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**DEVELOPMENT OF ELECTROANALYTICAL METHODS
FOR STUDYING MICROBES**

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SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES

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**DEVELOPMENT OF ELECTROANALYTICAL METHODS
FOR STUDYING OF MICROBES**

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School of Physical and Mathematical Sciences

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Summary

This thesis explores the utility of electrochemical biosensors for studying and detecting microbes. It consists of five chapters: a mini-review and four independent experimental works. Chapter 1 reviews the current state of novel biosensing techniques for ultrasensitive detection of microbes with highly promising application for disease diagnosis. Because of their remarkable specificity, sensitivity and response time, biosensors often present potential and powerful tools for quantitative and accurate detection of microbes. Recent advancements in nanotechnology, transduction system and genetic engineering provide various strategies to improve the detection performance of biosensors. In this chapter, I summarize concise and comprehensive lists of the recent bacteria and virus biosensors, and their analytical performance. In addition, I also include a brief summary of state-of-the-art molecular technologies for the detection of microbes.

In Chapter 2, a sensitive and specific electrochemical membrane-based nanobiosensor is reported for quantitative and label-free detection of *Escherichia coli* cells and analysis of the viable but nonculturable state of *E. coli* cells that remain mostly undetected using conventional methods. The sensing mechanism depends on the blocking of nanochannels of an alumina-modified platinum wire electrode coated with a layer of anti-*E. coli* polyclonal antibody (isotype: IgG), upon the formation of immunocomplexes at the nanoporous alumina membrane. The resulting obstacle to diffusive mass transfer of a neutral redox probe, ferrocenemethanol, toward the underlying platinum electrode decreases the Faradaic current response of the nanobiosensor, measured using cyclic voltammetry. Experimental parameters including loading amount of antibody and pH are

optimized. The membrane-based nanobiosensor gives a low limit of detection of 22 cfu mL⁻¹ over a wide linear working range from 10 to 10⁶ cfu mL⁻¹ ($R^2 = 0.999$). The nanobiosensor is specific toward *E. coli* with negligible cross reactivity to two other Gram-negative bacteria, *Serratia marcescens* and *Salmonella typhimurium*. Relative standard deviation for triplicate analysis of 2.5 % indicates good reproducibility. Differentiation of live, viable-but-non-culturable and dead *E. coli* cells are performed by monitoring of the bacterial enzyme catalytic activity using ferrocenemethanol as the alternative electron acceptor to oxygen, in the presence of glucose.

In Chapter 3, the same design of membrane-based nanobiosensor is employed for ultrasensitive detection of dengue type 2 virus (DENV-2). Anti-DENV-2 monoclonal antibody (clone 3H5, isotype IgG) is used as the biorecognition probe in this work. The stepwise construction of nanobiosensor and detection of DENV-2 are characterized using differential pulse voltammetry. A low limit of detection of 1 pfu mL⁻¹ with linear working range from 1 to 10³ pfu mL⁻¹ ($R^2 = 0.976$) can be achieved by the nanobiosensor. The nanobiosensor is specific toward DENV-2 with minimal cross reaction with other non-specific viruses such as Chikungunya virus, West Nile virus, and dengue type 3 virus. Relative standard deviation for triplicate analysis of 5.9 % reveals reasonably useful level of reproducibility. Additionally, I demonstrate the direct quantification of DENV-2 load in whole mosquito vector, *Aedes aegypti* using the nanobiosensor.

Chapter 4 reports the real-time monitoring of filamentous bacteriophage M13 infection of *E. coli* cells using an impedimetric microbial biosensor constructed from a gold electrode covalently coated with a self-assembled monolayer of anti-*E. coli* polyclonal antibody. After phage infection, damage to the lipopolysaccharide layer on the

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In Chapter 5, an impedimetric cell-based biosensor is fabricated from a poly-L-lysine-modified screen-printed carbon electrode to monitor the real-time DENV-2 infection of surface-immobilized baby hamster kidney fibroblast cells. Based on the described platform, DENV-2 induced cytopathic or cytopathogenic effects (degenerative morphological change, detachment, membrane degradation and death of host cells), which are indicated by a drastic decrease in impedance signal response, can be detected within ~ 35 hours post infection. A parameter that describes the kinetics of cytopathogenesis, CIT₅₀ (time taken for 50 % decrease in impedance signal response) reveals an inverse linear relationship to the logarithm of virus titer. It is also reported that CIT₅₀ values are delayed by 31.5 h for each order of magnitude decrease in multiplicity of infection. Therefore, the virus titer of given samples can be determined based on the measurement of impedance signal response and analysis of CIT₅₀.

Finally, the concluding remarks of these experimental works and the future outlook for biosensing techniques are included in the conclusion section.

Keywords: Electroanalytical method, electrochemistry, voltammetry, impedance, biosensor, antibody, virus, bacterium.

List of International Refereed Publications

1. Cheng, M. S.; Toh, C. S., Novel biosensing methodologies for ultrasensitive detection of viruses. *Analyst* **2013**, 138, (21), 6219-6229.
2. Cheng, M. S.; Ho, J. S.; Lau, S. H.; Chow, V. T. K.; Toh, C. S, Impedimetric microbial sensor for real-time monitoring of phage infection of *Escherichia coli*. *Biosens. Bioelectron.* **2013**, 47, 340-344.
3. Cheng, M. S.; Ho, J. S.; Tan, C. H.; Wong, J. P. S.; Ng, L. C.; Toh, C. S., Development of an electrochemical membrane-based nanobiosensor for ultrasensitive detection of dengue virus. *Anal. Chim. Acta* **2012**, 725, 74-80.
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5. Zhuo, L.; Huang, Y.; Cheng, M. S.; Lee, H. K.; Toh, C. S., Nanoarray membrane sensor based on a multilayer design for sensing of water pollutants. *Anal. Chem.* **2010**, 82, (11), 4329-4332.

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5. Cheng, M. S.; Ho, J. S.; Toh, C. S., Electrochemical biosensor for ultrasensitive detection of dengue virus and real-time monitoring of viral infection, *Singapore International Conference on Dengue and Emerging Infections*, 21-23 November 2012, Singapore.
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9. Cheng, M. S.; Zhuo, L.; Toh, C. S., Development of membrane-based electrochemical immunosensor for *Escherichia coli* cell detection, *Biosensors 2010 - 20th Anniversary World Congress on Biosensors*, 26-28 May 2010, Glasgow, United Kingdom.

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List of Abbreviations

Abbreviation	Meaning
<i>A. aegypti</i>	<i>Aedes aegypti</i>
Ab	Antibody
AC	Alternating current
BHK	Baby Hamster Kidney
BSA	Bovine serum albumin
cfu	Colony-forming units
CV	Cyclic Voltammetry
CIT ₅₀	Time taken for 50 % decrease in cell impedance
CPE	Constant phase element
CHIKV	Chikungunya virus
DNA	deoxyribonucleic acid
DENV	Dengue virus
DPV	Differential pulse voltammetry
<i>E. coli</i>	<i>Escherichia coli</i>
EDC	N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide
EIS	Electrochemical impedance spectroscopy
ELISA	Enzyme-linked immunosorbent assay
FcMeOH	Ferrocenemethanol
Fe(CN) ₆ ^{3-/4-}	Ferri/ferrocyanide redox couple

GDH	Glucose dehydrogenase
hpi	hours post infection
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LOD	Limit of detection
LPS	Lipopolysaccharide
MACA	Mercaptoacetic acid
MOI	Multiplicity of infection
NHS	N-hydroxysuccinimide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pfu	Plaque-forming units
Phage	Bacteriophage
PLL	Poly-L-Lysine
PNA	Peptide nucleic acid
RNA	Ribonucleic acid
RSD	Relative standard deviation
RT-PCR	Reverse transcriptase-polymerase chain reaction
SAM	Self-assembled monolayer
<i>S. marcescens</i>	<i>Serratia marcescens</i>
<i>S. typhimurium</i>	<i>Salmonella typhimurium</i>
SPE	Screen-printed electrode
SPCE	Screen-printed carbon electrode

VBNC

Viable but nonculturable

WNV

West Nile virus

List of Symbols

Symbol	Meaning	Unit (s)
A	Area of electrode	cm^2
A_w	Warburg coefficient	$\Omega \text{ s}^{-1}$
C	Concentration	mol dm^{-3}
D	Diffusion coefficient	$\text{cm}^2 \text{ s}^{-1}$
e	Base of natural logarithm	None
E	Potential versus a reference electrode	V
E_0	Potential amplitude in EIS	V
E_i	Initial potential	V
E_p	Peak potential	V
E_{pa}	Anodic peak potential in CV	V
E_{pc}	Cathodic peak potential in CV	V
E_t	Potential at time t	V
E_λ	Switching potential in CV	V
$E^{o'}$	Formal reduction potential	V
ΔE	Pulse amplitude in DPV	V
ΔE_p	$E_{pa} - E_{pc}$ in CV	V
F	Faraday constant	C mol^{-1}
f	Frequency	Hz
I	Current	A

I_0	(I) Current amplitude in EIS (Chapter 1)	A
	(II) Exchange current (Chapter 4)	A
I_{membrane}	Biosensor response in the absence of antibody	A
I_{cell}	Biosensor response in the presence of bacterium	A
$I_{\text{cell}=0}$	Biosensor response in the absence of bacterium	A
I_{virus}	Biosensor response in the presence of virus	A
$I_{\text{virus}=0}$	Biosensor response in the absence of virus	A
I_c	Charging current	A
I_f	Faradaic current	A
I_p	Peak current	A
I_{pa}	Anodic peak current in CV	A
I_{pc}	Cathodic peak current in CV	A
I_t	Current at time t	A
δI	Differential current in DPV	A
j	Imaginary number	None
K	Equilibrium dissociation constant	mol dm^{-3}
K_M	Michaelis-Menten constant	mol dm^{-3}
k	Heterogeneous electron transfer rate constant	cm s^{-1}
k'	Proportionality constant	s^{-1}
n	(I) Electron stoichiometry (Chapter 1)	None
	(II) Freundlich constant (Chapter 3)	None
	(III) Frequency independent exponent (Chapter 4)	None
R	(I) Resistance	Ω

	(II) Universal gas constant	$\text{J K}^{-1} \text{mol}^{-1}$
R_{et}	Electron-transfer resistance	Ω
R_{s}	Solution resistance	Ω
Q	Admittance	$\Omega^{-1} \text{s}^n$
T	Temperature	K
t	Time	s, min, h
ν	Scan rate	V s^{-1}
ω	Radial frequency	Radian s^{-1}
Z	Impedance	Ω
Z'	Imaginary impedance	Ω
Z''	Real impedance	Ω
Z_0	Magnitude of impedance	Ω
Z_{virus}	Impedance response in the presence of virus	Ω
$Z_{\text{virus}=0}$	Impedance response in the absence of virus	Ω
Z_{w}	Warburg impedance	Ω
θ	Surface coverage	None
θ_{max}	Maximum surface coverage	None
τ'	Start of potential pulse in DPV	V
τ	End of potential pulse in DPV	V
ϕ	Phase shift	$^{\circ}$

Introduction

1.1 Introduction

In recent decades, there have been increasing cases of emerging and re-emerging infectious diseases caused by microbes including bacteria, viruses, fungi, protozoa, and parasites. These microbes are capable of fast spreading and therefore possess on-going threats to global biosecurity. The complex interplay between different factors such as global movement of humans and animals, ecological and climatic changes, and demographic shifts contribute to the outbreak of numerous infectious diseases [1]. According to the World Health Organization (WHO), there are few billion cases of infection diseases and approximately ten million deaths caused by infectious diseases every year [2]. Thus, facing the challenges and threatening consequences brought about by the spread of infectious diseases, high throughput microbe detection and surveillance to achieve effective disease control have become the major concerns of public communities [3].

At present, biosensing techniques have been developed into rapid, sensitive and specific detection tools for a wide spectrum of microbes. Because of the advantages such as rapid analysis, inexpensive instrumentation, minimal or no sample pre-treatment and easy operation, biosensors are envisaged as a competent technique to modernize microbe detection methods [4]. Moreover, recent advancement in transduction system,

nanotechnology, biotechnology and genetic engineering offer various approaches to improve the detection performance of biosensors. This chapter presents an overview of the current states of novel electrochemical biosensing techniques for ultrasensitive detection of bacteria and viruses. Additionally, a concise summary of the state-of-the-art molecular technologies with regards to microbe detection is included.

1.2 State-of-the-art molecular technologies

Conventional microbe detection methods including immunofluorescence microscopy, enzyme immunoassays and conventional qualitative polymerase chain reaction (PCR) techniques are becoming obsolete for routine clinical practices. Enzyme immunoassays such as enzyme-linked immunosorbent assay (ELISA) [5], immunochromatographic ELISA [6], and enzyme-linked immunospotting (ELISPOT) [7] have been broadly used for multiplex analysis of microbiological samples. However, the laboratory application of these methods are limited by time-consuming analysis, false negative results and the need to obtain multiple serum samples from patients during different phases of infection for serological tests.

Current laboratory microbe detection tests rely exclusively on real-time PCR (RT-PCR) and multiplex PCR techniques. RT-PCR assays generally involve RNA extraction, reverse transcription, and polymerase chain reaction. The advantages of RT-PCR assays include high sensitivity and reduced chances of generating false positive results. However, the laboratory usage of RT-PCR is limited due to the fact that only a single microbe or gene can be detected in a single test. As a consequence, multiplex PCR assays emerged as a more powerful tool that is capable of simultaneous amplifying nucleic acids and

detecting multiple agents in a single specimen. These assays use multiple primers with each set of primer designed in such a way that an amplicon with specific size is produced for a target microbe. Recently, multiplex PCR has been integrated with RT-PCR for simultaneous detection of multiple specific-sized amplicons as they are produced in real-time [8, 9].

In addition, a new generation of isothermal nucleic acid amplification techniques such as insulated isothermal PCR, loop-mediated isothermal amplification, helicase-dependent amplification and nucleic acid sequence-based amplification techniques are gaining popularity recently as alternative molecular assays. These techniques involve rapid reaction, easy detection, and simple operation. Moreover, expensive instrumentation and the need of temperature cycling can be eliminated [10, 11]. Besides, different multiplex technologies have emerged, some of which allow rapid detection of multiple microbes in a single test. For example, ResPlex technology, which incorporates PCR for the amplification and purification of nucleic acids followed by subsequent detection using a liquid-phase bead-based array technique [12]. Other relevant integrated molecular systems include film array, Infiniti, Jaguar, scalable target analysis routine (STAR) and PLEX-ID technologies (See Ref. [12] for the detailed description and operation of each of these technologies).

1.3 Biosensors

1.3.1 Biorecognition probes and microbial targets

Biosensors are analytical tools that incorporate the sensitivity of analytical techniques and the inherent selectivity of biorecognition probes (the term “biorecognition probe” is

used interchangeably with “bioaffinity probes”). The interaction between biorecognition probes and microbial targets generates measurable signals that are proportional to the concentration of targets. Based on different types of biorecognition probes and microbial targets, biosensors employing bioaffinity interaction can be classified into five main categories namely antibody- (or immuno-), DNA-, antigen-, bioreceptor-, and cell-based biosensors (Figure 1.1). Bioaffinity interaction based on different types of bioaffinity probes and their relevant microbial targets is shown in Figure 1.2.

Figure 1.1 Diagrams of the biosensors based on different types of bioaffinity probes and the relevant microbial targets. (A) antibody- (or immuno-), (B) DNA-, (C) antigen-, (D) bioreceptor-, and (E) cell-based biosensors.

Figure 1.2 List of bioaffinity probes and their corresponding microbial targets.

In general, the analytical performance of biosensors is evaluated based on the specificity of biorecognition probes, sensitivity in terms of limit of detection (LOD), dynamic or linear working range, response time and reproducibility of biosensors. Comprehensive list of the recent virus and bacteria biosensors with reported analytical performance in terms of LOD and linear working range are summarized in Tables 1.1 and 1.2, respectively.

Figure 1.3 Structure of an immunoglobulin G (IgG) antibody. C_H : constant regions of heavy chains, V_H : variable regions of heavy chains, C_L : constant regions of light chains, and V_L : variable regions of light chains, S-S: disulfide bridge.

1.3.1.1 Immunosensors

Immunosensor or antibody-based biosensor is a subclass of affinity biosensor that relies on the specific interaction between antigens and antibodies to produce quantifiable signal response. Antibodies are usually produced in humans and animals (mouse/rat, rabbit, chicken, sheep/goat are the common host animals) by injecting small sub-lethal dose of antigens into host's bodies to trigger their immune response that produces specific antibodies against the invading antigens. Antibody belongs to the family of globular protein known as immunoglobulin. As shown in Figure 1.3, an immunoglobulin G (IgG) antibody is a Y-shaped molecule composed of four polypeptide chains: two heavy, H chains (~ 100 kDa) and two light, L chains (~ 50 kDa). Amino terminal ends of an antibody molecule with substantial variability in amino acid sequence are called variable,

V region. Whereas the amino acid sequence is identical at the caboxyl terminal ends and are called the constant, C regions. The two identical antigen binding sites at the amino terminal ends of both arms allow two antigenic determinants to be recognized by a single antibody molecule [13]. Immunosensing techniques had been widely employed in clinical application since 1970s to identify antigenic components and diagnose diseases. Over recent decades, successful isolation of specific antibodies for a wide variety of microbes has quickened the development of immunosensors.

Aptamers are single-stranded oligonucleic acids (ssDNA and RNA) or peptides (~ 40-60 bases in length) that recognize target antigens with excellent selectivity. Aptamers are normally selected from a pool of completely random oligonucleic acids using Cell-SELEX (systematic evolution of ligands by exponential enrichment) technique [14]. DNA aptamers typically adopt a three dimensionally preferred orientation that distinguishes antigens based on subtle structural difference [15]. On the other hand, peptide aptamers are made up of proteins that have been engineered to imitate the function of antibody. Peptide aptamers have its specific antigen binding sites located at the surface or loop of peptide [16].

1.3.1.2 DNA-based biosensors

Based on the principle of nucleic acid hybridization, DNA-based biosensors operate by relying on target-probe DNA base pairing. Construction of these biosensors involves the immobilization of short single stranded DNAs (ssDNAs, approximately 20-40 base pairs) on the sensor surface. Reactivity, accessibility, and stability of probe DNA are retained in the sensor construction process. Current efforts in DNA-based biosensing application

emphasize the use of peptide nucleic acids (PNAs) as the bioaffinity probe. PNA, first reported by Nielson *et al.* in 1991, is a structural mimic of DNA in which the sugar phosphate backbone is replaced by the pseudopeptide backbone comprising *N*-(-2-aminoethyl) glycine units linked by amide bonds [17]. PNAs demonstrate superior stability, and permit rapid, selective and strong nucleic acid hybridization, thus making them a promising tool in DNA detection [18, 19].

1.3.1.3 Antigen-based biosensors

Surface antigenic proteins derived from bacteria (capsular, flagellar and lipopolysaccharide antigens) or viruses (capsid, envelope, nucleoprotein, glycoprotein and nucleocapsid antigens) have been widely utilized as the bioaffinity probe [20-22]. Antigen-based biosensors generally involve the detection of microbe-specific antibodies obtained from the sera of infected patients. However, similar to that of the conventional serological tests and enzyme immunoassays, the utility of antigen-based biosensors are restricted by the amount of antibodies produced during different phases of infection.

1.3.1.4 Bioreceptor-based biosensors

Natural bioreceptors such as bacteriophages (phages) are typically utilized for detecting living microbes. Phages are viruses that recognize and infect bacteria with high selectivity, and capable of differentiating live and dead bacteria [23]. Furthermore, the production of phages can be conducted in a rapid, easy and cost-effective way, and does not depend on the animal host, thus making phages a promising alternative to antibody. The detection principle involves the initial binding of phages onto bacterial cell surface

receptors followed by the translocation of phage DNA. The eventual bacterial cell lysis releases a large number of phages and phage components into the surrounding environment [24].

1.3.1.5 Cell-based biosensors

These biosensors are usually employed as common alternatives to conventional animal testing for studying infectious diseases and investigating the efficiency of particular drugs and treatments. Principally, the detailed profile of cell adhesion in response to infection is generated using sensors coated with a monolayer of pre- or post-infected cells. Infection induces degenerative morphological changes, membrane degradation, detachment, and death of cells, which are collectively known as the cytopathic or cytopathogenic effects [25]. These effects are reflected quantitatively in the detected signals such as changes in the conductance, resistance, and reactance of cell-coated electrodes [26], thus allowing infection to be detected and monitored in a real-time fashion. This biosensing assay has an advantage over molecular techniques that is the capability of differentiating infectious microbes from non-infectious microbes.

1.3.2 Strategies for labeling and immobilization of biorecognition probes

Labeling or tagging of biorecognition probes with enzyme conjugates, nanomaterials, radioactive isotopes, fluorescence tags or redox probes is widely employed for amplification of signals or production of detectable signals in biological assays. Although labeled detection systems have shown remarkable sensitivity and popularity in biosensing application, these systems possess some limitations such as altered binding affinity of

Table 1.1 List of the recent electrochemical virus biosensing techniques and comparison of the analytical performance in terms of limit of detection (LOD) and linear working range.

Viral target	Probe	Approach	Transducer (s)	LOD	Linear range	Ref.
AIV, cDNA	DNA	Aptamer/hybrid nanomaterial-GE	Voltammetry	0.43 pM	5 pM-1 nM	[27]
AIV, cDNA	DNA	SAM thiolated DNA-GE	Voltammetry	24 pM	10-60 pM	[28]
DENV, NS1	mAb, IgG	Fuel cell PB nanotube membrane electrode	Amperometry	0.04 pfu mL ⁻¹	3-45 pfu mL ⁻¹	[29]
DENV, NS1	mAb, IgG	Alumina membrane electrode	Voltammetry	1 pfu mL ⁻¹	1-10 ³ pfu mL ⁻¹	[30]
DENV, NS1	mAb, IgG	Alumina membrane electrode	EIS	1 pfu mL ⁻¹	1-900 pfu mL ⁻¹	[31]
DENV, NS1	mAb	Protein A coated gold CD trode	Voltammetry	0.33 ng mL ⁻¹	1-100 ng mL ⁻¹	[32]
DENV, cDNA	DNA	Alumina membrane electrode	Voltammetry	9.55 pM	1 pM-1 μM	[33]
DENV, cDNA	DNA	Pencil graphite electrode	Voltammetry	0.92 nM	1-40 nM	[34]
EBV, LMP-1	Ab	Quantum dot/silica nanoparticle label-sandwich immunoassay	Voltammetry	1 pg mL ⁻¹	1 pg mL ⁻¹ -10 ng mL ⁻¹	[35]
HV, antigens	mAb	Protein A/nanogold-GE	Voltammetry	≤ 1 ng mL ⁻¹	~ 1-350 ng mL ⁻¹	[36]
HBV, IgG	Envelope protein	AuNP based magneto sandwich immunoassay	Chronoamperometry	3 mIU mL ⁻¹	5-69.2 mIU mL ⁻¹	[20]
HBV, surface antigen	ScAb	GBP/ScAb fusion protein-GE	EIS	0.14 ng mL ⁻¹	1 ng mL ⁻¹ -50 μg mL ⁻¹	[37]
HBV, cDNA	DNA	Multiprobe modified DNA/AuNP aggregate	Voltammetry	5 aM	10aM-100 fM	[38]
HBV, cDNA	DNA	PICAM/MWCNT composite-GCE	Voltammetry	2 fM	10 fM-1 pM	[39]
HBV, cDNA	DNA	SWCNT/AuNP array	EIS	1 aM	1 aM-1 μM	[40]
HCV, core antigen	Ab	Nanocomposite-GCE	Voltammetry	0.17 ng mL ⁻¹	2-512 ng mL ⁻¹	[41]
HCV, cDNA	DNA	AuNP coupled hairpin DNA	Voltammetry	~ 1 pM	0.01-2 nM	[42]
HCV, cDNA	DNA	BamHI/thionine/HRP/gold hollow sphere-GCE	Voltammetry	1 pM	0.01 nM-8 μM	[43]
HCV, cDNA	DNA	Thionine/AuNP/DNA-GCE	Voltammetry	0.31 zM	~ 1 zM-0.1 nM	[44]
HCV, cDNA	PNA	SAM thiolated PNA-GE	Voltammetry	0.57 pM	1-50 nM	[18]
HCV, cDNA	PNA	SAM thiolated PNA-GE	Voltammetry	1.8 pM	10-100 nM	[19]
HCV, RNA	PNA	TADC SAM PNA-GE	EIS	23 pM	< 10-500 nM	[45]

HCV, protease	Peptide	Ferrocene conjugated peptide-GE	EIS	5 pM	10-100 pM	[46]
HIV, capsid protein	pAb	AuNP/MWCNT/AEP film-GE	Voltammetry	6.4 pg mL ⁻¹	0.01-60 ng mL ⁻¹	[47]
HIV, capsid protein	mAb	AuNP-electroplated GCE	Voltammetry	8 pg mL ⁻¹	0.01-100 ng mL ⁻¹	[22]
HIV, cDNA	DNA	AgNP/CNT-gold microelectrode	Voltammetry	0.5 pM	1-100 pM	[48]
HIV, cDNA	DNA	Long range SAM DNA nanostructures	Voltammetry	2 aM	2 aM-10 pM	[49]
HIV, cDNA	DNA	Paramagnetic microparticle/CNT-SPCE	Voltammetry	20 ng mL ⁻¹	40-1250 ng mL ⁻¹	[50]
HIV, cDNA	DNA	Chitosan/Fe ₃ O ₄ nanoparticle-SPCE	Voltammetry, EIS	50 pM	50-300 pM	[51]
HIV, cDNA	DNA	PDI/graphene-GCE	EIS	0.55 pM	1 pM-1 μM	[52]
HIV, RT	Peptide	Ferrocene labeled lipoic acid/AuNP-SPCE	Voltammetry, EIS	0.8 pg mL ⁻¹	1-500 pg mL ⁻¹	[53]
HPV, capsid protein	Peptide aptamer	Polyaniline/MWCNT-platinum IDA	Voltammetry	0.49 nM	10-50 nM	[16]
HPV, cDNA	DNA	Thiolated DNA/bipodal thiol-GE array	Amperometry	0.17 nM	0.1-12 nM	[54]
HPV, cDNA	DNA	SWCNT/AuNP array	EIS	1 aM	1 aM- 1 pM	[40]
IAV, whole virus	pAb	Biotinylated Ab/thiol/ neutravidin-GE	EIS	8 ng mL ⁻¹	0-64 ng mL ⁻¹	[55]
IAV, cDNA	DNA	Biotinylated DNA/avidin-GCE	Voltammetry	85.1 fM	0.1 nM-0.1 pM	[56]
IAV, cDNA	DNA	Paramagnetic microparticle/CNT-SPCE	Voltammetry	12 ng mL ⁻¹	40-1250 ng mL ⁻¹	[50]
JEV, envelope protein	mAb, IgG	MWCNT/magnetic bead/HRP-magnetic GE	Voltammetry	2×10 ³ pfu mL ⁻¹	2×10 ³ -5×10 ⁵ pfu mL ⁻¹	[57]
JEV, antigen	Serum Ab	Protein A/glutaraldehyde-silanized interdigitated electrode	EIS	0.75 μg mL ⁻¹	1-10 μg mL ⁻¹	[58]
VACV, whole virus	DNA aptamer	SAM thiolated primer/DNA aptamer/AuNP-SPCE	Voltammetry	2×10 ³ pfu mL ⁻¹	5×10 ³ -3×10 ⁴ pfu mL ⁻¹	[59]
VSV, whole virus	DNA aptamer	DNA aptamer mediated immunoshielding of virus	EIS	10 ⁴ pfu mL ⁻¹	5×10 ⁴ -5×10 ⁶ pfu mL ⁻¹	[60]

Abbreviations: Ab: antibody, AEP: acetone-extracted propolis, AgNP: silver nanoparticle, AIV: avian influenza virus, AuNP: gold nanoparticle, CD: compact disc, cDNA: complementary deoxyribonucleic acid, CNT: carbon nanotube, DENV: dengue virus, DNA: deoxyribonucleic acid, EBV: Epstein-Barr virus, EIS: electrochemical impedance spectroscopy, GCE: glassy carbon electrode, GE: gold electrode, HIV: human immunodeficiency virus, HBV: hepatitis B virus, HCV: hepatitis C virus, HPV: human papillomavirus, HRP: horseradish peroxidase, HV: hepatitis virus, IAV: influenza A virus, IDA: interdigitated electrode array, IgG: immunoglobulin G, IU: international units, JEV: Japanese encephalitis virus, LMP: latent membrane protein, mAb: monoclonal antibody, MWCNT: multi-walled carbon nanotube, NS: non-structural protein, pAb: polyclonal antibody, PB: Prussian blue, PDI: *N,N*-bis-(1-aminopropyl-3-propylimidazol salt)-3,4,9,10-perylene tetracarboxylic acid diimide, PICA: poly(indole-6-carboxylic acid), pfu: plaque-forming unit, PNA: peptide nucleic acid, RT: reverse transcriptase, ScAb: single chain antibody, SAM: self-assembled monolayer, SPCE: screen-printed carbon electrode, SWCNT: single-walled carbon nanotube, TADC: 3-thiophene-acetamide-diaza-18-crown-6, VACV: vaccinia virus, VSV: vesicular stomatitis virus.

Table 1.2 List of the recent electrochemical bacteria biosensing techniques and comparison of the analytical performance in terms of limit of detection (LOD) and linear working range.

Bacterial target	Probe	Approach	Transducer	LOD	Linear range	Ref.
<i>Bacillus anthracis</i> , cDNA	DNA	EAM nanoparticle/sandwich hybridization-SPCE	Voltammetry	10 ng mL ⁻¹	~10-10 ³ ng mL ⁻¹	[61]
<i>Bacillus cereus</i> , antigen	mAb, IgG	Double layer AuNP/chitosan-GCE	Amperometry	10 cfu mL ⁻¹	50-5×10 ⁴ cfu mL ⁻¹	[62]
<i>Bacillus licheniformis</i> , protease	Peptide	SAM biotinylated peptide/streptavidin-GE	Voltammetry	0.16 µg mL ⁻¹	0.7-10 µg mL ⁻¹	[63]
<i>Bacillus thuringiensis</i> , cDNA	DNA	Biotinylated AgNP/thiolated DNA/streptavidin-GE	Voltammetry	10 fM	1 pM-1 µM	[64]
<i>Escherichia coli</i> , antigen	pAb, IgG	Alumina membrane electrode	Voltammetry	22 cfu mL ⁻¹	10-10 ⁶ cfu mL ⁻¹	[65]
<i>Escherichia coli</i> , antigen	pAb	Graphene-AgNP composite/SAM AuNP-GE	Voltammetry	10 cfu mL ⁻¹	50-10 ⁶ cfu mL ⁻¹	[66]
<i>Escherichia coli</i> , antigen	pAb	HRP/magnetic particle/flow channel-microband GE	Choro-amperometry	55 cells mL ⁻¹	10 ² -10 ⁸ cells mL ⁻¹	[67]
<i>Escherichia coli</i> , cDNA	DNA	AuNP/sol-gel-SPGE	Voltammetry	0.63 µM	2.1-40 µM	[68]
<i>Escherichia coli</i> , cDNA	DNA	ExoIII/thiolated DNA-GE	Voltammetry	8.7 fM	0.01 pM-1 nM	[69]
<i>Escherichia coli</i> , cDNA	DNA	Dendritic cysteine-GE	EIS	0.01 pM	0.01 pM-1 µM	[70]
<i>Escherichia coli</i> , cell	Phage	Phage/BSA-interdigitated gold microelectrode	EIS	10 ⁴ cfu mL ⁻¹	10 ⁴ -10 ⁷ cfu mL ⁻¹	[71]
<i>Escherichia coli</i> , cell	Phage	Phage/PDCT/cysteamine-GE	EIS	800 cfu mL ⁻¹	10 ³ -10 ⁹ cfu mL ⁻¹	[23]
<i>Escherichia coli</i> O157:H7, antigen	mAb, IgG	Ferrocene-thiolated chitosan-C ₆₀ composite/GOD loaded PtNC-GCE	Voltammetry	15 cfu mL ⁻¹	32-3.2 × 10 ⁶ cfu mL ⁻¹	[72]
<i>Escherichia coli</i> O157:H7, antigen	Ab	Biotinylated Ab/AuNP/streptavidin-graphene paper	EIS	150 cfu mL ⁻¹	150-1.5×10 ⁷ cfu mL ⁻¹	[73]
<i>Escherichia coli</i> O157:H7, antigen	pAb	SAM Ab/thiol-quartz crystal GE	EIS	10 ² cfu mL ⁻¹	10 ² -10 ⁵ cfu mL ⁻¹	[74]
<i>Escherichia coli</i> O157:H7, antigen	mAb	Electrospun nanofiber/EAM nanoparticle-silver electrode	Conductometry	61 cfu mL ⁻¹	~ 0-10 ⁴ cfu mL ⁻¹	[75]
<i>Legionella pneumophila</i> , cDNA	DNA	O ₂ plasma functionalized MWCNT electrode	Voltammetry	10 pM	10 pM-100 nM	[76]

<i>Legionella pneumophila</i> , cDNA	DNA	Thiolated hairpin DNA/ferrocene-GE	Voltammetry	23 fM	~ 10 fM-1 μ M	[77]
<i>Listeria innocua</i> , cell	Phage endolysin	Phage endolysin/EDC/NHS/thiol-SPCE	EIS	1.1×10^4 cfu mL ⁻¹	10^4 - 10^7 cfu mL ⁻¹	[24]
<i>Listeria monocytogenes</i> , antigen	Ab	Magnetic nanoparticle/microfluidic-interdigitated microelectrode	EIS	10^3 cfu mL ⁻¹	10^3 - 10^7 cfu mL ⁻¹	[78]
<i>Listeria monocytogenes</i> , cDNA	DNA	Dendritic AuNP/electrochemical reduced graphene-CILE	Voltammetry	0.29 pM	1 pM-1 μ M	[79]
<i>Mycobacterium</i> sp., cDNA	DNA	DNA-ALP labeled AuNP-ITO electrode	Voltammetry	1.25 ng mL ⁻¹	1.25-50 ng mL ⁻¹	[80]
<i>Mycobacterium tuberculosis</i> , IgG	LAM	AuNP labeled protein A-SPCE	Voltammetry	5.3 ng mL ⁻¹	15.6 - 10^3 ng mL ⁻¹	[21]
<i>Mycobacterium tuberculosis</i> , cDNA	DNA	Magnetic particle/dextrin coated AuNP-SPCE	Voltammetry	10 ng mL ⁻¹	10 - 10^4 ng mL ⁻¹	[81]
<i>Mycobacterium tuberculosis</i> , cDNA	DNA	Chitosan-ZrO ₂ composite/ITO coated glass plate	Voltammetry	0.78 nM	0.78-50 nM	[82]
<i>Mycobacterium tuberculosis</i> , cDNA	DNA	<i>MspI</i> /streptavidin-HRP/biotinylated DNA-thiolated GE	Voltammetry	2.3 pM	10 pM-100 nM	[83]
<i>Plasmodium falciparum</i> , histidine-rich protein 2	mAb, IgM	Magneto sandwich immunoassay-mGEC	Amperometry	0.36 ng mL ⁻¹	~0-250 ng mL ⁻¹	[84]
<i>Plasmodium</i> sp., lactate dehydrogenase	DNA aptamer	DNA aptamer-thiolated GE	EIS	1 pM	1- 10^3 pM	[85]
<i>Pseudomonas aeruginosa</i> , cDNA	DNA	Streptavidin-HRP/biotinylated DNA-thiolated GE	Voltammetry	12 μ g mL ⁻¹	0.3-600 ng mL ⁻¹	[86]
<i>Salmonella typhimurium</i> , antigen	pAb	Ag enhanced DNA barcode label/biotinylated Ab/avidin-SPCE	Voltammetry	12 cfu mL ⁻¹	10 - 10^6 cfu mL ⁻¹	[87]
<i>Salmonella typhimurium</i> , antigen	DNA aptamer	SAM thiolated DNA aptamer/AuNP-SPCE	EIS	600 cfu mL ⁻¹	10^3 - 10^5 cfu mL ⁻¹	[88]
<i>Salmonella typhimurium</i> , cDNA	DNA	Graphene oxide/chitosan-ITO electrode	Voltammetry	10 fM	10 fM-50 nM	[89]
<i>Staphylococcus aureus</i> , surface antigen	DNA aptamer	DNA aptamer-SWCNT based electrode	Potentiometry	800 cfu mL ⁻¹	800 - 10^8 cfu mL ⁻¹	[90]

<i>Staphylococcus aureus</i> , cDNA	DNA	Chitosan-Co ₃ O ₄ nanorod-graphene composite-CILE	Voltammetry	0.43 pM	1 pM-1 μ M	[91]
<i>Staphylococcus aureus</i> , cDNA	DNA	Polyaniline nanofiber-AuNP composite/HRP labeled DNA-GE	Voltammetry	0.15 fM	0.15 fM- 1 μ M	[92]
<i>Shigella flexneri</i> , antigen	Ab	HRP labeled Ab/MWCNT-sodium alginate composite-SPCE	Voltammetry	1100 cfu mL ⁻¹	10 ⁴ -10 ¹¹ cfu mL ⁻¹	[93]
<i>Streptococcus</i> sp., antigens	Ab	Graphene sheet/sandwich immunoassay-GCE	Voltammetry	~2.8-5.4 pg mL ⁻¹	~0.01-50 ng mL ⁻¹	[94]
<i>Vibrio cholerae</i> , cDNA	DNA	Chitosan/MgO-ITO electrode	Voltammetry	35.2 μ g mL ⁻¹	100-500 μ g mL ⁻¹	[95]
<i>Vibrio cholera</i> , cDNA	DNA	Carboxyl functionalized graphene oxide/poly-L-lysine-GCE	Voltammetry	0.169 pM	1 pM-1 μ M	[96]
<i>Vibrio cholera</i> , cDNA	DNA	Sandwich type double hybridization-SPCE	Choro- amperometry	0.488 nM	0.488-15.625 nM	[97]
<i>Yersinia enterocolitica</i> , cDNA	DNA	Chitosan-V ₂ O ₅ nanobelt-MWCNT composite-CILE	Voltammetry	1.76 pM	10 pM-1 μ M	[98]

Abbreviations: Ab: antibody, AgNP: silver nanoparticle, ALP: alkaline phosphatase, AuNP: gold nanoparticle, cDNA: complementary deoxyribonucleic acid, DNA: deoxyribonucleic acid, CILE: carbon ionic liquid electrode, EAM: electrically active magnetic, EDC: N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide, EIS: electrochemical impedance spectroscopy, ExoIII: exonuclease III, GCE: glassy carbon electrode, GE: gold electrode, GOD: glucose oxidase, HRP: horseradish peroxidase, IgG: immunoglobulin G, ITO: indium tin oxide, LAM: lipoarabinomannan, mGEC: graphite-epoxy composite magneto electrode, mAb: monoclonal antibody, NHS: N-hydroxysuccinimide, pAb: polyclonal antibody, PDCIT: 1,4-phenylene diisothiocyanate, PtNC: platinum nanochain, SPCE: screen-printed carbon electrode, SPGE: screen-printed gold electrode, SWCNT: single-walled carbon nanotube, MWCNT: Multi-walled carbon nanotube, SAM: self-assembled monolayer.

bioaffinity probes, possibility of non-specific labeling, and instability of detected signals (e.g. photobleaching of fluorescence tags) [99]. As a consequence, label-free detection methods, which are simple, rapid and cost-effective, are used preferably for quantitative detection of microbes.

The detection performance of biosensors is largely influenced by the stability, reactivity, and accessibility of biorecognition probes. Electrodes are usually made up of electrically conducting materials such as carbon, gold, platinum, silver, copper and metal oxides, which exhibit poor capability in loading biorecognition probes. Therefore, surface functionalization of these sensors is crucial for the specific adsorption of biorecognition probes while retaining their accessibility and reactivity. In general, immobilization of biorecognition probes can be conducted using two approaches: physical or chemical adsorption [100].

Physical adsorption (or physisorption) mainly involves direct, non-covalent attachment of biorecognition probes on the electrode surface. This surface functionalization approach is simple, rapid, easy to perform and typically produces a monolayer or multilayer of biorecognition probes on the electrode surface. Physisorption relies on weak electrostatic or hydrophobic interaction and van der Waals forces, where immobilized biorecognition probes are easily detached upon extensive washing. In addition, physisorption is an uncontrollable process that may result in random orientation of biorecognition probes, thus reducing their accessibility toward the microbial targets. Besides, the use of hydrophobic surface can cause denaturation of proteins [101].

On the other hand, chemical adsorption (or chemisorption) makes use of strong covalent attachment to anchor biorecognition probes in specific orientation on the electrode surface. Chemisorption offers several advantages over direct physisorption approach such as improving the conformational and structural stability of biorecognition probes, and longevity and sensitivity of biosensors. Covalent immobilization strategies that are commonly employed include thiol and organosilane self-assembled monolayers (SAMs), polyethylene glycol layers, hydrogels, agarose, and carboxymethylated dextran supports [100, 102]. Presently, engineered fusion protein with metal binding property offers an alternative approach for direct gold-protein surface interaction apart from the classic gold-thiol methodology [37]. Gold-binding polypeptides (GBPs) are composed of repeats of 14-30 amino acid sequences in which the polar groups of peptide i.e. threonine and serine are believed to be responsible for the interaction with gold [103]. Besides, polyhistidine tags demonstrate high efficiency for the immobilization of proteins on ligand-modified metal electrode surface attributed to their considerable metal binding affinity toward nickel, copper and cobalt [104]. Apart from that, intermediate cross-linkers such as proteins A/G, derivatives of avidin and hydrazine-containing cross-linkers are often incorporated to orientate the biorecognition probes in specific position toward microbial targets [55, 58]. In some immunosensing methods, the non-specific adsorption of interfering substances is commonly avoided by applying ~ 1 % bovine serum albumin (BSA) on the antibody-coated electrode surface [35, 65].

1.4 Electroanalytical techniques

1.4.1 Voltammetric techniques

Voltammetry comprises a group of electroanalytical techniques that measures current in response to the varying potential excitation signals. To complete the electrical circuit, a third electrode known as counter or auxiliary electrode is commonly incorporated in an electrochemical cell to facilitate the flow of current at working electrode while maintaining a stable referencing potential. Auxiliary electrodes are usually made up of electrochemically inert materials such as gold, platinum or graphite. Different types of voltammetric techniques include linear sweep voltammetry, staircase voltammetry, alternating current (AC) voltammetry, cyclic voltammetry, normal and differential pulse voltammetry, square wave voltammetry, stripping voltammetry, and hydrodynamic voltammetry [105]. Voltammetry has proven useful as an initial screening tool for acquiring information about a complicated or novel system [106]. Cyclic voltammetry and differential pulse voltammetry will be the main focus in this chapter since these techniques are the major electroanalytical tool employed in the experimental work.

1.4.1.1 Cyclic voltammetry

Cyclic voltammetry (CV) is one of the most versatile electroanalytical techniques that provide useful quantitative information about redox reactions in a form which is easily obtained and interpreted. CV involves the application of triangular potential excitation signals to a working electrode, which is immersed in an unstirred analysis solution, and measuring the current response (Figure 1.4). This potential excitation signal sweeps the

Figure 1.4 Cyclic voltammetric excitation signal as a triangular potential waveform. E_i is the initial potential, $E_{\lambda 1}$ and $E_{\lambda 2}$ are the switching potentials.

electrode potential between two values, $E_{\lambda 1}$ and $E_{\lambda 2}$, which are known as the switching potentials. The excitation signal causes the potential to scan linearly from an initial value, E_i to a pre-selected potential, $E_{\lambda 1}$ where the direction of scan is reversed. The voltammetric scanning process can be stopped anywhere or cycle between $E_{\lambda 1}$ and other pre-selected potential, $E_{\lambda 2}$ as monitored using an electrochemical analyzer/workstation.

Typical cyclic voltammogram, a display of current as a function of potential is shown in Figure 1.5 B. The key parameters in a cyclic voltammogram including anodic peak current (I_{pa}), cathodic peak current (I_{pc}), anodic peak potential (E_{pa}), and cathodic peak potential (E_{pc}) are defined and labeled in Figure 1.5. Ferrocenemethanol ($\text{Fc}^{\text{II}}\text{MeOH}$ and $\text{Fc}^{\text{III}}\text{MeOH}$) and ferri/ferrocyanide ($\text{Fe}(\text{CN})_6^{4-}$ and $\text{Fe}(\text{CN})_6^{3-}$) are examples of electrochemical reversible redox couples in which both species rapidly exchange electrons at a working electrode.

Figure 1.5 (A) Triangular potential waveform as a function of time, and (B) the corresponding cyclic voltammogram.

The formal reduction potential ($E^{o'}$) of a redox couple is calculated as an average of E_{pa} and E_{pc} , given that the electron transfer process is reversible and the diffusion coefficients (D) of the oxidized and reduced forms of redox couple are similar [105, 107-109]. The electron stoichiometry (n) for a redox couple in an electrode reaction can be estimated from the separation between cathodic and anodic peak potentials as follows,

$$\Delta E_p = E_{pa} - E_{pc} \approx \frac{0.059}{n} \quad (1)$$

Quantitative information of a reversible system is obtained from the Randles-Sevcik relation, which at 25 °C, the peak current or peak height (I_p) is given as follows,

$$I_p = (2.69 \times 10^5) n^{3/2} A D^{1/2} C \nu^{1/2} \quad (2)$$

where I_p is the peak current, n is the electron stoichiometry, A is the area of electrode in cm^2 , D is the diffusion coefficient in $\text{cm}^2 \text{s}^{-1}$, C is the concentration of redox probe in mol cm^{-3} , and ν is the scan rate in V s^{-1} .

1.4.1.2 Differential pulse voltammetry

Differential pulse voltammetry (DPV) is another electroanalytical technique that is designed to reduce the interfering charging current. More important, DPV enhances the sensitivity of voltammetric techniques. As displayed in Figure 1.6 A, the differential pulse voltammetric excitation signal is a sequence of small amplitude pulse in which the applied base potential varies steadily with small increment. Two current signals are sampled during each pulse, one at time τ' , before the pulse, and the second at time τ , late in the pulse. The stepped potential is in the range from 10 to 100 mV and is maintained at a constant level against the base potential. As shown in Figure 1.6 B, a typical differential pulse voltammogram shows the differential current signal, $\delta I = I(\tau) - I(\tau')$ as a function of base potential. Important parameters including peak current (I_p) and peak potential (E_p)

A

B

Figure 1.6 (A) Differential pulse voltammetric excitation signal, and (B) the corresponding voltammogram in differential pulse mode.

can be extracted from differential pulse voltammogram [105, 106, 110]. The peak current (I_p) is calculated as follows,

$$I_p = \frac{nFAD^{\frac{1}{2}}C}{\pi^{\frac{1}{2}}(\tau - \tau')^{\frac{1}{2}}} \left(\frac{1 - \sigma}{1 + \sigma} \right) \quad (3)$$

where n is the electron stoichiometry, F is the Faraday constant, A is the electrode area, D is the diffusion coefficient, C is the concentration of redox probe, $(\tau - \tau')$ is the time measured from pulse rise, and σ is given as follows,

$$\sigma = \frac{nF}{RT} \cdot \frac{\Delta E}{2} \quad (4)$$

where ΔE is the pulse amplitude, R is the universal gas constant, and T is the absolute temperature.

1.4.2 Electrochemical impedance spectroscopy

Electrochemical impedance spectroscopy (EIS) is often employed for studying electrode processes, resolving double-layer capacitance, and characterizing complex interface such as electrode surface covered with a confluent layer of cells. Electrochemical impedance is normally measured by monitoring of the AC current in response to small amplitude sinusoidal excitation signal, typically in the range from 5 to 10 mV. Measurement of the

Figure 1.7 (A) AC potential excitation signal, and (B) sinusoidal current response in a linear or pseudo-linear electrochemical system.

impedance signal response is generally conducted in a range of AC frequency and therefore, the name “electrochemical impedance spectroscopy” was adopted.

In a linear or pseudo-linear electrochemical system (Figure 1.7), the current signal response is sinusoidal at the same frequency but shifted in phase, ϕ ($^{\circ}$) with respect to the AC potential waveform as follows,

$$E_t = E_0 \sin(\omega t) \quad (4)$$

$$I_t = I_0 \sin(\omega t + \phi) \quad (5)$$

where E_t is the potential at time t , E_0 is the potential amplitude, I_t is the current at time t , I_0 is the current amplitude, and ω is the radial frequency expressed in radian s^{-1} .

By substituting equations (4) and (5) into the expression of Ohm's Law ($R = \frac{E}{I}$), the impedance of an electrochemical system can be determined as follows,

$$Z = \frac{E_t}{I_t} = \frac{E_0 \sin(\omega t)}{I_0 \sin(\omega t + \phi)} = Z_0 \frac{\sin(\omega t)}{\sin(\omega t + \phi)} \quad (6)$$

Therefore, the impedance signals can be expressed in terms of magnitude (Z_0) or phase shift (ϕ). Bode diagram which consists of the magnitude of impedance (primary y-axis) and phase shift (secondary y-axis) against the logarithm of AC frequency is shown in Figure 1.8 A. Furthermore, based on the Euler's formula that describes the relationship between a complex exponential function and a trigonometric function,

$$e^{j\phi} = \cos \phi + j \sin \phi \quad (7)$$

where e is the base of natural logarithm, and j is the imaginary number.

The excitation signal and current response can be expressed as follows,

$$E_t = E_0 e^{j\omega t} \quad (8)$$

$$I_t = I_0 e^{j\omega t - \phi} \quad (9)$$

Thus, impedance can be described as a complex number as shown below,

$$Z = \frac{E_t}{I_t} = \frac{E_0 e^{j\omega t}}{I_0 e^{j\omega t - \phi}} = Z_0 e^{j\phi} = Z_0 (\cos \phi + j \sin \phi) \quad (10)$$

Clearly, the expression of impedance comprises real and imaginary parts. By plotting the imaginary impedance (Z'') values on y-axis versus the real impedance (Z') values on x-axis, a Nyquist plot as shown in Figure 1.8 B is obtained. Figure 1.8 B describes an interfacial electrochemical reaction in the presence of semi-infinite linear

A

B

Figure 1.8 (A) Bode diagram, and (B) Nyquist plot of a mercaptoacetic acid-modified gold electrode in the presence of 2.5 mM ferricyanide ($\text{Fe}(\text{CN})_6^{3-/4-}$).

diffusion of electroactive species. Moreover, information on the electron-transfer kinetics at high frequency region ($\sim 10 \text{ Hz}$ - 1 MHz) and the diffusion characteristics at low frequency region (~ 0.1 - 10 Hz) of an electroactive species can be extracted from Bode diagram and Nyquist plot. The mechanism of mixed kinetic and diffusion controlled electrochemical reactions at the electrode/solution interface is well described by the Randles equivalent circuit model [105, 106, 111, 112]. Fitting of the experimental data with Randles equivalent circuit model and definition of each electrical circuit element are discussed in Section 4.2.2.

1.5 Electrochemical biosensors

Electrochemical biosensors employ electroanalytical techniques to monitor electrical signals generated from the biomolecular interaction between biorecognition probes and chemical or biological analytes. Since the first biosensor was invented in 1962 by Clark and Lyons for the detection of blood glucose using a glucose oxidase coated electrode [113], there have been increasing interests in utilizing biosensors for environmental monitoring, industrial, agricultural, and clinical applications. Electrochemical biosensors have been extensively reported in detecting small molecules (DNA, glucose, heavy metal ions, organic compounds, neurotransmitters, reactive organic species, explosives, and pesticides) and biological analytes (proteins, viruses, and bacteria) [114-119]. Biosensors based on electrochemical transducers possess advantages such as high sensitivity, cost effectiveness, portability, possibility of miniaturization with micro-liter sample volume, simple instrumentation and operation. In principle, the biomolecular interaction or biorecognition process in electrochemical biosensing assays generate measurable current at varying potential (voltammetry) or at a fixed potential (amperometry), quantifiable potential or charge accumulation without drawing substantial current (potentiometry), change in the conductivity of electrolyte in water or solvent (conductometry) or opposition of electrical circuit to the flow of current (impedance). Biosensing methodologies based on voltammetric and impedimetric techniques will be the focus of my thesis because these techniques are sensitive to the affinity interaction between biorecognition probes and microbial targets.

1.5.1 Voltammetric biosensors

Biosensors based on voltammetric techniques have been widely used for the detection of a wide variety of bacteria and viruses. Voltammetric biosensors constructed using screen-printed electrodes (SPEs) are frequently employed as an *in situ* detection tool for analyzing microbes because of its disposability, cost-effectiveness, and minimal sample requirement. Also, problems commonly encountered in electrochemical measurements using conventional electrodes such as electrode fouling and memory effect in the analysis of multiple biological samples can be eliminated. Furthermore, SPEs avoid the need of time-consuming electrode pre-treatment process [120, 121].

Nanoscale materials exhibit tunable spectral and chemical properties by varying their shapes and sizes [122]. Some of these nanomaterials serve as an efficient conducting interface of electrocatalytic activity, thus functionalization of these materials with biorecognition probes is commonly employed for the amplification of electrical signal. Nanoparticles such as gold nanoparticles (AuNP), silver nanoparticles (AgNP), and iron oxide nanoparticles ($\text{Fe}_3\text{O}_4\text{NP}$) provide attractive features such as excellent conductivity, biocompatibility and adsorption capability [38, 40, 51]. These nanoparticles are regularly used as electrocatalytic labels in which the electrical currents are derived from the metal oxidation or reduction of its oxide form. By using a nanocomposite material composed of $\text{Fe}_3\text{O}_4\text{NP}$ and chitosan as the stabilizing agent, Lam *et al.* have developed a square wave voltammetric DNA biosensor for the detection of human immunodeficiency virus (HIV) DNA and reported a dynamic working range of 50 to 300 pM [51]. In another work, Chen *et al.* have reported a voltammetric sandwich immunoassay using cadmium telluride quantum dot (QD) capped silica nanoparticles (SiO_2NP) label to enhance the

assay sensitivity. Epstein-Barr virus (EBV) can be detected down to 1 pg mL^{-1} using the described platform [35]. Other types of nanomaterials such as nanofiber [92], nanorod [91], nanobelt [98] nanochain, and fullerene (C_{60}) [72] have been used lately as electrical signal amplifiers attributed to their large surface area and excellent electron transfer efficiency.

Micro-structured materials such as dendrites also provide an appealing matrix for the construction of biosensors. Due to the favorable conformation (tree-like structure), abundant surface functional groups, great structural and chemical properties, dendrites appear as an excellent material which considerably improves the detection sensitivity of biosensors [70]. Sun *et al.* have described a DNA biosensor based on dendritic AuNP and electrochemically reduced graphene carbon ionic liquid electrode (CILE) for the detection of *Listeria monocytogenes* DNA. A low LOD of 0.29 pM with a wide linear working range from 1 pM to 1 nM have been achieved [79]. CILE is known as a high performance working electrode which possesses excellent electrochemical features of different carbon electrodes (glassy carbon, graphite, carbon-paste, and carbon nanotube electrodes) such as high sensitivity, significant electrocatalytic activity, low cost and anti-fouling ability [98].

Electrode surface modification is frequently employed to enhance the electrode conductivity, sensitivity and reversibility of biosensors. Presently, graphene has received great attention due to its remarkable electron transfer efficiency, superior structural and mechanical properties. In graphene, carbon atoms are closely packed in a sp^2 -bonded atomic-scale hexagonal honeycomb lattice [73, 91]. Besides, graphene oxide which displays significant water dispersibility is also broadly used to modify biosensor platform

[66, 89]. Recently, a nanocomposite film fabricated from carbon nanotube (CNT) and AuNP offer a promising interface for ultrasensitive detection of microbial targets. CNT exhibits high electrical conductivity, mechanical strength, chemical and thermal stability. There are two main types of CNT, known as single-walled carbon nanotube (SWCNT) which are made up of a single sheet of hexagonal honeycomb rolled in around itself to form a hollow tube, and multi-walled carbon nanotube (MWCNT) which consists of multiple concentric tubes of graphene [116]. CNT/AuNP nanocomposite film provides an appropriate surface for immobilizing biorecognition probes and eliminating the non-specific adsorption of interfering biological components. The synergistic interaction between CNT and AuNP considerably enhances the rate of electron transfer reactions at the electrode/solution interface [47]. Another nanocomposite film constructed from zirconia nanoparticle (ZrO_2NP), AuNP and chitosan has been reported by Ma *et al.* for the detection of hepatitis C virus (HCV) core antigen and achieved a low LOD of 0.17 ng mL^{-1} . ZrO_2NP shows excellent thermal stability, chemical inertness and high binding affinity toward proteins [41].

Our research group has fabricated platinum electrodes modified with an approximately 300-500 nm thick nanoporous alumina substrate. This substrate provides a suitable surface for the direct adsorption of biorecognition probes. Alumina substrate is a highly rigid and densely porous material with adjustable pore sizes ranging from 10 to 200 nm. Additionally, alumina possesses high pore density of approximately 1×10^{10} pores cm^{-2} , and is thermally and chemically stable [123]. Based on the described platform, ultrasensitive detection of dengue virus (DENV) surface antigen (non-structural protein 1, NS1) and DNA with low LODs of 1 pfu mL^{-1} [30] and 9.55 pM [33] respectively, have

Figure 1.9 Scheme of construction and operation of a nanoporous alumina membrane-based biosensor for the detection of bacteria and viruses.

been achieved. This nanobiosensor has also been used for the detection of *Escherichia coli* (*E. coli*) and *Legionella pneumophila* DNA with reported LODs of 22 cfu mL^{-1} [65] and 23 fM respectively (See Figure 1.9 for the scheme of construction and operation) [77]. In addition, we have demonstrated the feasibility of this nanobiosensor for assessing the cells viability by monitoring of the current derived from bacterial enzyme catalytic reaction using redox probes as the alternative electron acceptor [65].

Alkaline phosphatase (ALP) [124] and horseradish peroxidase (HRP) [43] are among the most widely used enzyme conjugates for signal amplification in electrochemical assays. In comparison to ALP, HRP possesses higher turnover rate and

greater stability. Furthermore, HRP uses a wide variety of organic compounds as electron acceptors or donors. In a recent work, Tang *et al.* have reported a DNA-based biosensing system for the detection of HCV. This biosensor relies on dual enzymatic activity: site specific cleavage by *Bam*HI endonuclease and signal amplification by HRP. Authors have demonstrated that the high electron transfer efficiency and enzymatic activity are preserved through encapsulation of HRP in nanogold hollow sphere [43]. Luo *et al.* recently introduced an application of *E. coli* Exonuclease III (Exo III) aided target recycling for electrical signal amplification in the detection of *Enteriobacteriaceae* bacteria *Lac Z* gene. The detection system relies on the selective digestion of captured target DNA by Exo III, which releases the target to undergo a new cycle of hybridization and cleavage at the electrode/solution interface [69].

1.5.2 Impedimetric biosensors

EIS has recently gained popularity among the biosensor community. This technique involves the application of small sinusoidal potential excitation signal to an electrode, which causes only minimal perturbation to the electrochemical system, and measuring the resulting reactance or resistance of the biosensor. EIS measurements are generally performed over a range of AC frequency to produce impedance spectra from which information about the diffusion characteristics and electron transfer kinetics of electroactive species or redox probes can be obtained [112]. EIS techniques have demonstrated many useful traits including non-invasive measurement, availability of quantitative and time-dependent data, and use on highly resistive materials. Another attractive feature of EIS is that a mechanistic model about an electrochemical system can

be developed by fitting the experimental data to an equivalent electrical circuit. Each circuit element such as resistor, capacitor and inductor in the equivalent electrical circuit has a basis in the physical electrochemistry of an electrochemical system. For example, in our recent work on impedimetric detection of DENV, the impedance data is fitted to an equivalent circuit to delineate the processes that occur at the electrode/solution interface during antigen-antibody interaction [31].

Bacteriophage is broadly used as an alternative to antibody for the detection and assessment of the viability of bacteria. Mejri and co-workers have developed an impedimetric *E. coli* biosensor constructed using an interdigitated gold microelectrode physisorbed with a layer of bacteriophage T4 and BSA. Analysis of bacterial cell viability and sensitive detection of *E. coli* have been achieved based on the monitoring of dual signals, which were composed of an initial increment in impedance signal caused by the binding of bacteria followed by subsequent decrease in impedance due to the phage-induced bacterial lysis, [23]. In another work, Tolba *et al.* made use of bacteriophage-encoded peptidoglycan hydrolase or endolysin, which targets on the bacterial cell wall domain, for the detection of *Listeria innocua*. Authors reported LODs of 1.1×10^4 and 10^5 cfu mL⁻¹ for cells in pure culture and 2 % milk respectively [24].

To address the problem of weak adsorption and random orientation of biorecognition probes on the electrode surface, deposition of thiol self-assembled monolayer (SAM) on gold electrodes is employed. Thiol groups (-SH) are chemisorbed on the gold surface through strong gold-sulphur bonds [125]. These groups allow covalent immobilization of biorecognition probes, forming a uniform, oriented, densely packed and highly ordered monolayer on the electrode surface [55]. Similarly,

silanization is another electrode surface modification approach that produces well-organized SAM of silane. However, the target electrode surface has to be polar and possessing, for examples, oxide or hydroxyl groups [126].

Affinity-based capture elements are widely used to capture biorecognition probes in specific orientation on the electrode surface. Among them, avidin and its derivatives (ExtrAvidin[®], neutravidin and streptavidin) which are derived from both amphibians and avians are the most popularly used capture element attributed to their substantial affinity toward biotin. Avidin-biotin interaction is known as the strongest non-covalent binding with a dissociation constant of $\sim 10^{-15}$ M [127]. Furthermore, avidin-biotin complexes are highly stable, unaffected by the extreme temperature and pH, denaturing agents and organic solvents. Based on the use of biotinylated bioaffinity probes and avidin-modified electrodes, ultrasensitive detection of influenza A virus's (IAV) DNA and antigens with reported LODs of 85.1 fM and 8 ng mL⁻¹ respectively, have been achieved by Hassen *et al.* [55, 56]. Besides, protein A is commonly used as the affinity-based capture element between SAM-modified electrode and antibody in some immunosensing techniques. Protein A is known as a surface protein (56 kDa) derived from the cell wall of bacterium *Staphylococcus aureus*. It exhibits excellent loading capability, biocompatibility and binding affinity toward antibody molecules. A recent work has been conducted using a protein A/glutaraldehyde coated silanized immunosensor for the detection of Japanese encephalitis virus (JEV) antigens and reported an acceptable LOD of 750 ng mL⁻¹.

In recent years, impedimetric cell-based analytical techniques have shown great potential in automated, real-time investigation of cellular behavior and response toward viruses and drugs. The operation of these techniques involves initial culturing of cells on

the electrode surface. The effects of viral infection on cells' growth and adhesion are subsequently monitored in real-time. Cell membranes are essentially non-conducting and therefore, variation in the impedance signal response can be correlated with the infection-induced changes in size or coverage of cells on the electrode surface [128]. Killerich-Pederson *et al.* has reported a microfluidic system based on the conductive polymer microelectrode array to monitor human cytomegaloviral infection of human foreskin fibroblast cells [26]. Interdigitated microelectrode array basically consists of repetitions of working and reference electrode pairs spaced a micrometer apart. Interdigitated microelectrode array-based biosensors offer advantages such as low resistance, diminished interference, low sample consumption and rapid achievement of steady state [129].

Extensive research on aptamer-based biosensor (aptasensor) for electrochemical detection of microbes has been performed by Berezovski and co-workers. The sensing platform is constructed using thiolated DNA primers which hybridize to the complementary end of DNA aptamer probes. This hybrid is then self-assembled on the gold electrode surface. Electrode surface modification and capturing of target microbe are characterized using both EIS and voltammetric techniques (See Figure 1.10 for the scheme of construction and operation). Authors have demonstrated the feasibility of the electrochemical aptasensors for quantifying bacteria and viruses, immunoshielding and viability assessment [59, 60, 88, 130].

Figure 1.10 Schematic diagram of the aptamer-mediated electrochemical detection of live *Salmonella typhimurium* bacteria. Reprinted from Ref. [88] with permission from American Chemical Society (Copyright 2012 American Chemical Society).

1.5.3 Other electrochemical biosensors

Biosensors based on other electroanalytical techniques such as amperometry, conductometry and potentiometry comprise a group of analytical methods that is not yet widespread but possesses a great potential in detecting and studying microbes. Amperometry measures current at a fixed potential as a function of time. Our group recently developed a novel fuel cell DENV biosensor using DENV-specific antibody coated Prussian blue nanotube (PB-*nt*) membrane electrode. This biosensor operates without the need of voltage power input but relies on the presence of hydrogen peroxide

for powering the PB-*nt* biosensor. An extremely low LOD of 0.04 pfu mL^{-1} with a linear working range from 3 to 45 pfu mL^{-1} has been reported [29].

Chronoamperometry is another electroanalytical technique that measures current as a function of time while the potential is stepped. The advantage of technique is that time resolution is only limited by the data collection frequency of the electrochemical

Figure 1.11 (A) Scheme of the experimental procedure performed. Hepatitis B surface antigen (HBsAg) captured on the surface of magnetic beads, incubation with human serum containing anti-HBsAg immunoglobulin G (IgG) antibodies and recognition with gold nanoparticles (AuNPs) conjugated with goat-human IgG antibodies. (B) Scheme of the electrochemical detection procedure based on the electrocatalytic hydrogen generation. Reprinted from Ref. [20] with permission from Elsevier (Copyright 2010 Elsevier).

analyzer/workstation. However, the reliability and accuracy of the quantitative detection system are restricted by the low chemical selectivity [105]. By immobilizing hepatitis B virus (HBV) onto magnetic beads (MBs), HBV-specific immunoglobulin G (IgG) antibody can be detected down to 3 mU mL^{-1} based on a magnetic sandwich chronoamperometric immunoassay (See Figure 1.11 for the scheme of construction and operation) [20]. MBs are recognized as a versatile tagging tool for numerous biorecognition probes. Targets are concentrated onto the surface of biorecognition probes-modified MBs and subsequently separated from the biological or chemical media by applying a magnetic field. In doing so, signal interference caused by the matrix effect can be significantly reduced [131].

Conductometry measures the conductivity of electrolyte between electrodes. Technically, conductivity is a measure of the ability of materials to conduct electric current, and is expressed in the unit of Siemens per centimeter (S cm^{-1}). Luo *et al.* have constructed a conductometric biosensor based on electrospun nanofibers for the detection of *E. coli* O157:H7 (an enterohemorrhagic strain of *E. coli*). Electrospinning is an effective method to produce biocompatible nanofibrous membranes. The capillary action and biomolecular capture ability of these membranes are further enhanced by O_2 plasma treatment. Based on the immunomagnetic concentration approach and conductivity measurement, *E. coli* O157:H7 cells can be detected down to 61 cfu mL^{-1} [75].

Finally, potentiometric techniques measure the potential of a working electrode versus a reference electrode either at open circuit or by applying small anodic or cathodic current to the working electrode. A potentiometric biosensor based on SWCNT and DNA aptamer has been reported by Zelada-Guillen and co-workers for detecting

Staphylococcus aureus (*S. aureus*). The potentiometric response in terms of the changes in electromotive force (EMF) is monitored following the stepwise incubation of *S. aureus* cell solutions on the biosensor surface. Authors revealed that the changes in EMF are linearly dependent on the concentration of *S. aureus* cells over eight orders of magnitude [90].

1.4 Scope of study

The aims and objectives of this thesis are to present the utility of electrochemical biosensors for the detection of microbes (bacteria and viruses) and monitoring of their activities with an emphasis on virus (or bacteriophage)-host interaction. The experimental work is divided into four chapters in which the summary of experimental data and interpretation of results are presented. The scope of work for each chapter is described as follows.

In chapter 2, an electrochemical nanobiosensor based on membrane-based alumina-modified electrode is constructed for the detection of *E. coli* cells. Anti-*E. coli* polyclonal antibody is physically adsorbed on the alumina-modified electrode surface for specific capturing of *E. coli* cells. The biosensing mechanism relies on the blocking of alumina nanochannels upon the formation of immuno-complexes at the nanoporous alumina membrane. In this work, cyclic voltammetry is used to characterize the biosensor construction and *E. coli* detection steps in the presence of a neutral redox probe, ferrocenemethanol (FcMeOH). Optimization of experimental parameters such as antibody loading and pH, and selectivity test against non-specific bacterial species are performed. Additionally, bacterial cells viability is assessed by probing the

electrocatalytic current generated from the bacterial enzyme catalytic oxidation of glucose using FcMeOH as the artificial electron acceptor.

In Chapter 3, the same design of nanobiosensor is employed for detecting dengue type 2 viruses (DENV-2). Capturing of DENV-2 viral particles by anti-DENV-2 monoclonal antibodies is monitored using differential pulse voltammetry. Similarly, the specificity of nanobiosensor toward DENV-2 is examined against other non-specific viruses. Furthermore, the direct quantification of viral load in DENV-2 infected *Aedes aegypti* mosquitoes using the electrochemical membrane-based nanobiosensor is demonstrated.

In Chapter 4, real-time monitoring of the interaction between filamentous bacteriophage (phage) M13 and *E. coli* is reported using an impedimetric microbial biosensor constructed from a gold electrode covalently grafted with a monolayer of antibody. The effects of phage infection on bacterial surface charge and morphology are reflected in the change of electron-transfer resistance as studied using the EIS technique. Fitting of experimental EIS data with the Randles equivalent circuit allows the diffusion characteristics and electron transfer kinetics of redox probe at the electrode/solution interface to be investigated.

In chapter 5, DENV-2 infection of mammalian fibroblast cells is investigated using a cationic polymer-modified screen-printed carbon electrode (SPCE). Electroanalytical methods are employed to examine the stability and adherence of the cationic polymer, poly-L-lysine (PLL) on the surface of screen-printed carbon electrode and the cell capture capability of this modified electrode surface. Viral induced cytopathic or cythopathogenic effects including degenerative morphological change,

detachment, membrane degradation and death of cells, as indicated by a drastic change in the impedance signal response, are monitored in real-time using the EIS technique. Furthermore, dependency of the cytopathic effects on virus titer is determined in this work.

Finally, a concise summary of this work and the future outlooks for biosensing techniques are presented in the conclusion section.

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Development of electrochemical membrane-based nanobiosensor for the detection of *E. coli* and analysis of cells viability

2.1 Introduction

In recent decades, development of methods for the detection of bacterial species have attracted great interest attributed to the urgent needs to study and identify disease-causing microbes or pathogens arising from the contamination in food and water sources, terrorism acts or widespread proliferation of these microbes under favorable environmental condition. Every year, approximately 1.5 million deaths are caused by pathogens in drinking water and foods [1]. One highly relevant and regularly targeted indicator for routine analysis of contaminated food and water sources is the bacterium *Escherichia coli* (*E. coli*) [2]. *E. coli* is a rod-shaped, facultative anaerobic, non-sporulating, Gram-negative bacterium in the Enterobacteriaceae family. The presence of *E. coli* in food and water sources often indicates fecal contamination, deterioration of microbiological water quality and possible existence of pathogenic microbes including *E. coli*. Though most *E. coli* strains are harmless, there are several strains which have been shown to cause urinary tract and gastrointestinal infection, inflammation, diarrhea and peritonitis in immunosuppressed patients, particularly children under age of ten and elderly people. *E. coli* O157:H7 is one of the most important enterohemorrhagic serotype,

which is known to cause foodborne illness and life threatening complication such as hemolytic uremic syndrome and hemorrhagic colitis that may lead to severe bloody diarrhea, occasionally kidney failure, and even death [3]. Conventional methods for the detection of *E. coli* include plate count, multiple tube fragmentation, membrane filter and Most-Probable-Number. These methods normally involve selective culturing and cell isolation followed by subsequent serological and biochemical confirmation, which are highly reliable and accurate but are also mostly time-consuming, labor intensive, lack of specificity, and require multiple-step procedure [4]. In addition, turbidity assay using spectrophotometer is one of the most commonly used methods in estimating bacterial numbers and measuring cell growth rate [5].

Many microbes including bacteria respond to harsh environmental condition by entering a physiological state in which the microbes have lost their culturability on standard microbiological media but retained their viability. It was previously presumed that microbes are dead when failed to grow on microbiological media. In many circumstances, these microbes are able to regain normal metabolic activity and culturability upon resuscitation during this dormant phase, which is now known as the viable but nonculturable (VBNC) state [6, 7]. More important, VBNC microbes constitute a reservoir for infection that can evade detection by many conventional methods [8]. As a consequence, one major challenge for reliable and accurate detection of bacterial population number is the inability to distinguish between live, VBNC and dead bacteria.

Several methods have been reported for physiological characterization of bacteria such as direct viable count [9], double staining method using epifluorescence microscopy

[10], RT-PCR [7], flow cytometry [11], and commercial LIVE/DEAD *BacLight* bacterial viability kit [12]. Furthermore, electrochemical monitoring of cellular metabolism such as respiration provides an alternative effective way for identifying VBNC microbes and enhances the accuracy in quantifying microbes. The approach is to probe the reductive or oxidative current generated by added redox probes such as ferri/ferrocyanide, $(\text{Fe}(\text{CN})_6)^{3-/4-}$, which functions as the alternative electron acceptor to oxygen for cellular processes. For instance, the concentration of $\text{Fe}(\text{CN})_6^{3-/4-}$ near the cell surface reveals important information on toxic effect of silver ion, (Ag^+) on *E. coli* cells [13]. In another work, $\text{Fe}(\text{CN})_6^{3-/4-}$ is used to monitor the bioelectrochemical oxidation of carbon sources such as acetate and glucose in *E. coli* cells under anaerobic condition [14].

Herein, this chapter describes the strategy for sensitive, specific and label-free electrochemical detection of *E. coli* cells using alumina membrane-based nanobiosensor. The detection mechanism relies on the monitoring of Faradaic current response toward a redox probe, which is sensitive to the formation of immnocomplexes that impedes the diffusive mass transfer of redox probe. Alumina is a rigid material with a high aspect ratio, high pore density of approximately 1×10^{10} pores cm^{-2} , and nominal pore sizes ranging from 10 to 200 nm [15, 16]. The pore size of alumina membrane layer can be easily controlled through chemical etching and its rigid surface provides a suitable platform for the attachment and growth of microbes. Other appealing features include high stability at elevated temperature of up to 400 °C, chemical inertness in corrosive environment, and inexpensive fabrication compared to lithographic techniques [17, 18]. This work reports the first demonstration of using the same membrane-based nanobiosensor to assess bacterial cell viability by probing the electrocatalytic current

derived from the bacterial enzyme reaction, mediated by ferrocene derivative in the analysis solution.

2.2 Experimental

2.2.1 Reagents and Materials

Rabbit anti-*E. coli* polyclonal antibody (isotype: IgG, 4.0 mg mL⁻¹) was obtained from AbD Serotec. Bovine serum albumin (BSA, ~99 %), ferrocenemethanol (FcMeOH, 97 %), D-(+)-glucose (C₆H₁₂O₆, ≥ 99.5%), sodium hypochlorite solution (NaOCl, 6-14 % active chlorine basis) and oxalic acid (H₂C₂O₄, ≥ 99 %) were purchased from Sigma-Aldrich. Phosphoric acid (H₃PO₄, 85%) was purchased from Lancaster Synthesis. Potassium chloride (KCl, ≥ 99.5 %) was purchased from Sinopharm Chemical Reagent. Alumina powder (particle size: 0.3 and 1.0 µm) were obtained from Allied High Tech Products. All *E. coli* and protein solutions were prepared in 10 mM phosphate buffered saline (PBS, containing 10 mM phosphate, 2.7 mM potassium chloride and 140 mM sodium chloride, pH 6.8). Ultrapure water (Barnstead Nanopure Ultrapure Water System) was used for all preparation, unless otherwise stated. All solvents and chemicals of analytical grade were used as received.

2.2.2 Culture and plating of *E. coli*

Pure culture of *E. coli* O1:K1:H7 (ATCC[®] 11775) was grown in Luria Broth (LB, containing 10 g tryptone, 5 g yeast extract and 10 g sodium chloride) on an orbital shaker at 37 °C for 18 h. For enumeration, bacterial culture was serially diluted with 10 mM PBS, pH 6.8, spread plated on LB agar plate and incubated at 37 °C for 24 h. *E. coli* colonies

formed on the agar plates were then counted to determine the number of viable cells in colony-forming unit per milliliter (cfu mL⁻¹).

2.2.3 Fabrication of aluminum-modified platinum wire electrode

Homemade platinum wire electrodes were fabricated using micropipette tip, copper wire and platinum wire (76 µm diameter) embedded in epoxy resin. The electrode tip was polished with alumina slurries with decreasing particle size (1.0 µm then 0.3 µm). Sub-micrometer (0.3-0.5 µm) thick aluminum film was sputter-coated onto the surface of electrode tip (~ 3 mm diameter) using 99.999 % purity aluminum target and Denton discovery[®] 18 sputtering system (Denton Vacuum, New Jersey, United State of America) with sputtering power of 100 W in an atmosphere of research-grade argon at low pressure of 5×10^{-3} Torr. All electrodes were rinsed with ultrapure water and air-dried.

2.2.4 Surface contact anodization

Conversion of aluminum film to nanoporous alumina was performed using the surface contact anodization method developed in our research laboratory [19]. Anodization of aluminum-coated platinum wire electrodes was conducted using Apelex electrophoresis power supply model P304 minipac II (Apelex, Evry Cedex, France). Anodization was carried out by positioning a glass pipette coated with ca. 40 nm thick platinum film over the tip surface of an aluminum-coated electrode. The interior rim of glass pipette was insulated with polydimethylsiloxane to prevent the direct contact of anodizing solution, 0.1 M oxalic acid with the platinum film. 0.1 M oxalic acid was added into the interior portion of platinum-coated glass pipette. The contact junction between electrode and

Figure 2.1 Diagram of the fabrication procedure for alumina-modified platinum wire electrode using the surface contact anodization method.

Figure 2.2 Homemade alumina-modified platinum wire electrode.

glass pipette was subsequently sealed with Teflon tape to avoid solution leakage. As depicted in Figure 2.1, the anodization set-up consists of a power supply, a platinum mesh auxiliary electrode immersed in anodizing solution as the cathode and aluminum film as the anode. When anodizing voltage was applied, the area of aluminum film within the pipette contact region was progressively anodized. A homemade membrane-based alumina-modified platinum wire electrode is shown in Figure 2.2.

2.2.5 Construction of nanobiosensor and procedures for the detection of *E. coli*

After anodization, chemical etching of alumina-modified electrode was performed in 3 % H_3PO_4 for 15 min to enlarge the pore size of alumina nanochannels. The scanning electron micrographs of an unetched and a 15 min etched alumina-modified electrode are shown in Figure 2.3 A and B, respectively. Longer etching time is not desirable as it

Figure 2.3 Scanning electron micrographs of (A) an unetched nanoporous alumina electrode and (B) alumina surface after 15 min etching in 3 % phosphoric acid. Reprinted from Ref. [20] with permission from Elsevier (Copyright 2012 Elsevier).

would cause membrane disintegration. Etched electrode was cleaned with ultrapure water and air-dried. 10 μL of 0.1 mg mL^{-1} anti-*E. coli* antibody was applied onto the electrode tip and stored at 4 $^{\circ}\text{C}$ overnight. The electrode tip was subsequently rinsed with 10 mM PBS, pH 6.8 to remove unbound antibodies, followed by blocking of unreacted and non-specific adsorption sites by physical adsorption of 1 % (w/v) BSA in 10 mM PBS, pH 6.8 at room temperature for 1 h. The nanobiosensor was ready for use after rinsing with 10 mM PBS, pH 6.8. The electrochemical signal response of *E. coli* nanobiosensor was derived from the cyclic voltammetric (CV) signal toward 1.0 mM ferrocenemethanol (FcMeOH) in 10 mM PBS, pH 6.8. FcMeOH was selected as the redox probe because of its neutral charge and electrochemical reversible behavior [19]. The electrochemical measurement set-up comprised an Autolab PGSTAT128N potentiostat/galvanostat with Nova 1.6 software (Eco Chemie, Utrecht, Netherlands), the prepared nanobiosensor as the working electrode, silver/silver chloride (Ag/AgCl in 1 M KCl) as the reference electrode and platinum mesh (2 mm diameter) as the auxiliary electrode. Ten-fold serial dilution of *E. coli* cell culture from 10^7 to 10 cfu mL^{-1} was performed using 10 mM PBS, pH 6.8. Detection of *E. coli* was carried out by spreading 10 μL of *E. coli* solutions with different cell numbers onto the nanobiosensor surface and incubated at 37 $^{\circ}\text{C}$ for 1 h. Non-specifically bound *E. coli* cells and proteins were removed by rinsing the nanobiosensor surface with 10 mM PBS, pH 6.8. The nanobiosensor was then characterized using ten repetitive potential cycles at a scan rate of 50 mV s^{-1} and potential range from 0 to 0.6 V vs. Ag/AgCl in the analysis solution containing 1.0 mM FcMeOH.

2.2.6 Measurement of cells viability

Cells viability was monitored by probing the rate of enzyme catalytic oxidation of glucose using FcMeOH as the electron acceptor. A batch system was used in this study in which the nanobiosensor with captured *E. coli* was kept immersed in 25 mL of 1.0 mM FcMeOH in 10 mM PBS, pH 6.8. Concentration of glucose in the analysis solution was varied by adding aliquots of 1.0 M glucose stock solution containing 1.0 mM FcMeOH. The solution was mixed thoroughly prior to each electrochemical measurement. The CV signal response at different concentrations of glucose (10, 20, 40, 60, 80 mM) was recorded for 10^6 cfu mL⁻¹ live *E. coli* cells. The electrocatalytic response of VBNC and heat-killed *E. coli* cells were measured using the same procedures as stated above. VBNC *E. coli* cells were prepared using a chemical disinfection method, which was carried out by adding 1.0 mg L⁻¹ NaOCl solution to *E. coli* cell culture and incubated for at least 5 min.

2.3 Results and discussion

2.3.1 Electrochemical characterization of membrane-based *E. coli* nanobiosensor

The construction and operation of nanobiosensor are depicted in Scheme 2.1. Anti-*E. coli* antibody is physically adsorbed on the membrane surface and channel walls of alumina nanopores. The remaining empty sites are subsequently backfilled using BSA to block the non-specific binding. The nanobiosensor is rinsed with copious amounts of 10 mM PBS, pH 6.8 followed by repetitive potential cycling in the range from 0 to 0.6 V (vs. Ag/AgCl reference electrode) in 1.0 mM FcMeOH to obtain stable voltammetric current

Scheme 2.1 Schematics of the design of an electrochemical membrane-based nanobiosensor and its principle of operation in *E. coli* cell analysis. The enlarged drawing shows the enzyme catalytic oxidation of glucose in the presence of redox probe, FcMeOH shuttling between the nanobiosensor and cytoplasmic membrane of a captured *E. coli* cell.

response. The constructed nanobiosensor is then incubated with *E. coli* cell solution, and CV is carried out in the presence of 1.0 mM FcMeOH. Our research group has previously shown that the signal response of the same membrane-based nanobiosensor toward protein and virus relies on the amount of redox probe moving through the alumina nanochannels [21, 22]. This work shows that *E. coli* cells can also block the alumina nanopores that results in a change of the nanobiosensor signal response.

Figure 2.4 shows the typical cyclic voltammograms obtained at the membrane-based alumina-modified electrode after each step of the nanobiosensor construction, in an analysis solution containing 1.0 mM FcMeOH in 10 mM PBS, pH 6.8. As expected, the CV peak current increases after chemical etching attributed to the enlargement of pore openings and nanochannel diameters. The cross-sectional area of alumina nanochannels are reduced upon the immobilization of antibody and BSA, which obstruct the movement of redox probe [21]. A significant decrease in CV peak current is clearly observed after 1 h incubation of the nanobiosensor with 10^6 cfu mL⁻¹ *E. coli* cells at 37 °C. Due to the structural variation of nanoporous alumina membrane layers, the CV peak current responses of nanobiosensors toward 10^6 cfu mL⁻¹ *E. coli* cells vary in the range from 16.71 ± 1.53 nA for three different alumina-modified electrodes. In contrast, the signal response in the presence of *E. coli* cells ($I_{E. coli}$) when normalized against the signal response derived from the same nanobiosensor after antibody-BSA immobilization in the absence of *E. coli* cells ($I_{E. coli=0}$) give a standard error of 2.5 %. Therefore, normalization of nanobiosensor signal response against blank signal (after antibody-BSA immobilization) significantly enhances the precision and eliminates the need for calibration of individual nanobiosensor from the same batch of construction.

Figure 2.4 Cyclic voltammograms of an alumina-modified electrode after each step of the nanobiosensor construction procedure in the presence of 1.0 mM FeMeOH in 10 mM PBS, pH 6.8. Conditions: scan rate = 50 mV s^{-1} , potential range = 0-0.6 V, antibody concentration = 0.1 mg mL^{-1} , *E. coli* concentration = 10^6 cfu mL^{-1} .

As shown in Figure 2.5, a platinum electrode without the alumina membrane overlayer but surface coated with antibody and BSA gives relatively constant normalized signal response at increasing concentration of *E. coli*. On the contrary, the nanobiosensor constructed from a 15 min etched alumina-modified electrode coated with antibody and BSA produces typical decreasing normalized signal response, which is linear in relation to the logarithm of *E. coli* concentration. This indicates the alumina membrane overlayer is critical for sensitive biosensor performance during *E. coli* detection. Another control experiment using a nanobiosensor constructed without BSA

Figure 2.5 Normalized nanobiosensor signal response of (♦) a platinum electrode immobilized with antibody-BSA, (●) an unetched alumina-modified electrode immobilized with antibody-BSA, (■) an etched alumina-modified electrode immobilized with antibody without BSA, and (▲) an etched alumina-modified electrode immobilized with antibody-BSA. Nanobiosensor signal response toward *E. coli* cells ($I_{E.coli}$) are normalized against the signal response derived from the same nanobiosensor in the absence of *E. coli* cells ($I_{E.coli=0}$).

also shows response toward *E. coli* cells but of slightly lower sensitivity (smaller slope). In another control study, a nanobiosensor prepared from an unetched alumina-modified electrode with $\sim 10\text{-}30$ nm pore sizes [21] does not respond to *E. coli* cells at concentration below 10^5 cfu mL⁻¹, unlike the 15 min etched alumina-modified electrode ($\sim 50\text{-}100$ nm pore sizes). The poor working range of the nanobiosensor constructed from an unetched alumina-modified electrode is due the presence of remnant alumina barrier

layer, which reduces the heterogeneous rate constant for the electrochemical reaction of FcMeOH at electrode surface [19].

2.3.2 Analytical performance of *E. coli* nanobiosensor

The voltammetric current response derived from the nanobiosensor decreases in the presence of increasing concentration of *E. coli* ranging from 0 to 10^7 cfu mL⁻¹ (Figure 2.6 A). The normalized nanobiosensor signal response is logarithmically linear from 10 to 10^6 cfu mL⁻¹ *E. coli* ($R^2 = 0.999$) with an extremely low LOD of 22 cfu mL⁻¹ *E. coli* (Figure 2.6 B). The LOD is determined from the minimum *E. coli* cell concentration that produces a signal reduction equivalent to three times the standard deviation of signal in

A

B

Figure 2.6 (A) Cyclic voltammograms of a nanobiosensor in the presence of 1.0 mM FcMeOH in 10 mM PBS, pH 6.8 after capture of *E. coli* from solutions of different cell concentrations. (B) Calibration plot of normalized nanobiosensor signal response derived from the anodic peak current against the logarithm of *E. coli* cell concentration. Conditions: scan rate = 50 mV s⁻¹, potential range = 0-0.6 V, antibody concentration = 0.1 mg mL⁻¹.

the absence of *E. coli* cells. Other highly attractive features of the nanobiosensor are the simple procedure based on bioaffinity interaction between *E. coli* cells and antibodies with rapid analysis time of ca. 90 min, which is much shorter compared to the conventional ELISA-based methods (6-7 h) [23]. In addition, the nanobiosensor eliminates the need of additional labeling procedure such as the use of fluorescent dye-

Table 2.1 Comparison of the analytical performance in terms of limit of detection (LOD), linear working range, and analysis time of biosensing techniques, PCR, and ELISA.

Detection method	LOD	Linear range	Analysis time	Reference
Electrochemical membrane-based nanobiosensor	22 cfu mL ⁻¹	10-10 ⁶ cfu mL ⁻¹	1.5 h	This study
Amperometric indirect sandwich enzyme-linked immunosensor	6 × 10 ² cfu mL ⁻¹	6 × 10 ² -6 × 10 ⁷ cfu mL ⁻¹	1 h	[24]
Amperometric sensor based on PtNP coated gold nanoporous film	10 cfu mL ⁻¹	20-10 ⁶ cfu mL ⁻¹	-	[25]
Amperometric tyrosinase biosensor based on Fe ₃ O ₄ nanoparticle/CNT nanocomposite	10 cfu mL ⁻¹	20-10 ⁵ cfu mL ⁻¹	4 h	[26]
Electrochemical immunosensor based on Cu/AuNP labeled Ab	30 cfu mL ⁻¹	50-5 × 10 ⁴ cfu mL ⁻¹	2 h	[27]
Gold screen-printed-based EIS immunosensor	3.3 cfu mL ⁻¹	5-10 ⁸ cfu mL ⁻¹	1 h	[28]
EIS immunosensor based on anodized alumina membrane	10 cfu mL ⁻¹	10-10 ⁵ cfu mL ⁻¹	-	[29]
QCM immunosensor based on BIMP	23 cfu mL ⁻¹	-	4 h	[30]
Lectin-based surface plasmon resonance biosensor	3 × 10 ³ cfu mL ⁻¹	3 × 10 ³ -3 × 10 ⁸ cfu mL ⁻¹	-	[31]
Evanescent wave absorbance-based fiber optic biosensor	10 ³ cfu mL ⁻¹	10 ³ -10 ⁶ cfu mL ⁻¹	-	[32]
SERS-based multiplex sensor based on Ab-functionalized nanoparticles	10 ² -10 ³ cfu mL ⁻¹	-	45 min	[33]
SERS-based flow-through microarray immunosensor	4.5 × 10 ³ cfu mL ⁻¹	4.3 × 10 ³ -4.3 × 10 ⁵ cfu mL ⁻¹	-	[34]
5' nuclease direct quantitative PCR assay	1 cfu mL ⁻¹	-	< 3 h	[35]
Indirect ELISA	10 ⁴ cfu mL ⁻¹	-	6-7 h	[23]

Abbreviations: Ab: antibody, AuNP: gold nanoparticle, BIMP: beacon immunomagnetic nanoparticle, cfu mL⁻¹: colony-forming unit per milliliter, CNT: carbon nanotube, Cu: copper, EIS: electrochemical impedance spectroscopy, ELISA: enzyme-linked immunosorbent assay (ELISA), PCR: polymerase chain reaction, PtNP: platinum nanoparticle, QCM: quartz crystal microbalance, SERS: surface-enhanced Raman scattering.

labeled or enzyme conjugated [36, 37] secondary antibody. The detection performance of the membrane-based nanobiosensor outperforms ELISA methods, and optical biosensors using surface plasmon resonance (SPR), fiber optic and surface-enhanced Raman scattering (SERS) that generally give LOD in the range from 10^2 to 10^4 cfu mL⁻¹. Furthermore, the LOD of nanobiosensor is comparable to biosensors, which monitor redox products derived from enzyme reactions, highly sensitive impedimetric biosensors, piezoelectric biosensors based on quartz crystal microbalance, and quantitative PCR assay (Table 2.1). In terms of reproducibility, three different nanobiosensors prepared under identical condition are found to give a relatively constant and excellent relative standard deviation (RSD) of 0.6-2.5 % over five orders of magnitude change in *E. coli* cell concentration.

2.3.3 Nanobiosensor optimization

Analytical performance of the membrane-based nanobiosensor depends on two critical parameters, loading of antibody and nanochannel dimensions that influence the movement of redox probes. In this study, the nanobiosensor signal response is optimized with regard to the concentration of antibody and pH of solution. These are expected to affect the nanochannel dimensions and the capture of *E. coli* cells. The effect of different antibody concentrations and pH values on the loading amount of antibody on the nanobiosensor surface are shown in Figure 2.7 A and C. The antibody loading is measured from the change in nanobiosensor signal response before and after antibody immobilization. The signal response toward 1.0 mM FcMeOH after antibody immobilization is normalized against the signal response derived from the same

nanobiosensor prior to antibody immobilization, where no change in the normalized signal response (100 %) indicates zero antibody loading. The sensitivity of nanobiosensors prepared under these conditions is determined from the slope of normalized nanobiosensor signal response after incubation with *E. coli* solutions of different cell numbers (Figure 2.6 B and D).

By doubling the concentration of antibody from 0.05 to 0.1 mg mL⁻¹, the sensitivity of nanobiosensor (or signal response slope) derived from the logarithmic plot enhances by approximately 1.5 times. However, change in sensitivity is minimal when the concentration of antibody used to prepare the nanobiosensor is greater than 0.2 mg mL⁻¹ (Figure 2.6 B). The improved sensitivity is obtained at an intermediate antibody concentration of 0.1 mg mL⁻¹ (Figure 2.7) is thus, selected as the optimal condition in which low LOD and wide linear working range can be achieved.

On the other hand, influence of pH on the loading of antibody and the nanobiosensor signal response are investigated over five different pH values as shown in Figure 2.7 C and D, respectively. The loading amount of antibody increases when pH is increased from 3.8 to 6.8 but decreases at higher pH of 8.3 and 9.8. Maximum loading of antibody takes place at pH 6.8 (Figure 2.7 C), which is about midway between the isoelectric point of rabbit anti-*E. coli* antibody (isotype: IgG) at ~ pH 6.1-6.5 [38] and the known neutral pH of alumina at ~ pH 7.2 [39]. The trend compares favorably with a previously reported membrane-based virus nanobiosensor using virus-specific immunoglobulin M (IgM) antibody instead of immunoglobulin G (IgG), thus indicates similar interaction between antibody and alumina membrane surface, which are mainly

A

B

C

D

Figure 2.7 Effect of antibody concentration on (A) loading amount of antibody, and (B) nanobiosensor signal response toward *E. coli*. Effect of pH on (C) loading amount of antibody, and (D) nanobiosensor signal response toward *E. coli*. Loading amount of antibody is measured from the change in nanobiosensor signal response before and after the antibody immobilization. All nanobiosensor signal responses are normalized against the signal response derived from the same nanobiosensor before antibody immobilization (I_{membrane}) for (A) and (C) or absence of *E. coli* cells ($I_{E.coli=0}$) for (B) and (D). Deviations of $I_{E.coli=0}/I_{\text{membrane}}$ and $I_{E.coli}/I_{E.coli=0}$ from 100 % and 1 indicate antibody loading and presence of *E. coli* cells, respectively.

electrostatic with insignificant hydrophobic contribution [22]. In general, the trend in nanobiosensor sensitivity correlates with the change in antibody loading. However, at extreme pH of 8.3 and 9.8 (Figure 2.7 D), the nanobiosensor sensitivity shows considerable deviation which may be due to the variations in surface charge and structure of antibody proteins [40].

2.3.4 Specificity study

Specific detection of *E. coli* is highly important for accurate and reliable measurement of coliform bacteria, which used as an indicator for microbiological water quality and fecal contamination. In addition, high specificity performance of biosensor is useful for unambiguous quantitative determination of particular pathogenic bacteria. In this study, the specificity of nanobiosensor toward *E. coli* is evaluated against two other Gram-negative bacteria, *Salmonella typhimurium* (*S. typhimurium*) and *Serratia marcescens* (*S. marcescens*). The normalized signal response of three different nanobiosensors constructed using anti-*E. coli* antibody and after 1 h incubation with bacterial species

over four different concentrations (10^3 , 10^4 , 10^5 and 10^6 cfu mL⁻¹) are shown in Figure 2.8. Nanobiosensor response in terms of absolute current toward *E. coli* and two non-specific bacteria is shown in Table 2.2. At cell concentration of 10^6 cfu mL⁻¹, the change in nanobiosensor signal response toward *S. marcescens* and *S. typhimurium* constitute 22.6-28.4 % of the nanobiosensor signal change in the presence of *E. coli*. Moreover, the

Figure 2.8 Specificity test to compare the normalized nanobiosensor signal response toward *E. coli* and two other non-specific bacterial species, *S. marcescens* and *S. typhimurium* at four different concentrations (10^3 , 10^4 , 10^5 , and 10^6 cfu mL⁻¹). Control experiments carried out in the absence of any bacterial species is included for reference. Nanobiosensor signal response toward bacterial species (I_{cell}) is normalized against the signal response derived from the same nanobiosensor in the absence of bacterial species ($I_{\text{cell}=0}$).

Table 2.2 Absolute current response of nanobiosensor toward *E. coli*, *S. marcescens* and *S. typhimurium* at four different concentrations (10^3 , 10^4 , 10^5 and 10^6 cfu mL⁻¹).

Bacterium	Absolute current (nA)			
	10^3 cfu mL ⁻¹	10^4 cfu mL ⁻¹	10^5 cfu mL ⁻¹	10^6 cfu mL ⁻¹
<i>E. coli</i>	19.93 ± 1.17	18.83 ± 1.21	17.77 ± 1.51	16.71 ± 1.53
<i>S. marcescens</i>	22.45 ± 0.68	21.88 ± 1.15	21.29 ± 1.29	21.16 ± 1.27
<i>S. typhimurium</i>	23.14 ± 1.05	22.67 ± 1.02	22.25 ± 1.06	22.04 ± 1.21

interference from these non-specific bacterial species at cell concentration of 10^3 cfu mL⁻¹ is considerably lower at 8.2-12.4 % of the nanobiosensor signal change toward *E. coli*. In contrast, several immunosensing methods reported for the detection of *E. coli* suffer from severe interference (up to 50 %) with non-specific bacterial species [23, 27, 41]. The higher specificity of the electrochemical membrane-based nanobiosensor toward *E. coli* compared to non-specific bacterial species would be useful in the determination of *E. coli* in surface water sources, where the typical concentration of bacterial species is in the range from 6 to 1000 cells mL⁻¹ (*E. coli*) and 0.001 to 58 cells mL⁻¹ (*Salmonella* sp.) [42]. It is possible that a change in biosensor signal can be due to either lower concentration of *E. coli* or very high concentration of non-specific bacteria. In such a rare situation, microscopic estimation of cell numbers or measurement of total cell count can be employed to distinguish between different bacterial species.

2.3.5 Analysis of cells viability

In this work, D-glucose and neutral redox probe, FcMeOH are added to the bacterial cell solution in order to probe the activity of redox enzyme, D-glucose dehydrogenase (GDH)

found on the outer surface of cytoplasmic membrane of *E. coli* cells captured on the nanobiosensor. GDH oxidizes D-glucose to D-gluconolactone within the periplasmic space [43] using FcMeOH as the alternative electron acceptor to oxygen. Channel proteins located in the outer cell membrane allow free diffusion of low molecular weight species ($MW \leq 1000$) into the periplasmic space [44]. Hence, reduced and oxidized forms of FcMeOH can freely diffuse between the enzymes sites at the outer cytoplasmic membrane and the electrode surface. The mediated electron transfer process [43] is shown below:

VBNC *E. coli* cells were derived from live *E. coli* cells after immersion in 1 mg L⁻¹ NaOCl solution for at least 5 min. The effect of chemical disinfection on the culturability of *E. coli* cells at different incubation time with 1.0 mg L⁻¹ NaOCl solution was examined using plating method. It was found that live *E. coli* cells treated with 1.0 mg L⁻¹ NaOCl solution have completely lost their culturability after 5 min incubation. In contrast, control experiments using live *E. coli* cells in 10 mM PBS, pH 6.8 show typical culturability in standard microbiological media. The respective linear sweep voltammograms of live, VBNC, and dead *E. coli* cells upon successive addition of glucose solutions in 1.0 mM FcMeOH are represented in Figure 2.9 A, B, and C. Figure 2.9 A and B indicate increases in the nanobiosensor current response toward FcMeOH in the presence of glucose for live and VBNC *E. coli* cells, respectively. These increments in current are attributed to the redox cycling of FcMeOH by active redox enzyme, GDH

A

B

C

Figure 2.9 (A) Linear sweep voltammetric response of nanobiosensors with captured (A) live, (B) VBNC, and (C) dead *E. coli* cells toward successive addition of 10 and 80 mM glucose solutions. (—) Binding of *E. coli*, (---) addition of 10 mM and (···) 80 mM glucose solutions.

on the outer surface of cytoplasmic membrane, in the presence of glucose. In the potential range from 0.24 to 0.28 V (vs. Ag/AgCl) between the anodic and cathodic peaks, the membrane-based nanobiosensor is most sensitive toward the bacterial cell viability as the current response toward successive addition of glucose is significant. This is due to the coupling of enzyme catalytic reaction at the cytoplasmic membrane to the redox reaction at the electrode surface. Enzyme-glucose reaction generates the reduced form of FcMeOH, which subsequently diffuse out of the periplasmic space to the platinum electrode where it is oxidized. Increased current response is expected because higher amount of FcMeOH, which diffused from both the bulk solution and periplasmic space of cells, are undergoing redox reaction at electrode surface. Thus, changes in the enzyme kinetics can affect the kinetics of heterogeneous electron transfer and diffusive mass transfer of FcMeOH, which are limiting in this potential range. At potentials higher than 0.28 V, the current response observed for live and VBNC *E. coli* cells can be very similar and less reproducible due to the non-steady state situation which leads to depletion of current at high potentials. From 0.24 to 0.28 V (vs. Ag/AgCl), the change in current response for live (9.2 to 13.9 %) and VBNC (3.4 to 14.0 %) *E. coli* cells upon successive additions of 10 and 80 mM glucose solutions are higher than the RSD of 1.4 % for ten repetitive potential scanning at the same nanobiosensor. On the contrary, control experiment using heat-killed *E. coli* cells produces negligible decrease in signal response

Figure 2.10 Normalized signal response of nanobiosensors with captured (■) live and (◆) VBNC *E. coli* cells toward successive addition of glucose solutions (10, 20, 40, 60, and 80 mM). Lines are non-linear curve fits using Michael-Mentelis equation.

(-2.8 to 0.5 % as shown in Figure 2.9 C). Figure 2.10 shows the normalized nanobiosensor signal response of live and VBNC *E. coli* cells toward successive addition of glucose solutions. Non-linear curve fitting of the electrocatalytic current response at 0.28 V toward FcMeOH in the presence of glucose, normalized against the blank signal (after antibody-BSA immobilization), shows apparent Michaelis-Menten constant, K_M (D-glucose) values of 7.1 ± 1.2 mM and 45.3 ± 20.7 mM for live and VBNC *E. coli* cells, respectively (Figure 2.10). The similarity between apparent K_M value of GDH enzyme in live *E. coli* cells and reported K_M value of GDH enzyme (2.1 ± 0.2 mM) indicates this method is relevant to the monitoring of GDH enzyme activity in *E. coli* cells [45]. On the

other hand, the larger apparent K_M value observed in VBNC *E. coli* cells is likely due to either limitation by transport of glucose into the *E. coli* cells or lower enzyme activity, which requires larger amount of glucose to attain the maximum rate of enzyme activity.

2.4 Conclusions

In conclusion, the feasibility of electrochemical membrane-based nanobiosensor for sensitive and specific detection of *E. coli* cells using nanoporous alumina-modified electrode is demonstrated in this work. The excellent analytical performance includes a low LOD of 22 cfu mL⁻¹ with five orders of magnitude linear working range from 10 to 10⁶ cfu mL⁻¹, a rapid analysis time of ca. 90 min, and is able to identify *E. coli* from two other non-specific bacterial species at high concentration of 10⁶ cfu mL⁻¹. Potential application of the nanobiosensor in future works includes on-site measurement of water quality and development of sensitive biosensors for a wide variety of microbes where specific antibodies can be harvested. In addition, VBNC *E. coli* cells in the dormant state such as in some treated waters can be distinguished from live and dead *E. coli* cells using the same nanobiosensor design by monitoring of the enzyme catalytic oxidation of glucose using ferrocene species as the alternative electron acceptor to oxygen.

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Development of electrochemical membrane-based nanobiosensor for ultrasensitive detection of dengue virus

3.1 Introduction

Dengue virus (DENV) is a mosquito borne flavivirus, which is transmitted by mosquito vectors, particularly *Aedes aegypti* (*A. aegypti*) and *Aedes albopictus* [1]. Dengue diseases are prevalent in urban and suburban areas of tropical and subtropical regions including Asia, Africa, Central and South America, and the Pacific. The diseases are endemic with an estimated 2.5 to 3 billion people at risk from DENV infection in more than 100 countries worldwide, and there are approximately 100 million cases of dengue fever (DF) and 0.5 million cases of dengue haemorrhagic fever (DHF) annually [2]. Dengue viruses carry a single-stranded RNA genome, and occur as four closely related but genetically distinct serotypes, DENV-1, DENV-2, DENV-3, and DENV-4 [3]. DENV infection can be asymptomatic or causing undifferentiated fever, DF or life-threatening complication including DHF and dengue shock syndrome (DSS) [4]. Among different dengue serotypes, secondary infection with DENV-2 has proven to be responsible for the more severe symptoms of dengue diseases [5, 6]. Furthermore, DENV-2 is one of the most prevalent serotypes locally and hence, is an excellent model for the development of dengue serotype-specific detection tool. Apart from the virus detection in human serum

samples, mosquito surveillance is also useful for reliable and accurate detection of virus to reduce the infection rate in mosquito vector population, and to give early warning against an imminent outbreak. Additionally, it is an essential tool for the implementation of effective mosquito control strategy, and is a useful indicator of virus activity and the status of infectious disease transmission [7].

Conventional DENV detection methods include virus isolation and serological tests. Virus isolation provides a direct and reliable approach to detect virus. However, the time-consuming virus cultivation and identification procedures restrict the utility of this method in early disease diagnosis. Serological tests such as hemagglutination inhibition (HAI) test and enzyme immunoassays rely on the detection of virus-specific IgG and IgM antibodies. These methods possess several drawbacks such as considerable cross reactivity among flavivirus antibodies and need for multiple serum samples derived from the acute and convalescent phases of viral infection during serological tests, thus making it less efficient [8]. As discussed in Chapter 1, state-of-the-art molecular techniques such as RT-PCR and multiplex PCR are employed almost exclusively in laboratory and clinical detection of virus due to their high sensitivity, reliability, accuracy and potential for high throughput testing. However, these techniques involve complicated procedures and operation, and expensive instrumentation. Recently, a rapid, cost-effective and qualitative dengue NS1 antigen strip[®] test kit is commercially available to help guide mosquito control operation in the prioritization of resources for effective control of DENV transmission [9].

To date, biosensing techniques using nanopore technology have received great interests because of their suitability in detecting different biomolecules (i.e. DNAs and

proteins) and microbes (i.e. bacteria and viruses) [10, 11]. Niedzweicki *et al.* have developed an electrical biosensor for the detection of HIV biomarker using silicon nitride (SiN) membrane with nanostructure morphology [12]. Freedman *et al.* have reported a detection system based on multilayer nanopore with graphitic polyhedral edges for the detection of both single and double-stranded DNAs [13]. In another work, a thin SiN nanopore sensor has been reported by Wanunu *et al.* for rapid electronic detection of probe-hybridized microRNAs from cellular RNAs [14]. Joung *et al.* have fabricated a label-free impedimetric immunosensor based on hyaluronan-modified anodized alumina membrane for the detection of *E. coli* O157:H7 in PBS and whole milk samples with LODs of 10 cfu mL⁻¹ and 83.7 cfu mL⁻¹, respectively [15]. Bandila *et al.* have developed an electrochemical immunoassay for the detection of EBV-specific antibody using EBV antigen coated-nanoporous gold electrode and alkaline phosphatase-labeled secondary IgG antibody [16]. de la Escosura-Muñiz *et al.* have described an anodized alumina nanochannel/nanoparticle-based filtering and immunosensing platform for direct detection of cancer biomarker in blood samples [17].

Herein, the same nanobiosensor design described in Chapter 2 is adopted for the detection of DENV-2. This nanobiosensor has shown its capability in monitoring of the sensitive change in Faradaic current response toward a redox probe moving in and out of the alumina membrane nanochannels. The diffusive mass transfer rate of redox probe relies on the extent of blocking by the virus-antibody immunocomplexes formed within alumina nanochannels. In addition, direct detection of DENV-2 load in infected whole *A. aegypti* mosquito, and specificity test against two other non-specific viruses and other

commonly occurring serotype, DENV-3 reveal the potential application of this membrane-based nanobiosensor for mosquito surveillance in the field.

3.2 Experimental

3.2.1 Reagents and Materials

Mouse anti-dengue type 2 virus monoclonal antibody (clone: 3H5, isotype: IgG, 1.0 mg mL⁻¹) was obtained from Millipore. Bovine serum albumin (BSA, ~ 99 %), ferrocenemethanol (FcMeOH, 97 %), and oxalic acid (H₂C₂O₄, ≥ 99 %) were purchased from Sigma-Aldrich. Phosphoric acid (H₃PO₄, 85%) was obtained from Lancaster Synthesis. Potassium chloride (KCl, ≥ 99.5 %) was obtained from Sinopharm Chemical Reagent. Alumina powder (particle size: 0.3 and 1.0 μm) were obtained from Allied High Tech Products. Dengue type 2 virus (DENV-2), dengue type 3 virus (DENV-3), Chikungunya virus (CHIKV), and West Nile virus (WNV) were kindly provided by Prof. Vincent T. K. Chow, Prof. Mary M. L. Ng, and Suk Hiang Lau from the Department of Microbiology, Yong Loo Ling School of Medicine, National University of Singapore (NUS), and Dr. Katja Fink from the Singapore Immunology Network-Agency for Science, Technology and Research (SigN-A*STAR). All virus and protein solutions were prepared in 10 mM phosphate buffered saline (PBS, containing 10 mM phosphate, 2.7 mM potassium chloride and 140 mM sodium chloride, pH 7.4). Ultrapure water (Barnstead Nanopure Ultrapure Water System) was used for all preparations, unless otherwise stated. All solvents and chemicals of analytical grade were used as received.

3.2.2 Fabrication of alumina-modified platinum wire electrode and nanobiosensor

Alumina-modified platinum wire electrode (76 μm diameter platinum wire embedded in ~ 3 mm diameter inert epoxy resin sheath) was constructed following the procedures described in Sections 2.2.3 and 2.2.4. After anodization, alumina-modified electrode was etched in 3 % H_3PO_4 for 15 min to enlarge the pore openings of alumina nanochannels to ~ 50 - 100 nm [18], which is suitable for the immobilization of anti-DENV-2 IgG antibody (~ 16 - 19 nm dimension) and for the capture of DENV-2 particles (~ 40 nm diameter). Anti-DENV-2 antibody was physically adsorbed on the outer surface of alumina membrane overlayer and nanochannel walls by incubating 5 μL of 0.2 mg L^{-1} anti-DENV-2 antibody on the electrode tip for 1 h. Unbound antibodies were removed by rinsing the electrode tip with 10 mM PBS, pH 7.4. The antibody-immobilized electrode was subsequently treated with 5 μL of 1 % (w/v) BSA at room temperature for 30 min to block the non-specific and unreacted adsorption site. After rinsing with 10 mM PBS, pH 7.4, the nanobiosensor was ready for use.

3.2.3 Procedures for the detection of DENV-2

Detection of DENV-2 was conducted by applying 5 μL of virus solutions with different plaque-forming units per milliliter (pfu mL^{-1}) onto the nanobiosensor surface and incubating it at room temperature for 30 min. The nanobiosensor was then rinsed with 10 mM PBS, pH 7.4 to remove non-specifically bound viruses and proteins. The signal response of nanobiosensor toward 1.0 mM FcMeOH in 10 mM PBS, pH 7.4 was monitored using differential pulse voltammetry (DPV). The electrochemical set-up consists of a CHI 1220B potentiostat/galvanostat with data acquisition software (CH

Instruments, Texas, United State of America), the constructed nanobiosensor as the working electrode, silver/silver chloride (Ag/AgCl in 1 M KCl) as the reference electrode and platinum mesh (2 mm diameter) as the auxiliary electrode. All DPV measurements were conducted at 50 mV s⁻¹ scan rate, 25 mV pulse amplitude, 0.2 s pulse width, 0.06667 sampling width, and potential range from -0.2 to 0.6 V.

3.3 Results and discussion

3.3.1 Electrochemical characterization of membrane-based DENV-2 nanobiosensor

The design of nanobiosensor and its principle of operation in DENV-2 are depicted in Scheme 3.1. The typical differential pulse voltammograms in the presence of redox probe, FcMeOH, after each step of the construction procedure are shown in Figure 3.1 A. Similarly, the significant increase in peak current response observed after chemical etching in 3 % H₃PO₄ is due to the enlarged alumina pore openings and nanochannels, which permit more FcMeOH to reach the platinum electrode surface. Thereafter, successive addition of anti-DENV-2 antibody followed by BSA narrows the alumina nanochannels, and impedes the diffusive mass transfer of FcMeOH, therefore results in the reduction of DPV peak current response [18-20]. Physisorption of antibody onto the alumina membrane overlayer often leads to random orientation of antibodies. As a consequence, BSA is applied to backfill the empty sites and block the non-specific binding on nanobiosensor surface. The movement of FcMeOH to the platinum electrode surface below the alumina membrane is further blocked upon the subsequent binding of

Scheme 3.1 Schematics of the construction of an electrochemical membrane-based nanobiosensor and its principle of operation in DENV-2 detection.

A

B

Figure 3.1 Differential pulse voltammograms of alumina-modified electrode obtained in 1.0 mM FcMeOH in 10 mM PBS, pH 7.4 (A) after each step of the nanobiosensor construction procedure, DENV-2 concentration = 10^3 pfu mL⁻¹, and (B) after capture of virus from solutions of different virus concentration.

DENV-2 viral particles from a 100 pfu mL⁻¹ DENV-2 solution within the alumina nanochannels and nearer the pore openings, thus produces a sharp decrease in the signal response of nanobiosensor derived from DPV peak current [20]. Figure 3.1 B reveals that increasing concentration of DENV-2 leads to corresponding decrease in nanobiosensor signal response, which shows that this nanobiosensor can be used for quantitative detection of DENV-2. To further understand the analytical relation between nanobiosensor signal response and virus concentration, I performed fitting of the nanobiosensor signal response with a simple physical adsorption model.

3.3.2 Physical adsorption model for the nanobiosensor signal response

Due to the structural variations in alumina nanopore dimensions ($\sim 50\text{-}100\text{ nm}$) and aluminium thickness ($\sim 0.3\text{-}0.5\text{ }\mu\text{m}$) in different nanobiosensors, DPV peak current response for three different nanobiosensors ($I_{\text{DENV-2}}$) toward 100 pfu mL^{-1} DENV-2 gives a poor RSD of 18.6 %. Conversely, normalization of these signal responses against the signal response derived from the same nanobiosensor after antibody-BSA immobilization in the absence of DENV-2 ($I_{\text{DENV-2}=0}$) gives lower RSD of 1.1 %. Figure 3.2 A shows that the decrease in normalized nanobiosensor signal response $\{1-(I_{\text{DENV-2}}/I_{\text{DENV-2}=0})\}$ changes upon the capture of more DENV-2 viral particles from solutions containing higher virus concentrations, and then becomes less sensitive (smaller slope) at high virus concentration of 10^3 pfu mL^{-1} . The decrease in normalized nanobiosensor signal response reveals important information about the amount of captured DENV-2 viral particles within the alumina nanochannels of nanobiosensor. In a previously reported work on West Nile virus detection, such relation between the amount of captured viral particles and the normalized signal response of an Immunoglobulin M (IgM) antibody-based nanobiosensor was modeled using simple Langmuir isotherm, and expressed as a function of the viral antigen-IgM equilibrium dissociation constant, K [20] as follows,

$$\frac{1 - \left(\frac{I_{\text{virus}}}{I_{\text{virus}=0}} \right)}{\theta_{\text{max}}} = \frac{K[\text{virus}]}{1 + K[\text{virus}]} \quad (12)$$

where $\{1-(I_{\text{virus}}/I_{\text{virus}=0})\}$ (or surface coverage, θ) is normalized against the maximum surface coverage (θ_{max}) to provide the differences in the adsorption of viruses on the IgM

antibody-coated nanochannels at different virus concentration. Both θ and $\theta_{\max}$ preclude binding sites at the outer surface of alumina membrane overlayer, which also capture viruses but do not influence the overall signal response of nanobiosensor.

Equation (12) suggests negligible interaction between the adjacent viral antigen-antibody binding sites, and that every immunocomplex formed within the alumina nanochannels produces equivalent signal output. This can be explained by the large size of pentameric IgM antibody (35-39 nm) relative to the alumina nanochannel dimension (~ 50 -100 nm) such that when one immunocomplex is formed within each nanochannel, subsequent formation of immunocomplexes within the same nanochannel does not affect the overall DPV current response. In fact, only formation of immunocomplexes in different nanochannels is registered by the nanobiosensor.

On the contrary, the smaller monomeric IgG antibody used in this work is less effective in blocking the alumina nanochannel upon the formation of a single immunocomplex. As a consequence, the formation of second, third, or more immunocomplexes within the same alumina nanochannel can further decrease the nanobiosensor signal response. The magnitude of reduction in subsequent signal responses is expected to be lesser than the first signal response recorded during formation of the first immunocomplex. In the following, the nanobiosensor signal response are described using the Langmuir-Freundlich isotherm, which explains surface adsorption of molecules, and assumes that the most favorable surface binding takes place first, which also gives the largest change in surface coverage in comparison to the subsequent binding events. In this DENV-2 biosensing model, the binding extent of DENV-2 viral particles on the IgG antibody-coated alumina membrane nanochannel (θ) at a particular virus

concentration ([DENV-2]) can be correlated to the equilibrium dissociation constant (K) between DENV-2 and anti-DENV-2 antibody as follows:

$$\frac{1 - \left(\frac{I_{DENV-2}}{I_{DENV-2=0}} \right)}{\theta_{\max}} = \frac{K[DENV-2]^{\frac{1}{n}}}{1 + K[DENV-2]^{\frac{1}{n}}} \quad (13)$$

where $\{1 - (I_{\text{virus}}/I_{\text{virus}=0})\}$ (or surface coverage, θ) is measured from the decrease in normalized nanobiosensor signal response $\{1 - (I_{DENV-2}/I_{DENV-2=0})\}$. Maximum coverage ($\theta_{\max}$) represents the maximum decrease in normalized nanobiosensor signal response, and Freundlich constant, $n < 1$ as described in the Langmuir-Freundlich isotherm.

Rearrangement of equation (13) into the logarithmic expression (14) allows the experimental data to be plotted in a linear form,

$$\log \left(\frac{\theta}{\theta_{\max} - \theta} \right) = \log K + \frac{1}{n} \log [DENV - 2] \quad (14)$$

Non-linear curve fit between experimental data and equation (13) as represented in Figure 3.2 A suggests the binding of DENV-2 viral particles within the IgG antibody-coated alumina membrane nanochannels is well described by the Langmuir-Freundlich isotherm. The linear logarithmic calibration plot based on equation (14) is shown in Figure 3.2 B. More importantly, the equilibrium dissociation constant between DENV-2 and anti-DENV-2 antibody derived from the non-linear curve fitting is $1.45 \pm 0.44 \mu\text{M}$,

which is close to the general equilibrium dissociation constant of DENV-IgG antibody (1 nM-0.1 μ M) [21] based on a reported viral particle-to-pfu ratio of 100,000.

3.3.3 Analytical performance of DENV-2 nanobiosensor

Figure 3.2 B shows that the nanobiosensor signal response derived from the DPV peak current is logarithmically linear from 1 to 10^3 pfu mL^{-1} DENV-2. Similar to the detection performance of *E. coli* nanobiosensor reported in Chapter 2, the complete coverage of DENV-2 viral particles on the alumina membrane overlayer produces the same maximum decrease in normalized nanobiosensor signal response of 25 %. The LOD of nanobiosensor is determined from the minimum concentration of DENV-2 that produces

A

B

Figure 3.2 (A) Plot of nanobiosensor signal response toward DENV-2 in the presence of 1.0 mM FcMeOH in 10 mM PBS, pH 7.4. Line is non-linear curve fit using equation (13). Deviation of decrease in normalized nanobiosensor signal response $\{1-(I_{\text{DENV-2}}/I_{\text{DENV-2=0}})\}$ from 0 indicates the presence of DENV-2. (B) Logarithmic calibration plot of $\{\theta/(\theta_{\text{max}}-\theta)\}$ against DENV-2 concentration with best fit linear line (14). Symbols are average values of nanobiosensor signal response derived from three different alumina-modified electrodes.

a signal reduction equivalent to three times the standard deviation of signal in the absence of DENV-2. The extremely low LOD of 1 pfu mL^{-1} outperforms those techniques using ELISA and RT-PCR assays, and is comparable to the recent biosensing methods, nucleic acid sequence-based amplification (NASBA) assay and multiplex RT-PCR technique (Table 3.1). Most of the reported techniques depend on the detection of DENV nucleic

acids and products of molecular amplification. Hence, the whole virus sensing by this electrochemical membrane-based nanobiosensor offers an alternative promising platform for direct detection of viral particles. Three different nanobiosensors prepared under identical conditions give a significantly low RSD of 0.5-5.9 % over three orders of magnitude change in DENV-2 concentration, thus reveal good reproducibility of the nanobiosensor signal response. Furthermore, direct and label-free detection of DENV-2 viral particles eliminates the need for additional labeling procedures such as utilization of avidin-biotin interaction, fluorescent probes, nanomaterials, and enzyme conjugates. Other attractive features of this nanobiosensor include simple analysis procedures and rapid analysis time of ca. 50 min, which is much shorter than the conventional virus isolation, ELISA and state-of-the art molecular methods (Table 3.1).

3.3.4 Specificity study

High specificity performance is necessary for accurate identification of particular infectious viruses. The specificity of this electrochemical membrane-based nanobiosensor is evaluated against two other non-specific arthropod-borne viruses, Chikungunya virus (CHIKV) and West Nile virus (WNV) with prevalent occurrence in urban and suburban areas. The nanobiosensor specificity toward DENV-2, CHIKV and WNV derived from the difference in normalized nanobiosensor signal response toward viruses and a control comprising virus-free culture medium is shown in Figure 3.3 in which no change in the normalized signal response indicates zero virus loading. The decrease in normalized nanobiosensor signal responses $\{1-(I_{\text{virus}}/I_{\text{virus}=0})\}$ toward three viruses at concentration of 100 pfu mL⁻¹ are derived from the consecutive addition of culture medium (control),

Table 3.1 Comparison of the analytical performance in terms of limit of detection (LOD) and analysis time of biosensing, state-of-the-art molecular and conventional detection techniques.

Detection approach	Viral target (s)	LOD	Analysis time	Reference
Electrochemical membrane-based nanobiosensor	DENV-2, surface antigen	1 pfu mL ⁻¹	50 min	This study
EIS membrane-based nanobiosensor	DENV-2, surface antigen	1 pfu mL ⁻¹	50 min	[22]
Suction-type microfluidic immunosensing chip	DENV-2, surface antigen	10 pfu mL ⁻¹	30 min	[23]
Piezoelectric QCM biosensor based on DNA functionalized AuNP	DENV-2, cDNA	2 pfu mL ⁻¹	1.5 h	[24]
Fluorogenic RT-PCR	DENV-1-4, RNA	20-50 pfu mL ⁻¹	3-4 h	[25]
Fourplex real-time RT-PCR	DENV-1-4, RNA	100 pfu mL ⁻¹ (DENV-1) 0.4 pfu mL ⁻¹ (DENV-2-4)	3 h	[26]
Integrated micro RT-PCR	DENV-2, RNA	100 pfu mL ⁻¹	4 h	[27]
SYBRGreen I-based real time RT-PCR	DENV-1-4, RNA	4-10 pfu mL ⁻¹	-	[28]
NASBA assay	DENV-1-4, RNA	1-10 pfu mL ⁻¹	~ 5 h (20 samples)	[29]
ELISA	DENV-2	10 ³ pfu mL ⁻¹	> 4 h	[23]
RT-PCR	DENV, RNA	1-10 ³ pfu mL ⁻¹	Hours	[30]
Real-time RT-PCR	DENV, RNA	1-50 pfu mL ⁻¹	Hours	
Conventional virus isolation	Viable DENV	1-10 infectious DENV	Days-weeks	

Abbreviations: AuNP: gold nanoparticle, cDNA: complementary deoxyribonucleic acid, DNA: deoxyribonucleic acid, DENV: dengue virus, DENV-1: dengue type 1 virus, DENV-2: dengue type 2 virus, DENV-3: dengue type 3 virus, DENV-4: dengue type 4 virus, EIS: electrochemical impedance spectroscopy, ELISA: enzyme-linked immunosorbent assay, NASBA: nucleic acid sequence-based amplification, pfu: plaque-forming unit per milliliter, RNA: ribonucleic acid, RT-PCR: reverse transcriptase-polymerase chain reaction, QCM: quartz crystal microbalance.

CHIKV, WNV, and DENV-2 (Figure 3.3 A). The procedures involve addition of 5 μ L of culture medium or virus solutions onto the nanobiosensor surface and incubation for 30 min. The nanobiosensor surface was subsequently rinsed with copious amount of 10 mM PBS, pH 7.4 followed by DPV measurement in 1.0 mM FcMeOH. Nanobiosensor signal responses after the addition of each virus (I_{virus}) is normalized against the nanobiosensor signal response derived from a virus-free culture medium after antibody-BSA immobilization ($I_{\text{virus}=0}$). Figure 3.3 A shows that the decrease in normalized nanobiosensor signal response $\{1-(I_{\text{virus}}/I_{\text{virus}=0})\}$ toward DENV-2 is approximately 2.5 times higher compared to CHIKV and WNV. The same experiment was repeated in reverse order by introducing DENV-2 prior to the addition of CHIKV and WNV. As

A

B

Figure 3.3 (A) Decrease in normalized nanobiosensor signal response $\{1-(I_{\text{virus}}/I_{\text{virus}=0})\}$ toward DENV-2, CHIKV and WNV. I_{virus} represents the nanobiosensor signal response after capture of virus from 100 pfu mL^{-1} virus solution and $I_{\text{virus}=0}$ represents the signal response of the same nanobiosensor after 30 min exposure to a virus-free culture medium after antibody-BSA immobilization. 30 min incubation and subsequent DPV measurement for DENV-2, CHIKV, and WNV are conducted respectively in consecutive steps using the same nanobiosensor. (B) The same procedure was performed in reverse order using second nanobiosensor.

shown in Figure 3.3 B, it can be noticed that in the reverse order, the nanobiosensor exhibits excellent sensitivity toward DENV-2, but shows insignificant change in the signal response after subsequent exposure of the same nanobiosensor to CHIKV and WNV. This is due to the specific binding of DENV-2 viral particles onto the IgG antibody-coated alumina pore openings and nanochannels, which prevents the entry and

non-specific binding of CHIKV and WNV inside farther regions of the alumina nanochannels.

Differentiation of the dengue serotypes remains a challenge for scientists because all four serotypes of DENV share common amino acid sequences and epitopes [31, 32]. To date, there are few methods that are capable of distinguishing different dengue serotypes besides RT-PCR and other related molecular techniques [25-28]. To examine the utility of the membrane-based nanobiosensor in differentiating DENV-2 from dengue type 3 virus (DENV-3), which is the other commonly occurring dengue serotype in the Southeast Asia region [7, 33, 34], the nanobiosensor signal responses toward consecutive addition of virus-free culture medium (control), DENV-3 and DENV-2 are obtained

A

B

Figure 3.4 (A) Decrease in normalized nanobiosensor signal response $\{1 - (I_{\text{virus}} / I_{\text{virus}=0})\}$ toward DENV-2 and DENV-3. I_{virus} represents the nanobiosensor signal response after capture of virus from 100 pfu mL^{-1} virus solution and $I_{\text{virus}=0}$ represents the signal response of the same nanobiosensor after 30 min exposure to a virus-free culture medium after antibody-BSA immobilization. 30 min incubation and subsequent DPV measurement for DENV-2 and DENV-3 are conducted respectively in consecutive steps using the same nanobiosensor. The nanobiosensor signal responses toward DENV-2 and DENV-3 are significantly different at 90 % confidence interval. (B) The same procedure was performed in reverse order using second nanobiosensor.

using the same procedures as described in Figure 3.3. Figure 3.4 A shows that at virus concentration of 100 pfu mL^{-1} , the change in nanobiosensor signal response toward DENV-2 is 1.6 times greater than toward DENV-3. Using t -test, these two signal responses are significantly different from each other at 90 % confidence interval.

Conversely, the consecutive signal response of second nanobiosensor indicates different results when the order of DENV-3 and DENV-2 is reversed. Figure 3.4 B reveals negligible change in the nanobiosensor signal response between DENV-3 and DENV-2, thus indicates the nanobiosensor is sensitive and selective to DENV-2.

A further control experiment was conducted using an alumina-modified electrode constructed in the same way as described in Figure 3.2 but without the immobilization of anti-DENV-2 antibody. The nanobiosensor signal response toward DENV-2 remains fairly constant over virus concentration range from 0 to 10^3 pfu mL⁻¹ (data not shown). This indicates that the nanobiosensor signal response arise from the capture of DENV-2 viral particles within the nanoporous matrix is strongly influenced by the specific viral antigen-antibody interaction.

3.3.5 Detection of DENV-2 in *Aedes aegypti* mosquito infected with DENV-2

To demonstrate the utility of the nanobiosensor in virus detection, standard addition method was employed to determine the viral load of a DENV-2 infected *A. aegypti* mosquito. 100 µL of 10 mM PBS, pH 7.4 was added into a microcentrifuge tube containing a single heat-inactivated DENV-2 infected mosquito, and the mixture was ground to homogeneity. The solution was centrifuged at 14,000 rpm for 3-5 min, and the supernatant solution was harvested to form the test sample for subsequent analyses. The test sample was further divided into three equal volume solutions, and two of these solutions were spiked with different volumes of solution containing 100 pfu mL⁻¹ of DENV-2 to make up two different spiked concentrations. All three solutions were eventually made up to equal volumes using 10 mM PBS, pH 7.4. The nanobiosensor

signal response from a non-spiked sample and two spiked samples were plotted as a multiple standard addition calibration plot and extrapolated to $\{1-(I_{\text{DENV-2}}/I_{\text{DENV-2=0}})\} = 0$ to derive the unknown concentration of test sample. Using this method, the DENV-2 load in an infected mosquito sample was found to be 2968 pfu mL⁻¹. In contrast, the same measurement performed on two uninfected mosquitoes gave considerably lower results at 124 ± 28 pfu mL⁻¹. To compare with quantitative real-time RT-PCR assay, DENV-2 RNA was extracted from the same infected mosquito sample using QIAamp viral RNA mini kit (QIAgen, Hilden, Germany). Real-time RT-PCR was conducted in LightCycler 2.0 (Roche, Mannheim, Germany) using QuantiTech SYBR Green RT-PCR kit (Qiagen, Hilden, Germany) that targets a 177 base pairs region of the non-structural protein 5 (NS5) gene [9]. The viral load of DENV-2 in the same infected mosquito sample was determined to be 1.43×10^8 RNA copies mL⁻¹, which gives a viral particle-to-pfu ratio of ca. 48,000 when compared with the nanobiosensor result. This is consistent within an order of magnitude with the reported viral particle-to-pfu ratio of 100,000 [23].

3.4 Conclusions

In conclusion, an ultrasensitive and specific electrochemical membrane-based nanobiosensor is developed for the detection of DENV-2 using nanoporous alumina-modified electrode. The nanobiosensor achieved an extremely low LOD of 1 pfu mL⁻¹ with a three orders of magnitude linear working range from 1 to 10³ pfu mL⁻¹. Other excellent performance of the nanobiosensor includes short analysis time of ca. 50 min and selective detection of DENV-2 against three other non-specific viruses, DENV-3, CHIKV and WNV. Furthermore, the specific identification and quantification of DENV-

2 load in infected mosquito samples suggests the potential development of this nanobiosensor into rapid, cost-effective, and portable test kit for dengue disease diagnosis at point-of-care, besides profiling the virus activity in mosquito vector populations.

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Impedimetric microbial biosensor for real-time monitoring of bacteriophage infection of *E. coli*

4.1 Introduction

Filamentous bacteriophage (phage) M13 (~ 6 nm diameter and ~ 0.8 μm length) is a rod-shaped non-lytic virus, which selectively infects male *E. coli* that carry F-pili [1, 2]. Unlike most lytic phages, release of progeny phage M13 particles does not lyse the infected host cells, which continue to grow and propagate at a slower rate [3]. Infection begins with the adsorption of phage M13 to the tip of F-pili found on the surface of *E. coli*. F-pili subsequently retract bringing phage M13 closer to the bacterial co-receptor TolA, followed by translocation of phage circular single-stranded DNA (ssDNA) into the host bacterium. Thereafter, the DNA and RNA polymerases of host convert the ssDNA into a super-coiled double-stranded DNA (dsDNA), known as the replicative form. These dsDNA serve as templates for phage gene expression, resulting in further replication (Scheme 4.1) [4-7]. In addition, non-lytic phages are widely used as molecular tools to generate specific biochemical reagents for high-throughput assays in therapeutic applications [8], and for gene transfer using genetic recombinant technologies [9].

Scheme 4.1 Schematic diagram illustrates the infection mechanism of filamentous bacteriophage M13.

Traditional plaque assay which has been widely employed for the quantification of virus titer is time-consuming, incapable of operating in real-time, and not feasible in detecting non-lytic viruses such as filamentous phages M13 and f1. Until now, study of phage-bacterium interaction is mainly conducted using *in-situ* microscopic methods such as confocal laser scanning [10], fluorescence and optical [11] microscopy. These methods remain inadequate because non-lytic phage infection often leads to negligible change in the bacterial cell surface morphology, which is hardly detectable using these microscopic

methods. On the other hand, electron microscopy is highly relevant for *ex-situ* visualization of the structural change in bacterial cell surface caused by phage infection. However, this microscopic method uses complicated procedure and operation, and not feasible in monitoring and visualizing real-time phage infection of bacteria [10, 12, 13]. Atomic force microscopy (AFM) serves as a promising tool for the investigation of phage-bacterium interaction based on the change in surface rigidity of bacterium after phage infection [14, 15].

In spite of the enormous contribution made by microscopic methods in analyzing phage-bacterium interaction, the physical and morphological changes of phage M13 infected *E. coli* cells are hardly distinguishable from uninfected *E. coli* cells. After phage DNA and coat protein have integrated into the *E. coli* cells, the progeny phages M13 that assembled at the bacterial cell membrane are secreted by *E. coli* along with the release of lipopolysachharide (LPS) found on the outer membrane surface of *E. coli* [16]. It is believed that the presence of LPS, which are anchored to the bacterial outer membrane via their lipid moiety facilitate bacterial adhesion through the formation of hydrogen bonds. The core region of LPS comprising negatively charged carboxylic and phosphate groups that give an overall negative charge to the bacterial outer surface. The outer polysaccharide part of LPS is made up of O-antigens responsible for the immunological interaction of Gram-negative bacteria [17, 18]. In 1970, Roy and Mitra have shown that there are insignificant changes in bacterial cell structure, growth rate, gross chemical composition, average volume, and size of *E. coli* cells after infection with filamentous phages. However, the infected *E. coli* become fragile and softer after losing their surface rigidity due to the damage and release of LPS [14, 19].

Herein, the strategy adopted to investigate phage M13 infection of *E. coli* is to construct an electrochemical impedance spectroscopic (EIS) biosensing system using mercaptoacetic acid (MACA)-modified gold electrode with a self-assembled monolayer (SAM) of anti-*E. coli* antibody. EIS techniques offer sensitive, non-invasive and automated approach for the analysis of phage infection where the impedance signal response, which is sensitive to the small changes in bacterial morphology and surface charge, is monitored in real-time throughout the course of phage infection. The appealing features of this impedimetric microbial biosensor include a monolayer of bacteria that provide rapid response to phage infection. Secondly, the surface immobilized monolayer of covalently attached antibody would be expected to achieve a short preparation time of approximately 30 min, which is sufficient for near equilibrium bacterium-antibody binding. Additionally, this biosensing approach can be generalized to other virus-host system provided specific antibodies can be obtained for different bacterial species. Furthermore, microbial biosensors operating at specific potentials can reduce signal interference and improve biosensor sensitivity, even without the cell labeling and modification steps [20]. Apart from the cells viability analysis by monitoring of the electrocatalytic currents derived from the bacterial enzyme activity as described in Chapter 2, this microbial biosensing system provides an alternative approach to the monitoring of bacterial cells viability based on filamentous phage infection.

4.2 Experimental

4.2.1 Reagents and Materials

Rabbit anti-*E. coli* polyclonal antibody (isotype: IgG, 4.0 mg mL⁻¹) was purchased from AbD Serotec. *E. coli* O1:K1:H7 (ATCC® 11775) was obtained from ATCC. Phage M13 was obtained from New England Biolabs. Hydrochloric acid (HCl, 37 %), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC, ≥ 97 %), potassium hexacyanoferrate (III) (K₃Fe(CN)₆, ~ 99 %) and mercaptoacetic acid (MACA, ≥ 98 %) were purchased from Sigma-Aldrich. Ethanol (C₂H₅OH, ≥ 99.9 %), hydrogen peroxide (H₂O₂, 30 %), N-hydroxysuccinimide (NHS, ≥ 97 %) and sulfuric acid (H₂SO₄, 95-97 %) were purchased from Merck. Potassium chloride (KCl, ≥ 99.5 %) and sodium hydroxide (NaOH, 99 %) were obtained from Sinopharm Chemical Reagent. Alumina powder (particle size: 0.05, 0.3 and 1.0 μm) were purchased from Allied High Tech Products. All *E. coli*, phage and protein solutions were prepared in 10 mM phosphate buffered saline, pH 7.4 (PBS, containing 10 mM phosphate, 2.7 mM potassium chloride and 140 mM sodium chloride, pH 7.4). Ultrapure water (Sartorius Ultrapure Water System) was used for all preparations, unless otherwise stated. All solvents and chemicals of analytical grade were used as received.

4.2.2 Pretreatment of gold electrode

A gold electrode (2 mm diameter, CH Instruments, Texas, United State of America) was polished with alumina slurries (1.0 followed by 0.3 and 0.05 μm particle size) for approximately 20 min respectively and sonicated in water for 5 min. The polished electrode was pretreated with 1 M NaOH for 20 min followed by 1 M HCl for 5 min and

Piranha solution (1:3 mixture of 30 % H_2O_2 /concentrated H_2SO_4) for 5 min to obtain a clean and activated gold surface. After pretreatment, the electrode was rinsed with ethanol and ultrapure water successively. Finally, the electrode was cleaned in 50 mM H_2SO_4 using CV (Parameters: 12 cycles, scan rate = 100 mV s^{-1} , potential range = - 0.4-1.4 V) and dried in a stream of nitrogen gas. This electrochemical polishing step produces a reproducible gold surface.

4.2.3 Construction of microbial biosensor and monitoring of phage infection

SAM-modified gold electrode was fabricated by immersing a pretreated gold electrode in 1.0 % (w/v) MACA in ethanol solution for ca. 24 h. After rinsing with ethanol and drying, the MACA-modified gold electrode was treated with a solution containing 0.4 M EDC and 0.1 M NHS for 1 h to convert the terminal carboxylic acid group to active NHS ester group. The electrode was subsequently rinsed with water and dried. $10 \mu\text{L}$ of 1.0 mg mL^{-1} anti-*E. coli* antibody was added onto the electrode surface and stored at 4°C overnight. The O-antigen containing LPS can be specifically recognized by the anti-*E. coli* antibody selected in this study (selective toward all O- and K-antigenic serotypes of *E. coli*). Unbound antibodies were removed by rinsing the electrode with copious amount of 10 mM PBS, pH 7.4. The microbial biosensor was then constructed by applying $10 \mu\text{L}$ of 10^4 cfu mL^{-1} *E. coli* (Culture and plating of *E. coli* cells was described in Chapter 2 Section 2.2.2) onto the electrode surface and allowing incubation for 1 h. Non-specifically bound antibodies and *E. coli* cells were removed by rinsing the electrode with 10 mM PBS, pH 7.4. Phage M13 infection was monitored using a batch system in which the prepared microbial biosensor was kept immersed in 10 mL of $2.5 \text{ mM Fe(CN)}_6^{3-/4-}$ in

10 mM PBS, pH 7.4 at room temperature. 100 μL of stock solution of 5×10^6 phage mL^{-1} phage M13 was added into the analysis solution and infection up to 20 h was monitored using EIS technique. CV and EIS measurements were carried out using Autolab potentiostat/galvanostat PGSTAT128N and FRA2 module with Nova 1.6 software (Eco Chemie, Utrecht, Netherland) in one-compartment three-electrode cell comprising the microbial biosensor as the working electrode, silver/silver chloride (Ag/AgCl in 1 M KCl) as the reference electrode and platinum mesh as the auxiliary electrode. CV was conducted at a scan rate of 50 mV s^{-1} in a potential range from -0.2 to 0.7 V while electrochemical impedance spectra were recorded at an excitation amplitude of 10 mV, sine-modulated AC potential of 200 mV, and frequency range from 0.1 Hz to 1MHz.

4.3 Results and discussion

4.3.1 Electrochemical impedance spectroscopic characterization

Schematic diagram for the construction of a SAM-based microbial biosensor and monitoring of phage infection is depicted in Scheme 4.2. Figure 4.1 shows the typical Nyquist plots obtained using a gold electrode after each step of the construction procedure in $2.5 \text{ mM Fe(CN)}_6^{3-/4-}$. A monolayer of MACA is self-assembled on the gold electrode surface via strong gold-thiolate bond. The thiol groups form a reproducible, oriented, well-ordered and ultrathin layer with the carboxylic tail groups exposed at the SAM-liquid or SAM-air interface [21]. As expected, electron transfer resistance (R_{et}) value increases (semi-circle in the Nyquist plot becomes larger) after the formation of MACA-SAM attributed to its insulating nature, which suppresses the penetration of

Scheme 4.2 Schematics of the construction of microbial biosensor and the *in-situ* monitoring of phage M13 infection using electrochemical impedance spectroscopy. “FC” represents $\text{Fe}(\text{CN})_6^{3-/4-}$, “LPS” represents lipopolysaccharide.

Figure 4.1 EIS Nyquist plot of a microbial biosensor after each step of the construction procedure in the presence of 2.5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ in 10 mM PBS, pH 7.4. Lines are fitted using the Randles equivalent circuit. Conditions: frequency range = 0.1 Hz-10 MHz, excitation amplitude = 10 mV, sine-modulated AC potential = 200 mV, phage concentration = 5×10^4 phage mL^{-1} .

redox probe, $\text{Fe}(\text{CN})_6^{3-/4-}$ at the gold electrode surface [22]. After MACA-SAM is activated with NHS/EDC, the negatively charged carboxylic tail groups of MACA are replaced by the positively charged NHS ester groups. The electrostatic interaction between reactive NHS ester intermediate and negatively charged redox probe, $\text{Fe}(\text{CN})_6^{3-/4-}$ enhances its penetration toward the underlying gold electrode, thus resulting in a slight decrease in R_{et} value. In the next step, the NHS ester groups are replaced by the anti-*E. coli* antibodies through amine coupling between the primary amine group of antibody and the carboxylic group of MACA. Amine covalent binding of antibody stabilizes the biorecognition layer by preventing solvent mediated desorption [23]. Finally, microbial

biosensor is constructed through the specific binding between *E. coli* and immobilized antibodies. Immobilization of antibody and subsequent specific binding of *E. coli* impede the electron transfer process at the electrode/solution interface to greater extents, and result in considerable increases in R_{et} values due to the insulating and hydrophobic nature of protein and bacterial cell membrane.

In this work, *E. coli* cells are infected with phage M13 at a high concentration because of the weak adsorption of phage M13 to the host cell [24]. As shown in Figure 4.2 A, decreases in the R_{et} values (semi-circle in the Nyquist plot becomes smaller) are observed after 5 h and longer infection time, and these reductions could be attributed to several possibilities. Firstly, damage to the O-antigen containing LPS shatters the antigen

A

B

Figure 4.2 (A) EIS Nyquist plot of the *in-situ* monitoring of phage M13 infection of *E. coli* in the presence of 2.5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ in 10 mM PBS, pH 7.4. Lines are fitted using the Randles equivalent circuit. (B) Reciprocal plot of the electron transfer resistance values at different phage infection time normalized against the control R_{et}^0 value obtained prior to phage infection. Conditions: frequency range = 0.1 Hz-10 MHz, excitation amplitude = 10 mV, sine-modulated AC potential = 200 mV, phage concentration = 5×10^4 phage mL^{-1} .

-antibody binding, thus reduces the blockage to the penetration of $\text{Fe}(\text{CN})_6^{3-/4-}$ to the underlying gold electrode. Secondly, the release of negatively charged LPS reduces cell adhesion, and facilitates the penetration of negatively $\text{Fe}(\text{CN})_6^{3-/4-}$ toward the gold electrode surface [17]. Thirdly, the removal of LPS, which is believed to be responsible for the structural rigidity of bacteria, induces slight surface morphological change in the

Figure 4.3 Randles equivalent circuit of the impedimetric microbial biosensor.

infected *E. coli* [14, 25], and this may possibly allow aggregation of redox probes in the space between *E. coli* and underlying gold electrode. These three hypotheses are further verified in the following section by fitting the EIS experimental data with the Randles equivalent circuit as represented in Figure 4.3 using the EIS Spectrum Analyzer version 0.1 b software.

4.2.2 Fitting of EIS data with the Randles equivalent circuit

Randles circuit is an equivalent electrical circuit that describes an electrode undergoing a heterogeneous electron transfer process. It consists of an ohmic resistance of the bulk solution (R_s), constant phase element (CPE), electron transfer resistance (R_{et}), and Warburg impedance (Z_w). Z_w is a diffusion circuit element commonly used to model the

semi-infinite linear diffusion of redox probe to the electrode surface. It has a constant phase angle of 45° , which is independent of frequency. The magnitude of Z_w is inversely proportional to the square root of radial frequency (ω) as follows,

$$Z_w = \frac{A_w}{\sqrt{\omega}} - \frac{A_w}{j\sqrt{\omega}} \quad (15)$$

$$|Z_w| = \sqrt{2} \frac{A_w}{\sqrt{\omega}} \quad (16)$$

where A_w is the Warburg coefficient, and j is the imaginary number, and ω is related to frequency (f) as follows,

$$\omega = 2 \pi f \quad (17)$$

In addition, the non-ideal behavior of double layer capacitance at the antibody-coated gold electrode surface is modeled using constant phase element, which consists of admittance (Q) and frequency independent exponent (n). Constant phase element is more appropriate compared to capacitor in this work because the antibody-coated electrode surface is expected to be not uniform and comprises regions with different localized charges due to the coating of antibody. Also, the wide variation in the antibody protein surface charge and charge density can contribute significantly to the difference in the overall electrode surface capacitance under the influence of frequency.

All currents that pass through uncompensated resistance of the bulk analysis solution is made up of Faradaic current (I_f) and capacitive or non-Faradaic current (I_c) in the branch circuits where constant phase element is connected in series with a parallel combination of Z_w and R_{et} . Table 4.1 shows the fitted values of all equivalent circuit elements for each step of the biosensor construction procedure and different phage infection time. From Table 4.1, R_s remains almost constant throughout the experiment since it represents the properties of bulk solution and thus, not affected by the reaction taking place at the electrode surface. In contrast, the initial increment in A_w at early phage infection stage indicates enhanced resistive obstruction to the diffusive mass transfer of $Fe(CN)_6^{3-/4-}$ toward underlying gold electrode, which could be possibly due to the blockage of diffusion paths of $Fe(CN)_6^{3-/4-}$ by the negative charge of phages or non-specifically bound phages. On the other hand, R_{et} and constant phase element depend on the insulating and dielectric features at the electrode/solution interface, respectively. The somewhat unchanged constant phase element values suggest negligible change in the electrode surface charge during phage infection. Decrease in the fitted R_{et} values after 5 h phage infection is consistent with the decreasing semi-circle diameter in the Nyquist plot (Figure 4.2 A), and verifies the above statements where the rate of electron transfer gradually increases with phage infection time. This electron transfer rate constant, k relies on the surface coverage (θ) of the blocking film of *E. coli* according to equation (1) as follows [26],

$$k = k' (1 - \theta) \quad (18)$$

where k' represents the heterogeneous electron transfer of redox probe at the unblocked electrode surface.

The heterogeneous electron transfer rate constant (k) can be determined from the R_{et} values in Table 4.1 using the relation between R_{et} and exchange current (I_0), and the dependence of I_0 on k and concentration of redox probe (C) as follows,

$$R_{et} = \frac{RT}{FI_0} \quad (19)$$

$$I_0 = FAkC \quad (20)$$

where R is the universal gas constant, T is the temperature, A is the area of electrode, and F is the Faraday constant.

Electron transfer resistance (R_{et}) is inversely proportional to the electron transfer rate constant (k). Due to the blocking effect of surface bound *E. coli*, k for $\text{Fe}(\text{CN})_6^{3-/4-}$ at the *E. coli*-coated gold electrode is estimated to be $2.2 \times 10^{-4} \text{ cm s}^{-1}$ (2mm diameter electrode and 2.5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$). On the contrary, k for $\text{Fe}(\text{CN})_6^{3-/4-}$ at the bare gold electrode and IgG antibody-immobilized gold electrode are found to be 1.6×10^{-2} and $5.6 \times 10^{-4} \text{ cm s}^{-1}$, respectively. Therefore, it is estimated that ~ 96 % of the electrode surface is covered with IgG antibody and after which, ~ 61 % of the IgG antibody-immobilized electrode surface is covered with *E. coli* prior to phage infection. The electron transfer rate constant (k) increases further upon phage infection as LPS is removed from the bacterial outer membrane and this reduces cell adhesion because of the decreased LPS-

Table 4.1 Fitted values and fitting errors of elements in the Randles equivalent circuit for an experiment using a microbial biosensor in the presence of phage M13.

	$R_s(\Omega)$	$R_{ct}(k\Omega)$	$A_w(k\Omega s^{-1/2})$	$Q(\mu\Omega^{-1} s^n)$	n
Bare gold	114.16 $\pm$ 0.59	0.21 $\pm$ 0.01	1.65 $\pm$ 0.01	21.72 $\pm$ 1.05	0.64 $\pm$ 0.00
Coated with MACA	128.50 $\pm$ 0.35	12.49 $\pm$ 0.04	1.73 $\pm$ 0.01	1.27 $\pm$ 0.03	0.94 $\pm$ 0.00
NHS/EDC activation	126.07 $\pm$ 0.27	0.94 $\pm$ 0.07	1.66 $\pm$ 0.01	1.01 $\pm$ 0.04	0.92 $\pm$ 0.01
Immobilized with IgG	124.05 $\pm$ 0.39	6.01 $\pm$ 0.07	2.35 $\pm$ 0.01	1.57 $\pm$ 0.06	0.86 $\pm$ 0.00
Binding of <i>E. coli</i>	105.77 $\pm$ 1.59	15.28 $\pm$ 0.09	4.28 $\pm$ 0.04	1.88 $\pm$ 0.06	0.83 $\pm$ 0.00
Phage infection at 1 h	111.20 $\pm$ 1.51	15.51 $\pm$ 0.08	5.33 $\pm$ 0.06	1.94 $\pm$ 0.07	0.83 $\pm$ 0.00
Phage infection at 3 h	112.96 $\pm$ 0.84	15.17 $\pm$ 0.05	6.27 $\pm$ 0.07	2.00 $\pm$ 0.06	0.83 $\pm$ 0.00
Phage infection at 5 h	112.77 $\pm$ 0.96	14.25 $\pm$ 0.03	6.69 $\pm$ 0.08	2.08 $\pm$ 0.06	0.83 $\pm$ 0.00
Phage infection at 7 h	111.91 $\pm$ 1.05	13.54 $\pm$ 0.04	7.05 $\pm$ 0.09	2.25 $\pm$ 0.05	0.83 $\pm$ 0.00
Phage infection at 10 h	106.12 $\pm$ 1.22	13.16 $\pm$ 0.01	6.70 $\pm$ 0.04	1.79 $\pm$ 0.03	0.83 $\pm$ 0.00
Phage infection at 14 h	101.15 $\pm$ 0.04	12.35 $\pm$ 0.01	6.14 $\pm$ 0.17	1.66 $\pm$ 0.01	0.84 $\pm$ 0.00
Phage infection at 20 h	98.82 $\pm$ 0.12	10.09 $\pm$ 0.02	5.56 $\pm$ 0.01	1.66 $\pm$ 0.03	0.84 $\pm$ 0.00

Table 4.2 Fitted values and fitting errors of elements in the Randles equivalent circuit for a control experiment using a microbial biosensor in the absence of phage M13.

	$R_s(\Omega)$	$R_{et}(k\Omega)$	$A_w(k\Omega\ s^{-1/2})$	$Q(\mu\Omega^{-1}\ s'')$	n
Immobilized with IgG	203.63 $\pm$ 16.35	10.53 $\pm$ 0.31	2.29 $\pm$ 0.01	0.78 $\pm$ 0.01	0.89 $\pm$ 0.00
Binding of <i>E. coli</i>	62.18 $\pm$ 4.68	23.69 $\pm$ 0.12	3.14 $\pm$ 0.00	0.61 $\pm$ 0.00	0.89 $\pm$ 0.00
Phage infection at 1 h	57.80 $\pm$ 5.00	23.84 $\pm$ 0.08	3.21 $\pm$ 0.08	0.60 $\pm$ 0.03	0.89 $\pm$ 0.00
Phage infection at 3 h	60.07 $\pm$ 7.64	24.62 $\pm$ 0.07	3.21 $\pm$ 0.07	0.63 $\pm$ 0.00	0.89 $\pm$ 0.00
Phage infection at 5 h	57.47 $\pm$ 4.85	25.27 $\pm$ 0.02	3.23 $\pm$ 0.09	0.64 $\pm$ 0.00	0.89 $\pm$ 0.00
Phage infection at 7 h	50.63 $\pm$ 4.51	26.63 $\pm$ 0.01	3.40 $\pm$ 0.01	0.68 $\pm$ 0.00	0.88 $\pm$ 0.00
Phage infection at 10 h	43.92 $\pm$ 3.24	27.13 $\pm$ 0.02	3.44 $\pm$ 0.01	0.69 $\pm$ 0.00	0.88 $\pm$ 0.00
Phage infection at 14 h	40.05 $\pm$ 6.64	27.55 $\pm$ 0.11	3.39 $\pm$ 0.13	0.73 $\pm$ 0.01	0.88 $\pm$ 0.00
Phage infection at 20 h	47.94 $\pm$ 5.87	27.61 $\pm$ 0.06	3.44 $\pm$ 0.01	0.75 $\pm$ 0.00	0.87 $\pm$ 0.00

antibody interaction. Since electron transfer rate constant (k) is inversely proportional to R_{et} , equation (18) can be expressed as a function of the phage infection time (t) as follows, by assuming that the surface coverage varies linearly with the phage infection time,

$$\frac{R_{et}^0}{R_{et}} = 1 + k't \quad (21)$$

where k' is the proportionality constant.

The reciprocal plot of R_{et} values at different phage infection time as shown in Figure 4.2 B is obtained from three separate phage infection monitoring experiments. The R_{et} values at different phage infection time are normalized against the control R_{et}^0 value obtained using the same microbial biosensor prior to phage infection. The good linear relation between R_{et}^0 / R_{et} and phage infection time (t) with RSD $\sim$ 1-10 % indicates that there exists a direct relationship between the phage infection time and the extent to which *E. coli* cells are adhering on the electrode surface through the bacterial LPS-antibody interaction. This shows that, EIS analysis can be a useful tool for monitoring *E. coli* cell damage caused by filamentous phage infection.

4.3.3 Cyclic voltammetric characterization

Cyclic voltammograms of a gold electrode after each step of the microbial biosensor constriction procedure are shown in Figure 4.4 A. These cyclic voltammograms reveal decreasing CV peak current response due to the electrode surface passivation by insulating surface activation reagents, antibodies and *E. coli* cells, and is consistent with the EIS results as shown in Figure 4.1 A. The plateau-shaped cyclic voltammogram

obtained after the binding of *E. coli* cells on the IgG antibody-coated electrode surface indicates that the typical hemispherical diffusion of redox probes from and to the underlying gold electrode is possibly due to the micrometer-sized spaces between the surface bound *E. coli* cells. Decrease in the CV current response after phage infection (Figure 4.4 B) reveals that the diffusive mass transfer of $\text{Fe}(\text{CN})_6^{3-/4-}$ is impeded by *E. coli* cells and phage particles, and is consistent with the changes of A_w in Table 4.1. Therefore, EIS technique appears as a potential and sensitive tool to detect and monitor real-time phage infection of *E. coli* cells by analyzing the impedance changes that take place at the electrode/solution interface.

A

B

Figure 4.4 Cyclic voltammograms of a gold electrode obtained in 2.5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ in 10 mM PBS, pH 7.4 (A) after each step of the microbial biosensor construction procedure and (B) *in-situ* monitoring of phage M13 infection. Conditions: scan rate = 50 mV s^{-1} , potential range = 0-0.6 V, phage concentration = $5 \times 10^4 \text{ phage mL}^{-1}$.

4.3.4 Control study

In comparison, microscopic images of the infected *E. coli* cells at different phage infection time obtained using LabRAM Raman confocal microscope (HORIBA, Kyoto, Japan) cannot be distinguished (Figure 4.5). A separate control experiment was carried out using the same procedure as described in Figure 4.1 and 4.2 A to prepare the microbial biosensor but in the absence of phage M13 (represented by red-colored line with square symbols in Figure 4.2 B) further confirms that phage M13 infection is critical

Figure 4.5 (A) Raman confocal microscopic images of (A) uninfected *E. coli* cells, (B) 3 h phage infected *E. coli* cells, and (C) overnight phage infected *E. coli* cells.

to bring about the decrease in R_{et} value. The fitted values of all equivalent circuit elements at different incubation time with analysis solution in the absence of phage M13 is summarized in Table 4.2. This control experiment shows an opposite trend to the phage infection monitoring experiment (represented by black-colored line with diamond symbols in Figure 4.2 B), which is due to the continual self-proliferation of *E. coli* cells on the IgG antibody-coated electrode surface that increases the R_{et} values with time. Using the student- t test with a p -value cutoff of 0.05, the p -analysis reveals that the phage

infection monitoring experiment and the control experiment in the absence of phage M13 (errors of the control experiment are estimated at a maximum of 10 % RSD of the fitted R_{et} values at different incubation time with analysis solution) are significantly different from one another at infection time beyond 5 h with p -values < 0.05 : infection time at 5 h ($p = 0.049$), 7 h ($p = 0.019$), 10 h ($p = 0.015$), 14 h ($p = 0.008$), and 20 h ($p = 0.004$). In contrast, there is no statistical difference at lower infection time of 1 h ($p = 0.795$) and 3 h ($p = 0.234$).

The high specificity of Rabbit anti-*E. coli* polyclonal antibody toward the *E. coli* O1:K1:H7 (ATCC[®] 11775) used in this work was shown in Chapter 2. Several control experiments were carried out to demonstrate the utility of the impedimetric microbial biosensor in monitoring of the filamentous phage infection of viable *E. coli* cells. A control experiment using autoclaved *E. coli* cells captured on the IgG antibody-coated electrode surface gives almost constant R_{et} values in the presence of phage M13 (Figure 4.6 A). *E. coli* cells were autoclaved by exposing to a pressurized steam of 15 psi and high temperature of 121 °C, which causes denaturation and damage to the macromolecular and subcellular components such as nucleic acid, protein and cytoplasmic membrane [27], thus prevent phages from attaching to the surface of host cell and replicate. A further control experiment using a biosensor constructed without *E. coli* cells produces increased R_{et} values upon phage addition (Figure 4.6 B), which could be attributed to the impeded electron transfer process caused by the non-specific adsorption of phage on the IgG-antibody-coated electrode surface. In another control experiment, a microbial biosensor prepared using viable *E. coli* cells but infected with heat-inactivated phage M13 generates a higher R_{et} response over time (figure 4.7 C).

Heat-inactivated phages have lost their infectious activity, and the higher R_{et} values are due to the self-proliferation of *E. coli* cells, similar to the results obtained in the control experiment conducted in the absence of phage M13 (Figure 4.2 B). In the last control experiment using lower phage concentration at 1×10^4 phage mL^{-1} , the effect of phage infection is not apparent even after a long infection time of 7 h in which the R_{et} values remain higher than the initial R_{et} value measured prior to phage infection time (Figure 4.6 D). This is due to the insufficient amount of phage for effective bacterial cell infection.

As a summary, in all these control experiments, the R_{et} values or semi-circle diameters in the Nyquist plots do not decrease below the initial R_{et} value measured prior to phage infection (R_{et}^0), throughout the experiment. Furthermore, initial increases in the R_{et} values are observed in all control experiments and these could be attributed to the

A

B

C

D

Figure 4.6 EIS Nyquist plots of control experiments obtained in 2.5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ in 10 mM PBS, pH 7.4. (A) An autoclaved *E. coli* cells captured biosensor, (B) an antibody-immobilized biosensor without *E. coli* cells, (C) monitoring of phage-bacterium interaction using heat-inactivated phage and (D) phage solution of lower concentration at 1×10^4 phage mL^{-1} instead of 5×10^4 phage mL^{-1} used in (A)-(C). Lines are fitted using the Randles equivalent circuit. Conditions: frequency range = 0.1 Hz-10 MHz, excitation amplitude = 10 mV, sine-modulated AC potential = 200 mV.

electrode surface passivation or fouling caused by the self-proliferation of *E. coli* cells, adsorption of insulating phage particles, or other solution reagents in the phage sample. Conversely, the experiments using viable *E. coli* cells and sufficiently high concentration of phage display initial increases in R_{et} values followed by large reductions such that after 5 h of infection produce initial increases in the R_{et} values followed by significant

decreases such that after 5 h of phage infection, the R_{et} values are considerably lower than the R_{et}^0 value.

4.4 Conclusions

In conclusion, a sensitive impedimetric microbial biosensor constructed from SAM-modified gold electrode with covalently bound antibody is reported for real-time monitoring of phage M13 infection of *E. coli* cells. The short detection time of phage infection at 5 h outperforms the conventional microscopic methods, which hardly observe the negligible change in bacterial cell surface morphology caused by filamentous phage infection. This impedimetric approach is expected to overcome the challenging obstacles of *in-situ* monitoring of the minimal surface charge and morphological changes of the host cells during phage-bacterium interaction, hence is potentially relevant to future detection and diagnostic purposes.

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Impedimetric cell-based biosensor for real-time monitoring of viral induced cytopathogenesis

5.1 Introduction

Detection of dengue virus (DENV) using electrochemical biosensor has been reported in Chapter 3. Virus samples usually contain high viral particle-to-pfu ratio of viruses (~100,000 as reported in Chapter 3). Technically, this ratio measures the fraction of viral particles in a sample that are viable or infectious. High viral particle-to-pfu ratio may implies that minority of viruses in a given sample is infectious, presence of non-infectious viruses with mutated or damaged genomes, or most of the viruses failed to succeed infection due to the complexity of infectious cycle [1]. Conventional laboratory and clinical detection methods such as molecular techniques [2, 3] are not feasible in identifying infectious viral particles because of the fact that nucleic acids originated from both infectious and non-infectious viruses are equally detected. Proof of infectious viral particles is highly important and can only be done using the traditional cell culture assays which is often time-consuming and labor intensive. Hence, it is essential to develop an effective and simple detection tool for the assessment of virulence and identification of infectious viral particles to overcome the adverse consequences caused by the widespread infectious diseases.

Cell-based assays using mammalian cell lines such as fibroblast and endothelial cells have been reported in toxicological and virological study [4, 5]. Since the first application of electrochemical impedance spectroscopy (EIS) in cell culture study was conducted by Giaver and Keese in 1984 [6], this technique has been widely employed for *in vitro* monitoring of the dynamic response of cells toward drugs, toxic agents and pathogenic microbes [7-9]. In principle, EIS cell-based analytical system involves quantitative monitoring of the attachment, morphology and viability of adhered cells in real-time. Formation of a confluent cell monolayer on the electrode surface constricts the flow of electric current due to the low conductivity of cellular membrane. By applying a constant alternating current (AC) electric field to the electrode, the resulting changes in impedance signal response can be continuously monitored to obtain information on the growth or coverage of cells on the electrode surface [10]. Ultrasensitive impedimetric methods have recently reported non-invasive cell-based analysis of viral infection including herpes simplex viral induced cytopathic or cytopathogenic effects in Vero cells [11], human cytomegaloviral infection of human fibroblasts, [8] and bluetongue viral infection of bovine endothelial cells [12]. Lately, impedimetric monitoring of cellular stress induced by cytotoxicants such as acetic acid and hydrogen peroxide has been described by Qureshi and co-workers [13]. Cytotoxicant-induced deformation of the charge distribution on cellular surface can be studied using a specialized analyzer/workstation which operates at ultrahigh AC frequency region (50 MHz-1 GHz). On the other hand, it is reported that moderate AC frequency region (1-10 kHz) is well-suited for probing the morphological change of cells caused by viral infection or cytotoxicants [11, 14, 15].

Invasion of host cells by lytic viruses such as DENV will ultimately results in degenerative change and damage to the hosts which is known as the cytopathic effects. Viral induced cytopathic or cytopathogenic effects are generally characterized by degenerative morphological change, detachment, membrane degradation and eventual death of cells. Typically, a combination of decreased tight junction between cells and increased separation between cell and electrode take places as a result of viral induced cytopathogenesis. A corresponding decrease in impedance or increase in conductance signal response is observed. In comparison to the conventional inspection methods such as microscopy and plaque assay that rely on observable changes in the morphology and surface coverage of cells, EIS techniques offer a more promising platform for investigating virus-host interaction based on the real-time measurement of cytopathic effects-induced impedance signal response [11].

The performance of impedimetric techniques in real-time monitoring of filamentous bacteriophage infection of bacteria was demonstrated in Chapter 4. Herein, an impedimetric cell-based biosensor constructed from a cationic polymer-modified screen-printed carbon electrode (SPCE) is developed to immobilize fibroblast cells and subsequently monitor the real-time viral infection of surface-immobilized fibroblast cells. This *in vitro* analytical method in which the biosensor is immersed in the culture medium monitors viral induced cytopathic effects by the gradual decrease in the impedance response. The sensitivity of biosensor is enhanced using SPCE which constricts the currents to flow within a small area of working electrode [14, 16]. Furthermore, adherence of a cationic polymeric film, poly-L-lysine (PLL) onto SPCE significantly improves the attachment and spreading of cells. The superior performance in terms of

rapid detection time, automated analysis, high sensitivity and capability of identifying infectious viral particles are some desirable features of this impedimetric cell-based approach for wide range of application in real-time biosensing.

5.2 Experimental

5.2.1 Reagents and Materials

Screen-printed carbon electrode (SPCE, carbon working electrode, silver pseudo-reference electrode and platinum auxiliary electrode on ceramic substrate) was purchased from DropSens. Poly-L-lysine solution (PLL, MW 70,000-150,000, 0.01 % w/v) and potassium hexacyanoferrate (III) ($K_3Fe(CN)_6$, ~ 99 %) were purchased from Sigma-Aldrich. Fetal bovine serum (FBS), and Dulbecco's phosphate buffered saline (DPBS) without calcium and magnesium were obtained from Biowest. trypsin-ethylenediaminetetraacetic acid (Trypsin-EDTA, 0.1 M) and Penicilin/Streptomycin (1 M) were obtained from PAA Laboratories. Roswell Park Memorial Institute (RPMI) 1640 medium was purchased from Thermo Scientific Hyclone. Avicel RC-591 (1.2 % solid) was purchased from FMC BioPolymer. All cell cultures and virus stocks were kindly supplied by Prof. Vincent. T. K. Chow and Suk Hiang Lau from the Department of Microbiology, Yong Loo Ling School of Medicine, National University of Singapore.

5.2.2 Fabrication of PLL-modified SPCE

10 μ L of 0.01 % (w/v) sterile-filtered aqueous solution of PLL was applied onto the carbon electrode and pipette tip was used to spread the solution evenly over the entire carbon electrode surface. The electrode was inverted and placed in a drying oven at 80 °C

for at least 5 min to ensure the transformation of PLL into a stable β -sheet configuration and its adherence to the carbon electrode surface. Excess of PLL solution was aspirated and electrode surface was thoroughly rinsed with sterile ultrapure water. The PLL-modified SPCE was characterized using cyclic voltammetry (CV) in the presence of 1.0 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ in 10 mM PBS, pH 7.4 at scan rate of 100 mV s^{-1} and potential range from -0.8 to 0.8 V.

5.2.3 Culturing and maintenance of BHK-21 cells

Baby hamster kidney (BHK-21, ATCC[®] CCL-10TM) cells were cultured in a T25 tissue culture flask containing 3 mL of growth medium (RPMI 1640 medium supplemented with 10 % FBS and $1 \times$ Penicillin-Streptomycin solution). Cells were grown in a humidified incubator at 37°C and 5 % CO_2 . When the cells are 80-90 % confluent (after 1-2 days of incubation), growth medium was removed from the flask. Cells were washed once with 5 mL of 10 mM DPBS to remove traces of serum that contain inhibitors of trypsin. 3 mL of 10 mM trypsin-EDTA solution was added to the cell monolayer and incubated at 37°C and 5 % CO_2 for approximately 5 min until cells detach. The detached cells were flushed to break up the clumps of cells. Trypsin-EDTA solution was removed and the cells were subsequently resuspended in 2 mL of growth medium. Cell suspension was centrifuged at 6,000 rpm for 1 min. The supernatant was discarded and the cell pellets were resuspended in 3 mL of growth medium. A subcultivation ratio of 1:2 was used to propagate BHK-21 cells.

5.2.4 Preparation of virus stock

Dengue type 2 virus (DENV-2, New Guinea C strain) was propagated in BHK-21 cells maintained in the maintenance medium (RPMI 1640 medium supplemented with 5 % FBS, 1 × Penicillin-Streptomycin solution). Virus with multiplicity of infection (MOI) of 0.1 was inoculated and the tissue culture infectious fluid (TCIF) was harvested after 4-5 days upon the observation of cytopathic effects. MOI represents the ratio of the number of infectious agents (e.g. virus or phage) to the number of infection targets (e.g. bacterium or cell). A control using uninfected cells was included for comparison. The TCIF was subsequently centrifuged at 6,000 rpm for 1 min. The supernatant containing viruses was collected and diluted with RPMI 1640 medium to achieve the desired MOI for viral infection monitoring experiments. Plaque assay using avicel (1.2 % solid) and crystal violet staining was performed to determine the virus titer in unit of pfu mL⁻¹.

5.2.5 Construction of cell-based biosensor and monitoring of DENV-2 infection

Experimental set-up consists of a sterilized polypropylene vessel integrated with a PLL-modified SPCE (Figure 5.1) and a CHI 750D potentiostat/galvanostat (CH Instruments, Texas, United State of America) with a data acquisition software. Total cell count was obtained using TC10™ Automated Cell Counter (Bio-Rad, California, United State of America). Cell suspension with viable cell count of > 95 % was harvested and diluted with growth medium if necessary to obtain a cell concentration of 5×10^5 cells mL⁻¹. 50 µL of BHK-21 cell culture was seeded on the PLL-coated carbon electrode surface (0.5 cm² surface area), allowed to attach and spread on the electrode surface for approximately 2 h. Thereafter, 10 mL of growth medium was gently added to the vessel. Cells were

Figure 5.1 Experimental set-up comprising a sterilized polypropylene vessel integrated with a poly-L-lysine (PLL)-modified screen-printed carbon electrode (SPCE, carbon working electrode, silver pseudo-reference electrode, platinum auxiliary electrode).

allowed to proliferate in a humidified incubator at 37 °C and 5 % CO₂ for 18 h. Subsequently, 500 µL of DENV-2 stock solution was added and mixed gently with cell medium. BHK-21 cells exposed to different MOI of DENV-2 were incubated at 37 °C and 5 % CO₂ for 5 days. All apparatus were properly sterilized and preparation works were performed in a biosafety level 2 cabinet. The impedimetric properties of cell monolayer before and after viral infection were monitored using EIS technique. Electrochemical impedance spectra were recorded at an open circuit potential, frequency of 4 kHz, and excitation amplitude of 10 mV.

5.3 Results and discussion

5.3.1 Cyclic voltammetric characterization of PLL-modified SPCE

The carbon working electrode of a SPCE is modified with a cationic polymeric film, poly-L-lysine (PLL) using a method resembles that described by Anson and co-workers. The configuration of PLL in solution changes from random coil to α -helix at ca. pH 10. Subsequent heating to 80 °C promotes the transformation of PLL film into a stable β -sheet configuration which is believed to comprise chains that are partially cross-linked by hydrogen bonding [17]. The cyclic voltammograms of a PLL-modified SPCE obtained in the presence of 1.0 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ in 10 mM PBS, pH 7.4 (Figure 5.2 A) indicate fairly constant CV peak current response for 100 repetitive potential cycles. RSD values of 5.7 % and 2.9 % are achieved for anodic and cathodic peak currents, respectively. This

A

B

Figure 5.2 (A) Repetitive potential cycling of a screen-printed carbon electrode (SPCE) modified with 0.01 % (w/v) poly-L-lysine (PLL). (B) Comparison of the cyclic voltammetric current response between a bare SPCE (—) and a PLL-modified SPCE (—). Conditions: scan rate = 100 mV s^{-1} , potential range = -0.8-0.8 V, analysis solution containing 1.0 mM Fe(CN)_6^{3-} in 10 mM PBS, pH 7.4.

excellent signal stability reveals the strong adherence of PLL film on the carbon working electrode surface. In comparison to other electrode surface such as platinum and glassy carbon, PLL has shown to be more resilient and strongly adhered on the rough surface of screen-printed carbon [18]. The contrasting behavior of a bare SPCE and a SPCE modified with 0.01 % (w/v) PLL toward the redox probe, $\text{Fe(CN)}_6^{3-/4-}$ is shown in Figure 5.2 B. The cyclic voltammogram show that a more reversible current signal response is obtained for the PLL-modified SPCE. Coating of the electrode surface with a layer of

cationic polymer, PLL reduces the electrode overpotential and facilitates the redox reaction of anionic redox probes such as $\text{Fe}(\text{CN})_6^{3-/4-}$ at the modified-electrode surface. The enhanced anionic exchange capability of cationic polymer-modified electrode is demonstrated by the 60.7 % increase in CV peak current response compared to a bare electrode (Figure 5.2 B).

Electrode surface coating methods such as gelatin coating [14] and equilibration with culture medium [15, 19] are commonly employed to promote the attachment and spreading of cells on the electrode surface. In spite of the good biocompatibility, the utility of these methods is limited by their instability, weak adherence to electrode surface and poor cell capture capability. Herein, the positively charged PLL coating is employed in this work because of its excellent cell capture capability and strong adherence to carbon electrode surface. Various studies have reported that the adherent cell lines such as fibroblast and endothelial cells possess a negative surface charge that facilitates their adhesion to positively charged electrode surface [20-22]. This negative surface charge could be possibly attributed to the carboxylic groups of aspartic and glutamic acid ($\text{pK}_a = 3.9\text{-}4.2$) of proteins, carboxylic groups of sialic acid ($\text{pK}_a = 2.8$) of glycoproteins, phosphate groups ($\text{pK}_a = 2.0$), and sulfate groups ($\text{pK}_a = 1.9$) of sulphated proteoglycans or glycoproteins [21]. Another explanation is that their extracellular matrix is rich in negatively charged glucosaminoglycans [22]. Thus, this suggests that the observed behavior of cell adhesion is mainly electrostatic in nature. The enhanced cationic property of PLL-modified carbon electrode is therefore responsible for the strong adhesion of BHK-21 cells. Furthermore, problems commonly encountered in analyzing biological samples such as electrode surface passivation or fouling and non-specific

interference can be avoided using polymer coating and disposable screen-printed electrode [23].

5.3.2 Real-time impedimetric monitoring of DENV-2 infection

In this work, the interaction between dengue type 2 virus (DENV-2) and baby hamster kidney (BHK-21) cells is studied using EIS technique. BHK-21 cells is a type of adherent fibroblast cells which originally derived by single-cell isolation from the kidneys of five Syrian golden hamsters, *Mesocricetus auratus* [24]. This cell line is susceptible to DENV,

Figure 5.3 EIS Bode diagram shows the impedance signal response of a cell-based biosensor measured prior to infection (●) and during day 5 of infection using virus titer in MOI of 5 (♦). EIS data were recorded at an open circuit potential, frequency range of 100 Hz to 1 MHz and excitation amplitude of 10 mV. Growth medium constitutes the background electrolyte.

vesicular stomatitis virus, reovirus and human adenovirus. Bode diagram as shown in Figure 5.3 is obtained in a frequency range from 100 Hz to 1 MHz. In the absence of electron mediator or redox probe, electrode impedance of cells is limited by the stray capacitance at high frequency and electrode polarization at low frequency. The intermediate frequency in which the impedance signal is found most affected by the cellular behavior is ~ 4 kHz (Figure 5.3) because the impedance signals measured between cells prior to virus addition and during day 5 of viral infection ($\text{MOI} = 5$) are considerably different from one another within this frequency range. Thus, the EIS data recorded at 4 kHz appear to provide more meaningful results of the morphological dynamics of cells and kinetics of viral induced cytopathogenesis. In the following experiments, monitoring of the impedance readings for BHK cell adhesion and subsequent DENV-2 infection are recorded at 4 kHz for every 30 min up to 160 h.

Due to the variation of cell coating on the electrode surface, impedance response at 25 hours post infection (hpi) of four different cell-based biosensors range between 355.4Ω and 508.5Ω with a poor RSD of 16.5 %. Normalization of impedance signal response in the presence of virus (Z_{virus}) against the impedance signal response derived from the same biosensor in the absence of virus at 0 hpi ($Z_{\text{virus}=0}$) gives a relatively low RSD of 4.6 %. This improvement is consistent with the experimental works on membrane-based virus and bacteria nanobiosensors as described in Chapter 2 and 3 in which the normalization of voltammetric current response against the blank signal enhances the reproducibility and precision of results, and avoids the need to calibrate individual biosensor from the same batch of construction.

Dengue viral infection involves several stages: attachment of viral envelope glycoprotein to the cellular receptors which include dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN), heparin sulfate or cell surface immunoglobulin [25, 26]. After fusion with plasma membrane, the virus is enveloped in an acidified endocytic vesicle known as endosome and subsequently releases RNA into the cytoplasm. The growth of virus would eventually trigger membrane degradation and releases viral progeny into the extracellular environment. In general, the invasion of host cells by virus induces a series of intra- and intercellular remodeling processes, which ultimately lead to changes in the tight junction between cells and distance between cell and electrode.

A

B

Figure 5.4 (A) Biosensor impedance signal response as a function of viral infection time at different multiplicity of infection (MOI). Impedance signal response in the presence of virus (Z_{virus}) is normalized against the impedance signal response derived from the same biosensor in the absence of virus at 0 hpi. Colored dotted lines for each virus titer in MOI indicate the onset of cytopathic effects. EIS data were recorded at an open circuit potential, frequency of 4 kHz and excitation amplitude of 10 mV. Growth medium constitutes the background electrolyte. (B) Plot of CIT_{50} against the logarithm of virus titer. CIT_{50} refers to the time taken for 50 % decrease in cell impedance.

The normalized impedance signal response as a function of viral infection time at different MOI (Figure 5.4 A) reveals important information on the attachment and growth of cells on the electrode surface, morphological dynamics of cells and kinetics of viral induced cytopathogenesis. Due to the low conductivity of cellular membrane, the flow of moderate AC frequency current at the cell/electrode or cell/cell interface is sensitively

influenced by few nanometer changes in the adhesion region or tight junction between cells [10]. Also, the presence of cells on the electrode surface exerts a strong influence on the local ionic environment at the electrode/solution interface. As shown in Figure 5.4 A, a gradual increment in the electrode impedance as observed during cell culture indicates that cells are propagating and densely populate on the PLL-modified carbon electrode surface. Electrode impedance keeps on increasing after virus addition as cells continue to grow until viral infection induces a series of cellular remodeling processes that lead to alteration of the cellular morphology and adhesion on the electrode surface. Viral induced cytopathogenesis often results in morphological and physical changes, detachment of cells from surface, and eventual cell death [14]. From Figure 5.4 A, DENV-2 induced cytopathic effects which are indicated by decreases in the normalized impedance response can be detected within ~ 35 hpi (MOI of 10). Based on the measurement of electrode impedance, EIS technique allows the detection of small changes in cellular morphology and adhesion which are hardly observed using the conventional inspection methods. Besides, it can be found that after the observed cytopathic effects, the normalized impedance response of cell-based biosensor declines by 50 % at ~ 69 hpi (MOI = 10). Technically, this particular time taken for 50 % drop in the cell impedance response is termed CIT_{50} , a kinetic parameter typically used to characterize the viral induced cytopathic events [27]. To further characterize the viral induced cytopathogenesis, CIT_{50} is regressed as a function of virus titer in logarithm of MOI. Figure 5.4 B shows an inverse linear relationship between the virus titer in MOI and CIT_{50} values ($R^2 = 0.955$). The resulting regression indicates that CIT_{50} is delayed by 31.5 h for each order of magnitude decrease in MOI and thus verify the dependence of

cytopahtic effects-induced impedance response on viral infectious dose. Based on the analysis of CIT₅₀, DENV-2 titer of virus samples can be estimated from the measured impedance response.

5.3.3 Control study

Several control experiments were carried to assess the performance of impedimetric cell-based biosensor for investigating virus-host interaction. A control study using BHK-21 cells in the absence of DENV-2 (represented by black-colored line in Figure 5.4 A)

Figure 5.5 Control experiment for the comparison of biosensor impedance signal response toward DENV-2 infection of Vero cells (—) and BHK-21 cells (—) using virus titer in MOI = 5. EIS data were recorded at an open circuit potential, frequency of 4 kHz and excitation amplitude of 10 mV. Growth medium constitutes the background electrolyte.

produces a gradual increment in the impedance response over time. This suggests that BHK-21 cells remain viable and actively propagate on the electrode surface. The normalized impedance signal response reaches its maximum after ~ 5 days of incubation with growth medium in which the electrode surface is fully covered with cells. A further control experiment using a PLL-modified SPCE immersed in growth medium in the absence of BHK-21 cells and DENV-2 gives a fairly constant impedance signal response over a period of 6 days. In another control experiment, the impedance response of a biosensor prepared using Vero cells toward DENV-2 infection in MOI of 5 was studied. Vero cell is one of the commonly used continuous mammalian cell lines which is derived from the kidney epithelial cells of African monkeys, *Cerrcopithecus*

A

B

Figure 5.6 Microscopic images of (A) BHK-21 cells after 5 days of infection with DENV-2 in MOI of 1, and (B) control using BHK-21 cells in the absence of DENV-2 after 5 days of incubation with growth medium.

aethiops. This cell line has been extensively used in cytopathological and cytotoxicological study, and screening for Shiga toxin produced by *E. coli* [11, 27-29]. Figure 5.5 indicates slow attachment and spreading of Vero cells on the PLL-modified carbon electrode surface during day 1 of incubation with growth medium. Electrode impedance subsequently increases as Vero cells are growing and reaches a plateau from day 3 to day 6 of infection using virus titer in MOI of 5. DENV-2 induced cytopathic effects cannot be observed with Vero cell line even at high MOI value. This suggests that the impedance response is specific to BHK-21 cells and may not be relevant to other cell

lines. In summary, decrease in impedance signal response can only be detected upon the occurrence of viral induced cytopathogenesis on BHK-21 fibroblast cell line.

In microscopic investigation, DENV-2 infected BHK-21 cells seeded in a PLL-coated culture flask were observed for cytopathic effects under EVOS[®] FL digital fluorescence microscope (Life Technologies, California, United State of America). Microscopic images were taken using a Sony high-sensitivity monochromatic CCD camera. Contrast and brightness were adjusted to improve the quality of microscopic images. A control using BHK-21 cells in the absence of DENV-2 was included for comparison. The microscopic image of BHK-21 cells after 5 days of infection with DENV-2 in MOI of 1 shows typical cytopathic effects such as considerable change in cellular morphology and detachment of cells from the surface (Figure 5.6 A). On the other hand, Figure 5.6 B shows a densely populated BHK-21 cell monolayer which remains almost intact after 5 days of incubation with growth medium in the absence of DENV-2.

5.4 Conclusions

In conclusion, an impedimetric cell-based biosensor constructed from a cationic polymer-modified SPCE has proven to be useful in real-time monitoring of the viral induced cytopathogenesis on surface-immobilized fibroblast cells. Viral induced cytopathic effects as indicated by decreases in impedance signal response can be detected at ~ 35 hpi (MOI = 10). The described platform outperforms the conventional inspection methods which depend on the observable changes in morphology and surface coverage of cells after 4-5 days of viral infection. Other attractive features of this impedimetric cell-based

biosensor include possibility of automation and non-invasive measurement, reduced time frame of diagnosis of DENV-2 infection, mediatorless and label-free monitoring procedure, and capability of identifying infectious viral particles. In addition, virus titer of given samples can be estimated from the measured impedance signal response based on the analysis of CIT₅₀. Future works include the specificity test of the impedimetric cell-based biosensor against different types of viruses (e.g. orbivirus, flavivirus, adenovirus and reovirus) or other dengue serotypes (e.g. DENV-1, DENV-3 and DENV-4).

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Conclusions

Concluding remarks

This thesis showed the promising utility of biosensors based on different electroanalytical techniques for the detection and analysis of microbes. In Chapter 2 and 3, detection of *E. coli* cells and DENV-2 viral particles is reported using an electrochemical membrane-based nanobiosensor constructed from a nanoporous alumina-modified platinum wire electrode. The electrochemical sensing mechanism relies on monitoring of Faradaic current response of the electrode in the presence of neutral redox probe, FcMeOH. The method is sensitive to the formation of immunocomplexes within the alumina membrane nanopores. Other appealing features of the membrane-based nanobiosensor include label-free detection, minimal cross reaction with non-specific microbes, rapid response time and good reproducibility of electrochemical measurements.

With electrochemical membrane-based *E. coli* nanobiosensor, I reported a low LOD of 22 cfu mL⁻¹ with a wide linear working range from 10 to 10⁶ cfu mL⁻¹. The analytical performance of *E. coli* nanobiosensor outperforms enzyme-linked immunoassays and optical methods such as fiber optic, SPR and SERS. On the other hand, the LOD of *E. coli* nanobiosensor is comparable to EIS, piezoelectric biosensing methods and quantitative PCR assays. Differentiation of VBNC or dormant *E. coli* cells from live and dead *E. coli* cells can be achieved by probing the electrocatalytic current

derived from the bacterial enzyme catalytic oxidation of glucose using a neutral redox probe, FcMeOH as the alternative electron acceptor.

In another study on DENV-2 detection using the same membrane-based nanobiosensor, an extremely low LOD of 1 pfu mL⁻¹ with three orders of magnitude linear working range from 1 to 10³ pfu mL⁻¹ is achieved. The membrane-based DENV-2 nanobiosensor surpasses enzyme-linked immunoassays and RT-PCR assays, and comparable to novel biosensing methods, NASBA and multiplex RT-PCR assays in terms of detection performance. Additionally, direct quantitative detection of viral load in DENV-2 infected *A. aegypti* mosquito is demonstrated.

We also explored impedimetric microbial and cell-based biosensors for investigating virus (or phage)-host interaction. In the study of phage-bacterium interaction (Chapter 4), a microbial biosensor fabricated from a gold electrode covalently grafted with a SAM of anti-*E. coli* antibody is employed to study the filamentous phage M13 infection of viable *E. coli* cells. By monitoring of the electron transfer resistance which is sensitive to the minimal change in bacterial morphology and surface charge, phage infection can be detected within 5 h. This microbial biosensor outperforms the traditional microscopic methods which barely observe the slight morphological changes caused by filamentous phage infection. Moreover, this biosensing system offers an alternative approach that is useful in assessing the cells viability besides probing the bacterial enzyme catalytic activity as described in Chapter 2.

In Chapter 5, DENV-2 infection of surface-immobilized baby hamster kidney (BHK-21) fibroblast cells is monitored using an impedimetric cell-based biosensor constructed from a poly-L-lysine-modified screen-printed carbon electrode (SPCE).

Based on the measurement of impedance signal response, DENV-2 induced cytopathic effects can be detected within ~ 35 hpi. It is also found that a kinetic parameter, CIT₅₀ that corresponds to the time taken for cell impedance to decrease by 50 %, is delayed by 31.5 h for each order of magnitude decrease in MOI. Furthermore, this cell-based approach is capable of identifying infectious viral particles, which cannot be done using the conventional molecular techniques. The shorter detection time of phage infection compared to that of viral infection could be possibly due to the shorter life cycle of a phage (~ 30 min) than an infectious virus (days). Overall, EIS technique has demonstrated its ultrasensitivity in detecting and monitoring of the impacts brought about by viruses (or phages) on hosts.

Future outlooks for biosensing techniques

In recent decades, biosensing technologies have achieved great improvement for the detection of microbes in terms of LOD, dynamic working range, response time, sensitivity and selectivity. In spite of these achievements, biosensing techniques still remain nascent in diagnosing infectious diseases. The growing demands for effective disease surveillance and high-throughput diagnostic tests reveal the need to develop biosensing devices into point-of-care detection tool which is rapid, cost-effective, selective and sensitive in analyzing multiple samples. Among all the available detection methods, biosensors are possibly the best candidate for development into handheld pen-size detection tool because of their possibility of miniaturization, disposability, portability and fast response time. In urban and suburban areas, problems exist where the infectious diseases spread well beyond the initial affected regions and the control of diseases at this stage has often become a huge logistic issue for veterinary services to deal with. As a consequence, rapid pen-size field tests may become critical for the implementation of initial disease control. These approaches would eliminate the delay in diagnosis as a result of sending routine samples to specialized laboratories and clinics, and time taken to deliver the test results.

Elimination of additives such as labeling probes and electron mediators in biosensing assays help to simplify measurement procedure and avoid possible contamination. Label-free and mediatorless biosensors constructed using electrochemically active bacteria offer an interesting and promising approach for bioelectrochemical analysis. As a part of the energy conservation strategy, Gram-negative bacterium, *Shewanella* sp. is able to convert its chemical energy into electricity and

mediates the extracellular electron transfer processes. This biosensing assay can be potentially used in environmental monitoring and ecotoxicity testing where the identity and source of toxicants, pollutants, and other harmful substances cannot be predicted.

In addition, further research to design and discover new biocompatible materials such as nanoscale materials, composites and polymers may improve the stability and accessibility of biorecognition probes. Apart from that, optimization of experimental parameters including loading amount of biorecognition probe, pH, temperature, incubation time and ionic strength of background electrolyte is crucial to ensure that the biosensors are always operate in the best condition and deliver superior performance. Furthermore, more studies have to be carried out to overcome the common problems encountered by most of the biosensors such as adsorption of non-specific biocomponents and interfering substances that present in the complex biological samples (e.g. human serum and blood) in order to meet the demands of clinical chemistry for accurate and reliable detection of microbes. Recent technological advancement would undoubtedly accelerate the development of biosensors into an essential, well established detection tool to accomplish the need of clinical laboratories and hospitals for routine investigation of infectious diseases.