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DEVELOPMENT OF LAB ON A CHIP PLATFORMS FOR BACTERIA DETECTION BASED ON LOOP-MEDIATED ISOTHERMAL AMPLIFICATION

Par

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“As we express our gratitude, we must never forget that the highest appreciation is not to utter words, but to live by them.”

John F. Kennedy

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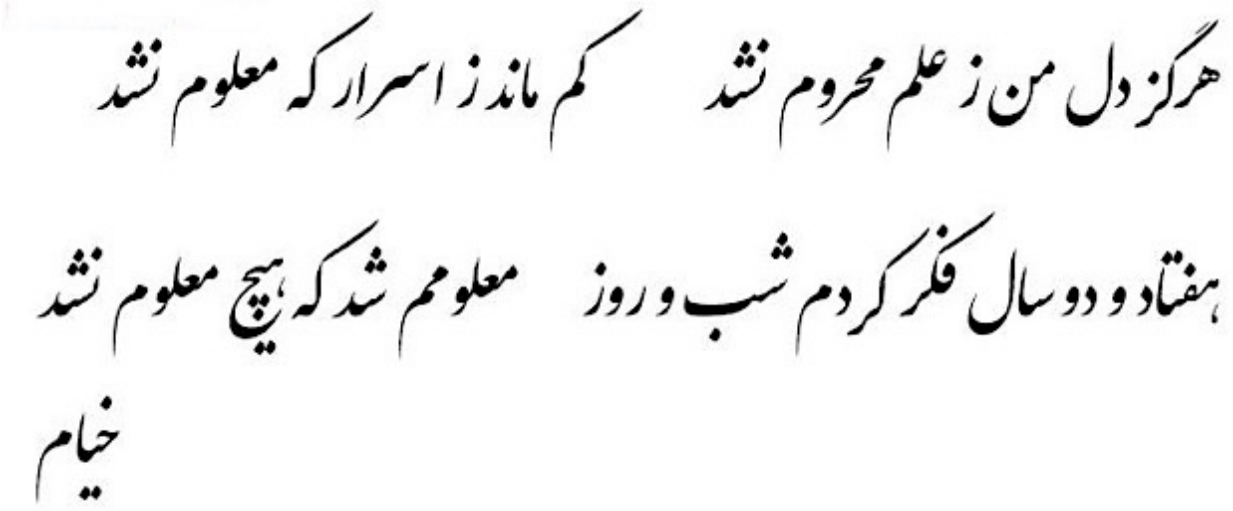
DEDICATION

I dedicate this thesis to my parents, Who thought me tenacity, passion and hard work.

"Out beyond ideas of wrongdoing
and rightdoing there is a field.
I'll meet you there.

When the soul lies down in that grass
the world is too full to talk about."

Rumi



ABSTRACT

Infectious disease accounts for nearly 50 million illness incidences around the world. Applications of lab on chip devices in conducting biomedical research have been drawn substantial interests from scientists and researchers all over the world. These lab-on-a-chip systems create clinically useful technologies and have a number of competitive advantages over the conventional biomedical instruments due to the reduced reagents/samples consumption, decreased analysis times and operational costs. In addition, these systems facilitate the development of the portable devices and the possibility of automatically performing multiple assay processes. Early detection and notification of pathogenic bacteria, is therefore of considerable significance in disease control. DNA-based amplification technique, known as a precise diagnostic tool for detecting pathogens, has presented a distinctive advantages over the conventional microbiological culture-based methods for pathogen detection such as high sensitivity, specificity and rapidity, especially in the detection of bacteria in samples containing low concentrations. over the past decade, loop-mediated isothermal amplification (LAMP) has caught significant attention as it is provides rapid amplification with high sensitivity and specificity of the target gene. In this thesis, various lab on a chip based platforms have been developed combining DNA LAMP amplification for detection and quantification of pathogens. In the first part, a microfluidic device designed for amplification of malB gene of *E.coli* bacteria followed by electrochemical detection of the amplified product. Through amplification time optimization, microfluidic chip could detect and quantify 48 cfu/ml of bacteria in 35 min employing immobilisation-free electrochemical transduction. In the second part of thesis, we have invented a cassette device for high throughput detection of various bacteria types (gram negative & gram positive). The cassette consists of two aluminum reels and a plastic ribbon, which has an array of chambers. one reel act as the provider of ribbon whilst the other one act as collector. The collector reel is connected to a heater to provide adequate temperatures for the amplification. LAMP solutions with *E.coli* (as gram negative model for bacteria) was applied to each reservoir and a plastic tape cover the chambers and rolls into the collector reel. After 1 hour - amplification of *E.coli* was detected using a colorimetric

method employing Hydronaphthol blue (HNB) with a 30 CFU/ml limit of detection. The color of HNB changed from purple to blue in the presence of bacterial amplified product. Through procedure modification we were able to detect as low as 200 CFU/ml of *S.aureus* as an example gram-positive bacteria using Calcein.

In the third part of thesis, the cassette's ribbon has been modified by using flexible screen-printed electrode attached at the bottom of each chamber. Using Osmium redox and immobilisation-free of electrochemical technique, we could monitor the amplification of DNA of *E.coli* and *S.aureus* bacteria in a real-time analysis and quantification.

In the forth part of thesis, a novel biosensor platform has been developed to detect the viability of bacteria using T4 bacteriophage and label free impedance spectroscopy. We have shown that we could monitor viability of bacteria in the range of 10^3 -10^9 CFU/ml, while using LAMP amplification and linear sweep voltammetry technique we could detect 10^2 CFU/ml within 40 min response time.

In the last part of the thesis, we summarize our findings, and provide contribution of these researches to the knowledge as well as health. In addition, future possible path to the research work will be discussed.

TABLE OF CONTENT

LIST OF TABLES

LISTE OF FIGURES

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Appendix 1

Appendix 2

ABREVIATIONS

ASSURED……..Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free and Deliverable to end users

CE…….. Capilary electrophoresis

CFU …….Colony Forming Unit

DL …….Double layer

dCTP…….Deoxycytidine triphosphate

DEP……Disposable Electrochemical Printed

DNA …….Deoxyribose nucleic acid

DPV …….Differential Pulse voltametry

dsDNA……....double stranded DNA

EIS ……..Electrochemical impedance spectroscopy

ELISA …….Enzymatic linked immunosorbent assay

HDA …… Helicase dependant amplification

HNB……Hydronaphthol Blue

LAMP …….Loop-mediated isothermal amplification

LOC……Lab on a Chip

LOD……Limit of detection

LSV ……..Linear sweep voltametry

NA ……..Nucleic Acid

NASBA …….Nucleic acid sequence based amplification

PBS…….Phosphate-buffered saline

PCR …….Polymerase Chain Reaction

PDMS…… Poly dimethylsiloxhance

PMMA ……..Poly methyl metha acrylate

POC ……..Point of Care

qPCR……Quantified Polymerase Chain Reaction

RCA ……..Rolling Circle amplification

RNA ……...Riboxy nucleic acid

SWV ……Square wave voltametry

SPE……..Screen printed electrodes

ssDNA…… Single stranded DNA

SAM……Self assembled monolayer

WHO……World health organization

XPS……X-ray photoelectron spectroscopy

CONTRIBUTION OF AUTHORS

This thesis is prepared by the collection of articles written by the candidate with the contribution from the co-authors. Chapter 1 presents the scope of thesis. In the chapter 2, general literature survey and background knowledge has been studied. Chapters of 3-6 represent the results, which have been either published or submitted in a peer-reviewed scientific journals. Finally, Chapter 7, summarize and concludes the findings and prospects the key elements for future works. Dr. Mohammed Zourob appears to be the co-authors of the paper for contribution in designing the research projects, leading the scientific research, helping to analyze the data and involvement in manuscript correction and submission. In Chapter 3, Dr. Minhaz Uddin Ahmed helped to accomplish the experiments and thought how to run various biological and electrochemical assays. On the chapter 4&5, Dr. Ahmed contributed to the project by designing the primers for loop mediated isothermal amplification, correcting the manuscript and discussing the data and achievements, Dr. Andy NG contributed effectively on manuscript correction and modifications. In Chapter 4, Esen Sokullu helped to run various biological assays, and Dr Breascau analyzed the finite element analysis data. In Chapter 5, Dr Chaker Tlili had the contribution by performing the experiments of viability test using electrochemical impedance spectroscopy measurements and writing the manuscript.

1 CHAPTER 1 : INTRODUCTION

1.1 Background

Effective bacteria detection and *in vitro* diagnostics are essential for clinical practices. These bioassays use body fluid such as blood, urine, and saliva to detect pathogens. The golden standard for bacteria detection relied on culture techniques, which can detect a single bacterium by growing it onto a specific media to form a distinct visible colony. However, it requires highly skilled personnel to conduct, it is time-consuming and depending on the bacterial type. It can take from 24 hours to weeks for some bacteria.

Immunoassays are biochemical tests that are used to detect various pathogens and viruses in different matrices. The assay is based on the high affinity and specificity in binding of an antigen and its corresponding antibody for the detection and quantification of this antigen in biological fluids. The immunoassays are available in various formats such as enzymatic linked immunosorbent assay (ELISA) and lateral flow assays that are developed for detection of various types of bacteria, virus, and also for spores detection [1]. However, these techniques are laborious, multi-step and semi quantitative, which does not provide high sensitivity to diagnose diseases caused by low concentration of infectious doses that most of the bacterial infections require another disadvantage, is cross-reactivity. Therefore, development of other types of biosensors is necessary.

Among other assays, DNA hybridization and amplification techniques provide accurate and reliable results. Microarrays spotters and nucleic acids amplification based point of care (POC) devices are two important tools that are commonly used to perform diagnosis. Microarrays spotters print millions of short nucleic acids or protein probes on the surface of glass or other substrates such as nitrocellulose which makes it a viable platform for pathogen detection [2]. The probes are designed in a complementary way to sequences of target organism biomarker. Various techniques have been used to spot onto the array such as mechanical deposition [3], inkjet spray using printer tip as well as

photo catalytic reaction [4] . In order to analyze samples with these arrays, nucleic acids first should be extracted. The DNA can be amplified by different amplification techniques such as polymerase chain reaction (PCR) and labelling. The labeled DNA is incubated for several hours to hybridized with the probe. Later, the array is washed to remove unbound DNA. The fluorescent intensity of the bound DNA is scanned with the bright sections representing the probes that are complementary to the DNA.

The most common and sensitive technique for the amplification of nucleic acids and genomic analyte is PCR. Since it was first published in 1985 [5], PCR demonstrated a promising technique for detection of organisms at low concentrations. Since it detects the target based on the amplification rather than the original signal, it is much less prone to false positive result. The principle of PCR is based on three sequential steps and it requires different temperature cycles. The high temperature, commonly, 90°C, is used to denature DNA template and form the single strand template. The lower temperature (~50°C) is for annealing of the primers to the target section of DNA and the intermediate temperature (~70 °C) which lies between the previous two temperatures is used to help the polymerase enzymes to generate huge number of copies of the target DNA[6].

Miniaturization of conventional assays can provide many advantages such as using minute volumes of samples as well as reagents. In addition, it increases the surface to volume ratio and provides better mass transfer in the reaction and reduces the time of the assay. Moreover, it provides portability and ease of use. The World Health Organization (WHO), set the remarkable criteria called ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free and Deliverable to end-users) as the ideal criteria for POC diagnostic devices. As a results, significant efforts have been accomplished toward meeting these criteria [7].

POC diagnostics consist of different sections of liquid handling devices, which controls the sample, and in some cases interfaces. The interface is designed for sample preparation (i.e. PCR processing), signal quantification and demonstration of the signal results. The main components of the liquid handling device is to a reaction chamber, where reagents can react together and a detection unit, where the signal is obtained at the interface of signal transducer. In the centralized laboratories, all these steps take

place in separate time-consuming multi-steps processes. Despite the fact that, it is cumbersome to satisfy all the ASSURED criteria, the liquid handling or the assay process can be designed in such a way to be close to ASSURED with minor use difficulties. Thus, the main challenge is to provide POC liquid handling devices that operate in a multi function fashion while satisfying the ASSURED requirements [8].

Our research objective is to develop a novel POC diagnostic device that is both accurate and rapid in detection of bacteria. The first technology described here is development of a novel microfluidic system for the detection and quantification of *E.coli* bacteria. We first developed a microfluidic device, with different components like valves, and fluidic reaction chamber and electrochemical detection components. Afterwards, we ran the electrochemical assay based on amplification of malB gene for *E.coli* detection, which can be used in the POC devices. LAMP is used as an amplification method with high sensitivity and specificity. We have detected the amplicon using linear sweep voltammetry (LSV). This device has a high potential to be used for detection of any gram-negative bacteria.

In the second part, we present cassette as a novel device for POC diagnostic device. We fabricated a roll of plastic and designed it to be used as a cassette format. Firstly, the plastic polyethylene ribbon composed of reaction chamber to implement the LAMP for detection of multiple targets of *Tuf E.coli* gene. Secondly, the cassette operation was modified in order to detect *S.aureus* bacteria. Through numerical simulation, the heat transfer around the collector reel was simulated in order to estimate the number of samples that can be analyzed in a high throughput format.

In the third part, the cassette was modified by attaching the flexible SPE at the bottom of each reservoir of flexible ribbon. The osmium redox was employed in the electrochemical detection and enabled the device to monitor real-time detection of amplicon in the solution. We were able of detection same levels of detection, previously achieved by colorimetric detection for *E.coli* and *S.aureus* bacteria in 30 min and 37 min, respectively followed by bacteria quantification. The cross reactivity test was employed, to check that the assays are highly specific to the target genes.

The aim of the last part is to provide a novel detection mechanism for future biochip design to test the viability of pathogens. Liveability of *E.coli* was tested using T4 bacteriophage as the natural *E.coli* bacteria receptor and monitoring the viability of *E.coli* using label free impedance spectroscopy. The assay provides confirmation of bacteria using LAMP following by LSV technique.

1.2 **Research Objectives**

Our research objective is to develop a novel POC diagnostic device that is both accurate and rapid in detection of bacteria. The first technology described here is development of a novel microfluidic system for the detection and quantification of *E.coli* bacteria. We first developed a microfluidic device, with different components like valves, and fluidic reaction chamber and electrochemical detection components. Afterwards, we ran the electrochemical assay based on amplification of *malB* gene for *E.coli* detection, which can be used in the POC devices. LAMP is used as an amplification method with high sensitivity and specificity. We have detected the amplicon using linear sweep voltammetry (LSV). This device has a high potential to be used for detection of any gram-negative bacteria.

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1.3 **Thesis overview**

This thesis covers our work in the development of various platforms of POC diagnostic systems and is organized as follows:

Chapter two provides introduction of the thesis and covers basic principles of bacterial detection. We focus mainly on amplification techniques as the most prominent and reliable method for bacterial detection. We specifically focus on the loop-mediated isothermal amplification. Various electrochemical detection mechanism that are suitable for POC diagnostics are reviewed. Also it covers a review of LAMP techniques in microfluidic platforms.

Chapter three initiates the result of the microfluidic electrochemical platform for detection and quantification of *E.coli* bacteria using disposable screen-printed electrodes (SPEs). Electrochemical detection was used based on interaction of Hoechst 33258 and DNA amplification products using LSV. We used a novel method to simultaneously lyse the bacteria followed by amplification of released DNA in the sample. The assay was tested using bacteria *E.coli* sample in the LB broth media without purification. LOD of 24 cfu/ml in 1 hour. amplification time was achieved. Afterward, we optimized the amplification time and 48 cfu/ml was detected in 35 min following by quantification of the bacteria. We have tested our chip and showed that we could detect the bacteria in various turbid media such as low concentration filtered urine.

Chapter four demonstrates the novel architecture as a proof of concept for POC diagnostic device using the naked-eye bacteria detection in a cassette format. The cassette consists of two aluminum reels as a provider and collector of the flexible

ribbons that has multi reservoirs for sample processing. We used a colorimetric assay with two different colorimetric dyes (HNB and Calcein) for detection of *E.coli* (as a gram-negative bacteria model) and *S.aureus* (gram positive model), respectively. We could first detect 20 cfu/ml of *E.coli* bacteria. The operational process for *S.aureus* bacteria detection was modified and 200 cfu/ml of *S.aureus* bacteria in 1 hour amplification time was achieved. Using numerical simulation, temperature profile around the collector reel a minimum of 32 samples can be analyzed simultaneously.

Chapter five, introduces the novel process for real-time bacteria detection with high sensitivity and specificity. The flexible ribbon in the cassette was modified using flexible screen-printed carbon electrode attached to the bottom of each reservoir. Then we used the Osmium redox to provide real-time monitoring of redox intercalating with amplification products. We have shown that we could detect 20 CFU/ml of *E.coli* and 200 CFU/ml of *S.aureus* bacteria following real-time analysis. Since all the lysis step as well as amplification was implemented in sealed chips, costly post processing is not required and as a consequence, it reduces the cost and potential of contamination for the environment.

Chapter six introduces the novel detection mechanism for the viability test of *E.coli* bacteria using T4 bacteriophage. Two different assays have been integrated to provide viability, screening and confirmation of bacteria in the sample. The first assay provides a viability test by monitoring of impedance spectroscopy of bacteria detection using T4 bacteriophage as the natural receptor with the sensitivity range between 10^9- 10^3 CFU/ml. Then the assay's sensitivity was increased using LAMP amplification technique followed by electrochemical detection using Hoechst 33258 redox based on LSV technique. While the linear relation response of LSV was achieved in the range of 10^2-10^7 CFU/ml with the LOD of 8×10^2 CFU/ml in less than 15 min and 10^2 CFU/ml within a response time of 40 min was achieved.

Chapter seven provides discussion, summary of findings and outline for the future prospects of these projects.

2 CHAPTR TWO: BACKGROUND KNOWLEDGE

2.1 Principle of bacteria detection

Development of a rapid, sensitive and accurate bacterial detection device is essential for controlling infectious disease. In general, identifying the bacteria in samples is the most critical step in order to cure patients and protecting individuals [9-11]. The development of rapid and sensitive platforms to detect pathogenic bacteria is essential for providing a rapid response to an outbreak [12]. This rapid response is more difficult in the area of biodefense and bioterrorism where immediate, appropriate preventive procedures are required.

Generally, there are various techniques for bacterial identification. The golden standard technique for bacteria detection relies on morphological characteristics of the microorganisms as well as bacteria's ability to grow in different media. This technique involves culturing and assessing growth of individual viable bacteria using either non selective media, such as trypticase soy agar or selective ones i.e Xylose, lysine desoxyscholate, MacConkey agar [13]. However, culturing technique is time consuming and depends on a specific bacterial strain, it takes between several hours to even several weeks to culture the baceria colonies. Moreover, culturing technique for bacteria detection will be more challenging if low number of bacteria exists in the sample. Pre-enrichment step is required to increase the concentration of bacteria.

Immunoassays are biochemical tests that are used to measure the concentration of one or many bio-analytes in a biological sample by means of antigen-antibody reactions. The assays take the advantage of the high-affinity and specificity in binding between an antigen and its corresponding antibody to detect and quantify the antigen in biological fluids. Many types of immunoassays have been developed and used for bacteria, virus, and spores detection. Figure 2.1 illustrates the schematic of an immunoassay, commonly used in the Immuno-chromatographic assays (i.e. Solid phase sandwich ELISA-type). In this format, the capture antibody is first immobilized on the surface. Subsequently, the sample is flowed over the capture antibody, and the analyte (antigen) in the sample is captured by the immobilized protein (antibody). Finally, by passing the

detection antibody, which is conjugated with a label [14] over the captured analyte pathogen can be detected. Despite using very short assay time in comparison with culture techniques, unfortunately this technique cannot detect microorganism in real time. Besides, this technique has some major drawbacks like poor sensitivity as well as being a multistep process. Other method for bacteria detection relies on detection of the nucleic acid located intracellular which refres to nucleic acid (NA) test. Whilst, the first method provides faster, and less manipulation technique, with lower specificity, NA tests provide higher sensitivity and accuracy.

The detection of pathogens in clinical samples has several technical challenges due to the fact that a) biological samples has a complex structure, b) samples are in very limited quantities, c) it might contain a very few pathogens (early infection and diagnostics).

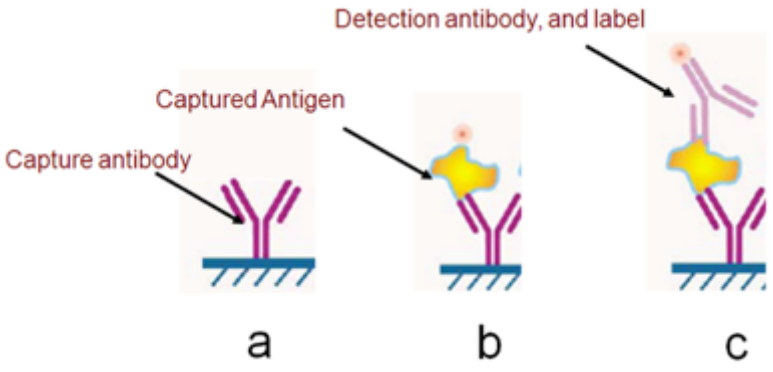


Figure 2.1 **Schematic of a Solid phase sandwich ELISA-type process used to measure the concentration of proteins in the solution. (a) The capture antibody (CAb) is immobilized on the surface. (b) Later on, we flow the sample over the capture antibody; as a result the analyte (antigen) in the sample is captured by the CAb. (c) Finally, we pass the secondary detection antibody combined with a label over the captured protein to detect the desired protein*.**

2.1.1 Nucleic acid based bacteria detection

Nucleic acids (NA) tests are the most reliable technique for pathogen detection. Generally, NAs can be detected by using microarray or amplification techniques. DNA Microarrays emerged as a viable platform for pathogen detection [4] by immobilizing thousands of genes specific probes, which were attached to the surface of solid

substrate such as wafer, glass or nylon. The probes are designed in such a way to be complementary to the sequences of target organism's genomic DNA. Then, the sequences are labeled by fluorescent or radioactive labels [15]. Once the sample is added either in bulk or using liquid handling device such as microfluidics to the microarray, hybridization takes place. The array will then be scanned to visualize the fluorescent pattern tilled on the surface of the substrate and the pattern is read out by microarray reader.

Various techniques have been used in order to spot probes onto the array such as mechanical deposition, inkjet spray using printer tip as well as photo catalytic reaction [16]. In order to analyze samples with this type of array, nucleic acids first should be extracted from the cells at the bench top. Probe design and selection is one of the important factors and a good design can help in resolving many issues to provide proper specificity and sensitivity. These issues are the cross hybridization, orthogonal probe binding to the target DNA, uniformity of the annealing temperature (GC content) and length of the probe [17]. All these issues result in false positive or false negative responses, which can have significant negative effects particularly when dealing with the deadly pathogens.

Over the past decade, combining microfluidic and DNA microarray technology has been exploited simultaneously for pathogen detection analysis. The main advantages of microfluidic technology, in this regard, is that first, it requires a small volume of sample to initiate the process in protein and DNA assays a very small amount of sample volume is provided which can not be implemented in the bulk analysis. Microfluidics provide a solution to analyze these materials in portable LOC devices. Moreover, surface hybridization of the DNA in microfluidic can be accelerated in comparison with bulk samples. Conventional DNA microarray with approximately 30-µl sample requires overnight incubation in order for the hybridization to take place. Initially, the kinetic of the hybridization is based on the reaction limited phenomenon [18]. After that, the probe depletion is based on a diffusion based limited phenomenon [19]. Since the diffusion coefficient (D) of the nucleic acid is 10^{-7} cm^2/s, and the length scale (L) such that the NA are moving according to ($L = \sqrt{Dt}$), is in the order of 1 mm in 24h. Yet, the time scale

(t) for precipitation of the DNA to the microarray will be much faster due to the fact that the convection forces in the micro-channel will enhance the diffusion of the nucleic acid and consequently increase the hybridization efficiency of DNA. Generally speaking, in conversional microarray, the DNA sample is loaded on to the surface of the microarray and incubated for several hours. In order to to overcome the barrier of the diffusion, various driving forces can be exploited. Initially, the negatively charged DNA is driven by electro-kinetic forces in the microfluidic channels [20]. Another intuitive approach to drive the flow into the microarray chamber is to render the pressure driven flow using a typical syringe pump. That is because the hybridization is more efficient if the solution-containing target DNA moves toward oligonucleotide probes in the small microarray chamber and can encounter every single probe. However if the microarray chamber is large, a series of approaches can take it into the consideration such as using peristaltic pump to recirculate the target DNA sample [21] or chaotic mixing of the DNA sample [22].

Other types of driving forces in the microfluidic platforms are rotational force in a CD based microfluidic [23] devices. Peytavi *et al* developed a microfluidic CD platform for DNA/PCR amplicon hybridization in less than 15 min for detection. it distinguished 4 different clinically relevant *Staphylococcus specious* that differ by as little as 1 base pair mismatch with a high sensitivity for as low as 10 copy of genome DNA. The captured probes were immobilized on 4 linear arrays with the size of 5-µm×75 µm. Table 2.1 shows the different platforms of microfluidic for DNA microarrays. Capillary force is another type of driving force, which was used in the microarray microfluidic systems. Sabourin *et al* developed a microfluidic chip made of PMMA to detect human beta globin (HBG) gene, which contains a mutation for 18 patients. The 60 µl sample were filled into the microchannel using capillary force and was detected based on fluorescent analysis [24]. Recently, conjugated beads with the target DNA have been used in the coated micro-channels with DNA probes. Javanmard *et al* developed a microfluidic system for DNA detection based on conductance measurement. The DNA beads were then injected to the microfluidic channels and were bound to the surface of the microarray, which partially clogged the channel. Electrical impedance measurements

Table 2.1 Microfluidic DNA microarrays

Sample Volume	Sample type	Driving force	Throughput analysis	Chip material	Hybridization time	Reference
60 µl	cDNA,PCR product	Capilary force	18 Samples	PMMA	37°C for 2 hrs	[24]
15 µl	PCR product,	Capilary force	1	PC	86.5°C for 2 min, 35 cycles of 86.5°C for 45 s, 50°C for 1 min 30 s, and 65°C for 5 min.	[25]
--	Oligonucleotide	Capilary force	3	Glass	45°C at 5 min	[26]
1 µl	PCR product	Centrifugal based	High throughput	PDMS	42 °C for 2 h.	[27]
1 µl	PCR product, Oligonucleotide	Centrifugal based	3	PDMS	45 °C for 2 hr	[28]
2 µl	PCR product	Centrifugal based	4	PDMS	5 min room temperature	[29]
1 µl	DNA, PCR product	Centrifugal based	High throughput	PDMS	40 min at room temperature	[30]
16 µl	DNA	Pnumatic pump	1	PDMS	42 °C for 5–300 s.	[31]
25 µl	PCR product,	Syringe pump	2	PC	15 min & 30 min	[32]
--	cDNA, Single base paair mismatch,	syringe pump (bead based)	1	PDMS	15 min	[33]

1 µl	DNA	syringe pump	1	PMMA	500 sec 42°C	[34]
90 µl	PCR product	syringe pump	3	PDMS	40°C for 10 min	[35]
30 µl	PCR product	syringe pump	1	PMMA	40-60°C for 5 min	[36]
30 µl	mRNA	syringe pump	--	PDMS	45–50 °C	[37]
25 µl	cDNA	syringe pump	4	PDMS	UV	[38]
--	cDNA	syringe pump	3	PDMS	45 °C	[39]
50 µl	DNA	syringe pump	4	COP	65°C	[40]

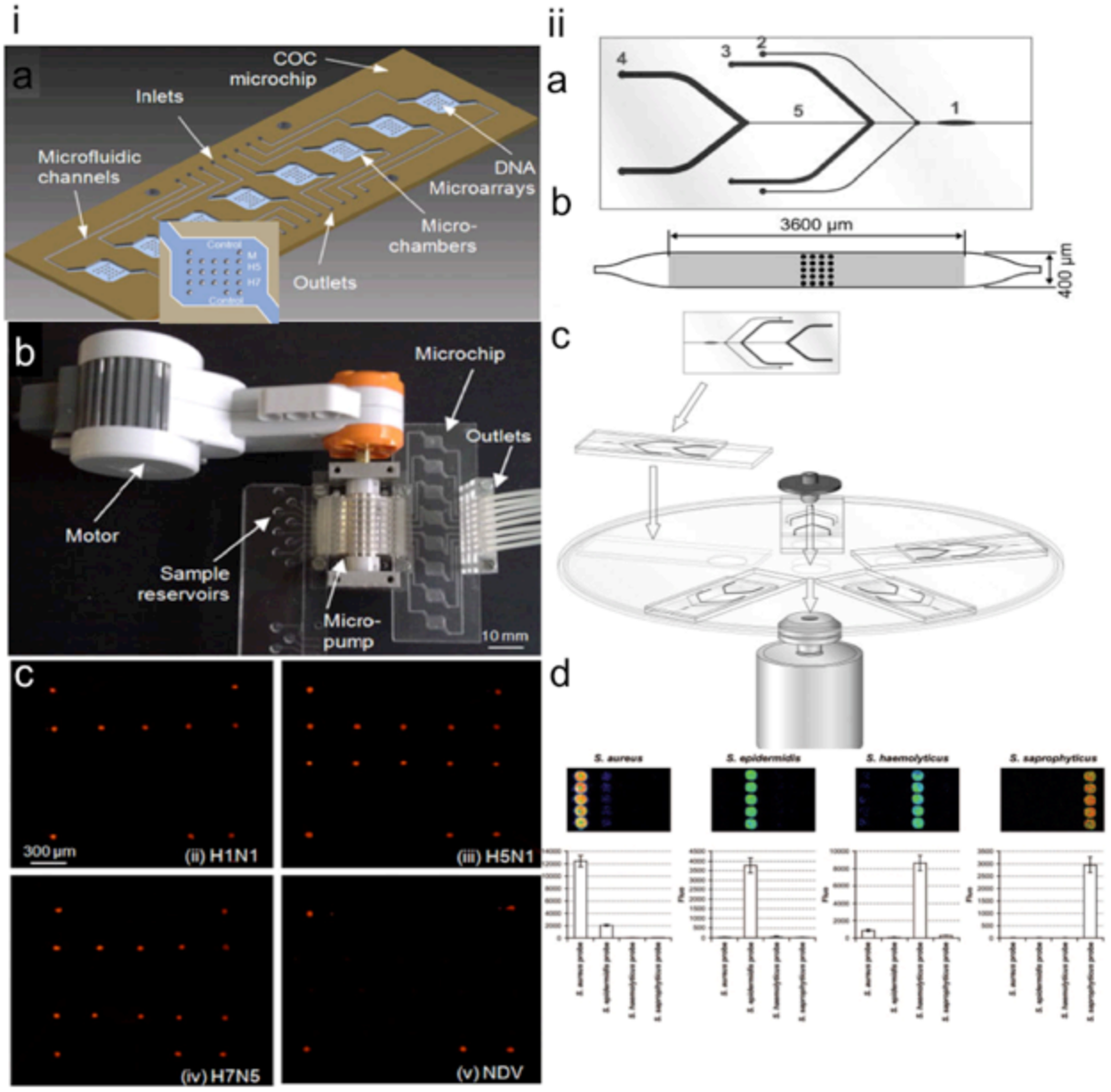
were taken between the two sides of the micro-channels using two electrodes fabricated at each side of the micro-channel. Optical microscopy was exploited to prove that the electrical changes took place as result of the beads binding between the electrodes. Figure 2.2 shows different types of microfluidic microarrays based on different driving forces of sample fluid. Despite attractiveness of providing precision, microarray is not suitable for POC diagnostics due to the fact that it requires scanner facilities as well as a costly microarray reader. Consequently, more efforts have been focused on other types of techniques for NA detection. Since the quantity of NA in bio-samples is relatively small, the amplification technique plays a critical role in the bacteria diagnostics. Generally speaking, amplification techniques are performed by DNA synthesis through a thermal reaction. Depending on the amplification techniques, differentiation can be categorized into two different subsection based on thermal cycling system (multi-thermal amplification) and isothermal amplification.

2.1.1.1 Multi thermal amplification

The most commonly used technique for NA amplification is PCR. PCR has been demonstrated as a promising technique for detection of micro-organisms. Since it detects the target based on amplification, rather than the signal thus it is much less prone to false positive results. The principle of PCR primarily uses three different temperatures to control various sequential steps (denaturing, annealing, extension). The high temperature, commonly 90°C, is used to denature DNA template and form the single strand template. Lower temperature (~50 °C) is for annealing of primers to the target sections of DNA and at intermediate temperature (~70 °C) between the previous two temperatures the polymerase enzymes generate huge number of copies of target gene in DNA [5].

PCR has distinct advantages over culture method and other standard methods and provides high sensitivity, specificity, rapidity and ability to detect small amounts of DNA in the sample. Various PCR methods have been developed based on their application for amplification. For example, quantitative real-time PCR can be used for quantification of DNA concentration by detecting the intensity of florescent signal [41]. RNA quantification can be achieved using reverse transcriptase PCR (RT-PCR), nested PCR

can be used to prevent contamination of product due to unexpected primers binding sites [42]. Long range PCR is for the nucleic acid amplification for more than 5k base pair. Multiplex PCR can amplify various DNA templates simultaneously [43]. Table 2.2 shows various type of PCR techniques.



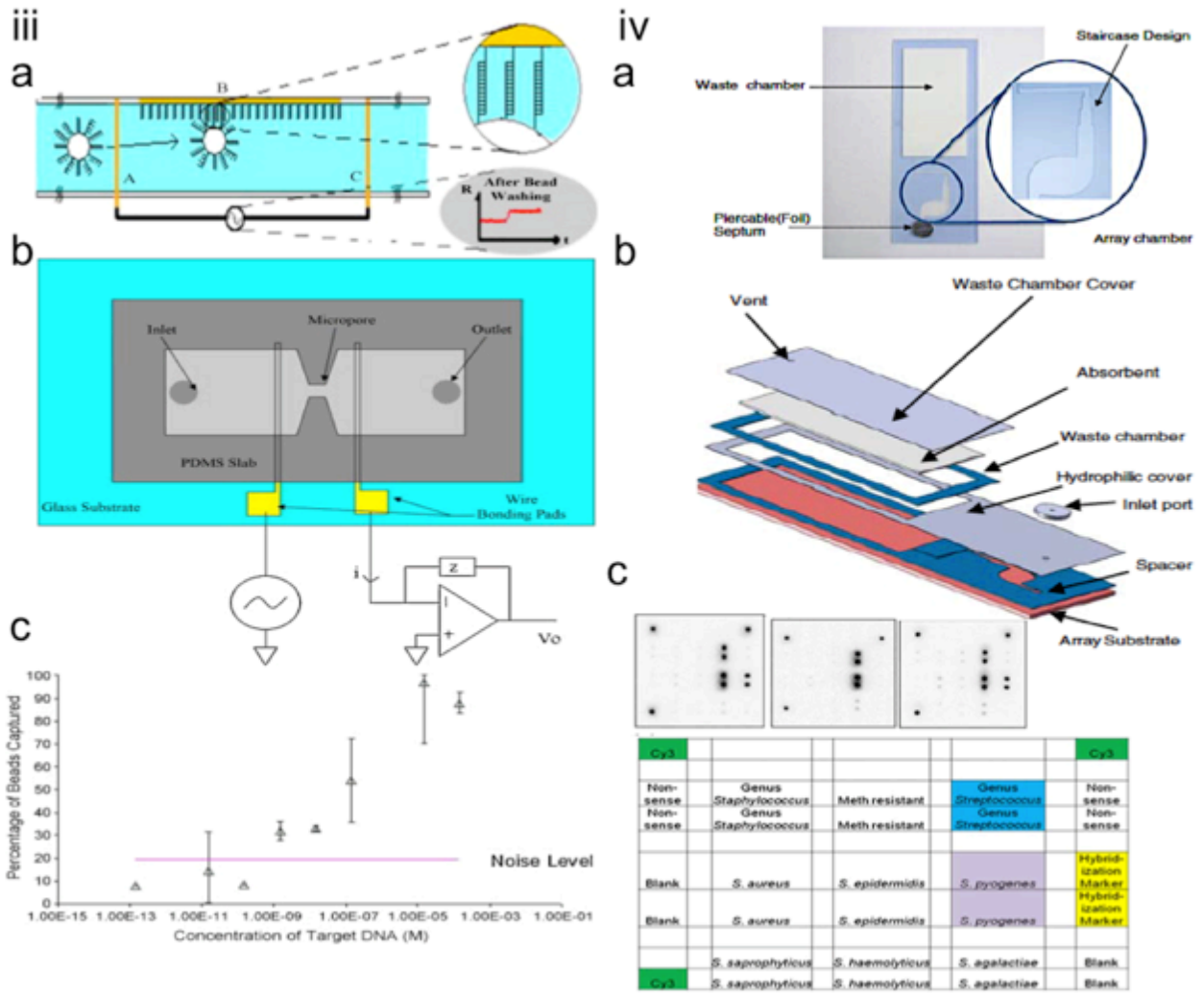


Figure 2.2 **Microfluidic microarrays for bacteria pathogenic detection based on various driving forces (i) Microfluidic microarray using active pump for injecting samples. (a) Schematic of the microfluidic chip consisting of the series of the microarray chamber. (b) Image of the microfluidic chip, which was connected to the step motor for injecting the sample into the chip. (c) Image of the fluorescent for detection of various influenza various subtypes. (ii) CD based microfluidic using centrifugal force.(a) schematic of the microfluidic platform for microarray hybridization. (a) PDMS microfluidic unit attached on a glass slide consisting of chambers 2 (3.5 µl), 3 (12 µl), and 4 (10 µl), which allow the reagents to flow through a middle micro-channel 5 to reach the hybridization chamber 1(140 nL). (b) Schematic view of the hybridization chamber showing the area of the chamber that can keep up to 150 nucleic acid capture probes spotted onto a glass slide. (C), the PDMS microfluidic chip is attached to a glass slide on which the capture probes are arrayed. The glass slide is placed on a CD support that can hold up to 5 biochips. The hybridization reagents are positioned to be pumped sequentially through the hybridization chamber using centrifugal force starting with chamber 2. (d) Detection of four different Staphylococcus subtypes and the fluorescent intensity.(iii) Bead based microfluidic microarray for DNA detection and quantification based on electrical impedance measurement. (a) Schematic of the bead, which the DNA was attached to the surface. The beads are injected through the microfluidic channel and are attached to the surface of the microarray. (b) Two**

electrodes are fabricated at each side of micro-channel to measure impedance. Hybridization of the DNA into microarray causes the increasing of the resistant between two sides of the micro-channel. (c) Optical measurement and quantification of the DNA detection. The detection limit is 1 nM. (iv) Capillary based microfluidic microarray. (a) Schematic of the capillary microfluidic microarray. (b) The microfluidic consists of substrate of array, a spacer tape to provide flow pathway, a hydrophilic film, which enhance the substrate to fill by capillary force, inlet port and waste chamber. (c) The assay result and the array map corresponding to the image of each test (Adapted from [24],[29],[33],[38]).

Table 2.2 Various non-isothermal PCR amplification techniques.

Multi-thermal	description	Reference
PCR	--	[5]
Real-time PCR	Measure real-time monitoring of fluorophore during PCR amplification	[41, 44-46]
RT-PCR	Reverse transcriptase PCR which transform RNA into cDNA	[47-50]
Nested PCR	Increasing the specificity by reducing of background of non-specific amplification	[42, 51]
Ligase PCR	Provide better specificity by using small DNA linkers ligated to DNA and multiple primers annealing to DNA linkers	[52, 53]
Long range PCR	Amplify templates more than 5k base pairs.	[54, 55]
Multiplex PCR	Have multiple primers and	[43, 56]

	amplify multiple target in one solution.	
Solid phase PCR	Amplify target on the surface of the substrate by bounding primers on the surface and enhance parallel amplification on single chip.	[57, 58]
Hot start PCR	Reduce non-specific amplification by increasing the pcr component to denaturation temperature.	[59, 60]
Touchdown PCR	Reducing the non-specific background by gradually lowering the annealing temperature during PCR process.	[61, 62]

2.1.1.1.1 PCR microfluidics

PCR microfluidics offers a great benefit in many applications especially in POC. Mainly, it reduces the cost of tests as well as time-consuming sample preparation as well as post processing processes. It also increases portability and multiple parallel sample amplification and analysis.

With the advances and development of the PCR technique in 1986, there was no immediate implementation of miniaturization using PCR, probably due to the lack of experience with silicon microchips. Around 20 years after the first microfluidic chip was developed by Northrup *et al* [63], the concepts of microfluidic and PCR merged together by development of various different platforms. With the development of capillary electrophoresis (CE) in the chip [64] , the first CE-PCR was developed in 1996. Despite

the fact that CE and PCR was not implemented in the single chip in the integration platform, it opened up the path for integration of PCR with other pre/post DNA and amplicon processing in a single chip with a silicon and glass substrate.

In addition, main effort were focused on flow through PCR initially in Manz group by developing the idea of passing the PCR solution into the micro-channel passing through three different temperature zones (90°C, 50°C, 70°C) to provide adequate temperatures for denaturing, annealing and, extension, respectively [65]. This process, significantly reduce the temperature ramping time from one temperature to another, which reduces the amplification time significantly.

For microfluidic PCR, there are various primary designs, which were developed over the past two decades, which has been described in the Table 2.3.

Table 2.3 Microfluidic PCR design and description.

Design	**Description**	**References**
Single/multi chamber Stationary PCR	The PCR solution is amplified in a single/multi chambers	[63, 66-69]
Flow through PCR	PCR flow solution is injected and passes through three different temperature zone as follow :denaturation temperature→extension temperature→annealing temperature	[65, 70-75]
Circular arrangement PCR	Arrangement of three themperature zones in a circular format	[76-80]
Thermal convection PCR	Using Rayleigh-Bénard convection to flow the sample between a cylendrical cavity and the temperature cycling was obtained as the flow continiously drive the fluid vertically into two temperature zone	[81-84]

Rotary chamber PCR	Using rapid motor , microfluidic chip is rotating between three different temperature zone for each amplification cycle.	[85-87]
Droplet based PCR	Generating a droplet of PCR mixture and passeing through three different temperature zone or three different thermal cycle.	[88, 89]

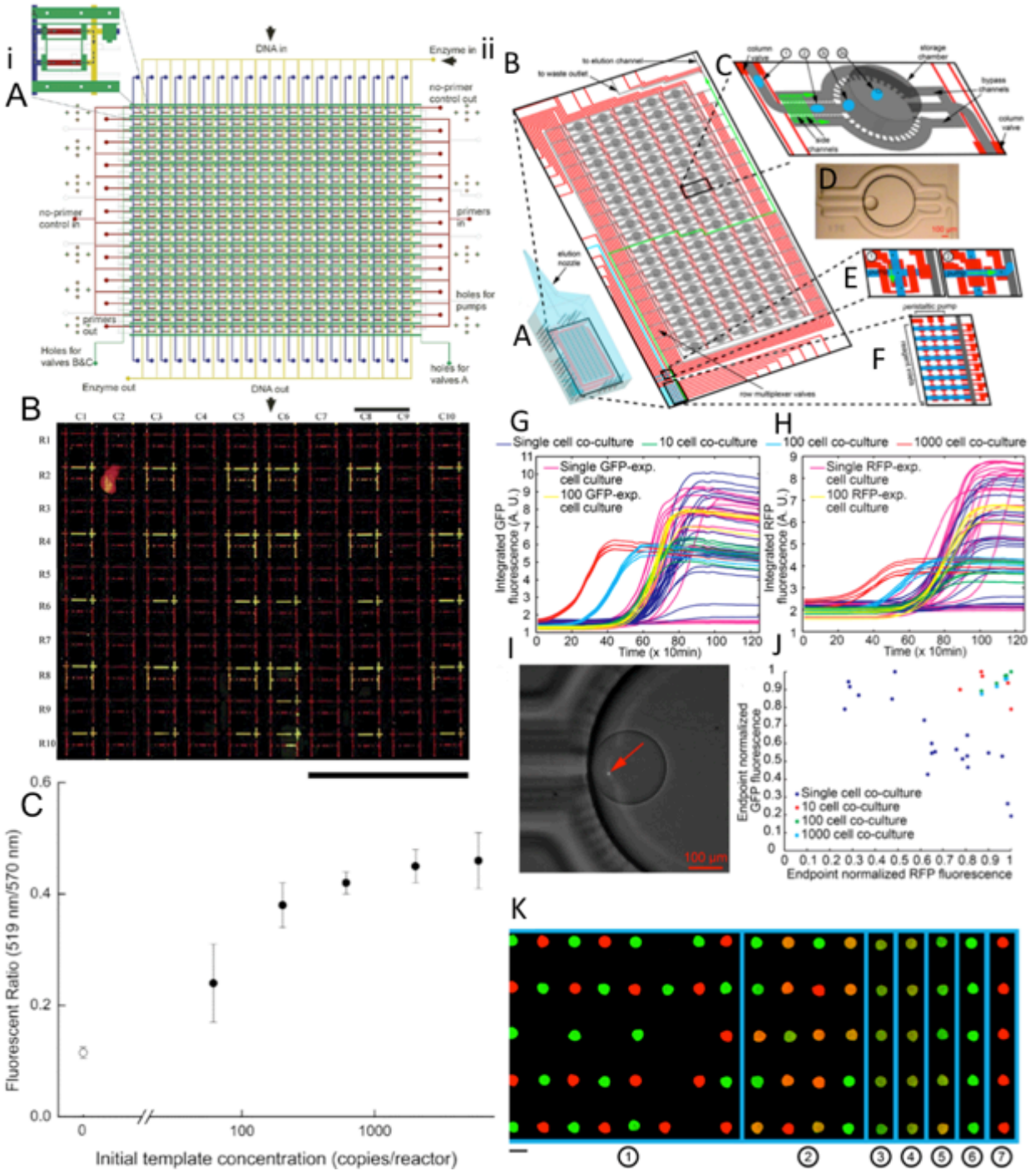
2.1.1.1.2 Nano/Pico litter PCR

The importance of miniaturization as well as reducing the sample size from microliter to pico liter and nano-liter provides the possibility of high throughput analysis as well as multiplexing in a limited volume sample. However, the main obstacle using PCR in the nano-liter volume is that sample may be los due to heat and evaporation. In order to overcome this obstacle, various engineering approaches have been developed. Liu *et al* developed a matrix microfluidic chip with three district function layer [90]. The matrix microfluidic layer was sandwiched between two layers with integrated hydraulic valves and pneumatic pumps. Each vertex of the matrix has 3nl reservoir for PCR reaction and the reagents are loaded from the rows and the DNA templates are injected from the column. The third layer provides 20×20 array of rotary pump to facilitate mixing of the reagents as well as DNA templates. The whole process of PCR using three thermal cycle takes place for 1 hour following by fluorescent colorimetric detection. Despite reducing the aliquot sampling as well as high throughput analysis, fabricating array of pumps and valves makes the platform costly and expensive and does not make the platform suitable for low cost analysis.

Droplet based microfluidics, can form small droplet size with nano-liter using pulse pressure which can be confined in a water-immiscible liquid such as mineral oil and completely prevent sample loss due to the evaporation [91]. For example, Leung *et al* fabricated a programmable, high throughput droplet-based microfluidic chip for cell sorting, cultivation and bacterial genome identification [92]. The chip contains 2D addressable array of chambers, reagent meter, cell sorting platform as well as integrated nozzle for automated collecting the reagent product without cross

contamination and can produce 133 pico-liter volume-droplets for detection and genome analysis of *Salmonella typhimurium*. Shen *et al* developed the slipChip® made out of glass for multiplexed PCR analysis with sample size of 30 nl for simultaneous detection of 5 different bacterial or fungal species [93]. Figure 2.3 shows various microfluidic chip designs for nano/pico litter PCR.

However, these methods have some drawbacks as well. In most of the cases, having three different precise thermal cycles is one obstacle in engineering aspect of LOC as it needs several thermal controller heating systems. Nevertheless, it requires highly equipped laboratory and skilled personnel. Also, the DNA extraction processing as well as purification, which is a highly time-consuming process. In order to apply this method in routine biosensor device for environmental or POC applications, there is an immense need of a compacting the device that can accomplish all the processes in a short period of time. Recently, great efforts has been put to accomplish the development of PCR microchip to meet the requirements of miniaturized micro- total- analysis system (microTAS) and can be applied as diagnostics [94].


i
A
DNA in
Enzyme in
no-primer control out
no-primer control in
primers in
holes for pumps
primers out
Holes for valves B&C
Enzyme out
DNA out
holes for valves A
ii
B
to elution channel
to waste outlet
C
column valve
storage chamber
bypass channels
side channels
column valve
D
elution nozzle
E
peristaltic pump
F
row multiplexer valves
B
C1 C2 C3 C4 C5 C6 C7 C8 C9 C10
R1 R2 R3 R4 R5 R6 R7 R8 R9 R10
C
Fluorescent Ratio (519 nm/570 nm)
0.6
0.4
0.2
0.0
0
100
1000
Initial template concentration (copies/reactor)
G
Single cell co-culture
10 cell co-culture
Single GFP-exp. cell culture
100 GFP-exp. cell culture
Integrated GFP fluorescence (A. U.)
Time (x 10min)
H
100 cell co-culture
1000 cell co-culture
Single RFP-exp. cell culture
100 RFP-exp. cell culture
Integrated RFP fluorescence (A. U.)
Time (x 10min)
I
100 μm
J
Endpoint normalized GFP fluorescence
Endpoint normalized RFP fluorescence
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10 cell co-culture
100 cell co-culture
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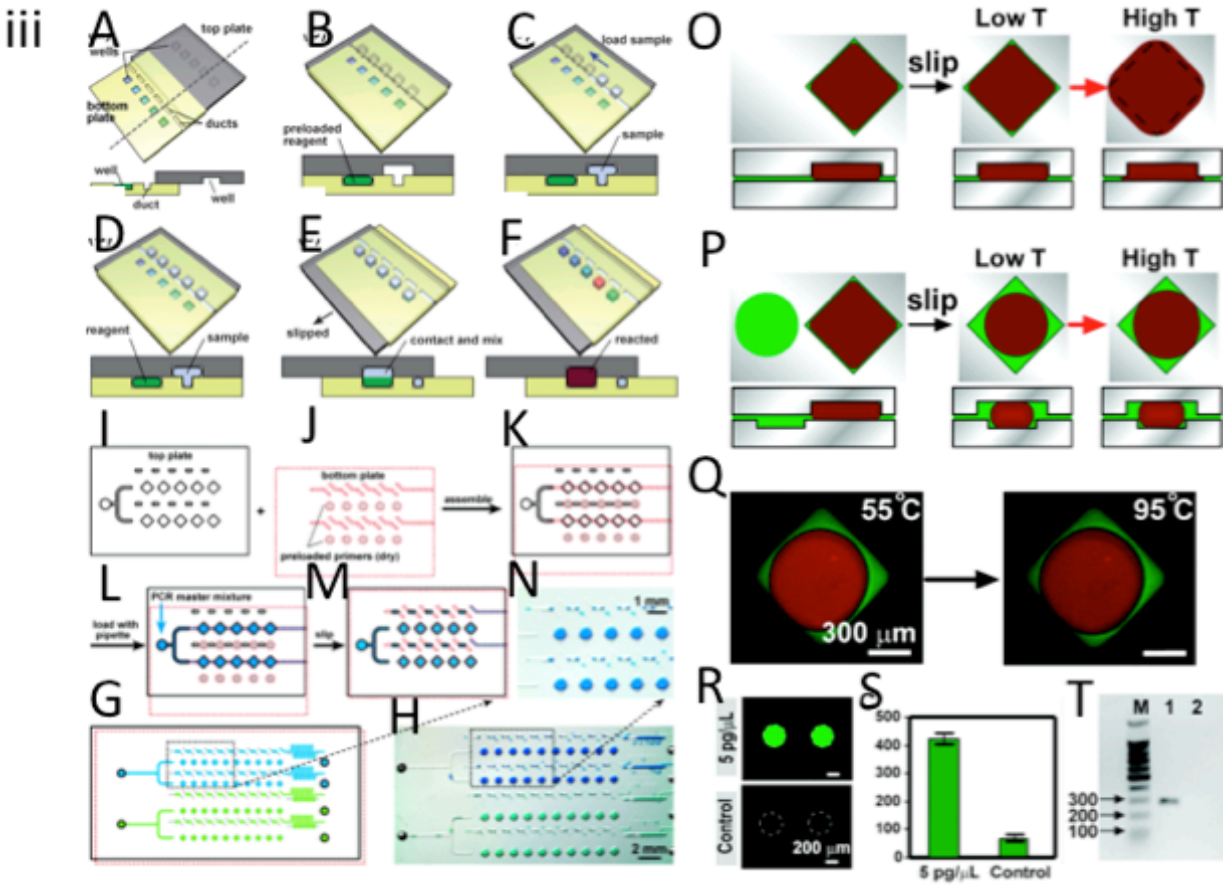

Figure 2.3 **Microfluidic NanoPCR Chips. (i) Microfluidic matrix chambers for high-throughput nano PCR chips. (A) Schematic representation of matrix chip with N=20 and showing the input and output layouts. The small reactor unit has been shown in the upper left and different colors show with green (control line) , DNA polymerase (yellow), primers (red), rotary pump (white). (B) Two color fluorescence emission image for PCR analysis and Yellow represent the positive signal and no primer/ negative control shows red the negative one. The odd rows were filled with the forward primer and column even numbers was filled with reverse primer. The correct combination of forward and reverse primer in one chamber only shows that PCR sample works properly. (C) Fluorescence intensity ratio vs initial cDNA template. The fluorescent intensity was taken after 20 cycle of the PCR. (ii) Programmable droplet based microfluidic for single bacteria analysis. (A) Microfluidic device schematic showing the elution nozzle. (B) Schematic of microfluidic addressable array consist of 95 chamber organized in 19 rows and 5 columns. (C) Schematic of the single chamber for droplet immobilization and coalescence using flow controlled wetting. (1) Before passing the sample to the chamber, the walls were lubricated to prevent wetting. (2) Side channels provide a bypass for the oil, which was shown by green arrows. (3i) Below the critical velocity wetting takes place and the droplet positioned at the entrance of the chamber. (3ii) Above the critical velocity, the droplet does not wet and it goes through the chamber. (D) Micro image of one chamber consist of 2.7 nl of water sample. (E) Schematic of cell sorting module. The cell-sorting module consists of a peristaltic pump and (1) a single cell suspension is pumped down the sorting channel. Later on (2) the cell is encapsulated in a droplet and transported to the array of chamber for further analysis. (F) Schematic of reagent-metering system.**

On chip culturing of single bacteria in a chamber microfluidic. Growth trend of each sample with different cell numbered based on (G) green fluorescent protein (GFP), and (H) red fluorescent protein (RFP) with respect to time. (I) Combined bright field and fluorescent image of single RFP expressing cell in a droplet. (J) Normalized plot of GFP and RFP fluorescent in scattered micro-channel for mixed cultures seeded with different number of both bacteria strain. (K) Confocal image of cultured array which was stored in droplets, with various seeded cells (1) a single cell, (2) a single cell of each strain, (3) 1000 cells of each strain, (4) 100 cells of each strain (5) 10 cells of each strain, (6) 100 GFP expressing cell (7) 100 RFP expressing cell. (iii) SlipChip schematic representation. (A-B) Schematic of the preloaded bottom wells, ducts at the bottom level as well as wells at the top chip. (C-D) Loading the sample in the duct and bottom wells as well as upper well chip. (E-F) Slipping the top layer to the bottom layer disconnects the upper samples from the duct and overlap bottom and top samples together which cause the mixing two samples together. (G-N) Schematic of the digital PCR using SlipChip.(O) Analysis of thermal expansion of chambers during thermal cycling by using different geometries. The master mix PCR solution (red) and mineral oil (green) was filled in the chamber. By slipping, mineral oil surround the PCR master mix in the low temperature (low T) and by increasing the temperature, the solution enter between the gap between the two plates of the SlipChip (High T) (P) Top and side view of schematic representation of shallow circular well. By slipping, the aqueous PCR mater mixes form to droplet due to surrounding by hydrophobic chamber. By increasing the temperature in the chamber, mineral oil moves outside of the well and goes between the two plates of the chip (High T). (Q) Micrograph of experiment, which was represented on (P). (R) Micro-image of fluorescent intensity in PCR result of S. aureus DNA. Negative Control (NC) shows no fluorescent intensity. (S) Analysis of fluorescent intensity between amplified PCR sample and NC.(T) Gel electrophoresis image of PCR amplicon in SlipChip with 100 bp DNA ladder . The first column contains the sample from PCR product taken from SlipChip and shows 270 bp band. The second column consist the sample from negative control, which shows no band. Adopted with the permission from [91-93].

2.1.1.2 Isothermal amplifications

Limitations of PCR have led to the use of isothermal amplification, which provides genomic amplification in a rapid and low-cost manner at single temperature only. Various isothermal amplification methods have been developed over the past decade, which meets the demand, of needs of nucleic acid amplification. Isothermal amplifications are different from PCR techniques in a sense that the rate of enzyme activity is a limiting factor for amplification whereas in PCR, it is the rate of cycling. Consequently, it is unlikely for microfluidic isothermal amplification to increase the rate of enzymatic reaction by changing the thermal cycling.

2.1.1.2.1 Nucleic acid sequence based amplification (NASBA)

Nucleic acid sequence based amplification is an isothermal amplification technique, which amplify the target gene based on rotavirus RNA replication. NASBA is highly

specific for the target RNA sequence and has gained popularity for amplification in clinical and environmental applications since introduction in 1991[95]. The whole process is based on using two primers and three reverse transcriptase enzymes, namely, R Nase H, T7 RNA polymerase. The first primer is attached to the complementary site at 3' end of the template. Subsequently, reverse transcriptase destroys the RNA template and forms the complementary DNA strand and the second primer attaches to the 5' site of the cDNA template. T7 RNA polymerase continuously polymerizing the templates which takes place at 41°C. The NASBA amplicon can be detected by various means and methods such as real time optical technique, lateral flow assay, electrochemiluminescence, or electrochemical techniques. The simplicity, sensitivity as well as amplification at low temperature make it highly desirable for portable diagnostic devices. However, using three enzymatic reaction as well as, using RNA as a template makes this technique limited in some application that use DNA as the template.

2.1.1.2.2 Rolying cycle amplification (RCA)

Rolling cycle amplification (RCA) is another amplification technique that employs one enzyme and one primer only. The padlock probe first anneals to the single stranded DNA, forming a circle with small gap that is sealed by ligation. The RCA reaction is initiated using a polymerase enzyme. Subsequent strand displacement forms a long chain with the template of ssDNA in a sphere shape. RCA requires 37°C temperature for amplification in less than an hour. Simplicity as well as using one primer makes it very attractive method for amplification. However, lack of specificity is a main drawback of this system and development of specific molecular probe is required to increase the assay's precision.

2.1.1.2.3 Strand displacement amplification (SDA)

Rolling cycle amplification (RCA) is another amplification technique that employs one enzyme and one primer only. The padlock probe first anneals to the single stranded DNA, forming a circle with small gap that is sealed by ligation. The RCA reaction is initiated using a polymerase enzyme. Subsequent strand displacement forms a long chain with the template of ssDNA in a sphere shape. RCA requires 37°C temperature

for amplification in less than an hour. Simplicity as well as using one primer makes it very attractive method for amplification. However, lack of specificity is a main drawback of this system and development of specific molecular probe is required to increase the assay's precision.

Strand displacement amplification (SDA) uses two primers for amplification and like NASBA it requires multiple enzyme to proceed amplification. SDA requires multiple enzymes (e.g., a thermo-stable polymerase and restriction enzyme). However, it requires multiple primers in a specific order (four total) to amplify the target sequence and displace the copied sequence. An additional difference is its use of a chemically modified deoxynucleotide base (thiolated dCTP). The amplification process uses two phases: the target generation phase and the amplification phase. In the target generation phase, an engineered primer that has a restriction enzyme site incorporated into it binds to its complementary target and initiates strand synthesis using a thermo stable polymerase. A bumper primer displaces the strand generated from the primer containing the restriction enzyme site. Because the newly generated strands incorporate thiolated dCTP, they are not susceptible to restriction enzymatic digestion. A thermo-stable restriction enzyme introduces a single-strand nick in the double-stranded molecules. The thermo-stable polymerase then extends the new strand and thereby displaces the strand 3′ to the nick. Ultimately, new strands that incorporate this restriction enzyme site lead to the exponential generation of target copies.

2.1.1.2.4 Helicase dependent amplification (HDA)

Helicase-dependant amplification (HDA) is another isothermal amplification technique, which makes the helicase enzyme available to open the double stranded DNA in order to facilitate the primers hybridization, extention and formation of two copies. This mechanism operates at the same temperature range as LAMP, but it is simpler because it requires two enzymes and like PCR, only two specific target oligos. However, compared to the LAMP method, it takes longer time to copmplete.

2.1.1.2.5 Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is one of the most common isothermal amplification methods. The final result can be analyzed either by fluorescence intensity

measurement or by the naked eye for turbidity due to the precipitation of magnesium pyrophosphate as a by-product, which makes it suitable for locations with limited resources. The entire LAMP method is accomplished at a static temperature (60-65 °C). The target DNA is amplified by the use of multiple primers, including loop primers and *Bst* polymerase, resulting in an amplification rate of 10^9 target nucleotides (i.e., 1 billion DNA copies) within 15–60 minutes at a single, static temperature. The whole principle of LAMP is based on using one polymerase enzyme with de-strand displacement activity and 4-6 sets of primers which was first published in 2000 [96]. There are two outer primers (F3, B3) and two inner primers (FIP, BIP). FIP and BIP are forward and backward two inner primers. LAMP amplification has two initial step and cycling step. In the initial step all four primers take part to the amplification. Yet, in the cycling step only inner primers continue the amplification. The mechanism start with the inner primer of FIP which hybridize in the specific region and outer primer hich is a bit shorter than FIP hybridize to the specific region of DNA and with the *bst* polymerase with displacement activity initial de-stranding the dsDNA into ssDNA template. Later on, from the other side of the template, BIP and B3 hybridize to the template and form a loop structure. This loop structure acts as the initial template for the cycling step. By subsequent self-prime annealing of primers a billion copies of the loop structure molecule is formed with the cauliflower loop structure. The use of four–six sets of primers makes this technique very specific to the target DNA in comparison with other amplification technique such as PCR. Figure 2.4 shows the schematic of various isothermal amplification techniques. Recent advances in LOC devices based on LAMP have been developed over the past years and it is reviewed in Appendix I.

2.1.2 Electrochemical techniques

Electrochemical biosensors use various electrochemical techniques to detect bio-analytes, which are mainly potentiometric, voltametric and impedance. Potentiometric measures collected potential/charges at the surface of the electrode with respect to a reference when there is no current flow and provides data on ion activity. Impedimetric sensors measure impedance usually resistance or capacitance change. All the devices developed in this project are based on Voltammetry. The basic of voltammetry depends

on measurement of the generated current on the surface of a sensor in response to the applied voltage. Generally, most electrochemical biosensors are composed of three-configuration electrodes of working electrode (WE), Counter electrode (CE) and reference electrode (RE). WE is the electrode where electrochemical reaction takes place. The purpose of CE is to complete the electrical circuit which is responsible of transfering the electrochemical signal from WE to the Potentiostat, completing the circuit to perform current measurement and control potential variance that cause damage of sensitive surface of WE.

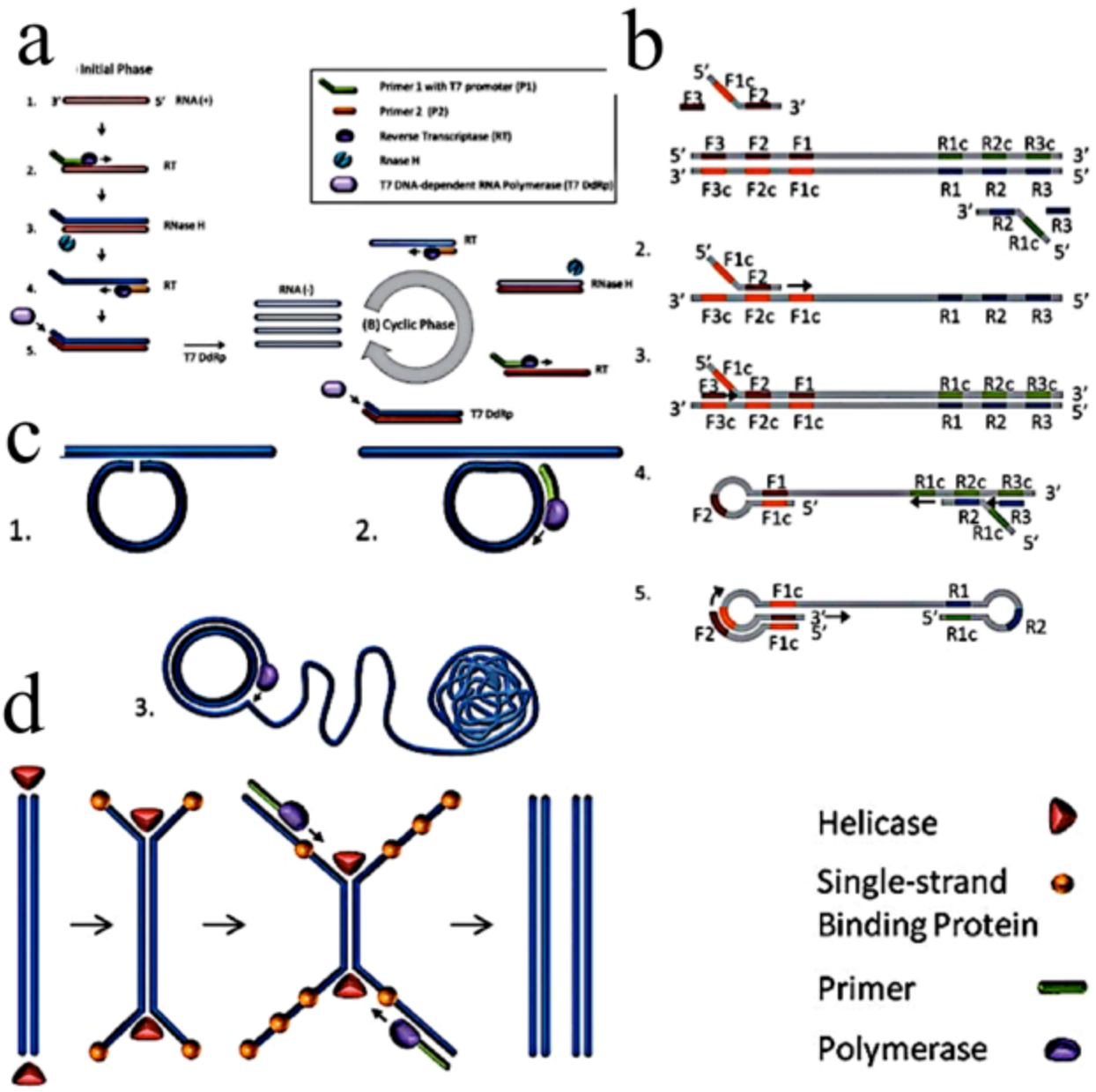

Figure 2.4 **Schematic representation of various isothermal amplification reaction processes. (a) Schematic process of NASBA has been shown. It mimics retrovial replication mechanism (1-6). First the RNA template forms into the cDNA. Then the template is amplified using T7 polymerase enzyme. (b) Schematic demonstration of LAMP process. Double stranded DNA is single stranded using DNA polymerase with the strand displacement activity. Then 6 different primer sets are hybridized to the specific target of the single stranded template and form a loop shape template. Subsequent self- primed annealing produces the billion copies in less than an hour and forms a cauliflower shape structure. (c) Schematic representation of RCA. Padlock probe annealed to the ssDNA forming the circle with the gap that filled with the ligation. The RCA reaction is initiated using a polymerase enzyme. Subsequent strand displacement forms a long chain with the template of ssDNA.(d) Schematic of HDA process. The double stranded DNA is un-wound using a helicase enzyme. Two primers are hybridized to the target template using a**

polymerase enzyme. Two copies of the double stranded target are produced. (Reproduced with the permission from [97])

RE provides stable potential reference point to which a potential is applied against the redox reactions of interest to the surface of WE. It usually consists of Ag/AgCl because its easy to fabricate, is of low cost, non toxic and has proper compatibility with bio-analyte samples. Generally, a potentiostat applies the voltage to the WE and measure current flowing from WE. Various voltammetry techniques such as cyclic voltammetry (CV), Linear sweep voltammetry (LSV) and square wave voltammetry (SWV) were used in this project, which we will briefly take an overview.

CV is one of the most common techniques. The principle is based on applying the linear sweep voltage with respect to RE. The obtained current of electro active species (for example: $Fe(CN)_6^{3-}$) is plotted with respect to applied voltage as shown in Figure 2.3. Various points in C-V graph shows different status of oxidation/reduction of $Fe(CN)_6^{3-}$. At the start point of (a) there is not enough potential to reduce $Fe(CN)_6^{3-}$. At (b) the potential is enough to reduce $Fe(CN)_6^{3-} + 1e \rightarrow Fe(CN)_6^{4-}$ (b-d). The cathodic peak starts to decrease where all the $Fe(CN)_6^{3-}$ are consumed in diffusive region and currents are obtained by transporting new $Fe(CN)_6^{3-}$ from the surrounding into the diffusive region. Then the reverse scan is performed and provides enough potential to reduce $Fe(CN)_6^{3-}$ in (f-g) region. At point h the potential of WE has been reduced enough so that $Fe(CN)_6^{4-}$ can be oxidized (h-j). The anodic peak starts to increase rapidly to oxidize and consumes all the $Fe(CN)_6^{4-}$ in a diffusive layer near WE. Then, it starts to decrease the anodic current due to the oxidizing of the remaining $Fe(CN)_6^{4-}$ in the diffusive layer. Figure 2.5 A&B shows the schematic of CV diagram.

CV can provide useful information about the characteristic of a bio-analyte on the surface of WE. Concentration of target analyte can be determined by oxidation/reduction peak. In addition, CV can determine electro-active surface of various analytes of WE and the compatibility of electron transport to the electro-active moieties. Reversibility of redox reaction can also be analysed by CV as irreversibility can easily be distinguished by disappearance of reverse current.

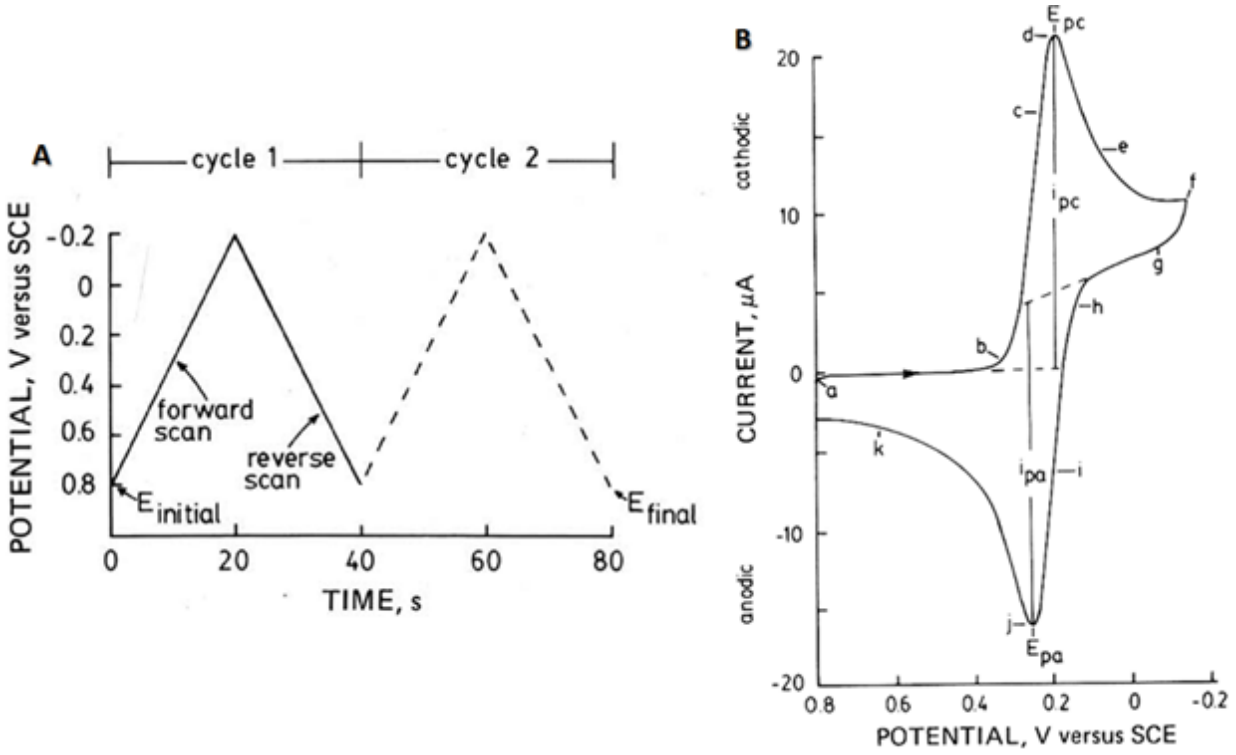

Figure 2.5 Schematic diagram of CV. (A) applied voltage. (B) CV diagram[98].

Linear sweep voltammetry (LSV) is similar to CV, except the voltage is scanned from lower to upper limits and the current is measured and plotted with respect to the applied voltage. The obtained current peak can be calculated by Randles-Sevic equation (25°C) as follows [99]:

$$i_p = (2.69 \times 10^5)\, n^{3/2} A\, D^{1/2} C_i v^{1/2}$$

where i_p is the current peak, n is number of electron transferred in redox reaction, A is electrode area, D is diffusion coefficient, C concentration, v is the scan rate.

Square wave voltammetry (SWV), is another voltammetry technique in which a symmetrical waveform imposed on staircase potential is applied on WE, and the current is sampled twice, once at the end of forward pulse and once at the end of reverse one. The difference between the two scans is plotted vs. the base staircase potential. The peaks in the differential current is proportional to the concentration of the redox species and is obtained by:

$$\Delta i_p = \frac{nFAD_0^{1/2}C_0}{(\pi t_p)^{1/2}}\Delta\Psi_p$$

where t_p is the pulse width, $\Delta\Psi_p$ is the dimensionless parameters gauges the peak height in SWV relative to the limiting response of normal pulse voltammetry. Electrochemical Impedance spectroscopy (EIS), is a resistance measurement of a sample by applying the AC voltage and obtaining AC current from the cell. In a linear system, the current response of the sinusoidal voltage will be sinusoidal at the same frequency but shifted in phase. Considering the excitation signal of $E = E_0 \sin\omega t$ and $\omega = 2\pi f$, the response current is shifted φ with a magnitude of I_0. The current value can be obtained by $I = I_0 \sin(\omega t + \varphi)$. Magnitude of the impedance can be calculated by:

$$Z = \frac{E}{I} = \frac{E_0 \sin\omega t}{I_0 \sin(\omega t + \varphi)} = Z_0 \frac{\sin\omega t}{\sin(\omega t + \varphi)}$$

The impedance can be expressed as the real and imaginary part plotted in Nyquist diagram by changing the definition of $E = E_0 \sin\omega t = E_0 e^{i\omega t}$ and $i = i_0 e^{j\omega t - \varphi}$.

$$Z = \frac{E}{I} = Z_0 e^{j\varphi} = Z_0(\cos\varphi + j\sin\varphi) = Z' + jZ''$$

The main shortcoming of Nyquist plot is that it does not provide information about frequency in which the impedance was used to plot the data point [100]. Another useful technique to analyze EIS, is to plot Z vs. log frequency which is called Bode diagram. EIS can be analyzed by equivalent circuit. The equivalent circuit analysis implemented on this thesis work has been explained in Chapter five.

2.1.3 Detection Mechanism

Sensing and quantification of bio-analyte is extremely important issue in POC diagnostic technology. Traditional methods such as gel electrophoresis and membrane blots lag behind the demand for detection of bio-analyte for more information in a shorter time manner. Biosensors offer the promising approach for rapid, faster, simpler and lower cost nucleic acid assay. Biosensor devices consist of major subsections of bio-analyte,

analyte recognition, transducer and readout. Range of various sensing technologies such as optical, surface plasmon resonance, electrical and mass spectroscopy have been employed for detection of various analytes. Despite providing promising technology to detect the bio analyte target, some of these techniques are expensive, complicated, and slow and are not disposable. Therefore, it cannot be implemented in POC diagnostic technologies. In contrast, electrochemical and colorimetric detection have the tremendous market growth for POC devices, as well as, the possibility for miniaturization and analysis of minute sample volume in a rapid, sensitive and specific manner.

2.1.3.1 Electrochemical Detection

Electrochemistry provides a robust and precise tool for detection of bio-analyte in solution and has been used for NAs and PCR amplification product detection for quite a while. It has many advantages over other detection techniques e.g optical techniques such as high sensitivity, rapidity, easy to use, portable, disposable, low cost and able of detecting a bio-analyte in highly turbid solutions. Electrochemical detection can be divided into two different subsection of direct and indirect detection techniques.

The direct detection of NAs consists of electrochemical detection of DNA hybridization usually involves in current response resulting from Watson-Crik base pairs NA recognition event under controlled potential conditions [101]. Such hybridization can be detected usually by oxidation or reduction of base pairs and the obtained current can be increased by binding electro-active indicators to DNA duplex such as redox active molecules or measurements of other electrochemical parameters such as capacitance or conductivity. There are major steps for electrochemical bio-sensing of DNA hybridization namely the design of NA recognition layer, the hybridization event as well as transforming the hybridization event into the electrochemical signal. The success achievement of this device requires a proper probe surface immobilisation step, which plays the critical role in sensitivity and high specificity of the assay. The probes are usually short oligonucleotides (25-40 mers) which are capable of hybridizing with specific region of NA [102].

Common probe immobilisation techniques are self assembled monolayer (SAM) of thiol group on gold surface electrode [103], biotin functionalized group to avidin surface [104], use of conducting polymer [105], carbamide covalent binding to activated surface [106], adsorption of carbon paste or carbon electrode or thick film carbon electrode [107]. For example, DNA can be immobilized by developing the thiol group on the gold surface and using thiol derivation single stranded oligonucleotide and 6-mercapto-1 hexanol. The thiol group causes the single stranded oligonucleoticd probe to be positioned in a standing situation. Later on, using a monolaye 6-mercapto1-hexanol (Hydrophilic linker) causes the avoidance of non-specific adsorption. Figure 2.6 schematizes DNA hybridization on the gold electrode surface. The nature of intermolecular binding as well as quality of DNA hybridization can be measured by surface characterisation techniques such as XPS, reflectance IR ellipsometry.

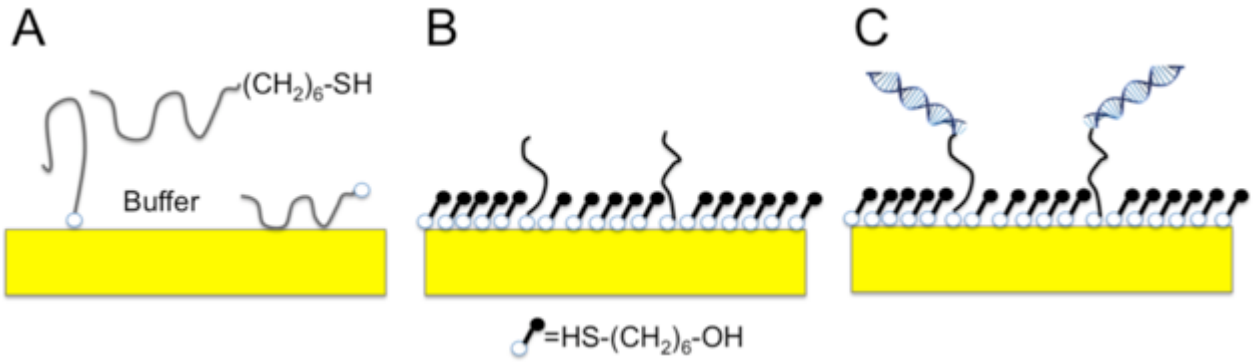

Figure 2.6 **Schematic representation of DNA hybridiation on gold substrate. (A) Thiol-linked single stranded oligonucleotide was immobilised on the surface. (B) Monolayer 1-mercapto6-hexanol was formed and make the nucleotid probe in a « up right « position. (C) DNA hybridization (Levicky 1998).**

The development of electrochemical detection in a biosensor needs proper attention to experimental parameters such as PH of the sample, temperature, amount of various salt concentrations as well as the length and base pair composition (G+C percentage) in the oligonucleotide probe [108]. The hybridization usually can be detected by increasing the current signal obtained from redox indicator binding. Such redox molecules are the small electro-active DNA intercalator which also bind to the DNA grooves. By increasing the concentration of these active molecules on the surface of electrode during hybridization, the electrochemical response increases due to oxidation (or reduction)

phenomenon. This can be monitored with various electrochemical techniques such as linear sweep voltammetry (LSV) or square wave voltammetry (SWV)[109].

Various redox molecules have been used for NA detection. For example, Wang *et al* used $Co(phen)_3^{3+}$ redox in carbon paste using chronopotentiometry with PNA probe for detection of single base in P54 gene[110]. Ferrocyanide naphthalene diimide (FND) is another redox molecule that binds tightly to the double stranded DNA and has very low affinity to single stranded probe [111]. Another alternative method for DNA detection is using *Ferrocene tag*, which has been used initially in a CMS eSensor (Motorola Inc), using SAM technique for immobilising the molecular probe for detection of mismatches in *Hfe* gene as a model [112]. In this assay, firstly, the electrode array was modified with a probe that perfectly matches with the mutant allele of *H63D.* Amplicon containing the mismatches was then hybridized with the probe in the presence of electrochemically active *Ferrocene tag* label. The electrochemical detection was implemented using voltammetry technique and the signal to noise ratio was optimised at the operational hybridization temperature (48°C). Interestingly, Fan *et al* developed reagent less Ferrocene tag DNA loop structure. Such molecular beacon labeled DNA caused the induction of the signal of hybridization due to the fact that, in absence of the target, the Ferrocene tag brings down the oligonucleotide probe closer to the surface of electrode. Yet, in the hybridization event a large signal was achieved because the Ferrocene is separated from the electrode surface [113]. Figure 2.7 shows the schematic of the Ferrocene molecular beacon labeled DNA.

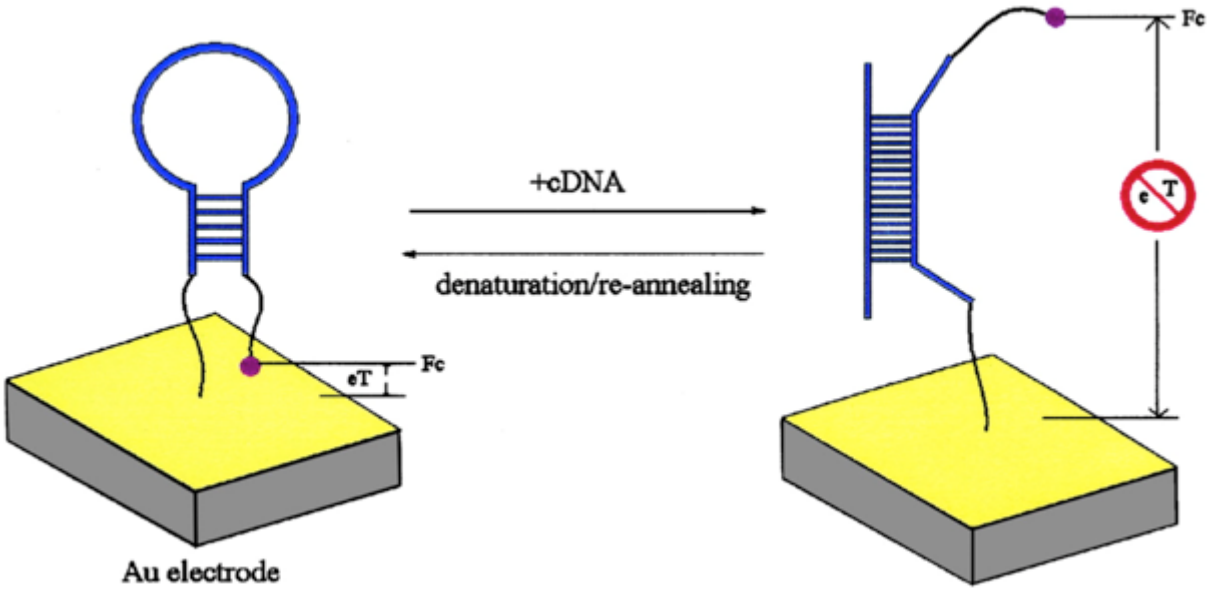

Figure 2.7 **Schematic of electrochemical DNA hybridization detection based on molecular beacon. The hybridization changes the distance of the label from the surface of electrode (Reproduced with the permission from [113]).**

Direct redox detection also can be used for NA detection. Redox active molecules can bind to ds-DNA , ss-DNA or PCR amplicon. In the absence of DNA in the solution, there is a high oxidation peak due to existing of high concentration of redox on the surface of electrode. By adding NAs, redox binds to minor grooves of DNA, which cause the major reduction in the concentration of free redox in the solution.

Label free detection is another interesting method that has caught significant attention in NA detection. Generally, electro-activity of DNA was shown over 40 years ago by Plaćek and his coworkers [114]. However among all the base pairs Guanine has been shown to be the most easily oxidized base pair and is the most suitable for label-free electrochemical detection of NAs. The Guanine oxidation is the irreversible process which has been shown previously using pulse voltammetry technique[115] and consists of two consecutive steps. The first step involves an irreversible two electron/two proton oxidation to 8-oxoguanine (8-oxoG) at E_p=0.8V (PH=4.5) with respect to Ag/AgCl reference electrode. The second part is a reversible oxidation of 8-oxoG at 0.55V, which takes place at the surface of electrode and can be shown in Figure 2.8.

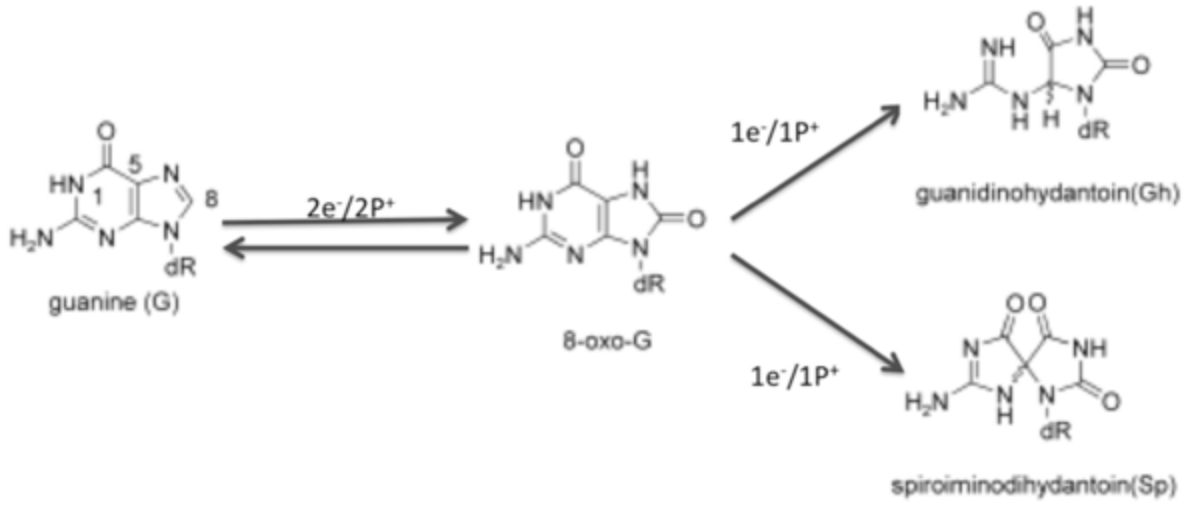

Figure 2.8 Schematic of the guanine oxidation.

NA detection can also be implemented in a non-immobilisation technique. In this method, redox intercalator is detected at the surface of the electrode before adding the NA in the sample. By adding NA, redox molecule intercalates with NA, resulting to decrease in the redox concentration on the surface of electrode. Figure 2.9 shows the schematic process of NA detection based on intercalating with redox.

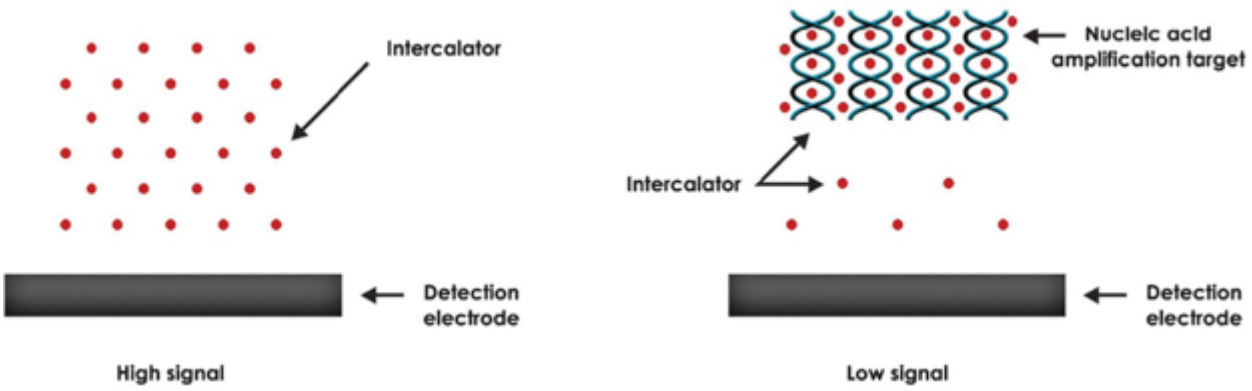

Figure 2.9. Schematic of electochemical detection of NA. When NA is not present , the signal is high due to all redox interclator molecule are free to react on surface of electrode. When NA is present, the signal is low as significant amount of redox binds with NA[116].

2.1.4 Microfluidics

Microfluidics is the manipulation and analysis of minute volume of fluid in micro-scale conduits [117]. It has emerged as a powerful technology with many established and relevant applications within biological sciences. In recent decades, a remarkable

improvement has been made in the field of miniaturization of existing methods as well as the development and integration of novel analytical approaches. Scientists have made it possible to miniaturize all kinds of systems—mechanical, fluidic, electromechanical and thermal—down to sub-micrometric sizes [118, 119]. This scaling provides superior control in a wide range of reaction parameters such as rapid flow mixing rate, short resistant time and precise reaction temperature [120]. These properties are the result of fast heat transfer in micro-scale due to better temperature profile and better mass transfer due to effective convection and short mixing time scale. Microfluidic devices, offer several distinct advantages such as decreasing reagent consumption, easy handling, faster analysis, reducing the reaction time by increasing the surface to volume ratio and decreasing cost. Figure 2.10 shows the length scale of various biomolecules and micro-organism with 1 mm as a scale.

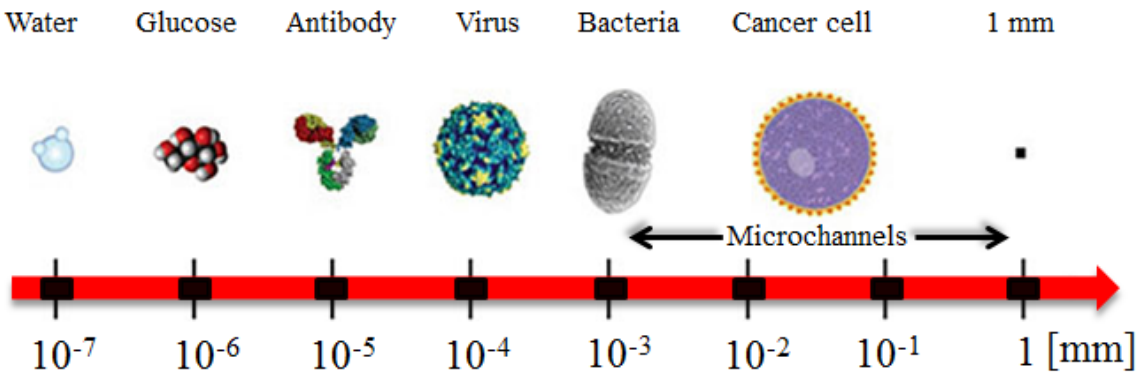

Figure 2.10 **Schematic of various biomolecules and their length scale ranging from angstrom (water) to tens of micrometer (cancer cells)[121].**

3 CHAPTER THREE: MICROFLUIDIC ELECTROCHEMICAL ASSAY FOR RAPID DETECTION AND QUANTIFICATION OF *E.COLI*

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ABSTRACT

Microfluidic electrochemical biosensor for performing Loop-mediated isothermal amplification (LAMP) was developed for the detection and quantification of *Escherichia coli*. The electrochemical detection for detecting the DNA amplification was achieved using Hoechst 33258 redox molecule and linear sweep voltammetry (LSV). The DNA aggregation and minor groove binding with redox molecule cause a significant drop in the anodic oxidation of LSV. Unlike other electrochemical techniques, this method does not require the probe immobilization and the detection of the bacteria can be accomplished in a single chamber without DNA extraction and purification steps. The isothermal amplification time has a major role in the quantification of the bacteria. We have shown that we could detect and quantify as low as 24 CFU/ml of bacteria and 8.6 fg/μl DNA in 60 min and 48 CFU/ml of bacteria in 35 min in LB media and urine samples. We believe that this microfluidic chip has great potential to be used as a point of care diagnostic (POC) device in the clinical/hospital application.

3.1 Introduction

Escherichia coli, one of the most versatile bacteria in the world [122], has a number of unique features. It can exist as a beneficial probiotics in the commensal of the digestive tract [123] as well as a poisonous pathogen present in food and the environment. This bacteria is the major cause of many nosocomial diseases such as food poisoning, Meningitis [124] and Urinary tract infection (UTI) [125-127].

The conventional method for detection and identification of bacterial cells is culture technique. However, due to the time consuming (few days to several weeks) and labor-

intensive protocols, it is not practical for rapid point of care (POC) diagnostics. Enzyme-linked immunosorbent assay (ELISA) is another technique that has widely been used [128]. However, it is a semi-quantitative, multiple steps and has poor sensitivity which limits its reliability and accuracy. Rapidity, accuracy and sensitivity are the foremost elements for POC diagnostics devices[10]. Recently, DNA hybridization and gene amplification techniques have widely been investigated for the rapid detection of *E.coli* with higher sensitivity and specificity [129]. Among them, molecular diagnostic based on gene amplification techniques such as polymerase chain reaction (PCR) has become a well-developed method for accurate detection of bacteria. The sensitivity of PCR for *E.coli* DNA detection is 1 ng/μL (4 hours) with high specificity [130]. Nevertheless, PCR requires demanding hardware capabilities such as precise temperature control, temperature ramping and cycling [129]. In addition, time-consuming processes for sample preparation, nucleic acid extraction and post processing fluorescence imaging make it difficult and complicated to be applied as a convenient technique in point of care diagnostic device[97].

Consequently, isothermal amplification techniques, which implement the amplification of the target nucleic acids in a constant temperature, have attracted significant interest for the rapid detection of bacteria[97]. However, all these amplification techniques have limitations. They need either multiple enzymes for strand displacement of the DNA template or precision instruments for amplification or elaborative method, due to low specificity [96].

LAMP is an alternative isothermal amplification technique that is accurate, fast, cost-effective with high sensitivity and high specificity [96]. It can amplify a few copies of DNA to 10^9 copies in less than an hour at 60-66°C [131]. LAMP process have been used for detection of various pathogens such as *H1N1 Influenza* [129], *Staphylococcus aureus* [132], *E. coli [133]*, *West Nile* Virus [134], *Salmonella enterica* [135]. Moreover, LAMP amplicons can be measured optically or colorimetric using DNA-interacting dye[136]. However, conventional florescent-based optical measurements need flurochrome labeling and expensive fluorescence analysis equipments. Colorimetric assays are less sensitive and they only provide qualitative result and analyze the existence of the DNA template in the samples [128, 137].

Despite the attractiveness of LAMP technique, there are still some problems and drawbacks, hindering its application in routine POC diagnostics. The sample preparation and nucleic acid extraction need precise protocol and highly skilled personnel and is a time-consuming procedure. In addition, DNA amplification is labor-intensive and requires laboratory based equipment and bulky controllers. Therefore, there is an immense demand for a compact device that can accomplish all the processes in a short period of time. This has led to the advance of microfluidics, which has been widely applied in the molecular diagnostics and gene amplification in the past years [8, 138]. Microfluidics refers to the manipulation of minute, amount of liquid, which takes place in closed micro-scale channels [64, 139]. It reduces the consumption of the costly reagents. By increasing the surface to volume ratio, it enhances the undergoing reactions. Microfluidic is a reliable tool for the development and application of miniaturized biosensors. Yet, it requires a robust sensing technique in order to be implemented for the labeling rules [140]. Over the past decade, many sensing techniques such as optical, colorimetric or electrochemical methods have been developed to provide new approaches to monitor biorecognition. Among them, electrochemical-based techniques provide sensitivity, selectivity, and low cost detection of the amplified DNA sequences. In fact, the advantages of the electrochemical detection over the optical detection techniques include not only the inherent miniaturization and portability, but also the independence from sample turbidity, extremely low-cost/low-power requirements and compatibility with microfabrication technology [140, 141].

Here, we integrate the merit of the LAMP, microfluidic and electrochemical detection to construct an inexpensive portable device for the rapid detection of *E.coli*. First, the design and fabrication of the microfluidic device is demonstrated. Then, the electrochemical detection of *E.coli* on chip is presented, followed by an analysis of the sensitivity and specificity of the chip. The amplification time is optimized and the chip is calibrated and established as the robust, consistent diagnostic device.

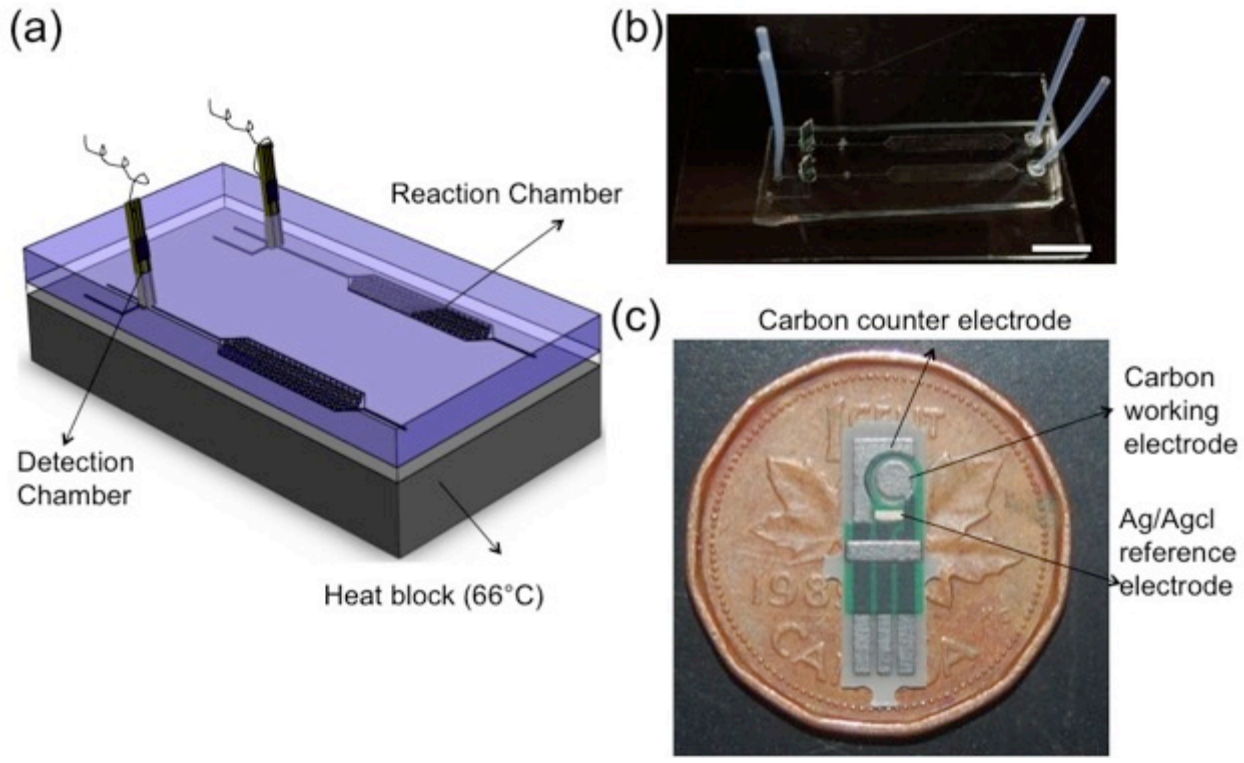

Fig. 3.1 **Schematic representation of the microfluidic chip for E.coli detection. (a) Microfluidic chip composed of a heat block as the heat source to provide 66 °C, glass slide as the substrate and PDMS chip. The PDMS chip contained two parallel microfluidic chips for negative control and DNA detection. The microfluidic chip composed of a reaction chamber, an active valve (are not shown here) and an electrode chamber. (b) Image of the microfluidic chip composed of two parallel microfluidic chip and capillary tubes that connect chip to the syringe pump (The syringe pump is shown here). The scale bar is 15 mm. (c) Micrograph of the DEP chip used for electrochemical detection.**

3.2 Materials and Methods

3.2.1 Bacteria preparation and DNA extraction

E.coli was freshly prepared and grown overnight (12 hours) in 2% LB broth media. *E.coli* DNA was extracted using GenElute™ DNA extraction kit from Sigma Aldrich (MO,USA). The final extracted DNA solution contained 35.3 ng/µL concentration. Hoechst 33258 ([20(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,50-bi (1Hbenzimid-azole)]) was purchased from Sigma Aldrich (MO,USA). The Hoechst solution was prepared with a concentration of 100 µM and was kept in the dark at 4 °C.

3.2.2 Microfluidic Chip

We have fabricated a microfluidic chip using soft lithography techniques which is made of Poly (dimethyl siloxhane) (PDMS) [142]. The PDMS was purchased from Dow Corning Corporation (Michigan, USA). The heat block was made from aluminum and the controller unit was purchased from Omega Engineering Inc. (USA). The glass slides were obtained from VWR International (Quebec, Canada). Disposable electrochemical printed (DEP) chips were fabricated of carbon working electrode, carbon counter electrode and Ag/AgCl reference electrode and were purchased from Bio device Technology (Ishikawa, Japan). The chip was connected to syringe pumps (New Era Pump Systems Inc., New York, USA) by capillary tubes (Upchurch Scientific, USA).

3.2.3 LAMP reaction

We used the modified protocol as mentioned previously [133] to detect the *malB* gene in *E.coli*. The primers were purchased from IDT (USA). 25 μL of reaction master mix contained 3.0 μL of 0.6 M Betain (Sigma Aldrich, USA), 2.5 μL of 10× Thermopol buffer, 0.75 μL of 3 mM $MgSO_4$, 2.0 μL of *Bst Polymerase* (1600 units), 8000 U/ml (New England BioLab, USA), 0.4 mM concentration 0.4 μL dNTP (BioShop Canada Inc., Ontario, Canada), 5.75 μL of distilled water, 0.2 μL of 0.2 μM outer primers (F3, B3), 1.8 μL of 1.6 μM inner primers (FIP, BIP) and 0.8 μL of 0.8 μM loop primers (Loop F, Loop B).

3.2.4 Electrochemical Detection

All electrochemical experiments were implemented using the LSV method. We used the Autolab PGSTAT 101 (Eco Chemie, The Netherland). The potentiostate was configured with computer using the NOVA 1.6 software. The measurements were repeated at least three times and all the experiments were run at room temperature (22°C - 27°C). The step potential and the scan rate were 0.00244 and 0.1 V/S, respectively. DNA was detected by using the mixture of the LAMP solution with the PBS buffer and 20 μM of the Hoechst 33258 molecule.

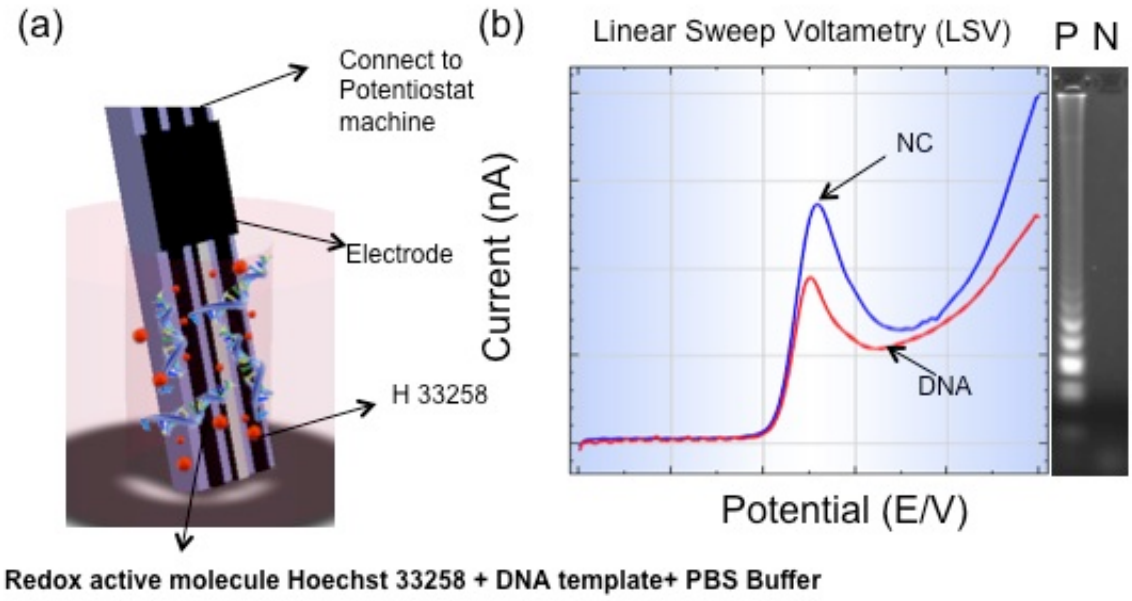

Fig. 3.2 **Schematic of the electrochemical detection in the electrode chamber on microfluidic chip. (a) The redox active molecule Hoechst 33258 binds to the DNA minor groove, which causes the major reduction of electron on the surface of the electrode. (b) LSV is used as an electrochemical method demonstrating major drop in DNA detection in comparison with negative control sample. Gel electrophoresis image of negative sample, N, and positive sample, approves the DNA amplification.**

3.3 Results and Discussion

3.3.1 DNA detection

In this developed microfluidic assay, electrochemical detection of the amplified DNA was first implemented. This was followed by the analysis of the extracted DNA detection on a chip. The chip is composed of an aluminum heat-block as a source of thermal energy, glass substrate and PDMS chip. Each chip composed of two parallel microfluidics to detect the negative control and sample with DNA template. Microfluidic chip has the following dimensions: channels with 200-μm width and 200-μm height, a reaction chamber and detection chamber for electrochemical measurement. The reaction chamber's volume is 35-μL volume and contains many cylindrical shape outgrowth with 2-μm diameter width to increase surface to volume ratio. The detection chamber is 3 mm diameter in width. Fig. 3.1 shows the schematic of the microfluidic chip. Each 30 μL of the LAMP consisted of 25 μL master mix and 5 μL DNA template [143].

Based on this ratio, 35 µL of LAMP solution was prepared and was injected into the reaction chamber using syringe pump with a flow rate of 15 µL/min. The solution was kept in the reaction chamber using an active valve. The active valve was made by a piece of PDMS, which was set vertically to block the channel. The sample remained in the reaction chamber to amplify the DNA target gene. Then, it was released to the detection chamber for electrochemical measurements. Meanwhile, the PBS buffer and redox solution from another entrance of the electrode chamber were added to the solution. Each 6 µL of the LAMP product was mixed with 12 µL of PBS buffer and 12 µL of the redox solution [143]. Experimentally, we gained 30 µL of amplicon from the reaction chamber. Based on this mixing ratio, we added 120 µL of PBS buffer and redox solution to the detection chamber. This amount of solution covered the surface area of the electrode in the detection chamber. Then LSV measurement was performed and illustrated the anodic oxidation of the bio-sample [144]. The difference in the maximum peak of the anodic oxidation between the negative control and the DNA sample was used for the detection and quantification of the amplified DNA. Fig. 3.2a shows the detection mechanism using the Hoechst 33258 redox molecule. When DNA was amplified, the redox molecule bound more with the DNA minor groove which caused a significant drop in the anodic oxidation of LSV as shown in Fig. 3.2b.

The drop in the peak maxima in the LSV with respect to the negative control for different concentrations of DNA were plotted for clarity as bars as shown in Fig. 3.3 It was found that the high concentration of the DNA has almost the same current peak (bar height) while the DNA concentration decreases the current peak (bar height) increases (Fig.3. 3a). This behavior for the drop in the current with varying DNA concentration has been reported previously in the literatures [143, 145]. For high concentration of DNA, the aggregation of the redox solution and amplicons reaches the saturation level. This aggregation causes a major reduction of the electrons on the surface of the electrode, which results in a significant drop of the peak maxima of the anodic oxidation. On the other hand, decreasing the DNA concentration causes less redox molecule to aggregate with the amplicons and accordingly, more electrons exist on the surface of the electrode. In turn, the anodic oxidation peak goes to the higher value as shown in Fig. 3.3a and Fig. 3.3b.

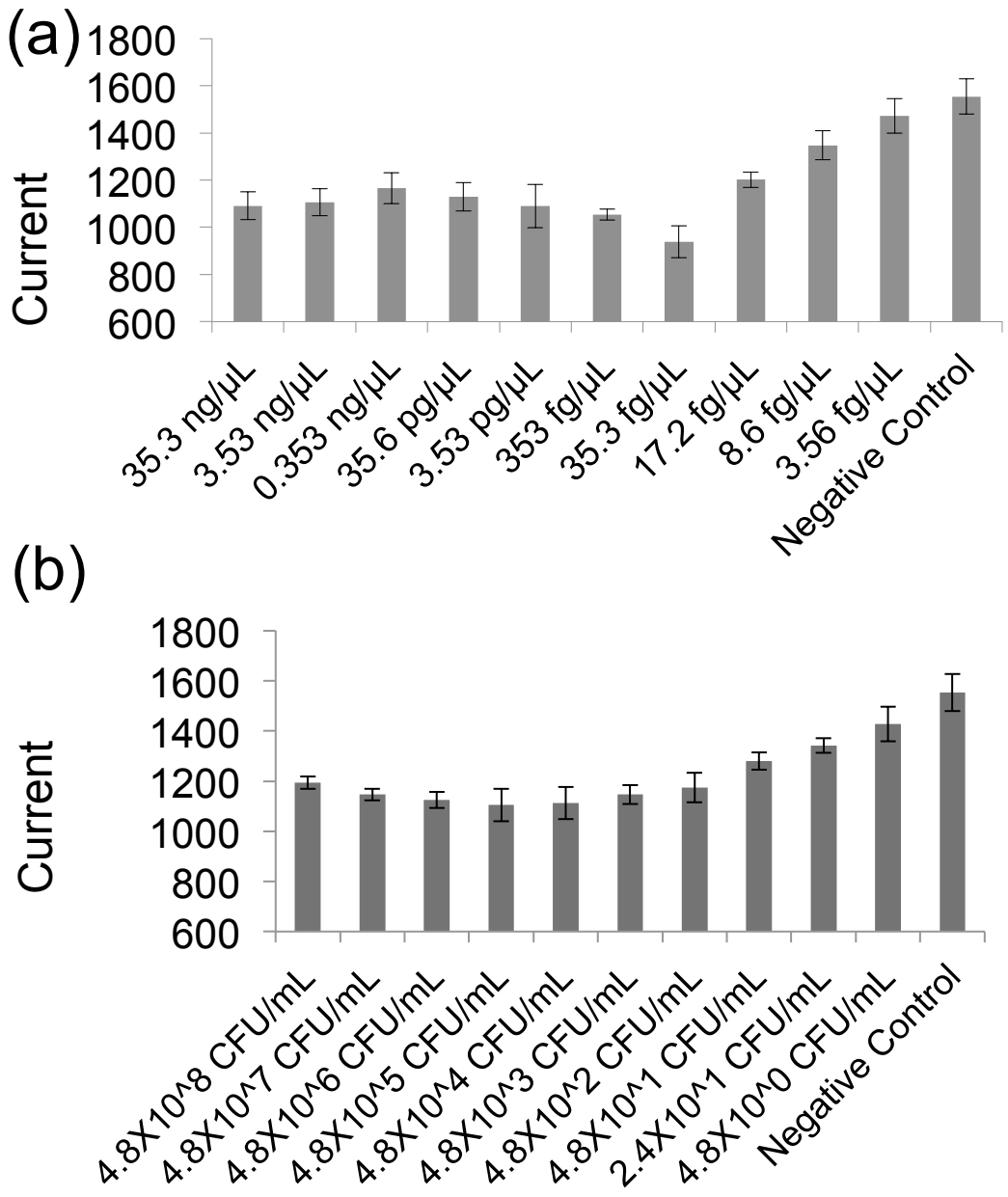

Fig. 3.3 **Electrochemical detection of *E.coli* in the microfluidic chip (a) Maximum peak current in LSV for different extracted E.coli DNA concentration. The detection limit on the chip is 8.6 fg/µL. (b) Maximum peak current in LSV for different bacteria concentration. The detection limit for the bacteria detection on chip is 24 CFU/ml.**

3.3.2 Bacteria detection

The detection of bacteria at different concentrations was also assessed as seen in Fig. 3.3b. Many diagnostic and theranostic devices have different sections/steps on the chip for bacteria lysis, DNA extraction and immobilization before the detection step. This

makes the structure and design of the chip complicated and increases the fabrication cost. For example, Wang *et al* used a microfluidic platform to lyse *Staphylococcus aureus* and extract the DNA and purify from the cell component and amplify the target DNA using the LAMP method, followed by optical detection[146]. Nakamura *et al* used the LAMP to detect six different nucleotides for gene analysis using electrochemical detection with Hoechst 33258 redox molecule [147]. The detection was based on the immobilization of the probe sequence on the electrode surface, which requires multi-steps treatment as well as time consuming/costly process. Furthermore, the detection took 2 hrs and it required hybridization and washing steps. Conversely, the microfluidic chip presented here does not need any pretreatment step(s) for bio-samples. Isothermal temperature at 66°C provides enough thermal shock to lyse the *E. coli* bacterial target in this study. Subsequently, the DNA from the bacteria was amplified and was detected using LSV without any pretreatment steps. Fig. 3.3b shows the detection of various concentrations of *E.coli* without any pretreatment steps and reagents. The detection limit for the *E. coli* bacterial cells is 24 colony-forming units (CFU/mL) in 1 hour.

3.3.3 Time optimization

A series of experiments have been done to optimize the *E.coli* LAMP products under isothermal amplification for different time periods (0,10, 20, 30, 40, 50, 60 min.), using the LSV electrochemical detection technique (Fig. 3.4). 35.3 ng/μL of the DNA template concentration was used in the experiment while water containing no DNA is used in the negative control experiments. It was found that LAMP product can be detected in 20 min. and the LAMP amplicons reached the saturation level after 40 min. as seen from the constant anodic peak as shown in Fig. 3.4. Therefore, based on Fig. 3.4 the DNA template can be detected in less than 20 min. without immobilization and purification step. However, it is expected to require a longer amplification time for lower concentrations of DNA, since more time is required to amplify the DNA to reach detectable levels.

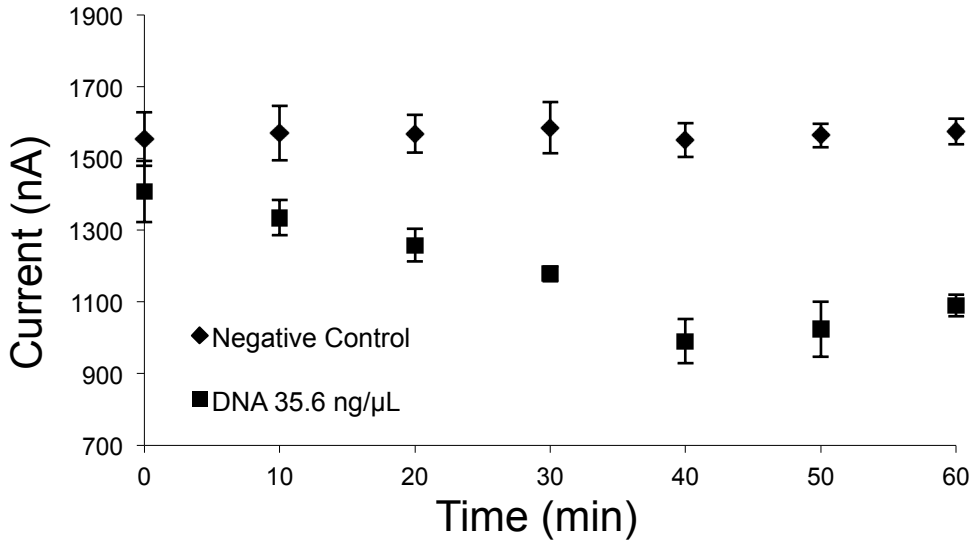

Fig. 3.4 **Time optimization of DNA sample on the chip. The (♦) illustrates the negative control and the (n) data represents the DNA sample. The chip detects the target DNA in 20 minutes. The DNA concentration is 35.6 ng/µL. Each measurement is carried out in separate chip, which reflects the reproducibility and stability of data.**

3.3.4 Chip calibration

E.coli has been widely reported as the major cause for the gastrointestinal disease globally [123]. More importantly, the bacterial *E.coli* cells can be found in the human body fluids such as blood or urine. Therefore, detection of the *E.coli* in the biological sample is necessary. A major improvement in disease control can be made if the target bacteria are rapidly detected and quantified accurately with high sensitivity by this microfluidic assay. In order to apply this device as a POC diagnostic device, calibration of the bacteria detection in the chip is required. We have reduced the resident time to 35 min and measured the LAMP product at different concentrations of *E.coli*. The urine sample was filtered off-chip using 0.2 µm filter to purify and remove the impurities from the sample prior spiking with bacteria. It was reported before [133, 148] that the level of the urine sample dilution plays a critical role in the success of the LAMP amplification since certain urine concentrations in the sample could inhibit the amplification reaction. For example, Kaneko *et al* showed that presence of the 2% urine in the sample inhibits LAMP amplification [149]. Our LAMP primers are not inhibited in the presence of 20 % of urine in the sample [133]. 5 ml of the urine in 25 ml of the master mix was used,

which is lower than 20% of urine inhibition limit. Fig. 3.5 shows such a linear relationship between the current (anodic peak maxima for each bacterial concentration) and the logarithmic scale of the bacteria concentration in different media.

Fig. 3.5a and Fig. 3.5b shows the results for the bacteria in LB broth media and filtered urine respectively. The bacterial concentrations varies from 48 CFU/mL to 4.8×10^5 CFU/mL. For accurate calibration the urine samples were freshly prepared from healthy people and sterilized using autoclave for 30 minutes at 121 °C.

3.3.5 Cross reactivity tests

Identification of the bacteria with high specificity on the chip is very important in the detection process. The specificity of the microfluidic chip was established by cross reactivity test using three different bacteria *E.coli*, *Listeria* and extracted DNA of *S. aureus*. The LAMP reactions were carried out using the primer sequences specific for the *E.coli* bacteria with *S. aureus DNA* and *Listeria* for 1 hour. Water without DNA template was used as the negative control. No major anodic oxidation drop has been observed for non-*E.coli* bacteria, which reflects the fact that the *E.coli* primers cannot amplify other types of bacteria (Fig. 3.6).

3.4 Conclusion

In this paper, we proposed a simple microfluidic chip for detection and quantification of *E.coli*. The microfluidic assay detected the bacteria electrochemically using LSV method with high sensitivity and specificity. The sensitivity of the assay was 48 CFU/ml in 35 min. The specificity of this device was examined by using different bacteria such as *Listeria* and *S. aureus* DNA. This device has a high potential for POC applications. It is much faster and more sensitive and consumes much smaller amounts of reagents in comparison with the other commercially available devices such as Galacto-Light Plus™ and TaqMan®. This approach enables us to detect various gram-negative bacteria in multiple parallel chips without using multi-sequences, costly, complicated methods.

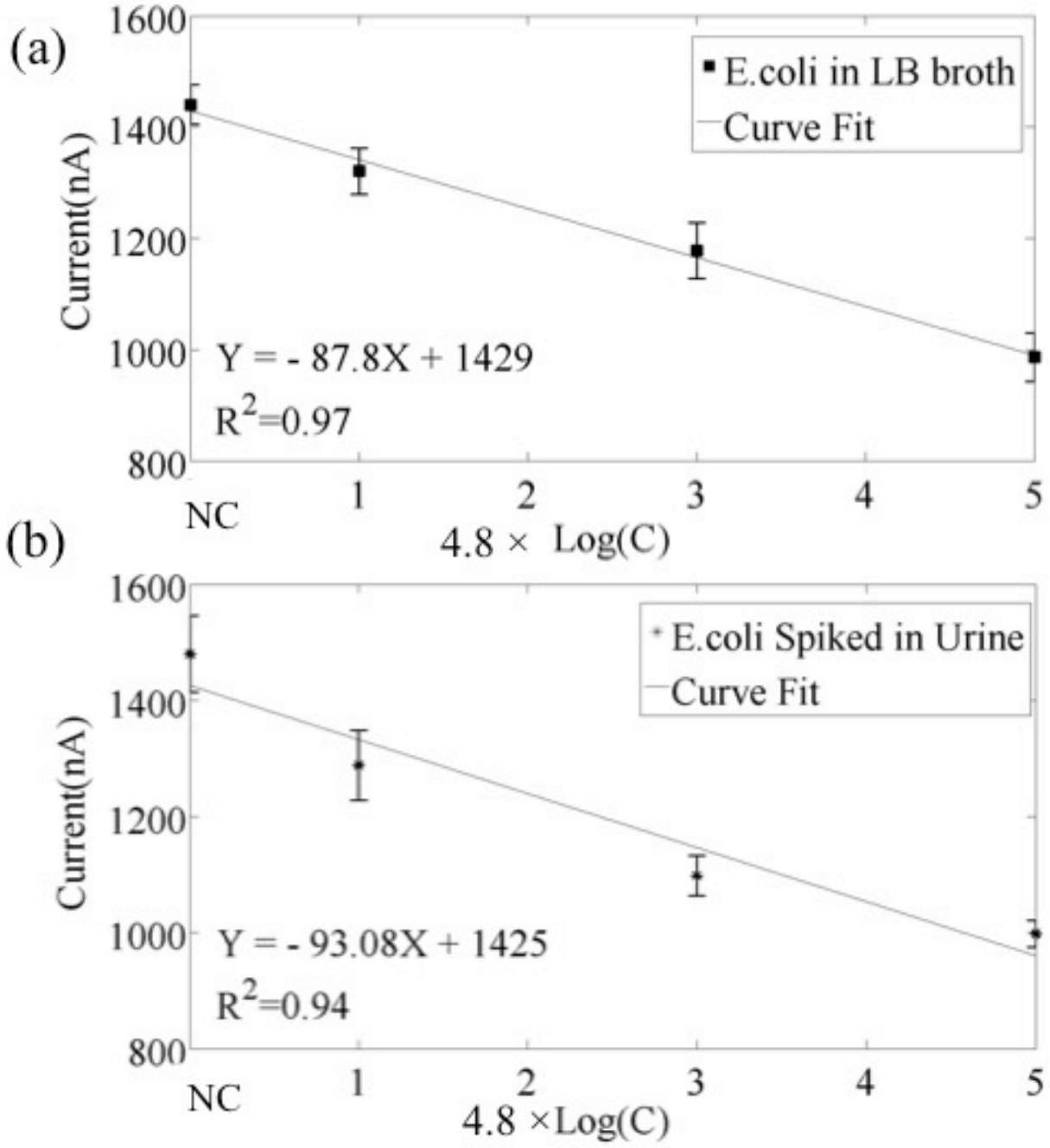

Fig.3. 5 **Quantification of E.coli bacteria in different media. Microfluidic chip is calibrated with different samples of Bacteria spiked in (a) LB Broth (b) urine samples using 35 min. amplification time. Linear relationship exists between the logarithmic concentration and the anodic oxidation current. The R^2 for the LB Broth and urine media are 0.97 and 0.94, respectively.**

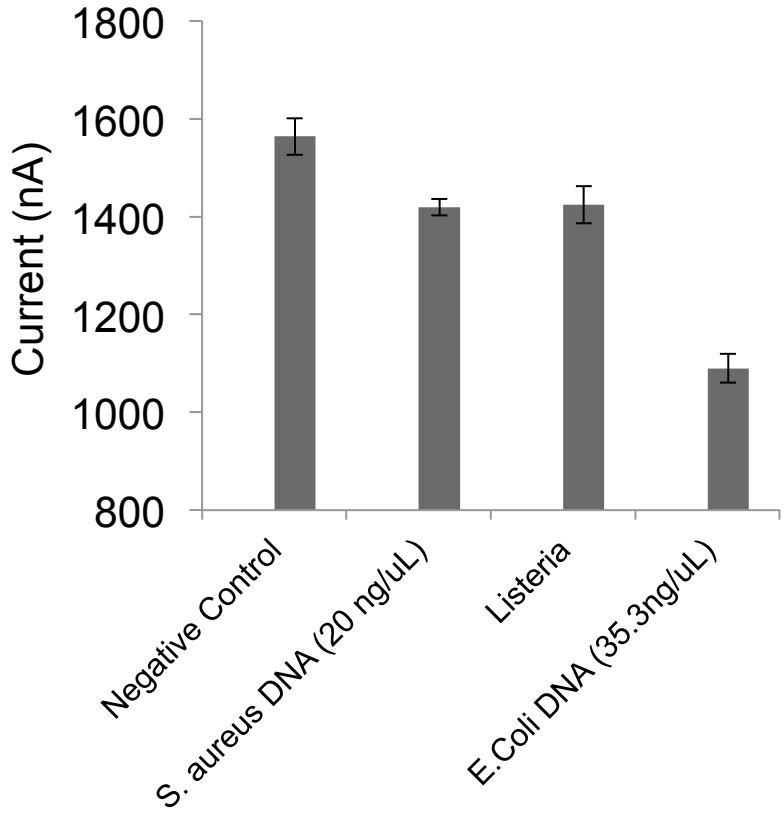

Fig. 3.6. **Cross reactivity test for the E.coli primes in the chip. Negative control contained of water with no E.coli DNA template. Due to existence of DNA and binding to redox molecule with its minor groove there is a small drop in the anodic oxidation peak current. The major drop is distinguishable between the E.coli DNA sample and other bacteria.**

Acknowledgement

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4 CHAPTER 4 : A SIMPLE CASSETTE AS POINT-OF-CARE DIAGNOSTIC DEVICE FOR NAKED-EYE COLORIMETRIC BACTERIA DETECTION

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ABSTRACT

Effective pathogen detection is inevitable for treatment of infectious disease. Point of care (POC) devices have tremendously improved the global human heath. However, design criteria for sample processing POC devices for pathogen detection with limited infrastructure are challenging and can provide significant contribution in global health by providing rapid and sensitive detection of bacteria in food, water, and patient's sample. In this paper, we demonstrate novel portable POC diagnostic device that is simple to assemble for amplification of specific bacterial's gene or detection of pathogens in real sample. The device is made, using very simple and easy to access materials and is highly simple and low cost for preparation. We have shown that we could detect 30 CFU/mL of *E.coli* and 200 CFU/mL of *S.aureus* bacteria in less than 1 hour using flexible substrate of polyethylene ribbon in colorimetric manner. Using numerical simulation we have shown that we can analyze and detect a minimum of 36 samples simultaneously in a high throughput manner. Since all fabrication processes as well as materials can be prepared and accomplished by low skilled personnel it can be used as highly sensitive, specific and robust POC device for multiple bacterial detection in developing countries and low resources settings.

4.1 Introduction

Infectious disease accounts for one third of the death of patients around the world [150]. Most of these diseases are easily treatable by current medications once diagnosed

properly, in a timely manner. Diagnostic assays are the cornerstone of the global health and have revolutionized diagnostic technology by providing, rapid, accurate, easy to use, bedside test in a short time manner [8, 150] . It is estimated that diagnostic companies spend only 3-5% of healthcare cost, yet their contribution to the health care decision are estimated well beyond 70% [151]. This significant impact provides better health care system in the global health care and specifically, in the area with poor and low resources. Despite the tremendous achievements in development of diagnostic devices, the culture method as well as immunoassay tests such as ELISA, lateral flow tests (LFT) remain the golden methods that diagnostic devices for bacteria detections rely on. Significant portion of POC devices are based on LFT, which provides rapid and accurate tests. However, these tests are faced with major obstacles for bacteria/pathogen detection, as well as the lack the sensitivity and specificity [10]. Alternatively, the advent of PCR techniques provides high sensitivity and specificity and revolutionized genetic and molecular diagnostics. Several POC devices have been developed that amplify targets using PCR, RT-PCR [65, 152-155]. However, major barriers exist in the face of rendering these methods in POC devices and providing precise, three different thermal cyclers, using complicated costly temperature controllers, unwanted evaporation of PCR reagents in the devices as well as engineering difficulties of heat transfer to the samples. In order to circumvent major traditional PCR problems, isothermal amplifications such as LAMP [96], RPA[156], HDA[157] , RCA[158] have been used for amplification in POC technologies, which do not need complicated infrastructures and provide similar/higher sensitivity and specificity. In order to apply these techniques, portable, easy to use, and simple design devices are highly in demanded.

Various technologies have been developed the past decade to manipulate minute volume of sample, within a biochip [159]. Microfluidics has caught a lot of attention for handling fluid as well as samples in POC technologies since it provides the ability of, sample purification, integration with a detection system, reducing the potential of contamination and human error as well as minimizing sample size [117, 160, 161]. However, despite having extraordinary benefits for sample processing in POC technologies it has several drawbacks. It requires costly, time-consuming fabrication

processes and it has to be implemented in the costly and expensive facilities of clean rooms. Most of the elastomers are inherently inexpensive, however the process by which to make them compatible with device's function increase the costs [118]. These difficulties make the fabrication technology cumbersome to implement in developing countries and low resources areas in order to develop ultra sensitive POC devices. In contrast, plastic sheets are very low costs and abundant across the world.

Currently, there is an extraordinary demand in large health care centers such as hospitals and doctors clinics, which requires analysis of many samples simultaneously in a low cost manner. Various detection methods have been used such as optical [162], or electrochemical [163], mass spectroscopy [164] methods in diagnostic technology. These methods are robust techniques for analyte detection. However they requires additional equipments and readers to monitor the phenomenon. Yet, colorimetric detection does not require any readers as well as fabrication of electrochemical electrodes or optical chips for interpretation of data. Various colorimetric dyes have been used to interchelate with LAMP amplicons such as Hydroxy naphthol blue (HNB)[137] , SYBR GREEN[165], Calcein[166], propodium iodide [133]. Therefore, providing a POC devices that can analyze several samples at the same time with high sensitivity and specificity with colorimetric detection methods can meet the demand and fulfill the current need in large health care centers.

Here, we developed a novel POC device that renders LAMP and colorimetric detection using HNB and Calcein dyes for real sample pathogen detection by the flexible substrate in a reel-to-reel Cassette format. We, first, demonstrate the assembly of the Cassette and fabrication of the chambers inside the flexible substrate.. Then, the results of colorimetric assays are shown for for *E.coli* as a gram- negative model and *S. aureus* as a gram-positive bacteria in real sample detection.

4.2 Material and Methods

4.2.1 Cassette fabrication and operation

The Cassette comprises of a collector and a provider reels with a diameter of 150 mm and thickness of 11mm. The collector provider is connected to the electric heater and controller unit to provide precise temperature (66°C). 5 ul of bacteria real sample with 20 ul of LAMP solution master mix were perfectly mixed initially and applied in to each reservoir. Then, the chambers were covered by tape and roll into the collector reel for further amplification. Figure 4.1 shows the schematic of the Cassette operation.

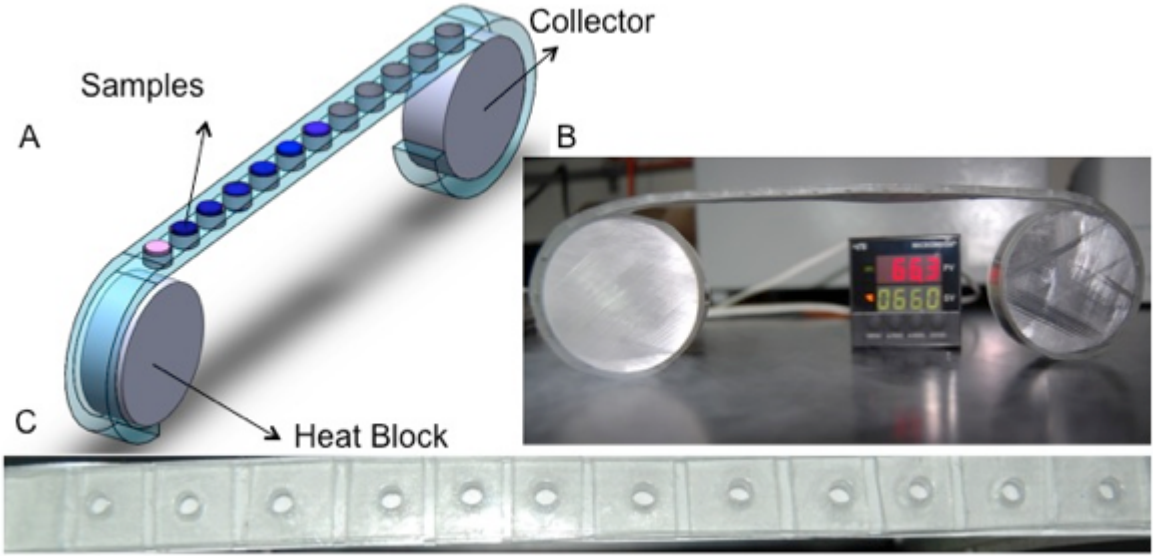


Fig 4.1 **Overview of Cassette device and its components. (A) Schematic of the Cassette. Cassette consists of two aluminum reels to provide and collect samples, a heat controller and heater to provide precise temperature for amplification and the flexible substrate where the chambers are fabricated. (B) Image of the flexible substrate. (C) Image of the flexible substrate.**

4.2.2 Flexible substrate fabrication

Flexible substrate has been fabricated based on Figure 2 and provide a unified volume reservoir with 35 µl each. Polyethylene strip has been used as a substrate width of 10 mm and the thickness of 3 mm. Two layers of poly styrene strips have been cut and were attached on top of each other using double sided Kapton ® tape. One-sided tape

was rendered as a bottom layer and paper has been used to enhance the observation of assay's results.

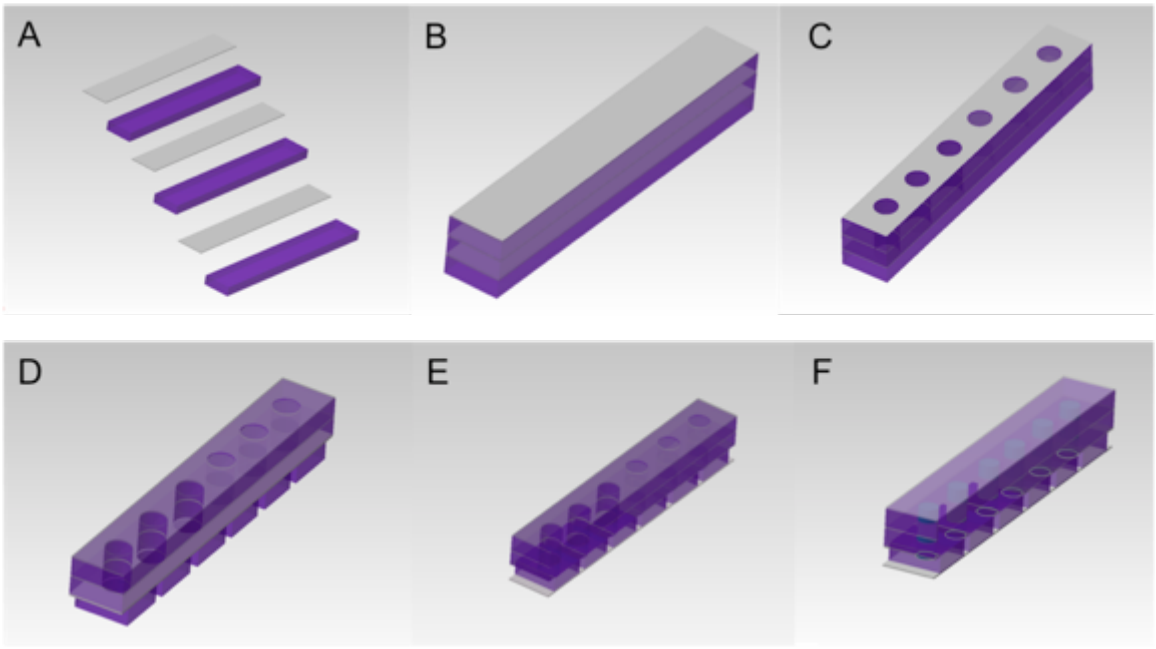


Fig 4.2 **Fabrication of flexible substrate. (A) Flexible substrate consists of three layers of polyethylene ribbons and double-sided tapes. (B) The plastic ribbons are attached on top of each other using double-sided tape. (C) The flexible substrate has been punched to provide uniform reservoirs. (D) The residues have been removed to increase the flexibility of ribbon. (E) A white paper has been attached at the bottom of ribbon in order to enhance visualization of the assay's result. (F) After applying the samples in each reservoir, samples have been covered using the tape.**

4.2.3 LAMP reaction

LAMP protocol is used to amplify *Tuf* gene of *E.coli* bacteria using hydronaphtol blue dye. The protocol has been optimized in order to be compatible with our primers. 20 µl of master mix composed of 3.2 µl of 0.6 µM Betaine, 2.5 µl of 10X Thermopol Buffer, 1 µl of *Bst Polymerase* (1600 units), 8000 U/ml, 1.25 µl of 5mM $MgSO_4$, 0.6 mM concentration 0.6 µl of 0.6 µM dNTP, 0.15 µl of 120 µM HNB, 0.25 µl of 0.2 µM outer primers (F3, B3), 2.0 µl of 1.6 µM inner primers (FIP, BIP) and 1.0 µl of 1.0 µM loop primers (LF, LB). Table S1 shows the six primers sequences of the LAMP reaction for *Tuf* gene.

The protocol has been optimized in order to amplify *Mcat* gene of the *S. aureus*. This protocol, materials and concentrations are identical to *E.coli Tuf* gene amplification

except, we have used 0.75 µl of 3mM of $MgSO_4$, 0.4 µl of 0.4 µM dNTP and 3.125 µl of 200 µM Calcein and 4 mM $Mncl_2$. or post processing sample analysis, a NanoDrop 2000 spectrometer (Thermo Scientific, Delaware, USA) and a NanoDrop 3300 (Thermo Scientific) were used. Table S1 from Appendix II shows the primer sequences of the LAMP reaction for Tuf gene amplification in *E.coli* and Mcat gene for *S.aureus*, respectively.

4.2.4 Finite element method modeling

A 3D time dependant heat transfer numerical model was implemented with a finite element method (FEM) using COMSOL Multiphysics 3.5 (MA, USA). The time-dependent temperature profiles at the surface of different layers were computed using the general heat transfer module for a 60 min period. Boundary conditions were set as follows: for reel perimeter T = 66 °C; far from the cassette T_{Inf} = 25 °C. Internal boundary conditions were set based on continuity of heat transfer between different layers and environments. The heat transfer simulation was run for various ribbon rounds around the heater.

4.3 Results

4.3.1 Colorimetric detection of *E.coli*

Detection of bacteria in real samples based on genomic amplification has been challenging due to the multi-step process required: cell lysis, DNA extraction, purification and amplification [9]. In our previous study [167, 168], we have shown that we could detect *E.coli* bacteria in various media without purification by lysis and amplification of the target DNA in one microfluidic reaction chamber. Subsequent detection of DNA amplicons was detected electrochemically. Despite providing a rapid, highly sensitive chip for bacteria detection, it has limitations for high throughput analysis of many samples in the low-income budget countries due to multistep operation as well as high fabrication cost. On the other hand, colorimetric detection is an easy-to-use, low-cost and qualitative technique to confirm the presence of DNA amplicons. In this work, we used HNB, which develops a purple color in the presence of Mg^{2+}. In the

amplification process, a significant amount of insoluble magnesium pyrophosphate is produced which causes major reduction of Mg^{2+} in the solution. This reduction changed the color of HNB from purple to blue [137]. We have added 25 µL of LAMP solution including 5 µL of bacteria sample with concentrations ranging from 3×10^8 CFU/ mL to 30 CFU/mL. After 1 hour of DNA amplification, the ribbon was expanded to visualize the result. We could detect *E.coli* at a concentration as low as 30 CFU/mL. The advantage of this process is that it does not require any reader/accessories and all the data can be interpreted by the naked eye. Fig. 3 shows the result of the colorimetric assay. In order to perform a more precise analysis, samples were extracted from the reservoirs and UV light absorbance was conducted.We have scanned UV light in various wavelengths ranging from 230 nm to 700 nm. As shown in Fig. 3B, there was a major difference in absorbance at 655 nm between the negative control and positive samples. This difference is due to the color change of HNB from purple to blue as well as the increased turbidity in the samples containing DNA amplicons (Fig. 4.3C). Since the chambers are of adequate size, direct observation by the human eye is straightforward without any accessories, allowing unambiguous distinction between negative and positive results. The assembly and, in particular, the operation of the device is also very easy. Personnel without specialized skills would appreciate the simplicity of the device's construction and its ease of operation.

4.3.2 Colorimetric Detection of *S.aureus* Bacteria

S.aureus is a gram-positive bacteria and the lysis step requires various hard to lyse. Colorimetric detection provides qualitative results for detection of pathogen and is highly applied due to use of low cost detection system that can provide result directly from sample to answer. High sensitive detection of *S.aureus* require more intense lysis technique since gram positive bacteria are hard to lyse in comparison to gram negative ones. Various lysing techniques exist such as chemical, thermal, electrical and mechanical [169, 170].

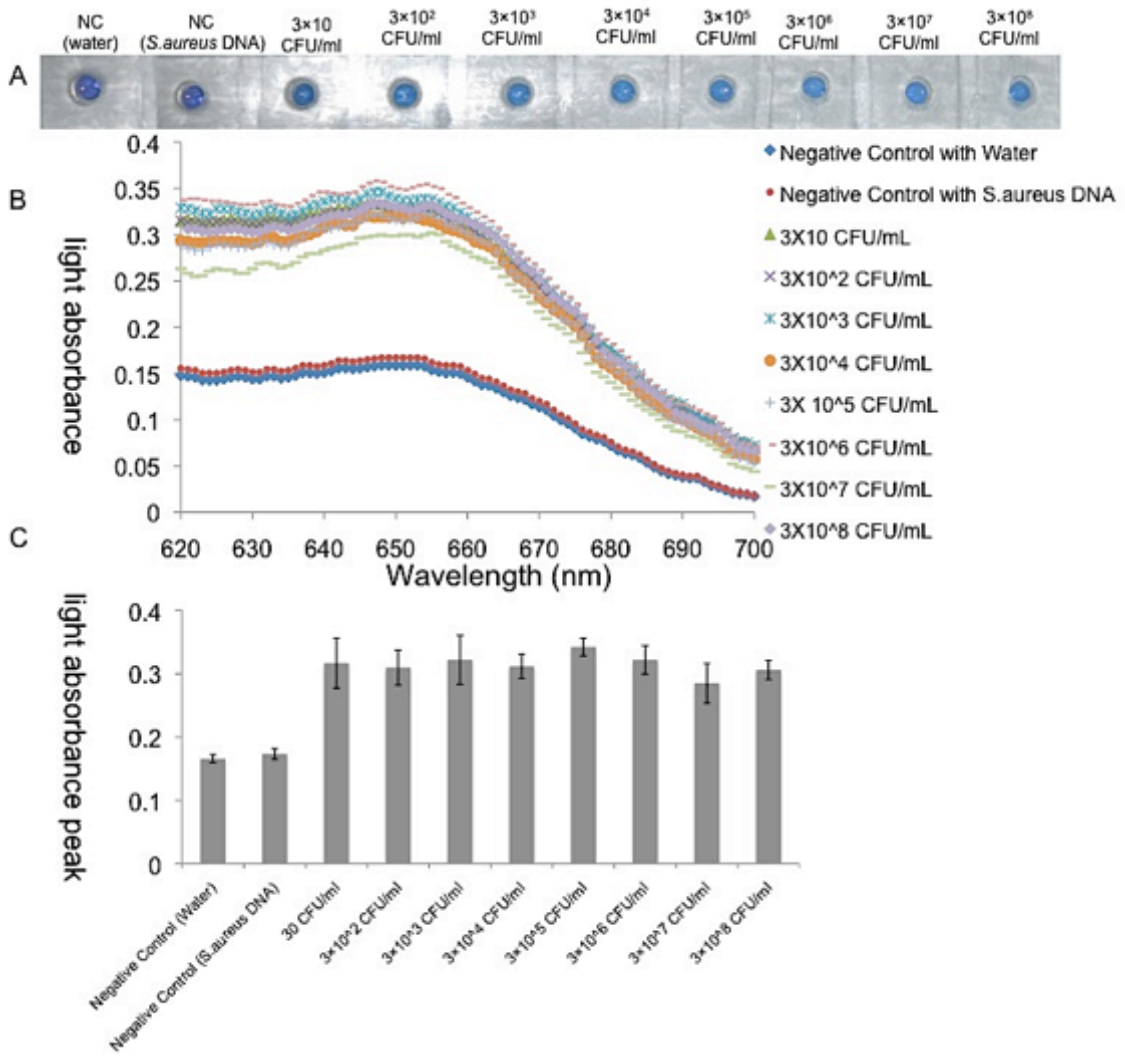


Fig 4.3 ***E.coli*** **real sample detection assay on flexible substrate. (A) Colorimetric assay of** ***E.coli*** **detection using HNB dye. Various tests has been implemented for different E.coli concentration ranging from 30-3×10^8 CFU/mL. The LOD is 30 CFU/mL. The positive samples are blue and negative tests are purples. The specificity of assey has been tested using** ***S.aureus*** **DNA and the negative result reflects the fact the assay is specific to the target** ***Tuf*** **gene in** ***E.coli*** **DNA. (B) Scanning of UV light absorbency for wavelength range from 620 nm- 700 nm. (C) UV Light absorbance peak has been measured at 650 nm wavelength for various samples and shows the gap in the light absorbency between negative and positive samples.**

Chemical lysis is a useful, low cost method for lysing bacteria but it needs a post-processing step to remove the inhibitor agent from the lysis solution, which does not make it suitable for amplification process simultaneously. Electrical and mechanical techniques need expensive apparatuses as well as expensive fabrication process and are not suitable for low cost system. On the other hand, thermal shock lysis is easy to

use, does not require any extra facilities due to using a heater in the amplification process in the device. It also can be used for lysis of *S.aureus* in a short time manner. To exploit this process, we have loaded 5 µl of *S.aureus* bacteria in one chamber and covered the chamber with tape and roll it to the collector reel and heated it for 5 min at 90°C. Then the samples were rolled back, uncovered and 20 µl of LAMP solution was applied into the chamber and mixed properly by pipette, followed by covering with tape again. The samples are rolled into the collector and heated at 65°C to amplify. Calcein colorimetric dye was used for qualitative detection. The color of Calcein has changed from yellow to green by emitting fluorescence in presence of amplicon. Figure 4 shows the colorimetric assay result for various concentrations ranging from 2×10^{8}- 200 CFU/mL. using this technique, we could detect 200 CFU/mL of real sample without any pretreatment in less than 1 hour. Further analysis of the samples have been implemented to measure fluorescence emitting. We have scanned fluorescent light with various wavelengths between 500-580 nm. Based on Figure 4B, there is a major gap in the fluorescent absorbance at 510 nm. This gap can be observed in Figure 3C between the negative controls and various samples with different concentration of *S.aureus* bacteria. The specificity of the assay has been examined using *E.coli* bacteria and it has been approved that the LAMP primers are highly specific to the *Mcat* target gene of *S.aureus*.

4.3.3 Heat transfer effect on Cassette reel

LAMP amplification method operates for temperature range between 60°C-66°C. Therefore, providing temperature within this range can initiate an amplification process for samples in chambers that are imposed in this temperature range. Understanding the heat transfer in the provider reel is the key element to provide the potential of sample processing, simultaneously. We, therefore performed the numerical simulation to understand the heat transfer and temperature profiles around the aluminum reel and flexible substrate and determine how many rolls of ribbon can be swatched around the aluminum reel in order to assure for detection of target pathogen. Details regarding the umerical calculation has been explained in the Appendix II.

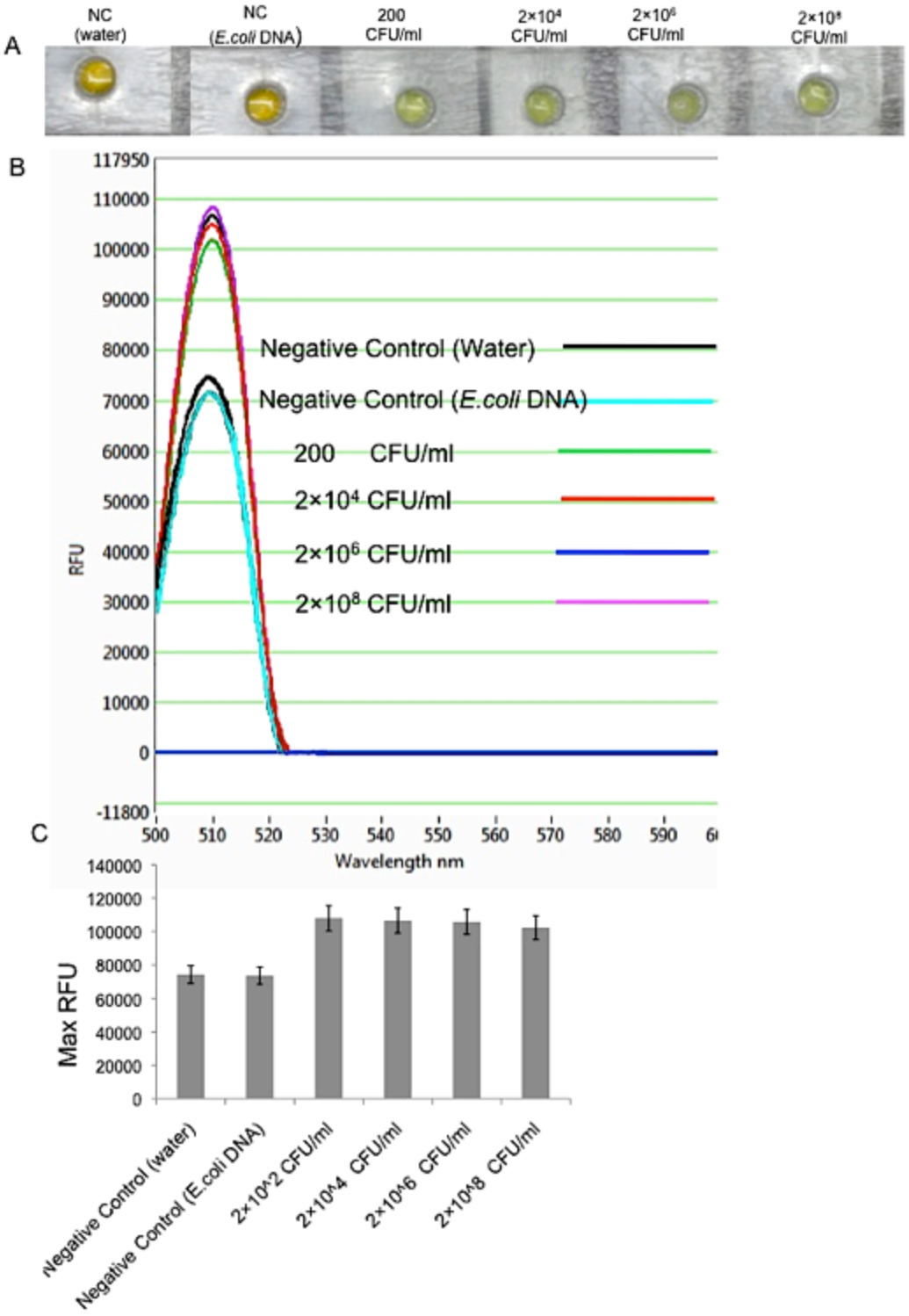


Fig 4.4 ***S.aureus*** **real sample detection assay. (A) Colorimetric detection of** ***S.aureus*** **using Calcein. The LOD is 200 CFU/mL. The specificity of assay was tested with** ***E.coli*** **DNA and shows that the assay is specific to** ***Mcat*** **gene only. (B) Scanning of Fluorescent absorbance from 500 nm-590 nm. Relative fluorescent unit (RFU) has**

been measured for different samples (C) Max RFU has been shown for different *S.aureus* concentrations and negative control.

Aluminum reel (K=205 W $m^{-1}k^{-1}$, ρ=2700 kg m^{-3}, C_p=897 J kg^{-1} K^{-1}) acts as the source of the heat and the heat transfer through the poly ethylene ribbons (K=0.42 W $m^{-1}k^{-1}$, ρ=900 kg m^{-3}, C_p=2307.2 J kg^{-1} K^{-1}) and the air (K=0.0257 W $m^{-1}k^{-1}$, ρ=1.205 kg m^{-3}, C_p=1005 J kg^{-1} K^{-1}) was set as environment material. 3D Time-dependant heat transfer FEM modeling has been implemented for various ribbon cycles with the 3 mm thickness for each round in order to understand whether the heater can provide enough temperature in the range of 60°C-66°C for minimum of 50 min. Figure 4.5 provides the result of heat transfer in the cassette. We have found that the heat apparatus can provide 3 round of ribbons for further amplification and detection. This number of rounds provides at least 36 samples processing and amplification simultaneously.

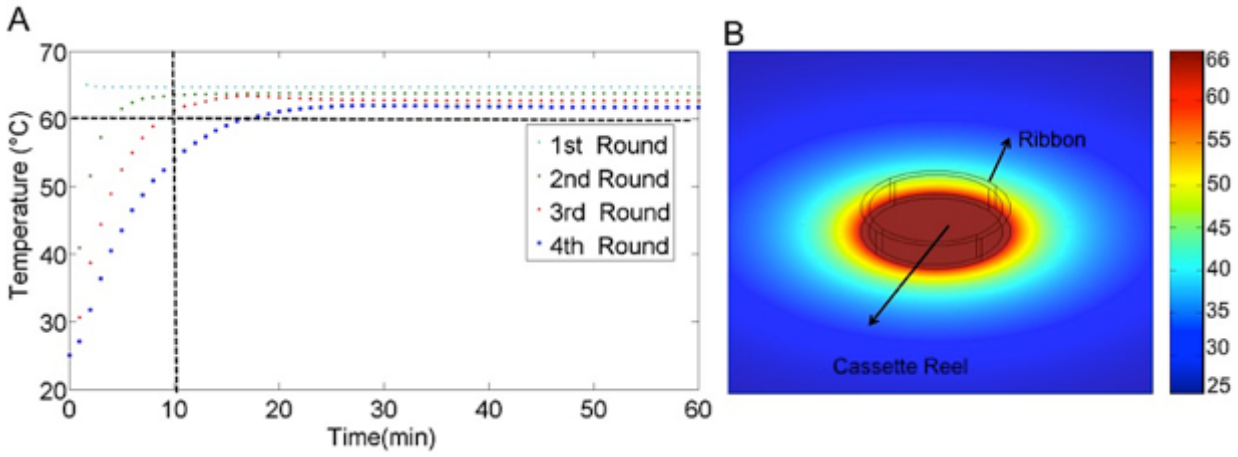


Fig 4.5 **Numerical heat-transfer simulation around the Cassette reel and ribbon. (A) 3D time-dependent heat transfer simulation has been implemented for different rounds of ribbons around the heater reel. The heater can provide minimum 60°C for at least 50 min, which requires for amplification and colorimetric detection. (B) Temperature profile around the heater and ribbon.**

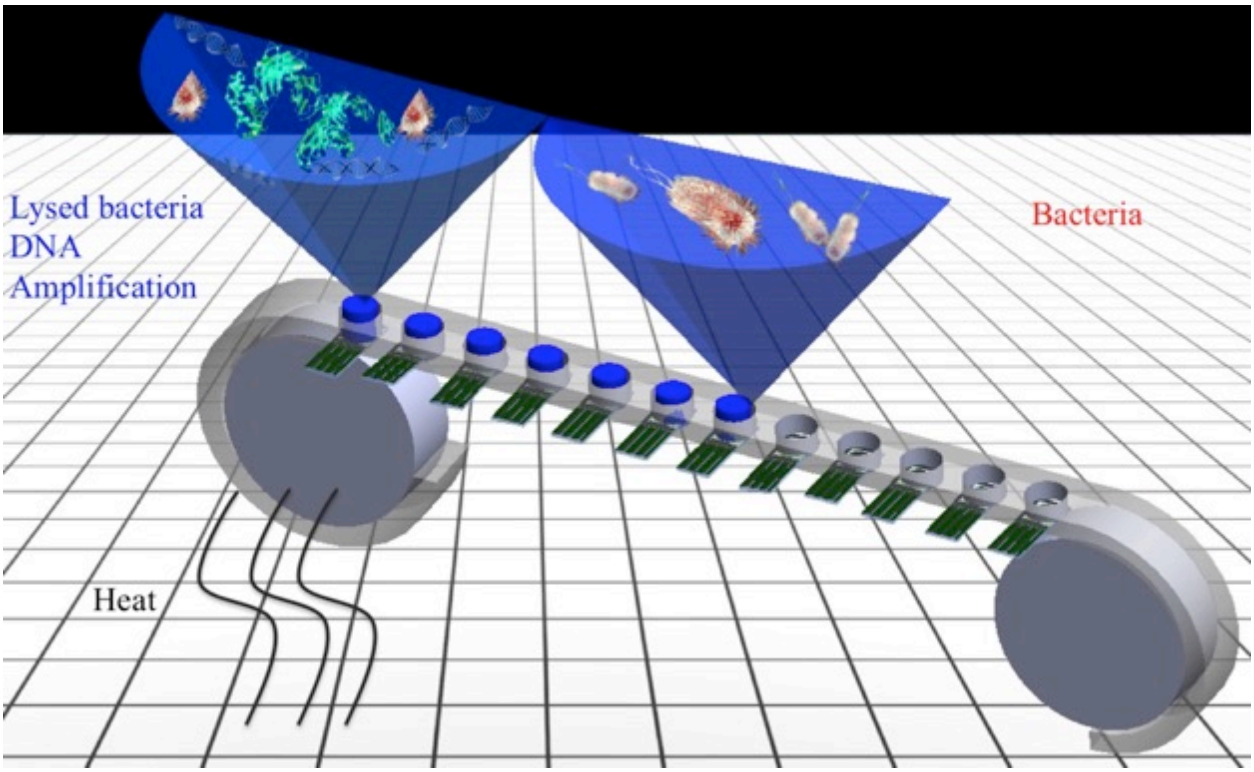
4.4 Conclusion

we have demonstrated a novel, easy-to-fabricate, easy-to-assemble diagnostic device capable of bacteria and pathogens detection with high sensitivity and specificity. This device can detect Gram-negative bacteria as well as a number of Gram-positive bacteria with the same characteristic as *S. aureus*. This device does not require any

expensive reader to process and interpret results. Since the device requires only one temperature controller, manufacturing cost is extremely low. This device can be integrated with an electricity free heating cartridge [171, 172] and is particularly attractive for bacterial pathogen detection in lowresource areas. In addition, this approach can also be implemented in a high-throughput manner, with high potential to meet the increasingly heavy demand for disease diagnostics in large health care centers. However, several problems need to be considered in order to be applied as POC devices. Usually a real sample does not have a pure sample of bacteria but instead, a mixture of various species/strains. If the assay predetermines the target species/strain based on the primer design, then DNA from other species/strains present in the sample, are not susceptible to amplification by the specific primers used. Therefore, the pathogens will be undetected which could be a dangerous approach for the clinician. A potential solution to this would be using multiple primers to target as many possible species/strains that could be present in the sample. However, then the cost of assay will go higher and this should be considered for POC applications in resource-depleted areas.

5 CHAPTER 5 : HIGH-THROUGHPUT REAL-TIME ELECTROCHEMICAL MONITORING OF LAMP FOR PATHOGENIC BACTERIA DETECTION

This chapter was published as : Safavieh, M., Ahmed, M.U., Ng, A., Zourob, M., High-throughput real-time electrochemical monitoring of LAMP for pathogenic bacteria detection, Biosens Bioelectron, 2014, 58, 101–106.



HIGHLIGHTS

- Cassette point-of-care (POC) diagnostics device for pathogens device developed using flexible substrate.
- The device was tested for LAMP amplification and real-time electrochemical detection.
- D.L. was 30 CFU/mL of *E. coli* and 200 CFU/mL of *S. aureus* in both real-time and end point detection.

ABSTRACT:

One of the significant challenges in healthcare is the development of point-of-care (POC) diagnostics. POC diagnostics require low-cost devices that offer portability, simplicity in operation and the ability for high-throughput and quantitative analysis. Here, we present a novel roll-to-roll ribbon fluid-handling device for electrochemical real-time monitoring of nucleic acid (NA) amplification and bacteria detection. The device rendered loop-mediated isothermal amplification (LAMP) and real-time electrochemical detection based on the interaction between LAMP amplicon and the redox-reactive osmium complex. We have shown the detection of 30 CFU/mL of *Escherichia coli* (in the range between 30 -3×10^7 CFU/mL) and 200 CFU/mL of *Staphylococcus aureus* (in the range of 200-2×10^5 CFU/mL) cultured samples in both real-time and end point detection. This device can be used for the detection of various gram-negative and a number of gram-positive bacterial pathogens with high sensitivity and specificity in a high-throughput format. Using a roll-to-roll cassette approach, we could detect 12 samples in one assay. Since the LAMP and electrochemical analysis are implemented within sealed flexible biochips, time-consuming processing steps are not required and the risk of contamination is significantly reduced.

5.1 Introduction

Diagnostic technologies were developed and achieved rapid detection and identification of various pathogens and diseases. Over the past decade, significant improvement toward development of rapid, accurate diagnostic tests have taken place, which significantly decreased unnecessary hospitalization and associated healthcare costs [10, 173, 174]. Among these tests, nucleic acid (NA)-based detection offers high sensitivity and specificity. Polymerase chain reaction (PCR) and isothermal amplification techniques are the most common high sensitive techniques for NA detection [161]. In fact, real-time PCR has become an indispensible tool in molecular diagnostic, forensic analysis, tumor diagnostic and determination of specific bacteria in samples. The key success of real-time monitoring of target gene amplification is that the amplicon is

quantified as it accumulates as a result of the reaction. This effectively avoids time-consuming and costly post processing such as fluorescent measurements, as well as possible potential of contamination using open cap viral samples [44, 175]. However, the requirement of three thermal controls as well as temperature cycling makes real-time PCR too costly and complicated as POC devices. For this, isothermal amplifications such as Recombinase polymerase amplification (RPA) [176],Nucleic acid sequence based amplification (NASBA) [95, 177] , Helicase dependent amplification (HDA) [178] , Smart amplification process v2 (SMAP2) [179], LAMP [96], which operate at a single temperature, have been developed. Nevertheless, many of these techniques are only suitable for amplification of short oligonucleotide and single nucleotides probes (SNP) and not suitable for amplification of large DNA template. In contrast, LAMP can utilize large DNA template and provides high sensitivity and specificity [180].

The most commonly used techniques for real-time analysis of amplicons rely on fluorescence as well as turbidity measurement of the sample which required bulky, costly as well as delicate equipments and limit their implementation within lab-on-a-chip POC devices. Alternatively, electrochemical detection is a fast, highly sensitive, easy to use, low-cost method amenable to miniaturization. Electrochemical methods have been exploited in detection of NA for POC applications [181-184]. Overall, electrochemical detection of NA is divided into direct and indirect detection techniques. The most popular direct detection of nucleic acid is through the oxidation of guanine bases in the sequence of DNA [185]. Kara *et al* used direct detection of PCR amplicon by immobilizing DNA probe on graphite electrode for the detection of Hepatitis B virus DNA [186]. However, direct electrochemical detection of NA requires probe immobilization on the electrode surface, which is a long and laborious multi-step process. Conversely, redox probes do not need surface immobilization and are also able to provide rapid and stable measurements [141, 187]. Various redox molecules have been utilized for electrochemical detection of DNA. These molecules include Methylene Blue (MB) [188], Hoechst 33258 [189], Ruthenium Hexamine (RuHex) [190] and Osmium redox [191-193]. Hoechst 33258 inhibits the LAMP reaction is therefore not suitable for real-time monitoring. Methylene blue, on the other hand, exhibit low binding activity with DNA in

comparison with other redox molecules [190]. In contrast, osmium redox has shown high binding activity as well as stability during the isothermal amplification [191].

Generally, sample detection is the ultimate goal in POC analysis. In order to design nucleic acid based POC, several criteria should be considered: (i) proper thermal precision for amplification (ii) detection of amplification product (iii) ability to integrate with the sample processing to implement sample processing steps such as cell lysis, DNA extraction and purification step if the sample has inhibitor agents. State-of-the-art microfluidic technologies have led to the design and fabrication of micro/nano chips which significantly reduced sample size, facilitated sample manipulation and enhanced the performance of amplification reactions [117, 194, 195]. Despite several efforts on fabrication of microfluidic devices without cleanroom facilities [196, 197], this technology is still faced with major obstacles before deployment in low-resource society due to the costly materials and complicated fabrication process. Consequently, the demand for high-throughput, fully integrated sample-to-answer microchip devices remains unanswered.

Here, we introduce a novel, compact, easy-to-use, POC device for high-throughput electrochemical real-time detection of bacterial pathogens. We used *E.coli* and *S.aureu*s as models of gram-negative and gram-positive bacteria, respectively. We first demonstrate the device fabrication and assembly using a flexible substrate. Based on the Osmium redox-LAMP amplicon interaction, real-time monitoring of *E.coli* bacteria detection was monitored by square wave voltametry (SWV). The device operation was further modified for detection of *S.aureu*s in cultured sample. Finally, cross-reactivity was assessed to confirm the high specificity of the assay.

5.2 Materials and Methods

5.2.1 Chemical reagents and Materials

E.coli was freshly prepared and cultured overnight (12 hours) in 2% LB Broth. Osmium redox ($Os[(bpy)_2DPPZ](PF_6)2$) was synthesized and prepared by Prof. Daimien Marchal (CNRS, France). Osmium redox was kept at room temperature in the dark condition.

Prof. Monique Lacroix from INRS-Armand Frapier kindly provided *S.aureus*. Disposable carbon flexible screen-printed electrode (FSPE) was purchased from BioDevice (Ishikava, Japan). Polyethylene sheets, Heater rods and temperature controller were bought from Omega (Connecticut, USA). Salmon Testes DNA was purchased from Sigma Aldrich (MO, USA).

5.2.2 Cassette fabrication and operation

The cassette consists of two collector and provider reels with a diameter of 150 mm and a thickness of 11 mm. The collector reel is connected to an electric heater and controller unit to provide precise temperature (65°C). The provider reel, acts as the source of the flexible ribbon. A flexible ribbon was fabricated as described in Figure A3.1 in Appendix III, providing reservoirs with a uniform volume of 35 µl. Three layers of polystyrene strips were cut and layered on top of each other using double-sided Kapton ® tape (3M,USA). Carbon FSPEs fabricated on flexible polyethylene strip were used as a substrate with 10 mm width and 3 mm thickness and is attached to the bottom of each reservoir. Figure 1 schematizes the cassette device and operation process.

5.2.3 Electrochemical Assay

Electrochemical assay was implemented by attaching the carbon FSPE at the bottom of each reservoir (see cassette fabrication in Appendix III ,Fig S1). Then, the electrode was connected to the potentiostate machine and the measurements were implemented using SWV in a real-time manner due to high sensitivity and rapid scanning. For this analysis the scanning rate was 100 mV/s within the range of -0.2 to 0.8 V to achieve the oxidation peak. This measurement was accomplished by measuring each sample one by one in the flexible substrate.

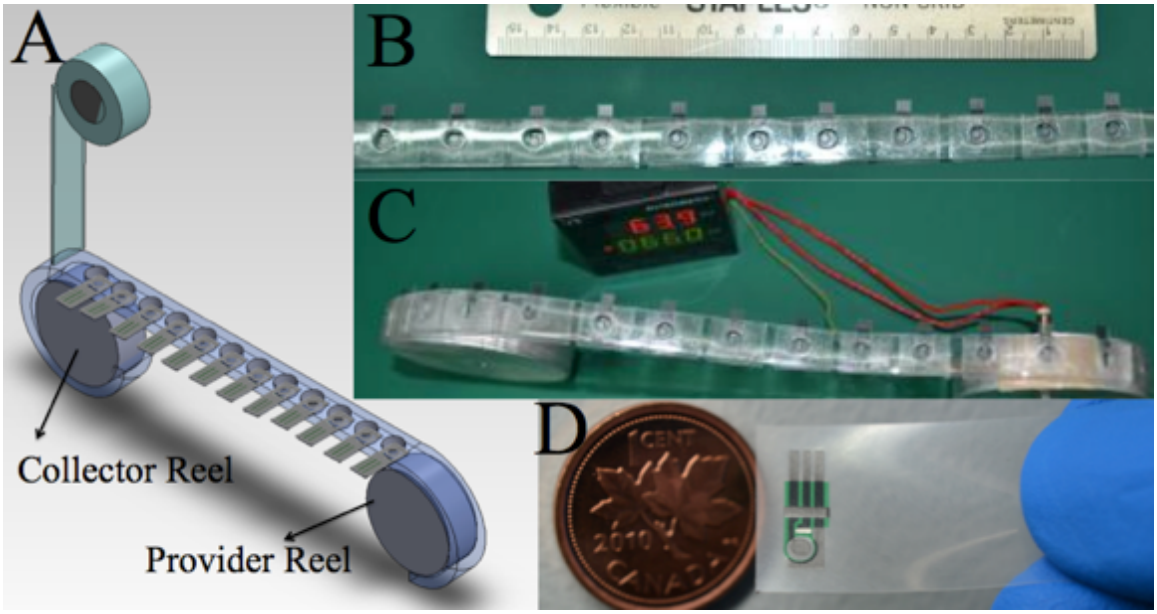


Fig 5.1 **Schematic representation of the cassette. (A) The cassette consists of two aluminum reels and a flexible ribbon in which uniform reservoirs were fabricated. After sample loading and turning into the collector reel, a tape covers all the samples and the ribbon turns into the collector reel to make the samples ready for electrochemical analysis. (B) Image of the fabricated reservoirs on flexible ribbon made of polyethylene. (C) Image of the cassette connected to the electrical heater and controller. (D) Image of the flexible carbon electrode consisting of a carbon working electrode, a carbon counter electrode and an Ag/AgCl reference electrode fabricated on flexible polyethylene sheet.**

Once the flexible substrate surrounded the perimeter of collector reel (which acted as a heater as well) and the tests for all assays are completed, it was cut and removed from the process. 0.2 µl of Osmium redox ($Os[(bpy)_2DPPZ](PF_6)_2$) was added to a final concentration of 0.5 µM in the LAMP protocol. This concentration provided a very distinct signal in the redox peak [192] and was used for real-time monitoring of the LAMP amplification.

5.2.4 LAMP assay

LAMP assays were performed targeting the *Tuf* gene of *E.coli* and *Mcat* gene in *S.aureus.* 20 µl of master mix composed of 2.5 µl of Thermopol Buffer (New England Biolabs, Beverly, MA, USA), 1.25 µl of 5mM $MgSO_4$, 3.2 µl of 0.6 µM Betaine (Sigma–Aldrich, MO, USA), 1 µl of *Bst Polymerase*, 8000 U/ml (New England Biolabs, MA, USA), 0.6 µl of 0.6 µM dNTP, 0.25 µl of 0.2 µM outer primers (F3, B3), 2.0 µl of 1.6 µM

inner primers (FIP, BIP) and 1.0 µl of 1.0 µM loop primers (LF, LB). Table S1 in supplementary information shows the primers sequences. For gel electrophoresis, 2% agarose gel was prepared and stained with Safe-T-Stain (Bioshop, Canada). For gel analysis, 5.0 µl of LAMP amplicon was mixed with 1.0 µl of loading dye (New England Biolabs) and before loading into the gel wells.

5.3 Results and Discussion

5.3.1 Cassette Operation

The carbon FSPE was attached on the bottom of each reservoir. The cassette and flexible ribbon were assembled as shown in Figure 5.1D. After sample loading, the reservoirs were covered with tape to protect and prevent the samples from evaporating. Fabricated ribbon and arrays of reservoirs demonstrate proper integrity

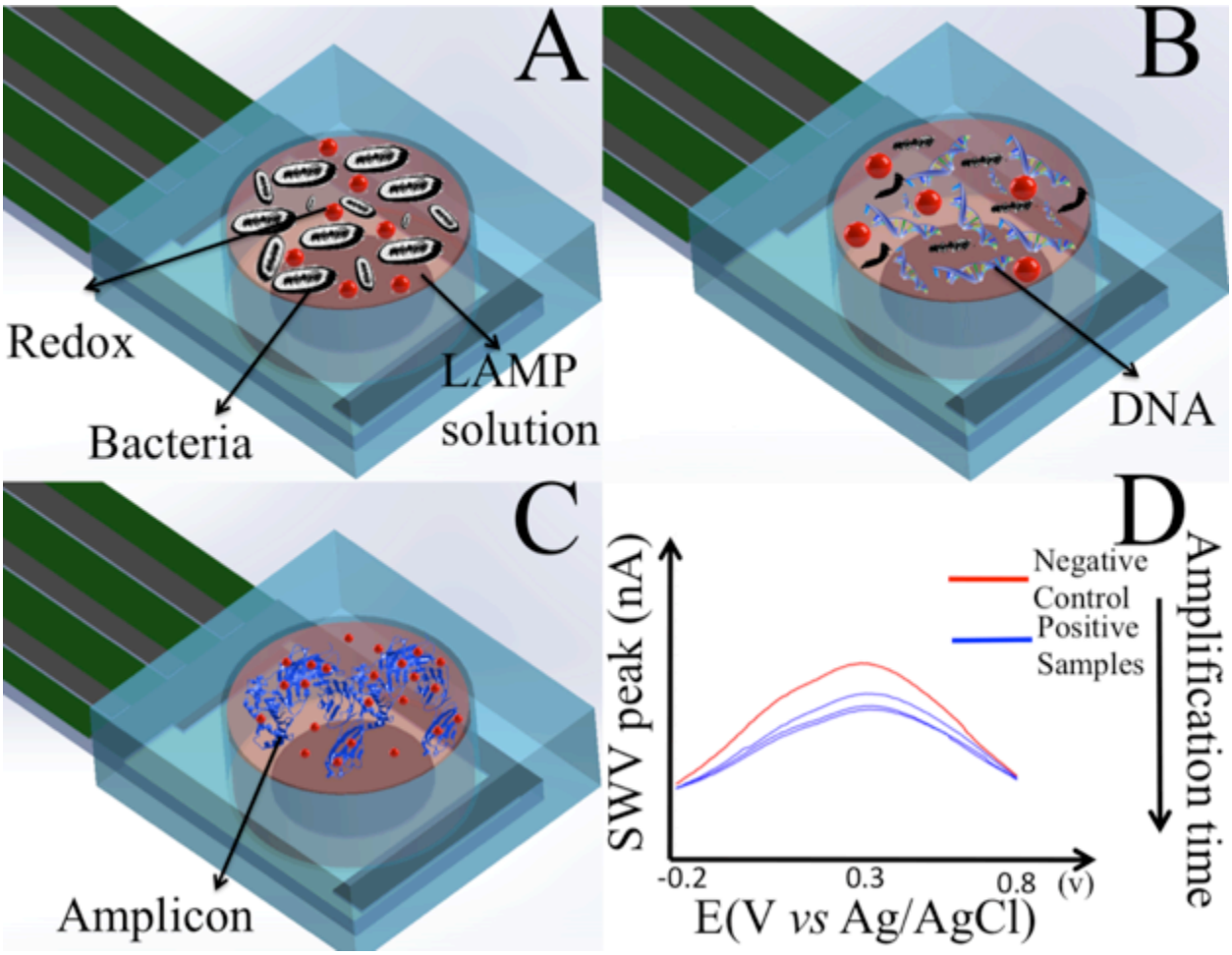


Fig 5.2 **Schematic of the real-time electrochemical monitoring of LAMP amplicon. (A) Bacteria, redox molecules and LAMP solution before amplification. (B) Thermal shock lyses the bacteria and the DNA released in the solution (C) LAMP amplification process initiates and the redox molecules intercalates with the amplicons (D) Redox interaction with amplicon is shown in the electrochemical measurements using SWV. The oxidation peak is reduced due to the fact that more redox molecules binds with the amplicons and less redox molecules are oxidized during amplification process.**

during the amplification reaction and its appropriate temperature (65°C). During the NA analysis, 25 µl samples were loaded onto each of the reservoir and then the ribbon was turned into the collector reel to heat and initiate the amplification process. Each chamber was connected to the potentiostat for real-time monitoring of electrochemical analysis of LAMP amplicons. The analysis can be implemented one sample at a time or simultaneously by connecting several samples to the potentiostat. The collector reel provides precise temperature for amplification for up to 12 samples simultaneously. At the end of the analysis the used portion of the flexible ribbon was separated from the source roll and discarded. The advantage of the roll-to-roll heating system over ordinary rectangular plate heater is the precision achieved during simultaneous heating of several samples, as well as the potential of multiple connections to the electrochemical reader without replacing samples, which significantly increases the portability as well as high throughput analysis. The heat transfer and temperature distribution around the reel has been studied previously [198] and through numerical analysis we have shown the consistent temperature in the reservoirs in the flexible ribbon.

5.3.2 Real-time Detection of *E.coli*

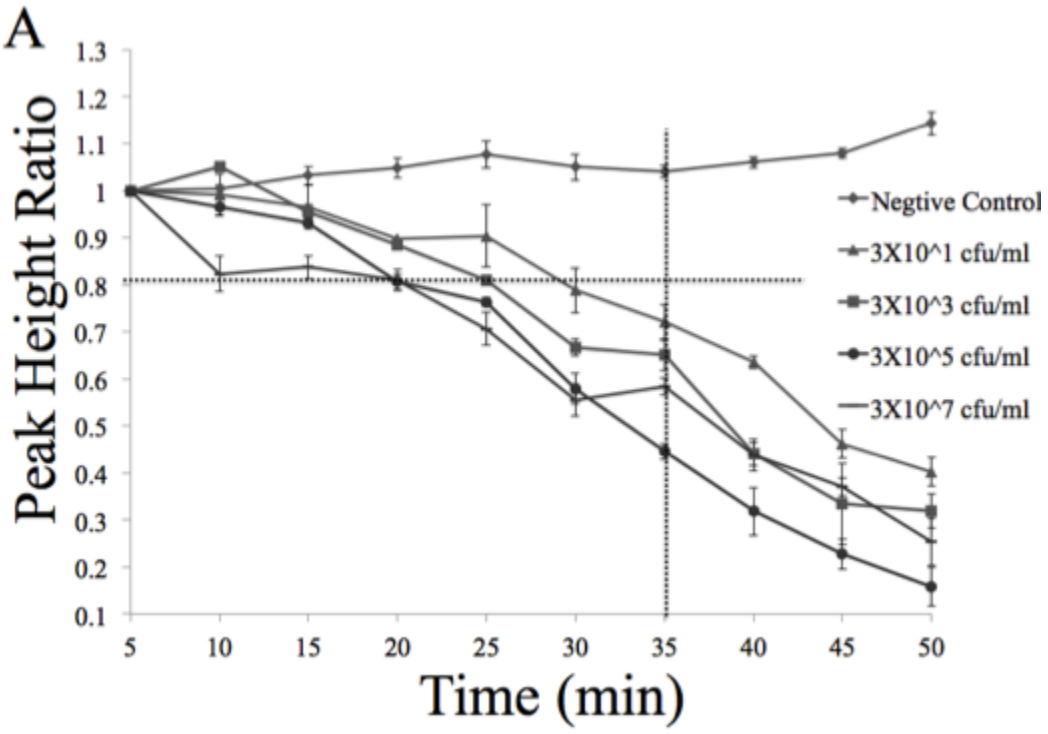
A 25 µl LAMP solution including 5 µl of *E.coli* bacteria and 0.5 µM of Osmium redox were loaded into the chamber and the electrode was connected to the potentiostate machine for electrochemical detection using SWV method. The amplification reaction was monitored every 5 min for a total of 50 min amplification time. The micro chamber within the flexible ribbon was rolled around the aluminum cylindrical collector reel which provided heating for a 65°C reaction temperature. Our previous studies have shown that a 65°C LAMP reaction temperature provide enough thermal shock for bacteria lysis [168, 199] prior to the amplification. Thereby, thermal lysis and NA amplification can be implemented in one reservoir as long as no polymerase inhibitor agents exist in the

solution[168, 199]. In addition, a previous study has shown that cell debris does not inhibit the LAMP amplification process [148]. The entire detection process consists of bacteria lysis, NA amplification and amplicon detection, integrated within a single chamber as shown in Figure 5.2.

One of the most critical parameters in electrochemical detection of NA amplicon by redox molecule is the maintenance of a consistent temperature. Researchers have shown that temperature has a significant effect on redox amplicon intercalating behavior [191]. In order to eliminate the temperature tolerance effect on the data, we started the SWV scanning process 5 min after loading the samples so the samples were equilibrated to a consistent amplification temperature. To simplify the results, peak height ratio has been defined as the ratio of the SWV peak at an amplification time to SWV at the initial point. The SWV method has been chosen due to high sensitivity, being fast with good reproducibility and has been implemented previously in PCR amplicon real-time monitoring [181]. Each scan takes precisely 23 sec and each 5 min, 3 scans have been implemented to provide precise monitoring of redox amplicon interaction. Figure 5.3 shows the real-time monitoring of *E.coli*. The redox molecule was intercalating with the increasing amount of amplicon during the amplification process, as a result, the concentration of the free redox in the solution decreased. At high concentration of bacteria, more DNA templates exists in the solution, which resulted in a more rapid generation of the amplicon when compared to samples containing lower bacteria concentration (Figure 5.3A). In order to determine required amplification time to detect the particular *E.coli* bacteria concentration, we have set the threshold value for the peak height ratio at 0.8 to distinguish between negative control and positive samples. Figure 5.3b shows that 29 min amplification time is required to achieve the limit of detection (LOD) of 30 CFU/mL of *E.coli* bacteria. There is a linear relationship between that amplification time and logarithmic concentration of *E.coli* with the slope of -3.15 and the R^2 of 0.94. By setting the amplification time to 35 min, we could quantify *E.coli* bacteria by plotting peak height ratio *vs* logarithmic *E.coli* concentration as in Figure 5.3C. The regression line calibrates the *E.coli* quantification assay with the slope of -0.04 and R^2 of 0.88.

5.3.3 Real-time Detection of *S.aureus*

Although gram-negative bacteria can be easily lysed by thermal shock at 60-66°C, this range of temperature does not provide enough shock for gram-positive bacteria such as *S.aureus,* for which effective lysis requires at least 5 min heating at 90°C. Nevertheless, the major shortcoming of the use of this high-temperature lysis is that the *Bst* polymerase in the LAMP reaction is irreversibly inactivated at temperature above 70°C. For this, we have modified the procedure by carrying out the lysis of the *S.aureus* bacteria at 90°C for 5 min on the collector reel, prior to addition of the LAMP reagents. After lysis, the ribbon was advanced to cool the lysis mixture, then the LAMP solution was added, covered by tape again and rolled back onto the collector reel following by heating at 65°C to initiate the amplification process. Real-time monitoring of LAMP



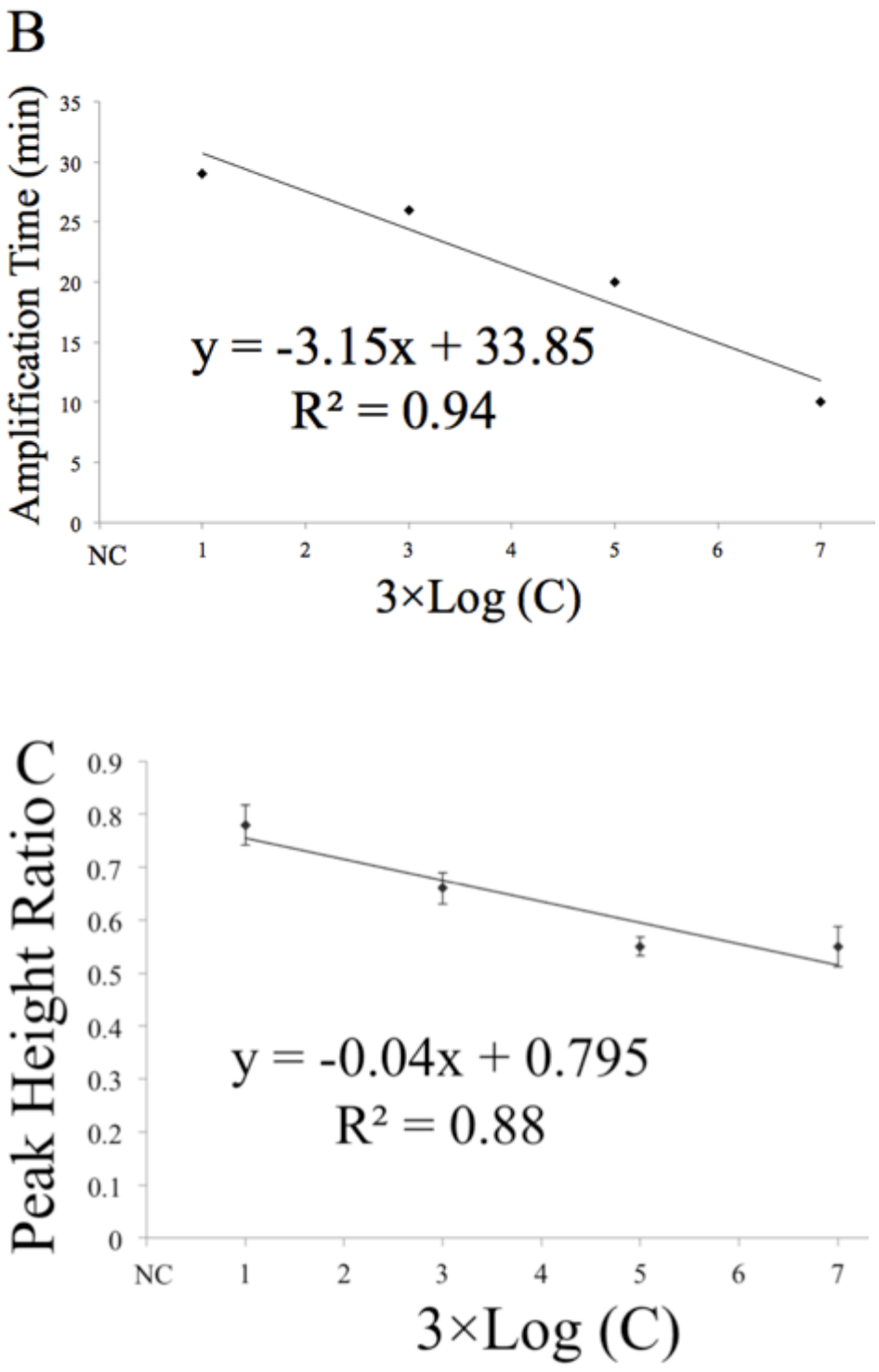
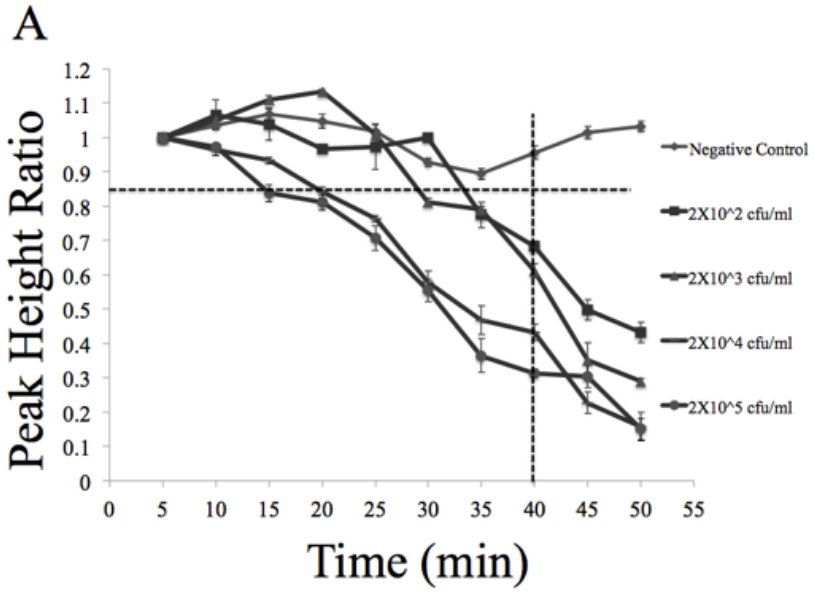


Fig 5.3 **Real-time measurement of *E.coli* using 0.5 µM Osmium redox. (A) Peak height ratio of SWV vs amplification time. Different bacteria concentrations were used as the template and electrochemical measurements were carried out at 5 min intervals for three consecutive times (n=3). The threshold value was set to 0.8 (horizontal dotted line). (B) Amplification time vs logarithmic concentration of *E.coli* bacteria. 30 CFU/ml of bacteria can be detected in 29 min. (C) Quantification of *E.coli* bacteria**

in 35 min amplification based on peak height ratio (Vertical dotted line). The RSD% amounts have been calculated in the supplementary information. These analyses can be used as the calibration curves for quantification of *E.coli* bacteria.

amplicon for various *S.aureus* bacteria concentrations was tested for 50 min amplification time (Figure 5.4A). As expected, amplicon generation reached the detection threshold sooner with high concentration bacteria samples in comparison to samples with lower bacteria concentration. In order to optimize the amplification time for the detection of specific bacteria concentration, peak height threshold value has been set to 0.85 in Figure 5.4A. The amplification time *vs* logarithmic concentration of *S.aureus* plotted as shown in Figure 5.4B. It was found that there is a linear relationship between the peak height value and amplification time for various *S.aureus* concentrations with the slope of -7.4 and R^2 of 0.97. The LOD of 200 CFU/mL was achieved within 34 min amplification time.

The peak height ratio at 40 min after the initiation of amplification was plotted against bacteria concentration as a standard curve for *S.aureus* quantification (Figure 5.4C). A linear relationship was obtained between peak height and the logarithmic concentration of *S.aureus* bacteria with a slope of -0.12 and R^2 of 0.99. After 40 min amplification time, samples of higher concentration of bacteria reach to the saturation amplicon amount in the sample, which yields a consistent peak signal. Consequently, it cannot be rendered as the appropriate amplification time set for the bacteria quantification.



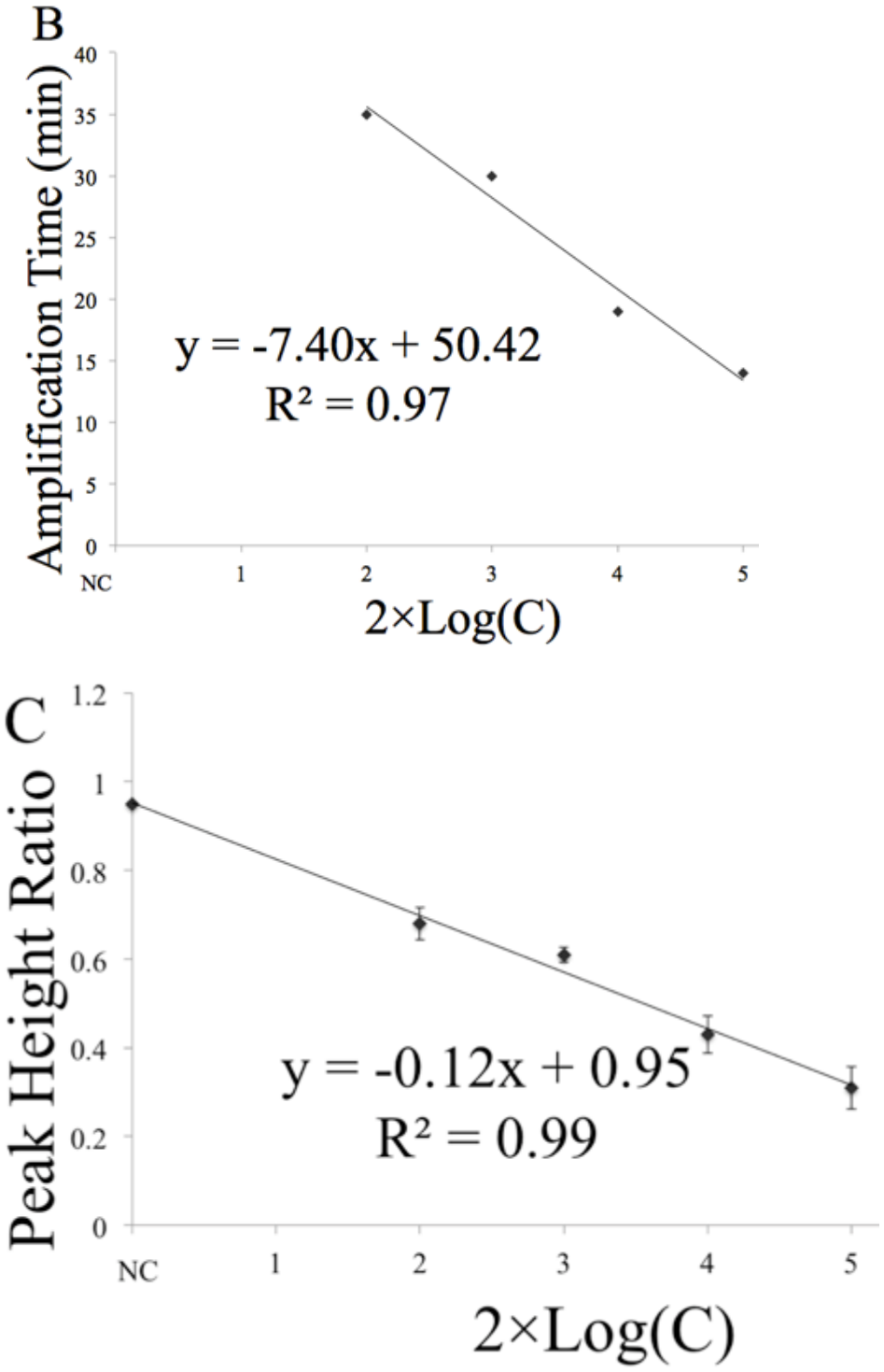


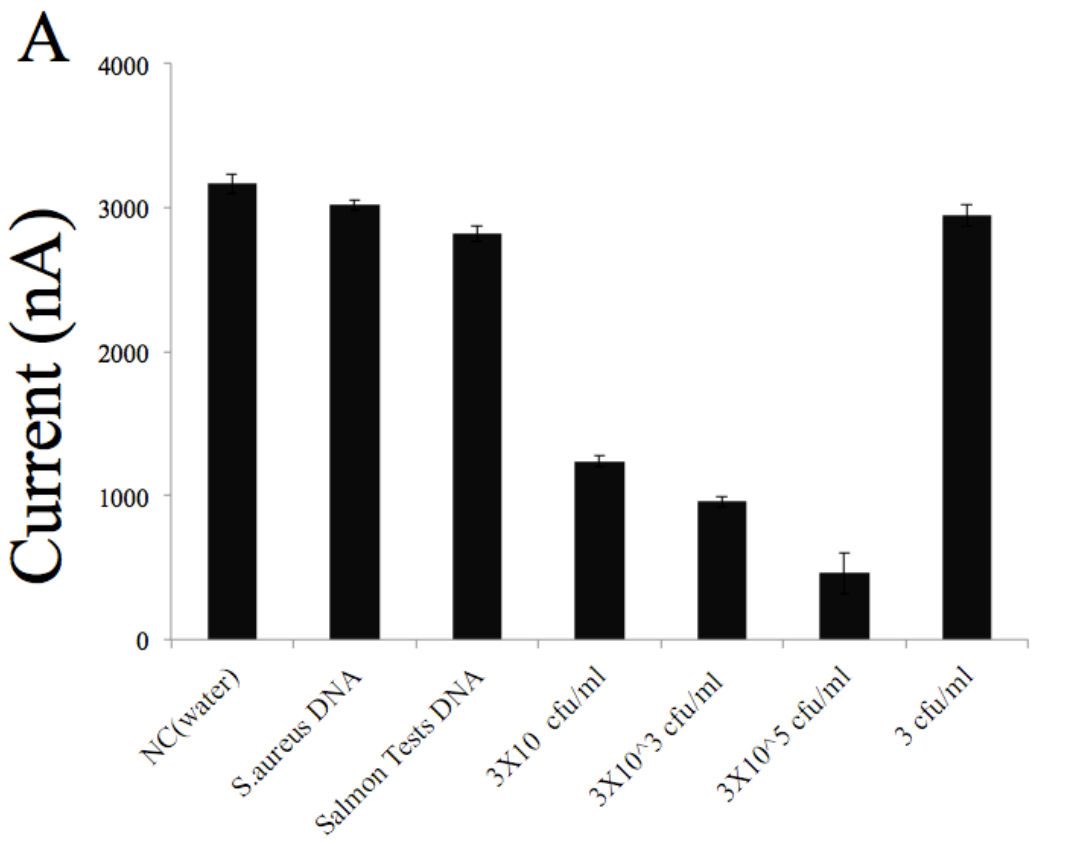
Fig 5.4 **Real-time measurement of *S.aureus* bacteria. (A) Real-time monitoring of LAMP amplicon using Osmium redox probe. *S.aureus* at different concentrations were detected in a real-time manner and electrochemical measurement at 5 min intervals for during the 50 min analysis. The threshold value was set at 0.85 (horizontal dotted line).The number of scan for each 5 min was 3 (n=3). (B) Amplification time vs concentration of *S.aureus*. A 35 min amplification time was required for detection of 200 CFU/mL of *S.aureus* (Vertical dotted line). (C) Quantification of the**

***S.aureus* based on peak height ratio. Theere is a linear relation ship between peak height ratio and logarithmic concentration of *S.aureus* (R=0.99). The RSD% amounts have been calculated in the Appendix III.**

5.3.4 End point detection and cross-reactivity

Generally, samples usually consist of several types of pathogens. To further investigate the performance of the device in detecting trace amount of pathogen, we chose to use end-point detection of LAMP amplicon. Measurement of end point detection was implemented by an analysis of the maximum peak of SWV, which indicates the extent of LAMP amplicons-Osmium redox interaction for different concentration of bacteria at 50 min amplification time. Additionally, the cross-reactivity test was performed to assess the specificity of the detection. Figure 5.5A shows the end-point detection of *E.coli* and *S.aureus* bacteria. Maximum anodic SWV peak was measured for 30 CFU/mL, 3×10^3 CFU/mL and 3×10^5 CFU/mL *E.coli* and the specificity of the assays was tested with *S.aureus* DNA, as well as Salmon testes DNA. It was found that the detection device was sensitive to as few as 30 CFU/mL of *E.coli* in real samples. No significant anodic peak change was observed for other the non-targeted DNA, confirming that the developed assay is highly specific.

The end-point detection as well as cross-reactivity tests were repeated for *S.aureus* with the same lysis and amplification steps as in the real-time measurements. It was found that the system was able to detect as low as 200 CFU/mL of *S.aureus* in 50 min. The specificity of the assay was assessed with, Salmon testes DNA as well as *E.coli* DNA and no significant peak change has been observed as shown in the Figure 5.5B.


A
Current (nA)
4000
3000
2000
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NC(water)
S.aureus DNA
Salmon Tests DNA
3X10 cfu/ml
3X10^3 cfu/ml
3X10^5 cfu/ml
3 cfu/ml

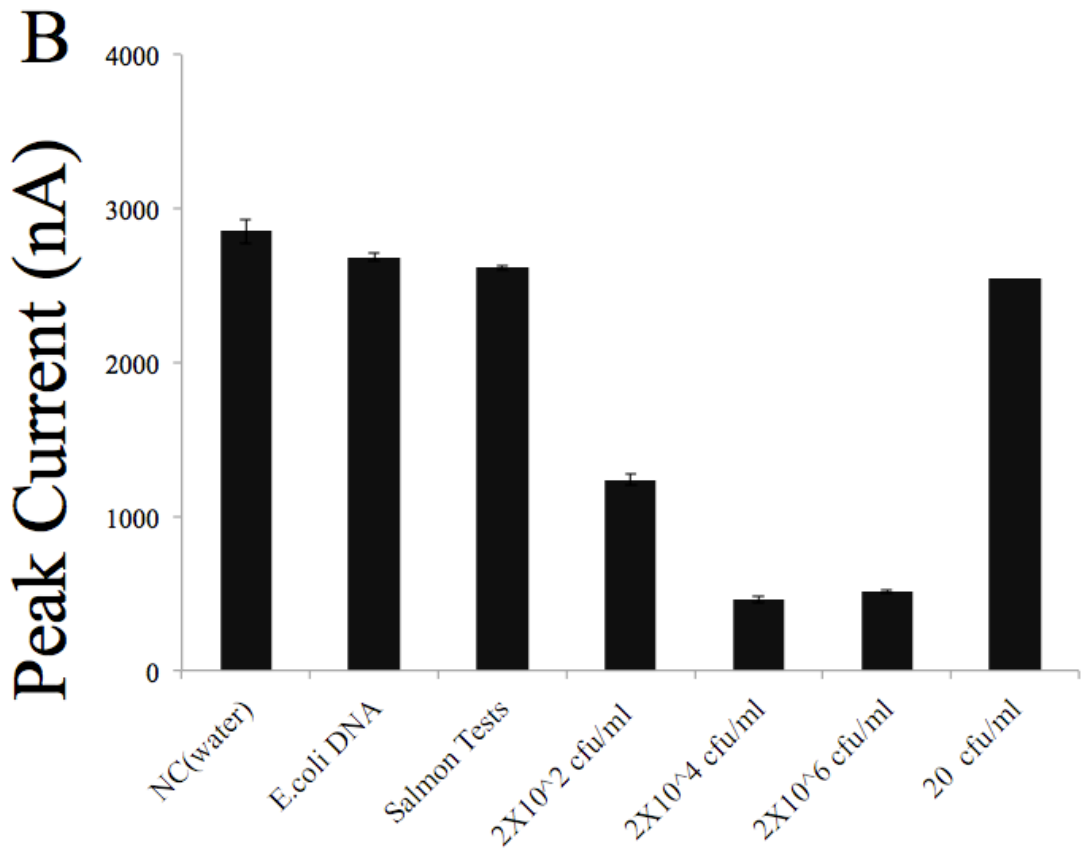
B
Peak Current (nA)
4000
3000
2000
1000
0
NC(water)
E.coli DNA
Salmon Tests
2X10^2 cfu/ml
2X10^4 cfu/ml
2X10^6 cfu/ml
20 cfu/ml

Fig 5.5 **End-point detection of bacteria at different concentration and cross-reactivity tests. (A) 30 CFU/mL of *E.coli* bacteria (B) 200 CFU/mL of *S.aureus* bacteria were detected after 50 min amplification time. The cross-reactivity test were performed for E.coli detection (A) using *S.aureus* DNA and *Salmon testes* DNA and (B) using *E.coli* DNA, *Salmon testes* DNA which reflect the fact that the assays are highly specific to the amplified targets.**

5.3.5 Comparison between Gel image and Electrochemical result

In order to validate our results, electrochemical data has been compared with the gel electrophoresis image. We have taken 5 µl of the LAMP amplicon from each reservoir of the flexible ribbon after 50 min amplification time, and run the gel electrophoresis following by UV imaging. The results are shown in Figures A3.2 and A3.3 in supplementary information. For the amplified samples ladder band have been observed which was in good agreement with the small SWV peak electrochemical signal due to the fact that OS redox intercalate with the amplicons. No ladder band has been observed in the negative control, which was in good agreement with the high SWV peak reflecting the fact that there is lack of amplicon in the sample and provides high specificity toward the target gene. Since the samples were collected after reaching the saturation limit (50 min amplification time), no dose dependent ladder bands were observed.

5.4 Conclusion

In this work, we combined the merit of LAMP in high-throughput format and real-time electrochemical monitoring of amplicon using OS redox. We demonstrated a novel roll-to-roll device that was easy to fabricate and assembled, with high sensitivity and specificity. The ability of real-time monitoring of amplicon, as well as sensitive end-point detection with the potential of being fully portable bacteria detection device, makes it highly robust for electrochemical pathogen detection. Using thermal shock lysis followed by isothermal NA amplification, we could amplify the *Tuf* gene of *E.coli* and detect as low as 30 CFU/mL; and the *Mcat* gene of *S.aureus* with a detection limit of 200 CFU/mL as a model for gram-positive bacteria. The portability, as well as simplicity of the technology helps to provide high-throughput ultra-sensitive POC for pathogen detection, capable of detection of up to 12 samples in a real-time manner.

6 CHAPTER SIX: BACTERIA SCREENING, VIABILITY, AND CONFIRMATION ASSAYS USING BACTERIOPHAGE-IMPEDIMETRIC/LOOP-MEDIATED ISOTHERMAL AMPLIFICATION DUAL-RESPONSE BIOSENSORS

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ABSTRACT

Here, we integrate two complementary detection strategies for the identification and quantification of *Escherichia coli* based on bacteriophage T4 as a natural bioreceptor for living bacteria cells. The first approach involves screening and viability assays, employing bacteriophage as the recognition element in label-free electrochemical impedance spectroscopy. The complementary approach is a confirmation by LAMP to amplify specifically the *E.coli Tuf* gene after lysis of the bound *E.coli* cells, followed by detection using linear sweep voltammetry. Bacteriphage T4 was cross-linked, in the presence of 1,4-phenylene diisothiocyanate, on a cysteamine-modified gold electrode. The impedimetric biosensor exhibits specific and reproducible detection with sensitivity over the concentration range of 10^3–10^9 CFU/mL, while the linear response of the LAMP approach was determined to be 10^2–10^7 CFU/mL. The LOD of 8×10^2 CFU/mL in less than 15 min and 10^2 CFU/mL within a response time of 40 min were achieved for the impedimetric and LAMP method, respectively. This work provides evidence that integration of the T4-bacteriophage-modified biosensor and LAMP can achieve screening, viability, and confirmation in less than 1 hour.

6.1 Introduction

Bacterial infection has been one of the major threats for public health and food safety for decades and remains the leading causes of outbreaks of diseases in the developing

and underdeveloped nations. In the United States, the Centers for Disease Control and Prevention (CDC) estimates that approximately more than 48 million illnesses, 128000 hospitalizations, and 3000 deaths occur each year because of food-borne and waterborne pathogens [200]. Besides, treatment for food-borne and waterborne diseases are extremely costly. The Economic Research Service (ERS) of the United States Department of Agriculture (USDA) indicated that the annual medical cost, productivity loss, and human disease caused by the more common food-borne pathogens ranged from 5.6 to 9.4 billion dollars [201]. Among all pathogens, *E. coli* is one of the highly relevant and commonly targeted indicator for routine analysis of contaminated water and food sources. It is a natural inhabitant of the intestinal tracts of humans and warm-blooded animals, which is characterized by diarrhea, urinary tract infections, inflammations, and peritonitis in immune-suppressed patients [202].

Over the past decade, the detection and identification of bacteria mainly relied on specific microbiological and biochemical methods, including culture and colony counting of bacteria [203], immunology-based methods (e.g., ELISA) based on antibody–antigen interactions [204], and polymerase chain reaction (PCR) which involves DNA amplification [205]. These conventional methods are highly selective and sensitive but the main challenges facing these techniques is that they require not only long incubation times to obtain results but also highly trained personnel or specialized equipment that make them impractical for field conditions, plus they are expensive. Moreover, more modern tests such as immunoassays and PCR do not distinguish between viable and nonviable bacteria cells, which can potentially lead to false-positive results and consequently inaccurate estimation of bacteria concentration in samples [206]. In order to overcome the issues associated with the current diagnostic techniques, there is an increasing demand toward diagnostic technologies that are rapid, reliable, low-cost, and user-friendly. These new technologies need to discriminate between closely related pathogenic and nonpathogenic microorganisms, detection of small quantities of a target within a complicated background matrix, a higher degree of stability over time, and the capacity of incorporation into a real-time monitoring system [207, 208].

Within the past few years, various biosensors have been developed as an alternative to the conventional methods for the detection of *E.coli* in food and water. These

biosensors include surface plasmon resonanance (SPR) [209, 210], optical fiber, surface enhanced Raman spectroscopy (SERS) [211], cantilever sensors [212], fluorescent-based methods [213], and quartz crystal microbalance (QCM) [214]. Each of these approaches features some combination of excellent sensitivity, good selectivity, fast time-to-result, and demonstrated clinical or biological applicability. However, they are time and labor intensive and require expensive instrumentation, which limits their use in laboratory and point-of-care applications [214].

In this regard, electrochemical biosensors hold a great potential as the next-generation detection strategy due to their high sensitivity, low cost, and ease of integration into miniaturized devices. Among them, electrochemical impedance spectroscopy (EIS) is a very powerful tool for the analysis of interfacial properties related to biorecognition events occurring at the modified surfaces. One of the advantages of EIS is the small amplitude perturbation from steady state, which makes it a nondestructive technique [215]. In this method, the immobilization of the bioreceptor is the key process in the construction of impedimetric biosensors, since the efficiency of bioreceptor immobilization on the electrode surface can profoundly affect the analytical performance of impedance biosensors. For this, antibody-based biosensing systems were widely used along with immobilization methods such as physical adsorption [216], the biotin–avidin system [217], covalent attachment through self-assembled monolayers (SAMs) [218], and a covalently linked antibody on a conducting polyaniline (PANI) film surface [219]. One of the major limitations of antibody-based immunosensors is the lack of discrimination between viable and nonviable bacteria cellsdue to the fact that antibodies can bind to the antigen regardless of whether the bacteria was dead or alive [220, 221]. Moreover, environmental factors or stress may affect antibody-specific antigen expression of bacterial cells, perturbing the capturing efficiency of the biosensors [222]. Yet, the development of impedimetric biosensors with an excellent selectivity against bacteria strains and dead bacteria still remains a challenge. Among the various bioreceptors that have been investigated, bacteriophage is emerging as a promising alternative due to its sensitivity, selectivity, discrimination between dead and alive cells, low-cost production, and higher thermostability than antibodies [223-225]. More recently, Mejri *et al* [226] developed a bacteriophage-based impedimetric biosensor for

the detection of *E.coli*; their results show that 10^4 CFU/mL can be detected, and minimal interference was observed upon incubation with non-target *Lactobacillus*.

To address this challenge, in this work we demonstrate for the first time a novel label-free impedimetric biosensor, integrating screening assay, viability testing, and confirmation assay using loop-mediated isothermal amplification (LAMP). This biosensor can be packaged within a portable microbioanalytical system for on-the-spot bacterial monitoring. The general approach (Figure 6.1) can be extended to develop biosensors for a wide variety of bacteria, and the methodology can easily be applied in a relatively short period of time at low cost, with higher sensitivity and specificity.

6.2 Experimental setup

6.2.1 Chemical and Materials

Cysteamine hydrochloride (Cyst), 1,4-phenylene diisothiocyanate (PDITC), *N*,*N*-dimethyl formamide (DMF), sulphuric acid, ethanolamine (EA), pyridine, potassium ferrocyanide ($K_4[Fe(CN)_6]$), potassium ferricyanide ($K_3[Fe(CN)_6]$), disodium hydrogen orthophosphate, and potassium dihydrogen orthophosphate were obtained from Sigma-Aldrich (Ontario, Canada). Hoechst 33258 20(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,50-bi (1*H*-benzimidazole) was purchased from Sigma-Aldrich (St. Louis, MO). Potassium chloride, sodium hydroxide, sodium chloride, hydrochloric acid, and Luria–Bertani (LB) Broth (Miller) and LB Agar (Miller) were purchased from Bioshop (Ontario, Canada). Bacteriophage T4 (HER27) and its host *E. coli* B were obtained from The Felix d'Herelle Reference Center for Bacterial Viruses (Laval University, Quebec, Canada) and molecular granulated agar was purchased from Fisher Scientific (Ontario, Canada). All chemical were of extra pure analytical grade and used as received without further purification.

Gold disk electrodes (2 mm diameter Au disk), Ag/AgCl reference electrodes, and a platinum wire counter electrode were purchased from Bioanalytical Systems (BAS, West Lafayette, IN, USA). Alumina pounder (1.0, 0.3, and 0.05 μm) and polishing cloth were purchased from Buehler (Ontario, Canada).

A 10 mM phosphate buffer saline (PBS) stock solution (pHs 7.4 and 8.5) was prepared using 8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, 137 mM NaCl, and 2.7 mM KCl. All aqueous solutions were prepared with deionized distilled water obtained from a Milli-Q water purifying system (18 MΩcm).

6.2.2 Pretreatment of the Gold Electrode and Surface Modification

Prior to cysteamine modification, the gold disc electrodes were mechanically polished with 1.0, 0.3, and 0.05 μm alumina/water slurry separately on a polishing cloth to a mirror finish, followed by washing thoroughly with deionized distilled water and acetone, respectively. Then, the polished electrodes were cleaned for 5 min with fresh piranha solution (7:3 v/v concentrated H_2SO_4 and 30% H_2O_2; CAUTION: piranha solution reacts violently with most organic materials and must be handled with extreme care) to remove hydrocarbon contamination and were rinsed intensively with distilled water. Subsequently, the electrodes were electrochemically cleaned by cycling the potential between −0.2 and 1.5 V (vs Ag/AgCl) at 100 mV/s for 25 cycles in a 100 mM H_2SO_4 solution and extensively rinsed with distilled water and dried with N_2 stream. The gold electrodes were then immersed in a 10 mM cysteamine solution overnight at room temperature to form a self-assembled monolayer (SAM), followed by rinsing with distilled water and ethanol to remove unbounded cysteamine molecules. The activation was done by an incubation of the cysteamine-modified gold electrode in a freshly prepared solution of PDICT in DMF/pyridine (9:1 v/v) at room temperature for 2 hour. The activated surfaces were washed successively with DMF and distilled water to remove unbound PDICT molecules and then dried under a stream of nitrogen. Next, the covalent immobilization of the T4 bacteriophage was accomplished via coupling their primary amines to the thiocyanate group in the surface of the modified gold electrode. The electrode was immersed in a 10 mM PBS solution (pH 8.5) containing 10^{10} PFU/mL T4 phage overnight at ambient temperature. Finally, the remaining thiocyanate groups were blocked with 0.1 M ethanolamine (pH 8.5) for 30 min. After the rinsing step with distilled water and PBS, the biosensor was installed in the electrochemical cell for the detection experiments.

6.2.3 Culture of *E. coli* Cells

An inoculum from frozen glycerol stock (kept at −80 °C) was streaked onto a LB agar plate and incubated at 37 °C overnight. A single colony from the plate was inoculated into 5 mL of LB broth and incubated overnight at 37 °C with shaking at 200 rpm. The viable bacterial number was calculated by preparing serial dilutions from the overnight culture and plating on LB agar plates. In order to prepare bacterial suspensions with different concentrations, the overnight culture was centrifuged at 10000 rpm for 5 min, and the pellet was resuspended and diluted in sterile PBS (pH 7.4). The biosensor was tested with skim milk, which was bought from a local store, and it was diluted 1:10 in PBS. The biosensor was also tested with a filtered and sterilized water sample from a local lake and spiked with various concentrations of bacteria. Further information about experimental protocols is available in the Appendix IV.

6.2.4 Apparatus

Cyclic voltammetry (CV), linear sweep voltammetry (LSV), and electrochemical impedance spectroscopy (EIS) were performed using a model 660D potentiostat/galvonastat (CH Instrument Inc.), controlled by a personal computer via CH Instruments. The electrochemical measurements were measured in a three-electrodes electrochemical cell configuration with a gold disc (2 mm) working electrode, a platinum wire as the counter electrode, and an Ag/AgCl (filled with 3 M KCl) reference electrode. Cyclic voltammetry was recorded between −0.2 and 0.5 V with a scan rate of 100 mV/s, in order to determine the midpoint between the oxidation and the reduction of the redox couple, which can be used as an applied DC potential for further EIS measurements. The impedance spectra were carried out in the frequency range of 1 to 10^5 Hz, using an

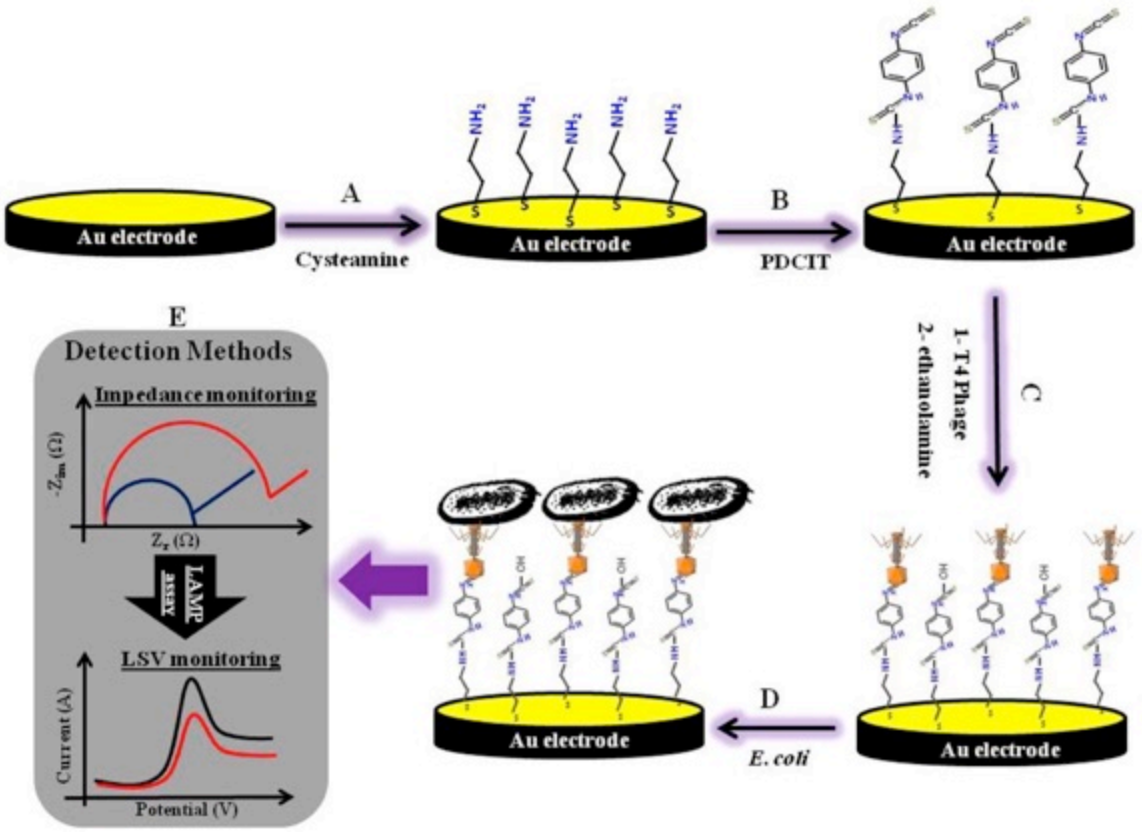


Fig 6.1 **Schematic representation of the T4-bacteriophage biosensors. (A) Cysteamine-assembly on gold electrode. (B) Activation with 1,4-dithiocyanate (PDICT) cross-linker. (C) Immobilization of the T4 phage and blocking with ethanolamine. (D) Capturing of the *E. coli* cells. (E) Detection method for *E. coli* based on the impedimetric/LAMP dual-response.**

alternative voltage with amplitude of 10 mV, superimposed on the DC formal potential of the redox couple. The impedance data were plotted in the form of a complex plane diagram (Nyquist plot) with a sampling rate of 5 points per decade. The obtained spectra were fitted using the CH instrument fitting program. The CV and EIS measurements were performed in 10 mM of PBS buffer (pH 7.4) in the presence of 10 mM of $Fe(CN)_6^{3-/4-}$. In order to minimize the electrical noise, all the electrochemical measurements were done in a Faraday cage.

The most critical step in biosensors is the immobilization of the biomolecules on the solid support, a process where the orientation of the probe toward the analyte in solution plays a key role in the performance of the biosensors. SAMs on a solid surface have been considered as one of the most suitable functional interlayers for the

immobilization of biomolecules, since it can easily be prepared by spontaneous chemisorption of molecules, on a metallic surface with a high degree of orientation, organization, packing, and stability [227]. Alkanethiols with various terminal groups can be used without affecting the self-assembly process or the SAM stability[228]. Cysteamine has been frequently employed as bifunctional building blocks, where the sulfur atoms of the molecules bind to the gold surface, while the amino groups can be used for the attachment of biological recognition agents [229]. The stepwise assembly of the bacterial biosensors was illustrated in Figure 6.1 and was monitored using multicharacterization techniques such as cyclic voltammetry and electrochemical impedance spectroscopy (EIS).

6.3 Result and Discussion

6.3.1 Electrochemical Characterization of the Gold Electrode and Surface Modification

Cyclic voltammetry is an efficient, well-accepted analytical method and is commonly used to monitor surface modification, since it provides a rapid and simple method for initial characterization of the modified electrode using a redox couple system [230]. The reversible redox probe $Fe(CN)_6^{3-/4-}$ was used to investigate the different steps of the gold electrode modification. Figure 6.2A shows the cyclic voltammograms of 10 mM $Fe(CN)_6^{3-/4-}$ at the bare gold electrode, cysteamine-modified gold electrode, immobilization of T4 bacteriophage, and afterward, blocking with ethanolamine. As expected, the $Fe(CN)_6^{3-/4-}$ redox probe exhibited a reversible behavior on the bare gold electrode with a peak-to-peak separation of 80 mV. The formal potential of 0.23 V was estimated from the mean of the anodic and cathodic peaks of the cyclic voltammogram of the bare gold electrode. This value can be used as the fixed DC potential for all the electrochemical impedance spectroscopy experiments. On the cysteamine-modified gold electrode, the peak current was slightly increased and the peak-to-peak separation decreased to 62 mV, due to the electrostatic attraction between the positively (surface pK_a 7.6) charged amine groups of the cysteamine-modified gold electrode and the negatively charged of the redox probe [231]. However, the peak current of the redox

probe was considerably decreased, and the peak-to-peak separation was increased after each step of the electrode modification: immersion in PDICT solution, immobilization of T4 bacteriophage, and ethanolamine blocking. The decreases in the peak currents might be attributed to the fact that the T4 bacteriophage and ethanolamine insulated the surface and effectively enhanced the electron transfer barriers.

Electrochemical impedance spectroscopy (EIS) was also used to further characterize the stepwise assembly of the bacterial biosensors, since it was reported as a nondestructive and an effective method to monitor the surface features, allowing the understanding of chemical transformation and the interfacial properties of the modified electrode [232]. Figure 6.2B illustrates the results of the Nyquist plot ($-Z_{im}$ vs Z_r) of the bare gold electrode, cysteamine-modified gold electrode, activation with PDCIT, and immobilization of the T4 bacteriophage in the presence of a 10 mM $Fe(CN)_6^{3-/4-}$ solution as a redox probe in 10 mM of PBS (pH 7.4). As shown in Figure 6.2B, an obvious semicircle was observed at high frequencies, corresponding to the electron transfer-limited process and followed by a straight line with a slope close to unity at lower frequencies, resulting from the diffusion limiting of the redox species from the electrolyte to the electrode interface. However, after cysteamine self-assembly on the gold electrode, the semicircle diameter was decreased because of the electrostatic interaction between the positive amino group of the cysteamine and the negatively charged redox probe. When the cysteamine-modified gold electrode was activated with PDCIT, the diameter of the semicircle was increased as expected. This is due to the chain length increase of the SAMs and the generation of neutral charge after the association of the amino groups of the cysteamine with the thiocyanate group. This increased chain length may introduce a certain hinderance to the flow of the redox probe to the surface of the gold electrode, as represented by a slight increase in the diameter of the semicircle. After the T4-bacteriophage molecules were attached to the activated electrode, the diameter was significantly increased in comparison with the previous modified electrode surfaces. The reason is that the T4-bacteriophage

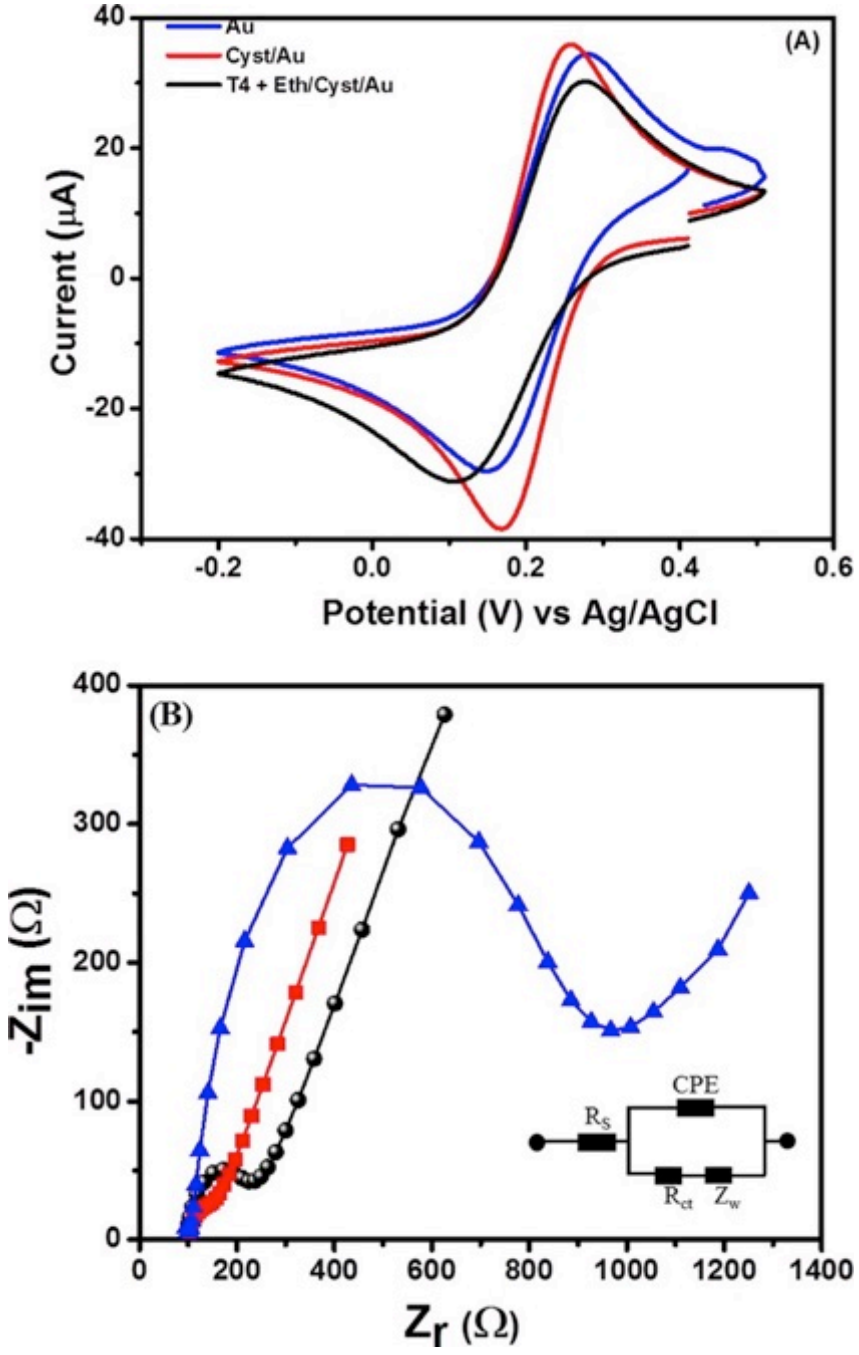


Fig 6.2 **T4-bacteriophage biosensor characterization on Au electrode (A) Cyclic voltammetry (CV) showing the steps of the T4-bacteriophage biosensors construction: (blue) bare gold electrode (Au), (red) cysteamine-modified gold electrode (blue ▲), and (black) immobilization of the T4 bacteriophage and blocking with ethanolamine in 10 mM PBS (pH 7.4) solution (T4+Eth/Cyst/Au), containing 10 mM Fe(CN)63–/4– at a scan rate of 100 mV/s. (B) Nyquist plot of the impedance spectra for bare gold electrode (Au) (●), cysteamine-modified gold electrode (Cyst/Au) (red ■), and immobilization of the T4 bacteriophage and blocking with ethanolamine (blue ▲), in the presence of millimeters of Fe(CN)63–/4– as a redox probe and a 10 mM PBS (pH 7.4) solution, over the frequency range**

from 105 to 1 Hz, a bias potential of +0.23 V vs Ag/AgCl (3M), and an ac signal of 10 mV. The inset is the Randle's equivalent circuit used to fit the impedance data.

molecules on the electrode acted as an insulting layer because they were negatively charged (pI 4–5) at the working pH 7.4 [233]. Consequently, they significantly hindered the diffusion of redox probe toward the electrode surface, resulting in a higher electron-transfer barrier and therefore enlarges the diameter of the semicircle.

The impedance spectra showed a semicircle and linear portion. The semicircle portion at higher frequencies corresponds to the electron-transfer limited process, and the linear portion at lower frequencies represents the diffusion-limited process. The equivalent circuit for an electrode undergoing heterogeneous electron transfer is usually described on the basis of the Randles equivalent circuit [234]. Due to the lack of surface regularity of biosensors and the nonhomogeneity of the current distribution, double layer capacitance can be replaced by a constant phase element (CPE). Therefore, the modified Randles equivalent circuit can be used as an ideal equivalent circuit to analyze the impedance spectra in this work [235]. As illustrated in the inset of Figure 6.2B, the equivalent circuit consists of a background solution resistance,R_s, a charge-transfer resistance, R_{CT}, the Warburg impedance, Z_W, resulting from the diffusion of the redox couple from the bulk of the solution to the electrode interface, and a CPE, representing the electrical double layer capacitance, which leads to a depressed semicircle in the corresponding Nyquist impedance plot. The impedance of the CPE is given by the following equation:

$$Z_{CPE} = Q^{-1}(j\omega)^{\alpha}$$

where ω is the electric field frequency, Q^{-1} is the modulus of the constant phase element, measured in Farads $cm^{-2}s^{\alpha-1}$, and the exponent α varies from 0 to 1 and reflects the ideality of the capacitor [236]. The double layer capacitance can be estimated from [237]

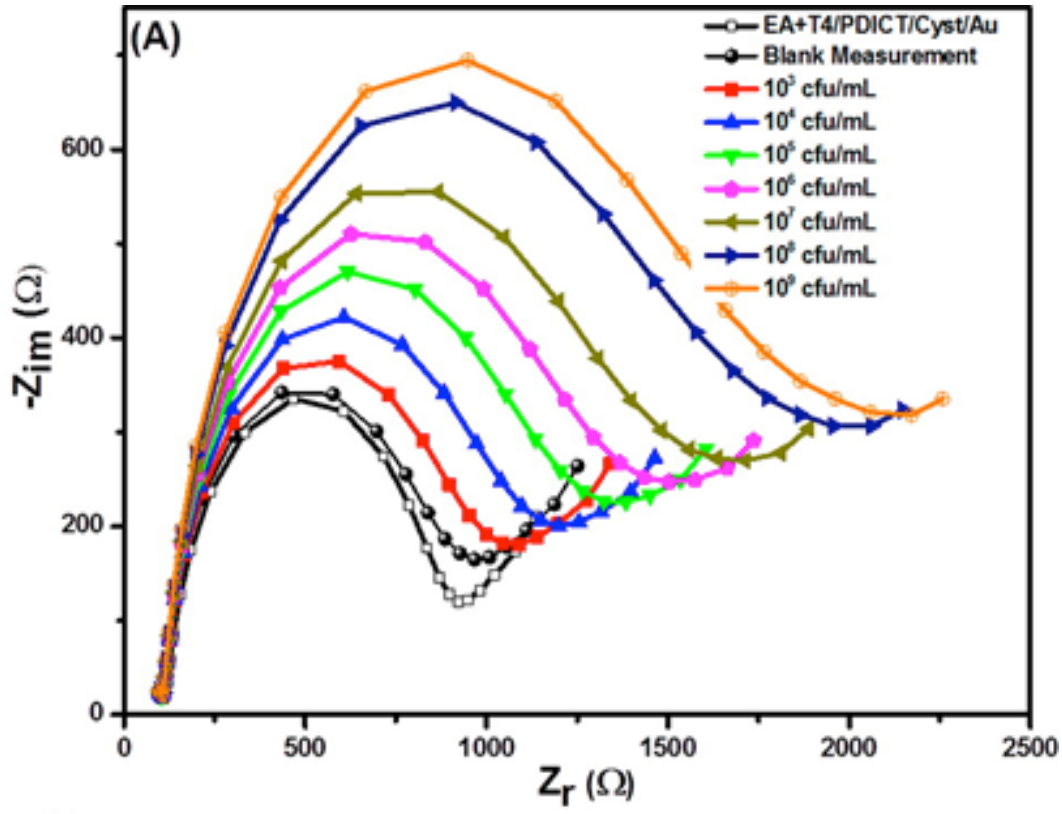
$$Q = C_{dl}^{\alpha}(R^{-1}{}_{s} + R_{ct}{}^{-1})^{1-\alpha}$$

R_s and Z_W represent the bulk properties of the electrolyte solution and diffusion features of the redox probe and ions in solution. These two components are not affected by

chemical modification occurring at the electrode surface. On the other hand, R_{ct} and CPE depend on the dielectric and insulating features at the electrode/electrolyte interface, respectively, hence they are controlled by the electrode surface modification. For Faradic impedance measurement, the R_{ct} is the most sensitive and straightforward parameter that can be used to characterize the events occurring at the interface between the immobilized probe on the electrode and the analyte in the solution [238]. Moreover, the variation in CPE is almost unnoticeable compared to the change in R_{ct}. R_{ct} for a bare Au electrode was estimated to be 220 Ω and decreased to 90 Ω when the electrode was modified with cysteamine, peripheral amine groups of which produced positive charge that accelerated the electron transfer for the $Fe(CN)_6^{3-/4-}$ redox probe. Subsequently, assembly of T4 bacteriophage on the aminated electrode after activation of the amine groups with the PDCIT cross-linker, generated an insulating layer on the electrode surface that functioned as a barrier to the interfacial electron transfer. This was reflected by the increased R_{ct} to 865 Ω, which was in agreement with the results reported by Yang *et al* [239]. All these data show that the stepwise modifications, cysteamine, PDCIT, and T4 bacteriophage, were successfully assembled on the gold electrode surface and formed a tunable kinetic barrier. The results obtained from EIS measurements are consistent with those extracted from cyclic voltammograms and confirm the successful immobilization of T4-bacteriophage molecules on the surface of the modified gold electrode.

6.3.2 Detection of *E. coli* Bacteria by Electrochemical Impedance (EIS)

The impedance measurement can be performed in the presence or absence of a redox couple, which is referred to as Faradaic and non-Faradaic impedance measurements, respectively[235]. Faradaic and non-Faradaic measurements are widely utilizedmethods that allow the detection of bacteria cells using immobilized antibodies on the electrode surface [240, 241]. The major drawbacks associated with antibodies as a bioreceptor is the low capturing efficiency of the biosensors [240], cross-reactivity with nontarget bacterial cells, instability due to environmental fluctuations, and high-cost


(A)
EA+T4/PDICT/Cyst/Au
Blank Measurement
10^3 cfu/mL
10^4 cfu/mL
10^5 cfu/mL
10^6 cfu/mL
10^7 cfu/mL
10^8 cfu/mL
10^9 cfu/mL
-Z_im (Ω)
Z_r (Ω)
0
200
400
600
0
500
1000
1500
2000
2500

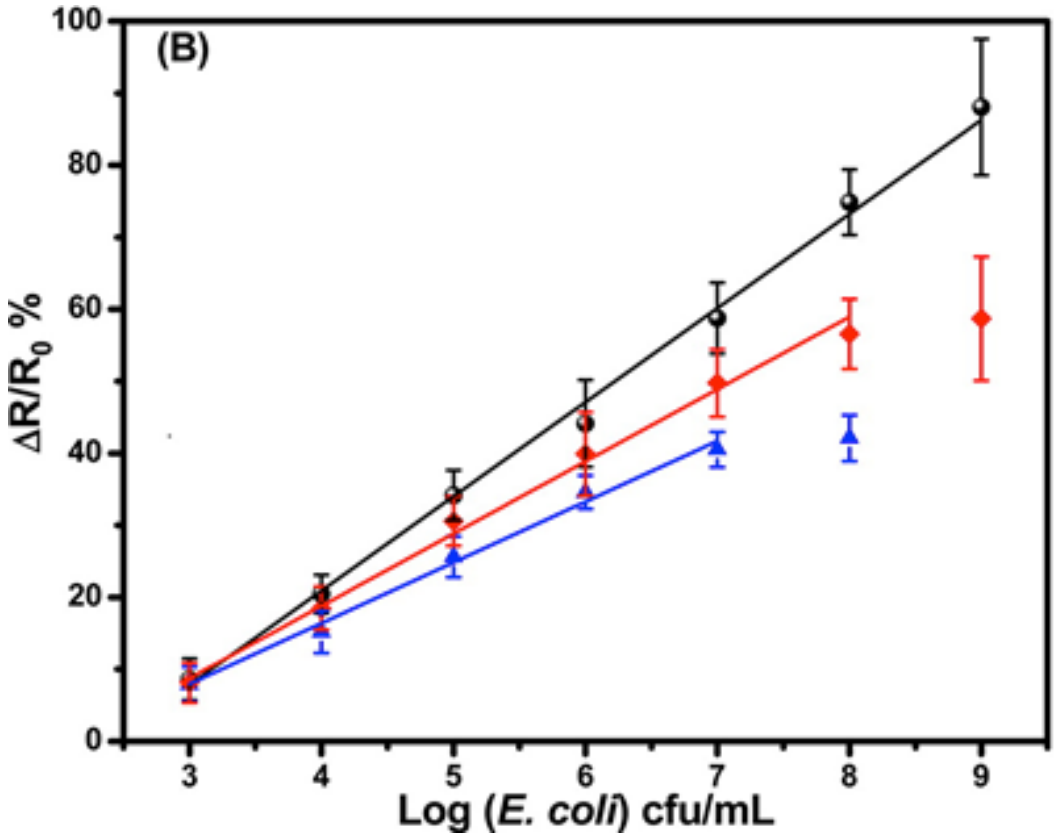

(B)
ΔR/R_0 %
Log (E. coli) cfu/mL
0
20
40
60
80
100
3
4
5
6
7
8
9

Fig 6.3 **(A) Nyquist impedimetric diagram of T4-bacteriophage-modified gold electrode interface at different concentrations of E. coli. Measurements were performed in 10 mM PBS (pH 7.4) containing 10 mM $Fe(CN)_6^{3-/4-}$: 0 (●), 10^3 (■), 10^4 (▲), 10^5 (▼), 10^6 (◆), 10^7 (◂), 10^8 (▸), and 10^9 (⊕) CFU/mL, (B) Normalized response, ΔRct (%), as a function of varied logarithmic concentration of the E. coli bacteria cells in PBS (●), skim milk (blue ▲), and lake water (⧫). Δ*R*ct (%) values obtained from the fitting of three independent impedance data sets.**

production and long analysis time, which can limit their long-term storage and their field applicability [220, 221]. Therefore, these issues need to be addressed by developing Faradaic impedance methods with immobilization of a new bioreceptor on the electrodes. Recently, bacteriophages are employed as biorecognition elements for the identification of various pathogenic microorganisms.

In order to demonstrate the practical utility of the proposed biosensor, the T4-bacteriophage-modified electrode was exposed to various concentrations of nonpathogenic *E.coli* B. The T4-bacteriophage biosensors were first incubated for 15 min with 50 µL of the appropriate *E.coli* concentration in 10 mM of PBS (pH 7.4), washed three times with PBS, and then the washed biosensor was placed into the conventional electrochemical cell containing a 10 mM of $Fe(CN)_6^{3-/4-}$ (pH=7.4) buffer solution for impedance measurement. The corresponding Nyquist plot of the impedance spectra is shown in Figure 6.3A. It appears that the diameter of the semicircle increased with increasing *E.coli* B concentrations. Gram-negative bacteria such as *E.coli* theoretically have an overall negative charge due to the charges present in their external membrane in the lipopolysaccharide [242]. Thus, their binding to T4 bacteriophage can act as a barrier for the electron transfer between the anionic redox species and the electrode surface. To complete the impedance investigation, the obtained results were fitted with the proposed modified Randle's equivalent circuit (inset of Figure 6.2B). As expected, we observed a significant change in the charge transfer resistance, R_{ct}, while the changes in the R_s, CPE, and Z_w were very small and irregular upon an increase of the *E.coli* concentration, supporting the use of R_{ct} as a quantitative parameter to evaluate the analytical performance of the developed T4-bacteriophage biosensor. In order to compare the relative responses of the developed biosensor and to suppress the small variation between electrodes, the signal values were normalized with respect to the charge transfer resistance (R_{ct}) of the immobilized T4 bacteriophage.

The normalized response, ΔR_{ct} (%) = [R_{ct}(phage bacteria) –R_{ct}(phage)]/R_{ct}(phage), was plotted as a function of *E. coli* concentration on a logarithmic scale (Figure 6.3B). R_{ct}(bacteria phage) and R_{ct}(phage) were the charge transfer resistance in the presence and the absence of *E. coli* cells, respectively. The change in the normalized response was linear with the logarithm of *E. coli* concentration from 10^3 to 10^9 CFU/mL and had a regression equation of ΔR_{ct} (%) = 13 log (*E. coli*) – 30.6, with a correlation coefficient of 0.998. The LOD was estimated to be 8×10^2 CFU/mL in 15 min time, which was calculated using the $3S_D/m$ equation, where m is the slope of the linear part of the calibration curve, and S_D is the standard deviation of the blank measurement.

In order to verify the ability of the phage biosensor to detect *E. coli* cells in a complex biological matrix, the performance of the biosensor was evaluated with skim milk and lake water spiked with *E.coli* B cells. On the other hand, the T4-bacteriophage biosensor exhibited a linear range between 10^3–10^8 CFU/mL, with LOD of 10^3 CFU/mL. The linear regression equation was ΔR_{ct} (%) = 10 log [*E.coli* (CFU/mL)] – 21.4 with a correlation coefficient of 0.993. The biosensor in lake water was saturated at 10^8 CFU/mL and showed no significant change with higher *E.coli* concentration.

The impedimetric label-free method described here for the detection and identification of nonpathogenic *E.coli* B cells represents a promising method. The dynamic range and the response time of this approach are much wider and extremely shorter, respectively, than those of methods reported previously. A short detection time is the most important demand concerning assays of polluted water sources and even more critical when bioterrorism is suspected. For comparison, Ruan *et al* [240] reported an impedimetric immunosensor for *E.coli* O157:H7 detection using horseradish peroxide enzyme-labeled for signal amplification. The corresponding calibration curve of their immunosensors was linear over the range from 6×10^4 to 6×10^7 CFU/mL; the LOD was 6×10^3 CFU/mL, and the overall assay time was 90 min. However, this method has significant drawbacks, such as the high cost of the reagents involved in the immunoreaction and the need of labeled antibodies. Another approach has been reported by Geng *et al*.[218] for the detection of *E.coli*, by immobilizing anti-*E. coli* antibodies on EDC/NHS-activated carboxyl groups, and then EIS was used to follow the specific binding between the antibodies and the bacteria. The authors showed that

there is a linear relationship between the charge transfer resistance (R_{ct}) and the logarithmic of the *E.coli* concentration in the range from 3×10^3 to 6×10^7 CFU/mL, and a concentration as low as 10^3 CFU/mL can be ambiguously detected within 60 min. More recently, the use of lectin as the biorecognition element has been proven as an effective tool for the detection and identification of bacteria. Gamella *et al* [243] reported biotinylated lectin-based screen-printed gold electrodes for the impedimetric label-free detection of *E.coli*. The proposed biosensor was able to detect *E.coli* in the range from 5×10^3 to 5×10^7 CFU/mL with a LOD of 5×10^3 CFU/mL within 60 min. A novel attempt for the detection of *E.coli* based on the recognition properties of the carbohydrate was reported recently by Guo *et al* [244]. The sensing layer was first constructed by mixed self-assembly of a long-chain, thiol-terminated α-mannoside and thiol-terminated oligoethylene glycol "spacer" molecule onto a clean gold electrode surface, and then the interaction between the α-mannoside and *E.coli* was monitored by EIS. The authors show that the calibration plot curve exhibits a linear relationship between the charge transfer resistance (R_{ct}) and the logarithmic concentration of the *E.coli* over the range from 10^2 to 10^3 CFU/mL. However, the LOD and time of detection were not determined in their work.

6.3.3 Specificity, Reproducibility, Stability, and Viability Test

To further investigate the performance of the T4-bacteriophage-based impedimetric biosensors, a series of experiments was performed on the specificity, reproducibility, stability, and viability test. The specificity of the T4-bacteriophage biosensor was investigated by verifying the normalized response of the proposed biosensors in relation to *Listeria* cells. As expected, no significant change in the normalized response (7.0 ± 2.8%) was observed to 10^8CFU/mL of *Listeria* cells, which is 10-fold lower than the response to the same concentration of the specific bacteria (Figure 6.4). An additional experiment was used as a negative control with a T4-bacteriophage-free electrode (i.e., the isothiocyante groups were capped with ethanolamine under similar solution conditions for T4-bacteriophage immobilization). In this case, a 4.5 ± 1.8% change in the normalized response was noticed (Figure 6.4). This supports that the changes of the charge-transfer resistance were induced by the specific binding between the

immobilized T4 bacteriophage and the bacteria in solution and not due to the nonspecific adsorption of the bacteria on the modified gold electrode.

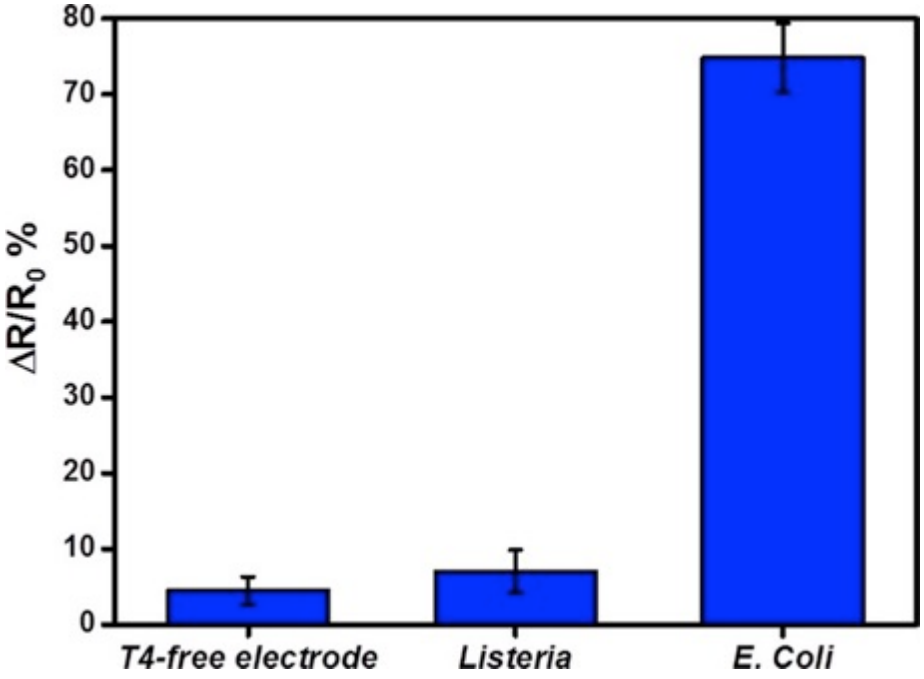


Fig 6.4 Figure 4. T4-bacteriophage biosensors selectivity against *Listeria* cells.

Repeatability and reproducibility of a proposed biosensor is always of great interest, and it appears to be a challenge. It depends mainly on the stability of the biological recognition element and the preparation of the biosensor itself. The repeatability and reproducibility of the present biosensor were investigated with intra- and interassay coefficient of variation (%CV). The intra-assay precision of the biosensor was evaluated, by measuring five times the *E.coli* B with a concentration of 10^8 CFU/mL. The interassay precision was assessed, by assaying the *E.coli* at the same concentration (10^8 CFU/mL) with three different biosensors prepared independently at the same experimental conditions. The coefficient of variation of intra- and interassay obtained from 10^8 CFU/mL *E.coli* B were 4.3% and 10.7%, respectively. Besides repeatability and reproducibility, stability of the biosensors was also investigated. As expected, no obvious change was observed after 7 days of biosensors storage in PBS solution at 4 °C. These results demonstrate the acceptable repeatability, reproducibility, and stability of the proposed T4-bacteriophage biosensors.

Real-time detection of alive bacteria using a portable instrument remains a challenge to many scientists and engineers, since the presence of dead bacteria is not considered a health risk. The impedemitric response for alive and dead *E.coli* B cells was tested after incubation of 10^8 CFU/mL of a mixture of alive and dead cells on the T4-bacteriophage-modified electrode for 15 min, followed by washing with PBS. The biosensors were transferred to the electrochemical cell, and the Faradaic impedance was consecutively measured over 1 h to determine the viability of the bacteria. Figure 6.5A depicts the normalized response change versus time for both cells. The normalized response of the biosensors with an alive cell was increased by 82% and reached a steady-state condition within 15 min, indicating the binding of the alive cells on the T4 bacteriophage. This response then started to decrease gradually, confirming the infection and lysis of the alive bacteria cells by the immobilized T4 bacteriophage. In contrast, a slight increase of 8.6% was observed in the case of the biosensors with dead cells, as can be seen from the curve (b) in Figure 6.5A, revealing that the T4 bacteriophage cannot recognize the dead *E.coli* B cells. These results are in good agreement with the previously reported data, where the author has shown that the T4 bacteriophage cannot attach to the acetone-ether-treated cells because the adsorption was inhibited by the lipopolysaccharides hydrolysis [245, 246]. Moreover, it was reported a long time ago that the adsorption rate constant of bacteriophage to bacterium is found to change between wide limits, depending on the physiological state of the bacterium [247]. Recently, it was found that the adsorption rate in alive host cells was much larger than that in dead host cells [248]. In our case, the small variation in the normalized response might be due to a short incubation time of 15 min and/or to the denaturation of the bacteriophage attachment receptors on the bacteria cell surface. This clearly confirms that our developed T4-bacteriophage biosensor can be used for excellent discrimination against the dead *E.coli* cells. Furthermore, more experiments have been performed for different ratios of alive/dead cells (alive:dead, 100:0, 80:20, 50:50, 30:70, and 0:100); the final concentration was 10^8 cfu/mL bacteria cells. As shown in Figure 6.5B, there is a small variation in the normalized response (ΔR_{ct} (%)) between the 100% of alive cells and the other mixed concentrations (80%, 50%, and 30%). This result is expected since

the different ratios of alive/dead cells present high concentration of live cells (more than 3×10^7 cfu/mL).

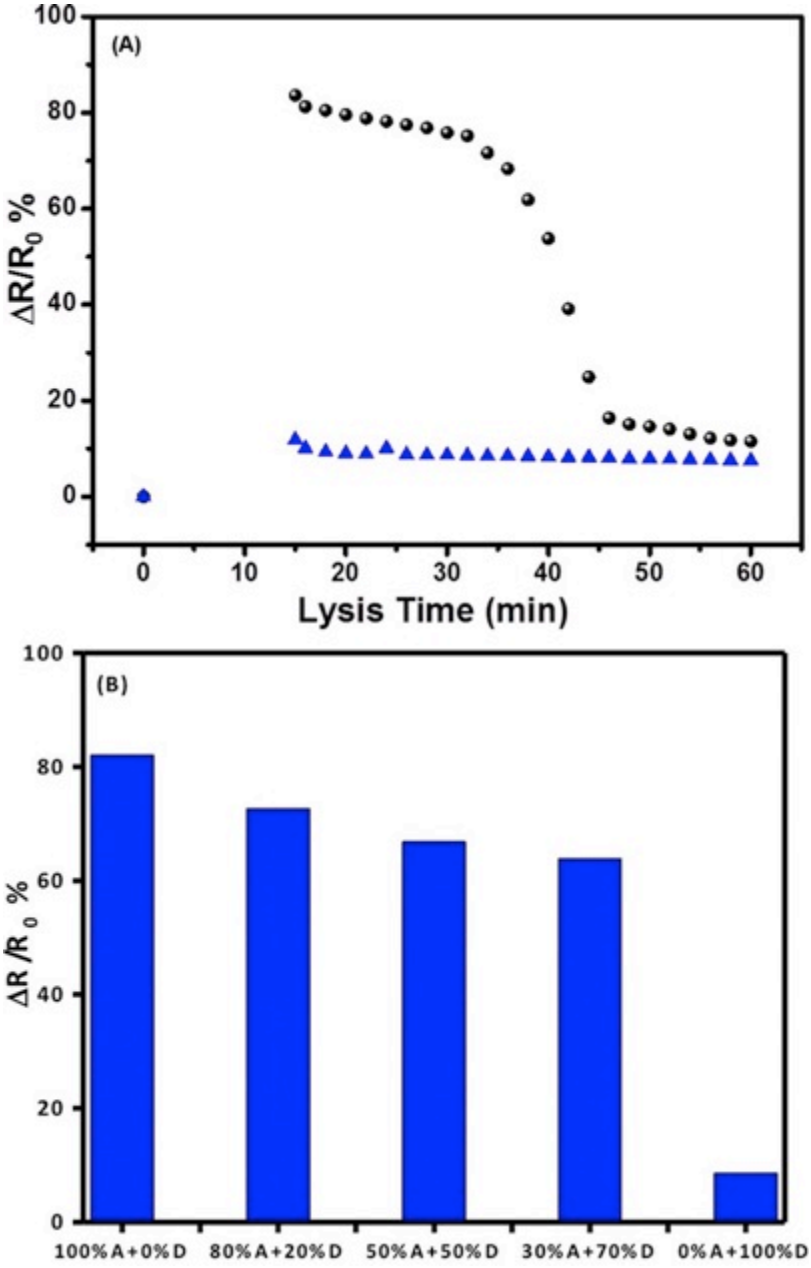


Fig 6.5 **(A) Viability test of live (●) and dead (blue ▲) E. coli bacteria cells (B) shows the sensor response for different ratios of alive/dead cells (alive:dead, 100:0, 80:20, 50:50, 30:70, and 0:100); the final concentration was 108 cfu/mL.**

6.3.4 LAMP-Based *E. coli* Biosensors

The concentration of the bacteria allowed by the Environmental Protection Agency regulation in treated drinking water should contain no (zero) bacteria in 100 ml [249]. Hence, a biosensor must be able to provide LOD as low as a single bacteria in 100 ml of potable water [250]. In this regard, the combination of the LAMP-based method and the related release of intrinsic substances following lysis of the cell by the specific bacteriophage can provide a powerful confirmation technology for highly specific and sensitive detection of *E.coli* and allowing the LOD to be pushed down. To demonstrate the signal amplification of the LAMP method, the binding of *E. coli* B to the T4-bacteriophage-modified electrode was removed from the electrochemical cell after finishing the impedance experiments, followed by washing with PBS and water, and then it was incubated in 200 µL of the LAMP master mix at 65 °C for 40 min before being subject to a LSV scan; the LAMP reaction protocol is described in Appendix IV. The time was already optimized in our previous work, since it plays a major role in bacteria quantification [251]. After that, the electrochemical signal (LSV) of the intercalating agent Hoechst was measured in a mixture containing 6 µL of the amplicon, 12 µL of Hoechst redox, and 12 µL of PBS (10 mM) buffer and then was plotted against the *E.coli* concentration. As shown in Figure 6.6A, a gradual decrease in the redox peak of the Hoechst was clearly observed with increasing *E. coli* concentrations. This can be attributed to the intercalation of the Hoechst molecules within the double-stranded LAMP reaction products[250]. From Figure 6.6B, it can be seen that the normalized response [ΔI (%) = (I_0 – I (LAMP))/I_0, where I_0 and I(LAMP) are the current peak at 0.5 V vs Ag/AgCl in the absence and the presence of LAMP product, respectively] has a linear relationship with the logarithmic scale of *E. coli* concentration over the range from 10^2 to 10^7 CFU/mL and had a regression equation ΔI (%) = 4 log (*E.coli*) + 7.36 with a correlation coefficient of 0.998. The LOD was 100 CFU/mL and with total assay time less than 45 min. With this strategy, LOD was enhanced by at least 1 order of magnitude.

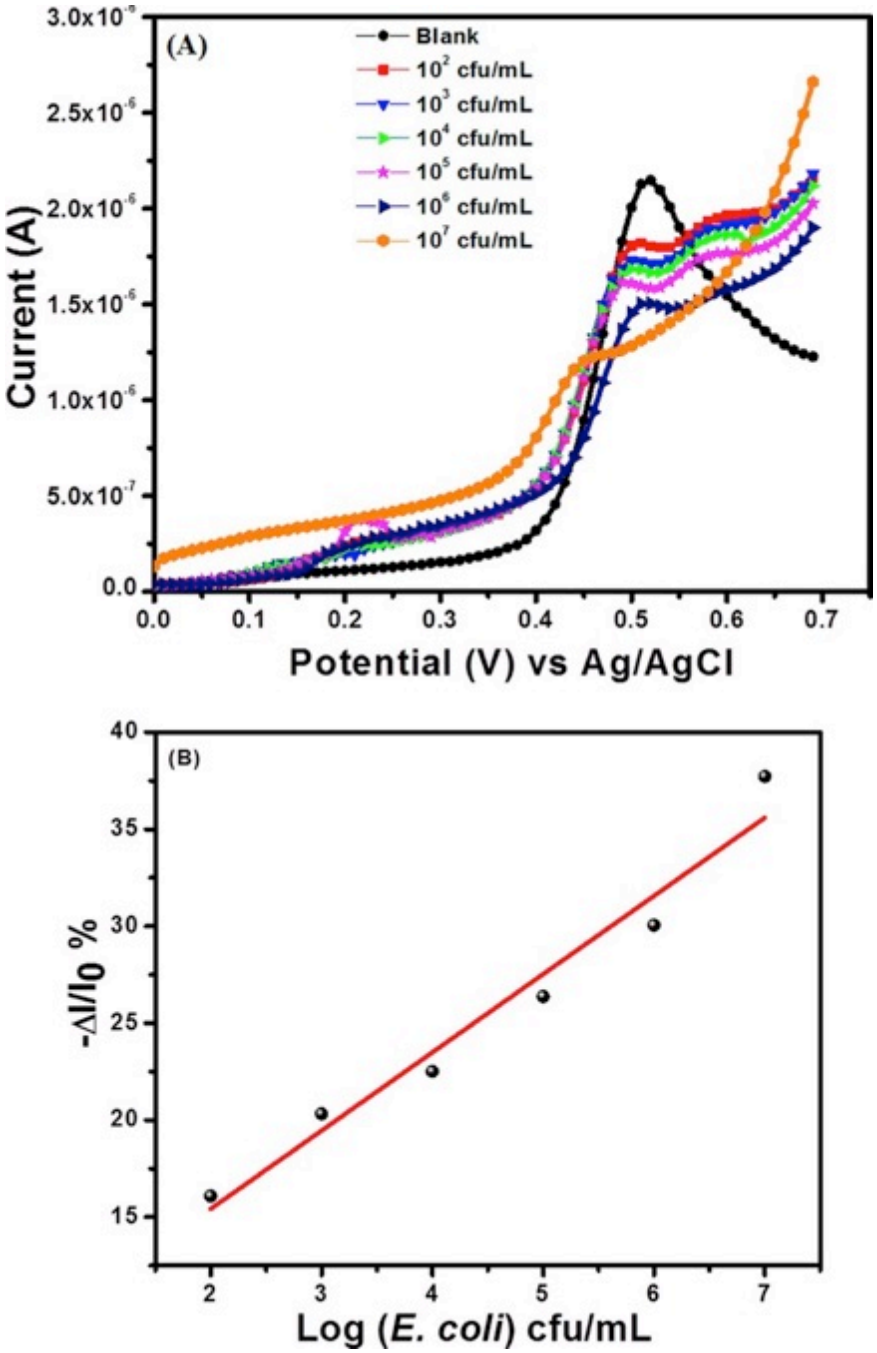


Fig 6.6 **(A) Linear Sweep voltammetry (LSV) monitoring the LAMP amplification of the *Tuf* gene target. (B) Calibration curve of the LAMP assay.**

6.4 Conclusion

A robust and convenient biosensing platform could provide a promising tool for rapid detection of pathogenic infection. Toward this goal, we have demonstrated for the first

time a fast, selective, and highly sensitive assay for the detection of *E.coli* B, using bacteriophage-based EIS/LAMP dual-response biosensors. The impedimetric assay incorporates a bacteriophage, a virus that can be used as a “capturing” agent, identifies, and lysis only one bacterial species among mixed populations, thereby releasing the intracellular *Tuf* gene that can be amplified by LAMP method and monitored by the LSV technique. Our electrochemical impedance results reveal that the *E. coli* bacteria can be detected quickly and accurately without preconcentration or enrichment steps at the level of 800 cfu/mL with excellent discrimination against other bacteria or even dead *E. coli* bacteria. The LOD corresponding to the LAMP assay was almost 1 order of magnitude lower. The time required for detection was approximately 15 min for the screening assay using EIS and 40 min for the LAMP confirmation assay. Given the advantages arising from the specificity, stability, and capture efficiency of the bacteriophage over antibodies, we expect that this assay could form a generic platform for advanced bacteria sensing, with a high promise in practical applications.

7 CHAPTER SEVEN : SUMMARY AND FUTURE WORK

7.1 Summary of findings

So far, isothermal amplification techniques have demonstrated alternative amplification methods over traditional "gold standard" PCR. The LAMP method has been used more than any other isothermal amplification technique for bacteria and virus detection due to being a robust tool for amplification of various gene targets with different base pair sizes. Microfluidic platforms provided the opportunity to perform all the traditional analytical steps such as sample purification, NA extraction, and detection [252]. Moreover, Despite the fact that several novel detection techniques such as giant magneto-resistive (GMR) [253] and bioluminescent [254] have been tried and shows silver lining detection mechanism for LAMP monitoring, it seems optical colorimetric and electrochemical detection analyses are the most frequently used detection mechanisms in LAMP based LOCs, both in academic research and commercial POC devices.

This work presented four different novel platforms based on LAMP technique for pathogen detection. These platforms include: (i) microfluidic electrochemical device for detection,(ii) novel high throughput cassette platform based on colorimetric pathogen detection, (iii) real-time electrochemical monitoring of LAMP reaction for bacteria detection, (iv) liveability test for *E.coli* bacteria using T4 bacteriophage with dual response impedimetric/LAMP biosensor.

First, we designed and fabricated LOC device for detection of LAMP amplicon based on end point electrochemical technique. Here, we developed two parallel microfluidic for negative control and sample with bacteria detection. Samples with *E.coli* bacteria were lysed and amplified in reaction chamber isothermally (66°C) and amplified product transferred to detection chamber containing disposable screen-printed electrode. The redox solution was added to the sample for electrochemical detection. Through amplification time optimisation, we quantified the bacteria based on the LSV peak signal corresponding to the amplicon concentration in the solution (Chapter three).

Next, we developed a LOC device for high throughput pathogen detection with colorimetric technique. Here, we used flexible plastic to fabricate array of chambers and

transformed into a cassette format. HNB and Calcein dyes were used for pathogen *E.coli* and *S.aureus* bacteria detection with LOD of 30 CFU/ml and 200 CFU/ml, respectively. Subsequently, we have lysed *S.aureus* at 95°C before amplification. Using this device, common shortcomings of LAMP such as lack of high throughput analysis have been compromised (Chapter four). We further excelled the detection mechanism of this device by attaching a flexible electrode at each reaction chamber and using Osmium redox complex for real-time bacteria detection (Chapter five). An Important issue for the current POC platforms based on LAMP amplification is the cost of the current LOCs and their reader devices, which still is high and limits its applicability in resource-limited settings due to their fabrication costs as well as the materials used for the chip. A possible way to circumvent this problem is to fabricate chips from abundant materials that are readily available all over the world at low cost for which this device provides an answer to this need.

We also advanced a biosensor platform for high sensitive liveability test by combining T4 bacteriophage as a natural bio-receptor for live *E.coli* bacteria by impedance measurement. The sensitivity of assay increased by using LAMP technique with LOD of 10^2 CFU/ml.

The original contributions to the knowledge of this thesis were mainly (1) novel design and fabrication of microfluidic chip for high sensitive quantification of *E.coli* bacteria in less than an hour (35 min) based on LAMP. (2) Novel design of high throughput LOC device for colorimetric and electrochemical real-time detection of gram-negative and some of gram-positive bacteria. (3) Novel biosensor as a proof of concept for high sensitive liveability *E.coli* bacteria test.

7.2 **Recommandations for future direction**

Following the advancement in development of LOCs based on LAMP, several challenges remain unresolved. For example, bacteriophage/LAMP biosensor in chapter 6 can be applied in a plastic chip made of out PMMA. Possible design of microchip can be based on fabrication of two gold electrodes on the glass substrate using e-beam lithography and lift-off techniques. The PMMA chips consist of multiple parallel

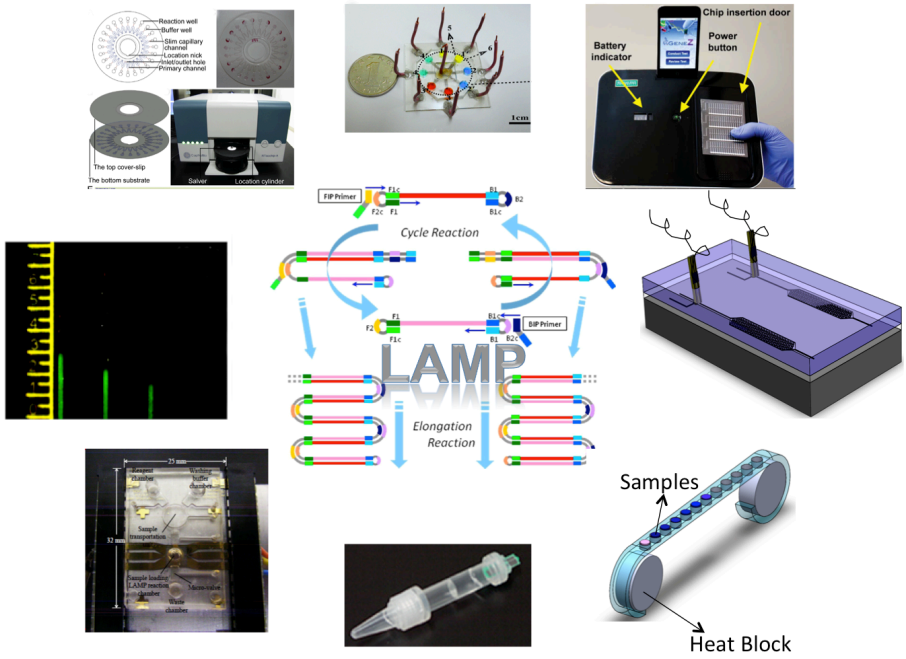
chambers with inlet and outlet channels for applying various reagents and specific bacteriophages for electrode surface modification as shown in Figure 6.1. The potential chip can be used as a portable high sensitive, multiplex liveability test for pathogens.

Another interesting prospect for future research is to use of cellulose paper as a substrate for LAMP amplification. Current LOC devices use plastic polymer for amplification, which increases the cost of the chip manufacturing. Development of paper-based microchip with colorimetric detection can reduce the cost and increase the sensitivity to as low as single cell detection.

Overall we believe that the platforms of LOCs based on LAMP we demonstrated in this thesis can be a step forward towards enabling these systems in the context of point of care diagnostics.

APPENDIX I

ADVANCES ON LAB ON A CHIP DEVICES BASED ON LOOP-MEDIATED ISOTHERMAL AMPLIFICATION: RECENT NOVEL STRATEGIES AND DETECTION MECHANISM



8 ABSTRACT

Rapid, sensitive, and selective pathogen detection is of paramount importance in infectious disease detection and treatment monitoring in global health. Currently available diagnostic assays based on PCR and ELISA are time-consuming, complex, and relatively expensive, which are ill suited to meet the current requirements for middle-/low- income countries. LAMP technique has shown a great promise to revolutionize point-of-care molecular diagnostics and has been used extensively to develop rapid and sensitive diagnostic assays for pathogen detection and nucleic acid analysis. Here, we reviewed novel LAMP-based LOC diagnostic assays that have been developed for pathogen detection over the past recent years. In addition, various LOC

platforms based on their design strategies for pathogen detection have also been presented. LAMP-based platforms in the development pipeline and commercialization were also discussed.

8.1 Introduction

The detection of nucleic acid (NA) using point-of-care (POC) micro total analysis systems (μTAS) is a rapidly growing field due to the numerous clinical and industrial applications [8, 138, 255-259]. The concept of μTAS, benefits nucleic acid amplifications by increasing the surface area to volume ratio, enhancing the heat transfer in lab on a chip (LOC) devices, and providing portable devices for diagnostic applications[260]. Development of POC diagnostic platforms based on nucleic acid detection require several rounds of cell lysis, NA extraction, and purification that need to be implemented prior to amplification[261]. NA amplification is of paramount importance in detecting small quantities of NA in finger-prick volumes of biological samples. Polymerase chain reaction (PCR) is the most common method to amplify DNA; however, it involves several thermo-cycling steps. Isothermal amplification of NA and DNA eliminates the need for thermo-cycling steps, thus reducing the cost and increasing the quality of the assay [97, 252, 262].

Among various amplification techniques including rolling circle amplification (RCA) [263], strand displacement amplification (SDA) [264], signal mediated amplification of RNA technology (SMART) [265] , nucleic acid sequence-based amplification (NASBA) [266], single primer-triggered isothermal amplification [267], helicase-dependent amplification (HDA)[268], and Cross Priming amplification (CPA) [269] which were reviewed extensively in the past [161, 270]; loop mediated isothermal amplification (LAMP) [271-273] has shown a relatively higher stability, faster amplification, and higher sensitivity and specificity for nucleic acid detection. Considering the nature of the reaction it can produces at least 50 times more amplicon, as compared with PCR-based techniques [274]. It has the ability to amplify medium to long-range template strands of nucleic acids (less than 300 bps), which makes it suitable for amplification of various DNA from pathogens and viruses [271]. Moreover, it can amplify the target NA in more

complex substrates and works despite the presence of substances that usually inhibit PCR reactions such as blood components of hemoglubin, IgG, IgM [275] or some food ingredients [254, 276, 277]. The specificity of LAMP is much higher than PCR, because LAMP uses four to six different primers that bind to specific sites on the template strand (in comparison with PCR which uses only two primers)[161] . LAMP amplifies NA at temperatures between 60-66°C by leveraging *Bst* polymerase enzyme with high strand displacement activity [271, 278, 279]. It is worth noting that LAMP has been shown to work even without proper NA extraction and purification as long as cell lysis takes place.

Here, we review LAMP-based microchip technologies that have been developed for NA detection. We first review various detection mechanisms for LAMP reactions such as colorimetric, electrochemical, optical, electrophoresis and pH based sensing. Then, novel LOC design concepts based on LAMP amplification are covered.

8.2 **Colorimetric Detection**

The basic concept of colorimetric detection of amplicon is based on the principle of LAMP reaction and the production of magnesium pyrophosphate ($Mg_2P_2O_7$) as a side product of the reaction of dNTP and $MgSO_4$. Magnesium pyrophosphate is insoluble and will precipitate out of solution, which causes the turbidity of the solution to increase[280]. Despite the fact that this technique requires no instrument for analysis, it is not a proper method to be used in POC diagnostics as it is sometimes hard to distinguish by naked eye on chip. Conversely, various colorimetric dyes can be used to detect the presence of amplicon in the solution on- chip by the naked eye. These dyes should have two main characteristics: 1) Dyes, should not inhibit the amplification reaction.. (2) The color change should be easily distinguished by the naked eye. As Shown in **Table A1**, there are various dyes used for LAMP amplicon detection such as fluorescent probes and pH sensitive dyes[281], that have been used for LAMP amplification detection. Calcein and hydronaphthol blue (HNB) are the main dyes that can be added in the pre-amplification sample without inhibiting the LAMP reaction[137]. Other dyes such as Propodium Iodine, SYBER GREEN I and Picogreen can be used after amplification, as they inhibit LAMP and terminate amplification.

Table A1.1 Various Dyes used for LAMP detection.

Dyes Name	Color before amplification	Color in presence of amplicon	Inhibit LAMP	References
HdroxynaphtolBlue (HNB)	Violet	Blue	No	[137]
Calcein	Yellow	Green	No	[280]
SYBR GREEN I	Dark Orange	Green	Yes	[282]
PicoGreen	Light Orange	Green	Yes	[283]
Propodium Iodine	Orange	White/Silver	Yes	[133]
SYTO-81, SYTO-9	Orange	Green	No	[284]
YO-PRO-1	Green	Green	No	[285]
Gene Finder	Orange	Green	Yes	[286]
Evagreen	Orange	Green	No	[287]
Ethidium Bromide	No Color (Under UV)	Yellow/Red (Under UV)	No	[285, 287]
AuNP	Red	Purple	No	[288], [289]
Gold nano rod (GNR)	Red	Purple	No	[290]
Fluorescein Isothiocyanate	Yellow-Orange	Green	No	[291]

(FITC)				
Leuco triphenylmethane	No color	Violet	TBD	[292]
SYBR Premix Ex TaqTM II	Red	Green	Yes	[286]

Dou *et al* have developed a hybridized paper/plastic microfluidic chip for the detection of *Neisseria meningitidis* using calcein that has a limit of detection of 3 DNA copies in a 26 µL sample[293]. The LAMP amplification was implemented in cerebrospinal fluid as LAMP was not inhibited in that solution.

In another study, a cassette device, consisting of two aluminum reels and a plastic ribbon with an array of chambers, was developed to detect *E. coli* (30 CFU/ml) and *S. aureu*s (200 CFU/ml) utilizing HNB and calcein dyes, respectively[198]. These pathogenic bacteria species and a LAMP reagent mixture were applied to the chambers, and then the array was covered by simple plastic tape. Cellular lysis was implemented through thermal shock at 65°C for *E. coli* and 90°C for *S. aureus* for 2 minutes[198]. The color of HNB changes from purple to blue and the color of calcein changes from yellow to green if the target species exist in the sample. A similar approach was also used to develop a plastic pouch microchip to detect HSV-1 and HSV-2 with detection limits of 6.08 copies/µl and 0.598 copies/µl, respectively [294]. The simplicity of the design, ease of fabrication, and use of a polyethylene sheet make these chips highly desirable for POC diagnostic for resource-limited settings. Wu *et al* developed a microfluidic chip made of glass for detection of NA using SYBER GREEN I dye [295]. The chip featured a flow control system to avoid LAMP inhibition via an interaction with solid phase extraction (SPE) reagents. Chemical lysis took place off chip and DNA extraction was performed by fabricating a pillar on the glass. By passivation of the LAMP chamber and extraction region, DNA extraction and amplification were completed with high sensitivity in 2 hours. **Figure A1.1** shows various chips based on colorimetric detection. Fang *et al* developed an octopus like

microfluidic chip for selectively detecting different subtypes of human influenza A virus. LOD of 10 fg/µl has been achieved from 2 µL of sample within 0.5 hour[296].

8.3 Electrochemical Detection

Electrochemical sensing is a modality that has shown great promise in developing rapid, sensitive, selective, miniaturized, and inexpensive POC diagnostic platforms [297-301]. In the medical devices application, electrochemical sensing, have been reported for diagnostic of single nucleic polymorphism[302, 303], nucleic acid[141, 300, 304] and amplification product[305, 306]. The principle of electrochemical sensing of LAMP relies upon the oxidation-reduction of the electro-analyte monitored by Linear Sweep

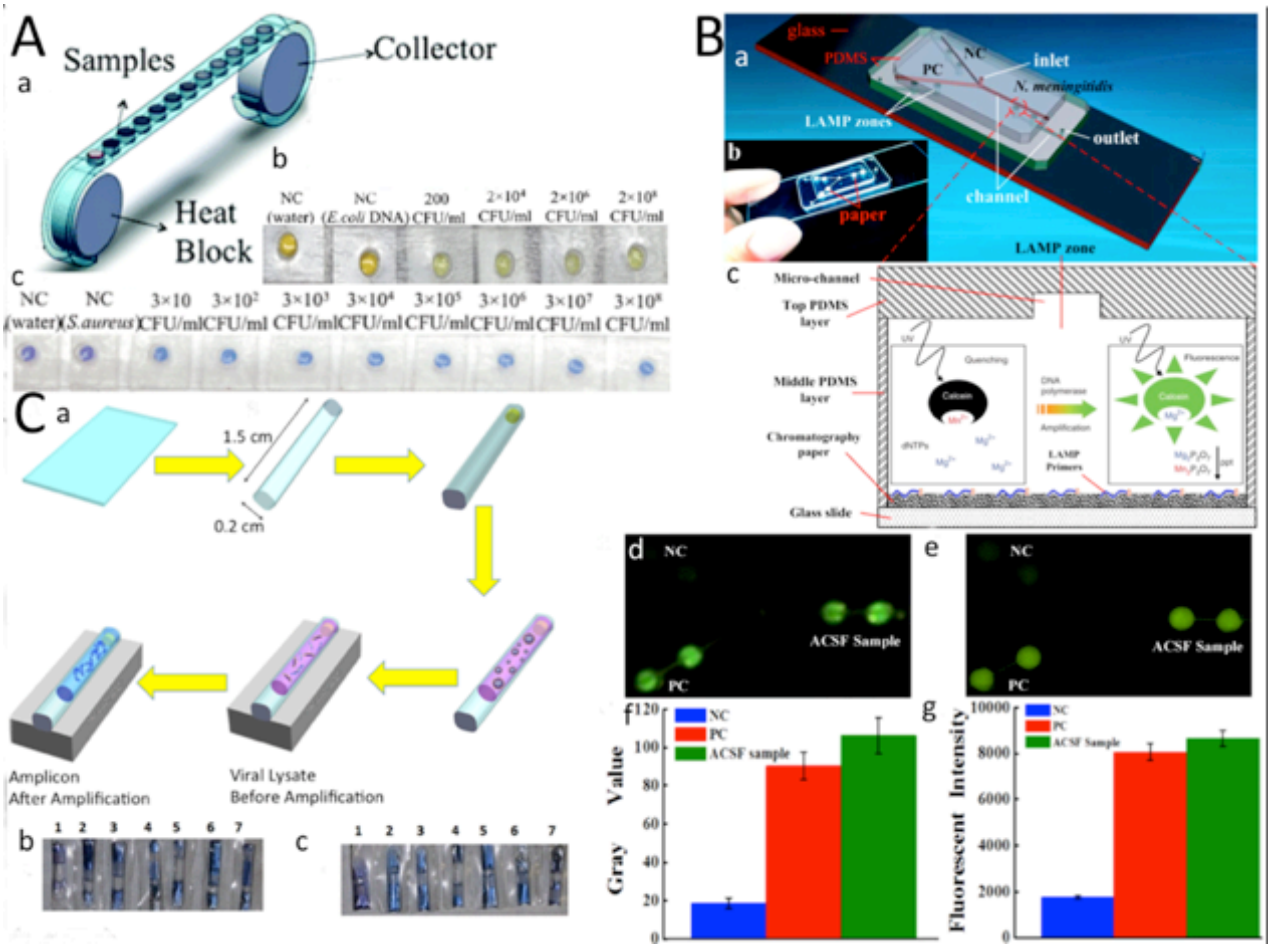


Figure A1.1 **LOC devices based on LAMP amplification and colorimetric detection (A) (a) Cassette device for high throughput bacteria detection, which has the array of chamber on flexible ribbon. (b) *S.aureus* pathogen was detected using Calcein with the LOD of 200 CFU/ml. (c) *E.coli* bacteria was detected using HNB dye with LOD of**

30 CFU/ml in 60 min amplification time. (B) Microfluidic paper plastic hybrid assay for high sensitive detection of H1N1 Virus. (a) Schematic representation of chip, which consists of PDMS chip, glass and paper chromatography paper. (b) Micro-image of microfluidic chip. Chromatography paper is placed at each LAMP zones to preload primers. (c) Schematic of color change during amplification using calcein. (d) Detection of purified *N. meningitidis* DNA by fluorescent detection with portable UV pen and (e) florescence microscopy. (f) Grey value of LAMP amplicon measured by Image J.(g) Fluorescent intensity of LAMP product measured by fluorescence microscopy. (C) Simple Polyethylene Pouch for detection of HSV-1&2 using HNB dye. (a) Schematic fabrication process of pouch. Simple polyethylene plastic is used to fabricate pouch by plastic sealer. LAMP reagent with HNB dye is mixed and is added to each pouch and target HSV is detected by changing color from purple to blue. Detection of (b) HSV-1 & (c) HSV-2 without DNA extraction/purification with LOD of 1 PFU/µl (Reproduced with the permission from [198, 293, 294]).

Voltammetry (LSV)[145], Square Wave Voltammetry (SWV)[306, 307], Differential pulse voltammetry (DPV)[308], or conductivity [309]. Electrochemical detection of LAMP amplicon is either achieved by redox labeling of immobilized LAMP amplicon on the surface of working electrode or by detection of redox molecule in the presence or absence of amplification product. Sun *et al* detected LAMP amplicon on the carbon ionic liquid electrode (CILE) with DPV technique. The electrode surface was modified with V_2O_5 nanobelt, multi-walled carbon nanotube (MWCNT), and chitosan for electrode surface modification. It was then set as a substrate for immobilization of a single stranded DNA probe for *Yersinia enterocolitica*. The assay had a LOD of 1.76 aM. Methylene Blue (MB) was used as an electrochemical redox for labeling the LAMP amplicon[308]. The second method is non-immobilisation technique of detection of redox molecule in presence and absence of amplification product.

Various electrochemical redox molecules and their potential peak commonly used for LAMP amplicon detection are listed in **Table A1.2**. Some of them, such as Hoechst 23458, inhibit the amplification reaction and cannot be rendered for real-time LAMP amplicon monitoring[310]. Others, such as methylene blue or ruthenium hexamine, can be used for real-time monitoring of LAMP reaction.

Table A1.2 Various redox has been used for LAMP detection.

Redox Name	Electrochemical technique	Electrode	E_p vs Ag/AgCl	References

Methylene Blue (MB)	DPV	Carbon	-0.2	[307]
Hoechst 23458	LSV	Carbon	0.52	[311]
Ruthenium Hexamine	SWV	Carbon	-0.275	[306]
$[(bpy)_2DPPZ^{2+}(Os)]$ DPPZ	SWV	Carbon	0.4	[310]
Daunomycin	DPV	Carbon	0.42	[312]
2′-deoxyguanosine 5′-triphosphated (GTP)	DPV	Carbon	0.81	[313]

Generally, LAMP amplicon can be detected either at the end point where the amplification reaction is terminated, or can be monitored in real-time. Ahmed *et al* have developed portable device for mazie CBH 351 GMO DNA detection and quantification based on LAMP amplicon in a tube-like chamber chip which has a wall to separate detection chamber from amplification one[145]. LAMP reagents with target DNA were amplified initially. By slight shaking, the wall between amplified chamber and detection chamber was damaged yielding to mix redox with amplicon for further electrochemical detection. The electrochemical chamber has screen-printed electrodes [314] and detects LAMP amplicons with LOD of 200 fg/μl. Safavieh *et al* developed a microfluidic device for the detection and quantification of *E.coli* based on linear sweep voltammetry (LSV) technique. This microfluidic device contained two parallel chips, which have a reaction chamber, an active valve, and an electrochemical detection chamber. The detection chamber has a carbon screen printed electrode, which was placed vertically. The mixture of an *E.coli* sample and LAMP reagents were injected into the chip. The *E.coli* lysis took place by thermal shock (66°C). Through time optimization of the LAMP reaction, 48 CFU/ml of *E.coli* bacteria was detected followed by quantification[168]. **Figure A1.2** shows various LOCs used electrochemical end point detection of LAMP amplicon. combination of LAMP and bacteriophage have been used for liveability test of bacteria. Tlili *et al* used a dual response biosensor for screening, liveability, and

confirmation of *E.coli* bacteria by leveraging T4 bacteriophage [199]. T4 bacteriophage was immobilised on a cysteamine modified gold electrode by cross-linking with 1,4-phenylene diisothiocyanate. The impedance biosensor shows reproducible and sensitive detection within a range of 10^3-10^9 CFU/ml. The impedimetric response of live and dead *E.coli* was tested after incubation of 10^8 CFU/ml live and dead cells on electrode after 15 min. After washing with PBS, faradic impedance was measured for the period of 1 hour to determine the liveability of bacteria. Using LAMP amplification for 40 min the sensitivity was increased to 10^2 CFU/ml within range of 10^2-10^7 CFU/ml.

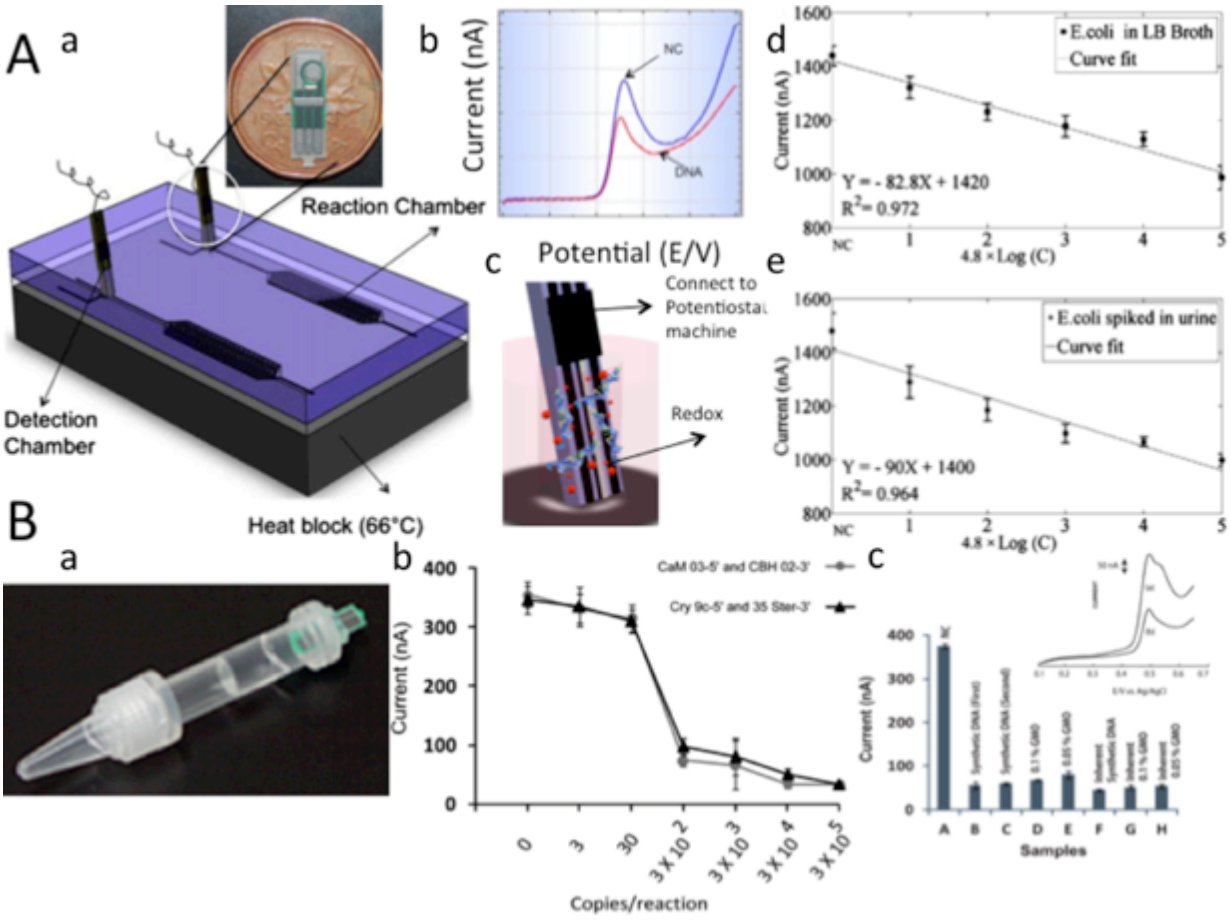


Figure A1.2 **Microchip devices for electrochemical LAMP assay at End point detection. (A) Microfluidic electrochemical assay for detection and quantification of *E.coli.* (a) Schematic of microfluidic chip for NC and sample detection. Each microfluidic chip consists of PDMS chip, glass substrate and aluminum heater. The detection chamber has disposable carbon screen printed electrode. (b) The LSV of Hoechst redox. (c) Schematic of binding redox and LAMP amplicon at the end of LAMP reaction which results of reduction of LSV peak. Quantification of *E.coli* bacteria in (d) LB broth media and (e) 20% urine sample at 35 min amplification time with LOD of 48 CFU/ml. (B) Microchip for electrochemical detection of maize CBH 351 GMO DNA. (a) Image of DNA stick. (b) Electrochemical LSV current peak for different**

concentration of LAMP product. (c) LSV behaviour of NC and various synthetic and real GMO DNA with different concentration.(Reproduced with the permission from[168, 305])

Hsieh *et al* fabricated another microfluidic device for real-time monitoring of DNA amplicon [315, 316]. Electrodes were fabricated at the bottom of the chip, which consists of a Au working electrode and Pt counter and reference electrodes. MB was used as a redox molecule as it does not inhibit the LAMP reaction where square wave voltammetry (SWV) scanning was implemented; the normalized current peak has been demonstrated with respect to amplification time. By taking the derivative of the normalized current with respect to time, the threshold time was obtained and was used to quantify of the assay, which had an LOD of 16 copies/μl of target amplicon that was detected in less than 50 minutes.

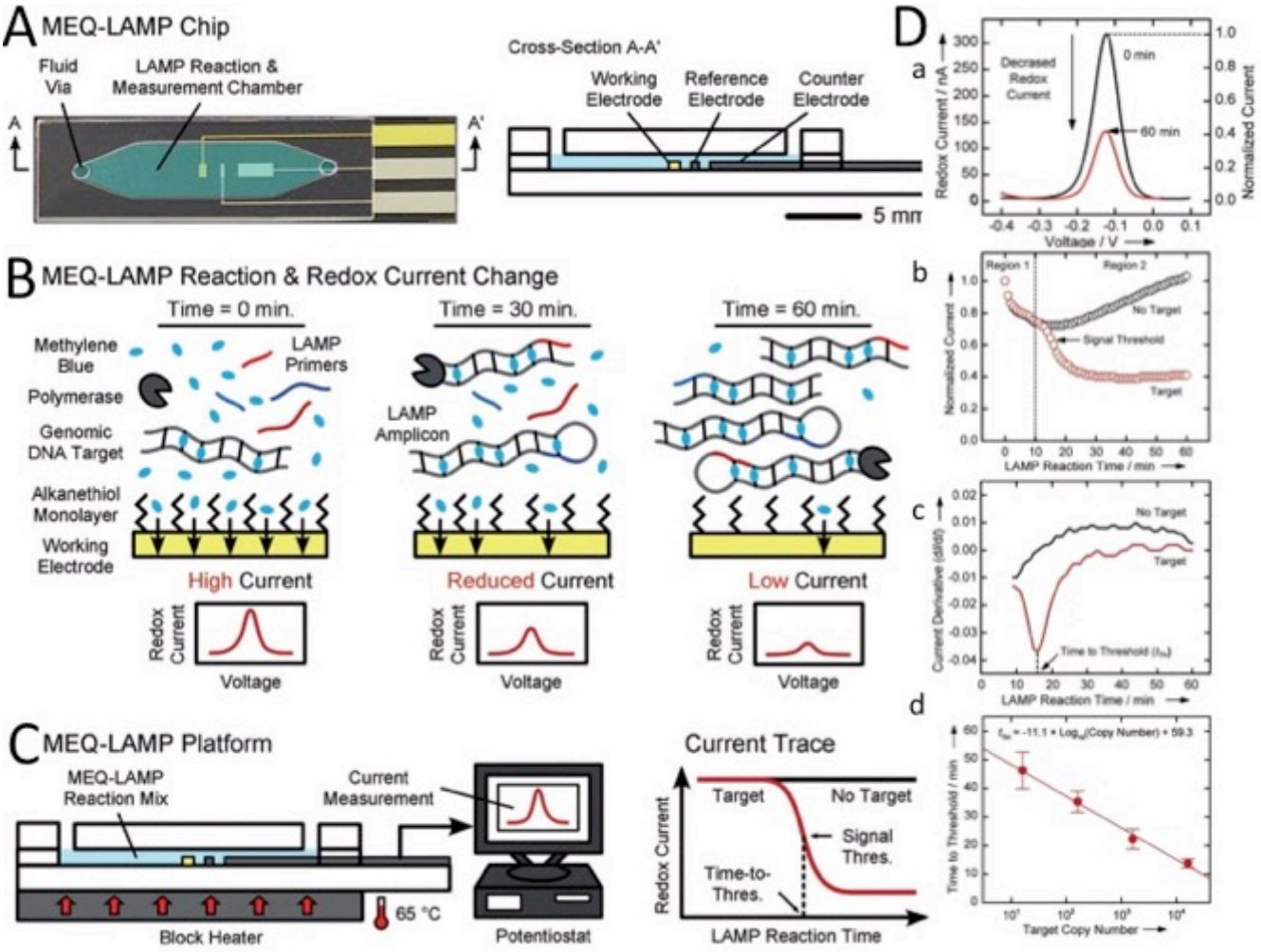


Figure A1.3 **Microfluidic electrochemical for real-time monitoring of LAMP. (A) Schematic of the LAMP chip and its cross section, which facilitates both LAMP reaction and electrochemical detection (B) Schematic of the electrochemical detection by methylene blue active redox compound. Prior amplification, methylene blue is free in the solution and generates high redox current due to rapid diffusion on the surface of gold working electrode. During LAMP reaction, MB intercalates with newly formed double stranded product which results of decreasing the current peak. (C) Microchip is placed on the heat block to provide enough temperature for amplification and simultaneously is connected to potentiostat for signal measurement. (D) SWV peak of the MB during the LAMP amplification. All signals are normalized with respect to initial signal. (b) Plot of normalized peak current of no target (Negative control) and target amplified S. *Typhimurium* gene. (c) Current derivative of the achieved peak current signal in (b) which shows a minimum point at specific amplification time which is set as a reliable time to reach threshold signal. (d) Calibration of the initial target copy number and threshold time with linear relationship and sensitivity as low as 16 copies and high specificity. (Reproduced with the permission from [315])**

With a similar approach, Luo *et al* fabricated an octopus-like microfluidic system with an indium tin oxide (ITO) electrode on the glass substrate for multiplexed DNA bacteria detection. Mycobacterium tuberculosis (MTB), Haemophilus influenza (HIN), and Klebsiella pneumonia (KPN) were detected with LOD of 28, 17 and 16 copies/μL respectively, with assay times between 30 minutes to 1 hour [317]. Safavieh *et al* also developed a cassette-type device for real-time electrochemical detection of *E.coli* bacteria as a gram-negative model and *S.aureus* as a gram-positive model. The device featured two aluminum reels and a plastic ribbon which formed an array of reaction chambers. Carbon screen-printed electrodes were fabricated at the bottom of each chamber. One of the aluminum reels acted as a ribbon collector and was connected to the heater to maintain a constant 65°C temperature for amplification. The other reel acted as a sample provider. When samples were added to the chamber, it was covered with heat tape to prevent sample evaporation. Then the sampled rolled around the collector reel to initiate amplification. Simultaneously, each electrode was connected to a potentiostat for signal processing. For *S.aureus* detection, the 65°C temperature could not lyse the bacteria. To overcome this problem, *S.aureus* was added to each chamber first. Each sample was then covered by tape and heated up to 90°C for 2 minutes. Then the tape was removed and LAMP reagents were added to each sample for further electrochemical monitoring and the temperature was set at 65°C [310]. **Figure A1.3** shows real-time electrochemical LOC device, which used LAMP amplification technique.

8.4 Optical Based Detection

Optical detection of LAMP amplicon can be monitored based on the production of the bi-product of the LAMP polymerase reaction, magnesium pyrophosphate ($MgPO_4$). Generating this bi-product can cause the turbidity in the sample to increase, which can be detected with a turbidimeter in real-time [318], optical fiber [319] ,surface plasmon resonance (SPR)[320], spectrophotometer [321], or fluorescent imaging by CCD camera [322] in microfluidic chips. Quantification of DNA copies can be achieved by plotting the turbidity against the amplification time. Fang *et al* developed eight-channel microfluidic chips made out of PDMS and glass for pseudorabies virus DNA detection and quantification. The chip utilized a digital fiber optic sensor to emit LED light at 640 nm and a phototransistor to measure turbidity. Using only a 0.4 μl sample, the LOD of 10 fg DNA sample was achieved after only an hour of amplification [319]. In a similar technique, more applicable for POC diagnostics, Stedtfeld et *al* developed a valve-less polyester film microfluidic chip fabricated using a hot embossing technique, and SYTO-81 dye was mixed with the reagents before applying the mixture to the microfluidic chambers (**Figure A1.4**) All primers and LAMP reagents were dehydrated in each chamber. Multiple genes were detected including stx2 and eaeA from *E.coli* and mecA and vicK genes of *S.aureus*. An LOD of 13 copies per sample was achieved by employing LED light at the bottom of each chamber [323]. The amplification time took 1 hour at a temperature of 63°C. The main drawback of the turbidity based detection mechanism is inhomogeneity of the magnesium pyrophosphate particle size, uneven spatial distribution, and re-dissolution of magnesium pyrophosphate particles [320, 324].

In development toward sample-to-answer device, Wang et al have fabricated a microfluidic chip for detection of *methicillin resistant staphylococcus aureus* (MRSA) bacteria in clinical samples. The chip had lysing, washing, and reaction chambers for sample pre-treatment prior to lysing, DNA extraction and purification steps. Initially, an oligonucleotide probe conjugated with magnetic beads was mixed with MRSA bacteria [146] . A temperature of 95°C lysed the pathogen then the probe conjugated with the magnetic beads bound to the released DNA. DNA was purified by allowing the magnetic beads to attach to a magnet on the underside of the cell lysis/DNA hybridization

chamber. LAMP reagents were applied to the chamber and amplification product was measured through the integration of a spectrophotometer. The entire sample processing and amplification procedure was carried out automatically and a LOD of 10 fg /µl of clinical sample was detected as shown in **Figure A1.5**. Liu *et al* developed simple sample to result cassette for an HIV viral load assay from saliva samples. The cassette microfluidic device consisted of a chamber, which has a flow that passes through a Flinders Technology Associates (FTA) Whatman® membrane for isolation and purification of RNA, as well as removing critical inhibitors that can significantly reduce the sensitivity of the assay. The assay utilized SYTO-9 green dye for amplicon and the cassette was integrated with a portable ESE fluorescent detector. A flexible, polyimide-based heater attached at the bottom of the microfluidic chamber provided temperature regulation for amplification. It was found that 10 copies of HIV were detected per sample. Through calculation of T_m (melting temperature of amplicon), the amplification reaction was examined to avoid primer-dimer problem[325].

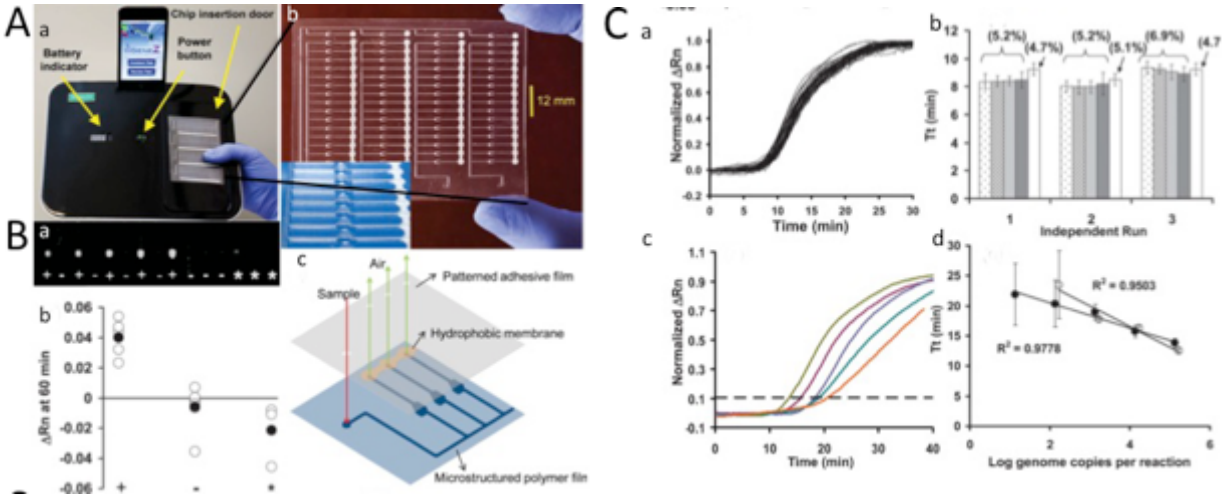


Figure A1.4 **Microfluidic integrated with Fiber optics. (A) Gene-z platform. (a) Image of Gene-Z device integrated with iPod dock and rechargable port and disposable microchip. (b) Photograph of the microfluidic chip with four parallel arrays. Close up of the microfluidic channels and its reaction wells. (c) Schematic structure of working principle of Gene-Z. Sample is loaded using pipette tips and fluid passes to all chambers and fill the reaction wells within several seconds. Air inside the reaction wells are purged out from the vents located downstream of each channels. (B) (a) Fluorescence image of 15 well array with positive and negative sample after amplification. (b) ΔR_n (Fluorescent intensity of sample - base line) for + as primers with DNA template, - as primers without DNA template, and * as wells without dehydrated primers and DNA. (C) Analysis of chip reproducability. (a) Amplification of reaction wells in one microchip. (b) Average and standard deviation and coefficient of deviation magnitude of threshold time for all reactions**

in each of four arrays which is tested for three separate chips. (c) Amplification graph for 10-fold serially diluted DNA sample. (d) Threshold time of the assay with respect to logarithmic concentration of LAMP amplicon and comparison with real-time PCR. (Reproduced with the permission from[323])

CCD based fluorescent imaging can be used for real-time monitoring of LAMP amplification. Ahmad *et al* developed a microfluidic chip for waterborne pathogen detection. SYTO-81 was used as florescent dye and real-time imaging was implemented for *Campylobacter jejuni* 0414 gene detection. Signal to noise ratio (SNR) and threshold time (T_t) were measured for the microfluidic chip. SNR was 8 times higher and T_t was halved in comparison with a commercially available real-time PCR instrument. A single copy of a gene was detected within only 19 min of threshold time [322]. The chip has been modified in order to detect multiple food and water borne pathogens (**Figure 6**) [326]. Chang *et al* developed an integrated microfluidic device for multiplex pathogen detection of *Streptococcus agalactiae*, *koi herpes virus*, *Irido virus*, and *Aeromonas hydrophila* utilizing an optical photomultiplier (PMT) sensor. DNA from cell lysate was isolated using magnetic beads coated with specific probes. The extracted DNA was amplified and then optically detected within 65 min of the start of the entire process with a LOD of 20 copies was achieved[327]. *Liu et* al developed a sample-to-answer portable microfluidic chip for determining the genotype of a malaria mosquito. The device was integrated with a CCD camera from a cell phone and a blue LED, which enhanced the image analysis of the detected target genome of malaria. The chip consists of three separate chambers to identify *Anopheles gambiae*, *Anopheles arabiensis,* and the negative control sample. A piece of the malaria mosquito leg was first placed on the FTA Whatman paper and crushed until it changed into a small disc shape sample. Then, the FTA paper was placed into each chamber and lysing reagent (AL lysing solution) was added to the sample followed by washing to remove debris and inhibitors from the sample. LAMP reagents along with SYTO-9 fluorescent dye were added to each chamber. LED light excited the intercalating dye, which made it easier to visualize by the naked eye [328]. A combination of real-time RT-LAMP and a handheld USB based fluorescent microscope on a microfluidic chip has been used for HIV viral load test [329]. As shown in **Figure A1.6,** the chip consists of a chamber and a diffusion-

reaction micro conduit. The sample laden with target DNA was introduced into the chamber and LAMP amplification was initiated thermally. The microconduit was filled with 4% (w/v) hydroxypropyl-methyl-cellulose (HPMC) to reduce the diffusion and confine the reaction front. As the amplification reaction progresses, the amplicon diffuses through the microconduit. The diffusion length of the amplicon is proportional to the amplification progress.

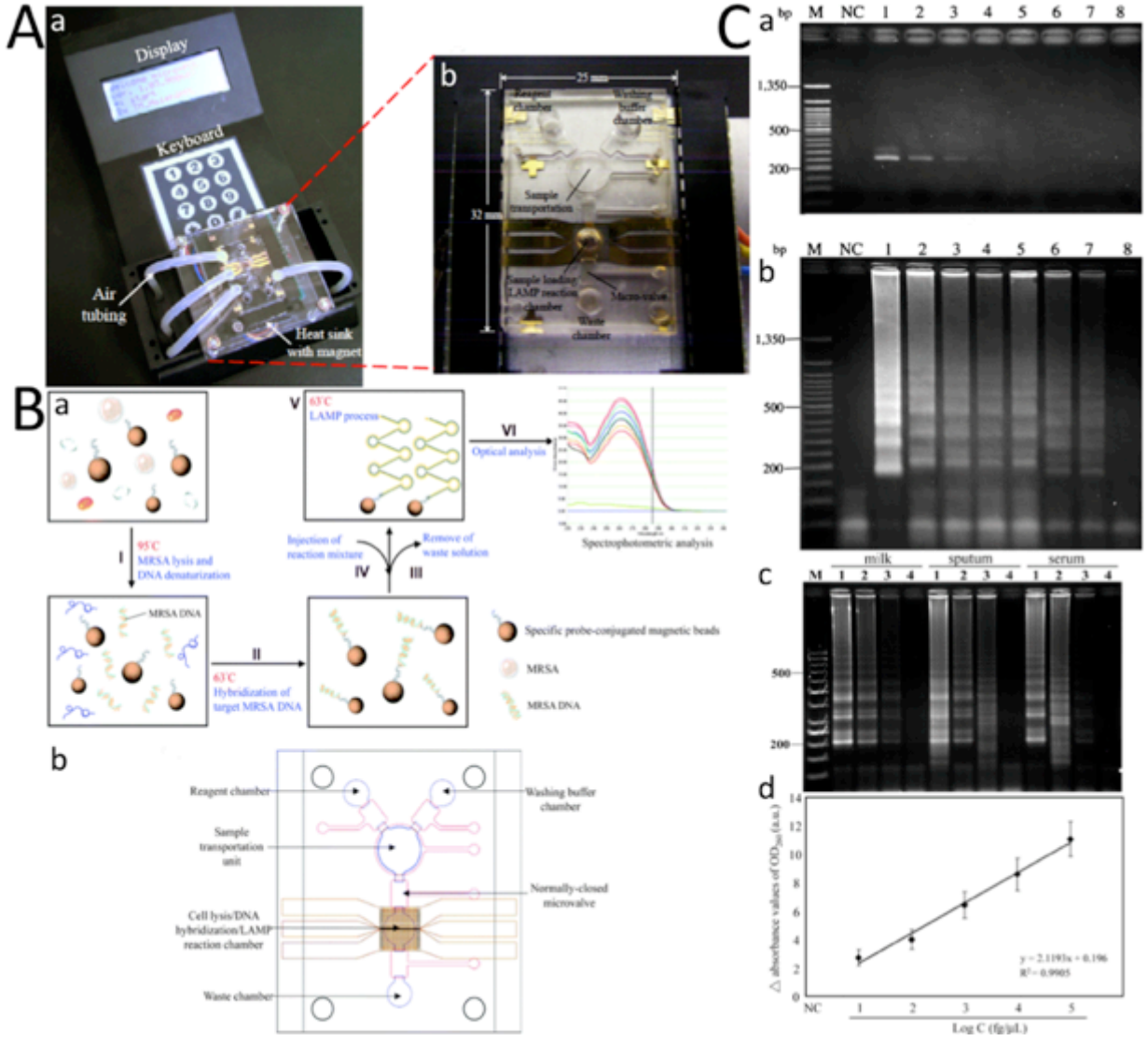


Figure A1.5 **Microchip LAMP bacteria detection integrated with Spectrophotometer. (A) (a) Image of integrated microfluidic chip which is connected to air vent and heat block and magnet. (b) Photograph of microchip. (B) Schematic representation of MRSA detection on chip. (a) MRSA bacteria is injected to the chip with specific probe conjugated with magnetic beads and is lysed in cell lysis chamber by applying 95C temperature. After releasing MRSA DNA, molecular probe is hybridized with DNA at 63C. By leveraging magnet, supernatants are washed away and LAMP reagent is**

added to the solution and further optical analysis is implemented with spectrophotometer. (b) Schematic of microchip and its different components. (C) Comparison of sensitivity of the assay for MRSA detection with (a) conventional PCR bench-top and (b) microchip LAMP assay with sensitivity as low as 1 fg/μl (c) Analysis and detection of MRSA in real sample of milk, spotum, serum. Different concentration are tested from Line 1 with 10^3 CFU/ml to line 4 with 1 CFU/ml. Calibration curve of absorbance data from spectrophotometer with linear relationship of logarithmic concentration from 100 pg/μl to 10 fg/μl (Reproduced with the permission from[330]).

SPR chips have been also used for LAMP amplicon detection[320, 331]. Chuang *et al* have developed an SPR chip made of a PMMA reactor chip and polycarbonate prism coated with 50 nm Au. HBV DNA was used as a template and mixed with 10 μl of LAMP reagent. As a result, an LOD of 2 fg/ml was achieved[320]. Integrating graphene on an SPR chip has also been used for LAMP amplicon detection. Single layer graphene was constructed on the surface of the Au SPR chip using a self-assembled monolayer (SAM) and has been used for tuberculosis bacillus (TB) DNA and LAMP amplicon detection with high sensitivity[332].

8.5 **Electrophoresis**

Electrophoresis is one of the most common techniques and a "gold standard" for separation of NA (DNA or RNA) and proteins based on their molecular size and charge[271, 280]. Gel electrophoresis (GE) has been used for LAMP amplicon detection by preparing 2% agarose gel as a matrix. In order to visualize amplicon on gel, ethidium bromide has been used as an intercalating fluorescent dye. By using a specific molecular size standard, the length of amplicon can be determined and quantified and, consequently, the risk of non-specific detection is reduced. GE is usually used as a post-processing method for amplicon detection and has not been used for on-chip detection analysis. However, electrophoresis technique was used for on-chip amplification detection analysis. Capillary electrophoresis (CE) has a lot of advantages such as multiplex detection while using very low power. Although it requires complex infrastructure and is time consuming, it can also provide end point detection while integrating other detection techniques [116]. Various LAMP microchips based on electrophoresis detection have been developed recently. Hataoka *et al* have developed

a PMMA chip making use of electrophoresis to analyze the LAMP product on the chip. Gene fragments of LAMP amplicon have been analyzed within 150 sec with an LOD of 23 fg/µl in 15 min amplification time [333]. Lam *et al* have also developed micro-reactor

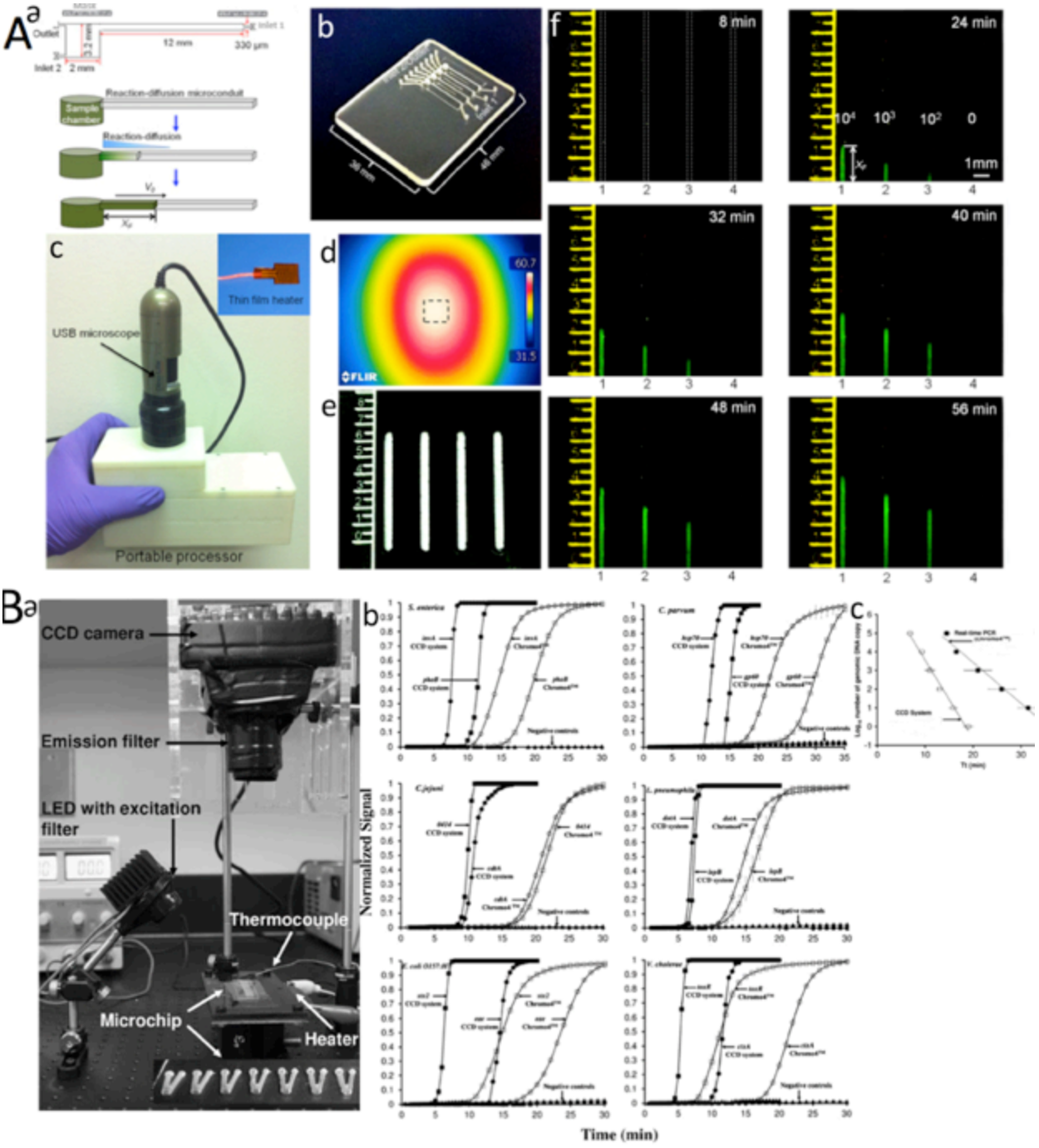


Figure A1.6 **LAMP Microfluidic chips integrated with CCD Camera. (A) The Nuclemeter. (a) Schematic of nuclemeter, which consists of reaction chamber and a microconduit where reaction-diffusion takes place. NA template is amplified and diffused**

through the channel. (b) Photograph of nuclemeter chip, which has four separate microfluidic channel for detection of different concentration. (c) Image of portable processor, which renders flexible thin heater and USB size microscope. (d) Thermograph of microchip taken by infrared camera. (e) a mask made by black Scotch 3M tape to remove background emission and a ruler set close by channel to position the reaction limit (X_F). (f) Various concentrations of HIV RNA and HIV viral load test based on reaction limit of LAMP amplicon for different amplification time. (B) Microfluidic chip for monitoring of various bacteria DNA. (a) Image of microfluidic platform . (b) Normalized signal of six different bacteria DNA amplification and comparing each of them with real-time PCR. (c) Calibration curve of microfluidic platform and comparing with real-time PCR(Reproduced with the permission from [322, 329].

chips for single molecule detection on polyacrylamide gel (PAA). Due to its porous structure, various ions diffused into the PAA gel matrix and initiated an amplification reaction. The chip consisted of a micro heater and a temperature sensor, both of which were fabricated at the bottom of each chamber. Amplification product can be visualized by fluorescent imaging analysis at the end of amplification reaction[334]. Isotachnophoresis (ITP) provides a robust technique to separate NA from complex samples utilising leading electrolyte (LE) and trailing electrolyte (TE) buffers and an electric field. Borysiak *et al* fabricated a chip which used minimum sample pipetting by leverage of a capillary valve. *E.coli* bacteria DNA were separated and purified from milk sample. LOD of 10^3 CFU/ml was achieved which in comparison of tube-LAMP with diluted milk was two order of magnitude more sensitive [335].

8.6 pH sensing platforms

During the LAMP polymerization reaction, the pH of the solution varies due to the release of ions into the solution. This phenomenon can be monitored by electrochemical or electrical sensing[336]. A simple pH meter can be used for pH monitoring of LAMP amplicon [337]. Tomazou *et al* have developed an ion sensitive field effect transistor (ISFET), which converts a chemical signal into an electrical signal. The ISFET was integrated with a temperature control sensor. The chip was placed at the bottom of the polymeric chip where the amplification reaction occurred. As the amplification reaction proceeded, a significant amount of proton ion (H+) was released into the chamber. The increase of the charge at the sensing surface caused an increase in the charge distribution between the source and drain grates of the ISFET, which leads to to an

increase in the IC output signal voltage [338, 339]. Electrolyte–insulator–semiconductor (EIS) has been also for pH sensing of the LAMP reaction as a label free technique. Veigas *et al* have developed a FET sensor made of Tantalum Oxide (Ta_2O_5). The FET sensor was integrated with AgCl as a reference electrode to make a cell. The generated protons (H+) were detected by employing a capacitance measurement in real-time during the amplification reaction. The measured voltages were calculated at 60% of the maximum obtained capacitance in a C-V curve [340]. Label free LAMP amplicon were quantified in a range of 10^8-10^{11} copies /ml as shown in **Figure A1.7**.

Most of the ion sensitive FET devices rely on establishment of fluid gate potential, which requires difficult microfabrication of reference electrodes and limits parallel detections. To overcome this problem, Salm *et al* developed a novel method for the fabrication of a solid state platinum quasi-reference electrode (QRE) coupled with PH insensitive field effect transistor (FET) for real-time pH monitoring of LAMP reaction. PVC has been used to cover ISFET and make it insensitive to pH change [341]. The pH solution of LAMP reagents has been optimised using HCl-Tris buffer, and the reaction was monitored with respect to pH change. An LOD of 10^2-10^3 was achieved in less than 30 min.

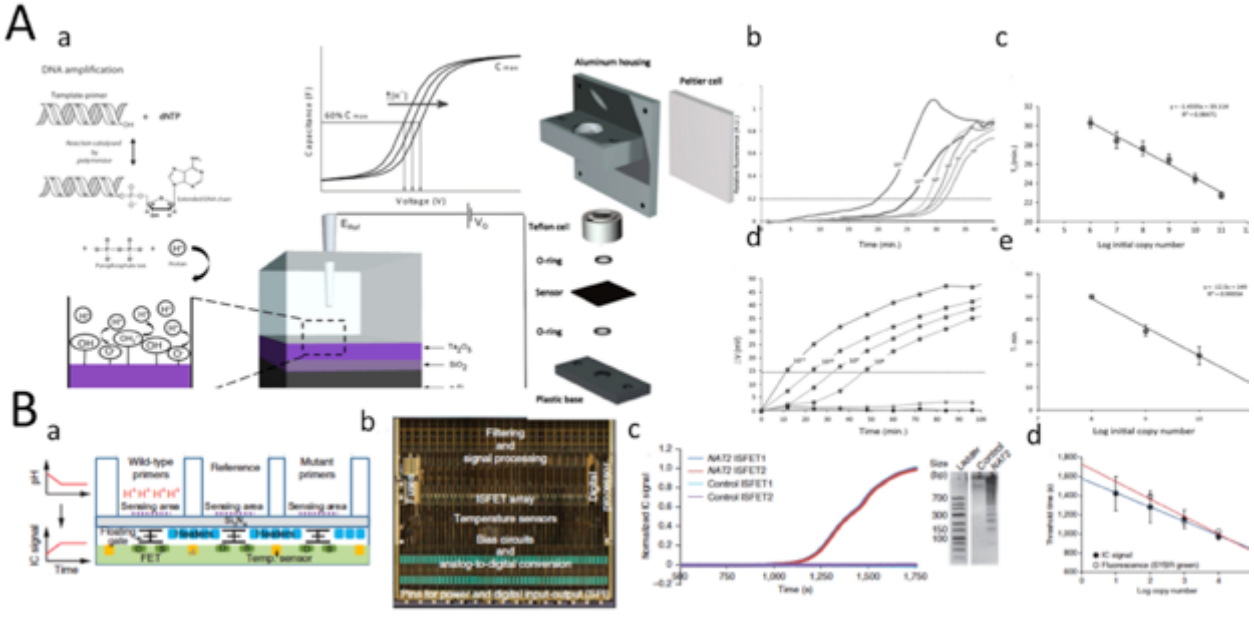


Figure A1.7 **PH sensing chip for LAMP monitoring. (A) Ion sensing platform and its component. (a) Representation of amplification process on c-v curve. The elongation process yields accumulation of proton, consequently makes the producing of the pH shift**

proportional to the number of nucleotides incorporated. The hydrogen accumulation can be measured by impedance spectroscopy. (b) RT-LAMP amplification curve based on fluorescent intensity with different DNA template concentration and its (c) calibration curve. (d) EIS RT-LAMP curve for different amplicon concentration ranging from 10^{11}-10^{8} copies and its (e) calibration curve. (B) Schematic of pH sensing with its ISFET platform. (a) Cross section of array of ISFET which consists of gate, source (S), drain (D) temperature sensors (yellow) and controlled heater (blue) which all are embedded at the underneath of silicon nitride sensing surface. The chip is placed at the bottom of fluidic chip, which has a reaction separate chamber having a unique primer set. (b) Micrograph of CMOS-fabricated chip. (c) On-chip amplification and detection using pH-LAMP ND ITS GEL IMAGE. (d) Sensitivity and calibration curve of pH-LAMP chip and its comparison with fluorescent-based LAMP. (Reproduced with the permission from [338, 340])

8.7 **Immunoassay -LAMP**

Immunoassays in two formats, lateral flow test (LFT) or ELISA, have been combined with LAMP. Lateral flow assays detect biotin labeled LAMP amplicon, which has been hybridized with a FITC labeled DNA probe conjugated with gold-based anti-FITC antibody. Non-hybridized FITC will bind to the gold-labeled anti-FITC forming a double complex without biotin and move forward to the test line [342-345]. The whole process of bio-analyte detection, excluding DNA extraction, takes around 40 min. The specificity of the LFT is high due to the fact that the probe is complementary to a specific amplicon. The obtained sensitivity of this technique was 2.4 fg/μl [346], 3 fg/μl [347] and 0.039 fg/μl [348]. A combination of LFT-RT-LAMP on a CD based microfluidic device was employed for detecting H1N1 virus through colorimetric detection. Jung *et al* fabricated a centrifuge micro-device consisting of three separate LFT to detect various H1 and M genes as well as negative control [349]. Each microfluidic device consists of an inlet for injecting LAMP reagents, a chamber for amplification, and a zigzag shaped dispensing micro channel, which was set to aliquot LAMP solution equally in three chambers. 2 μl of sample was injected into the inlet and filled the zigzag channel by capillary action. Using an air pump, the sample was transported to the reaction chamber and a siphon channel was connected to the chamber to prevent overflow of LAMP sample into LFT. The flow process has been shown in **Figure A1.8**. Using different RPM with various time as well as amplification, LAMP amplicon will reach to the LFT and different target gene in H1N1 was detected with LOD of 10 copies. LFT-

LAMP has great potential in POC diagnostic application by integrating sensitivity, and specificity of LAMP technique, with simplicity of lateral flow immunoassay as LFT does not require any trained personnel to handle the diagnostic test. Integration of this method with self-contained microfluidic platforms can also eliminate the potential of cross contaminations as well as providing high throughput sample analysis.

Combination of LAMP and ELISA provides a strong technique for detection of hundreds of samples (high throughput analysis) with high sensitivity. The achieved LOD has reached to 1 copy [350] and 10 CFU/ml, which is 100 times more sensitive than PCR and turbidity-based LAMP assays [351]. The basic principle of the LAMP-ELISA method relies on direct incorporation of labeled nucleotide on amplicon during the amplification process, the hybridization to a specific probe, and detection of captured antigen by immunoassay technique[351]. The main drawback of this method is the assay time which takes several hours and being a multistep analysis it renders the idea of a POC diagnostic device very unlikely. Conversely, the method does not require expensive reader equipment, which makes it interesting for resource-limited settings. Combination of phage with ELISA-LAMP can be used for specific protein detection such as organophosphorous pesticide. Phage g3p-displayed short peptide libraries were used as efficient tools for selecting the mimotope peptides of an array of compounds and provided the unique characteristic of the phage-borne peptide that allows it to bind to a specific target on a phage particle which contains NA [352]. This strategy makes the possibility of detection of small molecules, which do not have a DNA strand within the range of 2 to 128 ng/ml.

8.8 **Capillary tube**

A glass capillary tube has been shown to be a simple, easy to use liquid handling device for LAMP amplification. Due to having the robust, non-permeable, and chemically neutral structure of capillary glass, it was used as a liquid handling platform for amplification. Rafati *et al* have tested capillary glass for high sensitive detection of mycobacterium Tuberculosis [353]. Liu *et al* developed a capillary array device for detection of *Mycobacterium Tuberculosis* in less than 15 min. The device used

magnetic beads for sample processing and DNA purification [354]. Different zones of detection, magnetic area, and amplification have been categorized in capillary arrays. The magnetic area of the capillary glass has been sandwiched by two electrical magnets, which can be in on/off modes. The detection zone has an LED array with emission and excitation filters. Sample processing is implemented by syringe pump and water/oil/water plug into the capillary and move into different zone to perform DNA extraction and purification. After DNA purification, the sample droplet is transported into the reaction zone and amplifies the extracted DNA. The LAMP product is then moved into the detection zone where droplet fluorescence was excited by blue-light LED for further detection. LOD of 10 bacteria was achieved within 50 min including sample analysis [354]. By employing an FTA membrane, Zhang *et al* have developed an integrated capillary device. A small flake of FTA membrane was picked up by syringe needle and placed inside the capillary. Several reagents for purification and washing, including purification reagent, TE buffer, LAMP reagents and water, were injected from the other side of the capillary at various positions including purification reagent, TE buffer, LAMP reagents and water. 0.2 μl of blood sample was injected from the other side of the capillary and went through the FTA paper. The purification solution and TE buffer washed the extracted DNA in the FTA paper. The paper was then immersed in LAMP solution for further amplification [355].

8.9 **High throughput and multiplex analysis**

Rapid, high throughput multiplex detection of various pathogens require implementation of various parallel reaction chambers, micro valves, micro pumps, as well as micro dispensers for providing micro/nano droplet size samples. LOC devices can integrate all these technologies and provide automated devices, which can be used by non-skilled operators [356-358]. One of the main drawbacks of the LAMP technique in comparison with PCR is lack of multiplexing analysis, which can be resolved by developing LOCs. Zhou *et al* have fabricated centrifugal microfluidic devices for detection of 10 different bacteria. Real-time and end point fluorescent intensity measurement was implemented and an LOD of 0.4 pg per 1.414 μl of sample was achieved [359]. An octopus structure

microfluidic device was created for multiplexed real-time electrochemical analysis as well (three different bacteria)[317]. High throughput analysis based on end point colorimetric detection was developed in a cassette format device where 36 samples were analyzed simultaneously using HNB dye [198] with an LOD of 30 CFU/ml of *E.coli* bacteria. Similar devices have also been used for real time electrochemical detection of 12 samples[310].

SlipChip, is another microfluidic platform for high throughput nanoliter volumes. It consists of two plates, which are in contact with each other. The bottom plate has an array of chambers, which is filled with reagents as well as ducts. The top plate acts as a lead for the reservoirs as well as the series of wells. The sample is added through the fluidic channel and flows into the upper reservoirs. Then, by slipping the top plate, the sample can move toward the bottom well reagents for mixing and further reaction. This provides the ability for multiplexing, and numerous multistep sample analyses without having valve and pumps, as well as reducing the consumed samples down to pico/nanoliter volumes[360] . Sun *et al* used SlipChip based on digital RT-LAMP for quantification of HIV RNA using a Poisson distribution[361]. RNA molecules were compartmentalized and by performing reverse transcription on each RNA molecule independently a cDNAwas produced and later amplified.

Droplet based microfluidic devices are another high throughput platform which provides continuous flow of water-soluble samples in oil droplets. The droplet formation is based on inherent fluid formation phenomenon rather than hydraulic valving action[362]. Digital microfluidic chips have also been used for quantification of LAMP amplicon. Zhu *et al* has developed droplet-based microfluidics for self-priming compartmentalization used in digital LAMP[363]. The chip structure was completely valve free, and contains four separate panels, which have 1200, 6 nL chambers. The chip consists of four-microfluidic devices enabling detection of four separate samples. The device was mainly fabricated from PDMS and was vacuum packaged. The pressure difference of the air dissolved in PDMS provides the driving force for the liquid and oil sucked into the channels and micro wells. By leveraging the Poisson analytical model the fluorescent intensity of an array of various concentrations of DNA were detected. Continuous flow digital LAMP has been developed based on a droplet-based microfluidic device. The

microfluidic device consists of a micro-dispenser to produce a droplet. In fact, a pico-liter size droplet was formed using a micro dispenser. The droplets then flow to the serpentine chamber for incubation. The structure of the serpentine chamber allows the droplets to flow side by side, while narrowing the channel enhances the droplets flow to the detection region in which fluorescent detection was conducted through the use of confocal fluorescent spectroscopy [364].

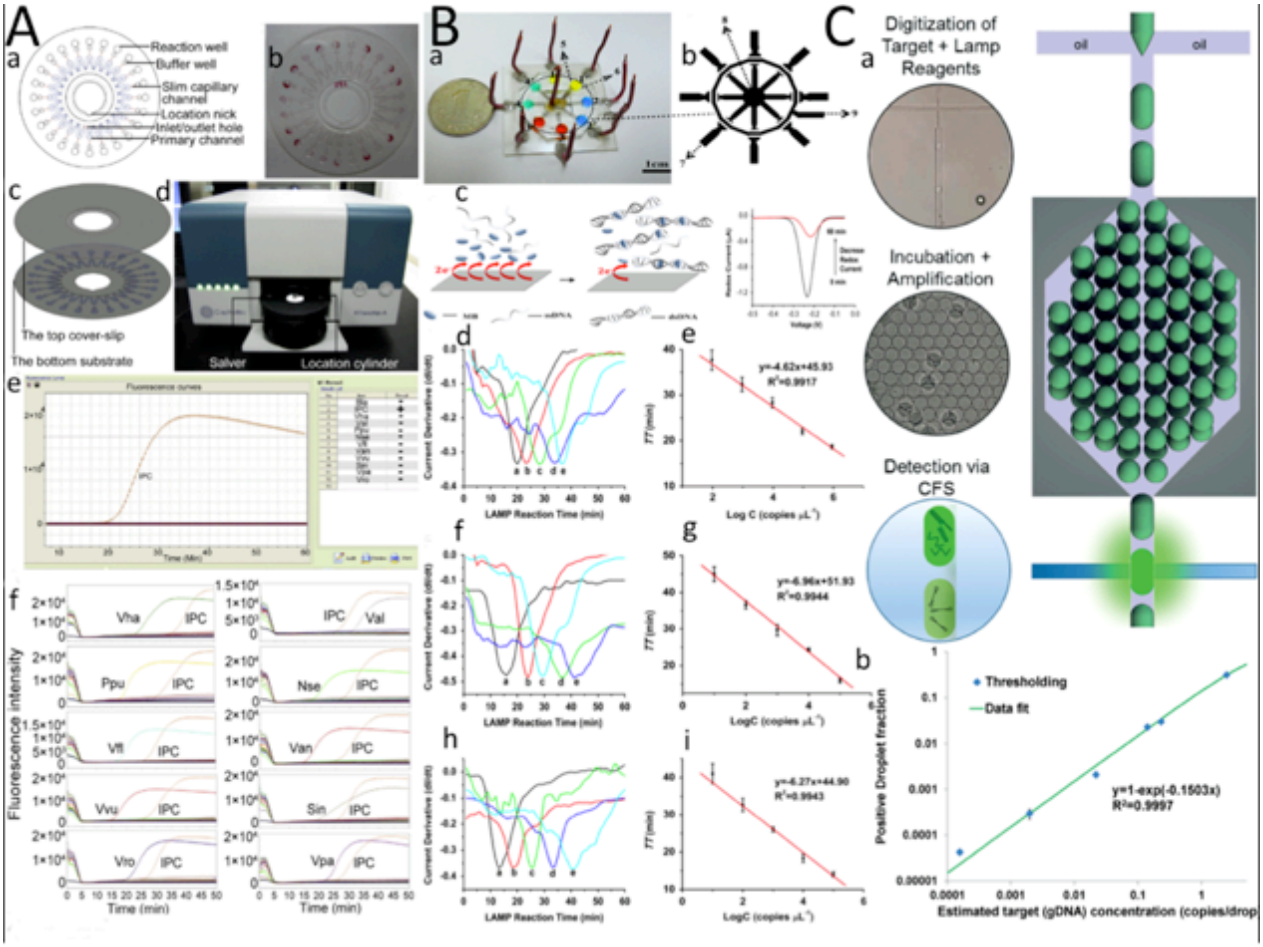


Figure A1.8 **Multiplexing and High throughput LAMP LOC devices. (A) CapitalBio RTisochip. (a) Microfluidic disc platform. Each disk consists of 24 reaction wells, which were attached to the buffer well. The buffer wells were connected with crooked primary channels with a slim capillary channel. The capillary channel was then cut off by thermal shock during the amplification process, which isolates the reaction well to protect the sample from potential contamination. Each reaction well had 1.414 μl and all the primers were first added to the wells and dehydrated. (b) Photograph of CD-based microfluidic. (Schematic of top cover slip as well as bottom substrate. (d) Image of the CapitalBio RTisochipplatform. (f) Detection of 10 different pathogenic bacteria DNA on chip. Fluorescence intensity of LAMP chip with DNA template of 10^3 pg/ul and inner positive control (IPC) is set at second reaction well to monitor reagent's performance. (B) Multiplexing electrochemical real-time microfluidic. (a) Image of microfluidic chip and (b) its schematic for detection of**

three different pathogen DNA (c) Schematic of electrochemical detection and signal between NC and amplified DNA. (d) Peak current derivative of different analytes with different DNA template concentration and (e) their calibration curve. (C) Microfluidic continuous flow based on digital LAMP. (a) Droplet based microfluidic device and Pico-liter size droplet formation and incubation are schematized. Amplified droplets are detected using Calcein fluorescent signal (CFS) analysis with confocal microscopy. (b) Quantification of amplified DNA in copies per droplet. (Reproduced with the permission from[364, 365] [359])

8.10 **Electricity free cartridges**

LAMP is a rapid and sensitive amplification technique, and has been used extensively in POC diagnostic platforms. One of the main obstacles for LAMP is that it relies on electricity in order to initiate amplification via heating. In order to be of benefit in resource-limited settings these platforms require electricity free cartridges. One of the main methods to provide consistent temperature for LAMP amplification is using an exothermal reaction. The reaction of calcium oxide (CaO_2) with water is an exothermic reaction and provides 63.7 kJ/mol [366-368]. By employing an engineered fat based compound or paraffin, a high capacity reaction chamber can be developed with a consistent temperature at a melting point of 65°C. Liu *et al* developed an electricity free detection method based on the Mg/Fe metal alloy reaction with water where the flow rate was controlled and adjusted using a filter paper. The cartridge contains a body where the reaction takes place and produces the heat for amplification, and a chip sits on top of the cartridge. The amplicon was detected by colorimetric fluorescent detection[369]. In a similar approach, Singleton *et al* combined an electricity free cartridge with LFT for HIV detection[370]. A pocket hand warmer can also be implemented for LAMP amplification[371, 372]. Zhang *et al* used a pocket warmer and capillary glass for multiplex detection. Various target DNAs and negative controls were injected at different positions of the channel and the two sides of the channel were sealed by epoxy glue. Then it was sandwiched between two hand-warmers for further detection. **Figure A1.9** shows various electricity free cartridges.

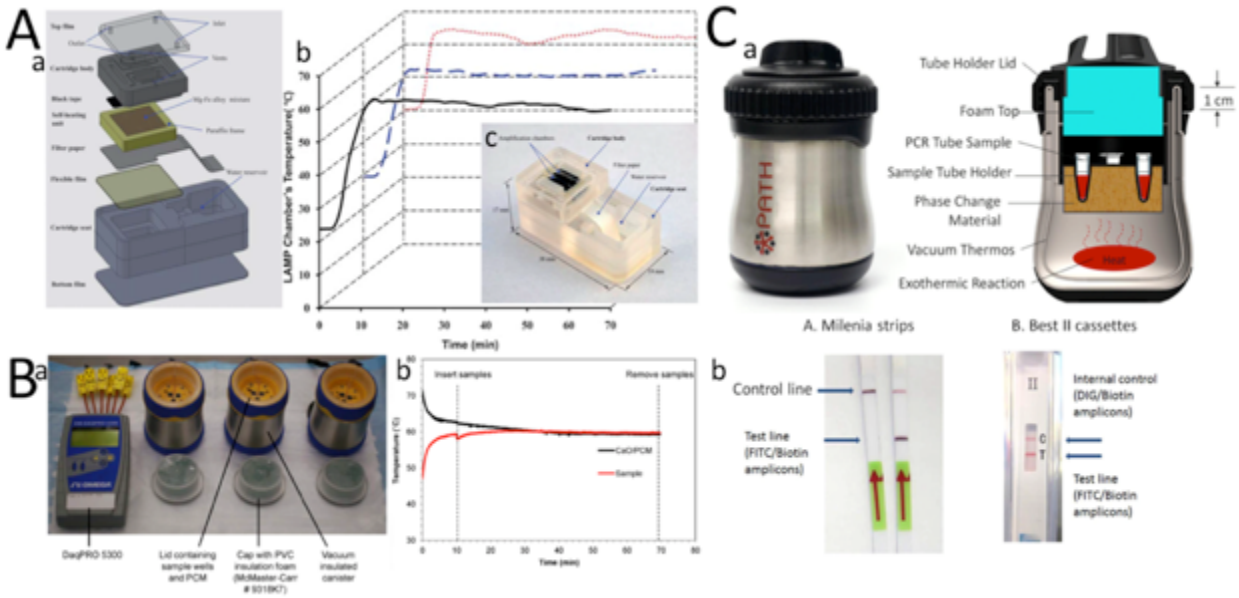


Figure A1.9 **Electricity free cartridges for LAMP amplification. (A) (a) The cartridge consists of cartridge body and cartridge seat. In the cartridge body which has heating chamber, Mg-Fe alloy and surrounded with custom cast paraffin. On the seat part microchip is placed for amplification. (b) Temperature profile at different ambient temperatures of black (22C) red dot (40C) and blue (30 C). (c) Photograph of cartridge. (B) (a) NINA electricity free cartridge which use CaO for heat generation and (b) its temperature profile for different amount of time. (C) (a) NINA-PATH cartridge. (b) The cartridge was integrated with lateral flow test for detection of HIV amplicon product after amplification.(Reproduced with the permission from[369] [370])**

8.11 Commercial companies using LAMP technology

With the significant development of various LOC devices based on LAMP, several diagnostic nucleic acid platforms have been developed and commercialized. Several of the platforms with the reader and their detection mechanism have been provided in **Table A1.3**.

Table A1.3 **LOC Companies used LAMP technology.**

Company Name	Product	Reader's Cost ($)	Cost per test ($)	Detection Mechanism	Throughput Analysis	Reference
Eicken	LA-320C	Discontin	Discontin	Turbidimeter	32	[373]

Chemicals		ued	ued			
Eicken Chemicals	LA-500	25000	3.3	Turbidimeter	16	[374]
CapitalBio	RTisochip-A	15000	1.25	Fluorescent	24	[359]
GeneZ	Gene-Z	TBD	2-10 for 24 tests	LED-optical fiber	15	[323]
Opti Gene	Genie® II	13000	4	Fluorescent	16	[375]
Opti Gene	Genie® III	17995	1.85	Fluorescent	8	[376]
QIAGEN	ESEQuant Tube Scanner	16000	10	Fluorescent	12	[377]
Meridian Bioscience, Inc	Meridian illumipro-10™	7500	28-30	Fluorescent	10	[378]
Diagenetix, Inc.	Smart DART v.30	2490	10-15	Turbidity	8	[379]

APPENDIX II

NUMERICAL CALCULATION OF THE HEAT TRANSFER AROUND THE CAASSETTE REEL

FEM modeling

3D time-dependent numerical simulation werre performed based on a finite element method using a commercial software (COMSOL Multiphysics 3.5a, USA). Heat transfer simulation was implemented using the general heat transfer equation as:

$$\rho c_p \frac{\partial T}{\partial t} + \nabla.(-k\nabla T) = Q$$

with proper boundry condition as follows: isothermal temperature (66°C) was set for the alluminium reel and for surrounding air temperature was set at 25°C. Time dependent simulation was implemented for 3600 sec with the time step of 1 sec. Temperature profile at the surface of the plastic ribbon was calculated over the time for 1st,2nd, 3rd and 4th ,respectively.

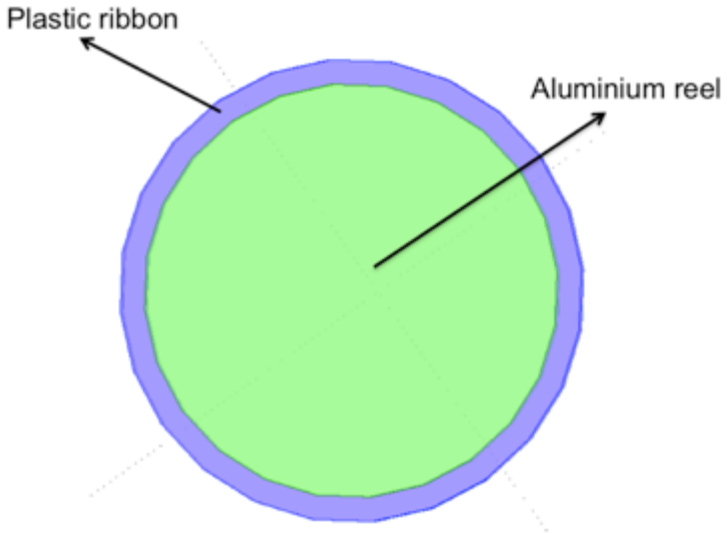


Figure A2.1 Schematic of the geometry of reel for heat transfersimulation.

Table A2.1 LAMP primers and primers sequences and size for *E.coli Tuf* gene and *Mcat S.aureus* gene amplification.

Primer name	Primers sequences	bp
	Ecoli Tuf gene	
F3	CTG CTG GGT CGT CAG GTA	18
B3	GGA TTT TCG CTT CCC ACT CT	20
LF	GGA TTT TCG CTT CCC ACT CT	20
LB	CGA CGA CAC TCC GAT CGT T	19
FIP	AGC AGC TCT TCG TCA TCA ACC AGG CGT TCC GTA CAT CAT CG	41
BIP	TGT CTC AGT ACG ACT TCC CGG GCG CTT TCA GAG CAG AAC CAC	42
	S.aures Mcat Gene	
F3	CTG GTG CAT TTG GGA CAT	18
B3	TTG TTC TAG GAT CTC GTT TCA C	22
LF	CTA CAG TAG AGA AAC GGG CAA	21
LB	CTG AAG AAG GGA ACT GGG ATT	21
FIP	TCA GCA GCA CCA CGT TCT CAG GTA AGC AAA CCG AAA TGT	39
BIP	GGA GCG TGA CAT TCG AGG ATT ACT GGT GTG TTA TTC CCT ACT A	41

APPENDIX III

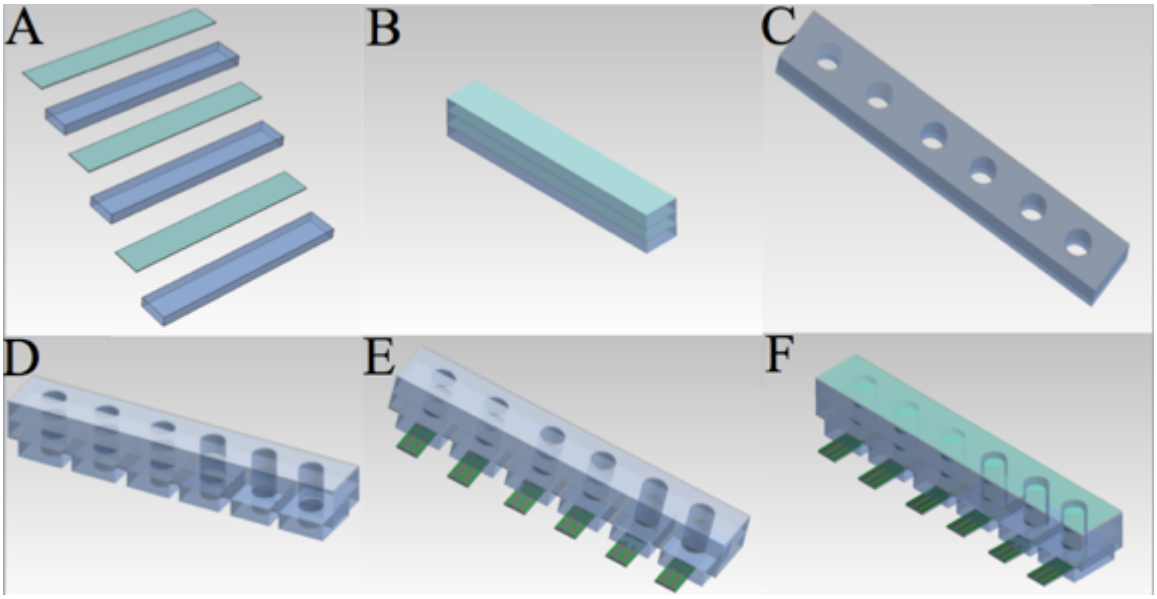


Fig A3.1: **Schematic of the fabrication process. (A) Flexible substrate consists of three layers of polyethylene ribbons, which was attached using double-sided tape. (B) The layers were attached on top of each other. (C) Punching the substrate to form reservoirs. (D) Removing the redundant to enhance bending and flexibility. (E) Attaching the flexible carbon electrode at the bottom of each reservoir. (F) Apply the samples and cover the reservoirs using the tape.**

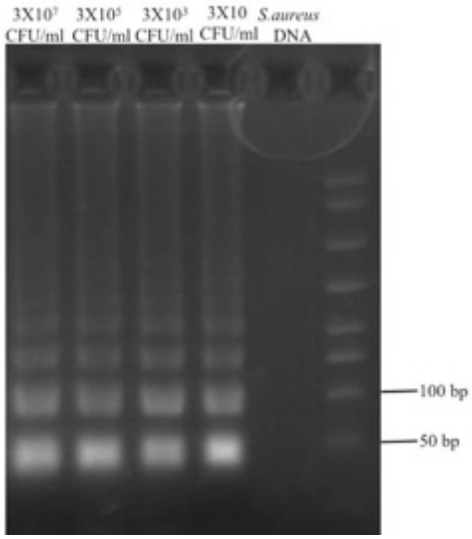


Figure A2.2: **Gel electrophoresis image for real time monitoring of *E.coli* bacteria after 50 min amplification time for various concentrations. Negative Control was set as *S.aureus* DNA.**

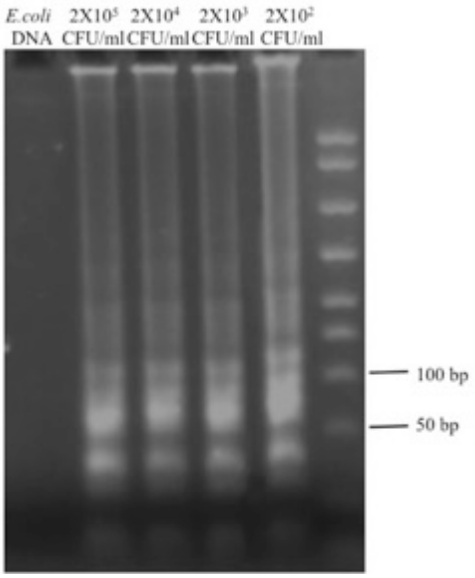


Figure A2.3: **Gel electrophoresis image for *S.aureus* bacteria after 50 min amplification time for various concentrations. Negative control was set as *E.coli* DNA.**

Table A3.1 **RSD % of real-time analysis for quantification of the asseys bacteria quantification of (A) *E.coli* (B) *S.aureus*.**

A

E.coli Bacteria Concentration(CFU/ml)	RSD% of peak height ratio
3×10^7	3.8
3×10^5	3
3×10^3	1.7
30	3.8

B

S.aureus Bacteria Concentration(CFU/ml)	RSD% of peak height ratio
2×10^5	4.8
2×10^4	4.2
2×10^3	1.7
2×10^2	3.7

APPENDIX IV

This file includes the experimental protocols of preparation of dead *E. coli* cells, T4 bacteriophage preparation, LAMP reaction, and testing the bacteriophage-biosensors in milk samples. In addition, one table is enclosed which illustrates Table 1S. LAMP primer's to amplify *E. coli Tuf* gene.

Preparation of dead *E. Coli* cells:

The killed *E. coli* were prepared according to the previous reported work in the literature.[48] Briefly, the dead *E. Coli* B cells were prepared by using the acetone-ether treatment adapted from the method used by Wang and Lin (48). *E. coli* cells were grown in LB broth overnight and collected by centrifugation at 3200 rpm for 15 min. The pellets were washed twice with 5 ml of 0.85% saline and were centrifuged at 3200 rpm for 15 min after each wash. After re-suspending the cells in 1 ml of 0.1 M tris (hydroxymethyl)-aminomethane (Tris)–HCl buffer (pH 7.4), 10 mL of cold acetone were added to the cell suspension. The mixture was vortexed for 5 minutes at an interval of 1-minute vortexing and 30 seconds chilling on ice. The suspension was centrifuged at 3200 rpm for 10 min and the acetone wash was repeated. Then 10 mL of cold diethyl ether were used twice to wash the suspension. Finally, cells were suspended in 5 mL of LB broth and stored at 4^0C.

Propagation of T4 bacteriophage:

T4 bacteriophage was propagated by using the soft agar overlay technique. Briefly, 100 µL of *E. Coli* B was added to 5 ml of molten top agar (LB broth containing 0.75% agar) and poured on the LB agar plates. After solidification of the top agar, 100 µL of bacteriophage stock ($2x10^{10}$ PFU/ml) was added to the plates and incubated at 37°C overnight. The top agar layer was scraped off by using λ buffer (50 mL of 1 M Tris-HCl (pH 7.5), 5.8 g/L NaCl, 2 g/L $MgSO_4$, 2% gelatin) and mixed with chloroform. The agar and bacterial debris were removed by centrifuging at 3200 rpm for 15 min. Sterile syringe filters with 0.45 µm pore size (Millipore) were used to filter the supernatant. In

order to determine the bacteriophage titer, serial dilutions of bacteriophage stock were prepared. The mixture of 20 µL of dilution and 100 µL of host bacteria was incubated for 5 min at 37°C and then added to molten top agar and spread over LB agar plates. For immobilization of T4 bacteriophage onto the sensor surface, the bacteriophage stock was centrifuged at 20000xg for 90 min and the pellet was re-suspended in sterile PBS (pH 8.5). The concentration of bacteriophage solutions was adjusted to 10^{10} pfu/ml.

LAMP reaction

LAMP was used to detect the *Tuf* gene in *E. Coli*. The protocol has been optimized in order to be compatible with our primers. 20 µL of master mix composed of 2.5 µL of 10X Thermopol Buffer (New England Biolabs, Beverly, MA), 3.2 µL of 0.6 µM Betaine (Sigma-Aldrich,MO,USA), 0.4 µL of 3 mM $MgSO_4$, 1 µl of *Bst Polymerase* (1600 units), 8000 U/ml (New England Biolabs, Beverly, MA), 0.6 mM concentration 0.6 µl of 0.6 µM dNTP (BioShop,Ontario,Canada), 5.65 µl of H_2O, 0.25 µl of 0.2 µM outer primers (F3, B3), 2.0 µl of

1.6 µM inner primers (FIP, BIP) and 1.0 µl of 1.0 µM loop primers (Loop F, Loop B). Table S1 shows the six primers sequences of the LAMP reaction.

Testing bacteriophage-biosensor in milk sample

Pasteurized skim milk was purchased from a local provider and they were inoculated with different known concentrations of *E. coli* B. The inoculated milk samples were then tested using the T4-bacteriophage functionalized gold microelectrode and electrochemical impedance spectroscopy as previously described. Three electrodes were used for each milk sample. Uninoculated milk was used as a control.

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RÉSUMÉ

La détection efficace des bactéries et le diagnostic *in vitro* sont essentiels pour les pratiques cliniques. Ces essais biologiques permettent aux liquides corporels tels que le sang, l'urine, et la salive de détecter des agents pathogènes. Les meilleurs moyens de détection des bactéries sont les techniques de culture. Ces techniques permettent la détection d'une bactérie unique par la croissance dans un milieu spécifique pour former une colonie évidente et distincte. Cependant, elle exige un personnel fortement qualifié pour être mise à l'œuvre. Elle est longue et dépendamment du type de bactéries, elle peut prendre entre 24 heures à plusieurs semaines pour certaines bactéries.

Les immunoessais sont des essais biochimiques employés pour détecter divers virus et agents pathogènes dans différentes matrices. Ces dit-s essais analyses utilisent cet avantage d'affinité et de spécificité très élevées en reliant un antigène et son anticorps pour détecter et mesurer l'antigène dans les fluides biologiques. Les immunoessais sont disponibles dans divers formats tels que le dosage d'immunoadsorption par enzyme liée (ELISA) et le test chromatographique qui se sont développées pour détecter divers types de bactéries, de virus, mais aussi pour la détection de spores [1]. Cependant, cette technique est laborieuse, exige le passage par plusieurs étapes et reste semi-quantitative, ce qui ne fournit pas la sensibilité nécessaire pour trouver les doses infectieuses pour la plupart des bactéries et souffre d'une réactivité croisée. Par conséquent, le développement d'un autre type de biocapteurs est nécessaire.

Parmi d'autres essais, l'hybridation de l'ADN et les techniques d'amplification fournissent des résultats précis et fiables. La technique la plus commune et la plus sensible pour l'amplification de l'acide nucléique et de l'analyte génomique est le PCR (Amplification en chaîne par polymérase). Depuis la première publication en 1985 [5], Le PCR s'est avéré être une technique prometteuse pour la détection des organismes à basses concentrations. Puisqu'il détecte la cible basée sur l'amplification plutôt que le signal, il est donc beaucoup moins enclin à donner à fausser un résultat positif.

Le principe du PCR est basé sur trois étapes séquentielles et il exige différents cycles de températures. La haute température, généralement à 90°C, est employée pour dénaturer le calibre d'ADN et le façonner en calibre monocaténaire. Une température plus basse (~50°C) permet l'hybridation ou l'appariement des amorces à 2 sections ciblées de l'ADN et à température intermédiaire (~70°C), (entre les deux températures précédentes) permets aux enzymes de polymérase de synthétiser le brin complémentaire de leur ADN matrice et de produire un nombre important de copies d'ADN cible [6].

Dans la plupart des cas, avoir trois cycles thermiques différents et précis est un problème qui rend ce processus pour obtenir de la précision relié à un cycle thermique très précis . Cependant il exige un laboratoire fortement équipé et un personnel hautement qualifié. En outre, le traitement de l'extraction d'ADN, aussi bien que la purification est un processus qui prend beaucoup de temps. Afin d'appliquer cette méthode dans des dispositifs de biocapteurs pour des applications environnementales ou de POC, il est important de disposer d'un dispositif compact qui peut accomplir tous ces processus dans une courte durée. Récemment, de nombreux efforts ont été accomplis afin de développer une puce PCR performante pour répondre aux exigences du dispositif micro-total d'analyses miniaturisées afin qu'il puisse être utilisé comme outil de diagnostic [96].

Les limitations du PCR ont ont conduit à utiliser l'amplification isotherme, qui peut fournir une 'amplification génomique de façon rapide et à un coût réduit. De diverses amplifications isothermes ont été développées au cours de la dernière décennie, qui peuvent exiger l'amplification de l'acide nucléique. Les amplifications isothermes sont différentes des techniques de PCR dans le sens où le taux d'activité de l'enzyme est un facteur limitant pour l'amplification, tandis que lors du PCR, c'est le taux de cyclisme qu'il l'est. Par conséquent, il est peu susceptible que l'amplification isotherme microfluidique augmente le taux de réaction enzymatique en modifiant le cycle thermique.

L'amplification isotherme mediée par Boucle (LAMP) est l'une des méthodes d'amplification isothermes les plus communes. Le résultat final peut être observé par la

mesure fluorescente de l'intensité ou à l'oeil nu pour la turbidité due à la précipitation du pyrophosphate de magnésium comme sous-produit, ce qui le rend approprié aux emplacements qui ont des ressources limitées. La méthode entière de LAMP se fait à une température de charge statique (°C) 60-66. L'ADN cible est amplifié en employant des amorces multiples, y compris les amorces de boucle et la polymérase *Bst*, ayant pour résultat un taux d'amplification de 109 nucléotides de cible (c.-à-d.,1 milliard de copies d'ADN) dans un délai de 15-60 minutes à une température simple et statique.

Le principe entier de la LAMP est basé sur l'emploi d'une seule enzyme de polymérase avec l'activité de déplacement de De-brin et 4 à 6 ensembles d'amorces qui ont été pour la première fois publiés en 2000 [98]. Il y a deux amorces externes (F-3 et B3) et deux amorces intérieures (FIP et BIP). La FIP et les BIP sont les deux amorces avant et arrière. L'amplification de LAMP a deux étapes initiales qui se font de manière cyclique. Dans la mesure initiale chacune des quatre amorces participe à l'amplification. Cependant, dans l'étape cyclique, seulement les amorces intérieures continuent l'amplification. Le début du mécanisme avec l'amorce intérieure de FIP hybride dans la région spécifique de l'ADN, tandis que l'amorce externe qui est un peu plus courte que le FIP hybride à la région spécifique de l'ADN et avec la polymérase *Bst,* l'activité initiale de déplacement de-strand le ADN dans le calibre ssADN.

Plus tard, de l'autre côté du calibre, BIP et B3 hybrident au calibre d'origine et forment la structure de boucle. Cette structure de boucle agit en tant que calibre initial pour l'étape cyclique. Par le recuit auto-principal suivant des amorces, des milliards de copies de la structure de boucle de la molécule sont formés avec la structure de boucle de chou-fleur. L'usage de quatre à 6 ensembles d'amorces rend cette technique très spécifique à l'ADN cible en comparaison avec l'autre technique d'amplification telle que le PCR. Dans cette thèse, nous montrons diverses plates-formes de LOC pour la détection d'agents pathogènes basée sur la technique de LAMP. La première section de la thèse concerne le développement de l'analyse électrochimique du micro-fluide pour la détection rapide et la quantification d'*E.coli* basée sur le LAMP. Le test électrochimique pour détecter l'amplification de l'ADN a été réalisé utilisant la molécule de redox de Hoechst 33258 et la voltammétrie à balayage linéaire (LSV).

L'agrégation d'ADN et le sillon mineur lié avec la molécule redox causent une baisse significative dans l'oxydation anodique de la LSV. À la différence d'autres techniques électrochimiques, cette méthode n'exige pas l'immobilisation de la sonde et la détection de bactéries peut être parfaitement accomplie dans une chambre simple sans étapes d'extraction et de purification d'ADN. Le temps isotherme d'amplification a un rôle important dans la quantification des bactéries.

Nous avons prouvé que nous pouvions détecter et mesurer 24 CFU/ml de bactéries et près de 8,6 fg/µl d'ADN de en 60 min et 48 CFU/ml de bactéries en l'espace de 35 minutes dans le milieu de culture LB et les échantillons d'urine.

Chaque puce est composée de deux puces microfluidiques parallèles pour détecter le contrôle négatif et l'échantillon avec le calibre d'ADN. La puce microfluidique a des canaux d'une largeur de 200 µm et d'une hauteur de 200 µm, une chambre de réaction et une chambre de détection pour la mesure électrochimique. La chambre de réaction a un volume de 35 µl et contient beaucoup de formes cylindriques d'un diamètre de 2 µm pour augmenter la surface par rapport au volume. La chambre de détection a un diamètre de 3 millimètres. La Figure 3,1 montre le schéma de l'analyse.

Se basant sur ce rapport, 35 µl de la solution de LAMP a été préparée et injectée dans la chambre de réaction utilisant une seringue à pompe ayant un débit de 15 µl/min. La solution a été maintenue dans la chambre de réaction en utilisant une valve active. La valve active est faite par un morceau de PDMS, qui a été placé verticalement pour bloquer le canal. L'échantillon est resté dans la chambre de réaction pour amplifier le gène de l'ADN cible. Il a été ensuite libéré dans la chambre de détection pour permettre les mesures électrochimiques. Entre temps, l'amortisseur PBS et la solution de redox provenant d'une autre entrée de la chambre d'électrode ont été ajoutés à la solution.

Chaque 6 µL du produit de LAMP a été mélangée avec 12 µL de PBS et 12 µl de la solution de redox [147]. Expérimentalement, nous avons gagné 30 µL de produit d'amplification de la chambre de réaction. Se basant sur ce rapport de mélange, nous avons ajouté le 120 µL de l'amortisseur PBS et de la solution de redox à la chambre de détection. Cette quantité de solution a couvert la superficie de l'électrode dans la chambre de détection. Ainsi, la mesure de la LSV a été effectuée et a illustré l'oxydation

anodique du bio-échantillon [148]. La différence dans le pic maximum de l'oxydation anodique entre le contrôle négatif et l'échantillon d'ADN a été employée pour détecter et quantifier l'ADN amplifié. Pour un ADN à concentration élevée, l'ajout de la solution de redox et des produits d'amplifications atteint son niveau de saturation. Cet ajout cause une réduction importante des électrons sur la surface de l'électrode, qui a comme conséquence une baisse significative des sommets d'oxydation anodique. D'autre part, diminuer la concentration en ADN fait agréger moins de molécules de redox avec le produit d'amplification et en conséquence, on trouve plus d'électrons sur la surface de l'électrode. La puce microfluidique présentée ici n'a besoin d'aucune étape de traitement préparatoire pour des bio-échantillons.

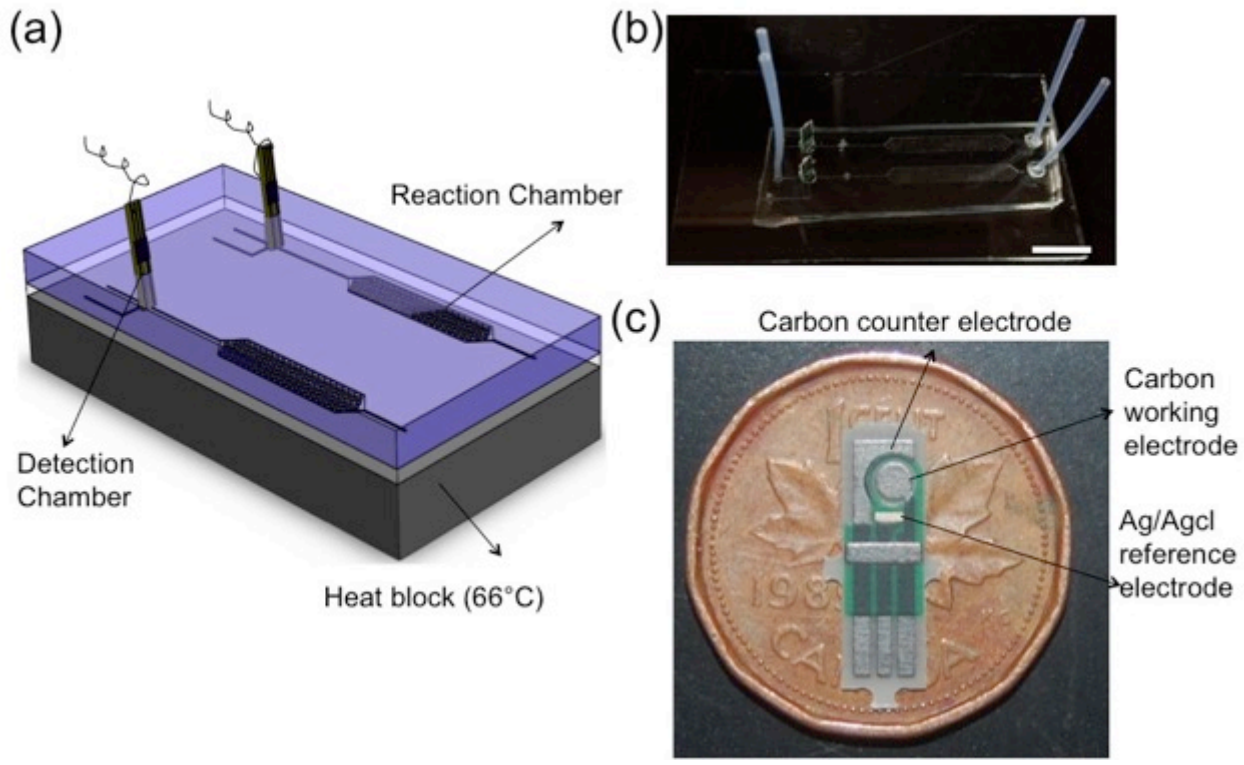

Fig. 3.1 **La représentation schématique de la puce microfluidique pour la détection d'E. coli. (a) la puce microfluidique composée d'un bloc de chauffage en tant que source de chaleur pour fournir 66 ° C, lame de verre comme substrat et la puce PDMS. La puce PDMS contient deux puces micros fluidiques parallèles pour le contrôle négatif et la détection de l'ADN. La puce micro fluidique est composée d'une chambre de réaction, une soupape active (non représenté ici) et une chambre d'électrode. (b) Image de la puce micro fluidique composée de deux puces micros fluidiques et des tubes capillaires parallèles qui relient la puce à la pompe à seringue (La pompe à seringue est montrée ici). La barre d'échelle est de 15 mm (c) la puce Micrographe DEP utilisée pour la détection électrochimique.**

La température isotherme à 66°C fournit assez de choc thermique pour lyser la cible bactérienne d'Escherichia coli dans cette étude. Plus tard, l'ADN des bactéries a été amplifié et détecté utilisant la LSV sans aucune étape de traitement préparatoire. Une série d'expériences a été faite pour optimiser les produits de LAMP d'*E.coli* sous l'amplification isotherme pour différents laps de temps (0,10, 20, 30, 40, 50, 60 min), utilisant la technique électrochimique de détection de la LSV (Fig. 3,4). 35,3 ng/mL de la concentration du calibre d'ADN ont été employés dans l'expérience, tandis que l'eau ne contenant aucun ADN est utilisée dans les expériences de contrôle négatives.

On a pu donc constater que le produit de LAMP peut être détecté en l'espace de 20 min tandis que les produits d'amplification de LAMPE atteignent leur niveau de saturation après 40 min. Une amélioration majeure dans la lutte contre la maladie peut être apportée si les bactéries ciblées sont rapidement détectées et exactement mesurées avec une sensibilité élevée grâce à analyse microfluidique. Afin d'appliquer ce dispositif comme dispositif diagnostique de POC, le calibrage de la détection de bactéries dans la puce est exigé. Nous avons diminué le temps résident à 35 minimum et avons mesuré le produit de LAMP aux différentes concentrations d'*E.coli*. L'échantillon d'urine a été filtré hors puce utilisant des filtres de 0,2 µm pour épurer et enlever les impuretés de clouer avant d'y clouter les bactéries. Il a été mentionné auparavant [136.152] que le niveau de la dilution d'échantillon d'urine joue un rôle critique dans le succès de l'amplification de LAMP puisque certaines concentrations en urine dans l'échantillon pourraient empêcher la réaction d'amplification [153].

Nos amorces de LAMP ne sont pas été inhibées en présence de 20 % d'urine dans l'échantillon [136]. 5 mL d'urine dans 25 ml du mélange principal ont été utilisés, dose inférieure à 20% de la limite d'inhibition d'urine. La Figure. 3,5 montre une relation linéaire entre l'existant (maximum des pics anodiques pour chaque concentration bactérienne) et l'échelle logarithmique de la concentration de bactéries dans un milieu de culture différent. La concentration de bactéries varie de 48 CFU /mL à 4.8×10^5 CFU/mL. La spécificité de la puce microfluidique a été établie par l'essai d'activité hétérospécifique utilisant trois bactéries différentes *E.coli*, Listeria et l' ADN extrait de *S. aureus*. Les réactions de LAMP ont été effectuées utilisant les ordres d'amorce spécifiques pour les bactéries d'*E.coli* avec l'ADN de *S.aureus* et Listeria durant 1

heure. L'eau sans calibre d'ADN a été employée pour le contrôle négatif. On n'a pas observé de baisse d'oxydation anodique majeure pour les bactéries n'appartenant pas aux-*E.coli*, ce qui démontre le fait que les amorces d'*E.coli* ne peuvent pas multiplier d'autres types de bactéries.

Dans la deuxième partie de la thèse nous avons développé une cassette simple comme dispositif de diagnostic pour la détection colorimétrique de bactéries à l'œil nu pour le système élevé de détection de séquence. Le dispositif est préparé, utilisant une méthode très simple et facile d'accéder aux matériaux de grande simple et de coût de préparation très bas. Nous avons prouvé que nous pouvions détecter 30 CFU/ml d'*E.coli* et 200 CFU/ml de bactéries de *S.aureus* en moins d'1 heure utilisant le substrat flexible du ruban de polyéthylène de manière colorimétrique.

En utilisant la simulation numérique, nous avons prouvé que nous pouvons analyser et détecter un minimum de 36 échantillons simultanément à rendement élevé.

Depuis que le procès de fabrication ainsi que les matériaux peuvent être parfaitement accompli et trouvés par un être personnel peu qualifié, elle peut être employée en tant que dispositif à haute sensibilité, un POC spécifique et robuste pour la détection de bactéries multiples dans les pays en voie de développement et les contextes où l'on manque de ressources. La cassette comporte deux bobines de collection et de fourniture dont le diamètre est de 150 millimètres et l'épaisseur de 11mm.

Le fournisseur de collecte est relié au contrôleur de radiateur électrique pour fournir la température précise (66°C). Les 5 µl du vrai échantillon de bactéries avec les 20 µl de la solution de LAMP ont été parfaitement mélangés au commencement et ont été placées dans chaque réservoir. Ensuite, les compartiments ont été couverts par une bande et un rouleau à l'intérieur de la bobine de collection pour une amplification supplémentaire. La figure 4,1 montre le schéma de l'opération de cassette.

Le substrat flexible a été fabriqué en utilisant la bande de polyéthylène avec une largeur de 10 millimètres et une épaisseur de 3 millimètres et fournit un réservoir d'un volume unifié avec 35 µl chacun. Deux couches de polystyrène ont été coupées et attachées l'une sur l'autre, utilisant le double scotch Kapton®. La bande a été utilisée comme couche inférieure et un papier a été utilisé pour clarifier l'observation des résultats de

l'analyse. La détection des bactéries dans de vrais échantillons basés sur l'amplification génomique est considéré comme laborieux dû au processus étapes multiples exigé : la cellule de lysis, l'extraction d'ADN, la purification et l'amplification [9].

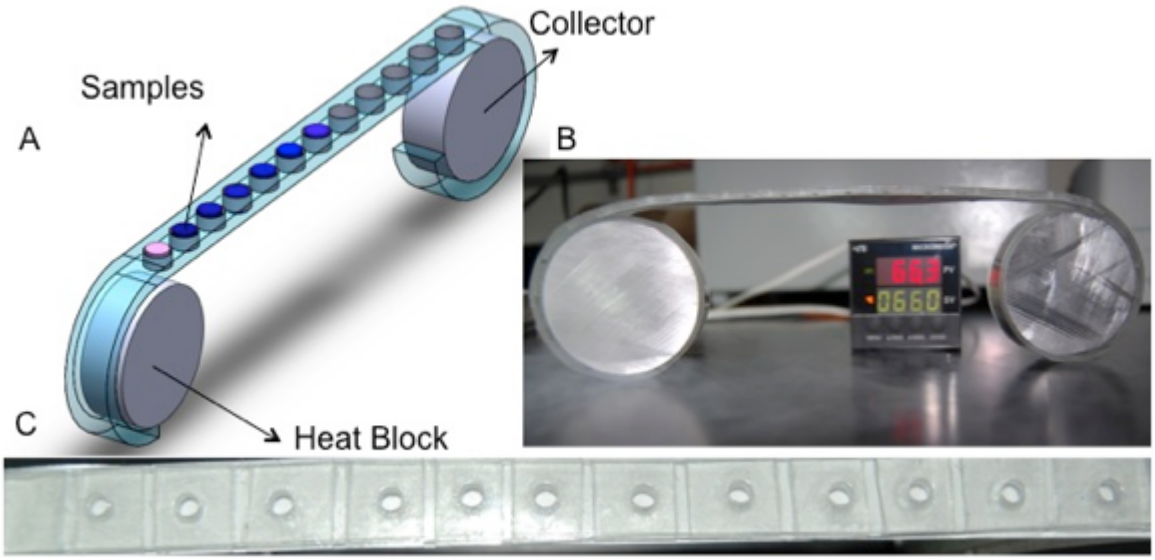

Fig 4.1 **Vue d'ensemble du dispositif de la cassette et de ses composants. (A) Représentation schématique de la cassette. La cassette se compose de deux bobines d'aluminium pour fournir et collecter des échantillons, un dispositif de commande de chaleur et un dispositif de chauffage à température précise pour fournir l'amplification et le substrat flexible, où les chambres sont fabriquées. (B) l'image du substrat flexible. (C) l'image du substrat flexible.**

Dans notre étude précédente [172-174], nous avons prouvé que nous pouvions détecter des bactéries d'*E.coli* dans des milieux de culture divers sans purification par lysis et amplification de l'ADN de cible dans un compartiment microfluidique de réaction.

La détection suivante des produits d'amplification d'ADN a été détectée électro-chimiquement. En dépit de fournir une puce, rapide et extrêmement sensible pour la détection de bactéries, elle a des limitations pour le système élevé de détection de séquence de beaucoup d'échantillons dans les pays à faibles revenus, dus aussi bien à l'opération étapes multiples qu'au coût élevé de fabrication. D'autre part, la détection colorimétrique est une technique facile à utiliser, bon marché et de grande qualité pour confirmer la présence des produits d'amplification d'ADN. Durant ce projet, nous avons utilisé du HNB, qui développe une couleur violette en présence de Mg^{2+}. Dans le processus d'amplification, on produit une importante quantité de pyrophosphate

insoluble de magnésium, qui cause la réduction principale de Mg^{2+} dans la solution. Cette réduction a changé la couleur du HNB du violet au bleu [169].

Nous avons ajouté les 25 µL de la solution de LAMP comprenant les 5 µL de l'échantillon de bactéries avec des concentrations allant de 3×10^8 CFU/mL à 30 CFU/mL. Après 1 heure d'amplification d'ADN, le ruban a été aggrandi pour mieux visualiser les résultats. Nous avons pu détecter Escherichia coli à une concentration aussi basse que 30 CFU/mL. *S.aureus* est une bactérie Gram-positives et l'étape de Lysis exige divers difficultés pour lyser. La détection à sensibilité élevée de *S.aureus* exige une technique plus intense de lysis puisqu'il est difficile de lyser les bactéries gram-positives comparée aux gram-négatives. Diverses techniques lysantes existent comme le chimique, le thermal, l'électrique le mécanique [175,176]. Le lysis chimique est une méthode utile, et peu coûteuse pour lyser les bactéries, mais elle nécessite une étape finale de traitement pour enlever les agents d'inhibiteur de la solution de lysis, ce qui ne la rend pas appropriée au processus d'amplification simultanément.

Les techniques électriques et mécaniques ont besoin d'appareils coûteux, aussi le processus de fabrication reste très cher et n'est pas approprié aux systèmes à bas prix. D'autre part, la lyse de choc thermique est facile à utiliser, n'exige aucun équipement supplémentaire dû à l'utilisation d'un appareil de chauffage dans le processus d'amplification dans le dispositif et peut être employé pour la lyse de *S.aureus* en peu de temps.

Pour exploiter ce processus, nous avons chargé les 5 µl des bactéries de *S.aureus* dans un compartiment et l'avons couvert de bande adhésive et l'avons roulé à la bobine du collecteur et chauffé pendant minute 5 à 90°C. Ainsi les échantillons ont été roulés à leur tour, découvert, puis nous avons rajouté les 20 µl de la solution de LAMP dans le compartiment et mélangé correctement à l'aide d'une pipette, en la couvrant de bande adhésive encore une fois. Les échantillons sont roulés dans le collecteur et sont chauffés à 65°C pour les amplifier. La calcéine a été utilisée comme colorant pour la détection qualitative. La couleur de la calcéine passe du jaune au vert par l'émission fluorescente en présence de produits d'amplification. La figure 4,4 donne le résultat de l'analyse colorimétrique pour différentes concentrations allant de 2×10^8 à 200 CFU/mL.

Avec cette technique, nous avons pu détecter 200 CFU/mL de vrai échantillon sans n'importe quel traitement préparatoire et ce en moins d'une heure.

La méthode d'amplification de LAMP fonctionne pour des températures ambiantes entre 60°C-66°C. Par conséquent, la fourniture de la température dans cette température peut lancer le processus d'amplification pour des échantillons dans les compartiments qui sont soumis à cette température ambiante.

Comprendre le transfert de chaleur dans la bobine du fournisseur est l'élément clé pour fournir le potentiel de l'échantillon traitant, simultanément. Nous avons donc exécuté la simulation numérique pour comprendre le transfert de chaleur et les profils de température autour de la bobine en aluminium et du substrat flexible et pour déterminer combien de rouleaux de ruban peuvent commutés autour de la bobine en aluminium afin de s'assurer de la détection de l'agent pathogène cible.

Le modèle 3D de transfert de chaleur dépendant du temps en utilisant la méthode des éléments finis (MÉF) a été mis en application pour différents cycles de rubans d'une épaisseur de 3 millimètres pour chaque tour afin de comprendre si l'appareil de chauffage peut fournir assez de température de l'ordre de 60°C-66°C pour au minimum 50 min. Nous avons constaté que l'appareillage de chaleur peut fournir 3 tours de rubans pour plus d'amplification et de détection. Ce nombre de tours fournit au moins 36 échantillons durant le processus et l'amplification en même temps.

Dans la troisième partie de la thèse, nous démontrons une surveillance électrochimique en temps réel à haut débit de LAMP pour la détection de bactéries pathogènes. Un des défis importants dans les soins de santé est le développement des diagnostics. Les diagnostics nécessitent des dispositifs à faible coût qui offrent la portabilité, la simplicité d'utilisation et la possibilité de haut rendement et d'analyse quantitative. Ici, nous présentons un dispositif de traitement de fluide pour une surveillance électrochimique en temps réel de l'amplification de l'acide nucléique (AN) et la détection de bactéries.

LAMP et la détection électrochimique en temps réel est basé sur l'interaction entre le produit d'amplification LAMP et le complexe d'osmium de redox-réactif. Nous avons démontré la détection de 30 cfu / ml d'*E.coli* (dans la gamme comprise entre 30-3x10^7 CFU/mL) et 200 CFU/mL de *Staphylococcus aureus* (dans la gamme de 200 à 2x10^5

CFU/mL) des échantillons cultivés à la fois en temps réel et la détection au point d'équivalence.

Ce dispositif peut être utilisé pour la détection de diverses bactéries à gram-négatif et un certain nombre de bactéries pathogènes à Gram-positives avec une haute sensibilité et une spécificité dans un format à rendement élevé. En utilisant une approche de bobinage, nous avons pu détecter 12 échantillons dans un seul essai.

Comme LAMP et l'analyse électrochimique sont mises en œuvre dans les bio puces souples et étanches, les étapes de traitement laborieux ne sont pas nécessaires et le risque de contamination est considérablement réduit. La figure 5.1 montre le schéma de l'appareil. Un dosage électrochimique a été mis en œuvre en attachant l'électrode sérigraphies de carbone (FSPE) au bas de chaque réservoir (voir la fabrication à l'Annexe III, figure A3.1). Ensuite, l'électrode est connectée à la machine de potentiostat et les mesures sont mises en œuvre en utilisant le SWV en temps réel en raison d'une sensibilité élevée et d'un balayage rapide. Pour cette analyse, la vitesse de balayage est de 100 mV/s dans la gamme allant de -0,2 à 0,8 V pour atteindre le pic d'oxydation.

Cette mesure a été réalisée en mesurant chaque échantillon, un par un dans le substrat flexible. Une fois que le substrat souple entourait le périmètre du collecteur de bobine, (qui a agi comme un dispositif de chauffage) et tous les tests de tous les essais sont complètement finis, elle a été coupée et a été retirée du processus.

0,2 µl d'osmium redox ($Os[(bpy)_2DPPZ](PF6)_2$) a été ajouté à une concentration finale de 0,5 µM dans le protocole de LAMP. Cette concentration a fourni un signal très distinct dans le redox [200] et a été utilisée pour la surveillance en temps réel de l'amplification de LAMP.

Pour simplifier les résultats, le rapport de la hauteur du pic a été défini comme le rapport du pic SWV à un moment d'amplification SWV au point initial. La méthode SWV a été choisie en raison de sa haute sensibilité, sa rapidité mais aussi parce qu'elle permet une bonne reproductibilité et a été mise en œuvre précédemment dans le suivi de produit d'amplification PCR en temps réel [188]. Chaque analyse prend précisément 23 secondes et chaque cinq minutes, 3 scans sont effectués pour assurer une

surveillance précise de l'interaction redox - produit d'amplification. La figure 5.3 montre le suivi en temps réel d' *E.coli*.

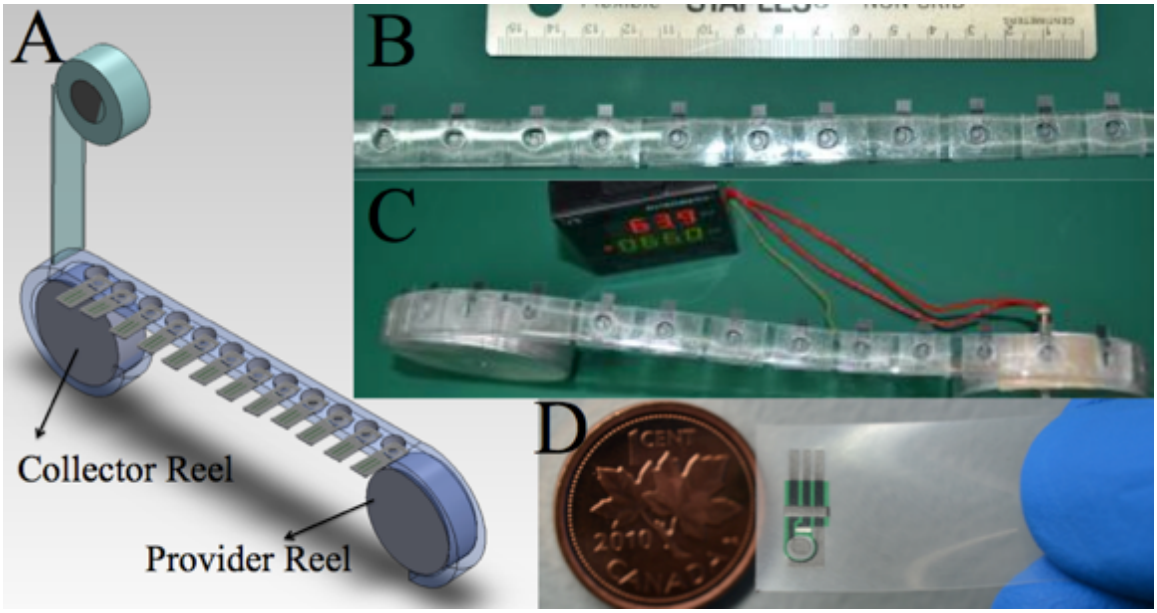

Fig 5.1 **Une représentation schématique de la cassette. (A) La cassette est constituée de deux bobines d'aluminium et un ruban souple dans lequel les réservoirs ont été fabriqués uniformément. Après le chargement de l'échantillon et après avoir tourné dans la bobine du capteur, une bande adhésive couvre tous les échantillons et le ruban tourne dans la bobine du capteur pour rendre les échantillons prêts à l'analyse électrochimique. (B) l'image des réservoirs fabriqués sur ruban flexible en polyéthylène. (C) l'image de la cassette reliée à l'appareil de chauffage électrique et le contrôleur. (D) l'image de l'électrode de carbone souple constitué par une électrode de travail en carbone, une contre-électrode de carbone et une électrode de référence Ag / AgCl fabriqué sur une feuille de polyéthylène flexible.**

La molécule rédox a été intercalée avec une quantité croissante de produit d'amplification pendant le processus d'amplification, par conséquent, la concentration du redox libre dans la solution a diminué. A une forte concentration de bactéries, on trouve d'autres matrices d'ADN dans la solution, ce qui donne lieu à une production plus rapide de produit d'amplification par rapport à des échantillons contenant une concentration inférieure de bactéries (Figure 5.3A).

Afin de déterminer le temps d'amplification nécessaire pour détecter la concentration de bactéries *E.coli*, nous avons fixé la valeur de seuil pour le rapport de la hauteur de pic à 0,8 pour distinguer entre le contrôle négatif et les échantillons positifs. La figure 5.3b montre que 29 minutes comme temps d'amplification sont nécessaires pour atteindre la limite de détection (LOD) de 30 CFU/ ml de bactérie *E.coli*. Il existe une relation linéaire

entre le temps d'amplification logarithmique et la concentration d'*E.coli* avec la courbe de -3,15 et le R^2 du 0,94. En réglant le temps d'amplification à 35 min, nous pourrions quantifier la bactérie *E.coli* en traçant le rapport de hauteur en fonction de la concentration de l'*E.coli* logarithmique comme celle qu'on peut observer dans la figure 5.3C. La droite de régression étalonne la quantification de l'*E.coli* avec une courbe de -0,04 et un R^2 de 0,88. Bien que les bactéries gram-négatives peuvent être facilement lysées par choc thermique à 60-66°C, cette gamme de température ne fournit pas assez de choc pour les bactéries gram-positives telles que S. aureus, pour lesquels il faut au moins 5 min chauffage à 90°C pour une lyse efficace. Cependant, le principal inconvénient de l'utilisation de cette lyse à haute température, c'est que la *Bst* polymérase dans la réaction de LAMP est inactivée de manière irréversible à une température supérieure à 70°C. C'est pour cette raison que nous avons modifié la procédure en réalisant la lyse des bactéries *S.aureus* à 90°C pendant 5 minutes sur la bobine collectrice, avant l'addition des réactifs de LAMP.

Après la lyse, le ruban fut promu pour refroidir le mélange de lyse, puis on a ajouté la solution LAMP, couvert par la bande à nouveau et nous l'avons roulé sur la bobine du collecteur en chauffant à 65 °C pour lancer le processus d'amplification. Une surveillance en temps réel du produit d'amplification LAMP pour diverses concentrations de bactéries *S.aureus* a été testée pendant les 50 min d'amplification (Figure 5.4A). Comme attendu, la production de produit d'amplification a atteint le seuil de détection plus tôt avec des échantillons de bactéries de concentration élevée en comparaison à des échantillons avec des concentrations plus faibles de bactéries. Afin d'optimiser le temps d'amplification pour la détection de la concentration des bactéries spécifiques, la valeur du pic de seuil de la hauteur a été définie à 0,85 tel qu'on peut le voir dans la Figure 5.4A. Le temps d'amplification vs. la concentration logarithmique de S. aureus apparaissent dans la figure 5.4B. Il a été constaté qu'il existe une relation linéaire entre la valeur de hauteur de pic et le temps d'amplification pour différentes concentrations de *S.aureus* à une courbe de -7,4 et un R^2 de 0,97. Le LOD des 200 CFU/ ml a été atteint en 34 minutes temps d'amplification. Le rapport de la hauteur du pic à 40 min, après le début de l'amplification a été tracé en contrepartie de la concentration des bactéries en tant que courbe d'étalonnage pour la quantification de *S. aureus* (Figure 5.4C)

Une relation linéaire a été obtenue entre la hauteur du pic et de la concentration logarithmique des bactéries *S.aureus* avec une courbe de -0,12 et un R^2 de 0,99. Après 40 minutes de temps d'amplification, les échantillons à concentration élevée de bactéries atteignent le degrés de saturation de produits d'amplification dans l'échantillon, ce qui aide à capter un signal constant de pic. Par conséquent, il ne peut être rendu en tant que le temps d'amplification approprié fixé pour la quantification des bactéries.

Pour approfondir la performance de l'appareil quant à la détection de traces d'agents pathogènes, nous avons choisi d'utiliser la détection du point final du produit d'amplification LAMP. Une mesure de détection de point d'extrémité a été mise en œuvre par une analyse du pic maximum de SWV, ce qui indique l'étendue de l'interaction produit d'amplification LAMP-osmium de redox pour différentes concentrations de bactéries à 50 minutes de temps d'amplification. En outre, le test de réactivité croisée a été effectué pour évaluer la spécificité de la détection.

La Figure 5.5A montre la détection du point final de la bactérie *E.coli* et *S.aureus*. Le maximum de pic SWV a été mesuré pour 30 CFU/ ml, 3 x 10^3 CFU/ ml et 3 x 10^5 CFU/ ml de *E.coli* et la spécificité des dosages a été testé avec de l'ADN de S. aureus, ainsi que l'ADN des testicules de saumon. On a constaté que le dispositif de détection est sensible à moins de 30 CFU/ml d'*E.coli* dans des échantillons réels. Aucun changement significatif de pic anodique n'a été observé pour d'autres ADN non ciblé, ce qui confirme que le test développé est très spécifique. La détection du point final ainsi que les tests de réactivité croisée pour *S.aureus* ont été répétés avec les mêmes étapes de lyse et d'amplification que dans les mesures en temps réel. Il a été constaté que le système est capable de détecter une quantité aussi basse que 200 CFU/ ml de *S.aureus* en 50 minutes. La spécificité du test a été évaluée avec l`ADN des testicules de saumon ainsi que l'ADN de *E.coli* et aucun changement de pic significatif n'a été observé, comme nous le montre la figure 5.5B.

Afin de valider les résultats, les données électrochimiques ont été comparées à l'image d'électrophorèse sur gel. Nous avons pris 5 µl de produit d'amplification LAMP de chaque réservoir du ruban flexible après 50 minutes de temps d'amplification, et avons

exécuté l'électrophorèse sur gel par imagerie UV. Les résultats sont présentés sur les figures A3.2 et A3.3 en tant qu'informations supplémentaires. Pour les échantillons amplifiés, la bande d'échelle a été observée, ce qui est en bon accord avec le petit signal électrochimique du pic SWV dû au fait que les OS redox intercalent avec les produits d'amplification.

Aucune bande d'échelle n'a été observée dans le contrôle négatif, ce qui est en bon accord avec le pic de SWV, reflétant le fait qu'il y a un manque de produit d'amplification dans l'échantillon et fournissant une haute spécificité envers le gène cible. Étant donné que les échantillons ont été prélevés après avoir atteint la limite de saturation (50 minutes de temps d'amplification), aucune bande d'échelle dépendante de la dose n'a été observée. Dans la quatrième partie de la thèse, nous intégrons deux stratégies de détection complémentaires pour l'identification et la quantification d'*E.coli* basés sur le bactériophage T4 en tant que bio-récepteur naturel pour les cellules de bactéries vivantes. La première approche implique le dépistage et la viabilité des essais, en utilisant un bactériophage en tant qu''élément de reconnaissance de la spectroscopie d'impédance électrochimique sans étiquette.

L'approche complémentaire est une confirmation par amplification isotherme facilitée par l'anneau (LAMP) pour amplifier spécifiquement le gène *E.coli Tuf* après la lyse des cellules liées d'*E.coli*, suivie d'une détection à l'aide de la voltamétrie à balayage linéaire. Le bactériophage T4 a été réticulé, en présence de 1,4-phénylène d'isothiocyanate, sur une électrode d'or cystéine modifiée.

Le biocapteur impédimétrique présente une détection spécifique et reproductible avec une sensibilité sur la gamme de concentration de 10^3 à 10^9 CFU/ml, alors que la réponse linéaire de l'approche de LAMP a été déterminée comme étant de 10^2 à 10^7 CFU/ ml. La limite de détection de 8×10^2 CFU/ml, en moins de 15 min et 10^2 CFU/ml dans un délai de 40 min de réaction ont été obtenus respectivement pour les méthodes impédimétriques ainsi que LAMP,. Ce travail fournit la preuve que l'intégration du biocapteur modifié de bactériophage T4-LAMP permet le dépistage, la viabilité et la confirmation en moins d'une heure.

La voltamétrie cyclique est une méthode analytique efficace, bien acceptée et qui est couramment utilisée pour surveiller la modification de surface, car elle fournit une méthode rapide et simple pour la caractérisation initiale de l'électrode modifiée à l'aide d'un système de couple redox [237]. La sonde redox réversible $Fe(CN)_6^{3-/4-}$ a été utilisée pour étudier les différentes étapes de modification de l'électrode. La figure 6.2A présente les voltamogrammes cycliques de 10 Mm $Fe(CN)_6^{3-/4-}$.

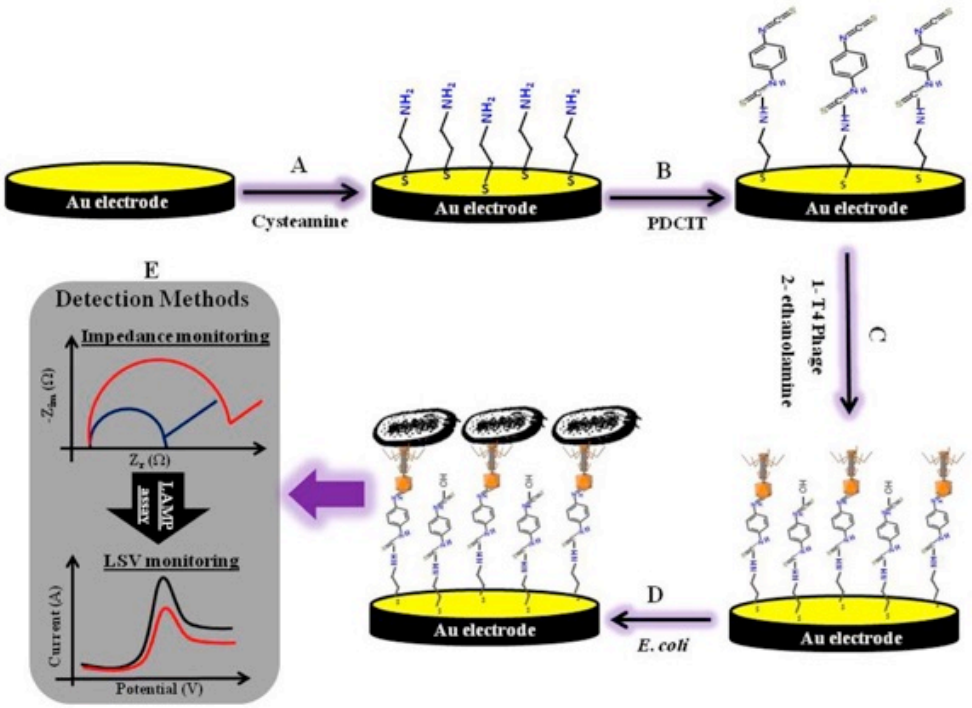

Fig 6.1 **La représentation schématique des biocapteurs bactériophage T4. (A) Montage de la cystéine sur l'électrode. (B) Activation avec du 1,4-dithiocyanate (PDCIT) Agent de réticulation. (C) Immobilisation du phage T4 et le blocage avec de l'éthanol aminé. (D) Capture des cellules de E. coli. (E) Procédé de détection de E. coli basé sur les double réponse impédimétrique / LAMP.**

$Fe(CN)_6^{3-/4-}$ à l'électrode d'or, l'électrode d'or cystéine modifiée, l'immobilisation du bactériophage T4, et ensuite, le blocage avec de l'éthanol aminé. Comme prévu, la sonde $Fe(CN)_6^{3-/4-}$ redox a présenté un comportement réversible sur l'électrode nu avec une différence de pic à pic de 80 mV. Le potentiel formel de 0,23 V a été estimé à partir de la moyenne des pics anodiques et cathodiques du voltammogram cyclique de l'électrode d'or nu. Cette valeur peut être utilisée comme un point potentiel continu fixe pour toutes les expériences de spectroscopie d'impédance électrochimique.

Sur l'électrode d'or avec cystéine modifié, le courant du pica été légèrement augmenté et la différence de pic à pic a diminué de 62 mV en raison de l'attraction électrostatique entre les groupements aminés positivement (surface pK_a 7,6) chargés de l'électrode en or cystéine modifié et de la charge négative de la sonde redox [238].

Cependant, le courant de pointe de la sonde redox a considérablement diminué, et la différence de pic à pic a augmenté après chaque étape de la modification de l'électrode: une immersion dans une solution de PDICT, l'immobilisation du bactériophage T4, et le blocage de l'éthanol aminé ont été observés. Les diminutions dans les courants de pics peuvent être attribuées au fait que le bactériophage T4 et l'éthanol aminé ont été isolés de la surface et ont efficacement améliorés les obstacles de transfert d'électrons.

La spectroscopie d'impédance électrochimique (SIE) a également été utilisée pour caractériser davantage l'ensemble des étapes de fonctionnement des biocapteurs bactériens, car signalée comme non destructive et considérée comme un procédé efficace pour contrôler les caractéristiques de la surface, permettant la compréhension des transformations chimiques et des propriétés inter-faciales de l'électrode modifiée [239].

La Figure 6.2B illustre les résultats de la parcelle de Nyquist ($-Z_{im}$ vs Z_r) de l'électrode nu, de l'électrode d'or cystéine modifié, l'activation avec le PDCIT et l'immobilisation du bactériophage T4 en présence d'une solution de 10 mM $Fe(CN)_6^{3-/4-}$ comme sonde redox dans 10 mM de PBS (pH 7,4). Comme le montre la figure 6.2B, un demi-cercle a été observé à des fréquences élevées, ce qui correspond au processus de transfert limité des électrons et suivi pard'une ligne droite dont la courbe est proche de l'unité à des fréquences plus basses, résultant en la diffusion limitée de l'espèce redox depuis l'électrolyte jusqu'à l'interface de l'électrode. Cependant, après l'auto-assemblage de la cystéamine sur l'électrode d'or, le demi-cercle de diamètre a été réduit en raison de l'interaction électrostatique entre le groupe amino-positif de la cystéamine et la sonde redox chargée négativement.

Lorsque l'électrode en or modifié en cystéamine a été activée avec le PDCIT, le diamètre du demi-cercle a augmenté comme prévu. Cela est dû à l'augmentation de longueur de chaîne de SAM's et la génération d'un charge neutre après l'association

des amino-groupes de cystéamine avec le groupe Thio-cyanate. Cette longueur accrue de chaîne peut former une certaine entrave à l'écoulement de la sonde redox à la surface de l'électrode d'or, comme représenté par une légère augmentation du diamètre du demi-cercle.

Une fois que les molécules de bactériophage T4 ont été fixées à l'électrode activée, le diamètre a été augmenté de façon significative par rapport aux surfaces d'électrodes modifiées précédemment. La raison est que les molécules T4 bactériophage sur l'électrode agissent comme une couche isolante parce qu'elles ont été chargées négativement (pI 5.4) au pH de 7,4 [240]. En conséquence, elles ont entravés de façon significative la diffusion de la sonde redox vers la surface de l'électrode, ce qui forme une meilleure barrière de transfert d'électrons et élargit par le même biais le diamètre du demi-cercle.

Les spectres d'impédance ont démontré un demi-cercle et une portion linéaire. La partie de la portion en demi-cercle à fréquences élevées correspond au processus limité de transfert d'électrons, et la partie linéaire à fréquences inférieures représente le processus de diffusion limité. Le circuit équivalent pour un électrode subissant un transfert d'électrons hétérogènes est généralement décrit sur la base au circuit équivalent Randles [241]. En raison du manque de régularité de la surface des biocapteurs et de la non-homogénéité de la distribution du courant, la capacité de double couche peut être remplacée par un élément de phase constante (CPE). Par conséquent, le circuit équivalent au Randles modifié peut être utilisé comme un circuit équivalent idéal pour analyser les spectres d'impédance dans ce système [242].

Comme illustré dans l'encart de la figure 6.2B, le circuit équivalent est constitué d'une résistance de base de la solution, Rs, une résistance de transfert de charge, ECR, l'impédance de Warburg, ZW, résultant de la diffusion du couple redox à partir de la masse de la solution à l'interface de l'électrode, et un CPE, représentant la capacité de double couche électrique, ce qui conduit à un demi-cercle enfoncé dans la parcelle correspondante à l'impédance de Nyquist. L'impédance de la CPA est fournie par l'équation suivante:

$$Z_{CPE} = Q^{-1}(j\omega)^{\alpha} \ ,$$

où ω est la fréquence du champ électrique, Q^{-1} est le module de l'élément de phase constante, mesurée en farads cm-2sα-1, où l'exposant α varie de 0 à 1 et reflète la réalité du condensateur [243]. La capacité de double couche peut être estimée à partir de [244] $Q = C_{dl}^{\alpha}(R^{-1}{}_{s} + R_{ct}{}^{-1})^{1-\alpha}$

R_s et Z_w représentent les propriétés globales de la solution d'électrolytes et les caractéristiques de diffusion de la sonde redox ainsi que les ions en solution.

Ces deux composantes ne sont pas affectées par des modifications chimiques se produisant à la surface de l'électrode. D'autre part, le R_{ct} et le CPE dépendent respectivement des caractéristiques diélectriques et isolantes à l'interface de l'électrode / électrolyte, d'où ils sont contrôlés par la modification de la surface d'électrode. Pour la mesure d'impédance faradique, le RCT est le paramètre le plus sensible et le plus simple qui peut être utilisé pour caractériser les évènements qui se produisent à l'interface entre la sonde immobilisée sur l'électrode et l'analyte dans la solution [245].

En outre, la variation du CPE est presque négligeable par rapport à la variation du R_{ct}. R_{ct} pour un électrode d'or a été estimée à 220 Ω et a diminué atteignant 90 Ω lorsque l'électrode a été modifiée par la cystéamine, les groupes périphériques aminés desquelles est produite une charge positive accélère le transfert d'électrons pour le $Fe(CN)_6^{3-/4-}$ redox.

Par la suite, le montage du bactériophage T4 sur l'électrode aminé après l'activation des groupements aminés avec l'agent de réticulation, a généré une couche isolante sur la surface d'électrode qui fonctionne comme une barrière au transfert d'électrons inter faciaux. Cela se traduit par l'augmentation de R_{ct} à 865 Ω, ce qui n'est absolument pas en accord avec les résultats rapportés par Yang *et al*. [246] Toutes ces données montrent que les modifications étape par étape, la cystéamine, le PDCIT et les T4 bactériophages, ont été assemblés avec succès sur la surface de l'électrode et forment une barrière cinétique accordable.

Les résultats obtenus à partir de mesures relatives à l'SIE concordent avec ceux extraits des voltamogrammes cycliques et confirment le succès de l`immobilisation des molécules T4 bactériophages sur la surface de l'électrode en or modifiée. La mesure

d'impédance peut être réalisée en présence ou en l'absence d'un couple d'oxydo-réduction, dit respectivement mesures d'impédence faradique et non-faradique [242].

Les mesures Faradiques et non faradiques sont des méthodes largement utilisées qui permettent la détection de cellules bactériennes en utilisant des anticorps immobilisés sur la surface de l'électrode [247, 248] utilisé. Les inconvénients majeurs liés à des anticorps comme un bio-récepteur sont: la faible efficacité de capture des biocapteurs [247], une réactivité croisée avec des cellules bactériennes non ciblées, l'instabilité due aux fluctuations de l'environnement, la production à coûts élevés et le temps d'analyse considéré comme assez long, ce qui peut limiter leur stockage à long terme et leur applicabilité sur le terrain [227, 228].

Par conséquent, ces questions doivent être abordées par le développement de méthodes d'impédance faradique avec l'immobilisation d'un nouveau bio récepteur sur les électrodes. Récemment, les bactériophages sont utilisés comme éléments de bio reconnaissance pour l'identification de différents microorganismes pathogènes. Afin de démontrer l'utilité pratique du biocapteur proposé, l'électrode modifié du bactériophage T4 a été exposé à différentes concentrations d'*E.coli* non pathogène B. Les biocapteurs bactériophage T4 ont été d'abord incubées pendant 15 minutes dans 50 µl de E. avec une concentration appropriée de 10 mM de PBS (pH=7,4), on les lave ensuite trois fois avec du PBS, puis on y place le biocapteur lavé dans la cellule électrochimique classique contenant 10 mM de solution Fe $(CN)_6^{3-/4-}$ (pH = 7,4) pour la mesure d'impédance.

L'intrigue de Nyquist correspondante à des spectres d'impédance est illustrée dans la figure 6.3A. Il semble que le diamètre du demi-cercle ait augmenté avec l'augmentation des concentrations d'*E.coli* B. La bactérie à Gram négatif telles que l'*E.coli* ont théoriquement une charge globale négative en raison des charges présentes dans leur membrane externe dans le lipopolysaccharide [249]. Ainsi, leur liaison aux bactériophages T4 peut agir comme une barrière pour le transfert d'électrons entre les espèces redox anioniques et la surface d'électrode. Pour compléter l'enquête d'impédance, les résultats obtenus ont été comparés avec les circuits équivalents modifiés proposés par Randles (encart de la Figure 6.2B).

Comme prévu, nous avons observé un changement important dans la résistance de transfert de charge, R_{ct}, tandis que les changements dans la R_s, CPE, et Z_w étaient très petits et irréguliers lors d'une augmentation de la concentration d'*E.coli*, soutenant l'idée d'utilisation du R_{ct} comme paramètre quantitatif pour évaluer la performance analytique du biocapteur T4 bactériophage développé. Afin de comparer les réponses relatives du biocapteur développé et supprimer la faible variation entre les électrodes, les valeurs de signaux ont été normalisés par rapport à la résistance de transfert de charge (Rct) du bactériophage T4 immobilisé.

La réponse normalisée, ΔRct (%) = [R_{ct} (bactéries de phages) -R_{ct} (phages)]/R_{ct} (phage), a été tracée en fonction de la concentration de *E. coli* sur une échelle logarithmique (Figure 6.3b) .

R_{ct} (bactéries phage) et R_{ct} (phage) étaient respectivement, la charge de transfert de résistance en présence et en l'absence de cellules d'*E. coli*. La variation de la réponse normalisée était linéaire avec le logarithme de concentration de *E.coli* de 10^3 à 10^9 CFU/ml et a une équation régression de ΔR_{ct} (en%) = 13 log (*E.coli*) - 30,6, avec un coefficient de corrélation de 0,998. La limite de détection (LOD) a été estimée à 8×10^2 CFU / ml en 15 minutes. Elle a été calculée en utilisant l'équation 3SDm, où « m » est la pente de la partie linéaire de la courbe d'étalonnage, et SD est l'écart type de la mesure à blanc.

Afin de vérifier la capacité du phage du biocapteur pour détecter des cellules d'*E.coli* dans une matrice biologique complexe, la performance du biocapteur a été évaluée avec du lait écrémé et de l'eau du lac auquel on y a ajouté des cellules d'*E.coli* B. D'autre part, le biocapteur bactériophage T4 présentait une gamme linéaire entre 10^3 à 10^8 CFU/ml, avec LOD de 10^3 CFU/ml. L'équation de régression linéaire était ΔR_{ct} (%) = 10 log [*E. coli* (CFU/ml)] - 21.4 avec un coefficient de corrélation de 0,993. Le biocapteur dans l'eau du lac a été saturé à 10^8 CFU/ml et n'a montré aucun changement significatif à plus forte concentration de *E.coli*.

La méthode impédimétriques sans étiquette décrit ici pour la détection et l'identification de cellules non pathogènes *E.coli* B représente une méthode prometteuse.

La gamme dynamique et le temps de cette approche de réponse sont respectivement beaucoup plus larges et extrêmement courtes, , que ceux des méthodes indiquées précédemment. Un temps de détection court est la demande la plus importante concernant les dosages de sources d'eau polluées et encore plus critique lorsque le bioterrorisme est suspecté. A titre de comparaison, Ruan *et al* [247] ont rapporté un immuno-capteur impédimétrique pour *E.coli* O157:H7 en utilisant la détection de peroxyde de raifort marquée par une enzyme pour l'amplification du signal.

La courbe d'étalonnage correspondante à leurs immuno capteurs était linéaire sur toute la gamme de 6×10^4 à 6×10^7 CFU/ml; la limite de détection était de 6×10^3 CFU/ml, et le temps global du test était de 90 min.

Cependant, ce procédé présente des inconvénients importants, tels que le coût élevé des réactifs participant à la réaction immunitaire et la nécessité d'anticorps marqués. Une autre approche a été rapportée par Geng *et al*. [225] pour la détection de *E.coli* en immobilisant les anticorps anti-*E.coli* sur les groupes carboxyle EDC/NHS activés, puis l'EIS a été utilisée pour suivre la liaison entre les anticorps et les bactéries spécifiques. Les auteurs ont démontré qu'il existe une relation linéaire entre la résistance de transfert de charge (R_{ct}) et le logarithme de la concentration de *E.coli* dans l'intervalle de 3×10^3 à 6×10^7 CFU/ml, et une concentration aussi faible que 10^3 CFU/ml peut être détectée de façon ambiguë dans les 60 minutes.

Plus récemment, l'utilisation de la lectine comme élément de bio reconnaissance a été prouvé comme un outil efficace pour la détection et l'identification des bactéries.

Gamella et al [250] ont rapporté des électrodes biotinylés à base de lectine-sérigraphiée pour la détection impédimétriques sans étiquette d'*E.coli*. Le biocapteur proposé est capable de détecter les *E.coli* de 5×10^3 à 5×10^7 cfu / ml avec une limite de détection de 5×10^3 CFU/ml dans les 60 minutes. Une nouvelle tentative pour la détection d'*E.coli* basée sur les propriétés de reconnaissance de l'hydrate de carbone a été rapportée récemment par Guo *et al*. [251]

La couche de détection a été construite en utilisant un mélange d`auto-assemblage d'une longue chaîne, thiol à terminaison α mannoside, thiol à terminaison de molécule polyéthylène glycol dit :"molécule espaceuse" - sur une surface d'électrode en or

propre. Ensuite, l'interaction entre l'α-mannoside et *E.coli* a été contrôlée par l'EIS. Les auteurs montrent que la courbe d'étalonnage présente une relation linéaire entre la résistance de transfert de charge (R_{ct}) et la concentration logarithmique de la bactérie *E.coli* dans la plage de 10^2 à 10^3 CFU/ml. Cependant, la limite de détection et l'heure de la détection n'ont pas été déterminées dans leurs travaux.

La détection en temps réel de bactéries vivantes en utilisant un instrument portable reste un défi pour de nombreux scientifiques et ingénieurs, car la présence de bactéries mortes n`est pas considérée comme risquée pour la santé.

La réponse impédimétriques pour les cellules B de *E.coli* vivantes et mortes a été testée après l`incubation de 108 CFU/ml d'un mélange de cellules vivantes et mortes sur l'électrode de bactériophage T4 modifiées pendant 15 minutes, suivie d'un lavage avec du PBS.

Les biocapteurs ont été transférés dans la cellule électrochimique, et l'impédance faradique a été mesurée consécutivement pendant plus de 1 heure pour déterminer la viabilité de la bactérie. La Figure 6.5A représente le changement de réponse normalisée en fonction du temps pour les deux cellules. La réponse normalisée des biocapteurs avec une cellule vivante a été augmentée de 82% et a atteint un état stable dans les 15 minutes, indiquant la liaison des cellules vivantes sur le bactériophage T4.

Cette réponse a ensuite commencé à diminuer avec le temps, ce qui confirme l'infection et la lyse des cellules bactériennes vivantes par le bactériophage T4 immobilisé. En revanche, une légère augmentation de 8,6% a été observée dans le cas des biocapteurs avec des cellules mortes, comme on peut le voir à partir de la courbe (b) sur la Figure 6.5A, révélant que le bactériophage T4 ne peut pas différencier les cellules mortes d*'E. coli* B de celles vivantes. Ces résultats sont en bon accord avec les données présentées antérieurement, où l'auteur a montré que le bactériophage T4 ne peut pas se fixer aux cellules de l'acétone-éther traité parce que l'adsorption a été inhibée par l'hydrolyse lipopolysaccharides [252, 253].

En outre, il a été signalé depuis longtemps que la constante de vitesse de bactériophage d'adsorption de bactéries s`avère changer entre de larges limites, en fonction de l'état physiologique de la bactérie [254]. Récemment, il a été constaté que le

taux d'adsorption dans des cellules hôtes vivantes a été beaucoup plus grand que celle dans des cellules hôtes mortes [255]. Dans notre cas, la faible variation de la réponse normalisée peut être dû à un court temps d'incubation de 15 minutes et / ou à la dénaturation des récepteurs d'attachement de bactériophage sur la surface cellulaire des bactéries. Ceci confirme bien que notre biocapteur de bactériophage T4 développé peut être utilisé pour une excellente discrimination contre les cellules mortes d'*E.coli*. En outre, plusieurs expériences ont été réalisées pour différents rapports de cellules vivantes / mortes (en vie: morts, 100: 0, 80:20, 50:50, 30:70 et 0: 100); la concentration finale était de 10^8 CFU/ml des cellules bactériennes.

Comme le montre la figure 6.5B, il existe une petite variation dans la réponse normalisée (ΔR_{ct} (%)) entre le 100% de cellules vivantes et les autres concentrations mixtes (80%, 50% et 30%). Ce résultat est attendu étant donné que les différents rapports de cellules mortes / vivantes présentent une concentration des cellules vivantes (plus de 3 x 10^7 CFU/ml). La concentration des bactéries autorisées par le règlement du « Environmental Protection Agency » dans l'eau potable traitée ne doit contenir aucunes (zéro) bactérie dans 100 ml [256]. Par conséquent, un biocapteur doit être en mesure de fournir un LOD aussi bas qu'une seule bactérie dans 100 ml d'eau potable [257].

À cet égard, la combinaison de la méthode de la LAMP et la libération des substances intrinsèques suivants la lyse de la cellule par le bactériophage spécifique peuvent fournir une confirmation de la technologie puissante pour la détection hautement spécifique et sensible de *E.coli* permettant au LOD d'être poussé vers le bas. Pour mettre en évidence l'amplification du signal de la méthode de LAMP, la liaison de *E. coli* B à l'électrode de T4 bactériophage modifié a été retirée de la cellule électrochimique après avoir terminé les essais d'impédance, suivie d'un lavage avec du PBS et de l'eau, puis on l'a incubé dans 200 pi du mélange maître de LAMP à 65°C pendant 40 minutes avant d'être soumis à une analyse de LSV; le protocole de réaction LAMP est décrit dans l'annexe IV.

Le temps a déjà été optimisé lors de notre précédente expérience, car il joue un rôle majeur dans la quantification des bactéries [206]. Après cela, le signal électrochimique

(LSV) de l'agent d'intercalation Hoechst a été mesuré dans un mélange contenant 6 µl de produit d'amplification, 12 µl de Hoechst redox, et 12 µl de PBS (10 mM) et le tampon a été ensuite tracé en fonction de la concentration de *E.coli*.

Comme représenté sur la Figure 6.6A, une diminution progressive de la pointe d'oxydo-réduction du Hoechst a été clairement observée avec l'augmentation des concentrations d'*E.coli*. Cela peut être attribué à l'intercalation des molécules Hoechst dans les produits à réaction LAMP double brin [257].

D'après la Figure 6.6B, on peut voir que la réponse normalisée [ΔI (%) = (I_0 – I (LAMP))/I_0, où I_0 et I_{LAMP} sont respectivement la pointe courante à 0,5 V par rapport à Ag / AgCl dans l'absence et la présence de produit de LAMP, à une relation linéaire avec l'échelle logarithmique de la concentration de *E.coli* dans la plage de 102 à 107 cfu / ml et a une équation de régression ΔI (%) = 4 log (*E.coli*) + 7.36 avec un coefficient de corrélation de 0,998. L'LOD était de 100 CFU/ml et le temps total de dosage est moins 45 minutes. Avec cette stratégie, le LOD a été renforcé par au moins 1 ordre de grandeur.