-1} and I_p is in μA . A slope of 0.720 indicated that the electrode process was controlled simultaneously by heterogeneous electron transfer rate and adsorption.

Figure 3-13: Plot of $(I_p/v^{1/2})$ versus $\log v$ on a sol-gel CCE in 0.7 M phosphate buffer (pH=7.0), scan rate=25 mV s⁻¹.

Figure 3-14: Plot of $\log I_p$ versus $\log v$ for an adsorption test for 10 μM guanine on a sol-gel CCE in 0.7 M phosphate buffer (pH=7.0), scan rate=25 mV s⁻¹.

3.4.2 Effect of pH and ionic strength

The solution pH affects the stability of the guanine, and therefore the peak current as well as the peak potential. Both the oxidation peak current and potential (E_p) are dependent on pH of the buffer solution. The effect of pH on the oxidation peak current is shown in Figure 3-15. When the pH was low, peak current was low and it reached a

maximum value at pH 7.0, while beyond this pH, the oxidation peak current decreased. Beyond pH 7.0, the oxidation peak current decreased. When the pH is below 3, the sol-gel carbon composite electrode (CCE) surface is likely to be positively charged, and the guanine also behaves as positively charged cation, thus the repulsion from the CCE surface causes a low current response.

Figure 3-15: Effect of pH on the oxidation peak current (I_p) for 10 μM guanine; other conditions are as in Figure 3-11.

The effect of the ionic strength of the phosphate buffer solution on anodic peak current was investigated by CV over the range 0.01~0.5 M buffer (pH=7.0) for 10 μM guanine. Figure 3-16 shows that slightly larger currents were obtained with the 0.5 M phosphate buffer at the sol-gel CCE. This phenomenon is similar with the reported work on the determination of guanine on GCE [152].

Figure 3-16: Effect of ionic strength of pH 7.0 phosphate buffer on the anodic peak current for 10 μM guanine; other conditions are as in Figure 3-11.

3.4.3 Optimization of stripping procedure: E_{acc} and t_{acc}

To obtain the optimum conditions for the detection of guanine, important parameters to be investigated were the accumulation time (t_{acc}) and accumulation potential (E_{acc}) as these parameters affect the stripping current significantly. Figure 3-17 shows that I_p increases with t_{acc} initially for the first 60 s for guanine concentration of 10 μM and I_p attains saturation beyond 60 s. The effect of t_{acc} was investigated by cycling the potential of the sol-gel CCE between +1.20 V and -0.40 V at a scan rate of 20 $mV s^{-1}$ for 6 full cycles in between the stripping experiments.

Figure 3-17: Effect of accumulation time (t_{acc}) on the anodic peak currents obtained at a sol-gel CCE for 10 μM guanine solution in 0.5 M phosphate buffer (pH 7.0) using differential pulse stripping adsorption voltammetry with $E_{acc} = -0.20$ V vs. SCE and other conditions are shown as follows: pulse amplitude=50 mV, pulse width=25 ms, scan rate=5 $mV s^{-1}$.

The effect of E_{acc} on the stripping peak current was shown in Figure 3-18. I_p increased as the E_{acc} changed from +0.40 V to -0.20 V, at which a maximum peak current was achieved. As the potential was further lowered to -0.60 V, the peak current decreased. This trend on the effect of E_{acc} on I_p was similar to other reported work on the stripping experiments for the determination of guanine on denatured DNA on a GCE electrode [137]. Thus, the optimum E_{acc} of -0.20 V was selected for the remaining work.

Figure 3-18: Effect of accumulation potential (E_{acc}) on the anodic peak currents obtained at a sol-gel CCE for 10 μM guanine solution in 0.5 M phosphate buffer (pH=7.0); $t_{acc}=60$ s and other conditions are as in Figure 3-17.

3.4.4 Response characteristics and analytical application

Under optimum differential pulse stripping experimental conditions with $t_{\text{acc}} = 60\text{ s}$ and $E_{\text{acc}} = -0.20\text{ V}$ vs. SCE in a 0.5 M phosphate buffer (pH=7.0), a calibration for guanine in the range of 0.1 μM ~400 μM was shown in Figure 3-19. It was clear that as the guanine concentration increased, the peak current increased linearly and it reached a saturation level for higher concentration which was typical of adsorption stripping analysis. The insert in Figure 3-19 shows a linear calibration range of $2 \times 10^{-8} \sim 1 \times 10^{-6}$ M. The relationship between the peak current and guanine concentration was described by this equation: $I_p (\mu\text{A}) = 1.15[\text{guanine}/\mu\text{M}] - 0.0248$ ($R^2 = 0.997$). The limit of detection for guanine was 0.06 μM , based on $S/N = 3$.

The reproducibility of this stripping analysis was investigated by successive determination of 10 μM guanine ($n = 8$) ($t_{\text{acc}} = 60\text{ s}$ and $E_{\text{acc}} = -0.20\text{ V}$ vs. SCE) with relative standard deviations (RSDs) of 3.6% and 0.2% for I_p and E_p , respectively. The RSD of the peak current was comparable to other work [132]. It should be emphasized that our aim was to develop a simple, reliable and inexpensive stripping voltammetric procedure that could be used for the determination of guanine in a variety of samples. This work indicated for the first time that a sol-gel CCE is a possible suitable electrode for the determination of guanine based on differential pulse adsorption stripping analysis.

Figure 3-19: Calibration graph of guanine under optimum stripping conditions: $E_{acc} = -0.20$ V vs. SCE and $t_{acc} = 60$ s and other experimental conditions are as in Figure 3-17. The insert shows the calibration graph for the range of $2 \times 10^{-8} \sim 1 \times 10^{-6}$ M guanine.

Figure 3-20 shows the differential pulse stripping voltammograms of a mixture containing different amounts of uric acid, guanine and guanosine in 0.5 M phosphate buffer (pH=7.0) with $E_{acc} = -0.20$ V vs. SCE and $t_{acc} = 60$ s. The peak potentials of uric acid, guanine and guanosine were approximately 0.30 V, 0.70 V and 0.90 V, respectively. Thus clearly sol-gel CCE is a suitable electrode with good selectivity for the analysis of the above mixture.

Figure 3-20: Differential pulse stripping voltammograms of a mixture containing different concentrations of (a) uric acid, (b) guanine and (c) guanosine for (i) 1 μM , (ii) 2 μM and (iii) 5 μM of the analytes in 0.5 M phosphate buffer (pH 7.0) using a sol-gel CCE. Other experimental conditions: $E_{acc} = -0.20$ V vs. SCE, $t_{acc} = 60$ s, and other experimental conditions are as in Figure 3-17.

3.5 Summary

This work is the first reported electrochemical study of adenine and guanine on a sol-gel CCE. Cyclic voltammetry (CV) and differential pulse adsorption stripping voltammetry were used to study the electrochemical oxidation of DNA purine bases, i.e. adenine and guanine, at sol-gel CCE. From the scan rate and peak current study, there was an evidence of adsorption of DNA purine bases on the sol-gel CCE. The oxidation peak of guanine and adenine on the sol-gel CCE was well defined with a peak at approximately +0.70 V and +0.98 V vs. saturated calomel electrode (SCE), respectively. Both purine bases undergo irreversible oxidation at the sol-gel CCE. Typically, the adenine and guanine show more sensitive oxidation peak at the sol-gel CCE compared to carbon paste electrode. For the differential pulse adsorption stripping analysis, systematic optimization of accumulation potential (E_{acc}) and accumulation time (t_{acc}) was carried out. For guanine, under optimum conditions of $t_{acc} = 60$ s, and $E_{acc} = -0.20$ V vs. SCE, a linear calibration graph in the range of

$2 \times 10^{-8} \sim 1 \times 10^{-6}$ M was obtained. For adenine, under optimum conditions of with $t_{\text{acc}} = 60\text{s}$, and $E_{\text{acc}} = -0.10$ V vs. SCE, a linear calibration graph in the range of $2 \times 10^{-8} \sim 1 \times 10^{-6}$ M was obtained. The detection limits of the stripping method were calculated to be $0.06 \mu\text{M}$ and $0.09 \mu\text{M}$ for guanine and adenine, respectively, based on $S/N=3$. Under optimum conditions, the adsorption stripping method was used to determine a mixture of DNA purine bases and similar compounds, i.e. uric acid and guanosine. The CCE provided good resolution for the simultaneous analysis of the 3 compounds. This CCE was also useful for the simultaneous analysis of DNA purine bases from denatured DNA. DNA charge transport chemistry was found to provide a sensitive method for the development of electrochemical DNA biosensor. Subsequently, the work illustrated the selectivity and flexibility of the electrochemical method for analyzing DNA and other biomolecules.

Chapter 4

Electrochemical Detection of DNA Hybridization Based on Electrode Surface Modification

4.1 Introduction

In recent years, there have been considerable interests in developing DNA electrochemical biosensors for rapid and inexpensive diagnosis of genetic diseases and other applications [68, 73, 75, 77, 118, 153-157]. Such biosensors have shown both high selectivity and sensitivity in detecting a specific DNA sequence, since the surface of the electrode can be immobilized with a specific oligonucleotide. The immobilization step of the capture probe would lead to well-defined probe orientation, allowing the ready accessibility of the target. Thus, specific DNA probe-target interactions and charge transfer reactions would give an electrical signal directly which can be easily monitored. In addition, electrochemical devices offer certain advantages over the optical devices. They are easy to miniaturize, simple, rapid and inexpensive since electrochemical transduction is independent of solution turbidity or optical pathway.

The immobilization of DNA onto the transducer surface plays an important role in the overall performance of DNA biosensors. However, traditional methods in immobilization of DNA onto electrode surfaces have poor hybridization efficiency [83, 158]. A useful method to retain the biological activities of DNA, including its function of hybridization and its bio-affinity property is covalent coupling, whereby DNA strands are anchored onto the transducer surfaces [159]. Self-assembly monolayer (SAM)/Au – orientated system has been widely reported for the fabrication of biosensors [93]. However, gold-thiol bond can only withstand reasonably mild potentials and the alkanethiols are susceptible to thermal desorption and oxidative damage [71]. Typically, the precursors of SAMs might not be stable and are easily oxidized in air [160, 161], resulting in a gradual reduction in DNA immobilization. Moreover, the potential window of a gold electrode is limited to a

relatively positive range due to its low over-potential for hydrogen evolution. In contrast, carbon electrodes are without such a problem given their wider potential range. Carbon materials showed higher efficiency in covalent modification due to their alterable surface functionalities [162, 163], in addition to their easy availability in various physical structures.

Different carbon-based surfaces have been used as electrochemical transducers for DNA hybridization biosensors. Carbon paste [164], pencil lead [116] and screen-printed electrodes [165, 166] have been widely used by various groups to fabricate DNA biosensors. GCEs have well defined surface and various groups have reported on the covalent modification of the electrode surface, e.g. grafting of different functional groups for different applications [167-171]. Millan *et al* have reported on the covalent immobilization of DNA onto oxidized GC surfaces using a water-soluble carbodiimide reaction [95, 172].

Methylene blue (MB) has been widely used as an electrochemical intercalator to monitor the DNA hybridization reaction [25, 107, 173-176] as ssDNA and dsDNA have different affinity for MB. MB has been reported to bind specifically to the guanine bases [107, 174, 175, 177] and thus a lower current signal is observed upon hybridization since less MB can bind to dsDNA. This is due to the inaccessibility of the guanine bases in dsDNA. Kelly *et al.* [123] reported a strategy for the electrochemical detection of single-base mismatches in oligonucleotides, based on charge transport from the intercalated MB through self-assembled monolayers of oligonucleotides immobilized on the gold electrode surface. Erdem *et al.* have also investigated the interaction of dsDNA and ssDNA with MB using carbon paste electrode [107, 174, 175], gold electrode [178] and self-assembled alkanethiol monolayer on gold electrodes [176]. Their group have also performed the electrochemical detection of hybridization based on peptide nucleic acid probes with MB as an electroactive label on carbon paste and SAM modified gold electrodes [105, 106]. Thus, the decrease in the magnitude of the voltammetric reduction signals of MB reflects the extent of the hybrid formation [107].

The aim of this work is to develop a simple DNA immobilization strategy which provides a well defined recognition surface for hybridization. In the present work, we reported the covalent immobilization of a synthesized 21mer capture probe onto a carboxylate-terminated 4-aminobenzoic acid (4-ABA) monolayers modified on a GCE surface via 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydrosulfosuccinimide (NHS) cross-linking reaction for the voltammetric detection of a sequence-specific DNA. A 4-ABA monolayer was first formed on a GCE surface by forming an amine cation radical in anhydrous ethanol solution [161]. The ssDNA capture probe was then covalently attached to the 4-ABA/GCE surface using the cross-linking reaction between EDC and NHS. The immobilized capture probe was used to hybridize with complementary ssDNA, forming double-stranded DNA (dsDNA). MB was used as an electrochemical intercalator to monitor the DNA hybridization reaction. This approach has also been applied into real-sample from PCR assays. The capability of the sequence specific electrochemical detection for the identification and quantification of asymmetric PCR amplified real samples at end-point detection was also demonstrated using the above approach.

4.2 Experimental Procedures

4.2.1 Chemicals

MB and 4-ABA were purchased from Sigma Aldrich. The solution of 4-ABA was freshly prepared prior to the modification of the GCE surface. EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) and NHS (N-hydrosulfosuccinimide) from Sigma Aldrich were used without further purification. The composition of 1X TBE was 89 mM Tris, 89 mM boric acid and 2 mM EDTA. The following oligomers were synthesized by Proligo Singapore Pte Ltd. Their base sequences were as below:

DNA probe (21 mer base sequence A): 5'-NH₂-GAG GAG TTG GGG GAG CAC ATT-3'

Target DNA (21 mer base sequence B): 5'- AAT GTG CTC CCC CAA CTC CTC-3'

One base mismatch (21mer base sequenceB'): 5'-AAT GTG GTC CCC CAA CTC CTC-3'

Non-complementary (21mer base sequence B''): 5'-AAC GTG TGA ATG ACC CAG TAT-3'

The 21-mer base sequence B is complementary to 21 mer base sequence A; 21 mer base sequence B' is a mutant of the 21-mer base sequence B with one base changed, as indicated by underline.

DNA probe for PCR amplified products (21 mer base sequence A): 5'-NH₂-TGC ACC GAC AGG GGG AGC CCG -3'

The DNA oligonucleotide stock solutions were prepared with ultra-pure water (MilliQ Waters, Milford, MA) and kept frozen. Dilute solutions of DNA were prepared with this stock solution.

4.2.2 Instrumentation

Electrochemical experiments were performed with an AUTOLAB PGSTAT 30 electrochemical analysis system, with GPES4.9 and GPESSIX software package for multi-channel measurements (Eco Chemie B. V., Utrecht, Netherlands). A 3-electrode system was used: a GCE as the working electrode, a platinum wire as the auxiliary electrode and the SCE as reference electrode. A magnetic stirrer provided the convective transport.

Cyclic voltammetry (CV) of bare electrode was performed in 0.02 M phosphate buffer pH7.0. The scan rate was 20 mV s⁻¹. Cyclic voltammetry (CV) of 4-ABE/GCE was performed in 0.02 M phosphate buffer (pH 7.0) with a scan rate of 100 mV s⁻¹.

Square wave voltammetry (SWV) was performed with the following parameters; Frequency 150 Hz, amplitude 50 mV, step potential 0.15 mV, scan rate 22.5 mV s⁻¹.

The electrochemical impedance spectroscopy measurements were performed with a Solartron 1255 HF Frequency Response Analyzer and model SI1287 electrochemical interface, both controlled through a Dell PC compatible microcomputer. These measurements were carried out using a three-electrode electrochemical cell consisting of a GCE as working electrode, an Ag/AgCl (3 M KCl) reference electrode (CH Instrument, USA), and a platinum wire auxiliary electrode. The experiments were carried out at the open circuit potential (E_{ocp} = 220 mV) of the [Fe(CN)₆]³⁻ (5 mM)

redox couple in 0.02 M PBS (pH= 7.4) solution, with 10 mV (rms) sinusoidal excitation amplitude. The impedance data were measured and collected for 61 harmonic frequencies from 0.1 Hz to 100 kHz at 10steps/decade and then analyzed using the ZView software. The cyclic voltammetry measurements were also performed with a SI1287 electrochemical interface using the same three electrodes cell at the scan rate of 50 mV s⁻¹ from -0.10 V to 0.70 V (vs. Ag/AgCl).

4.2.3 Asymmetric PCR amplification

To improve sensitivity and reproducibility of our assay, an asymmetric PCR technique was used to generate an excess amount of single-stranded DNA targets [179, 180]. Asymmetric PCR assays traditionally employ forward and reverse primers in a 1:10 ratio [179]. This process leads to rapid depletion of the limiting primer during the exponential amplification, followed by linear amplification of the strand extended from the excess primer. Asymmetric PCR amplification was performed in a 10 μ L reaction volume containing 1 μ L of 10 ng/ μ L DNA template, 1 μ L of 1X PCR buffer (910 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM of MgCl₂), 0.6 μ L of 50 mM MgCl₂, 1 μ L of 2 mM of dNTPs and 0.03 μ M forward (DNA_nested_F208): 5'-CAG CTG GTC GAC ATT TTC GG-3' (20 mer) and 0.3 μ M reverse (DNA_nested_R697): 5'-TAT CAG TTC ATC CAG CGC GG-3' (20 mer) primers, 0.2 μ L of 5 U/ μ L Platinum[®] Taq DNA polymerase (Invitrogen) and top up to 10 μ L with ultra-pure water. The reactions were carried out in MJ DNA Thermal Cycler. Thermal cycling parameters were as follows: 95°C for 5 min; followed by 40 cycles of amplification with each cycle consisting of 1 min primer annealing at 60°C, 2 min of extension at 70°C, and 1 min of denaturation at 95°C. The amplification products of lambda DNA were 489 base pair (bp) fragments. Negative controls (PCR mixture without DNA template) were included in each set of reactions. The sequences of 489 bp Lambda DNA are as follows:

5'- ca gctggctgac atttcggtg cgagtatccg taccattcag aactggcagg aacaggggaat gcccggttctg cgaggcgggtg gcaagggtaa tgaggtgctt tatgactctg ccgccgtcat aaaatggtat gccgaaaggg atgctgaaat tgagaacgaa aagctgcgcc gggaggttga agaactgcgg caggccagcg aggcagatct ccagccagga actattgagt acgaacgcca tcgacttacg cgtgcgcagg ccgacgcaca ggaactgaag

aatgccagag actccgctga agtgggtggaa accgcattct gtactttcgt gctgtcgcgg atcgcaggtg
 aaattgccag tattctcgac gggctccccc tgcggtgca gcggcgtttt ccggaactgg aaaaccgaca
 tgttgatttc ctgaaacggg atatcatcaa agccatgaac aaagcagccg cgctggatga actgat -3'

The PCR amplified fragments were confirmed by fractionating the fragments on a 1.5 % agarose slab gel stained with ethidium bromide, visualized under UV transillumination 321 nm (Vilber Lourmat) and the images were photographed.

4.2.4 The preparation of surface of biosensor and its modification with DNA

The procedure for the preparation of DNA biosensor is schematically illustrated in Figure 4-1. DNA biosensor was fabricated based on the covalent bonding of capture probe on a chemically modified GCE via the electrooxidation of 4-ABA. A synthesized 21mer amino-modified single-stranded deoxyribonucleic acid (ssDNA) was then immobilized via covalent bonding onto a carboxylate-terminated 4-ABA monolayers modified GCE surfaces via EDC and NHS cross-linking reaction. Hybridization of ssDNA in the solution will only occur if the target is complementary to the probe.

Figure 4-1: Schematic diagram of the procedure for the fabrication of DNA biosensor based on the covalent bonding of capture probe on a chemically modified glass carbon electrode (GCE) via the electrooxidation of 4-aminobenzoic acid (4-ABA).

4.2.5 GCE modification to form 4-ABA/GCE

Before surface modification, the GCE was polished in sequential order with 1.0, 0.5 and 0.3 μm alumina (Alfa Aesar, USA), the electrode was thoroughly washed with ultrapure water, sonicated in ethanol and finally dried thoroughly under N_2 flow. The surface modification of the GCE was performed by procedures reported in reference [161]. Briefly, the electrochemical modification of the clean GCE was carried out using 4-ABA via C-N covalent bond in absolute ethanol solution containing 3 mM 4-ABA and 0.1 M LiClO_4 by cyclic scanning between 0.00 V and +1.40 V vs. SCE for 20 cycles at scan rate of 10 mV s^{-1} . The mechanism of the oxidation of 4-ABA was reported in [161].

4.2.6 Modification of 4-ABA/GCE with EDC/NHS (linker) and ssDNA

A versatile method for covalently attaching ssDNA to the 4-ABA/GCE was via the EDC and NHS linking reaction. The terminal carboxylic acid groups of the 4-ABA/GCE were activated by immersion in 50 mM PBS (pH 7.4) containing 2 mM EDC and 5 mM NHS for 1 hour. The linker/4-ABA/GCE was then rinsed with 50 mM PBS (pH 7.4) to wash off the excess EDC and NHS.

Synthetic ss-DNA immobilization onto the surface was performed by the following procedure: a sample of 3 μl of 100 ppm probe DNA was pipetted onto the chemically modified GCE. The probe droplet was left to dry overnight at 20°C [181]. Thus, a probe-modified GCE was obtained. This electrode ssDNA/4-ABA/GCE was designated as probe electrode. The electrode surface was then washed with ultra-pure water to remove the unbound oligonucleotides.

4.2.7 Hybridization

The hybridization was performed by pipetting 3 μl of different concentrations of the target DNA onto the probe-modified GCE electrode. The duration for the hybridization process was kept consistently at 5 min and 20°C [174]. Thus, a hybrid-modified GCE was obtained. The electrode surface was then washed with ultra-pure water to remove the unbound oligonucleotides. The same protocol as mentioned

above was applied at probe-modified GCEs for hybridization reactions of probe (21-mer sequence A) with one-base mismatch (21-mer sequence B') and also with non-complementary sequences (21-mer sequence B'').

4.2.8 Intercalation of methylene blue

MB was accumulated onto the surface of hybrid modified GCE by immersing the electrode into stirred 20 mM Tris-HCl buffer (pH 7.0) containing 20 μ M MB with 20 mM NaCl for 5 min without applying any potential [25]. For the intercalation conditions, we adopted the conditions reported in the following references and no further optimization was done [25, 177]. This concentration of MB was chosen as 20 μ M and the accumulation time of MB was set at 5 min for optimum analytical performance [25, 177]. After accumulation of MB, the electrode was rinsed with 20 mM Tris-HCl buffer (pH 7.0) in ultrasonic bath for 10 s to remove the non-specifically bound MB. MB was intercalated into the DNA to form DNA/MB system on the probe electrode after hybridization.

4.2.9 Detection procedures (voltammetric transduction)

The reduction signal of the accumulated MB on the electrode was measured by SWV with an initial potential of 0.50 V vs. SCE in 20 mM Tris-HCl buffer (pH 7.0) containing 20 mM NaCl. The parameters of SWV were as follows: frequency = 150 Hz, amplitude = 50 mV, step potential = 0.15 mV and scan rate = 20 mV s⁻¹. In square wave voltammetry, when the measured current is plotted as a function of the applied potential in a voltammogram (Figure 4-2, [182]), the negative anodic current (i_1) is subtracted from the positive cathodic current (i_2) producing a current (I) larger than both i_1 and i_2 . At the time of measurement, the charging current has been reduced significantly but the Faradic current is still relatively unaffected. Apart from the advantages of increasing the speed of the experiments, in many electroanalytical applications in the negative potential region, oxygen does not have to be excluded from solution when using SWV unless it interferes directly with the electrode reaction under study. At negative potentials in the limiting current region for oxygen reduction, the forward and reverse currents are equal, leading to a zero net current. For a scan in

the positive direction from negative potentials, the faster effective scan rate would mean a shorter time for electroactive species to diffuse to the electrode surface from the bulk solution. Thus, degassing of solution using nitrogen or argon prior to the experiment is not required, and this decreases the experimental time and simplifies the procedure. This results in other advantages such as lower consumption of electroactive species relative to DPV and reduced problems of electrode surface blockage. Consequently, SWV offers a faster scan rate, a higher sensitivity and a higher dynamic range compared to other techniques. Due to the greater sensitivity and better defined peaks of SWV relative to cyclic voltammetry, all measurements were performed using SWV. No base line correction in SWV was used.

Figure 4-2: Square wave voltammogram for reversible electron transfer : (i_1) forward, (i_2) reverse, and (I) net current [182].

4.2.10 Regeneration of modified electrode surface

The probe modified GCE surface denaturation/regeneration cycles were investigated.

Since both hybridization and detection involve reversible-binding processes, the biosensor is reusable for all the five cycles of hybridization and regeneration. Surface denaturation/regeneration cycles using MB as indicator were carried out as follows: A probe modified GCE electrode was first characterized by SWV in a stirred 20 mM Tris-HCl buffer (pH 7.0) containing 20 μ M MB with 20 mM NaCl for 5 min without applying any potential. The reduction signal of MB was measured as in Section 4.2.2. Then, hybridization of the complementary was performed as in Section 4.2.8 and intercalation of MB was performed as in Section 4.2.9. Subsequently, the reduction signal of MB was measured as in Section 4.2.2. After this measurement, the surface-immobilized dsDNA was then denatured in ultra-pure water at 95°C for 1 min, thus this gave rise to the probe modified GC electrode. Then a new cycle of denaturation/regeneration would start again.

4.3 Results and Discussion

4.3.1 Covalent modification of GCE with 4-ABA

Glassy carbon has many desirable properties for electrodes in voltammetry because it is impermeable to gases, highly resistant to chemical attack, electrically conductive and available in relatively high purity [95]. In fact, GCEs have one of the widest potential range of the many others carbon electrodes or other solid electrodes. In the common mineral acids the potential limit of glassy carbon in the cathodic region is the most negative in HNO₃, and in the anodic region the potential limit is the most positive in HCl. The widest potential limits are found in phosphate buffer about 1.30 to - 0.80 V (versus SCE). Furthermore, GCEs have a greater potential range in mineral acids than other carbon electrodes, such as wax-impregnated graphite, carbon paste and pyrolytic graphite. Therefore, modification of the GCE surface, so as to produce stable and compact monolayer, which can be used as charge-rich precursor films for subsequent fabrication, is an important objective in bioelectrochemistry.

The covalent modification of the GCE was performed by repeated cycling between the potential of 0.00 V and 1.40 V vs. SCE in 3 mM 4-ABA in 0.1 M LiClO₄ ethanol

solution at a scan rate of 10 mVs^{-1} for 20 cycles. Figure 4-3 shows the cyclic voltammogram of 3 mM 4-ABA in 0.1 M LiClO_4 ethanol solution on a GCE at 10 mV s^{-1} . The 4-ABA was found to oxidize at about +1.10 V. There is an irreversible oxidation peak attributed to the formation of amino cation radical and subsequently chemical bonding of the radical to GCE surface [161]. Typically, it is found that when the potential was repeatedly scanned, the peak gradually diminished. This indicates the formation of a coating on the electrode surface. Thus the GCE surface was modified with carboxyl-terminated monolayer, forming 4-ABA/GCE. Figure 4-4 shows the proposed mechanism of oxidation of 4-ABA and grafting on GCE.

Figure 4-3: Cyclic voltammograms on a freshly polished GCE in 0.1 M LiClO_4 ethanol solution with 3 mM 4-ABA. Scan rate= 10 mV s^{-1} .

Figure 4-4: Grafting of 4-ABA on a glassy carbon electrode. Formation of a carbon-nitrogen bond between the amine cation radical and an aromatic moiety of the glassy carbon surface is through the reactive radicals and rapid grafting on glassy carbon surface.

4.3.2 Blocking effect of the 4-ABA-modified GCE on electrochemical behaviour of redox probes

In order to study the blocking effect of the 4-ABA-modified GCE on the electrochemical behavior of redox probes, the effect of 4-ABA layer on GCE was investigated in 5 mM $\text{K}_3\text{Fe}(\text{CN})_6$ solution. Figure 4-5 shows the cyclic voltammograms for a (a) bare GCE, and (b) 4-ABA/GCE for 5 mM $\text{Fe}(\text{CN})_6^{3-}$ in 0.02 M phosphate buffer solution (pH 7.0) and (c) bare GCE in 0.02 M phosphate buffer solution with scan rate of 100 mV s^{-1} . Figure 4-5 clearly shows that the electron transfer of $\text{Fe}(\text{CN})_6^{3-}$ is completely blocked on the 4-ABA/GCE (Figure 4-5 (b)). The electrostatic repulsion prevented access of $\text{Fe}(\text{CN})_6^{3-}$ to the electrode surface and blocked the electron transfer to the electrode surface (Figure 4-6). The high negative charge density of the COO^- groups on the 4-ABA/GCE repels the $\text{Fe}(\text{CN})_6^{3-}$ groups from the surface of GCE. The dissociable terminal carboxylate groups on the 4-ABA/GCE played a major role in blocking the redox response of the anionic probe $\text{Fe}(\text{CN})_6^{3-}$. Next, amine-modified ssDNA can be covalently anchored on the COO^- groups from the surface of 4-ABA/GCE in the presence of EDC and NHS.

Figure 4-5: Cyclic voltammograms for a (a) bare GCE, and (b) 4-ABA/GCE for 5 mM $K_3Fe(CN)_6$ in 0.02 M phosphate buffer solution (pH 7.0) and (c) bare GCE in 0.02 M phosphate buffer solution. Scan rate = 100 mV s^{-1} .

Figure 4-6: A schematic showing the negatively charged ferricyanide ion being expelled from the 4-ABA modified GCE surface.

4.3.3 Electrochemical impedance spectroscopy (EIS) characterization

EIS is a simple and effective way to measure the charge transfer resistance (R_{et}) of the

electrochemical reactions. Figure 4-7 and Figure 4-8 show the representative Nyquist plots of the EIS spectra of a bare GCE and a 4-ABA modified GCE in the presence of $[\text{Fe}(\text{CN})_6]^{3-}$ as the redox probe, respectively. It can be seen that each of the impedance spectra includes a semicircular and a linear portion. The semicircular portion corresponds to the electron transfer process of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ in the high frequency range whereas the linear portion was due to the diffusion process in the low frequency range. The diameter of the semicircle represents the magnitude of R_{et} at the electrode surface. It is clear that the magnitude of R_{et} was larger for the 4-ABA modified GCE compared to that of bare GCE as shown in Figure 4-7 and Figure 4-8. The semicircle portion, observed at higher frequencies, corresponding to the electron-transfer-limited process. Hence, larger semi-circle in Figure 4-8 relative to Figure 4-7 implied that a high charge transfer resistance for 4-ABA modified GCE surface/solution interface.

Figure 4-7: Nyquist plots (Z_{imag} vs Z_{real}) for the Faradaic impedance measurement of a bare GCE, ($\text{pH} = 7.4$) containing $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (10 mM, 1:1). Scanning voltage amplitude = 10 mV around the equilibrium DC potential of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ in the frequency range from 0.1 Hz to 100 kHz.

Figure 4-8: Nyquist plots (Z_{imag} vs Z_{real}) for the Faradaic impedance measurement of 4-ABA modified GCE, (pH= 7.4) containing $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (10 mM, 1:1). Scanning voltage amplitude = 10 mV around the equilibrium DC potential of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ in the frequency range from 0.1 Hz to 10 MHz.

4.3.4 DNA immobilization

Although physical adsorption provides a simpler way to attach DNA onto solid surface, the attached DNA on the solid surface is not well ordered or tightly bound. Using the NHS/EDC coupling approach in contrast, produces a more ordered DNA immobilization with higher DNA surface density on electrode surface [183]. Ruffien *et al.* [166] reported a strategy for covalent immobilization of capture oligonucleotides probe on p-aminophenyl-functionalised carbon screen-printed electrodes. During the coupling reaction, the aromatic rings of various bases in the oligonucleotides undergo attack by the *in-situ* generated diazonium ions on the functionalized screen-printed electrodes. Kerman *et al.* reported on the voltammetric determination of DNA hybridization on self-assembled alkanethiol monolayer on gold electrodes based on NHS and EDC coupling reaction [176]. Millan *et al.* [95] reported that DNA was covalently immobilized onto oxidized GCE surfaces which had been activated using EDC and NHS, and the reaction was selective for immobilization

through deoxyguanosine (dG) residues. However, amine-modified ssDNA (capture DNA) was used in this work to anchor the amine groups from the 5' end terminal ssDNA to carboxylate groups of 4-ABA in order to achieve a more orderly layer of ssDNA on the surface of 4-ABA/GCE. A schematic diagram of the reaction process illustrating the roles of EDC and NHS in the anchoring of amine-modified ssDNA on 4-ABA/GCE is shown in Figure 4-9. In the presence of EDC and NHS, the 5'-terminal amine modified capture probe ssDNA forms a peptide bond with the COO⁻ groups from 4-ABA on the GCE surface. The activation of the 4-ABA modified GCE leaves the electrode surface terminated with a succinimide ester which is susceptible to nucleophilic attack from amine at the end terminal of the 5' ssDNA to form a peptide bond since amino-modified ssDNA is used in this experiment. Therefore, the probe must be covalently attached to the 4-ABA monolayer via the amines on the 5' end terminal. A popular and highly versatile method for covalently attaching ssDNA to the GCE/4-ABA is by using EDC, which couples amines to carboxylic acids. In the reaction, EDC converts the carboxylic acid into a reactive intermediate, which is susceptible to the attack by amines. EDC and NHS are used as they produced a more stable reactive intermediate, which has been shown to give a greater reaction yield. A phosphate buffer is used to prepare the NHS, EDC and DNA solutions. The use of a buffer solution is to overcome the electrostatic repulsion between the DNA polyanions and the negatively charged NHS esters originated from the sulfonate groups on the NHS esters. Such a step allows DNA molecules to approach to the vicinity of the NHS ester-containing SAMs so that the subsequent nucleophilic substitution of the NHS ester by the amino groups on DNA can occur.

Figure 4-9: A schematic of the reaction process where EDC and NHS are used and amine-modified ssDNA are anchored at the end of the COO^- groups of 4-ABA/GCE for the fabrication of DNA biosensor.

4.3.5 Voltammetry of methylene blue at oligonucleotide functionalized GCE electrodes

MB is an organic dye that belongs to the phenothiazine family (Figure 4-10). It is a redox indicator with the formal potential in the range of -0.10 to -0.40 V (versus SCE) in pH 4-11 medium, which is close to the redox potentials of many biomolecules, and thus it has been used as an electron transfer mediator. MB is known to undergo a reversible redox reaction and was studied in detail by Kelly *et al.* [123]. The reduced form of MB is known as leuco-MB. The reduction of MB involves the addition of two electrons and one proton under the experimental conditions to form leuco-MB [103]. MB could bind specifically to the guanine bases on DNA molecules. Detection of target DNA is thus achieved by measuring the cathodic peak current of MB on the DNA probe with SWV.

Figure 4-10: Chemical structure of methylene blue.

Detection of target DNA was monitored by measuring the cathodic peak current of MB on the DNA probe electrode with SWV. Typically, a low signal of MB was obtained on a 4-ABA modified GCE as shown in Figure 4-11 (a). The reduction peak potential for non-specifically adsorbed MB peak was at approximately -230 mV vs SCE. This is due to the relatively low quantity of MB adsorbed on the 4-ABA modified GCE surface. Such non-specific adsorption was also observed by Erdem *et*

al. [105, 174] with CPE, Tani *et al.* [173] and Yang *et al.* [177] on bare gold electrodes using cyclic voltammetry. The selectivity of this assay was investigated by using the 4-ABA/GCE capture probe to hybridize various DNA sequences [complementary oligonucleotide, non-complementary oligonucleotide sequence and one base mismatch oligonucleotide]. Figure 4-11 shows the SWV for the initial cathodic signal of MB at the capture probe (Figure 4-11 (b)), and after hybridization with the same amount of complementary DNA sequence (Figure 4-11 (e)), non-complementary sequence (Figure 4-11 (c)) and one base mismatch sequence (Figure 4-11 (d)), on the capture probe electrode. The concentration of the target DNA was $100 \text{ ng } \mu\text{L}^{-1}$. It was clear that Figure 4-11 (b) shows the highest intercalator peak current on the capture probe modified electrode GCE. There was a significant difference between the voltammetric signals of MB obtained with capture probe (Figure 4-11 (b)) and after hybridization with complementary DNA (Figure 4-11 (e)) on the capture probe electrode. The highest MB reduction signal was observed with capture probe on the electrode because MB has a strong affinity for the free guanine bases and hence the greatest amount of MB accumulation occurs at this surface (Figure 4-11 (b)). However, a significant decrease in the voltammetric cathodic peak current of MB was observed after complementary target sequence ssDNA was allowed to hybridize with the capture probe electrode. After hybridization, the current signal of MB decreased due to less MB binding to dsDNA, caused by the inaccessibility of MB to the guanine bases [107, 174, 175, 177]. This decrease is attributed to the steric inhibition of the reducible groups of MB packed between the bulky double helix of the hybrid. The decrease in the magnitude of the voltammetric MB intercalator peak current thus reflects the extent of the hybridization at the electrode surface.

Figure 4-11: Square wave voltammograms of 20 μM MB at a 4-ABA/GCE (a) and as the redox indicator for (b) a ssDNA probe-modified GCE electrode and after hybridization with 100 $\text{ng } \mu\text{L}^{-1}$ of (c) non-complementary sequence (d) mismatch sequence and (e) complementary target sequence. For SWV, modulation amplitude 10 mV, modulation time 50 ms, scan rate 20 mV s^{-1} . Buffer solution: 20 mM Tris-HCl buffer (pH 7.0) containing 20 mM NaCl.

Since it was observed that there was a significant difference between the voltammetric signals of MB obtained with dsDNA and ssDNA on the probe electrode, subsequently the capture probe was investigated for the response of non-complementary and mismatch oligonucleotides. Figure 4-11 (c) shows that the peak current almost did not decrease when the capture probe electrode (Figure 4-11 (b)) was exposed to the non-complementary oligonucleotides in the control experiment. This implied that there was no change occurring at the capture probe electrode surface and hence hybridization had not occurred. The result demonstrated that only a complementary sequence (Figure 4-11 (e)) could form a double-stranded DNA with the capture probe electrode and resulted in a significant decrease of the signal. No significant change in signal was observed for non-complementary sequence, which shows the high selectivity of the hybridization detection (Figure 4-11 (c)). For the response of mismatch oligonucleotides with guanine as the mismatched base on the modified GCE, the voltammetric signal (Figure 4-11 (d)) observed was just slightly higher than the one observed with fully complementary sequences (Figure 4-11 (e)). This slight

increase in signal is probably attributed to the interaction of MB with the one unbound guanine bases in the mismatch oligonucleotides and shows that complete hybridization was not accomplished. It is hard to distinguish the mismatch oligonucleotides from the complementary oligonucleotides. However, the response of MB on the probe electrode can be considered as an efficient intercalator to distinguish between hybrids and non-complementary oligonucleotides. There was a clear difference in current signal between the hybrids and the non-complementary oligonucleotides.

4.3.6 Calibration curve of DNA

The response in the presence of hybridization between DNA probe and increasing concentration levels of target was displayed in Figure 4-12, with constant MB. The response for the reduction of MB after hybridization with the target DNA decreased with increasing target concentration. The response levelled off at approximately 100 ng/ μ L. This may have indicated that all the available immobilized probes on the probe electrode surface were involved in hybridization. The decrease of the MB signal caused by the hybrid formation prevented the interaction of guanine base with MB. Fabrication reproducibility (evaluated as relative standard deviation over 3 independently modified capture probe electrodes, measured at 10 ng/ μ L of target DNA) was 9.70% and the detection limit was about 0.5 ng/ μ L of oligonucleotide target sequence.

Figure 4-12: The response of the capture probe with increasing concentration of target oligonucleotides. Other experimental conditions are as in Figure 4-11.

4.3.7 Regeneration of modified electrodes

Regeneration of modified electrodes with single-stranded oligonucleotides bound to the probe electrode surface was accomplished by rinsing the hybridized surfaces with hot (95°C) ultra-pure water for 1 min. Following this rinsing procedure, the peak current obtained for 20 μM MB was the same as those obtained at the modified GCE surface prior to hybridization. The reusability of the model biosensor was tested by repetitive hybridization with ssDNA and regeneration. Figure 4-13 shows the entire denaturation/regeneration process for a DNA biosensor using MB as indicator. Our results showed that no significant deterioration of cathodic peak currents occurs over the 5 cycles of hybridization/regeneration. The regeneration of the modified GCEs with single-stranded oligonucleotides was useful for continuous monitoring of the PCR amplicons in the future research.

Figure 4-13: Square wave voltammogram for a working cycle of a DNA probe using MB as a denaturation/regeneration indicator. The original ssDNA probe (a), hybridization (b) and regeneration of the DNA probe (c). Other experimental conditions are as in Figure 4-11.

4.3.8 Hybridization detection of PCR amplified real samples

PCR amplification is a useful technique for the diagnosis of diseases caused by genetic factors. In recent years, DNA-based diagnostic methods such as PCR have been developed for rapid identification of microorganisms from clinical specimens. Generally, the most common method for detecting PCR products in the research lab is by agarose gel electrophoresis and the results are analyzed by sizing the PCR amplification products formed for end-point detection. Although this method is simple and effective for the detection of PCR products, the interpretation of the results can be subjective and it is not possible to determine whether the sequence of the amplified target DNA is exactly the one intended for measurement. In addition to this, a carcinogenic chemical such as an ethidium bromide is used for this method. Therefore, gel electrophoresis is not suitable. Traditional methods only use agarose gels for PCR detection and obtain the results at the final phase of the end-point of the PCR reaction, hence it is very time consuming. The end-point detection results are based on size discrimination and a 10-fold concentration difference is required to accurately

differentiate between two samples. The sensitivity and specificity can be improved by transferring the PCR products to a nylon membrane and detection by hybridization with labelled specific probes. However, Southern blotting is not suitable for rapid analysis of many samples because the technique is labour intensive, involves multiple steps and reproducibility is sometimes lost in several steps, and the standardization of the procedure between laboratories is difficult.

There have been considerable interests in developing DNA electrochemical biosensors for rapid PCR products analysis to replace gel electrophoresis. Thus, we present herein an electrochemical detection system for the sequence-specific detection of PCR amplified real samples via the above mentioned approach. By adapting PCR products into capture probes, which are complementary to specific sequences in the target gene, specific and sensitive detection of PCR amplification products are facilitated. For the identification of PCR amplification products which were PCR amplicons based on the biosensor, ssDNA probe was covalently immobilized onto a carboxylate-terminated 4-aminobenzoic acid (4-ABA) monolayer modified glassy carbon electrode surface.

The efficiency for quantifying the amount of PCR amplicons from Lambda DNA template with the ssDNA/4-ABA/GCE was investigated. Asymmetric PCR predominantly produces ssDNA caused by two unequal primer concentrations. Since a forward to reverse primer ratio of 10 : 1 was used, majority of the final PCR product was ssDNA. We used the asymmetric PCR to ascertain that the copy numbers of ssDNA are substantially more than the dsDNA [184, 185]. The amplicons of asymmetric PCR, which were predominantly ssDNA as a result of two unequal primer concentrations, could hybridize with the DNA probe easily. Specific DNA sequences from asymmetric PCR amplicons without subsequent purification were detected based on the earlier approach we developed. Herein, the DNA probe that had specificity to the sequences of single-stranded PCR products was designed for probe immobilization.

The capability of identifying the presence of specific DNA sequence, using the capture probe that was covalently attached to the 4-ABA/GCE working electrode surface, was demonstrated in Figure 4-14. The specific sequence of the PCR amplicons will hybridize with the capture probe and the formed hybrids will bear less MB than the probe. Upon hybridization with the target PCR amplicons, a large change in the reduction peak current of MB was observed as shown in Figure 4-14. It can be seen that the electrochemical signal of MB was much lower for the reaction mixture which containing 10 ng/ μ L DNA template than that of the capture probe. The signal reduction can be explained by less MB accumulation on the capture probe after the asymmetric PCR amplicons were hybridized on the probe DNA.

Figure 4-14: SWV of 10 μ M MB (●) intercalated onto the capture probe working electrodes. The current signal was decreased when the PCR amplicons from PCR 40th cycle hybridized to the 4-ABA/GCE probe. (a) SWV current-potential scan for capture probe and (b) SWV current-potential scan for PCR amplicons from 40th cycle hybridized to the capture probe. For SWV, modulation amplitude = 10 mV, time = 50 ms, scan rate = 20 mV s⁻¹.

A series of experiments with various amounts of the PCR amplicon, after being amplified every 5 cycles, were collected. A plot of the peak current of the methylene blue (MB) versus cycle number of PCR was given in Figure 4-15. By using this approach, we were able to detect PCR amplicons after the 5th PCR cycle. Upon hybridization with the target PCR amplicons, a large reduction of peak current was

observed.

Figure 4-15: A calibration plot of the SWV peak current of the probe electrode versus number of PCR cycle for quantification of PCR amplicons every 5 cycle of amplification.

The sensitivity of the electrochemical detection was evaluated in comparison with standard agarose gel electrophoresis for detecting PCR products amplified from Lambda DNA (489 bp). With the conventional agarose gel electrophoresis and ethidium bromide staining method, the minimum amount of lambda DNA template, which produced a visible band, was only detected from 8th cycle for 10 ng/μL of DNA template as an initial concentration in the asymmetric PCR mixture (Figure 4-16).

Figure 4-16: Gel electrophoresis of PCR amplified real sample from lambda DNA. Lane L, 1 kb DNA ladder; lane 1, negative control (without DNA template); lane 2-21 corresponding to PCR cycles 5, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38 and 40, respectively.

The results obtained from the electrochemical detection method were in good agreement with that obtained from gel electrophoresis. In addition, electrochemical detection is more specific and sensitive in DNA detection compared to the traditional gel method. The electrochemical detection method allows detection of low quantities of PCR products obtained from 5 PCR cycles. In this study, an electrochemical DNA biosensor, which offers fast and reliable results for PCR amplified real samples, was described. The biosensor was able to detect the complementary sequence in the PCR amplified real samples by using MB as the hybridization indicator. This procedure also eliminated the use of toxic chemicals such as ethidium bromide, which was used in the gel electrophoresis step of the reference method in DNA analysis.

4.4 Summary

In this chapter, we developed a DNA biosensor based on the covalent bonding of capture probe on a chemically modified GCE via the electrooxidation of 4-ABA. A synthesized 21mer amino-modified ssDNA was immobilized via covalent bonding onto a carboxylate-terminated 4-ABA monolayers modified GCE surfaces via EDC and NHS cross-linking reaction. Since the NHS/EDC approaches produces a more uniform DNA immobilization with higher DNA surface density, covalent bonding can be the method of choice for developing DNA biosensors with high sensitivity and better hybridization efficiency. This immobilization method for the probe ssDNA has been demonstrated to be useful for monitoring the DNA hybridization with the complementary ssDNA on the GCE surface. The probe-modified GCE was shown to be an effective biosensor for the detection of hybridization by using MB as the electrochemical indicator. The peak current of MB was found to increase in the following order: hybrid-modified GCE, mismatch hybrid-modified GCE, non-complementary modified GCE. There was decrease of the reduction current of MB intercalator with increasing concentration of target DNA with the capture probe. Fabrication reproducibility for 3 independently made electrode was approximately 9.7%, measured at 10 ng/ μ L of target DNA. The detection limit of the DNA biosensor

was approximately 0.5 ng/ μ L for the target DNA. This approach had also been further applied into real-sample from PCR amplification. Using an asymmetric PCR and the electrochemical DNA detection scheme, single-stranded amplicons without subsequent purification can be rapidly identified. The decrease of the peak current of MB, an electroactive indicator, was observed upon hybridization of the probe with the target DNA. The reduction signal of MB was found to be effective for the discrimination of probe and target PCR amplicons/probe hybrids.

Chapter 5

In-situ Electrochemical Detection for Real-Time Quantification of PCR Amplicons

5.1 Introduction

Polymerase chain reaction (PCR) is one of the most widely used techniques in molecular biology [4, 5]. The PCR entails the *in vitro* exponential enzymatic amplification of specific DNA sequences to the level that are several orders of magnitude higher than the starting materials. Its ability to amplify and, therefore, isolate the genes of interest is used in many different areas. DNA amplification has become an essential step of most procedures used for nucleic acid detection, quantification and sequencing. For many applications involving PCR and reverse transcriptase PCR (RT-PCR), it is critical to accurately quantify the PCR products or PCR amplicons.

5.1.1 Real-time quantitative PCR (qPCR) using fluorescence detection

An end-point PCR measurement does not provide information regarding the initial amounts of the target molecules in the samples; such measurement can only distinguish a positive sample from a negative one. On the other hand, real-time quantitative PCR provides additional information beyond mere end-point detection of the DNA. It indicates not just whether a specific DNA segment is present in a sample, but also its amount information. This information is required in a number of applications ranging from the medical diagnostics testing through the target searches to the basic research. Most of the qPCR methods in use today are based on a discovery in 1992 by Higuchi, who used the dye EB as a fluorescence reporter [22]. After being intercalated into the double-stranded DNA, the EB fluorescence indicates the amount of the DNA formed. Since this method can monitor the PCR products at any time during the PCR reaction, it is named as real-time PCR. In this method, parallel runs are performed with the known quantity of the same kind of DNA to plot

a comparable curve for DNA characterization under the identical conditions.

The fluorescence-based detection instrument is suitable for the amplification, detection and quantification of the DNA and makes the real-time PCR a powerful technique. More recently, SYBR Green I, which is less toxic than EB, is widely used as a dye that can incorporate into the minor groove of the dsDNA (Figure 5-1) to greatly enhance the fluorescence signal [186, 187].

Figure 5-1: The DNA interaction dyes. (a) The Chemical structure of SYBR Green I. (b) When DNA fragment is denatured, SYBR Green I dye is attached to a single strand. (c) Single stranded DNA fragment is duplicated by polymerase and becomes double stand DNA when DNA amplification is completed; SYBR Green I dye binds to double stand DNA. When this occurs, the dye will emit fluorescent light.

During the PCR reaction, the amount of the double-stranded target will increase exponentially, paralleled by an increase in the SYBR Green I incorporation and fluorescence emission. In each cycle, the fluorescent emission will increase gradually during the extension phase of the reaction, and will become low or absent during the denaturation phase. This fluorescence signal increases in direct proportion to the amount of the PCR product in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during the exponential phase where the first significant increase in the amount of PCR product

correlates to the initial amount of the target template. The higher the starting copy number of the nucleic acid target is, the sooner a significant increase in the fluorescence is observed. Thus, the real-time PCR system is based on the detection and quantification of a fluorescent reporter [188]. Besides the SYBR Green I, there are other three chemistries: TaqMan[®] (Applied Biosystems, Foster City, CA, USA), Molecular Beacons and Scorpions[®] are available for the real-time PCR. All of these chemistries allow the detection of PCR products via the generation of a fluorescence signal similar to the principle of SYBR Green I.

5.1.2 Thermocycling profiles

A typical temperature profile of PCR is shown in Figure 5-2. When performing the PCR amplifications, reaction components and PCR parameters are not the only criteria that affect the quality of the results; differences may also arise from the capability of the thermal cyclers to maintain a consistent temperature both within and between the experiments. If the well-to-well temperature across the thermal block is not uniform, it can have a significant impact on the PCR. This is an important consideration especially for researchers performing quantitative or diagnostic PCR assays with multiple samples.

Figure 5-2: The PCR thermocycling profile.

5.1.3 Real-time PCR response curves

Real-time PCR is a homogeneous method that performs DNA amplification and analysis without the need of slab gels, radioactivity, or sample manipulation. Figure 5-3 shows a typical DNA amplification process, in which the terms and definitions

routinely used in the real-time PCR are illustrated.

Figure 5-3: The real-time PCR response curves [188]. A typical amplification of a real-time PCR reaction includes a baseline, an exponential phase, and a plateau phase. The threshold is determined by the variation of the baseline. The point at which the fluorescent intensity crosses the threshold is defined as C_T (threshold cycle). The C_T value is proportional to the initial target DNA copy number and thus can be used in a quantitative comparison between the samples.

During the initial cycles of the PCR reaction, there is little or no change in fluorescence signal. This stage is the baseline of the amplification plot. As the amount of the PCR product accumulates, a signal is initiated and increases exponentially during the initial PCR phase. There will be no significant increase in PCR product amounts after the signal levels off and saturates. The signal saturation is due to the running out of some critical components in the reaction, such as the primers, the fluorescence reporters, the polymerase molecules or the dNTPs [188, 189]. The real-time PCR response curve reflects the difference in the initial amounts of the target DNA. The difference is quantified by comparing the number of amplification cycles

required for the samples' response curves to reach a particular threshold fluorescence signal level. The number of cycles required to reach threshold is called the C_T value. C_T values decrease linearly with increasing input target quantity, so it is used as a quantitative measurement of the input target.

The most important parameter for real-time quantitative PCR is C_T , the cycle number that starts to reach the threshold level, which has an exponential relationship with the DNA copy number. A plot of C_T against the $\log_{10}$ of the initial copy number for a set of standard DNA gives a straight line (Figure 5-4). This curve is then used as a reference standard for extrapolating the quantitative information for the targets of unknown concentrations.

Figure 5-4: The real-time PCR standard curve [188]. (A) Real-time PCR response curves are shown in a logarithmic scale for five standard samples. The intersection points of these curves to the threshold line are their C_T values. (B) The C_T values are plotted vs. the logarithm of the initial number of template copies in the standard samples. The absolute copy number of an unknown sample can be determined by this standard curve.

The advantages of real-time PCR are exploited in the clinical diagnosis and the monitoring of infectious diseases such as tumours, the analysis of age-dependent disease, cytokine and tissue-specific expression, epigenetic factors like DNA

methylation, and food quality monitoring. The real-time PCR has also attracted a great deal of interests in the forensic DNA typing community in the last few years as this technique can rapidly detect low levels of the DNA in minimum hands-on time and minor sample consumption. The capability of quantifying specific human assays in criminal identification laboratories is important in order to meet federally mandated requirements to assess the quantity of human DNA in casework samples, particularly where bacterial contamination may exist. As the field of applications is still expanding rapidly, it suggests that real-time PCR is becoming one of the most important techniques in molecular life science and medical science.

5.1.4 *In-situ* electrochemical detection of PCR product

The routine quantitative real-time PCR using fluorescent detection method was described in Section 5.1.1. This has great impact on molecular research and diagnostics, given that enormous amounts of data can be obtained within a very short time. In particular, real-time PCR is highly attractive as it enables the real-time quantification of PCR through the concurrent analysis of the fluorescence generated during the reaction, thus relinquishes the need of conventional post-PCR analyses such as the gel electrophoresis. In recent years, it has become the dominant standard for the detection and quantification of DNA and has been adopted by most research and diagnostic laboratories. Nevertheless, although fluorescence detection method is sufficiently sensitive and specific for the PCR product detection under realistic field conditions, the corresponding systems are generally bulky and costly; because these require high-intensity laser sources and high numerical aperture optical components that preclude their use in truly miniaturized devices. Thus, electrochemical detection of the PCR products may be an alternative to the fluorescence detection scheme. As the response at the electrode in the electrochemical detection is not path length dependent, electrochemical detectors can be miniaturized without a loss in sensitivity.

Currently, the integration of PCR with EC detection is achieved only at the end-point stage of the PCR as reviewed in section 2.2.11 of Chapter 2. In this Chapter, we propose to develop an *in-situ* EC detection scheme for the quantification of PCR

products, instead of the EC based end-point PCR detection scheme. Specifically, we propose to develop a stationary electrochemical detection method for quantitative PCR, abbreviated as stationary EC-qPCR. Three platinum electrodes were inserted into a 0.2 mL eppendorf tube, acting as the working, reference and counter electrode, respectively. MB was added into the PCR mixture as intercalator for dsDNA. Free MB in the PCR mixture was scanned by SWV *in-situ* during the PCR amplification. During the amplification, the quantity of the PCR product increased exponentially. At the annealing cycle, MB will intercalate into the PCR products, thus reduces the amount of free MB in the solution. SWV is used to measure the concentration of this free MB. This *in-situ* online monitoring is different from the classical end-point assays, as they are designed to provide information concurrently while the amplification process is in progress, thus requiring no post-PCR manipulation.

5.2 Methodology

5.2.1 Approach of EC detection in quantitative PCR

A macro 3-electrode module is incorporated into the PCR tube in order to perform the EC detection during the PCR. Since the PCR mixture stays still in the PCR tube while experiences thermocycling profile, this device is defined as stationary EC-qPCR reactor, as shown in Figure 5-6. In the stationary EC-qPCR reactor, a 3-electrode module is used for an *in-situ* EC measurement of the PCR products inside the PCR tube, which consisted of 3 platinum metal wires with 0.1 mm diameter and the length is ca. 2 mm functioning as the working electrode, the counter electrode and the reference electrode, respectively. This 3-electrode module is then sealed and dipped in a 200 μ L PCR tube filled with 20 μ L PCR mixture. The loaded stationary EC-qPCR reactor consisting of the 3-electrode module and the PCR tube is then put in a thermal cycler PT-100 (MJ, USA), a programmable instrument which can rapidly alter temperatures and keep the PCR tubes at the desired temperatures with a preset temperature profile. The wires of the 3-electrode module are then connected to an EC workstation (Model 660, CH Instrument, USA) for the SWV measurements at every PCR cycle throughout the PCR process. Hence, the PCR amplification and the EC

measurements are executed concurrently. The electrochemically active DNA intercalator, MB, is incorporated into the PCR mixture to generate the electrochemical signal at every PCR cycle that relates to the quantities of the PCR products during the PCR cycling. The SWV method of EC technique is utilized to measure the peak current I_p of DNA intercalators at the extension phase where the dsDNA is formed. I_p at a PCR cycle versus the PCR cycle numbers is plotted, and the threshold line is used to obtain a C_T value. The C_T is the number of amplification cycle required for response curves of the samples to reach a particular threshold level of current measurement signal, while the threshold level is based on the variation of the background noise. In the tests, C_T can be determined for an unknown initial DNA template concentration sample by being compared with the standard curve, where the C_T is plotted against the $\log_{10}$ of a set of standard initial DNA template concentrations for extrapolating the unknown initial DNA template concentrations. Thus, the stationary EC-qPCR technique can be used to quantify the PCR products cycle-by-cycle in real time. Two main concerns in performing the stationary EC-qPCR are that the metal electrodes and the electrochemically active DNA intercalator must not cause significant inhibition to the PCR amplification since the platinum wires and the DNA intercalator are in contact with the PCR mixture. A study of inhibition on the PCR is performed on the platinum wires and DNA intercalators. To investigate the electrode inhibition, the platinum wires with different radiuses and DNA intercalators with different concentrations are incorporated into the PCR mixture throughout the PCR amplification, and the gel electrophoresis and microcapillary electrophoresis are used to characterize the yield of the PCR products.

The details of the methodology of the stationary EC-qPCR reactor are described as follows:

(a) Electrochemically active DNA intercalator

The mechanism of this EC approach is based on the reaction of the electrochemically active DNA intercalator with the PCR product, as depicted in Figure 5-5.

Figure 5-5: The mechanism of the reaction of the electrochemically active DNA intercalators with the PCR products. The EC peak current decreases as the PCR cycle number increases.

After the electrochemical active DNA intercalator is added into the PCR mixture, the EC measurement is taken during the extension phase of the PCR at various PCR cycles (e.g. cycle 0, 2, 10, 25 and 40) of the PCR. During the extension stage at around 70°C, the dsDNA is formed. Since the MB bounds to dsDNA and the bounded complex is electro-inactive, the diffusion coefficient of the intercalator-DNA complex will change. Before the PCR, a large portion of the DNA intercalator molecules remain in the PCR mixture solution, the diffusion of the DNA intercalator to the electrode surface remains constant. When the DNA amplification is started, the diffusion coefficient of the bounded DNA intercalator (DNA-intercalator complex) becomes lower than that of the free DNA intercalators. As a result, the anodic current of the free DNA intercalator decreases significantly in proportion to the increase of the PCR product concentration, the peak current of the intercalators decreases further as the concentration of PCR products increases, and the concentration of electroactive species decreases when the PCR cycle number increases [29, 190-192]. Thus, at the

end of a PCR cycle, the signal of the intercalator is the lowest compared to the signal at the beginning of the cycle. This *in-situ* EC detection approach makes it possible to perform EC DNA quantification during the PCR thermal cycling.

(b) Construction of the 3-electrode module and the stationary EC-qPCR reactor

Figure 5-6 shows a schematic of the 3-electrode module and the stationary EC-qPCR reactor inside the thermal cycler to conduct the stationary EC-qPCR. The 3-electrode module for the EC detection consists of 3 platinum metal wires with 0.1 mm in diameter functioning as the working electrode, the counter electrode and the reference electrode, respectively [193-195].

Figure 5-6: The scheme of the 3-electrode module and the stationary EC-qPCR reactor for performing the stationary EC-qPCR.

The details of the construction for the 3-electrode module in the stationary EC-qPCR set-up are as follows:

First, each platinum wire working as an electrode is soldered to a longer wire, which is used as an extension to connect the platinum electrode to the EC workstation for the SWV measurement. Secondly, each platinum electrode is protected by inserting it into a thin glass capillary with an outside diameter (OD) of 1.1 mm and an internal diameter (ID) of 0.8 mm, so as to prevent the metal wires from contacting each other. Thirdly, the void among the glass capillaries and between the interior surface of each glass capillary and its internal metal wire is sealed by PDMS as shown in Figure 5-7.

Figure 5-7: The top view of a 3-electrode module in the stationary EC-qPCR reactor; each electrode made of a platinum wire in 0.1 mm diameter is inserted into glass capillary in a 1 mm diameter glass, and the whole structure is glued together by PDMS.

The PDMS is prepared from a mixture of a base and a curing agent at the ratio 10:1, and is poured into the void among the glass capillaries and the metal wires. This structure is incubated in an oven for 3 hours at 80°C for PDMS to be cured. After the PDMS curing, the three glass capillaries are closely bonded together with a 1.3 mm separation between the two adjacent platinum metal wires. The bonded structure is then inserted together through the top cover of a 200 μ L Eppendorf tube filled with

20 μ L PCR mixtures for the EC measurements. At last, the cover of the tube is sealed with PDMS to prevent the evaporation of the PCR mixtures.

(c) System set-up for performing the stationary EC-qPCR

The 3-electrode module together with the Eppendorf tube are placed in the 96 wells PCR thermal cycler (PT-100™ Programmable Thermal Controller, MJ, USA) to perform the stationary EC-qPCR as shown in the experimental setup photos in Figure 5-8.

Figure 5-8: The equipment set up for performing the stationary EC-qPCR.

PCR relies on the use of different temperatures for the three steps of reaction – denaturation, annealing and extension. To amplify the target DNA, it is necessary to cycle through these temperatures 25-40 times depending on the application. In this approach, it was accomplished by using a thermal cycler, which is based on a thermoelectric cooler (TEC) for the rapid heating and cooling of the reaction tubes to a predetermined temperatures and holding the temperature for a specific time before

shifting to the next temperature. This automation is one of the important advances that made PCR widely accessible. Before thermal cyclers were invented, PCR was performed by using three water baths with the set temperatures where the reaction tubes were moved manually among the baths.

The wires of the 3-electrode module are then connected to the EC workstation (model 660, CH Instruments, USA) as shown in Figure 5-8. The PCR amplification is carried out in the PTC-100 thermal cycler up to 40 cycles with the electrochemical detection conducted simultaneously at the selected cycles. The total volume of the PCR mixture was 20 μL , and the exposed area of each metal wire to the PCR mixture solution was 0.1 mm in diameter $\times$ 2 mm in length. The SWV of 10 μM MB in PCR mixture is carried out by scanning the potential applied to MB from 0.00 V $\sim$ -0.50 V at an increasing step of 0.15 mV, with pulse height of 50 mV and pulse width of 25 ms at the frequency of 100 Hz. SWV offers a faster scan rate, a higher sensitivity and a higher dynamic range compared to other techniques [182, 196, 197]. The current-potential curve of MB is obtained from SWV measurement. The peak current, I_p , is then obtained by deducting the peak current from its base line of the current-potential curve during the PCR cycles.

(d) Construction of a standard curve for the stationary EC-qPCR

Figure 5-9 (a) shows the plot of the measured peak current, I_p versus the PCR cycle number. The entire experiment is repeated a few times with different initial DNA template concentrations. Hence, various profiles of peak current versus PCR cycle number are obtained. The C_T value is then obtained from the peak current versus PCR cycle number profiles. C_T is the number of amplification cycle required for the I_p curves of the PCR samples to reach a particular threshold level, and the threshold level is based on the variation of the background noise. Therefore, C_T is considered to be the point most reliably proportional to the initial concentration. The C_T differs for each DNA template because different amount of the starting DNA template needs different numbers of cycles to reach this detection limit. The greater the amount of the

initial DNA templates is, the smaller the C_T value will be. Such as in Figure 5-9 (a), the three I_p versus number of PCR cycle curves represent subsequently higher initial concentrations from the right curve to the left curve. A standard curve where the C_T is plotted against the $\log_{10}$ of a set of known initial DNA template concentrations can be created as shown in Figure 5-9 (b). This curve will then be used as a reference standard for extrapolating the quantitative information for the DNA targets with unknown concentrations. By monitoring the log-linear phase, it is possible to get an accurate quantification, since an increase in the EC signal during the log-linear phase corresponds directly to the increase of the amplified DNA.

Figure 5-9: Construction of a standard curve, C_T , versus $\log_{10}$ DNA concentration for the quantitative PCR. (a) a plot of I_p versus PCR cycle numbers curve measured in the stationary EC-qPCR, (b) a plot of C_T versus the $\log_{10}$ of the initial concentrations for a set of standard DNA template which gives a straight line.

5.2.2 Chemicals and PCR mixture preparation

The MB and iron 1, 10-phenanthroline ($\text{Fe}(\text{phen})_3^{2+}$) were purchased from Sigma-Aldrich. Other reagents used were all in the analytical reagent grade or better. The ultra-pure water prepared from an EASY Pure with a resistance of 18 M Ω cm was used for the solution preparations. The 10 μ L of PCR reaction mixture contained 1 μ L of 20 mM Tris-HCl (pH 8.4) with 50 mM KCl, 1 μ L of 2 mM dNTPs, 0.6 μ L 50 mM MgCl_2 , 0.3 μ M each of the forward and reverse primer, 1 μ g/ μ L of BSA, 0.1 U/ μ L of

Platinum *Taq* DNA polymerase (Invitrogen), 1 μL of 0.1 ng/ μL SARS DNA cloned in pGEM-3Z vector, 1 μL of 1X SYBR Green I (Cambrex Biosciences Rockland, Inc.) for the real-time PCR fluorescent detection or 1 μL of MB with final concentration of 20 μM for the EC detection. The PCR mixture was topped up to 10 μL with ultra-pure water. The PCR mixture was prepared as in Table 5-1. The concentrations of the template DNA varied from 0.1 ng/ μL to 100 ng/ μL .

The primer-pairs for PCR

The PCR primer pair was designed to amplify a 77 base-pair of the BNI-1 fragment of SARS and to flank the region of amplification. The primers were designed to avoid sequences which would produce internal secondary structures. Ideally, both primers should be annealed at the same temperature. The sequences of the forward and reverse primers were 5'-TTA TCA CCC GCG AAG AAG CT-3' and 5'-CTC TAG TTG CAT GAC AGC CCT C-3', respectively. Regardless of the primer choice, the final concentration of the primer in the reaction mixture was at a final concentration of 0.3 μM in a 10 μL reaction.

Table 5-1: *Recipe for the PCR mixture.*

Master Mixture	1X Reaction
Ultra-pure Water	3.7 μL
PCR buffer (10X)	1.0 μL
MgCl ₂ (50 mM)	0.6 μL
dNTPs (10 mM)	1.0 μL
BSA (10 mg/mL)	1.0 μL
SYBR Green (1X) or Methylene blue (20 μM)	1.0 μL
Pt <i>Taq</i> DNA polymerase (5U/ μL)	0.1 μL
Reverse Primer (10 μM)	0.3 μL
Forward Primer (10 μM)	0.3 μL
SARS DNA Template	1 μL
Total Volume	10 μL

dNTPs

The PCR nucleotide mixture (Promega Cat. # C1141, Promega corporation, US) was a premixed solution containing the sodium salts of dATP, dCTP, dGTP and dTTP, each at a concentration of 2 mM in the ultrapure water. This solution was ready to be used and was optimized for the standard PCR and specialty approaches including the hot-start and reverse transcription PCR. The final concentration of the dNTPs in the reaction mixture was 0.2 mM in a 10 μ L PCR reaction.

Bovine Serum Albumin (BSA)

The acetylated BSA (Promega Cat. #R3961, Promega corporation, US) could be used as an enzyme stabilizer or as a carrier protein. It was dialyzed extensively with deionized water to remove the impurities.

Taq DNA polymerase PCR Buffer

The Taq DNA polymerase PCR Buffer (Invitrogen Cat. No. 10966-018, Invitrogen Corporation, Carlsbad, California, US) was to be used with *Taq* DNA Polymerase only. It was supplied at a 10X concentration and thus was diluted by 1:10 (1 part buffer + 9 parts other components = 10 parts final reaction volume). The buffer composition (10X) was 200 mM Tris-HCl (pH 8.4) with 500 mM KCl.

Magnesium Chloride ($MgCl_2$)

The concentration of the magnesium is a crucial factor that affects the performance of Taq DNA polymerase. Reaction components, including template DNA, chelating agents present in the sample (e.g., EDTA or citrate), dNTPs and proteins, can affect the amount of free magnesium. Without adequate free magnesium, the *Taq* DNA polymerase is inactive. The magnesium ion can stabilize DNA duplex, and hence, the high concentration of $MgCl_2$ decreases the efficiency of PCR. For these reasons, it is important to empirically determine the optimal $MgCl_2$ concentration for each reaction. In this work, the concentration of $MgCl_2$ (Invitrogen Cat. No. 10966-018, Invitrogen Corporation, Carlsbad, California, US) was at a final concentration of 3 mM/ μ L in the reaction mixture.

Fluorescent Dye

SYBR Green (Cambrex Part No. 50513, Cambrex Corporation, US) is a highly sensitive fluorescent stain for detecting dsDNA and oligonucleotides. SYBR Green I fluorescent signal is greatly enhanced when the dye is bound to the nucleic acids. It is ideal for detecting the small amounts of DNA in low copy/target number PCR products, apoptosis studies, and heteroduplex analysis.

Ultrapure Water

Ultrapure water can be self-prepared by autoclaving the deionized water at about 121°C. It is highly purified water that is low in ions, particles, organic matter and colloids, which could affect the PCR reaction adversely.

Platinum® Taq DNA Polymerase

Platinum® Taq DNA Polymerase (Invitrogen Cat. No. 10966-018, Invitrogen Corporation, Carlsbad, California, US) is a recombinant Taq DNA polymerase complexed with a proprietary antibody that blocks the polymerase activity at ambient temperatures. The activity is restored after the denaturation step in the PCR cycling at 94°C, thereby providing an automatic hot start for the Taq DNA polymerase in PCR. Hot starts in PCR provide increased sensitivity, specificity, and yield, while allowing an assembly of reactions at room temperature. The use of this antibody helps to reduce the PCR optimization requirements, the reaction set-up time and effort, the handling of reaction components, and the contamination risks, thereby improves the PCR results. Platinum® Taq DNA Polymerase is supplied at a concentration of 5 units per µL.

Cloning of SARS DNA fragment

SARS RNA was quantified by using the Ribogreen kit from the Molecular Probes, Inc. (Eugene, OR). The reverse transcription was performed with Invitrogen's (Carlsbad, CA) Superscript First-Strand Synthesis System with the random primers according to the manufacturer's instruction. After the reverse transcription, the enzyme was heat inactivated and then the sample was treated with RNase H. The PCR was performed

separately by using the *Taq* DNA Polymerase from Promega (Madison, WI) and the SARS cDNA. The forward primer 5'-ggggtaccatacctcagcgtgatataaagttcaag-3' and the reverse primer 5'-agaattcgaagcctttatacatgagtggataagatg-3' were used to amplify a region between the nucleotide position 18031 and 18424 of the SARS coronavirus CUHK-AGO1 genome. This region encompassed the BNI-1 fragment described by Drosten *et al.* [198]. Additional nucleotide sequence on the primers was added to introduce Kpn1 and EcoRI restriction site, respectively.

DNA Template

400 base-pair fragment of SARS RNA was real-time PCR amplified and cloned into pGEM-3Z vector. The 77 base-pair fragment (TTATCACCCCGCGAAGAAGCTATTCGTCACGTTTCGTGCGTGGATTGGCTTTGATGTAGAGGGCTGTCATGCAACTAGAGA) was cloned between Kpn I and Eco R I restriction sites [198].

5.2.3 The PCR amplification

The amplification products of the SARS DNA were 77 base pair (bp) fragments. The negative controls (PCR mixture without DNA template) were included in each set of reactions. The same PCR mixture was carried out in the real-time Rotor-Gene 3000 machine. The PCR condition used on a PTC100 thermocycler (MJ Research) was as follows: initial 95°C for 2 min, 40 cycles of 95°C for 1 min, 58°C for 1 min and 72°C for 2 min, ended with 72°C for 10 min and 4°C for 30 min. After the PCR, the agarose gel electrophoresis was performed. The concentrations of the SARS DNA template were varied in each PCR mixture to obtain the standard curve. 20-500 μ M of MB was added together in the 20 μ L PCR mixture as an intercalator for the EC detection, and SYBR Green was added for the real-time fluorescence study.

5.2.4 Inhibition of the metals and the EC intercalators to the PCR amplification

The inhibition of the PCR amplification, i.e., the biocompatibility of all the substances in the reaction tube to PCR is an important issue in the development of the stationary EC-qPCR. In the stationary EC-qPCR reactor, both the platinum wires and DNA intercalators were in contact with the PCR mixture; thus, a study of inhibition on PCR

was performed on the platinum wires and DNA intercalators. Platinum wires with 3 different radiuses (0.05 mm, 0.125 mm and 0.25 mm) and various numbers of platinum wires were immersed into the PCR mixture and the PCR amplification was carried out for 40 cycles in the thermal cycler. The platinum wires were immersed for approximately 2 mm in the solution. Both gel electrophoresis and microcapillary electrophoresis were used to characterize the PCR products. The same inhibition study was repeated with two different DNA intercalators, methylene blue (MB) and iron 1, 10-phenanthroline ($\text{Fe}(\text{phen})_3^{2+}$) at various concentrations.

5.2.5 Agarose gel electrophoresis

The PCR amplified fragments were confirmed by fractionating the fragments on an agarose slab gel. Agarose based gel matrix was prepared by heating a solution of 3.0 g of agarose gel (SeaKem LE, USA) in 100 mL of 0.5X Tris-borate-EDTA (TBE) buffer (10X TBE contains 0.89 M Tris-base, 0.89 M Boric Acid, 20 mM EDTA (disodium), pH 8.3) for 3 minutes. Following this, 0.5 $\mu\text{g/mL}$ of EB (Bio-Rad, Hercules, USA) was added for the DNA visualization. In order to validate the length of the amplified PCR products, 500 base pair DNA ladder (New England Biolabs, Inc) was loaded into the agarose based gel matrix as a reference marker. The PCR product samples were loaded into the wells of the gel matrix after mixing it with 1X blue/orange loading dye (Promega Corporation, Madison, USA) (6X loading dye contained 15% Ficoll 400, 0.03% bromophenol blue, 0.03% xylene cyanole FF, 0.4% orange G, 10 mM Tris-HCl (pH 7.5) and 50 mM EDTA. The electrophoresis of the amplified products was carried out in a 3.0 % (w/v) agarose gels in a contour-clamped homogeneous electric field DRII system (Bio-Rad) with 1X TBE running buffer (pH 8.3). After the samples were loaded, the agarose gel matrix was submerged in 0.5X TBE running buffer solution and a 100 Volt CD voltage was applied at the ends of the submerged agarose gel matrix for 40 min. After this step, the gel matrix was taken out from the bath and the DNA bands in the gel matrix were visualized under UV transillumination (Vilber Lourmat Co., France) at 321 nm wavelength, and the images were photographed subsequently.

5.2.6 Microcapillary electrophoresis

The yields from the PCR products were analyzed by a DNA chip 500 (Caliper Lifesciences) and a Bioanalyzer 2100 (Agilent Technologies). The Agilent 2100 Bioanalyzer is a microfluidics-based platform for the analysis of DNA, RNA, proteins and cells based on the microcapillaries electrophoresis technology.

5.2.7 Real-time PCR fluorescence detection machine

A verification experiments for the EC-qPCR results were carried out in a commercial qPCR machine, real-time PCR Rotor-Gene 3000 fluorescence detection machine (Corbett Research, Australia).

5.3 Results and Discussions

5.3.1 PCR products analyzed by the gel-electrophoresis and the real-time PCR

The amplification was carried out in the PTC-100 thermocycler, MJ Research Inc. The parameters for the PCR were similar to those in Table 5-1. The results were checked by running a portion of the amplified PCR mixture (initial DNA concentration was 0.1 ng/ μ L) in agarose gel electrophoresis. A band representing the amplified DNA was visible around 100 bp (the SARS DNA template is at 77 base pairs) after an EB staining, as shown in Figure 5-10. The band could only be seen from PCR cycle 22 to cycle 40.

In addition, several recipes for the PCR mixture, each having different initial concentration of DNA template, were prepared. The PCR tests were carried out with the developed PCR thermocycler with three different PCR mixtures, and their results from the agarose gel taken after electrophoresis were as shown in Figure 5-11. The agarose gel results were obtained from the end point of the reaction. A typical endpoint detection is very time consuming, and its results are based on the size discrimination, which may not be very precise. Figure 5-11 shows that the gel electrophoresis is unable to resolve this variability in the PCR yield from the different initial DNA concentrations.

Figure 5-10: The gel electrophoresis image shows the amplification results of 77 bp SARS DNA collected at various cycles during the amplification. L: 1kb DNA marker; line 1: the negative control without SARS DNA template; line 2 to 22: the PCR cycle numbers of 5, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 37, 38, 39, 40, respectively. The band can only be seen after cycle 22.

Figure 5-11: The gel electrophoresis image shows 4 different concentrations of SARS DNA template in the PCR amplification. The lanes respectively represent for: 1kb molecular weight marker (L); negative control without DNA template (lane 1-2); PCR with DNA template concentrations of 0.1 ng/ μ L (lane 3-4); 0.01 ng/ μ L (lane 5-6); 0.001 ng/ μ L (lane 7-8); and 0.0001 ng/ μ L (lane 9).

The amplification of a similar batch of PCR mixture was carried out in the real-time PCR Rotor-Gene 3000 fluorescence detection machine. The parameters for the PCR run were the same to those in Table 5-1. Four different initial concentrations of the SARS DNA templates, 1 ng/ μ L, 0.1 ng/ μ L, 0.01 ng/ μ L, 0.001 ng/ μ L were used for the PCR amplification. The real-time PCR chemistries allow for the detection of PCR amplification products during the early phases of the reaction, also known as the exponential phase. In the exponential phase, the amount of material is doubling for each cycle, which is the optimal region for data analysis. As the PCR reagents are consumed, the reactions begin to slow down or reach a plateau, at which the traditional PCR quantification is done, i.e. end-point analysis. If measurements are taken at the plateau phase for the real-time PCR, the data will not truly represent the initial amounts of starting target material. This is due to the reason that the PCR products can no longer be doubled at each cycle. The quantification of DNA is based on the number of cycles required to reach threshold intensity, C_T . The C_T values obtained from the Rotor-Gene PCR machine for the 4 different starting SARS DNA concentrations were summarized in Table 5-2. Data from Table 5-2 clearly indicated that the greater the amount of starting DNA was, the sooner the threshold value would be reached. For example, the C_T value for the initial DNA concentration of 0.1 ng/ μ L was 10.9-11.2, but the C_T value for the concentration of 0.0001 ng/ μ L was 21.6-22.4. While slab gels electrophoresis may not be able to resolve this variability in the PCR yield (Figure 5-11, lane 8-9), the real-time PCR Rotor-Gene 3000 fluorescence detection machine is sensitive enough to detect these changes.

Table 5-2: The C_T values for 4 different concentrations of DNA template obtained from the real-time PCR Rotor-Gene 3000 fluorescence detection machine.

DNA Concentration (ng/ μ L)	Threshold Cycle, C_T
1	10.9-11.2
0.1	14.3-14.5
0.01	17.9-18.9
0.001	21.9-22.4

5.3.2 Inhibition effect of different sizes of platinum metal electrodes on PCR

The PCR may be inhibited by a wide range of compounds derived from the biological samples used for PCR, such as animal tissues and bodily fluids, including peripheral blood cells, urine, cell smears, hair roots, semen, cerebrospinal fluid, biopsy material, amniotic fluid, placenta, bacteria, forensic and archaeological samples and plant tissues. Often the PCR is performed on a relatively crude DNA preparations that contain unidentified inhibitory substances, and the appropriate PCR controls are essential to eliminate the possibilities of inhibition [199-202].

In the stationary EC-qPCR reactor, since the platinum wires and DNA intercalators were in contact with the PCR mixture, a study of inhibition on the PCR was performed on the platinum wires with different radiuses and DNA intercalators with different concentrations. Here, the inhibition of the metal wires on the PCR was first investigated. The platinum wires used as the working, reference and counter electrodes for the stationary EC-qPCR detection were dipped into the PCR mixture during the entire PCR amplification and the simultaneous EC detection. Thus, it was important to investigate the inhibition effect of the different sizes of platinum metal electrodes on the PCR process. Three sizes of platinum wires were selected for the inhibition study: 0.1 mm, 0.25 mm and 0.5 mm in diameter. Platinum wires with an immersion depth of approximately 2 mm in length for the working electrode, reference electrode and counter electrode, respectively. The volume of the PCR mixture was 10 μ L.

Figure 5-12 shows the gel electrophoresis image for the amplification results of the different surface areas of the platinum metal electrodes dipping in the PCR mixture during the amplification process for 40 cycles. There was an inhibition from the platinum electrode of 0.5 mm in diameter (total exposed area for 3 platinum electrodes is 10.02 mm²). On the other hand, the platinum electrodes of 0.1 and 0.25 mm in diameter showed no evidence of inhibition to the PCR amplification. Therefore, platinum electrodes of 0.1 mm in diameter were selected for the stationary EC-qPCR study.

Figure 5-12: The gel electrophoresis image shows the amplification results of the 200 μ M methylene blue and different surface area of the metal electrodes towards PCR. Lane L: a 1 kb DNA ladder; Lane 1: a no-template control; Lane 2: a positive PCR control; Lane 3: 20 μ M MB in the PCR mixture; Lane 4: 20 μ M MB in PCR mixture with 3 platinum electrodes of 0.1 mm in diameter; Lane 5: 200 μ M MB in PCR mixture with 3 platinum electrodes of 0.25 mm in diameter; Lane 6: 200 μ M MB in PCR mixture with 3 platinum electrodes of 0.50 mm in diameter.

The effect of the inhibition of the metal exposed to the PCR mixture was investigated by using microcapillary electrophoresis to analyze the concentration of the final yield of the PCR. Different sizes of the platinum metal wires were immersed in the PCR mixture during the whole process of amplification. From the experiments, the graph of the amplification yield versus the platinum surface/volume was plotted as shown in Figure 5-13. The larger exposed area of the metal in the PCR mixture decreased the PCR yield. This is due to the inhibition of the metal towards PCR. The PCR yield decreased exponentially with the increase of the metal wires exposed area in the PCR mixture. In this work, the platinum wire of 0.1 mm in diameter was thus selected, with the wire length of 2 mm submerged in the PCR mixture, for use as the working electrode, reference electrode and counter electrode, respectively. From the data obtained in Table 5-3, the PCR yields analyzed by microcapillary electrophoresis from the PCR product without platinum wire submerged in the 10 μ L of the PCR mixture and the PCR product with the three platinum wires of 0.1 mm in diameter submerged in the 10 μ L of the PCR mixture were 19.850 ng/ μ L and 16.420 ng/ μ L, respectively. Thus the inhibition caused by the three platinum wires of 0.1 mm in diameter is calculated by dividing the PCR yield difference of these two cases to the

PCR yield without the existence of the platinum wires, which is 17% after calculation.

Table 5-3: Inhibition of the metal on PCR yield.

Wire radius (mm)	Submerged wire length (mm)	Volume of PCR mixture	Number of platinum wire in PCR mixture	Surface of metal expose in the PCR mixture (mm ²)	Surface (mm ²) / volume (μL)	PCR yield (ng/μL)
0	0	10	0	0.00	0.000	19.850
0.05	2	10	1	0.64	0.064	18.850
0.05	2	10	2	1.27	0.127	16.610
0.05	2	10	3	1.91	0.191	16.420
0.125	2	10	1	1.62	0.162	14.000
0.125	2	10	2	3.24	0.324	6.950
0.125	2	10	3	4.86	0.486	2.943
0.25	2	10	1	3.34	0.334	3.830
0.25	2	10	2	6.68	0.668	1.100
0.25	2	10	3	10.02	1.002	1.120

Figure 5-13: The effect of exposed metal area on PCR amplification yield. 3 platinum wires of 0.1 mm in diameter with the wire length of 2 mm submerged in the PCR mixture was selected as working, reference and counter electrode for the stationary EC-qPCR study as indicated by the arrow symbol (↓).

5.3.3 Inhibition effect of the DNA intercalators towards the PCR process

DNA-intercalators binding in solution can be traced by electrochemical methods. As a result of specific interaction of an indicator with dsDNA, decrease of its

electrochemical signal is continuously observed. $[\text{Fe}(\text{phen})_3]^{2+}$ and MB (2-200 μM) were chosen as DNA intercalators for the subsequent inhibition and electrochemical studies. As shown in Figure 5-14 and Figure 5-15, $[\text{Fe}(\text{phen})_3]^{2+}$ and MB were biocompatible to PCR and did not cause inhibition to PCR.

Figure 5-14: The gel electrophoresis image shows the amplification results of different concentrations of methylene blue towards PCR. Lane L: 1kb DNA ladder; Lane 1: negative control; Lane 2: PCR without MB; Lane 3: PCR with 2 μM MB; Lane 4: PCR with 10 μM MB; Lane 5: PCR with 20 μM MB; Lane 6: PCR with 50 μM MB; Lane 7: PCR with 100 μM MB; Lane 8: PCR with 150 μM MB; Lane 9: PCR with 200 μM MB; Lane 10: PCR with 500 μM MB.

Figure 5-15: The gel electrophoresis image of the PCR amplification results of the different concentrations of $[\text{Fe}(\text{phen})_3]^{2+}$. Lane L: 1kb DNA ladder; Lane 1: the non-template control, Lane 2: a positive control of PCR without $[\text{Fe}(\text{phen})_3]^{2+}$, Lane 3, 4, 5 and 6: PCR products with the $[\text{Fe}(\text{phen})_3]^{2+}$ concentrations of 500, 200, 100, and 10 μM , respectively.

However, $[\text{Fe}(\text{phen})_3]^{2+}$ (metal complexes) are not good indicators when compared to organic intercalating compounds because their main binding mechanism is based on electrostatic interaction. The DNA binding of iron (II) mixed-ligand complexes containing phen ligands was strongly influenced by the salt concentration in the buffer solution [203]. Secondly, the oxidation potential for the metal complexes at the higher potential (+0.80 V and above) was also considered to be unsuitable for this work [77]. Figure 5-14 shows that 500 μM of MB had an inhibition effect on PCR process as there were no bands on the electrophotogram (lane 10).

A study was carried out to investigate the effect of the inhibition of various concentrations of MB towards PCR mixture. The inhibition of MB was investigated by using microcapillary electrophoresis to analyze the concentration of the final yield of the PCR. The detail of the experimental procedures can be found in the section 5.2.4 and 5.2.6. Different concentrations of MB in the range of 0-500 μM were added into the PCR mixture during the whole process of amplification. From the experiments, the graph of the amplification yield versus different concentrations of MB was plotted as shown in Figure 5-16. The PCR yield decreased gradually with the increase of the MB concentration in the PCR mixture, due to the inhibition of high concentration of MB towards PCR. According to the data listed in Table 5-4, the PCR yields drop from 27.19 to 1.70 $\text{ng}/\mu\text{L}$ when the MB added into the mixture with concentration varying from 0 to 500 μM . The percentage of inhibition by MB towards PCR yield is shown in Table 5-4. This percentage is calculated for each MB concentration by firstly subtracting the PCR yield obtained in the presence of a certain concentration of MB from the PCR yield obtained in the absence of MB in PCR mixture, and this difference is then divided by the PCR yield obtained in the absence of MB. The PCR products yield in the absence of the MB was set to be 100%.

In short, the % inhibition can be obtained from the equation (5.1)

$$\%inhibition = \left(\frac{x-y}{x} \right) \times 100\% \quad (5.1)$$

where,

x is the PCR yield in the absence of MB, in this case, x is 27.19 ng/ μ L.

y is the PCR yield obtained in the presence of a certain concentration of MB.

According to this study, 10 μ M of MB caused only 5.2 % of PCR inhibition, so 10 μ M of MB was used for the following experiments.

Table 5-4: Inhibition of the MB on PCR yield.

MB concentration (μ M)	PCR yield (ng/ μ L)	Percentage of PCR inhibition by MB (% inhibition)
0	27.19	0.0
2	25.20	7.3
*10	25.77	5.2
20	26.07	4.1
50	25.33	6.8
100	16.58	39.0
150	11.33	58.3
200	3.82	85.9
500	1.70	93.7

* Concentration of MB used for the stationary EC-qPCR study.

Figure 5-16: The effect of MB concentration on PCR yield. 10 μ M of MB was selected for the stationary EC-qPCR study as indicated by the arrow symbol ($\downarrow$).

5.3.4 Electrochemical detection for quantification of initial DNA concentration

Figure 5-17 shows the SWV curves of 10 μM MB when (a) with and (b) without 10 $\text{ng}/\mu\text{L}$ DNA in the solution. MB was one of the small molecules that bind to DNA through intercalative mode.

Figure 5-17: The square wave voltammograms of 10 μM MB (a) in the absence and (b) in the presence of 10 $\text{ng}/\mu\text{L}$ DNA after 40 cyclers of the PCR amplification. The voltammetry was performed by increasing the potentials with the step potential = 0.15 mV, the pulse height = 50 mV, and the frequency = 100 Hz.

The anodic current of the MB in the absence (Figure 5-17 (a)) and the presence of the DNA (Figure 5-17 (b)) were 0.28 (I_0) and 0.11 μA (I_{pa}), respectively. In the presence of the DNA, the I_{pa} decreased by 60% compared to that in the absence of DNA. The peak current of the MB intercalator during each PCR cycle for the negative control is shown in Figure 5-18. The peak current had a negligible increase throughout the 40 PCR cycles for the PCR mixture without DNA template as a negative control.

Figure 5-18: The electrochemical detection of 10 μM MB in the negative control (PCR without template). Other experimental conditions were the same as in Figure 5-17.

After the EC detection, the PCR sample was further analysed by gel electrophoresis method. From Figure 5-19, the PCR product can be seen clearly at the Lane 4 after the EC detection. This shows that the electrochemical detection is a non-destructive detection method for the PCR amplification.

Figure 5-19: The gel electrophoresis image shows the amplification results of the PCR. Lane L: 1kb DNA ladder; Lane 1: the negative control of PCR; Lane 2: PCR without MB and electrodes; Lane 3: 10 μM MB PCR with 3 platinum electrodes with 0.1 mm diameter; Lane 4: 10 μM MB PCR with 3 platinum electrodes in the diameter of 0.1 mm and with the electrochemical measurement.

Figure 5-20 shows the EC current level from the EC detection of the amplified DNA during the PCR process. The current values was measured by SWV in the presence of $10\ \mu\text{M}$ MB as intercalator.

With the initial of $0.1\ \text{ng}/\mu\text{L}$ template DNA, the peak current decreased further when increasing the number of PCR cycle. The I_p value of the MB with amplified DNA for the PCR cycle gradually decreased from cycle 30th, and then formed a plateau at around 35 cycles. There was an obvious decrease in peak current at the 15th cycle in the EC method. The EC detection results implied that this method is feasible for the DNA quantification during the PCR cycles. Moreover, EC detection method is a relatively simple and inexpensive, and the consumables needed for the measurements are cheaper as compared to fluorescence dyes.

Figure 5-20: The EC current level (I) of the amplified DNA ($0.1\ \text{ng}/\mu\text{L}$ DNA template) as a function of the number of PCR cycles by using $10\ \mu\text{M}$ MB as the DNA intercalator. The EC current starts to decrease at the 15th cycle.

In the presence of DNA, the current is mainly due to free species, as the diffusion rate of the bound species is small. The reason for the decrease in the peak current was that the apparent diffusion coefficient and the apparent concentration of the electroactive

species decreased. Before the PCR, a large portion of the DNA intercalator remained in the PCR mixture solution and the diffusion of the DNA intercalator to the electrode surface remained constant. After the PCR started, the diffusion coefficient of the bounded DNA intercalator (DNA-intercalator complexes) became lower than that of the free DNA intercalator. As a result, the anodic current of the free DNA intercalator decreased significantly. When the aromatic ring intercalated into dsDNA (dsDNA was electrochemically inactive), the electrochemically active center of MB was enveloped by the bulky DNA molecule and was not available for redox activity [106]. The peak current decreased further with increasing concentration of DNA. Thus, at the end of the PCR cycle, the signal of the intercalator was the lowest compared to the signal the beginning of the cycle. This approach makes it possible to perform an *in-situ* DNA quantification during the PCR cycles. Additionally, this EC PCR amplicon detection method eliminates the time-consuming and labour-intensive probe immobilization step. The amplification produces increasing amounts of dsDNA, which bind to MB, resulting in a decrease in the current signal of MB. By plotting the decrease in current signal versus cycle number, the system produces amplification plots that provide a more complete picture of the PCR process than assaying product accumulation after a fixed number of cycles. The decrease in the peak current of MB caused by the increasing of the PCR products can be employed to quantify PCR.

The real-time PCR chemistries allow for the detection of PCR amplification products during the early phase of the reaction, also known as the exponential phase (section 5.1.3). The quantitation of DNA is based on the number of cycles required to reach threshold intensity, C_T . The C_T values for 3 different concentrations of the DNA template (0.01, 0.1 and 1.0 ng/ μ L) and a no-DNA template as a negative control for the EC detection are shown in Figure 5-21.

Figure 5-21: Measured EC detection results with different concentrations of DNA template (0.01, 0.1 and 1.0 ng/μL) with 10 μM of MB and a negative control. (◆): the negative control corresponds to the PCR sample without DNA template, (✱): 1.0 ng/μL of the initial DNA template, (▲): 0.1 ng/μL of the initial DNA template and (■): 0.01 ng/μL of the initial DNA template.

Figure 5-21 shows that the C_T values were 11 for 1.0 ng/μL DNA template, 17 for 0.1 ng/μL DNA template, 34 for 0.01 ng/μL DNA template and 34.6 for the no-template control. A lower C_T value corresponds to a greater amount of the starting DNA. A set of C_T values can be converted as a function of the known initial DNA concentrations, so called a standard curve of quantitative PCR as drawn in Figure 5-22.

Figure 5-22: Standard curve obtained by the correlation of the C_T values and the initial DNA template concentrations from the amplification plot of Figure 5-21.

The standard curve in Figure 5-22 shows the relationship between the threshold cycle versus the log of the initial DNA concentrations of the running series. By using PCR threshold method, the PCR amplification is described by [2-3]:

$$N_o = N_t(\varepsilon + 1)^{C_T} \quad (5.1)$$

where N_o is the initial concentration of target DNA, N_t is the number of amplicon DNA at threshold, ε is the amplification efficiency, and C_T is the threshold cycle. By taking the logarithm of Eq. (1), the relationship between threshold cycle, C_T versus the logarithm of DNA sample concentrations, N_o can be obtained as

$$C_T = \left(\frac{-1}{\log(1 + \varepsilon)} \right) \log N_o + \frac{\log N_t}{\log(1 + \varepsilon)} \quad (5.2)$$

Based on Eq. (2), a linear regression line can therefore be derived by plotting the graph of C_T against $\log N_o$. The resulting graph clearly represents a standard linear equation $y = mx + b$, which can be ascertained by having a minimum of 3 data points falling on a straight line in the experiment. It showed a linear relationship between C_T value and the logarithm of the initial DNA template concentrations. A line was fit to the log linear portion of each amplification curve with R^2 value of 0.9578 was obtained by using the slope of the best-fitted line in Figure 5-22. There many factors that affected the linearity of the standard curve obtained in this experiment including the amplification efficiency and the sensitivity of the electrochemistry method. From Figure 5-22, the C_T values decreased linearly with the increase of the input target quantity. This could be used for the quantitative measurement of the unknown DNA sample.

EC detection incorporated into PCR was compared with fluorescence detection method by using the real-time PCR Rotor-Gene 3000 fluorescence detection machine (Corbett Research). Table 5-5 shows the comparison of the C_T values obtained from the fluorescence detection of the real-time PCR Rotor-Gene 3000 fluorescence detection machine and the electrochemical detection in the stationary EC-qPCR reactor for the negative control, 1.0 ng/ μ L DNA template, 0.1 ng/ μ L DNA template, and 0.01 ng/ μ L DNA template.

Table 5-5: The comparison of C_T values obtained from the fluorescence detection method and the EC detection method, for the 0.01 ng/ μ L, 0.1 ng/ μ L and 1 ng/ μ L concentrations of DNA template respectively.

Concentration of DNA template (ng/ μ L)	C_T obtained from fluorescence method	C_T obtained from EC-qPCR method
0.01	19	34
0.1	14	17
1	11	11

The C_T values from Table 5-5 suggests that the EC method can be used for DNA quantification and be comparable with the fluorescence detection method for PCR products. The EC method can be a substitution for fluorescence detection for the PCR product monitoring, due to its advantages such as low cost, simple instrumentation and portability. It can also be incorporated into other DNA chip based detection platform. However, its sensitivity must be improved to detect lower concentrations of the PCR products.

5.4 Summary

Currently, the real-time PCR utilizes commercially available thermocyclers with fluorescence detection capability to amplify the specific nucleic acid sequences and to measure their concentrations simultaneously. In this Chapter, we proposed and developed a sensitive and cost effective DNA analysis method by using *in-situ* EC detection for real-time PCR instead of the currently available fluorescence detection method.

In this work, we demonstrated an electrochemical detection approach called the stationary EC-qPCR for monitoring the PCR products during the amplification. The underlying principle of the EC real-time PCR method developed in this work was the current measurement of MB in the PCR mixture during the amplification process at the extension phase. In the extension phase of the PCR cycle, the dsDNA was formed,

MB bound to the dsDNA, and the the bounded complex was electro-inactive. The MB signal decreased with an increasing amount of products produced in PCR thermal cycling. This approach makes it possible to perform *in-situ* DNA quantification during the PCR cycling. The most prominent feature of this method is the possibility to detect the PCR products signal electrochemically cycle-by-cycle, enabling the quantification of PCR products. The method presented in this work offered a significant potential to substitute the current fluorescence optical detection.

The stationary EC-qPCR reactor contained three platinum metal wires of 0.1 mm in diameter, functioning as the working electrode, the counter electrode and the reference electrode, respectively. The three electrodes were inserted through the top cover of an Eppendorf tube (0.2 mL) filled with test samples, and sealed with PDMS to prevent evaporation. The electrodes together with the Eppendorf tube were then placed in the 96 wells PCR thermal cycler (MJ, USA). The electrodes were connected to the electrochemical workstation for the EC measurements. The PCR amplification was carried out in the thermal cycler for 40 cycles with simultaneous electrochemical detection.

Because the electrodes were incorporated into the PCR mixture, an inhibition study was carried out to investigate whether the platinum metal electrode causes inhibition to PCR. First, we immersed the platinum metal electrodes with different surface areas per volume into the PCR mixture and conducted PCR in thermal cycler. The results from the thermal cycling were checked by running the amplified PCR products in the gel electrophoresis and microcapillary electrophoresis. The platinum electrode with a high surface area per volume caused lower PCR yield. Thus, the total surface area of $1.91 \text{ mm}^2/\mu\text{L}$ platinum metal wires immersed in the $20 \mu\text{L}$ of PCR mixture was used subsequently in this work, as it caused less than 17 % of inhibition, as calculated in section 5.3.2. Regardless of the binding methods, the DNA binding intercalator for the EC real-time detection of PCR must not cause significant inhibition to PCR. Therefore, different concentrations of MB were incorporated into the PCR mixture and the amplified results after 40 PCR cycle run were checked in 3% agarose gel

electrophoresis. It showed that the MB concentration below 500 μM did not cause significant inhibition towards PCR because the PCR product bands in 3 % gel electrophoresis were visible under the UV transmitter. The inhibition of MB was further investigated by using microcapillary electrophoresis to analyze the concentration of the final yield of the PCR. The PCR yield decreased gradually with the increase of the MB concentration in the PCR mixture, due to the inhibition of high concentration of MB towards PCR. 10 μM of MB caused only 5.2 % of PCR inhibition, so 10 μM of MB was used for in this study.

The phenomenon of a decrease in the peak current of 10 μM MB caused by increasing the DNA during the PCR cycles was employed to quantitatively monitor the PCR. The EC detection method was compared with real-time PCR using fluorescence method. The both results showed a similar trend in signal increase with PCR cycle number. With the results demonstrated in this Chapter, it was clear that EC detection for the *in-situ* PCR monitoring can be a potential alternative for the current established fluorescence detection method. However, optimization needs to be carried out to further increase the sensitivity of the EC detection in PCR, e.g. searching of an electrochemical active DNA intercalator that can be used in PCR with lower inhibition and with increased sensitivity and selectivity to dsDNA as compared to the MB.

The methodology presented in Chapter 5 on the stationary EC-qPCR can be used as a basic framework to develop a miniaturized PCR systems. A microfluidic flow-through EC-qPCR microdevice based on the above methodology will be demonstrated in Chapter 6.

Chapter 6

Microfluidic Flow-through PCR Microdevices with Concurrent Electrochemical Detection

6.1 Introduction

Nucleic acid analysis techniques have become a significant set of tools for many important applications, besides the frequent molecular diagnoses of diseases and assessments of therapies in clinics and hospitals; they are also broadly applied in environment surveillances, food developments, agricultural researches, and forensic identifications. However, the amount of DNA analytes in the test samples is usually not sufficient for direct detection. Hence, DNA amplification becomes an essential step to increase the amount of the target sequence. The most common and established method used for this purpose is the PCR method, which creates the copies of specific fragments of DNA by cycling through three temperature steps. Each temperature cycle doubles the DNA numbers, and 20-25 cycles will produce millions of DNA copies. However, the conventional PCR devices only can achieve a temperature rate of 4°C/s in the temperature range of 50-94°C, and need more than an hour to run a complete PCR reaction. Conventional systems also require relatively high reaction volume (>25 µL) that contributes to a slower thermal cycle. Thus, the whole process with pre-PCR processing and PCR detection will take several hours with the cost of several hundred dollars.

In recent years, many devices for the PCR applications have been fabricated to demonstrate the benefits of miniaturization. These devices, commonly referred as ‘PCR chips’, was first described by Northrup *et al.* [204] on a silicon-based stationary system. Since then, more researches on the microfluidics-based PCR chip had been investigated and many research groups began to develop the chip-based PCR devices with significant advances. All kinds of PCR microfluidic technologies facilitate DNA amplifications with much faster rates because of the smaller thermal capacity, the

larger heat transfer rate between the PCR sample and temperature control system on the chips, and the faster ramping rates of the heating components. Miniaturization of PCR devices lead to many improvements, including high throughput with reduced fabrication cost; decreased time for DNA amplification and measurements; avoidable cross-talk in the PCR reaction [32]; minimized consumption of biological samples and reagents; increased portability; and the prospect of lab-on-a-chip or μ TAS (micro total analysis system) which integrates all necessary steps necessary in a single chip [36, 40, 56, 205].

The reported chip-based PCR systems so far fall into two main categories. In the first configuration, the reaction mixture is stationary-enclosed in a reaction chamber that is typically micromachined in silicon or glass [37, 39, 51, 61, 206, 207]. A second configuration of the chip-based PCR was reported by Kopp and Manz [42]. This involves a continuous-flow amplification system in which the reaction mixture, instead of being stationary in a thermocycling chamber, is pumped continuously through the chip and flows repeatedly through the different temperature phases to replicate the PCR reaction. The benefit of this system is that the cycle time of DNA no longer depends on the time required for heating or cooling the solution and its surroundings. The system exhibits rapid heat transfer and thermal cycling of the minute fluidic element due to the high surface-to-volume ratio and the short linear thermal diffusion distance in the microfluidic channels. The continuous-flow system is a true microfluidic device that is more suitable for high-throughput analysis and is easy to couple with upstream and downstream flow-through devices. Since the pioneering prototype, this type of flow-through PCR microfluidics has undergone various improvements, all with different properties in terms of the structure and surface-to-volume ratio of microchannel [43-46, 208-211].

As can be seen from the developmental history of flow-through PCR microfluidics in section 2.3.1 of Chapter 2, another “bottleneck” in the realization of a truly integrated DNA analyzer is to meet the physical constraints required by a small-sized, portable detection module for the on-line PCR product detection. Detection in the microPCR

via fluorescence is a widely used technique [49, 51]. Although this technique is sensitive and widely used in microarray applications, it is not easy to be simplified and integrated onto a small chip, given the inherent nature of the process that requires an optical detection system, which has a considerable size and is difficult to be miniaturized onto a monolithic microanalytical system. Compare with the optical detection techniques, electrochemistry-based detection is an ideal technique for incorporation into the miniaturized analytical systems. The sensitivity and selectivity of the electrochemical detection are comparable to that of the laser-induced fluorescence detection. In fact, the advantages of the EC detection over the optical techniques include not only the inherent miniaturization and portability, but also the independence from the optical path length, sample turbidity, extremely low cost, low power requirements and compatibility with microfabrication technology.

Lee *et al.* [67] developed the first microfabricated silicon/glass hybrid PCR chip with functionalities of the PCR amplification and sequence-specific voltammetry EC detection. In their work, a silicon microchamber with a volume of 8 μL was sealed by a glass wafer and the electrode materials were patterned onto the glass substrate. The integrated chip as then measured by EC detection. Gold working electrodes within the PCR chamber were used to immobilize the DNA probes, and either electrochemical intercalators or silver-coated gold nanoparticles were used to detect the concentration of the PCR products. This EC detection method had the ability to obtain the sequence information of DNA, which was accomplished by utilizing the hybridization between a DNA probe immobilized on an electrode transducer surface and the target DNA segment. The system was capable of detecting down to ten molecules of the starting DNA template. Such a system is easy to be miniaturized because it avoided the need of confocal optics and laser excitation. The fabrication of electrodes is also low cost and can be accomplished on the plastic or glass substrates amenable for mass fabrications. However, this EC detection has its limitations, namely the detection occurs only at the end-point and the products from PCR amplification are asymmetric PCR products (single-stranded PCR products). These asymmetric PCR products will

hybridize with the immobilized probe on the working electrode surface if a certain region of the amplified sequence is complementary to the probe. In short, such a system will encounter difficulties in differentiating similar DNA sequences.

In order to overcome the shortages in the end-point detection method proposed by Lee *et al.*, we intended to develop an integrated microfluidic flow-through PCR microdevice with concurrent DNA amplification and EC detection to quantify the PCR products in real-time. We implemented the concept of the *in-situ* EC detection in Study 3 into such a micro-device, in which both the amplification of the target DNA sequence and the subsequent EC detection of the PCR product were realized simultaneously at every PCR cycle. In this study, microfabrication technology was utilized to fabricate an integrated microelectrode array. The EC detection method was used in the scheme instead of the existing fluorescence detection method. Both the amplification of the target DNA sequence and the subsequent EC detection of the PCR product were carried out on the integrated device for the real-time PCR products monitoring. An array of platinum microelectrodes fabricated on a glass slide was integrated directly into the microchannels made of polydimethylsiloxane (PDMS) to form the microfluidic flow-through EC-qPCR microdevice. The microdevice was then connected to the EC workstation for an *in-situ* measurement during the PCR amplification in the microfluidic flow-through PCR channel. The underlying principle of the microfluidic flow-through EC-qPCR was based on the changes of current signal of methylene blue (MB) as an electrochemically active species DNA intercalator in the PCR mixture during the extension phase of the DNA amplification process. The most prominent feature of this strategy is the capability of detecting the PCR products signal electrochemically cycle-by-cycle to realize the PCR products quantification. The details of the detection scheme of this microfluidic flow-through EC-qPCR microdevice is to be discussed in the following methodology section.

6.2 Methodology

6.2.1 Approach of the EC detection in microfluidic flow-through EC-qPCR microdevice

Microfluidic flow-through EC-qPCR was a microdevice we developed that performed PCR amplification and detection with the moving segments of PCR mixture flow-through 3 different temperature zones. The overall scheme of the microfluidic flow-through EC-qPCR microdevice was shown in Figure 6-1. The DNA was amplified after the PCR mixture passes through the 3 temperature zones. The microfluidic channel loops crossed 3 different temperature zones with a micro-electrode array embedded underneath the microchannel at the extension phase of the selected PCR cycles. The position of each 3-electrode detection station corresponded to a specific cycle number of PCR. The DNA intercalator (MB) was incorporated in the PCR mixture to generate an electrochemical signal that was proportional to the concentration of the PCR products during a PCR process. The EC measurement was taken when the flow front of the PCR mixture segment in the microchannel fully submerged a particular 3-electrode EC detection station. The SWV measurement method of the EC technique was used to measure the peak current of the DNA intercalator (MB) at the detection station, and the measurement system was subsequently switched to the next EC detection station for measuring the corresponding peak current of the MB when the flow front of the PCR mixture segment submerged the next detection station. This EC measurement process continued until all the detection stations were used sequentially for the EC PCR measurement. In this way, the I_p versus PCR cycle numbers curve was plotted to obtain C_T , which was the number of the amplification cycle that the PCR products start to reach a particular threshold signal level. The microfluidic flow-through EC-qPCR microdevice measured the C_T of DNA template at an unknown concentration; the C_T value was then compared with the standard characterized curve to extrapolate the concentration of the initial DNA template. Because one scan of SWV only takes a few seconds, the microfluidic flow-through EC-qPCR microdevice can quantify the PCR products cycle-by-cycle in real time.

Figure 6-1: Scheme of the microfluidic flow-through EC-qPCR microdevice with the 11 of 3-electrode EC detection stations.

The details of the methodology of microfluidic flow-through EC-qPCR microdevice are described as follows:

(a) Microfabrication of the device

Microfabrication technology was utilized to fabricate the microfluidic flow-through EC-qPCR microdevice. The details of the microfabrication processes will be explained in Section 6.2.3. Basically, an platinum microelectrode array was fabricated on a bottom glass chip by lift-off, and integrated directly with microchannel made of poly(dimethylsiloxane) (PDMS).

(b) Flow injection

The microfluidic outlet was connected to a syringe pump to suck the solution to flow-through the microdevice. The suction from the outlet provided a smooth flow and

avoided uneven injection force, which might cause the leakage of the device. In the experiments, different flow rate was optimized to obtain the highest amplification yield.

(c) Electrochemical active DNA intercalator

The chemistry behind the microfluidic flow-through EC-qPCR microdevice method used in this work was based on the current measurement of MB, which was utilized as electrochemical active species DNA intercalator in the PCR mixture during the amplification process at the extension phase. The current measurement of MB changed corresponding to the amount of PCR products, as was explained in Chapter 5, Section 5.2.1.

(d) 3-electrode EC detection stations and subsequent measurements

There were 11 detection stations (3-electrode was present in each station) in the microfluidic flow-through EC-qPCR microdevice. As shown in Figure 6-1, from the inlet, the PCR solution flowed continuously from 1st detection station to the 11th detection station. Each detection station comprised of 3 platinum electrodes respectively served as the working electrode (WE), counter electrode (CE) and reference electrode (RE) for the EC detection. Thus, 33 individually addressable platinum electrodes were arranged on the chip and located in the extension phase for EC measurements where the dsDNA formed during the PCR cycle.

When the PCR experienced a temperature cycle of 95°C for denaturation, 60°C for annealing and 72°C for extension, it was regarded as one PCR cycle. According to our design (Figure 6-1), the 1st to 11th detection stations corresponded to their respective PCR cycle numbers as summarized in Table 6-1.

Table 6-1: The number of the detection stations corresponding to the PCR cycle numbers.

Detection station number	1	2	3	4	5	6	7	8	9	10	11
PCR cycle number	5	8	10	12	14	16	18	20	22	24	26

The microfluidic flow-through EC-qPCR microdevice was connecting to an EC workstation (Model 660, CH Instruments Inc, USA) for EC detections. All 11 contact pads, each from one 3-electrode detection station, were soldered by wires to one PCB board. On the PCB board, each detection station was connected to one single row test pins, which had 3 pins corresponding to the 3 electrodes of the detection station. Since the electrochemical workstation can only capture a single channel input, the measurements of the EC signal were conducted by manually switching the connection of each detection station to the EC workstation, in the sequence from number 1 to number 11, and each measurement was taken and saved accordingly. The measurement for each detection station was taken when the PCR solution was flowing through and just fully submerged the 3 corresponding electrodes area, as shown in Figure 6-2 (c). The potential-current of the EC scan was taken as the PCR reaction signal. The SWV measurement of the PCR was regarded as a fast real-time monitoring, because it only takes a few seconds to scan each detection station.

Figure 6-2: Photographed continuous-flow solution in one of the detection stations. (a) the PDMS channel without flow, (b) the flow approaching the detection station, (c) the flow exactly fully covers the 3-electrode detection station. The EC measurement should be taken when the flow starts to cover the full surface area of the 3 electrodes, as shown in (c).

(e) Extrapolation of microfluidic flow-through EC-qPCR

The scanned potential signal of the EC was collected and depicted in Figure 6-3 (a-b). The most important signal that indicated the PCR reaction was the peak current, I_p , which was obtained by deducting the peak current from its base line. Figure 6-3 (a) shows the scanned potential signal of EC and I_p obtained from the first detection station, and other subsequent detection stations. The 11 detection stations represented their respective PCR cycles (Table 6-1). The curve in Figure 6-3 (b) was obtained as I_p versus the PCR cycle numbers. The entire experiment was repeated for the different concentrations of initial DNA template.

Figure 6-3: The scanned potential signals. Figure 6-3 shows (a) the scanned potential signals of the EC detection collected sequentially from the different detection stations. (b) I_p signals of the 11 detection stations versus the PCR cycle numbers.

The I_p curve versus the PCR cycle numbers measured in real-time RT-PCR response reflects the initial amounts of target DNA is shown in Figure 5-9 (a). The difference in the initial amounts of template DNA was quantified by comparing C_T values.

A plot of C_T versus the $\log_{10}$ of the initial concentrations for a set of standard DNA template gives a straight line as shown in Figure 5-9 (b). This standard curve can be used as a reference standard for extrapolating the quantitative information for targets of unknown concentrations. This reflects the difference in their initial amounts of DNA template, and this method can be used in diagnostics purposes.

6.2.2 Design of the microfluidic flow-through EC-qPCR microdevice

This section describes the details of the AutoCAD drawing when both the

microchannel layer and electrode glass slide were combined. Figure 6-4 shows the design principles of the microfluidic flow-through EC-qPCR.

Figure 6-4: Integration of the platinum microelectrode arrays into the flow-through PCR. The total length of the PDMS microchannel is 322 mm, which is equivalent to a flow volume of 161 μL.

PCR required three temperature phases, in the developed chip ‘the extension phase’ was arranged in the middle of the chip with the ‘denaturation’ and ‘annealing’ phases on two sides. The overall dimension of the microfluidic flow-through EC-qPCR chip was 55 mm × 83 mm. The reaction chamber of the microfluidic flow-through EC-qPCR microdevice was made of PDMS and bonded to a glass slide. It was equipped with external heaters and sensors for temperature control. The PDMS channel with a meandering design guides the reaction mixture through the three temperature phases. The PDMS channel with approximately 3.2 m long (250 μm wide and 200 μm deep) folded microchannel was bonded on a thin (0.5 mm) glass substrate with oxygen

plasma technique. The chip was designed with channel loops for 26 amplification cycles of PCR including prolonging the initial denaturation and the final elongation steps. A round shape of channel loops supported not only the flow-through PCR, but also the homogeneous flow behaviour of a serial flow regime in a two-phase system with low sensitivity against disturbance such as gas bubbles.

The design of the PDMS-glass hybrid microfluidic flow-through EC-qPCR microdevice was shown in Figure 6-5. The chip had 2 layers, i.e. the top layer contained the PDMS microchannel and the bottom layer contained the electrode on glass layer (Figure 6-5). They were designed and fabricated separately, and the 2 mm thick PDMS replica and the 0.5 mm thick glass substrate were bonded to form the channels at the last step of the fabrication processes. Such a hybrid design omitted the expensive glass micro-fabrication process but still utilized the rigidity, flatness and higher heat conductivity of the glass ($\sim 1\text{W/m}\cdot\text{K}$ of glass V.S. $0.18\text{W/m}\cdot\text{K}$ of PDMS [32]) when heating and cooling the underneath elements for the thermal cyclings.

Figure 6-5: Illustration of the top and bottom layer of the microfluidic flow-through EC-qPCR chip being fabricated.

a. PDMS microchannel layer

Sylgard 184 Polydimethylsiloxane (PDMS) (Dow Corning, USA) was selected as the material for the fabrication of PCR microchannel in this work. PDMS is a widely used polymer material for the prototype devices and commercial devices in BioMEMS [212, 213] applications. The use of PDMS elastomer for the miniaturized bioassays has numerous advantages over silicon and glass. PDMS is an inexpensive and flexible material, structured PDMS surfaces are obtained quickly and transparent over the entire visible spectrum of light, and it can be sealed to virtually any flat material to form the channels. It is also compatible with biological studies because it is impermeable to water, non-toxic to coolers, and permeable to gases. The chemical structure of PDMS is shown as below:

Figure 6-6: Chemical structure of PDMS.

Figure 6-7 shows the design of the micro channel chip that was fabricated on the master mould (a 4-inch silicon wafer). The PCR mixture was pumped through the channel loops for an equivalent of 26 amplification cycles. The serpentine channel was 250 μm wide and 200 μm deep with a total length of 322 cm, which was equivalent to 161 μL of the reaction volume. The design was in such a way that the extension region had a longer channel length. This was to ensure that the DNA was amplified at that particular cycle. The length was designed in a ratio of 1:2:1 (denaturation: extension: annealing). Two circular chambers were designed for the input and output of the PCR solution. The experiment was performed under the following conditions: Heater temperature was set at 95°C for denaturing, 65°C for annealing and 72°C for elongation. The flow rate of the PCR mixture was 1 $\mu\text{L}/\text{min}$. Total 26 cycles of the PCR take about 40 min to complete.

Figure 6-7: Scheme of the microfluidic channel with an inlet and an outlet. It loops through the three temperature phases (A, B, C) of the microfluidic flow-through PCR chip. The microfluidic flow-through PCR microchannel is 250 μm wide $\times$ 200 μm deep $\times$ 3.2 m long. The overall chip size is 55 mm wide $\times$ 83 mm long.

b. Microelectrode array on glass slide

Microelectrodes were designed as shown in Figure 6-8, they were located in the extension phase for the EC measurement.

Figure 6-8: AutoCAD design of the microelectrodes on the glass slide.

Each set of the EC detection station comprised three platinum electrodes served as the working electrode (WE) [195], the counter (CE) and the pseudo-reference electrode (RE) [193, 195, 214, 215], respectively. The dimensions of the working area of the WE and RE in the 250 μm wide PDMS channels were $100 \times 250 \mu\text{m}^2$, and the CE electrode was $300 \times 250 \mu\text{m}^2$.

Typically, not all the PCR cycles were measured in our design. The platinum 3-electrode arrays were integrated in the PCR channel at the cycle number 5, 8, 10, 12, 14, 16, 18, 20, 22, 24 and 26. The chip was not designed to measure each PCR cycle lines in a few reasons. First, it is due to the chip's size and space limitation; secondly, it would be time consuming to finish all measurements if each cycle was measured. At the same time, PCR was expected not to change much from one cycle to the next cycle. For cost effective and demonstration purpose, thus, it was only measured at certain cycles in our microfluidic flow-through EC-qPCR microdevice. Commonly used working electrode materials for the EC detection include platinum, gold, mercury, and glassy carbon. However, platinum is a good material for a working electrode because it is a good thermal and electrical conductor, solderable, electrochemically inert, has a wide potential window, and has low stress when deposited, allowing relatively thick layers to be made. In addition, platinum metal layer is easy to deposit. These properties make platinum often used as an electrode in biochemical microsystems [214-216]. In this study, microfabrication technology was utilized to fabricate an integrated microelectrode array. Each microelectrode was connected to an external potentiostat by an insulated wire.

6.2.3 Device fabrication

In the present study for the microfluidic flow-through EC-qPCR microdevice the fabrication process adopted as follows:

- a. Bottom layer - electrode fabrication
- b. Top layer - microchannel fabrication

a. Bottom layer – electrode fabrication

The microelectrode chip was fabricated with three main processes: photolithography, sputtering and lift off process. Glass substrate Corning 7740 (55 mm × 83 mm) was used as a platform for the fabrication of the electrodes. Each detection station comprised three platinum electrodes served as the working electrodes, the counter and the pseudo-reference electrodes, respectively. Platinum electrode array was fabricated using photolithographic and lift-off techniques on the Corning 7740 glasses.

Lift-off process is a preferred metallization technique for depositing hard-to-remove metals such as platinum and tungsten. First, a layer of the photoresist was spin coated on the wafer and structured with the appropriate pattern by lithography. Then the desired metal or metal sandwich was deposited. Finally, when the photoresist was stripped, the metal that was applied to the photoresist-covered areas would be removed together with the photoresist, and the desired metal pattern was left over. Normally the photoresist coating should be thicker than the metal layer. During the stripping of the photoresist, the wafer was often ultrasonicated to facilitate the removal of the metal flakes and to obtain more efficient stirring. Lift-off produces well-defined, almost vertical sidewalls, which could not be obtained by wet etching of the metal. The final electrode patterns in the microfluidic flow-through EC-qPCR microdevice were formed via the lift-off process. The details of the sputtering and lift off process in the fabrication of the electrode arrays are described in the Figure 6-9.

Figure 6-9: Process flow diagram for fabricating the microelectrode arrays.

1. Cleaning of the substrate

The glass slides (Corning 7740) were cleaned in piranha solution (a mixture of 7:3 of H_2SO_4 and H_2O_2) for 30 min to remove all of the organic contaminants. The glass slides were then rinsed with distilled water and dried with nitrogen gas. Finally, the glass slides were placed on a 100°C hotplate. Hexamethyldisilazane (HMDS) gas as a bonding agent was drizzling onto the glass slides.

2. Spinning and soft bake of the photoresist

A layer of AZ7220 photoresist was spin-coated on the glass substrate by a spin coater (model P6700 series, Speciality Coating System Inc.) to achieve a desired thickness of $2\mu\text{m}$. The spin condition for the photoresist is listed in Table 6-2.

Table 6-2: Spin conditions for the AZ7220 photoresist.

Resist	AZ7220
Thickness (μm)	2
Spin speed (rpm)	3000
Time (s)	60

After the resist was applied to the substrate, it must be soft-baked to evaporate the solvent. The soft bake condition for 2 μm photoresist AZ7220 was 1 min at 100°C.

3. *Exposure under UV*

The resist was exposed in a UV mask aligner (OAI J500-IR/VIS) with an intensity of 10mW/cm² at the wavelength of 365 nm. The exposure time was 10 s. Following the exposure, a post exposure bake (PEB) must be performed to selectively cross-link the exposed portions of the film. The baking condition was 1 min at 100°C.

4. *Development of the photoresist*

The exposed AZ7220 resist photoresist was developed by soaking in the AZ[®] 300 MIF positive photoresist developer for 2 min, leaving the unexposed resist on the glass substrate. It was then rinsed with the distilled water for at least 1 min.

5. *Sputtering of the Ti/Pt metal layer*

A Magnetron sputter system (DC/RF) (National Scientific, Singapore) with both DC and RF sputtering heads, was used to deposit a 10 nm adhesion layer of titanium via a RF sputtering (pressure = 10 mTorr; power = 300W; t = 0.5 min) followed by a DC sputtering of 300 nm of platinum (pressure = 10 mTorr; power = 300W; t = 13 min) over the entire glass slides.

6. *Dissolution of the photoresist in acetone and lift off of the metal layer*

After the sputtering, the glass slides were soaked in acetone with ultrasonic vibration

conditions to accelerate/facilitate the lift-off process. This caused the underlying positive photoresist layer to dissolve faster. The Ti/Pt electrode remained only in the exposed recessed patterns on the glass slides. The fabricated electrode array on the glass slide is shown in Figure 6-10.

Figure 6-10: The fabricated platinum microelectrode arrays.

b. Top layer – microchannel fabrication

The microfabrication of the microfluidic flow-through EC-qPCR microdevice involved several steps. The PDMS microchannel was fabricated using a soft lithographic technique. Photolithography is the process of transferring geometric shapes on a mask to the surface of a material coated with photoresist. The steps involved in the photolithographic process are wafer cleaning; barrier layer formation; photoresist application; soft baking; mask alignment; exposure and development; and hard baking. In this section, PDMS microchannel was fabricated by first making a master mould by photolithography and deep RIE on a 4-inch silicon (Si) wafer, then the master mould was pressed onto the PDMS mixture to produce the PDMS PCR chip. A process flow diagram of the fabrication procedure of the top layer is illustrated in Figure 6-11.

Figure 6-11: The process flow diagram of the PDMS mould for the microfluidic EC-qPCR microdevice.

1. Cleaning of the silicon wafer

The silicon wafer (type P/Boron <100> with thickness of 455-575 μm) was cleaned in piranha solution (a mixture of 7:3 of H_2SO_4 and H_2O_2) for 30 min to remove all of the organic contaminants. The silicon wafer was then rinsed with distilled water and dried with nitrogen gas.

2. *Spinning and soft bake of the photoresist*

The Silicon wafer was then placed on the hotplate and a bonding agent known as hexamethydisilazane (HMDS) gas was drizzled onto the wafer before further coating of the photoresist. A layer of AZ9260 photoresist (AZ electronic materials) with the thickness of about 9 μm was spin-coated on the silicon wafer. A uniform coating was desired. The spin condition for the photoresist is listed in Table 6-3:

Table 6-3: *Spin conditions for the AZ9260 photoresist.*

Resist	AZ9260
Thickness (μm)	8
Spin speed (rpm)	1500
Time (s)	30

After the resist was applied to the substrate, it must be soft-baked to evaporate the solvent and densify the photoresist film. The soft bake condition for an 8 μm AZ4620 photoresist film was 4 min at 110°C.

3. *Exposure under UV*

The resist was exposed in a UV aligner (OAI J500-IR) with an intensity of 10mW/cm² at the wavelength of 365 nm. The exposure time was 40 s. Following the exposure, a post exposure bake must be performed to selectively cross-link the exposed portions of the film. The baking condition was 1 min at 100°C.

4. *Development of the photoresist*

The development process is to selectively remove the photoresist after the exposure. The photoresist was developed in AZ421K positive photoresist developer (AZ electronic materials) for 3 min and followed by a hard bake on a hot plate at 110°C for 15 min.

5. *Deep RIE*

STS Deep Reactive Ion-Etching (DRIE) from Surface Technology Systems (STS, UK), was used in fabricating the master mould for PDMS replication. DRIE is capable of achieving an aspect ratio of 30:1 and a silicon-etching rate of 2 to 3 $\mu\text{m}/\text{min}$. In this technique, the etched pit was coated with a passivation layer of polymer, followed by an ion bombardment. Ions were directed against the bottom of the pit, it etched the bottom polymer and a thin layer of underlying substrate, while left the sidewalls intact. This process was repeated numerous times until an etched pit of desired depth results. The wafer was etched to a depth of 200 μm at a rate of 1.8 $\mu\text{m}/\text{min}$ with 1 hour and 50 min. The etched wafer (master mould) was then soaked with acetone to remove the remaining photoresist.

6. *Replication of PDMS Layer*

The detailed fabrication steps for the PDMS microstructure were as follows:

- a. Cleaning of the master mould - A master mould structure was prepared and cleaned as much as possible. If the master mould was glass or silicon, the piranha solution was the preferred method of cleaning.
- b. Surface modification - The mould surface was modified to be hydrophobic by the following procedure: If the surface was not hydrophobic, it would not be possible to remove the PDMS from the mould. Dichlorodimethylsilane and toluene were mixed in the ratio ~3% of silane in toluene. Mould was dipped for at least 3 hours, or preferably overnight before being used. Finally, the mould was rinsed with ultrapure water and blown dry with nitrogen.
- c. Preparation and de-gassing of PDMS - Dow Corning PDMS Sylgard 184 was prepared according to the manufacturer's directions, by mixing 1 part of the catalyst with 10 parts of the base resin. The two solutions were mixed mechanically with a clean glass rod. The mixture was later left to settle until all the bubbles disappeared visually. The PDMS mixture was poured and left on the mould until all the bubbles disappeared.

d. Curing by heat - PDMS pre-polymer was poured onto the master mould to a thickness of a few millimetres and the dish was placed on a 60°C hot plate for curing. The device was covered by Petri dish to prevent any dust from entering the device.

7. *Release of the PDMS layer*

After 3 hours, the PDMS layer was removed mechanically from the mould by tweezers. The film should be kept on the clean flat glass surface. Figure 6-12 shows the top view of the flow-through PDMS microfluidic channel for 26 PCR cycles.

Figure 6-12: The top view of the PDMS microfluidic channel layer for 26 PCR-cycles.

8. *Plasma treatment of the PDMS bonding surface and the bonding of the treated PDMS layer to the electrode plate*

In order to permanently bond the microelectrodes glass slide to the PDMS microfluidic channel, the two substrates were treated with oxygen plasma with an RF power of 100 W, a pressure of 20 Pa, and a treatment time of 30 s. After the O₂ plasma treatment, the PDMS-glass hybrid biochip was pressed together as shown in Figure 6-13.

Figure 6-13: The photography of the microfluidic flow-through EC-qPCR microdevice.

The surface properties of PDMS before and after O₂ plasma treatment were found to be different. O₂ plasma surface treatment, which was required for PDMS bonding, changed the surface characteristic of PDMS from hydrophobic to hydrophilic. When water was dropped onto the surface, a difference in the droplet size was observed. The droplets on the PDMS surface after O₂ plasma treatment were much bigger than those before O₂ plasma treatment were due to the differences in hydrophobicity. The contact angles were measured by the Krüss Contact Angle Measuring System G10. The contact angles for the PDMS surface before (Figure 6-14(a)) and after (Figure 6-14(b)) O₂ treatment are 112° and 19°, respectively.

Figure 6-14: The contact angles over (a) a hydrophobic surface of PDMS (before oxygen plasma) (b) a hydrophilic surface of PDMS (after oxygen plasma treatment).

After bonding, the two reservoirs work as the inlet and outlet were drilled at the end of the channel. The dummy capillaries were pushed through the PDMS chips to ensure a through hole. Otherwise, thin sheets of PDMS might block the holes. For PDMS connection, pipette tip or glue cannot be used because the pressure by the tip or tube tightening will likely squeeze and deform the shape of the fluidic channel. This deformation may change the shape of the reservoir, resulting in the leakage. Fortunately, since PDMS is flexible, it can seal the tube by itself when the tube is inserted into the reservoir if the tube diameter and the reservoir size are almost identical. A good sealing is achievable when the tube is solid, such as stainless tubes. This is the easiest method to drive fluid in the PDMS microfluidic channel. This method is very simple and *yet* allows device or tubing change frequently. There is a strong possibility that the stainless tube also being tilted accidentally and deforming the reservoir shape, leading to the leakage. In order to avoid this, the only simple method is to keep the stainless tube in the vertical position. One way to support the tube stronger is to prepare a very thick PDMS such that the tube and PDMS has a larger contact area. The silicone tube is then connected to the biocompatible stainless connector as depicted in Figure 6-15.

Figure 6-15: The connection of the microfluidic flow-through EC-qPCR microdevice to the silicone tube.

6.2.4 Instrumentation and system set-up

The followings are brief descriptions of the components integrated in the microfluidic flow-through EC-qPCR microdevice. The *in-situ* quantification of the PCR products with the microfluidic flow-through EC-qPCR microdevice required the following equipment: Konics PID digital temperature controller, AC power supply, solid state relay to turn the heating element on and off, a single row PCB test pins that were soldered on the circuit board (15 cm × 6 cm) for connecting the 11 set of the electrode arrays from the platinum contact pad of the microdevice to electrochemical workstation. A 3-way plug works as the connector to link the terminal to and from the circuit board to the electrochemical workstation.

The system set-up for the microfluidic flow-through EC-qPCR microdevice experiments was shown in Figure 6-16. The microfluidic flow-through EC-qPCR microdevice was placed on the thermoelectric cooler (TEC) module heaters with the glass bottom in direct contact with the cooper thin plate on the TEC surface. This was to establish three different temperature zones. Because there was only thermal resistance of the 0.5 mm thick glass, the temperature difference between the liquid in the chamber and the TEC surface was minimized. Heat sink was applied between the cooper thin plate and the microfluidic flow-through EC-qPCR microdevice to

evaluate their effects on the temperature distribution and transient time. The temperature in each phase was detected by a thermocouple and controlled by a PID temperature controller (Konics, Korea) with a closed-loop system. Syringe pump (NE-1000, New Era Pump Systems Incorporated) was used to transport the fluid through the chip. Electrochemical measurements with the SWV and CV techniques for an *in-situ* real-time quantitative PCR detection were performed by direct measurements made on the platinum electrodes array, by using the electrochemical workstation.

Figure 6-16: The system setup for the microfluidic flow-through EC-qPCR microdevice experiments.

a. Heat source – TEC

The presence of a heat source is necessary for the thermal cyclings above room temperature. The heat sources must comply with certain constraints to realize a fast thermal cyclers for the PCR-chips. Since one of the intended advantages of PCR-chips is the purported low thermal mass, heat sources should not add excessive thermal

mass to the system in order to achieve a fast temperature transition. Although there exist some commercial alternatives (e.g. the use of small resistor mats), at present the main heat sources being used in passive PCR-chips research are Thermoelectric cooler (TEC) [41, 217, 218]. TEC exhibits dual operating regimes, as it can rapidly heat up or cool down its surfaces depending on the polarity of the applied voltage, and cools faster than the passive-cooled integrated thin-film systems. A typical single stage TEC consists of two ceramic plates with p- and n-type semiconductor material (bismuth telluride) sandwiched inside the plates. The elements of the semiconductor material are connected electrically in series and thermally in parallel. When a positive DC voltage is applied to the n-type thermoelement, electrons are transported from the p- to the n-type thermoelement and the cold side temperature will decrease as the heat is absorbed. The heat absorption (cooling) is proportional to the intensity of the current and the number of thermoelectric couples. This heat is quickly transferred to the hot side of the cooler, where it is dissipated into the heat sink and the surrounding ambient. Major specifications of the TEC (Melcor Corporation) are listed in Table 6-4.

Table 6-4: The specifications of TEC, Melcor Corporation.

Dimension (cm):	$6 \times 0.2 \times 0.9$ (L $\times$ H $\times$ W)
Maximum power (W):	14.7
Maximum current (A):	3.9
Maximum DC voltage (V):	6.57
Module resistance (Ω):	1.6
Maximum operating temperature ($^{\circ}\text{C}$):	110

b. Temperature control setup and calibrations for temperature measurement.

Three PID temperature controllers (*KONICS* controller series KC-24) (Figure 6-17) were used to adjust the power applied to the TEC so as to control the temperatures on the top surface of the TEC. Major specifications of the PID temperature controller

were listed in Table 6-5.

Table 6-5: The specifications of PID temperature controller, KONICS controller series KC-24.

Calibration accuracy and sensor conformity:	$\pm 0.3\%$ of span
Temperature Input Range:	-200 to 400°C
Sample Rates:	PID control with 4hz.
T/C (T type) resolution:	0.1°C

Figure 6-17: The temperature controllers and solid-state relays for the three operating temperatures of the PCR cycles.

c. Temperature sensor –thermocouples (type-T)

Three PCR temperatures were monitored by three identical thermocouples (type-T) with a small measurement tip in the diameter of 0.5 mm during the calibration process. The calibration was performed for 30 minutes each at the temperatures of 65°C, 72°C and 95°C, respectively. The thermocouples, based on the Seebeck effect, are typically more stable and offer better precision than thermistors. They are best suited for TEC surface temperature measurements with faster reaction times. The thermocouples

were used for accurate sensing of the temperature in the custom fast thermocycle, since they provide enough accuracy ($\pm 0.1^\circ\text{C}$) for PCR. Because of the high sensitivity of the thermocouples, we had to ensure the thermocouples have a full contact with TEC surface to avoid inaccurate temperature sensing. Figure 6-18 shows the recommended way of securing the thermocouple on the device. Thermal gel (Anabond Limited), a very good heat conductor gel, was used to ensure that the whole thermocouple was fully covered and the correct temperature was taken. Thermal gel was also applied between the microfluidic flow-through EC-qPCR microdevice and the TEC for good thermal transfer.

Figure 6-18: The thermocouple is attached onto the heater with an intermediate layer of the thermal gel.

a. Electrochemical workstation

An *in-situ* electrochemical measurement of the PCR products with the SWV and CV techniques were performed by using the electrochemical workstation (model 660, CH Instruments, Inc, USA). SWV is a well-established electrochemical technique with a small-amplitude voltage waveform that has higher sensitivity than those of the CV and LSV [196]. The total of 11 detection stations in the microfluidic flow-through EC-qPCR microdevice, as shown in Figure 6-10 were used, where each station containing one working electrode, one counter electrode and one reference electrode,

respectively. Each microelectrode was connected to an external potentiostat by an insulated wire. The details of the EC detection procedure are explained in Section 6.2.8.

6.2.5 PCR sample preparation

A 489 bp of the Lambda DNA was PCR amplified in the microfluidic flow-through EC-qPCR microdevice. Reaction mixture for PCR was prepared prior to the flow-through PCR operation. The sequences of the forward and reverse primers were 5'-CAG CTG GCT GAC ATT TTC GG -3' (20mer) and 5'- TAT CAG TTC ATC CAG CGC GG -3' (20 mer). The PCR master mixture consisted of 18 μ L of ultra-pure water, 12.5 μ L of BSA (20 mg/mL), 5 μ L of 25 mM $MgCl_2$, 1 μ L of 10 mM dNTP, 5 μ L of 20 mM the forward and reverse primers each, 5 μ L of 10 $\times$ PCR buffer solution, 2 μ L of lambda DNA template (0.1-20 ng/ μ L) as the PCR template, and 2.5 μ L of 5 unit/ μ L *Taq* DNA polymerase (Introvigen, Sg). 1 $\times$ SYBR Green I (Cambrex Part No. 50513, Cambrex Biosciences, USA) was added for fluorescence real-time PCR experiments. The same PCR mixture was used for PCR amplification on the chip except 20 μ M of MB was added as electroactive DNA intercalators for electrochemical real-time PCR experiments instead of 1 $\times$ SYBR Green I, which is used for conventional fluorescent detection. The sequence of the Lambda DNA template (489 base pair) is as follows: ca gctggctgac atttcggtg cgagtatccg taccattcag aactggcagg aacaggggaat gcccgttctg cgaggcggtg gcaagggtaa tgaggtgctt tatgactctg ccgccgtcat aaaatggtat gccgaaaggg atgctgaaat tgagaacgaa aagctgcgcc gggaggttga agaactgcgg caggccagcg aggcagatct ccagccagga actattgagt acgaacgcca tcgacttacg cgtgcgcagg cgcacgaca ggaactgaag aatgccagag actccgctga agtggtggaa accgcattct gtactttcgt gctgtcgcgg atcgcaggtg aaattgccag tattctcgac gggctcccc tgctggtgca gcggcgttt ccggaactgg aaaaccgaca tgttgatttc ctgaaacggg atatcatcaa agccatgaac aaagcagccg cgctggatga actgata.

The temperature zones of the flow-through PCR device were set at 95°C for denaturation, 60°C for annealing and 72°C for elongation. The flow rate for the temperature cycling was controlled by the syringe pump. Approximately 40 min was

needed for total 26 cycles. To compare and validate the PCR on the microfabricated flow-through EC-qPCR microdevice, the lambda DNA was firstly PCR amplified on a commercial real-time PCR machine by fluorescence detection (real-time PCR Rotor-Gene 3000 fluorescence machine, Corbett Research, Australia) with the following thermocycling profile: an initial denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 1 min, then annealing at 60°C for 1 min and extension at 72°C for 2 min. The desired PCR product was confirmed by agarose gel electrophoresis.

6.2.6 Slab gel electrophoresis

Although real-time PCR was conducted in our experiments and the fluorescence detection and EC detection were done for every PCR run, gel electrophoresis was still necessary to confirm the correct size of PCR product and the formation of primer dimers. The PCR amplified fragments were confirmed by fractionating the DNA fragments on an agarose slab gel. The agarose based gel matrix was prepared by heating a solution of 1.5g of agarose gel (SeaKem LE, USA) in 100 mL of 0.5X Tris-borate-EDTA (TBE) buffer (10X TBE contains 0.89 M Tris-base, 0.89 M Boric Acid, 20 mM EDTA (disodium), pH 8.3) for 3 min. Following this, 0.5 µg/mL of EB (Bio-Rad, Hercules, USA) was added for DNA band visualization. In order to validate the length of the amplified PCR products, a 500 base pair DNA ladder (New England Biolabs, Inc) was loaded into agarose based gel matrix as a reference marker. The PCR products were loaded into the wells of the gel matrix after mixing PCR products with 1X blue/orange loading dye (6X loading dye contained 15% Ficoll 400, 0.03% bromophenol blue, 0.03% xylene cyanole FF, 0.4% orange G, 10 mM pH 7.5 Tris-HCl and 50 mM EDTA; Promega Corporation, Madison, USA). The electrophoresis of the amplified products was carried out in 1.5% (wt/vol) agarose gel in a contour-clamped homogeneous electric field DRII system (Bio-Rad) with 1X TBE running buffer (pH 8.3). After the samples were loaded, the agarose gel matrix was submerged in 0.5X TBE running buffer solution and a 100 Volt DC electrical field was applied at the ends of the submerged agarose gel for 40 min. After this step, the gel matrix was taken

from the bath, and the DNA bands in the gel were visualized under UV transillumination (Vilber Lourmat) at 321 nm and the images were subsequently photographed.

6.2.7 Bioanalyzer 2100

The yields of the amplified PCR products from both the microfabricated flow-through EC-qPCR microdevice and the commercial real-time PCR machine (Rotorgene, Corbett Research, Australia) were compared, validated and analyzed by a DNA chip 500 (Caliper Lifesciences) and a Bioanalyzer 2100 (Agilent Technologies). The Agilent 2100 bioanalyzer is a microfluidics-based platform for the analysis of DNA, RNA, proteins and coolers based on the microcapillaries electrophoresis technology. It needs only 1 μL of sample volume for the analysis. Thus, the PCR products from the flow-through EC-qPCR was analysed and quantified with Agilent 2100 bioanalyzer.

6.2.8 *In-situ* electrochemical detection procedures

For each experiment, 100 μL PCR mixture was prepared (please refer to Section 6.2.5 for details), and 200 μM MB was added into the PCR mixture in the ratio of 1:10 to obtain a final concentration of 20 μM . The master mixture was subjected to the reaction chamber of the flow-through EC-qPCR chip. The microfluidic flow-through EC-qPCR microdevice was connected to an EC workstation (model 660, CH Instruments, Inc, USA). SWV current signal were measured at the cycle numbers of 5, 8, 10, 12, 14, 16, 18, 20, 22, 24 and 26, respectively. The SWV pulse has the amplitude of 50 mV, the pulse width of 25 ms, the scan rate of 50 mV/s, and the scan potential of 0.00 V $\sim$ -0.50 V. The measurement of the EC signal was conducted by manually switching the connection of each detection station to the EC workstation, in the sequence from number 1 to number 11, each measurement was taken and saved accordingly. The measurement for each detection station was taken when the solution was flowing through and just fully covered the 3 corresponding electrodes area.

6.3 Results and Discussion

6.3.1 Temperature control of the microfluidic flow-through EC-qPCR microdevice

The denaturation temperature, usually around 90°C to 96°C, is the temperature at which the single-stranded DNAs are released as the templates in the next round of DNA synthesis. The annealing temperature, usually around 40°C to 60°C, is the temperature at which the primers attach to templates. The extension temperature, usually around 70°C to 75°C, is the temperature at which the DNA synthesis occurs. Optimum temperatures or temperature ranges for each stage of the PCR reaction depend on factors such as the particular DNA or RNA being replicated, the number of cytosine-guanine hydrogen bonds versus the number of adenosine-thymine bonds, the enzyme species being used, and the length of the primer. Precision control of these optimum temperatures affects PCR reaction in numerous ways. Insufficient heating during denaturing is a common reason for a PCR reaction failure. Similarly, overheating of the sample during denaturing can result in excessive denaturation of the polymerase or other enzymes being used [28]. During the PCR cycle, the annealing temperature is the most important as it affects the specificity of the PCR reaction. DNA-DNA hybridization is a temperature dependent phenomenon. If the temperature is too high, no hybridization takes place; instead primers and templates remain dissociated, which results in a decreased yield of the desired product. However, if the temperature is too low, mismatched hybrids will occur, which are the ones not having all the correct base pairs. If these mismatches are tolerated, the number of potential hybridization sites for each primer is greatly increased, and the amplification is more likely to occur at non-target sites on template molecule, thus results in non-specific DNA fragments being amplified. Ideal annealing temperature must be low enough to enable hybridization between the primer and the template but high enough to prevent the mismatched hybrids from forming. Attaining the optimum temperature for the extension reaction is also important for obtaining the desired PCR results. Temperature may affect both the rate and accuracy of the extension reaction. If the rate of polymerase reaction is too slow, newly synthesized polynucleotide may not

contain a site for primer annealing. Additionally, the denatured polynucleotide sequence for amplification may contain one or more regions of secondary structure that may form or disappear according to the selected temperature. Several different enzymes with polymerase activity may be used. Each enzyme will have its own optimum temperature for the activity, stability, and accuracy of the polymerase reaction [10, 28]. Therefore, the determination of optimum temperatures is important in order to guarantee the success of PCR. In addition, any unnecessary temperature transition should be avoided. This inability to perform rapid transitions between temperatures causes inaccurate PCR results. A lag time between the denaturation and the annealing can result in unspecific annealing. Long transition time between the denaturation and the annealing promotes the premature inactivation of the polymerase enzyme, resulting in less specific reaction and lower product yield [10].

The system for our microfluidic EC-qPCR microdevice consisted of several constant and uniform temperature phases, and a looped long capillary was designed to carry PCR reagents on each temperature phases, instead of using the heat blocks and reaction tubes. The temperature control system consisted of a PID temperature controller, some temperature sensors and three heating modules. The microfluidic EC-qPCR microdevice had an external temperature sensors and heaters for accurate thermal cycling of the reaction chamber for the PCR. In order to achieve and control the three heating temperatures, we used three thermoelectric cooler (TEC) module heaters, which had a faster thermal response than other kinds. In order to monitor the temperature of the TEC surface, an external thermocouple sensor was connected to the temperature controller. The TEC was bonded together with wires and parallel connections. The precision of the temperature control was initially investigated. A copper thin plate ($100 \times 10 \times 1 \text{ mm}^3$) with high thermal conductivity was cut to fit the TEC dimensions and allowed an efficient and homogenous distribution of temperature across its surface (Figure 6-19). The copper thin plate was bonded to the TEC hot surface with the thermal gel (Anabond Limited, USA) in order to evaluate their effects on temperature distribution. Thermocouples were attached by the thermal gel

to each copper plate for the temperature monitoring.

Figure 6-19: The cooper thin film is attached to the TEC to allow an efficient and homogenous distribution of temperature across the surface.

Figure 6-20 shows the temperature distribution at the TEC surface for a set of temperatures at 60°C, 72°C and 95°C, as measured with an external thermocouple sensor. The results indicated a high precision in the temperature control. Temperature distribution on the surface of 1 mm copper thin plate was greatly enhanced when compared to the temperature distribution on the bare TEC surface. The maximum temperature gradients observed across the plate were less than 2.0°C, which was well below the tolerable range of conventional PCR thermocyclers.

Zone I (95 °C)

94.7	94.7	95.3	93.5	94.7	94.3	95.6	94.7	94.9	94.8	95.3	94.2	95.3	94.9	95.3
95.5	95.2	94.8	94.9	95.1	95.0	94.8	95.1	94.8	95.1	94.8	94.8	94.9	94.4	95.0

Zone II (72 °C)

71.9	72.1	71.9	72.4	72.2	72.0	72.3	72.1	72.1	71.8	71.9	71.8	72.0	72.0	71.8
72.1	72.1	72.0	72.1	72.0	72.1	72.0	71.9	71.9	72.0	72.1	71.8	72.1	72.0	71.9

Zone III (60 °C)

59.8	60.0	60.1	59.9	60.2	59.8	60.2	59.9	60.1	59.8	60.0	59.5	60.2	59.7	60.1
60.0	60.0	59.9	60.0	59.9	60.2	59.9	60.1	59.6	60.1	60.0	59.8	59.9	60.1	60.1

Figure 6-20: The temperature distribution on a 1 mm-thick cooper plate surface as measured with the external thermocouple sensor.

Figure 6-21: Correlation between the set temperature (T_{set}) and the glass slide surface temperature (T_{glass}).

Temperature control was one of the most critical factors in PCR. In order to perform the PCR successfully, the temperature should be maintained at a constant level in each temperature phase. In order to measure the temperature of the glass over the PCR mixture flows, a thermocouple was attached between the glass and the block. The

surface temperature (T_{glass}) of the glass slide was measured and compared with the preset temperature (T_{set}). The two temperatures (the T_{set} was shown on the LED display of the controller, and the T_{glass} as measured by the thermocouple) were well correlated with an r^2 value of 0.99, as shown in Figure 6-21. To study the temperature distribution of the microfluidic flow-through EC-qPCR microdevice in the three temperature zones, temperatures were measured with the thermocouple sensors, along the microchannel as shown in Figure 6-22. The points 1-4 were located in zone I (95°C), the points 6-9 were in zone II (72°C) and the points 11-14 (60°C) were in zone III. Figure 6-23 summarizes the temperature distributions over these temperature zones. The glass temperature was measured by the type-T thermocouple and was compared to the present cut-off temperature. The temperatures set on the individual Konics KC-24 temperature controller were 95°C, 72°C, and 60°C, respectively. The response time of the cut-off temperatures was based on the intelligent PID control. The temperature was uniform in each zone. At each zone, the temperature was almost constant, and varied only across the zones. The temperature at zone I was $95 \pm 0.30^\circ\text{C}$ (average of points 1-4) and this value was slightly lower than that of the set temperature. The temperature at zone II (average of points 6-9) and zone III (average of points 11-14) were $74 \pm 1.29^\circ\text{C}$ and $59 \pm 0.86^\circ\text{C}$, respectively.

Figure 6-22: The schematic drawing of the temperature-measuring points along the PDMS microfluidic channel.

Figure 6-23: The temperature distribution along the PDMS microfluidic channel vs. the set temperature. The glass temperature (T_{glass}) was measured by the thermocouple and was compared with the required preset point (T_{set}). The glass slide surface temperature measured at the zone I, II and III were $95 \pm 0.3^\circ\text{C}$, $72 \pm 1.29^\circ\text{C}$ and $59 \pm 0.86^\circ\text{C}$, respectively, while the individual Konics KC-24 temperature controller preset temperature were 95°C , 72°C and 60°C , respectively.

The temperature fluctuation over time was also examined by monitoring the temperatures for 30 min each at 60°C , 72°C and 100°C , respectively. The results were as shown in Figure 6-24. It was clearly that the cooper plate provided a stable temperature over the monitoring durations. Thus, the temperature distribution on each of the films was within the temperature variation tolerance for PCR.

Figure 6-24: The temperature distribution on a 1 mm copper plate surface as measured with the external thermocouple sensor at 3 different temperatures over 30 minutes. The temperatures at zone I, II and III were 95 ± 1.5 °C, 72 ± 1.1 °C, and 59 ± 0.7 °C, respectively.

6.3.2 PCR performance

The performance of the PCR master mixture was verified by a real-time PCR Rotor-Gene 3000 fluorescence machine (Corbett Research, Australia). With the conventional thermocycler, 5 μ L of the PCR master mixture was executed for 40 cycles over 90 minutes. This time included initial denaturation step at 95°C for 2 min, final elongation step at 72°C for 1 min and actual cycling with 40 repetition steps. The repetition step consisted of 95°C for 1 min for denaturation followed by 60°C for 1 min for annealing and 72°C for 2 min for elongation.

For PCR in the microfluidic flow-through EC-qPCR microdevice, the 50 μ L PCR samples were loaded into the inlet of the chamber by a syringe. When the PCR is performed in the PDMS chip, the major problem is bubble formation. First, because of the porosity of PDMS [219, 220], bubbles are easily generated during the thermal cycles. Secondly, during the PCR cycles, air bubbles can be occasionally generated in the chamber over 90°C. Thirdly, these bubbles may be generated by the evaporation of PCR buffer or the incomplete sealing in the fluid inlet and outlet passages [221-224]. For this case, generated and growing bubbles acted as an insulation layer within the

chamber, causing poor temperature uniformity over the chip and large temperature difference in the PCR sample. During the thermal cyclings, the bubbles would grow bigger, which might prevent the circulation of the PCR buffer in the reaction chamber and inhibit PCR. There are a few ways to reduce bubble formations in the PCR chip. For example, the microfluidic substrate is siliconized just before the substrate is used [223]. Alternatively, prior to loading the PCR master mixture into the PCR chip, the PDMS microfluidic channel was flushed with ultrapure water for several times with a high flow rate of 25 mL/min for 15 min to remove all of the air bubbles present in the channel and also to keep the channel surface wet. A pipette (Gilson, France) was placed in the inlet of the channel. The sample was pushed slowly while the vacuum suction was applied gently at the outlet, in this way the sample was subsequently loaded into the chamber without causing any bubble. During the amplification, only one syringe driver was used to push at the inlet end and another driver may be used to suck at the outlet end of the channel at the same flow rate as that of the input driver. This prevents the expansion of air bubbles formed in a channel during a reaction that could disrupt the flow of fluid in the channel. A thorough degassing of the reagents prior to the injection is important in biochemical reactions such as those involve thermal cycling in which significant levels of gas are produced at high temperatures.

The surface absorption of protein (*Taq* DNA polymerase) is another major obstacle in the microchip PCR [53, 202]. The PDMS surface is highly hydrophobic and causes hydrophobic species like *Taq* DNA polymerase to absorb onto its walls [32, 211]. One obvious solution for overcoming the problem of polymerase absorption on the hydrophobic PDMS microfluidic walls is to increase *Taq* DNA polymerase concentration until it saturates the medium and supersedes the dynamic equilibrium of absorption processes [211]. Nevertheless, this simple option is somewhat restricted in PCR applications. On the other hand, *Taq* DNA polymerase is the most expensive reagent used in PCR and increasing its concentration in a fair amount is costly. Several research groups have developed other methods for surface treatment to prevent surface absorption of the enzyme. Another suggested method for decreasing

Taq DNA polymerase absorption on the chip walls is to use additives such as Bovine serum albumin (BSA) and polyethylene glycol (PEG). Hence, in this research work, to prevent protein absorption onto the intact hydrophobic PDMS surface (walls in the microchannel), the PDMS surface was first treated with BSA. BSA in the PDMS microchannels prevents protein absorption by competing with polymerase for absorption at the active sites of chip walls. BSA supports the stabilization of the annealing of the primers and the polymerase to the DNA template. BSA is a coating widely used to reduce protein absorption to the sidewalls of microchannels, especially in large surface-volume ratios [225]. We found that BSA was very effective in preventing surface denaturation of polymerase on the microchannel. No amplification result was observed when BSA coating was not used inside the microchannel. In addition, a simple flushing of BSA in the microchannel to coat channel sidewalls was inadequate. Subsequently, 1 mg/mL BSA solution was injected into the PDMS microchannel and kept to stay for 30 minutes. After flushing out the BSA solution, PCR master mixture was then loaded into the microfluidic flow-through EC-qPCR microdevice. A syringe pump was used to load the PCR master mixture into the microfluidic flow-through EC-qPCR microdevice.

Next, experiments were performed to check the polymerase adsorption to the PDMS microchannel of the microfluidic flow-through EC-qPCR microdevice. Results from gel electrophoresis reflected no amplification when the PCR mixture contains only 1 unit/ μ L of *Taq* DNA polymerase. This observation confirmed that the reaction failure was due to the excessive absorption of *Taq* DNA polymerase. Hence, the concentration of the polymerase was increased by 5 times. Properties of both the glass surface and the PDMS microfluidic channel were compatible with PCR. Evidence for PCR amplification products in the microfluidic flow-through EC-qPCR microdevice was confirmed by the gel electrophoresis as shown in Figure 6-25. Results in lane 3 of Figure 6-25 showed the successful amplification of the samples with positive control. The 489 bp PCR products were clearly observed and non-specific products were not found. Thus, the microfluidic flow-through EC-qPCR microdevice performed PCR

amplification successfully.

Figure 6-25: The gel electrophoresis image shows the amplification result of the 1 ng/ μ L initial template of lambda DNA (489 bp) in the flow-through chip-based PCR experiments (lane 3), a negative control with no-DNA template (lane 2), and the 1 kb DNA ladder (lane 1), respectively.

The influence of the flow rate on the PCR yield in the microfluidic channel on the amplification efficiency was analyzed by a DNA chip 500 (Caliper Lifesciences) and a Bioanalyzer 2100 (Agilent Technologies) in order to accelerate the entire PCR procedure while yielding a good PCR amplification. In Figure 6-26, the effect of different flow rates on the signal intensity was shown in the gel-like image.

For the chip, an optimal flow rate of 2 μ L/min was determined, which represented a total procedure time of about 40 min for 26 PCR cycles. Significant product amplification was obtained at a flow rate of 2 μ L/min, as shown in the lane 4 of Figure 6-26. The increase in the flow rate up to 20 μ L/min led to a decrease in the signal intensity. We found that the amplification process required a certain minimum time for its completion. The period of one PCR process depends on the synthesis rate of the polymerase enzyme and the diffusion time. Due to the small channel cross-section, the diffusion time is almost negligible; therefore, the flow rate can be adjusted according to the synthesis rate of the enzyme.

Figure 6-26: The gel-like image of 489 bp PCR products run on a DNA labchip 500 using Agilent Bioanalyzer 2100. Lane L is a DNA ladder that contains 15bp lower marker and 600bp upper marker. The yields of the amplification products on microfluidic EC-qPCR microdevice at the flow rates of 20 $\mu\text{L}/\text{min}$ (lane 1), 10 $\mu\text{L}/\text{min}$ (lane 2), 5 $\mu\text{L}/\text{min}$ (lane 3), and 2 $\mu\text{L}/\text{min}$ (lane 4) are demonstrated in the gel-like image, respectively.

Figure 6-27 shows the PCR product obtained from the commercial Rotor-Gene thermocycler machine and the microfluidic flow-through PCR microdevice. The yield of PCR product amount from the real-time PCR Rotor-Gene 3000 fluorescence machine was 15.50 ng/ μL and the yield from the microfluidic flow-through EC-qPCR microdevice was 9.40 ng/ μL . The no-template control (NTC) contained all the PCR components except DNA template. A signal in the NTC was due to the formation of the primer-dimer synthesized when approaching the end of this experiment. The performance of the microfluidic flow-through EC-qPCR microdevice was less effective compared to the real-time PCR Rotor-Gene 3000 fluorescence machine. Many factors that contributed to this phenomenon, i.e. the PCR products accumulated on the PDMS channel wall, or the temperatures were not well optimized. However, the system can be further optimized to increase its efficiency of the DNA amplification. Nevertheless, the PCR yield of 9.40 ng/ μL was obtained within 90 min in the microfluidic flow-through EC-qPCR microdevice.

Figure 6-27: The yields of the amplified products from both a commercial real-time PCR machine (Rotor-Gene 3000, Corbett Research) and the microfluidic flow-through EC-qPCR microdevice. Lane 1 and 2 are positive control and negative control (without DNA template) using the commercial real-time PCR machine. Lane 3 and 4 are negative control and positive control using the microfluidic flow-through EC-qPCR microdevice.

Several problems such as bubble formation, sample evaporation, protein absorption and PCR reagents leakage are critical in the micro-PCR. Leakage on the chip could results in contamination of the sample and subsequently the inhibition of PCR. The risks of contamination would be reduced greatly in a well-sealed PCR microchip. In this case, PDMS channel must be sealed tightly to the glass substrate though the O₂ plasma surface treatment. The leakage test can be carried out by distilled water to fill the whole channel and see if there is any droplet occurred at the edges of the PDMS channel.

6.3.3 Characterization of the microelectrodes and the indicator

With the development of a truly portable PCR based DNA analysis system, the bulky optical detection system should be substituted by a simpler electrochemical detection system. Instead of using a single electrochemical sensor on the PCR chip, we fabricated an array of 11 EC detection stations along the PDMS microfluidic channel, each comprised one WE, one CE and one RE, respectively. Based on the electrochemical detection results of the PCR product by the *in-situ* EC detection approach in Chapter 5; we implemented the same detection methodology in this chapter and developed the microfluidic flow-through EC-qPCR microdevice. Platinum is a good choice for the electrode array material as it can easily be patterned onto the glass substrate by a lift-off process, and the adhesion of the platinum on the glass slide is strong enough to resist the denaturation temperature of the PCR. Electrodes array integrated in the microfluidic flow-through EC-qPCR microdevice is shown in Figure 6-28.

Figure 6-28: The microelectrodes arrays integrated in a microfluidic flow-through EC-qPCR microdevice.

Throughout this study, 11 detection stations were used. At the beginning, it was necessary to ensure that the active surface areas of all electrodes are similar. This was

significant in a practical point of view that micro-fabricated electrodes with identical electrode surface areas must have reproducible surface area coverage. The surface area coverage is defined as the surface of the electrode that makes the measurement in order to get the same surface measurement.

Figure 6-29 shows the simultaneous plot of the cyclic voltammograms in 0.1 M of PBS buffer (pH7.0) with the 11 platinum electrodes. The peak currents of the 11 curves were nearly indistinguishable with a relative standard deviation of 3.24%, indicating that the active surface areas of all 11 electrodes were approximately the same.

Figure 6-29: The cyclic voltammograms of 0.1 M PBS buffer of the 11 platinum microelectrodes, being scanned at the rate of 50 mV s^{-1} .

The properties of glass surface, PDMS microfluidic channel and microelectrode arrays are compatible with PCR. Evidence for PCR amplification in the microdevice was once again confirmed with gel electrophoresis as shown in Figure 6-30. The result from Figure 6-30 showed that the microelectrode array was compatible with

PCR mixture, thus no inhibition event was observed.

Figure 6-30: The gel electrophoresis image shows the amplification result of lambda DNA in chip-based PCR.

MB used as DNA intercalator, featured reversible oxidation with a peak potential of -250 mV at the platinum electrode (Figure 6-31). It could be positioned in the reaction mixture during thermal cycling without inhibiting the amplification. It could also withstand the extreme temperatures and maintain the labelling capability. Therefore, MB was chosen as the DNA intercalator for PCR reaction.

Figure 6-31: The peak current (I_p) of 20 μM methylene blue in the PCR solution obtained from platinum patterned working electrode. Under the conditions: Pulse amplitude = 50 mV, pulse width = 25 ms, scan rate = 50 mV s^{-1} .

6.3.4 *In-situ* PCR products detection using both electrochemical and fluorescence detection

One major disadvantage of end-point PCR measurements is that it cannot provide information about the initial amounts of target molecules that present in the samples; they can only distinguish between a positive and a negative sample. The capability to identify the presence of PCR products by our microfluidic flow-through EC-qPCR microdevice with the *in-situ* EC detection method was demonstrated in Figure 6-32.

Figure 6-32: Electrochemical detection results in the microfluidic flow-through EC-qPCR microdevice when using MB as intercalator. The (♦) is the EC detection result of the PCR products without DNA template, defined as no-template control (NTC). The (■) is the EC detection result of the PCR products with 2 ng/μL initial DNA template.

When the quantities of PCR product increased from cycle to cycle, the concentration ratio of DNA to MB in the solution increased, hence the concentration of free MB in the solution became lower. The peak currents of the MB in the presence of DNA decreased significantly; because the diffusion coefficient of bound MB was much lower than that of free MB. The C_T value for 2 ng/μL of the DNA template was around 15 cycles. The NTC contained all the PCR components except the DNA template. Although there should be no signal decrease in the negative control PCR mixture, a slight decrease of signal could also be detected under negative control. The

amplification in NTC was due to the formation of the primer-dimer, which had a C_T around 23 cycles. That was because some primer-dimer would be synthesized when approaching the end of this experiment.

To compare and validate the detection of the PCR products on the microfluidic flow-through EC-qPCR, a similar batch of the lambda DNA was PCR amplified on a commercial real-time PCR Rotor-Gene 3000 fluorescence machine (Corbett Research). Results from the real-time PCR Rotor-Gene 3000 fluorescence machine are shown in Figure 6-33.

Figure 6-33: The fluorescence intensity results of the PCR products without DNA template as a negative control and with 2 ng/ μ L initial DNA template. The fluorescence intensity started to increase at the 10th cycle.

From Figure 6-33, we observed that fluorescence profiles showed an increase in fluorescence intensity at the 10th cycle. However, for the electrochemical detection, profiles decreased in current changes began at cycle 15th, with the final concentration of 2 ng/ μ L template DNA. This means EC detection had a similar trend to the increase of the PCR products during the amplification. During the initial phase, although the amplification already occurred, the change in EC was below the

detection limit. After a certain number of cycles, the decrease in the EC current was detectable and exponential amplification was observed. Finally, the signal reached a plateau as reagents were fully consumed. We had developed the microfluidic flow-through EC-qPCR that had successfully detected PCR products at the same time during amplification. The results showed that the EC approach developed in this thesis is workable, although the detection limit is higher than detection limit achieved by the commercial PCR Roteo-Gene instrument. However, the others advantages of microfluidic flow-through EC-qPCR microdevices can compensate the shortage of its detection limit.

The quantification of the initial Lambda DNA concentration was also performed by the microfluidic flow-through EC-qPCR microdevice. To evaluate the chip sensitivity to DNA template concentrations, three recipes for the PCR mixture, each had different DNA concentrations, were prepared. PCR tests were carried out by loading the three samples with different concentrations and one negative control sample into the microfluidic flow-through EC-qPCR system, and controlled the samples under the same temperature profile. The MB at the concentration of 20 μM as an intercalate dye was used in the PCR assay. In Figure 6-34, the (■) curve represents the PCR sample with 0.2 ng/ μL DNA template final concentration; the (▲) curve represents the PCR sample with 2 ng/ μL DNA template final concentration; the (×) curve represents the PCR sample with 20 ng/ μL DNA template final concentration; the (◆) curve represents the PCR sample without DNA template final concentration. The PCR mixture of lower DNA concentration exhibited a decrease in current signal at the later cycles. It would be seen that a current signal started to decrease at approximately the 11th cycle in the case of 20 ng/ μL initial template DNA, the 15th cycle for the case of 2 ng/ μL and the 18th cycle for the case of 0.2 ng/ μL . A larger amount of the initial template DNA corresponded to an earlier onset of the exponential phase, or an earlier detectable decrease in the current signal. Since 0.2 ng/ μL of the target DNA were enough to give a readable signal, this concentration was determined as our limit-of-detection.

Figure 6-34: The electrochemical detection of PCR products by the microfluidic flow-through EC-qPCR microdevice with 20 μ M methylene blue and 3 different DNA template concentrations of 0.2 ng/ μ L (■), 2 ng/ μ L (▲) and 20 ng/ μ L (×), respectively. Amplification of 10-fold serially diluted lambda DNA template from 0.2 ng/ μ L to 2 ng/ μ L (positive control). The (◆): the PCR sample without DNA template as no-template control (NTC).

Figure 6-35 shows the real-time PCR conducted using SYBR[®] Green I with 3 different Lambda DNA templates concentrations of 0.2 ng/ μ L, 2 ng/ μ L and 20 ng/ μ L, respectively. According to the data in Figure 6-35, the threshold cycles of the positive control was comparable to that of the conventional real-time PCR Rotor-Gene 3000 fluorescence machine.

Figure 6-35: The fluorescence detection of real-time PCR Rotor-Gene 3000 fluorescence machine was conducted by incorporating SYBR® Green I into 3 different concentrations of 0.2 ng/μL, 2 ng/μL and 20 ng/μL Lambda DNA templates respectively. The no-DNA template control contained all the PCR components except DNA template as a negative control sample.

The cycle number at which the PCR efficiency is maximized is defined as C_T in this work. A set of C_T values can be converted as a function of the known initial DNA concentrations, so called a standard curve of quantitative PCR as drawn in Figure 6-36. Figure 6-36 showed a plot of the threshold cycle versus the initial DNA concentrations of the running series. It showed a linear relationship between C_T value and the logarithm of the initial DNA template concentrations. The C_T values for both the electrochemical detection (Figure 6-36 (a)) and the fluorescence detection (Figure 6-36 (b)) system decreased linearly with the increase of the input target quantity. This could be used for the quantitative measurement of the initial DNA target. R^2 values of 0.99 and 1.00 were obtained by using the slope of the best-fitted line. Hence, a disposable, inexpensive microfluidic flow-through EC-qPCR microdevice for the nucleic acid amplification and integrated detection was constructed and tested. The

PCR amplification was performed successfully, and the EC detection system was sensitive to the DNA template concentrations and proved to be an applicable alternative of the optical detection.

Figure 6-36: The quantitative analysis plots of both the EC detection using the microfluidic flow-through EC-qPCR microdevice and the fluorescence detection using real-time PCR Rotor-Gene 3000 fluorescence machine for 489 bp Lambda DNA. The analysis data were plotted as threshold cycle versus logarithmic initial DNA concentrations, based on the data of Figure 6-34 and Figure 6-35. (a) Standard curve for the electrochemical detection on the microfluidic flow-through EC-qPCR microdevice, and (b) Standard curve for the fluorescence detection by real-time PCR Rotor-Gene 3000 fluorescence machine.

The reproducibility of the EC measurements repeated on a single microfluidic flow-through EC-qPCR microdevice is shown in Figure 6-37. The average standard derivation for successive 5 runs was 1.61. This proved that the microfluidic flow-through EC-qPCR microdevice could be used at least 5 times for amplifying and detecting PCR products of a 2 ng/ μL initial DNA template subsequently.

Figure 6-37: The graph shows the reproducibility of the microelectrode arrays in the same microfluidic flow-through EC-qPCR microdevice ($n = 5$). The curve indicates average value of the current for 5 runs on the same device. An initial DNA template of, 2 ng/ μ L was used in these measurements.

6.4 Summary

Recently quantitative PCR (qPCR) has been developed into a popular technique in molecular biology. In qPCR, quantitation of the DNA is based on the number of cycles that is required to reach threshold intensity, and the cycle number is defined as C_T . The greater the amount of initial DNA, the sooner this threshold value will be reached. A calibration curve plotted as the logarithm of DNA template concentrations versus C_T can be obtained by using a series of standards. An unknown sample can be quantified with qPCR method and the specific C_T for this unknown sample is compared to the calibration curve to quantify the amount of DNA in this sample. Therefore, C_T values are reflective of the quantity of target DNA in any sample. This methodology yields much more precise quantitative results than end-point analysis.

Because a rapid detection of the nucleic acids was extremely valuable in particular clinical contexts to substitute traditional time-consuming laboratory assays. In this Chapter, we developed an integrated microfluidic flow-through EC-quantitative PCR (EC-qPCR) microdevice for the concurrent DNA amplification, PCR products EC detection and PCR products quantification instead of the current available fluorescence detection scheme. The EC detection strategy enabled the development of the fast, easy, accurate, and compact DNA detection systems, and the integration of the PCR amplification and detection functionalities in a single chip reduced the size of the PCR microchip. The microfluidic flow-through EC-qPCR microdevice was fabricated with the state-of-the-art microfabrication technology, by bonding a bottom glass substrate having a microelectrode array to a top glass cover having the microchannels made of PDMS material. Both the amplification of the target DNA sequence and the subsequent EC detection of the PCR products were carried out concurrently on the integrated device by real-time monitoring. The *in-situ* EC measurements were conducted at the cycle numbers of 5, 8, 10, 12, 14, 16, 18, 20, 22, 24 and 26, during the PCR amplification while the microfluidic flow-through EC-qPCR microdevice was connected to the EC workstation. The underlying principle of the microfluidic flow-through EC-qPCR method was based on the changes of current signal of methylene blue (MB), which worked as an electrochemically active species DNA intercalator in the PCR mixture, during the amplification process at the extension phase. The most prominent feature of this strategy was the possibility of detecting the PCR products signal electrochemically cycle-by-cycle, which enabled quantification of the PCR products.

The results shown in this work indicated that the nucleic acid analysis could be performed in a fast thermal cycling and true real-time quantitative electrochemical detection. The signal variation trends of the EC detection and the fluorescence detection were the same in our verification measurements for both methods, which suggested that the EC detection method was, in fact, feasible for this application. The C_T values for both the electrochemical detection and the fluorescence detection system

decreased linearly with increased input target quantity. Electrochemical detection system was incorporated onto the microfluidic flow-through EC-qPCR microdevice. It proved that this chip could be used as on-chip quantitative measurement of the initial DNA concentration, and the limit-of-detection for the fabricated microfluidic flow-through EC-qPCR microdevice was found to be 0.2 ng/ μ L for the initial concentration of the target DNA. Based on the concept and the system set-up established in this work, an automated EC detection system for the quantification of PCR products could be implemented with further developments.

In this work, the microfluidic flow-through EC-qPCR microdevice was presented, which renders a fast EC detection of the PCR products, and realizes the exponential accumulation of the PCR products and real-time quantifications.

Chapter 7

Conclusions and Future Work

7.1 Summary of the thesis

This thesis is a multidisciplinary study of different electrochemical detection methods for DNA analysis. The conclusions for the four studies in this thesis are summarized as follows.

Study 1: Voltammetry detection of the nucleic acid bases with sol-gel carbon composite electrodes

The first macro electrochemical detection study is based on the study of DNA purine bases - adenine and guanine, on a sol-gel carbon composite electrode (CCE). The purine bases, guanine (G) and adenine (A), and the pyrimidine bases, thymine (T) and cytosine (C), are fundamental components of biological systems. Besides participating in various processes such as energy transduction, metabolic cofactors and cell signalling, they are essential in building the blocks for DNA. Thus, determining the individual concentrations of guanine and adenine or their ratio in DNA is important.

In this work, preliminary analysis of adenine and guanine mixtures from the denatured DNA digest indicates that the two bases have oxidation peak potentials separated by more than 200 mV on a sol-gel CCE. It shows that adenine and guanine can be measured simultaneously on a sol-gel CCE by a sensitive differential pulse stripping absorption voltammetry. For guanine and adenine, under optimum conditions a linear calibration graph in the range of $2 \times 10^{-8} \sim 1 \times 10^{-6}$ M is obtained, with the limits of detection (LOD) by the differential pulse stripping absorption voltammetry method calculated to be 0.06 μ M and 0.09 μ M for guanine and adenine, respectively. This CCE is useful for the simultaneous analysis of the DNA purine bases from the denatured DNA. We demonstrate that the electrochemical pre-

treatment of a sol-gel CCE is simple, effective and sensitive for detecting purine in DNA. This method can be used to fabricate screen-printed DNA biosensor arrays or microelectrode arrays for the integration of portable DNA analysis devices in the future.

Study 2: Voltammetric detection of sequence-specific target DNA with capture probes covalently immobilized onto a glassy electrode surface

The aim of this work is to develop a simple DNA immobilization strategy, which provides a well-defined recognition surface on the GCE for DNA hybridization study. We have reported the fabrication of a macro DNA biosensor based on the covalent bonding of the capture probe on a chemically modified GCE via the electrooxidation of 4-ABA [226]. A synthesized 21-mer amino-modified single-stranded deoxyribonucleic acid (ssDNA) is immobilized via covalent bonding onto a carboxylate-terminated 4-ABA monolayer modified GCE surface via EDC and NHS cross-linking reaction. Since the NHS/EDC approach produces a more uniform DNA immobilization with higher DNA surface density, covalent bonding can be a good choice of developing DNA biosensors with high detection sensitivity and hybridization efficiency.

This immobilization method for the probe ssDNA on the GCE surface is demonstrated to be useful for monitoring the DNA hybridization with the complementary ssDNA. The probe-modified GCE is shown to be an effective biosensor for the detection of hybridization by using MB as the electrochemical indicator. The reproducibility for the three independently made electrodes is 90.3%, measured at 10 ng/ μ L of the synthetic target DNA, with the LOD of the DNA biosensor around 0.5 ng/ μ L. We further try this method on the real PCR sample instead of the synthetic DNA. The DNA analysis in this approach starts with asymmetric PCR amplification to produce single-stranded target products, following by the immediate sequence-specific recognition of the PCR product as they hybridize with the probe-modified electrode. This electrochemical detection method is sequence specific, and is able to detect the

minimum amount of PCR product after five PCR amplification cycles with the initial Lambda DNA template at the concentration of 1 ng/ μ L. The experimental results indicate that the established electrochemical hybridization transduction schemes, with the combination of asymmetric PCR and the MB, can be further developed and integrated into portable PCR microdevices for the sensitive and selective detection of the target DNA templates.

Study 3: In-situ EC detection for DNA quantification in a stationary EC-qPCR reactor

So far, the integration of PCR with electrochemical detection is achieved only at the end-point stage. The end-point PCR measurements do not provide any information about the initial amounts of the target molecules in the samples; but only able to distinguish a positive sample from a negative one. Quantitative real-time PCR provides additional information beyond the end-point detection of the DNA. It indicates not just the existence of a specific DNA segment in a sample, but also the amount of the DNA segment. This information is required in a number of applications in medical diagnostics tests, target searches and basic researches. Currently, commercial fluorescence detecting thermocyclers is already available for the real-time PCR detection, which amplifies specific nucleic acid sequences and measures their concentrations simultaneously. However, the optical detection systems are difficult to be miniaturized due to its complex and bulky optical components. Thus, our objective in the third study is to develop an *in-situ* EC detection method for the quantification of PCR product instead of using the existing fluorescence detection scheme. In short, for the above purpose, we developed a stationary electrochemical detection for quantitative PCR process, abbreviated as stationary EC-qPCR.

The *in-situ* EC detection of the PCR product is demonstrated in the stationary EC-qPCR reactor. A macro 3-electrode module is incorporated into the PCR tube in order to perform electrochemical detections of the PCR product. The PCR amplification is carried out in a thermal cycler for 40 cycles with simultaneous electrochemical

detection. Since the MB interacts with DNA in solution without immobilization on the electrode surface, thus the time-consuming probe immobilization step can be eliminated. The changes in the current signal of the MB are monitored in real-time during the amplifications by using SWV. When the concentration ratio of DNA to the MB in the solution is increased, the EC current decreases because most of the MB molecules in the solution are intercalated to the DNA, and the concentration of the free MB molecules in the solution will gradually decrease. The inhibition studies are also carried out to ensure that the 3-electrode module and the electrochemically active DNA intercalator in the stationary EC-qPCR reactor only inhibit the PCR process within a tolerable range. The C_T values obtained from this EC detection were 11 for 1.0 ng/ μ L DNA template, 17 for 0.1 ng/ μ L DNA template, and 34 for 0.01 ng/ μ L DNA template. This study is a basic framework that can be used to develop an advanced microfluidic flow-through EC-qPCR microdevice as described in Study 4.

Study 4: Microfluidic flow through PCR microdevice with concurrent EC detection

In recent years, many devices for PCR applications have been fabricated to demonstrate the benefits of miniaturization. Lee *et al.* [67] developed the first microfabricated silicon/glass hybrid PCR chip with the functionalities of PCR amplification and sequence-specific voltammetry EC detection. However, this EC detection has limitations, namely the detection occurs only at the end-point and the product from PCR amplification is asymmetric PCR product (single-stranded PCR product). The asymmetric PCR product will hybridize with the probe immobilized on the working electrode surface if a certain region of the amplified sequence is complementary to the probe. In short, such a system will inherently encounter difficulties in differentiating similar DNA sequences, such as those generated in forensic investigations through the amplification of short tandem repeats. Thus, we developed an integrated microfluidic flow-through EC-quantitative PCR (EC-qPCR) microdevice for the simultaneous PCR amplification, EC detection and quantification of PCR cycle-by-cycle, which eliminates the shortages of the device developed by Lee and his colleagues [67].

The microdevice implemented in this study includes a long microfluidic channel and EC electrodes. The PDMS microfluidic channel loops across 3 different temperature zones with underneath micro-electrode array embedded at the extension phase at selected PCR cycles, the position of each 3-electrode detection station corresponds to a specific cycle number of the PCR process. The MB used as DNA intercalator is incorporated into the PCR mix to generate the electrochemical signal, which is proportional to the concentration of the PCR product during the PCR cycles. The peak current measurement of the MB is taken with SWV method when the flow front of the PCR mix segment in the microchannel fully submerges a particular 3-electrode EC detection station. The EC measurement circuit is subsequently switched to the next EC detection station when the flow front of the PCR mix segment submerges the next detection station. This similar EC measurement process continues until all of the detection stations are sequentially measured. The peak current versus PCR cycle numbers curve for our real-time PCR system can be used to calculate the initial amounts of the target DNA, the difference in the initial amounts of template DNA is quantified by comparing different C_T values. During the EC detection process, the current signal is observed to decrease significantly at around 11, 15, and 18 cycles for the initial template DNA concentrations of 20 ng/ μ L, 2 ng/ μ L, and 0.2 ng/ μ L, respectively. The limit of detection for our microfluidic flow-through EC-qPCR microdevice is around 0.2 ng/ μ L. This limit of detection is higher than that obtained by using an optical fluorescence detection system. But considering the small size of the entire system and the potentials for further design improvements, this microfluidic flow-through EC-qPCR microdevice exhibits a new breakthrough in the concept and implementation of a portable, inexpensive, robust real-time qPCR. Our reported microfluidic flow-through EC-qPCR microdevice has the ability to quantify the PCR products by using electrochemically active DNA intercalator, which overcomes the limitations of the end-point PCR measurements.

7.2 Recommendations for future work

The development of the integrated DNA analytical microdevices is still at its early stage. A number of critical issues, such as sample introduction, precise fluidic control within microchannels, on-chip detection and signal processing capabilities, have to be carefully addressed in order to realize a sophisticated and user-friendly microsystem.

Optimization of the whole integration process remains an open challenge. The possible improvements of the microfluidic flow-through EC-qPCR microdevice can be categorized into three major aspects. First, a more functionalized design and fabrication of the microdevice will improve the performance of the device. For example, the heaters and temperature sensors can be incorporated into the microfluidic flow-through EC-qPCR microdevice for more efficient heat-transferring and rapid thermal cycling, thus increases the precision and efficiency of the PCR process. The second improvement solution is the selection of the more sensitive DNA intercalators. Other suitable DNA intercalators, which have lower inhibition to the PCR but higher duplex specific affinity, sensitivity and stability against higher temperature, are preferred to lower the detection limit of initial DNA template. The third solution is to improve the peripheral circuit detection system of the microdevice, such as adding an automatic electrical switch with precise time control to automate the cycle-to-cycle detection, using a noise filter to suppress of the background noise in the electronics system, or averaging the EC signal of a few measurements to increase the signal-to-noise ratio. It may be possible to increase the level of integration in the future by incorporating the detection system into a single PCR chamber. The integration of genomic DNA capturing, DNA amplification and detection in an array structure will further improve the portability of the overall system. This may herald a new field-operational clinical examination system within a high-throughput PCR microfluidic chip.

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