CMOS Biosensor for Rapid Detection of Bacteria and Antibiotic Susceptibility

by

Nasim Nikkhoo

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Electrical and Computer Engineering
University of Toronto

© Copyright 2015 by Nasim Nikkhoo
Abstract

CMOS Biosensor for Rapid Detection of Bacteria and Antibiotic Susceptibility

Nasim Nikkhoo
Doctor of Philosophy
Graduate Department of Electrical and Computer Engineering
University of Toronto
2015

The development of a low-cost, specific and sensitive integrated system for the detection and identification of bacteria, that can provide results rapidly has been a major research challenge. This thesis introduces a systematic approach to the selection and design of appropriate biological components combined with a microelectronics system, that provides rapid and highly specific detection results at a low sensor cost and using a small sample volume. The bacterial detection system achieves specific identification through the use of two types of biological recognition elements: bacteriophages and bacteriocins. Both systems utilize the rapid efflux of potassium ions to the sample as a result of the infection of the specific bacteria in the sample by the biological recognition elements. The potassium efflux is a transducing event detectable by a potassium selective field effect transistor implemented in CMOS, providing conclusive results in less than 10 minutes. Different strains of Gram-negative *E. coli* were tested using bacteriophages, and both Gram-negative and Gram-positive bacterial species were tested using bacteriocins. Measurement protocols and processing techniques are implemented to cancel the effect of ion-selective system DC baseline variations and drift. The implemented sensors achieve the minimum detection limit of $3 \times 10^7$ cfu/ml at 10-minute detection time using the PVC-based potassium-sensitive membranes with the sensitivity of $10^{-6}$M of potassium. Processing of the raw sample has been simplified to a less-than-5 minute assay that simply resuspends the raw sample into a constant suspension medium for measurements using a 100$\mu$l sample volume.
A predictive model is presented for the bacterial sensor system that captures the effects of biological, chemical and environmental parameters on the system output signal. The bacterial detection system can be repurposed to equivalently determine bacteria susceptibility to pore-forming antibiotics. Experimental results using polymyxin B antibiotic and different strains of Gram-negative *E. coli* are presented.
Acknowledgements

The work presented in this thesis could not be completed without guidance, help support and encouragement of so many people that I had the privilege of getting to know and interacting with during my years at University of Toronto. I hope to acknowledge them in a small way.

I would like to thank my supervisor professor Glenn Gulak for his invaluable guidance and support throughout this journey. His broad vision and encouragement has been a source of inspiration for me. I would also like to thank my thesis committee members professor Roman Genov, professor Yu Sun, professor Amr Helmy, professor Wai Tung Ng and external examiner professor Vamsy Chodavarapu for their valuable insights and comments that improved this thesis tremendously. I had the privilege of collaborating with Dr. Karen Maxwell’s group at department of molecular genetics and I feel greatly indebted to her for her support, kind mentorship and patience. I would like to thank Diane Bona that helped me with all biological setup and experiments step by step with kindness and patience. Special thanks to Alan Gulak for his help in designing antibiotic experiments.

I feel blessed to get to know so many friends throughout my years at University of Toronto. I learned many valuable lessons from each one of them. I would like to specially thank Kelly Reimer, Nichole Cumby, Senjuti Saha and Mostafa Fatehi for their true friendship, enjoyable discussions and encouragements. They generously taught me how to perform biological assays, spent so much time explaining the science and lent me their recipes, buffers, hard-made plates and more. I would like to thank Meysam Zargham and Michal Fulmyk for their true friendship, memorable moments and fun we had. Their presence made my final Ph.D years more productive and enjoyable. I would like to thank my fellow BA5000 and BA5158 graduate students, Mario Milicevic, Kevin Banovic, Alireza Nilchi, Sadegh Jalali, Mahdi Shabany, Samira Karimelahi, Dustin Dunwell, Mike Bichan, Alhassan Khedr, Aynaz Vatankhah, Behzad Dehghani, Zeynep Lulec, Dawei Song, Rosana Murugesu, Arshya Feyz for their friendship and support.

Special thanks to Sara Scharf for her wonderful comments on the thesis and Jeetendar Narsinghani for his help and support with lab setup and equipments.

No words are sufficient to express my gratitude and love for my mom and dad who have always been supportive, loving and have encouraged me to achieve the better and my sister, Neda, who has been cheering me up. I could never complete this work without their help.

I would like to thank my husband, Ali, who was always there for me and supported me. He helped me through the tough times during this work through his love and kindness.
Last but not the least, special thanks to my lovely little son, Elias, who has brought greatest joy and excitement in my life. He entered this world just in time to defend this thesis with me.
Contents

List of Figures xi
List of Tables xix
List of Acronyms xx
List of Symbols xxii

1 Introduction 1
  1.1 Motivation for Bacterial Detection and Antibiotic Testing 1
  1.2 Bacterial Detection and Antibiotic Testing Systems and Their Specifications 2
  1.3 Challenges and Objectives 4
  1.4 Outline of the Thesis 5

2 Fundamentals of Bacterial Sensing Systems 7
  2.1 Introduction to Basic Biological Components 7
    2.1.1 Bacteria 8
    2.1.2 Antibiotics 9
    2.1.3 Viruses 10
  2.2 Current Techniques for Bacterial Detection and Identification 10
    2.2.1 Visual Inspection 10
    2.2.2 Culturing Techniques 11
    2.2.3 Enzyme-Linked Immunosorbent Assay (ELISA) 13
    2.2.4 Phage Typing 13
    2.2.5 DNA Techniques 14
  2.3 Biosensors and Their System Block Diagrams 15
    2.3.1 Biological Recognition Element (BRE) 16
    2.3.2 Recognition-Event Translator 16
    2.3.3 Electrical Transducer 17
3 System Design and Modelling of the Bacteria Biosensor

3.1 Biological Recognition Element (BRE)

3.1.1 Bacteriophages ("Phages")

3.1.2 Bacteriocins

3.2 Recognition-Event Translator

3.2.1 Sensors Utilizing Bacteriophages

3.2.2 Sensors Utilizing Bacteriocins

3.3 Ion-Selective Electrode Systems as Electrical Transducers

3.3.1 Ion-Selective Electrode Systems (ISE Systems)

3.3.2 Non-Idealities in ISE Systems

3.4 Analog Front-End and Processing Unit

3.5 Complete Bacterial Sensor

3.6 Modelling the System Behaviour

3.6.1 Ion-Selective Electrode System Output Voltage versus Potassium Concentration

3.6.2 Maximum Ion-Selective Electrode Output Voltage Signal Variations with Bacterial Cell Concentration

3.6.3 ISE System Output Signal Over Time

3.6.4 Biosensor Calibration Curve

3.6.5 Extracting System Model Parameters from Measurement Results

3.6.6 System Model Predictability

3.6.7 System Model Summary

3.7 Experimental Design and Errors

3.7.1 Calibration

3.7.2 Control Experiments

3.7.3 False Positive and False Negatives

3.8 Summary

4 ISFET and Analog Front-End Design

4.1 Introduction to Ion-Selective Field Effect Transistors (ISFETs)

4.1.1 Ion-Selective Membrane

4.2 ISFET Design and Characterization and ISFET Readout Circuitry

4.2.1 Single-Ended vs. Differential ISFET System

4.2.2 ISFET Readout Circuitry

4.2.3 Analog to Digital Conversion
4.3 Design of the First Test IC ........................................ 68
4.4 Design of the Second IC ........................................ 73
  4.4.1 ISFETs Connected to a Voltage-Mode Drain-Source Follower Read-
        out ............................................................... 74
  4.4.2 ISFETs Connected to Current-Mode Fixed Source and Drain ... 80
  4.4.3 Differential ISFET plus Drain-Source Follower Readout ...... 82
4.5 Discussion and Summary ........................................ 83

5 Detection of E. coli Using Bacteriophages ....................... 84
  5.1 Sample preparation and Processing ............................. 85
  5.2 Initial Experiments using Commercial ISE .............. 85
  5.3 System Model Parameter Extraction and
       Predictive Model Estimation .................................. 89
    5.3.1 Estimation of ISE Output Voltage Signal versus Bacterial Cell Con-
           centration .................................................... 89
    5.3.2 ISE System Output Signal Over Time .................... 91
  5.4 Test Chip Experimental Setup and Procedures ............ 95
    5.4.1 Chip Implementation and Preparation ................... 95
  5.5 Experimental Results ........................................ 96
    5.5.1 Experimental Protocol .................................. 96
    5.5.2 Group A Experiments using T6 Phage as the biological recognition
           element ..................................................... 96
    5.5.3 Group B Experiments using λ Phage at 37°C ............... 99
    5.5.4 Group C Experiments using λ Phage at 23.7°C ............ 99
  5.6 Summary ..................................................... 100

6 Detection of Bacteria Using Bacteriocins ...................... 102
  6.1 Initial Plating Experiments of the Bacteriocin .......... 103
  6.2 Selection of Biological Probes Using a Commercial ISE .... 104
  6.3 System Model Parameter Extraction and Predictive Model Estimation ... 108
    6.3.1 Estimation of the ISE Output Voltage Signal versus
           Bacterial Cell Concentration .......................... 108
    6.3.2 Calibration Curve Using Commercial ISE .............. 111
    6.3.3 ISE system Output Signal Over Time ................... 112
  6.4 CMOS Chip Preparation ..................................... 116
  6.5 Experimental Results Using the CMOS Chip .............. 118
    6.5.1 CMOS Biosensor Specifications at 10-Minute Detection Time ... 122
6.5.2 Comparison to State-of-the-Art ........................................... 122
6.6 Summary ................................................................................. 124

7 Biosensor Application for Antibiotic Susceptibility Testing 125
  7.1 Antibiotic Categories and Resistance ................................. 125
  7.2 Model Antibiotics ................................................................. 126
  7.3 Experimental Design using Polymyxin B (PMB) Antibiotics ................................. 127
  7.4 Measurement Results Using CMOS Chip .............................. 131
    7.4.1 CMOS Biosensor Specifications at 10-Minute Testing Time ................................. 133
  7.5 Summary ................................................................................. 134

8 Contributions and Future Work 135
  8.1 Contributions ........................................................................ 135
  8.2 Publications ......................................................................... 138
  8.3 Future Work ....................................................................... 139
    8.3.1 Biological Recognition Elements ......................................... 139
    8.3.2 Multiple Detection and Identification on a Single CMOS Chip ................. 139
    8.3.3 Integration of ADC and Processing Unit ...................................... 139
    8.3.4 Membrane Optimization ..................................................... 140
  8.4 Summary ................................................................................. 140

Appendix A Protocols 141
  A.1 Buffers and Media ............................................................... 141
    A.1.1 Lennox Broth (LB) .......................................................... 141
    A.1.2 SM Buffer ................................................................. 141
    A.1.3 N-Minimal Medium ......................................................... 141
    A.1.4 Phosphate Buffer Saline .................................................... 142
  A.2 Bacteriophage Preparation .................................................. 142
    A.2.1 T6 Phage ...................................................................... 142
    A.2.2 λ Phage ...................................................................... 143
  A.3 Bacteriocin Preparation ......................................................... 145
  A.4 Raw Bacteria Sample Preparation ........................................ 145
  A.5 Bacteria Sample Processing .................................................. 146
  A.6 Potassium-Sensitive Membrane Preparation, Chip Preparation and Membrane Deposition ..................................................... 146
    A.6.1 Reagents ...................................................................... 146
    A.6.2 Protocol ................................................................. 146
List of Figures

2.1 Cultured agar plate of dilution showing (a) *E. coli* K12 Bw25113 and (b) *Staphylococcus aureus* 8325 used to quantify the concentration of the original undiluted sample. The round dot-like regions are the colonies grown from a single bacterium.  

2.2 A bacterial lawn with phage spotting creating clearings where specific phages have killed the bacteria being tested [1].  

2.3 Conceptual block diagram of a biosensor.  

3.1 Conceptual block diagram of a biosensor using electrochemical transducer and integrated electronics.  

3.2 (a) Left: electron micrograph of a *T*₄ phage [2]; right: structure of a typical phage. (b) Left: electron micrograph of phage attachment to *E. coli* cell membrane [3]; right: structure of phage attachment to bacterial cell membrane, DNA injection and efflux of potassium (K⁺) ions.  

3.3 The output of the recognition event translator when phages or bacteriocins are used as biological detection elements.  

3.4 Conceptual working principle of a conventional liquid-contact ISE system. E1, E2, E3 and E4 show the electrochemical voltages between the reference electrode-sample (E1), membrane-sample (E2), membrane-internal reference liquid (E3) and internal reference liquid-reference electrode (E4). All these voltages are constant except E2. The potential difference across the membrane (the potential difference between sample liquid and internal reference liquid) is called membrane potential $E_m$, which is equivalent to $E_2 + E_3$.  

3.5 Experimental procedure and the algorithm used by the PU (Processing Unit).
3.6 (a) Typical raw ISE output signal for 2 experiments, (b) ISE output signal after processing by the PU that removes DC baseline variations and compensates for drift.

3.7 Conceptual block diagram of the system (a) The bacteria in the sample are sensitive to the selected BRE. After the attachment of BRE, K⁺ ions leak out of the sensitive cells. The increased [K⁺] in the sample yields an increased CMOS ISFET signal (b) The bacteria in the sample are not sensitive to the selected BRE. After the BRE is added, no changes in [K⁺] occur and, hence, no changes in the CMOS ISFET signal are expected.

3.8 The output voltage of a potassium-selective probe versus the potassium concentration in a buffered sample.

3.9 The output voltage of a potassium-selective electrode system versus the potassium concentration in a buffered sample with overlay of the released potassium in the sample.

3.10 Proposed system model that determines the time domain output signal of the sensor. The figure shows three main components: 1) physical input parameters to the system, BRE, bacterial species, temperature and sample buffer, 2) two main blocks affecting the signal output (potassium efflux generator and ISE voltage converter, 3) system model parameters, [Kᵣ], d, and τ.

3.11 Pseudo-code for extraction of the model parameters from measurement results.

3.12 Temperature dependence of the initial rate of efflux (at time=0) or the time constant (τ) in our model to temperature.

4.1 Block diagram of the analog front-end.

4.2 (a) Cross-section of an ISFET implemented in CMOS with passivation oxide acting as the pH-sensitive layer, (b) Site-binding model representation of the SiO₂ oxide interface and the sample liquid [4, 5].

4.3 Conceptual development of ISFET shown in cross-section. (a) a pH-sensitive ISFET where the gate oxide is the pH-sensitive membrane (b) Ion-sensitive membrane is added on top of ISFET in (a) to make the ISFET ion-sensitive to ions other than H⁺ as determined by the chemical composition of the membrane. (c) A cross-section of a MOSFET (d) ISFET implemented in CMOS by depositing an ion-selective membrane on top of the passivation layer.
4.4 Chemical structure of valinomycin [6] with a K⁺ ion fitting inside the structure. .................................................. 58
4.5 Ionophore valinomycin traps the potassium ion from the membrane-sample liquid interface and provides a charge separation across the interface. .. 58
4.6 ISFET and ISFET electrode equivalent circuit. ......................... 59
4.7 (a) Representation of single-ended ISFET system using an ideal Ag/AgCl electrode, (b) A differential ISFET approach employing an identical membrane with one ISFET touching the sample liquid and another ISFET touching a reference liquid. ........................... 62
4.8 (a) Representation of single-ended ISFET system using an ideal Ag/AgCl electrode, (b) A differential ISFET approach using a noble metal (e.g. gold-bonded pad) as a reference electrode, having two separate ISFETs and readout circuitries. The final output is the difference between the outputs of the two ISFETs. ............................... 64
4.9 Voltage-mode drain-source follower readout circuit connected to the input PMOS ISFET. \( I_b \) is the bias current that generates \( I_b \times R \) that is equal to ISFET \( V_{ds} \) using the drain-source follower configuration. ................. 65
4.10 (a) Structure of an example current-mode fixed drain and source voltage readout circuit connected to the input NMOS ISFET, (b) An example of a simple implementation from [7]. ................................. 66
4.11 Representation of an ISFET cross-section in CMOS with the K⁺-sensitive membrane on the top surface. ................................. 69
4.12 Schematic of the ISFET readout circuit. The ISFET transistor implemented on the 0.18\( \mu m \) CMOS was connected to off-chip drain-source follower circuit implemented on a PCB. ............................... 69
4.13 Die photo of the first chip ........................................... 70
4.14 The 3-layer passivation parameters and the ISFET parameters implemented in the first prototype in 0.18\( \mu m \) CMOS. ................................. 71
4.15 (a) pH calibration curve for the implemented ISFET (24°C), (b) pK calibration curve for the implemented ISFET 24°C ................................. 72
4.16 Die micrograph of the second implemented chip illustrating Group 1 and Group 2 voltage-mode ISFETs and current-mode ISFETs with the electrode area. ................................. 73
4.17 (a) Block diagram of the implemented Group 1 ISFETs on the second test prototype. Notice the membrane is deposited on top of the metal electrode not the passivation layer. The metal electrodes are exposed using the conventional mask for opening pads. 75

4.18 ISFET and drain-source follower ISFET readout circuit. Six channels are implemented by the combination of 2 different-sized ISFETs and 3 different-sized electrodes. The opamp1 (Op1) and opamp2 (Op2) designs are described in Fig. 4.19 and Fig. 4.20. 76

4.19 Opamp 1 (Op1 in Fig. 4.18) circuit diagram and its specifications. 76

4.20 Opamp 2 (Op2 in Fig. 4.18) circuit diagram and its specifications. 77

4.21 Potassium sensitivity of the Group 1 ISFETs across 6 different channels at room temperature (24°C). 77

4.22 The aluminum oxide capacitance and the ISFET parameters implemented in Group 1 of the ISFETs showing the equivalent capacitors that affect the ISFET sensitivity. 78

4.23 Circuit diagram of the current-mode circuit (one channel) in the second prototype IC. 80

4.24 Potassium sensitivity of two current-mode ISFETs (24°C). 81

4.25 Structure of a proposed differential ISFET readout and difference circuit. 82

5.1 Overall system diagram using bacteriophages. 84

5.2 A commercial ion-selective electrode system. 86

5.3 Experimental protocol using the combination of $T_6$ phage and two strains of $E. coli$, that provide positive and negative control experiments. 87

5.4 ISE measurements using $T_6$ phage with BL21 and TSX- bacterial cells at 23.7°C. The OD of BL21 cells was 1.1 and for TSX- cells was 1.2. (a) The raw ISE outputs without DC baseline removal and no drift compensation, (b) The ISE output with DC baseline removal and drift compensation. 88

5.5 ISE Measurements using $\lambda cI_{857}$ phage and $E. coli$. Each experiment used a bacterial concentration corresponding to an OD = 1.2. 88

5.6 Calibration response of ISE at room temperature (24°C). 90

5.7 Fitted response of the ISE to the system model in Fig. 3.10 using the algorithm presented in Section 3.6.5. The experiment was performed using an ISE and combination of $E. coli$ BL21 OD of 1.1 with added phage $T_6$. The figure shows the response after phage $T_6$ addition at 23.7°C. 91
5.8 Extracted $\ln(1/\tau)$ versus inverse of temperature and a fitted Arrhenius plot. $\tau$ was extracted from experiments involving phage $\lambda$ and *E. coli* BW25113 $\Delta fhuA$ in Table 5.2. .................................................. 93

5.9 Extracted delay versus temperature and fitted line in temperature range from $24^\circ C$ to $37^\circ C$. Delay $d$ was extracted from experiments involving phage $\lambda$ and *E. coli* BW25113 $\Delta fhuA$ in Table 5.2. .................. 93

5.10 Estimated values of system parameters, delay $d$ and time constant $\tau$ and the extracted parameters from independent measurements at $37^\circ C$ and $30^\circ C$. The independent experiments were performed using the *E. coli* BW25113 and $\lambda$ phage. ................................................................. 94

5.11 Left: chip micrograph, specifications and encapsulated chip with exposed electrodes; right: test board plus other components of the measurement setup inside the dark chamber .......................................................... 95

5.12 Output $\Delta V$ voltage recordings for Experiment Group A: (a) raw $\Delta V$ output with drift, and estimated drift line (dotted) before the addition of the $T_6$ phage. (b) Estimated output $\Delta V$ after drift compensation. ............. 97

5.13 Slope of $\Delta V$ curve with respect to time for experiment Group A. ...... 98

5.14 Slope of $\Delta V$ curve with respect to time for (a) experiment Group B and (b) experiment Group C. .................................................. 100

6.1 Conceptual block diagram of the system utilizing bacteriocins. (a) The bacteria species in the sample are sensitive to the selected bacteriocin. (b) The bacteria species in the sample are not sensitive to the selected bacteriocin ................................................................. 103

6.2 Spotting assay performed with different bacteriocins to confirm bacterial sensitivity to them. For each plate, a bacterial lawn is grown and small droplets of the designated bacteriocin are added. (a,b) *E. coli* BW25113, (c) *Staphylococcus aureus* 8325, (d) *Pseudomonas aeruginosa* (PA01). . . 104

6.3 Commercial ISE output when different colicins are added to *E. coli* K12 BW25113 at $37^\circ C$. ................................................................. 105

6.4 Commercial ISE output showing colicin specificity to different *E. coli* K12 BW25113 cells with modified receptors at $37^\circ C$. ......................... 106

6.5 Initial experiments with commercial ISE at $26^\circ C$ with cells having OD = 0.8 . ................................................................. 107
6.6 Experimental results utilizing ISE for bacterial identification in artificial urine (AU) at 30°C. In the case of *E. coli* K12, the addition of colicin a9 results in a positive outcome, whereas in PA01, negative results occur.

6.7 (a) ISE output positive control results using 3 concentrations of *E. coli* K12 at room temperature (23.7°C), (b) calibration curve of the ISE showing the raw (without DC baseline removal) output voltage of ISE versus potassium concentration in the sample.

6.8 Calibration curve of the sensor using commercial ISE as ISE system. Experiments were performed with 3 different concentrations of *E. coli* K12 cells and colicin A9 as BRE at 23.7°C.

6.9 Fitted response of the ISE to the system model in Fig. 3.10 using the algorithm presented in Section 3.6.5. The experiment was performed using an ISE system and a combination of *E. coli* K12 at a concentration of $3 \times 10^8$ cfu/ml with added colicin A9 at 23.7°C. The figure shows the response after the colicin A9 was added.

6.10 Extracted $ln(1/\tau)$ versus inverse of temperature and a least squares fitted Arrhenius plot. The values of $\tau$ were extracted from experiments involving colicin A9 and *E. coli* K12 in Table 6.2.

6.11 Extracted delay versus temperature. The delay is extracted from experiments involving colicin A9 and *E. coli* K12 according to Table 6.2.

6.12 Estimated values of system parameters, delay $d$ and time constant $\tau$ and the extracted parameters from independent measurements at 37 °C and 23.7 °C (Experiment 1 and Experiment 2). The independent experiments were performed using the *E. coli* BW25113 and colicin A9. The OD of the cells were 1.14 and 0.9 in Experiment 1 and Experiment 2 respectively.

6.13 System components from fabricated CMOS IC to the test setup connection to PC. (a) Micrograph of the fabricated CMOS IC in 0.13μm IBM CMOS technology. CMOS IC electrodes are shown in the middle of the die, where they are connected to ISFET readers and controllers. (b) The packaged CMOS IC in a 69-pin PGA package. The bond wires are encapsulated with epoxy, leaving the CMOS IC electrode area exposed. A potassium-selective membrane is deposited on the exposed CMOS IC area. The sample liquid is in contact with the potassium-selective membrane deposited on top of the CMOS IC. (c) The system setup that mounts the packaged CMOS IC on a test PCB which connects to a PC.
6.14 Representation of the experiments performed on CMOS IC. Each experiment consists of a combination of one bacteriocin and one bacterial strain. Two bacteriocins (colicin A9 and lysostaphin) and three different bacterial samples are employed for a total of 6 experiments. The outcome of the experiment is either positive (increase in the potassium concentration in the sample), or negative (no potassium variations).

6.15 CMOS IC detection of 3 different bacteria strains using bacteriocins. The panels show the CMOS IC output signal from one of the available electrodes through a complete cycle of potassium efflux at 26.7°C. *S. aureus* + lysostaphin and *E. coli* + colicin A9 are expected positive results.

6.16 CMOS IC detection and identification capability in 10 minutes using the outputs of the array of electrodes after DC baseline removal and drift compensation at 26.7°C. The baseline and drift calibration has been performed using the IC signal for 1 minute prior to the addition of bacteriocin. The compensated and processed outputs are depicted for 10 minutes post-infection to provide positive/negative outcome. Error bars are computed from measurements taken from multiple electrodes.

7.1 Results of plating of both (a) *E. coli* K12 BW25113 ΔfuA (Keio WT) and (b) *E. coli* K12 BL21 (DE3 Δtail) (BL21). For each panel the right side is the plating of the sample bacteria alone. On the left side, PMB has been added to the sample suspended in SM. The panel (a) on the left side shows no bacteria is present because of bacterial sensitivity to PMB. On the left side of panel (b), there are many colonies of BL21 bacteria that have survived PMB treatment.

7.2 Experimental procedure and expected output for mixing 2 different strains of *E. coli* with PMB.

7.3 Experimental results using ISE with both combinations of positive and negative control at 37°C. PMB is added to the mixture at t= 10 minutes.

7.4 Experimental results using the CMOS test chip with both combinations of positive and negative control at 37°C. PMB is added to the mixture at the 10 minute mark.

7.5 Detection capability of the CMOS chip that provides conclusive results in less than 10 minutes after the addition of PMB across multiple electrodes. Error bars are determined from measurements from 4 to 6 exposed electrodes.
B.1 (a) Nanowell construction in CMOS process, (b) dimensions of the electrodes.

B.2 System block diagram of the sensor.

B.3 (a) Schematic of the amplifier, (b) schematic of the OTA.

B.4 Micrograph (a)(b) of the chip and the electrodes, and (c) chip specifications.

B.5 (a) Integrated circuit with encapsulated bonding wires, (b) test board.

B.6 The time domain measurement results of the input when sensitive and insensitive cells are mixed with λ phage, in a 50 second time window.

B.7 The power spectral density of the input-referred signal for positive and negative controls and the open loop test results. Also shown (for reference) is the PSD computed using 512-point FFT.

B.8 (a) Electron micrograph of R-type pyocin [8], (b) pyocin structure [8], (c) *Pseudomonas aeruginosa* [9].

B.9 A 90-second window of the time-domain input voltage fluctuations when PAC10 cells with OD=0.7 are mixed with PA01 pyocin (Time origin is from the start of recording time).

B.10 Power spectral density of the input voltage in dry test and when mixture PAC10 OD=0.7 and PA01 are applied.

B.11 PSD for a large number of sample points and also for a 512-point FFT for the mixture of two different concentrations of PAC64 with PA01 pyocin.
List of Tables

2.1 Application-specific sensor sensitivity and volume requirements . . . . . . 7
2.2 E. coli statistics [10] ........................................................................... 8

3.1 Concentration of ions in E. coli cells [10] . ........................................ 27
3.2 Relative concentration of interfering ions to potassium ion in sample buffer
that result in 10% error at the potassium ISE output [11]. ....................... 42

5.1 Parameters extracted from the ISE response using λ phage and BL21 and
BW25113 cells according to Fig. 3.10 of the system model. .................... 90
5.2 Parameters extracted from the ISE response according to Fig. 3.10 of the
system model. ....................................................................................... 92

6.1 Comparison of estimated ISE output voltage vs. measurements using E.
coli K12 with added colicin A9 at room temperature. ......................... 110
6.2 Parameters extracted from the ISE response according to the system model
presented in Fig. 3.10 using the algorithm presented in Section 3.6.5. ... 113
6.3 CMOS bacterial biosensor specifications at 10-minute detection time . . 122
6.4 Electrochemical Bacterial Biosensor Performance Summary and Comparison123

7.1 CMOS biosensor specifications at 10-minute detection time for antibiotic
testing ................................................................................................. 134
List of Acronyms

ADC  Analog to Digital Converter.

AFE  Analog Front-End.

AST  Antibiotic Susceptibility Testing.

AU   Artificial Urine.

BRE  Biological Recognition Element.

CMOS Complementary MetalOxideSemiconductor.

DNA  Deoxyribonucleic Acid.

DRC  Design Rule Check.

\textit{E. coli} Escherichia coli.

ELISA Enzyme-Linked Immunosorbent Assay.

EMA-PCR Ethidium Monoazide Polymerase Chain Reaction.

ESD  Electrostatic Discharge.

FET  Field-Effect Transistor.

FFT  Fast Fourier Transform.

IC   Integrated Circuit.

ISE  Ion-Selective Electrode.

ISFET Ion-Selective Field Effect Transistor.
LB  Lennox Broth.

MBC  Minimum bactericidal concentration.

MEMs  Microelectromechanical Systems.

MIC  Minimum inhibitory concentration.

MIP  Molecular Imprinted Polymer.

OD  Optical Density.

OPAMP  Operational Amplifier.

PBS  Phosphate Buffer Saline.

PCB  Printed Circuit Board.

PCR  Polymerase Chain Reaction.

PMB  Polymyxin B.

PSD  Power Spectral density.

PU  Processing Unit.

PVC  Poly Vinyle Chloride.

RE  Reference Electrode.

RNA  Ribonucleic Acid.

SELEX  Systematic Evolution of Ligands by Exponential Enrichment.

SPR  Surface Plasmon Resonance.

THF  Tetrahydrofuran.

UTI  Urinary Tract Infection.

WE  Working Electrode.

WHO  World Health Organization.
List of Symbols

$C_g$  Equivalent capacitance at the gate of the ISFET transistor

$C_m$  Membrane bulk capacitance

$C_{dl}$  Double-layer capacitance between membrane and liquid

$C_{gs}$  Gate-source capacitance

$C_{pass}$  Passivation layer capacitance

$DL$  Detection limit of the ISE system

$E_a$  Activation energy

$E_m$  Membrane potential

$E_{const}$  Constant part of the potential across the membrane

$E_{ref}$  Potential of reference electrode in the ISFET

$F$  Faraday constant ($9.64 \times 10^4$ C/mol)

$K_{in}$  Number of potassium ($K^+$) ions inside a single target bacterium

$N_A$  Avogadro constant ($6.02 \times 10^{23}$ mol$^{-1}$)

$P_{M_i^+}$  Membrane permeability towards ion $M_i^+$

$R$  Gas constant ($8.31446$ JK$^{-1}$mol$^{-1}$)

$R_L$  Contact resistance between the membrane and the ISFET surface

$R_m$  Membrane bulk resistance

$R_s$  Resistance of the solution in contact with the ISFET
$R_{dl}$ Ion-transfer resistance between membrane interface and liquid

$S$ Sensitivity of the ISE system in the linear region

$T$ Temperature

$T_{off}$ Temperature below which no membrane depolarization is activated after the addition of the BRE

$T_{sat}$ Temperature above which the rate of efflux stays constant

$V_b$ Baseline voltage at the output of the ISE

$V_g$ Gate voltage of ISFET transistor

$V_i$ Chemical reaction rate

$V_t$ MOSFET Threshold voltage

$V_{Ref}$ Reference voltage applied to a solution

$[C_b]$ Baseline potassium concentration in the sample (in mol/L) corresponding to $V_b$ in the ISE system calibration curve

$[C_s]$ Concentration of sensitive cells in a sample in cfu/ml

$[I_{in}]$ Concentration of the ion $I$ on the inner side of the membrane

$[I_{out}]$ Concentration of the ion $I$ on the outer side of the membrane

$[K_r](t)$ Time-dependent concentration of the released potassium ions from the infected cells in the sample liquid in mol/L

$[K_r]_i$ Total released potassium concentration in each step $i$ of the infection process in mol/L

$[K_r]$ The total released concentration of potassium ions in mol/L

$\Delta V$ ISE system output voltage changes after drift compensation

$\Delta V_k(t)$ ISE system output voltage changes without drift compensation

$\Delta \psi'$ Changes in $\psi'$ during the test interval

$\alpha$ Fraction of the internal potassium ions released during the whole infection
\( \beta \)  Surface buffer capacity of an oxide

\( \chi^{\text{sol}} \)  Constant dipole potential of the solvent

\( \psi \)  pH dependent surface potential

\( \psi' \)  Potassium ion (K\(^+\)) sensitive voltage across a potassium-selective membrane

\( \tau \)  Time constant of the infection process in the system model

\( d \)  Delay associated with the infection process in the system model

\( d_k \)  Drift at the output of the ISE system

\( z \)  Valency of an ion
Chapter 1

Introduction

1.1 Motivation for Bacterial Detection and Antibiotic Testing

There is increasing demand for systems that detect and identify bacteria, in applications ranging from medical diagnosis to water and food inspection. Timely diagnosis and treatment of infectious diseases is one of the greatest global health challenges. The World Health Organization (WHO) reported that infectious diseases cause 25.9% [12] of all global deaths and account for 45% of global disease burden in 2000. Large numbers of patients and the unavailability of rapid low-cost tools for pathogen identification have resulted in misdiagnoses and over-prescription of antibiotics, exacerbating global antibiotic resistance [13].

Specific applications of our current prototype system is to identify bacteria in urinary tract infection (UTI) and to identify bacteria during water safety inspections. Urinary tract infections are the most common type of bacterial infections [14] and are one of the top five most burdensome infectious diseases [15, 16].

Currently available techniques used for bacterial detection include culturing methods, polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA) [17]. Culturing techniques, where bacteria are grown on nutrients and then characterized according to their physical or biochemical features, are very reliable but slow (take several hours to days to yield results) and are labour-intensive. They require bulky and expensive lab equipment and highly trained personnel to use them. Both PCR and ELISA techniques have been successfully implemented in single integrated platforms providing rapid response in point-of-care systems [17, 18]. ELISA systems suffer from disadvantages associated with high costs of antibody production to achieve high specificity towards tar-
get bacteria. Many PCR systems are subject to high costs of biomarkers, many steps of sample processing (cell lysing, DNA/RNA extraction), high power consumption associated with thermal cycling and large devices (in the range of cube centimetres) [19]. In addition, PCR techniques cannot distinguish live from dead bacteria without introducing additional sample processing steps [20].

In this thesis, some of the challenges of today’s bacterial detection systems are addressed by utilizing techniques that combine microelectronic systems with new classes of biological components and sensing techniques. In particular, the focus is on the design of low-cost biological probes, miniaturized single-chip electronics and simple sample processing steps to reduce the biosensor system complexity, cost and response time.

Antibiotics are widely used for the treatment of bacterial infections. They are also regularly added to livestock feed and are used in the cultivation of fruits and vegetables [21]. Antibiotics have been used extensively in the past for various infections because of their wide killing spectrum and because of the lack of a rapid technique to identify the source of the infection. This has resulted in global antibiotic resistance, requiring careful diagnosis before any antibiotics administration [22]. A significant step towards this goal is to identify what kind of antibiotics needs to be administered even when the exact source of the infection is unknown. Techniques currently used for this purpose are based on culturing techniques very similar to bacterial detection methods [23]. In this thesis, a new method, for a specific class of antibiotics, has been developed to increase the speed of detection and provide ease-of-use and accuracy in antibiotic stewardship.

1.2 Bacterial Detection and Antibiotic Testing Systems and Their Specifications

Detection and/or identification of bacteria in a sample is performed by employing a set of analytic assays (procedures) that follow specific protocols and require one or more items of equipment. A bacterial sensing system is an integrated platform that performs one or multiple assays according to the required protocols with the goal of detecting the presence of bacteria in the sample and/or identifying the bacteria. In order to design an optimal bacterial sensing system, all the system constituents, including the detection technique, equipment and procedures to handle the sample and perform the assays, need to be designed for component compatibility and overall performance of the sensing system. In order to characterize and compare different bacterial sensing systems in this thesis, a review of the basic parameters that characterize each type of system as well as its
performance is provided below.

1. Qualitative vs. quantitative measurement: Qualitative tests can detect whether the target bacteria are present in the sample or not. Quantitative tests can measure the quantity (e.g. in cfu/ml) of the target bacteria present in the sample in addition to performing the detection.

2. Minimum detection limit/sensitivity: The minimum concentration of the target bacteria where the bacteria-sensing system detects the presence of the target. For systems performing qualitative measurements, any bacterial concentration below the detection limit results in no detection system output. The sensitivity of the sensing system is often reported as the minimum detection limit.

3. Amplification: In some sensing platforms, a method is utilized to increase the concentration of the bacteria, DNA, etc. to decrease the minimum detection limit. A simple example of amplification is providing nutrients to allow the sample bacteria to grow, divide and increase in concentration. The amplification period is the time required to increase the concentration of the bacteria in the sample prior to the measurements performed by the bacteria sensing system.

4. Detection time: the time required to give results during the test performed by the bacteria sensing system.

5. Specificity: How specific the bacteria sensing system is to the target bacteria. In chapter 2, we explain how bacteria is specifically identified. As an example for specificity, assuming the target bacterium is identified as *E. coli* O157:H7, the only positive detection response from the sensing system should come from this strain of bacteria. Some sensing systems are designed for wide-spectrum sensing; e.g., detection of all strains of *E. coli* bacteria. In this case, the specificity indicates if any detection outside the target *E. coli* species occurs.

6. Sample volume: The minimum sample volume required for the measurements performed by the sensing system. It might depend on the amplification, sample handling methods and sensitivity required. Also, the initial sample volume might differ from the final sample volume utilized in the final measurement step.

7. Cost: The total cost for one measurement. This includes the labour cost of sample handling, the costs of reagents and buffers, as well as those of biological reagents, the fabrication of various components of the sensing system and consumables and packaging.
Antibiotic susceptibility testing (AST) is performed by a sensing system similar to a bacterial sensing system, and equivalent specifications are required. In both cases the samples contain possible unknown bacteria. A bacterial sensing system needs to detect and/or identify the bacteria present. In an AST sensing system, the sensor is required to indicate the antibiotic that is able to kill the bacteria in the sample without necessarily requiring the identification of the bacteria per se.

1.3 Challenges and Objectives

One of the greatest challenges in bacterial detection, identification and AST approaches is the development of a low-cost and rapid integrated system that can be utilized in both clinical and laboratory settings. A significant step towards designing an integrated bacterial detection and identification sensing system ("bacterial sensor", for simplicity) requires new types of miniaturized sensors that can provide fast and reliable responses using small sample volumes and provide high specificity toward the target bacteria while maintaining good sensitivity/low detection limits. Optimizing the integrated circuit performance alone does not significantly enhance system performance, since all the system components, including biological, chemical and, more importantly, compatible biochemical detection techniques, affect the sensor performance significantly.

In this thesis, we focus on the following aspects of bacterial sensor design:

• **CMOS Integration:** We focus on bacterial detection and identification integrated in low-cost generic microelectronic fabrication processes. We specifically focus on complementary metal-oxide semiconductor processes where the high integration capability of electronic devices at a low cost is achieved because of their wide utilization in today’s consumer electronics. Integration of the whole detection system in an electronic platform would also provide a highly configurable, miniature and easy to use bacterial sensing systems.

• **High Specificity, Rapid Response, Low Sample Volume and Low Cost:** We mainly focus on a systematic design approach to the selection and design of sensor components. We focus on the selection and design of biological components, buffers and protocols that provide high specificity and very rapid responses—usually under 10 minutes—using small sample volumes. Reducing the cost of the biological components is also one of the first design constraints that we discuss in this thesis.

• **Simple and Rapid Sample Processing:** We concentrate on designing simple, efficient protocols that rapidly prepare biosensor-compatible samples from raw
liquid samples in under 5 minutes. We utilize a constant sample buffer for the biosensor throughout all experiments to minimize the effect of interference from present components in raw samples.

1.4 Outline of the Thesis

The outline of this thesis is as follows:

Review and Background of Bacterial Biosensors Presented in Chapter 2

Chapter 2 provides an introduction to relevant biological components for bacterial sensors, followed by a description of traditional techniques currently available for bacterial detection and identification. It provides some examples of available integrated biosensors and provides background information about different system components utilized in state-of-the-art biosensors.

System-Level Sensor Design and Development of a Behavioural Model Presented in Chapter 3

Chapter 3 describes the system-level selection and design of the components of our proposed bacteria biosensor system based on our required specifications. Chapter 3 includes the following:

- system-level block diagram of the sensor and selection and design of each block.
- description of the biological components and techniques used in the bacterial sensor.
- discussion on selection of the electronics components in the system. Further design details are provided in Chapter 4.
- design of the signal processing unit to cancel DC-offset and drift non-idealities in the circuit components.
- development of a behavioural system model of the bacterial biosensor.

Background, Design and Electrical Measurement Results of CMOS Circuit and Potassium-Selective Membrane Presented in Chapter 4

Chapter 4 provides background, specifications and constraints in the design of the analog front-end (one of the blocks in a bacteria sensor system). It also discusses the design
of the two test CMOS prototypes that are utilized in our bacterial sensors. Design of two test chips are discussed in this chapter. Either of the test chips can be used for experiments. Electrical measurement results of the chips are included in this chapter.

**Experimental Results of the system as a bacterial biosensor Presented in Chapter 5 and Chapter 6**

In Chapter 5, protocols and the first experimental results of the biosensor system using our first generation CMOS prototype for bacterial detection are provided. Chapter 6 discusses the usage of a new set of biological components and a second generation CMOS design for bacterial detection and identification, as well as the experimental results.

**Application of Biosensor for Antibiotic Testing Presented in Chapter 7**

Chapter 7 provides an introduction to antibiotics, followed by the protocols, experimental design and test results of our system for antibiotic susceptibility testing (AST).

**Contributions and Future Work Presented in Chapter 8**

Chapter 8 summarizes the contributions of this thesis and provides insights into possible extensions of this work, as well as future directions of research.
Chapter 2

Fundamentals of Bacterial Sensing Systems

This chapter provides a brief introduction to some of the biological components commonly used in this thesis. It then discusses available techniques for bacterial detection and identification. It finally defines the concept of biosensors and provides a basic block diagram illustrating their use in the context of bacterial sensing. Table 2.1 shows the important sensor specifications for some applications of bacterial detection systems.

Table 2.1: Application-specific sensor sensitivity and volume requirements

<table>
<thead>
<tr>
<th>Application</th>
<th>Sensitivity</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water quality inspection [24]</td>
<td>1 cfu/100ml</td>
<td>100ml</td>
</tr>
<tr>
<td>Sepsis detection [25]</td>
<td>1-30 cfu/ml</td>
<td>1ml</td>
</tr>
<tr>
<td>Urinary tract infection [26]</td>
<td>$10^5 - 10^8$</td>
<td>10ml</td>
</tr>
</tbody>
</table>

2.1 Introduction to Basic Biological Components

There are a number of biological entities that can be used to construct systems for bacterial detection, and identification and treatment. A brief introduction to relevant biological entities and chemicals that are essential in many existing systems is provided. New types of biological components related to bacterial detection systems will be introduced in Chapter 3.
2.1.1 Bacteria

Bacteria are unicellular microorganisms. Their genetic material is not enclosed in a specific nuclear membrane; hence they are part of the category of Prokaryotes. Eukaryotes, as opposed to Prokaryotes, are the organisms that have a nucleus with a well-defined membrane enclosing it. Bacteria species (like all Prokaryotic species) are defined as a population of cells that have similar characteristics and can interbreed [27]. A genus contains species that are different but are related by descent. Different clones or genetic variants of a single species of micro organism are called strains. The scientific name of an organism starts with genus as the first name that is also capitalized (e.g Escherichia) followed by species name that is not capitalized (e.g coli). Both of these names are written in italics. The strains are identified by numbers and letters following the species name of the organism (e.g O157:H7 or BW25113). The genus name is often abbreviated in the text (e.g. E. coli O157:H7 that completely identifies a specific strain of E. coli) [27].

Bacteria are typically between 0.5\(\mu\text{m}\) to 5\(\mu\text{m}\) in size. Bacteria are enclosed in cell walls of various shapes that consist of carbohydrate and protein complexes called peptidoglycans. The cell wall surrounds the fragile cytoplasmic membrane and protects the inner cell. The cell membrane is selectively permeable to ions and organic molecules and controls their transfer to and from the cell [28]. Table 2.2 shows some of the characteristics of Escherichia coli (E. coli), a common bacterium used in this thesis.

<table>
<thead>
<tr>
<th>Table 2.2: E. coli statistics [10]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell length</strong></td>
</tr>
<tr>
<td><strong>Cell diameter</strong></td>
</tr>
<tr>
<td><strong>Cell aqueous volume</strong></td>
</tr>
<tr>
<td><strong>Number of ions/cell</strong></td>
</tr>
<tr>
<td><strong>Number of K(^+) ions/cell</strong></td>
</tr>
<tr>
<td><strong>Speed at which E. coli move</strong></td>
</tr>
<tr>
<td><strong>Concentration of protein in cell</strong></td>
</tr>
<tr>
<td><strong>Concentration of RNA in cell</strong></td>
</tr>
<tr>
<td><strong>Concentration of DNA in cell</strong></td>
</tr>
</tbody>
</table>

Since one of the goals of this thesis is the development of techniques for the detection and estimation of a bacterial concentration in a sample, it is interesting to note how bacteria grow. Bacteria in a nutrient-rich environment increase in number by cell division.
Various growth factors contribute to the optimum growth and multiplication of bacteria, depending on the strain. Nutrients in the culture media, temperature, pH and osmotic pressure in the surroundings are some of the important factors. Generation time is the time required for a bacterial cell to divide, doubling the number of cells. As an example, the generation time for *E. coli* in an optimal growth environment at 37°C is 20 minutes. Small aliquots of bacteria are added to a solid or liquid nutrient medium and left for several hours or overnight to multiply in order to increase the concentration of the bacteria for further analysis. This process is known as incubation. Traditional laboratory-based techniques that assist in the detection and estimation of bacterial concentrations will be discussed in section 2.2.

### 2.1.2 Antibiotics

Antibiotics are agents that kill or inhibit the growth of microorganisms. Most antibiotics are derived from different species of bacteria that utilize these antimicrobial weapons against competing bacteria species [22]. There is a broad range of antibiotics with different activity spectrums. Some antibiotics have narrow-spectrum microbial activity meaning that they kill only a few bacteria species. Because the identity of the bacteria in a sample is often unknown, wide-spectrum antibiotics are desirable to ensure the bacteria are killed. The disadvantages of using wide-spectrum antibiotics include the unnecessary killing of a large proportion of normal, useful bacteria in the host (e.g., human intestine) and the emergence of “superbugs” (i.e. antibiotic-resistance bacteria). The normal microbes compete in the natural host environment providing normal growth. If antibiotics destroy certain organisms from the useful microbes and leave some of the competitors, the survivors may become opportunistic antibiotic-resistant bacteria.

In order to determine the efficacy of an antibiotic against different strains, two basic quantities are defined. The minimum inhibitory concentration (MIC) of an antibiotic is the minimum concentration of an antibiotic added to a sample that inhibits the growth of a bacterium after a given period of growth of the cultured sample in nutritious media [22]. Minimum bactericidal concentration (MBC), defines the minimum concentration of an antibiotics added to the sample that kills the bacteria or inhibits the growth of a bacteria after subculturing the sample in a new antibiotic-free nutritious media [29, 22]. More detailed explanation about antibiotics and their killing mechanism will be provided in Chapter 7.
2.1.3 Viruses

Viruses are very small (20nm to 1000nm) particles that are not cellular. They have a core made up of only nucleic acid, either DNA or ribonucleic acid (RNA), surrounded by a protein coating. Viruses are not considered living entities because they cannot reproduce on their own. They are inert outside their living hosts, which they require to reproduce. As part of their reproduction cycle, they infect bacterial cells, hijack the cells’ internal machinery to make copies of themselves, and ultimately result in lysis or death of their host. The lysis of the cells results in the release of internal cell components and many copies of the virus [27].

2.2 Current Techniques for Bacterial Detection and Identification

Techniques currently utilized to detect the presence of bacteria species and to further identify them are mainly based on a series of assays that systematically differentiate microorganisms through one or a combination of the following techniques. Some are used more efficiently in new generations of sensing systems. This section also introduces how some of these techniques are used to quantify the bacteria present in a sample.

2.2.1 Visual Inspection

Visual inspection of the sample under the microscope provides morphological (structural) characteristics of the microorganism, such as shape and size, that help in identification [27]. Techniques such as staining can also be used to differentiate species under the microscope. Direct visual inspection involves labor-intensive sample handling, purification and processing. The detection or identification also needs to be performed by trained personnel using desktop laboratory-based equipment. On the other hand, these methods provide direct visual observation of the cells.

Gram Staining Method: Gram-Positive and Gram-Negative Bacteria

Gram staining is a very popular staining technique that categorizes bacterial species into two groups named Gram-positive bacteria and Gram-negative bacteria. Hans Christian Gram invented the technique [30] where a primary stain (crystal violet) is applied to a heat-fixed bacterial culture and followed by the addition of iodine, and then a decolorization step, and, finally, counterstaining. After the decolorization step, Gram-positive
bacteria retain their purple color while Gram-negative bacteria lose their color. Gram-negative bacteria instead acquire a pink color following the counterstaining step.

The classification of bacteria into these groups is important since it differentiates two categories of species with distinct differences in cell wall structure and composition. The cell wall in a Gram-positive bacteria [27] contains a thick peptidoglycan, a mesh-like layer of sugars and amino acids, responsible for the retention of the violet dye that surrounds the cytoplasmic lipid membrane. Gram-negative bacteria have a thin peptidoglycan layer positioned between an outer membrane and an inner membrane [27]. It is important for a bacterial detection method to be able to detect and differentiate both categories of bacteria, especially for methods that rely on cell wall structure and membranes for proper functioning. Gram-positive bacteria are more susceptible to antibiotic treatment because of their single-membrane structure. The outer membrane in Gram-negative bacteria has a complex structure protecting the cells from damage, as well as penetration by many antibiotics.

2.2.2 Culturing Techniques

Culturing techniques are currently the most popular methods used for bacterial detection, identification and quantification. Culturing promotes growth (cell division) of the bacteria in a sample in a controlled nutritious media.

Culturing techniques take a very long time to provide results. They consume significant chemicals and supplies and the outputs need to be characterized by trained personnel. The advantages of culturing techniques are their relative ease of use and versatility.

Solid Culturing

In a simple solid culturing assay, a small aliquot of the sample is distributed onto a solid nutrient-rich agar plate and left to dry. The plates are then incubated at a certain temperature for a period of several hours to days so that each individual bacterium multiplies in number and results in a growth region called a colony. Selective and differential plates that suppress the growth of some organisms while encouraging the growth of the target cells are used for differential identification [27].

Using solid culturing, the direct measurement of the bacterial concentration of a sample is performed as follows [31]: the sample is diluted and spread on an agar plate and left for several hours (e.g. overnight for *E. coli*) at a certain temperature to grow and form colonies. The number of colonies are counted the next day in terms of the number
Chapter 2. Fundamentals of Bacterial Sensing Systems

Figure 2.1: Cultured agar plate of dilution showing (a) *E. coli* K12 Bw25113 and (b) *Staphylococcus aureus* 8325 used to quantify the concentration of the original undiluted sample. The round dot-like regions are the colonies grown from a single bacterium.

of present bacteria in the sample in units of colony-forming units per millilitre (cfu/ml) [32] and adjusted back to the undiluted concentration of bacteria in the sample. Figure 2.1 shows the photo taken after an overnight culturing of a diluted sample.

**Liquid Culturing**

Liquid culturing can also be performed, though the quantification of colonies that can be counted, as in solid culturing, is not possible. An indirect measurement of the number of cells in a liquid sample is performed using a spectrophotometer to measure the optical density (OD) of the liquid sample at a specific wavelength. In a spectrophotometer, a narrow beam of light is passed through a small sample volume and the reflection or transmission of the light through the sample is quantified. The output of the spectrophotometer cannot be used alone to quantify the concentration of the sample. The spectrophotometer is first calibrated to the OD of zero using a chosen buffer. Then the sample suspended in the same buffer is inserted. Since any impurities, including bacteria, result in scattering of the light, the output optical density is different from the sample buffer alone. The OD also changes with the concentration of the cells in the sample. This method is only utilized in laboratories when growing a known species to estimate the concentration of the available bacteria. To correlate the optical density with the actual concentration of the cells, separate experiments need to be done using solid culture...
techniques to map the OD numbers to the actual concentration in cfu/ml. The mapping curve also depends on the bacterial species because the light scattering in the sample depends on the morphological characteristics of its constituents. A wavelength of 600nm is the standard for measurements involving live bacteria [33] and it is used throughout this thesis to prepare samples at specific concentrations.

2.2.3 Enzyme-Linked Immunosorbent Assay (ELISA)

Immunological techniques use the selectivity of antibody-antigen interactions for the selection, enrichment and, ultimately, detection of bacteria. Antibodies are produced as a response to a specific foreign microorganism by bonding with the antigens on the surface of the microorganism [34]. Antibody-antigen bonding is very specific and can be used for rapid testing. Immunological techniques can be combined with other sensory systems to provide a complete, self-contained detection package, as, for example, found in ELISA systems. In ELISA systems, antibodies sensitive to the target bacterial antigen are produced and linked to an enzyme and attached to a substrate. The sample is applied to the substrate where possible binding occurs. The sample is washed and enzymes are added that result in a reaction with the bonded antibodies to produce a detectable signal, most commonly a color change [35]. ELISA systems combine the selectivities of antibodies with sensitivity of enzymatic detection. They have been extensively utilized in different sensor implementations, as reviewed in [36].

The extraction of antibodies can be quite expensive, depending on their target range [36]. This is one of the main costs of systems using antibodies in general. ELISA systems and their variations can be used in integrated systems. In general, systems relying on selective attachments can also form non-specific attachments hindering the accuracy of the results. In summary, high cost and potential non-specific reactions are the main disadvantages of this technique.

2.2.4 Phage Typing

Bacteriophage ("phage") is a type of virus that infects bacteria. Different phages are specific to different bacterial targets, allowing for their use in both narrow and wide-spectrum bacterial detection and identification. Phage typing is a technique that identifies the phages that the sample containing possible bacteria are sensitive to [37]. The bacterial culture is spread on a nutrient agar plate, as in solid culturing, and small droplets of different known phages are deposited at specific locations on top of the plate. After sufficient incubation of the plates, the bacterial colonies grow and cover the whole
agar plate except at the locations where they are sensitive to particular phages, because phages kill and inhibit the growth of the sensitive bacteria. By mapping the sensitivity of the sample to different types of phages, a sample bacterium can be characterized down to its specific strain. Figure 2.2 [1] shows an example of a phage typing plate where different phages have been applied. The clearing zones are the spots where the bacteria has not grown, showing its sensitivity to the applied phage. Though phages are inexpensive as compared to antibodies, phage typing technique still use culturing methods that take significant time to give results and, need to be performed in a laboratory setting using trained personnel. The advantage phage typing has over simple culturing is that it is much more specific in the identification of bacterial strains in the sample.

2.2.5 DNA Techniques

Extracting the genetic fingerprint of a microorganism is a sure way to identify it. With these techniques, the cells need to be lysed, purified and processed. Determining the entire genetic sequence is very expensive, takes time (several weeks) and is impractical for general purpose identification [36]. However, using restriction enzymes to compare the base sequences of different organisms and comparing them provides a certain degree of identification capabilities [36].

Advances in Polymerase Chain Reaction (PCR) [38] that amplify different DNA strands through rapid heating and cooling cycles have improved identification capabilities. The traditional DNA techniques test the amplified DNA strands against known
strands of bacterial DNA using gel electrophoresis [39]. Gel electrophoresis separates DNA strands by size and reactivity. DNA techniques that rely on PCR assays require special equipment for thermal cycling and annealing steps. They take hours to produce results and, in most cases, cannot distinguish between live and dead bacterial cells. Combinational techniques are available that mitigate this problem by combining additional steps with PCR to stop DNA replication of the dead cells as in Ethidium Monoazide PCR (EMA-PCR) [20].

In other DNA techniques [40], DNA probes are used to detect hybridization between amplified DNA strands and the target strands. Southern blotting technique uses electrophoresis-separated DNA strands heat-fixed onto a membrane, and subsequent strand detection by probe hybridization [40]. Techniques utilizing DNA hybridization are being developed for fast identification of microorganisms in the latest generation of sensors [18].

### 2.3 Biosensors and Their System Block Diagrams

The bacterial detection and identification techniques discussed in the previous section provide a comparison baseline of the methods available today. None of these methods satisfy the simultaneous requirements of a low-cost, portable, easy-to-use sensor that provides rapid results that are both specific and sensitive enough for the detection of live bacteria.

The trend in the detection and identification of biological entities is toward the usage of single or multipurpose integrated platforms called biosensors that could simplify the detection process using low-cost integrated devices that require little or no expertise to operate [16]. Biosensors have gained particular attention as a means to identify microorganisms due to their specificity, sensitivity and real-time handling of the information [41, 36]. By definition, a biosensor is a device that transforms biological information into an analytically useful signal [42], as shown in Fig. 2.3. Central to this concept is a biological transducer that transforms the biological information into an input to the electrical transducer where it is converted to an electrical signal. The electrical signal is then processed and the output decision is made according to the biosensor design target. The biological transducer consists of two main building blocks as described below: the biological recognition element and the recognition-event translator.
2.3.1 Biological Recognition Element (BRE)

A biological recognition element identifies the biological target of the biosensor. The specificity of the biosensor toward its target is determined mainly by the biological recognition element. Biosensor material cost is also heavily dependent on the selection of the recognition element. Commonly used recognition materials for bacterial detection are antibodies and DNA strands that can be mixed with the sample or immobilized on the sensor platform itself. Other new and powerful recognition elements are aptamers, molecular imprinted polymers (MIPs) and bacteriophages [36]. These biological recognition elements are chosen based on the target bacteria to be detected by the biosensor. A desirable feature would be parallel integrated measurement channels that would allow multiple bacterial targets to be identified in a sample at once.

Aptamers are molecules that are engineered to bind to specific target molecules. They are created by a series of selection and amplification steps using a target sample of DNA, a toxin, a protein or even a whole cell through a process called SELEX (systematic evolution of ligands by exponential enrichment) [43]. A large library of engineered aptamers is being created and they have been used in detection systems that previously used antibodies or DNA fragments [43, 44]. Molecular imprinted polymers or plastic antibodies, are artificially created receptor structures to serve as antibodies. So far they have not be used for pathogen detection, but are considered as candidates for future pathogenic sensors [36].

In this work, we use bacteriophages using a novel recognition-event translator, and introduce bacteriocins as another category of powerful recognition elements with variable specificity towards target cells. A more detailed explanations of these two BREs will be provided in Chapter 3.

2.3.2 Recognition-Event Translator

The recognition-event translator exploits specific physical, chemical or optical interactions between the biological recognition element and the physical input of the biosensor to provide an analytically useful signal for the electrical transducer. It utilizes specific
properties associated with the biological recognition element, selects the biological events that occur during the recognition process, and translates these events to meaningful time-dependent data. Selection of the recognition-event translator in a biosensor has the most impact on the detection speed and immunity to interference in biosensors.

Examples of recognition-event translator designs for bacterial sensors using antibodies and DNA strands as recognition elements are as follows. In systems using antibodies, the recognition-event translator includes antibodies immobilized on electrodes and uses the event of antibody-antigen binding to produce an analytically useful signal for the transducer [45, 46, 47].

For systems using DNA strands as biological detection elements, two categories of events can be used. The first is the binding (attachment) event between the immobilized strands and the sample. Non-specific absorption of the sample constituents onto the electrodes in the sensors that detect binding effects can result in significant interference; hence false positives and false negatives in these biosensors. The second is the chemical reactions that occur due to hybridization of the target strand and the sample (e.g. pH changes, as in [18, 48]). Hybridization is different from simple binding; it is the chemical process that produces non-covalent bonds between specific single complementary strands of DNA, producing a double-stranded complex.

### 2.3.3 Electrical Transducer

A variety of transducers are used to convert the output of the recognition-event translator to a readable electrical signal. Selection and design of the transducer is mainly defined by biosensor cost, ease of use, and sensitivity and detection limit. Three main categories of transducers used in bacteria recognition are optical, electrochemical and piezoelectric transducers [36].

**Optical Transducers**

Optical sensors detect the emitted/absorbed photons from the source as a result of a recognition-event transducer event [45]. Several categories of optical sensors for bacterial detection have been reported. A brief review of these methods will be discussed here. Optical sensors are costly because of the integration of optical components and the sensitivity requirements of these components.

**Fluorescence Detectors:** In this method, fluorescent-labeled molecular recognition elements interact with the sample and emit photons for detection. Fluorescence ELISA [36], for example, is a popular method that uses specific antibodies as recognition
elements. When light of the appropriate wavelength is projected upon the sample, the antigen/antibody complexes will fluorescence. The amount of fluorescence is proportional to the amount of antigen, thus concentration of the target bacteria [49, 50]. Numerous techniques exist for the detection of proteins, bacteria or toxins [51, 52].

**Spectroscopy:** Each chemical compound produces a unique spectrum when excited by electromagnetic waves. Infrared (IR) spectroscopy measures the absorption of the infrared light while Raman spectroscopy measures the light scattered following the excitation of the samples. Bacteria are composed a large number of chemicals, each having a unique spectral signature [53].

**Surface Plasmon Resonance (SPR) Sensors:** Surface plasmons are electromagnetic waves that travel in parallel to a metal/dielectric interface. The surfaces of the sensors are treated with bio-recognition receptors and the sample is applied to the surface. The surface is then optically excited, and the emitted light from the surface is received and analyzed. The absorption of the sample onto the surface changes the refractive index of the surface, so the emitted light can be used to detect the specific binding of the bacteria to the receptors [54, 55, 56]. Special sample purification and handling is required for the use of these sensors, and interference from non-specific surface attachments is problematic.

**Piezoelectric Transducers**

These transducers are mass detection systems that detect the changes in the oscillation frequency of a crystal or microelectromechanical systems (MEMs) cantilever [57] when it is coated with a biological reagent. The binding of the bacteria to the surface changes the overall mass of the crystal or cantilever, and hence the oscillation frequency. These transducers can be designed and implemented to be very sensitive to small surface variations, though the sample purification and application need to be tightly controlled [58, 59].

**Electrochemical Transducers**

Electrochemical transducers detect the electrical properties of the sample contacting one or multiple electrodes [60]. The advantage of electrochemical transducers over optical and piezoelectric transducers is their lower cost, high integration potential in microelectronic fabrication technologies, and versatility in detecting various electrochemical properties of a sample. We focus on electrochemical transducers in this thesis.

**Amperometric Transducers:** Amperometric transducers rely on electrochemically active media that are oxidized or reduced at the working electrode. Typical amper-
Amperometric systems consist of a 3 electrodes: a working electrode (WE), a reference electrode (RE) and an auxiliary electrode. A specific voltage is applied to the WE with respect to the RE and the current between the WE and auxiliary electrode is measured. If the current is observed while scanning the potential of the WE, a voltammetric measurement is performed. In most amperometric measurements the integral of the total current is computed, while in voltammetric systems, the time-dependent current measurements are performed. These systems often lack specificity alone. Specificity is obtained by applying selective membranes to the electrodes. Biosensors based on amperometric transducers for bacterial detection reported in [61, 62, 63], as examples, use antibodies, phages and bacterial DNA as recognition elements, respectively. These BREs are immobilized on special membranes providing selective membranes for sensor specificity.

Amperometric systems have widespread applications in biosensors. They require multiple-step assays which involves immobilization of biological recognition material, washing steps, and the addition of redox mediators to provide redox currents. The measurement of redox currents requires electrodes with noble metals such as gold or platinum, adding extra cost when integrated in CMOS. The amperometric techniques are heavily dependant upon the final sample media used and specificity can be compromised because of the interference from unknown chemicals in the sample without proper calibration.

**Impedance-Based Transducers:** These transducers measure the impedance between electrodes. Impedance transducers are further divided into three main categories. The first category detects the changes in interfacial capacitance between electrodes caused by the binding of the bacteria to the receptors on the surface of the probes [64]. The second category measures the faradaic resistance between the probes as a results of oxidation and/or reduction at the surface of the electrodes. The binding of the bacteria to the surface of the electrodes creates a barrier that increases this faradaic resistance. The third category is based on conductivity changes in the solution between the electrodes. As an example of this approach, antibodies can be introduced into the area between the electrodes. The binding of antigens to the surface antibodies will change the resistance between the electrodes.

Impedance-based transducers are very much prone to sample interference because of their inherent lack of specificity. On the other hand, impedometric systems are very powerful to measure the impedance between electrodes to monitor the sample medium, cell growth as well as binding of bio/chemical materials. They can be used to detect bacteria by selective binding of recognition elements on top of the electrodes and monitoring the impedance transformations as a result of specific binding to recognition elements. These systems also require washing steps and media calibration as well as noble metal
electrodes. Examples of using bacteriophages and antibodies with impedometric systems can be found in [65, 46].

**Potentiometric Sensors:** In potentiometric approaches, the voltage between the electrodes in the sample is recorded. The system that generates this voltage can be diverse. The most common type of potentiometric sensors are chemical or biological field-effect transistors (FETs), where the gate voltage of a FET is modulated by the voltage across selective membranes on the FET electrodes. Examples of selective membranes are ion-selective membranes, enzyme-selective membranes and immobilized DNA strands (DNA-selective membranes) [36]. For bacterial detection, genetic FETs—FETs with DNA selective membranes—that detect specific DNA hybridization at the surface of the FET electrode have been utilized [36]. The usage of potentiometric techniques using FETs have so far been limited to genetic FETs for bacterial detection, in which bacterial DNA is extracted and amplified separately [36, 18].

### 2.4 Summary

This chapter provided an introduction into some of the fundamental biological components utilized in systems for bacterial detection and identification. It provided detailed background on the techniques currently utilized for bacterial detection and identification. Each of these techniques use a detection method, a series of assays based on protocols, and various equipment. Though these techniques do not provide optimal solutions for bacterial detection and identification, new classes of integrated bacteria sensing systems, referred to as bacterial biosensors, utilize and integrate these techniques in various ways. This chapter introduced the concept of integrated systems called biosensors and described their constituent building blocks. For each building block, available component examples from the literature were introduced briefly, showing advantages and disadvantages of each component selection.

Based on the description of the biosensor blocks described in this chapter, Chapter 3 of this thesis provides step-by-step selection criteria for each building block in our biosensor based on the requirements of an integrated bacterial sensing system. The compatibility between the system building blocks and optimization of the components in each block will also be discussed in Chapter 3.
Chapter 3

System Design and Modelling of the Bacteria Biosensor

In this chapter, we discuss the selection of the building blocks of our biosensor as defined by the crucial parameters of detection time, cost, sensitivity and specificity towards target bacteria and ease of use. Figure 3.1, modified from Fig. 2.3, shows the constituent building blocks of our biosensor. The selection criteria for each of the bacteria biosensor building blocks will be discussed here.

Figure 3.1: Conceptual block diagram of a biosensor using electrochemical transducer and integrated electronics.

A behavioural macromodel of the system is also introduced, and the parameters that affect the output signal of the bacterial biosensor are discussed. An algorithm is presented to extracted system model parameters from the experimental results. In Chapters 5 and 6, where measurement results of the bacterial biosensor are presented, we extract the system model parameters using the algorithm presented in this chapter, compare the system model output with the measurement results, and estimate the biosensor output using the proposed predictive model.

At the end of this chapter, some experimental design definitions, practices and common errors are introduced to facilitate our discussion of the measurement results in Chapters 6, 7 and 8.
3.1 Biological Recognition Element (BRE)

The biological recognition element defines the specificity of the bacterial sensor to the target bacteria. It defines the range of strains that interact with the biological recognition element to produce a transducible event. Non-target bacteria ideally produce no transducible event.

Recognition elements are consumables that are often used in purified format and in high concentrations in biosensors. The cost of producing high-purity components scales with the number of products used and contribute to a significant portion of the overall sensor cost. Antibodies are one of the most popular recognition elements utilized specifically in bacterial biosensors. The production costs of antibodies are quite high, and dependent on antibody specificity and on patent licensing fees [66].

The ability of a bacterial sensor to differentiate between live and dead bacteria is dependent on its biological detection elements. Samples taken for diagnostics purposes or for food or water quality inspection services all need to be examined for live bacteria. Systems using genetic material (DNA or RNA) rely on a genetic finger-print of the bacterial sample. The DNA or RNA is usually obtained from a sonicated sample of lysed cells. Interference from genetic material already in the sample, such as from dead bacterial cells, contaminates the genetic material.

The transducible event that occurs due to the interaction between the recognition element and target bacteria is also an important factor to consider in the choice of the recognition element. The robustness of the transducible event, the time-frame of the event, and the robustness of the event against interferences directly affect sensor speed, detection time and specificity.

In this thesis, we first utilize bacteriophages as recognition elements. The background on bacteriophages and the parameters important in their selection will be discussed first. We then utilize bacteriocins as bio-recognition elements and discuss their superior characteristics. Both of these biological components are naturally occurring and can be modified synthetically for specific usages. Both bacteriophages and bacteriocins have distinct cost and specificity advantages over other available BREs (antibodies, DNA/RNA, aptamers) which we will discuss in more detail below.

3.1.1 Bacteriophages (“Phages”)

Bacteriophages (also known as phages) are a type of virus that infect bacteria. Phages are ubiquitous in nature and can be found in all reservoirs populated by bacterial hosts, such as soil, the intestine of animals, or sea water [67, 68]. Phages are highly specific
Figure 3.2: (a) Left: electron micrograph of a $T_4$ phage [2]; right: structure of a typical phage. (b) Left: electron micrograph of phage attachment to *E.coli* cell membrane [3]; right: structure of phage attachment to bacterial cell membrane, DNA injection and efflux of potassium ($K^+$) ions.

to particular bacterial strains and occur naturally, hence their easy production. Phage libraries with specificities for a wide variety of bacteria are available commercially [69, 70]. Phages are inexpensive and can be produced at high concentrations and low cost and stored for several years. Another important strong point of phage-based systems is that they detect only living bacteria, reducing the number of false positive results. Figure 3.2 shows the electron micrograph of a typical $T_4$ phage, as well as a drawing that shows the structure of a typical phage. When a phage is mixed with specific host (bacterial cell), it attaches to specific receptors on the surface of the cell using its tail fibers. This specificity means that a bacteriophage can only infect bacteria bearing the exact receptors that match the phage. The presence of such receptors determines the phage’s host range [71]. After attaching to the surface of the cell, the phage injects its DNA into the bacteria using its core as shown in Fig. 3.2.

When phages attack bacteria, the membrane of the bacteria goes through a membrane depolarization and cannot sustain the ion concentrations within the bacterial cell. This results in a transitory efflux of ions especially $K^+$ from inside the cell to outside of the cell in the sample. Depending on the combination of target bacteria and the attacking phage, the rate and extent of this ion efflux is variable. In [72], the duration of ion efflux has been hypothesized to correspond to the duration of the DNA injection of the phage into the host cell. DNA injection and efflux of ions happen within minutes of infection.
But the activity of phage against bacteria is not completed by DNA injection alone. Phages with a lytic cycle, attack the surface of bacteria first and inject their DNA into the cell within minutes. The next step in the lytic cycle is the multiplication of phages within the cells. The phage DNA inside the cell makes copies of its parent phage and ultimately will lyse the cell releasing many newly assembled phage entities. Phages with lysogenic cycle, inject their DNA and integrate their DNA into the cells. The cells replicate several times with the newly integrated DNA before they are lysed, releasing a large number of phages.

Bacteriophages are robust biological recognition components that have been used for bacterial identification assays such as phage typing [37], detecting the cell lysis after overnight incubation in petri dishes. In traditional assays using phages for detection, the lytic/lysogenic cycle of the phage that results in cell lysis of some sensitive cells is used for detection. Phages have been reliable, low-cost, selective, natural biological detection elements that provide multiple possible transducible events throughout their infection cycle that can be exploited in sensors.

### 3.1.2 Bacteriocins

Bacteriocins are a class of antimicrobial peptides (proteins) that are naturally produced by a variety of Gram-positive and Gram-negative bacteria [74]. These proteins kill or inhibit the growth of specific bacterial strains. We have selected bacteriocins as an alternative to bacteriophages because of their simple protein structure compared to the complex assemblies of multiple protein components in bacteriophages, simplifying the production of the biological detection element as well as reducing the cost. Databases of different bacteriocins and their classification and killing spectra are available through web databases such as BACTIBASE [75] and BAGEL [76].

The killing spectra of different bacteriocins are quite diverse, ranging from very narrow spectra, to which only certain strains are sensitive, to broad-spectrum bacteriocins that kill a large percentage of one or more species [77, 78]. Bacteriocins have many distinct mechanisms of action that can be broadly divided into two classes, one that acts primarily at the cell envelope and those that act on processes within the cell. The first class of bacteriocins act on their specific target by forming membrane channels (pores) through the cell membrane that disrupts the membrane potential and cause cell death within minutes [79]. The pore-formation in the membrane results in immediate leakage of internal cell contents into the extracellular fluid. Unlike bacteriophages, the pore-formation results in complete target cell death and no subsequent cell division cycle.
occurs. Bacteriocins have no DNA and do not replicate inside their host bacteria cells.

Although the cell envelope and membrane structure of Gram-positive and Gram-negative bacteria are different, bacteriocins affect both classes of bacterial cells. Bacteriocins have not previously been used for bacterial identification, but provide very favourable advantages compared to other recognition elements. They are easier and cheaper to produce, even compared to bacteriophages, because of their simple structure, and they are robust under a variety of environmental conditions. They also attack only live bacteria and are very selective in targeting particular bacterial strains. The variability of killing spectra among different bacteriocins as well as their simple chemical composition provide strong motivation for their usage in the clinical identification and isolation of different live bacteria. Though naturally quite diverse, synthetic variations of bacteriocins [80, 81, 82] are relatively simple to produce, because they are proteinaceous. Bacteriocins are very strong alternatives to bacteriophages and provide very similar transducing events. If immobilization is desired in a biosensor, especially in systems detecting multiple bacterial targets, bacteriocins are easier to use because techniques for protein immobilization on top of multiple platforms have been developed [83, 84, 85].

3.2 Recognition-Event Translator

A recognition-event translator utilizes the events that occur as a result of interactions between the biological recognition component and the sample containing bacteria. It can utilize one or a combination of the events provided by the biological recognition elements, which directly result in the time required by the sensor to provide detection results. Using a selection of phages and bacteriocins as biological detection components, we now introduce the event translator in our system.

3.2.1 Sensors Utilizing Bacteriophages

The infection of the bacterial cells by phages can be exploited by sensors in different ways. In [62, 65] phages are used as biological detection elements and the recognition-event translator is the lysis of the bacterial cells that occurs after phage infection, phage DNA injection and several division cycles of the cells. In [62], an amperometric transducer is used to measure the enzymes released from inside the bacterial cells upon cell lysis by measuring the redox current proportional to enzyme concentration. Authors in [65] measured the impedance changes resulting from cell lysis.

This work does not rely on cell lysis, but, rather, exploits the chemical changes tak-
ing place during the initial infection process, when the phage injects its DNA into the cell [86, 87]. In [86, 72, 87] the kinetics of phage DNA injection resulting in bacterial cell membrane depolarization, as well as bacterial cell ion-channels involved in the infection process, have been studied. Their results show that the phage first binds reversibly to the cell surface; then it recognizes the sensitive cell and binds specifically and irreversibly to the cell outer membrane receptors. The phage DNA is then released from the phage capsid and transported through the bacterial cell wall. The DNA transport has been measured indirectly by measuring the efflux of potassium ions from inside the cell to the outside sample which happens as a result of DNA transport and membrane depolarization.

Researchers in [86, 72, 87] have quantified the above potassium (K\(^+\)) efflux by using commercial ion-selective electrodes to measure the external potassium ion concentration in the sample. They have shown the dynamics of three different phages \(T_4\), \(T_5\) and \(T_7\) infecting \(E.\ coli\) bacteria. As shown in [72, 86, 88], the dynamics of the ion efflux as well as the rate of phage adsorption depends on the phage and temperature. The total efflux of ions from inside the cell to the outside takes between 30 seconds to 30 minutes depending on the type of phage and temperature. Our group has used potassium-selective electrodes to successfully quantify potassium efflux resulting from phage adsorption using other phages, including \(\lambda\) phage [89], \(HK97\) phage [90] and \(T_6\), all infecting \(E.\ coli\) cells. Other bacteria, such as \(Pseudomonas\ aeruginosa\) and \(Salmonella\) have also been successfully tested with multiple phage combinations. Our results are presented in Chapter 5.

The recognition-event translator in this design is the potassium ion efflux resulting from infection of bacterial cells by the addition of phages, our biological recognition elements. Phages have excellent specificity towards their targets and the initial potassium efflux is easy to measure and happens very quickly upon infection. Based on the target bacteria of interest, an appropriate sensitive phage is chosen as the biological recognition element. The selected phage is added to the sample to initiate the infection process, if the bacteria in the sample are sensitive to the phage.

The output of the biological transducer is an increase in the concentration of potassium ions in the sample, which occurs upon infection. As a representative example, Table 3.1 shows important statistics about the concentration of ions in \(E.\ coli\) cells that serve as our model bacteria used in the measurements. This also shows why potassium ions (75% of the total ions) among all other ions (\(Na^+\), \(Ca^{++}\), etc.) inside the bacterial cells are chosen for measurements [91].
Table 3.1: Concentration of ions in *E.coli* cells [10].

<table>
<thead>
<tr>
<th>Number of ions/cell</th>
<th>$12 \times 10^7$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of $K^+$ ions/cell</td>
<td>$9 \times 10^7$</td>
</tr>
</tbody>
</table>

### 3.2.2 Sensors Utilizing Bacteriocins

Pore-forming bacteriocins kill and lyse their target bacterial cells by weakening the membrane that results in efflux of inter-cellular ions into the sample medium. The events occurring following bacteriocin exposure are very similar to the event happening after phage infection. The primary difference is that cell lysis occurs much faster than with phages [92, 93]. Spotting the bacteriocins on a lawn of sample bacteria in a petri dish, as in phage typing, can be used for identification of sensitive bacteria after overnight incubation. In [92, 93], the kinetics of pore-forming bacteriocins (colicin A and colicin K) were observed by measuring the efflux of potassium ions from inside cells to the sample.

We have extensively tested and used different bacteriocins to which different species of bacteria are sensitive by measuring the potassium ion efflux resulting from exposure of the bacteriocin to the sensitive bacteria. Our experimental results are presented in Chapter 6. Therefore, our biological event translator is the efflux of potassium ions from sensitive cells.

Though the cell lysis resulting from bacteriocin exposure results in efflux of all intracellular ions, potassium ions are measured because of their high concentration inside bacteria cells (see Table 3.1). Figure 3.3 shows the output of the recognition-event translator when phages or bacteriocins are used as the biological detection elements.

Figure 3.3: The output of the recognition event translator when phages or bacteriocins are used as biological detection elements.

One of the difficulties with many previously-designed biosensors is that they cannot distinguish live from dead bacteria, a factor that plays a critical role in medical treatment decisions. While PCR-based methods incorporating chemicals such as propidium
monoazide [94] and reverse-transcription PCR [95] can differentiate DNA derived from live versus dead bacterial cells, these reagents are sensitive to inhibition by contaminants from biological samples, making the tests less robust and more difficult to perform. By contrast, the use of bacteriocins (as well as bacteriophages) for bacterial detection intrinsically identifies only live cells with no extra reagents or procedural steps, as the ion flux requires that the bacteria be alive and physiologically viable (i.e. they have membrane polarization). An additional advantage of our biosensor is that the detection of ion flux from a viable bacterial cell does not require that it be actively dividing. Many bacteria exist in a viable but nonculturable state [96], where they are metabolically static (and antibiotic insensitive) but can retain virulence following a return to an actively metabolizing state [97, 98]. These dormant bacteria have been shown to act as hidden reservoirs of disease, thus, the accurate detection that could be achieved with this biosensor is highly desirable.

3.3 Ion-Selective Electrode Systems as Electrical Transducers

To quantify the event of the ion efflux resulting from the interaction of bacteria with either bacteriophages or bacteriocins, multiple electrochemical transducers can be used. Potentiometric transducers are among the most suitable, with high integration capabilities. We have selected the ion-selective electrode system (ISE system) as the transducer for both of the biosensors utilizing bacteriophages and bacteriocins. The target ion in the ion-selective electrode system is potassium in both systems because of the high concentration of potassium ions inside bacterial cells in comparison to other ions (see Table 3.1), as well as previous research showing extensive leakage of potassium ions.

The ISE system measures the concentration of potassium ion in a sample. It converts the potassium ion concentration in the sample directly to an electrical voltage or current. The changes in the potassium concentration in the sample as a result of potassium efflux from the leaking cell membranes are measured in our bacteria biosensor. There are different ways to construct an ISE system. In this thesis, we are interested in integrating an ISE system in a conventional CMOS process.

In conventional ion-selective electrode systems, an ion-selective membrane is in contact with two liquid solutions. These ISE systems (liquid-contact) were developed long before solid-contact ISE systems and ion-selective field-effect transistor systems but their working principle is very similar. Here, an introduction to the working principle of liquid-
contact ISE systems will be provided along with a brief discussion of how solid-contact ISE systems are built.

### 3.3.1 Ion-Selective Electrode Systems (ISE Systems)

An ion-selective electrode system is a potentiometric electrical transducer that converts the activity of a specific ion dissolved in a solution into an electrical potential. Figure 3.4a shows the conceptual working principle of a conventional liquid-contact ISE system. Depending on the target ion to be measured, a specific ion-selective membrane is used to separate the outside liquid sample from an internal reference liquid which has a non-changing specific ion concentration. Two sets of reference electrodes contact the sample and the internal reference liquid respectively. The reference electrode in this thesis refers to an ideal electrode that keeps the voltage across the liquid-electrode interface constant, regardless of the ion composition of the liquid (E1 and E3 in Fig. 3.4b). The voltage across the two reference electrodes in Fig. 3.4 is measured by a voltage-sensing system and the voltage changes by variations in the concentration of the target ion. As shown in Fig. 3.4b, all the voltages across the interface between the reference electrodes and the liquids are constant except for the voltage across the ion-selective membrane that separates the sample liquid and the internal reference liquid.

![Reference Electrodes](image)
![Sample to be Measured](image)

**Figure 3.4:** Conceptual working principle of a conventional liquid-contact ISE system. E1, E2, E3 and E4 show the electrochemical voltages between the reference electrode-sample (E1), membrane-sample (E2), membrane-internal reference liquid (E3) and internal reference liquid-reference electrode (E4). All these voltages are constant except E2. The potential difference across the membrane (the potential difference between sample liquid and internal reference liquid) is called membrane potential $E_m$, which is equivalent to E2 + E3.
The voltage across a membrane separating the two liquid solutions is described by the membrane potential using Goldman-Hodgkin-Katz voltage equation [99]:

\[
E_m = E_{\text{const}} + \frac{RT}{F} \ln \left( \frac{\sum_i^N P_{M_i^+} [M_i^+]_{\text{out}} + \sum_j^M P_{A_j^-} [A_j^-]_{\text{in}}}{\sum_i^N P_{M_i^+} [M_i^+]_{\text{in}} + \sum_j^M P_{A_j^-} [A_j^-]_{\text{out}}} \right)
\]  

(3.1)

where the equation assumes all ions in the solution are monovalent ions. \( P_{M_i^+} \) is the permeability of the membrane toward ion \( M_i^+ \). \( E_m \) is the membrane potential, \( E_{\text{const}} \) is a constant voltage across the membrane, \( R \) is the gas constant, \( T \) is the temperature in Kelvin and \( F \) is the Faraday constant. When a membrane is selective to a specific ion, its permeability to that ion is close to 1 and its permeability to other ions is close to 0. This equation is often too complicated to be useful in actual designs. For an ion-selective membrane, the membrane potential is often specified using a simplified form of the Goldman-Hodgkin-Katz equation, that assumes zero permeability for all other ions except for a specific ion \( I \). This equation is described as the Nernst equation [100].

\[
E_m = E_{\text{const}} + \frac{RT}{zF} \ln \left( \frac{[I]_{\text{out}}}{[I]_{\text{in}}} \right)
\]  

(3.2)

In this equation, \( z \) is the valency of the ion \( I \). \([I]_{\text{out}}\) and \([I]_{\text{in}}\) are the concentrations of the ion \( I \) on the two sides of the membrane permeable to ion \( I \). A full Nernstian sensitivity of 58.2 mV/dec at 20°C is achieved for a monovalent ion such as K⁺.

The membrane potential (\( E_m \)) in the above equations is defined as the voltage between the two liquids in contact with the membrane. In fact the membrane potential consists of two potentials (E2 + E3) in Fig. 3.4b, where each potential is generated because of the chemical interaction between the liquid and the active sites on the membrane surface, \( E_i = E_{\text{const}} + \frac{RT}{zF} \ln[I]_i \). It becomes clear here that E3 in Fig. 3.4b is constant because of the non-changing composition of the reference internal liquid. The only contributing Nernstian voltage is generated at the membrane-sample interface (E2). This is the principle behind solid-contact ISE systems. In solid-contact ISE systems, the membrane is in contact with the target sample on one side and a solid electrode on the other side. The Nernstian voltage is generated at the membrane-sample interface for the ion-selective electrode system.

ISE systems are divided into two categories of liquid-contact or solid-contact ISEs. A special type of solid-contact ISEs are ion-selective field-effect transistors (ISFETs), that are integrated in CMOS processes. We used two kind of ISE systems: a commercial ion-selective electrode that is a liquid-contact ISE system for initial experiments and an
ISFET system fabricated in a conventional CMOS process for final system integration and measurement for bacterial detection and identification. A detailed description of the working principle of ISFET in a bacterial sensing system will be provided in Chapter 4.

3.3.2 Non-Idealities in ISE Systems

All ISE systems suffer from two major non-idealities in their output voltage signal: DC baseline variation and drift. ISE systems need to undergo calibrations frequently in order to compensate for these two effects to measure the absolute concentration of the target ion accurately. Aging of the ion-selective membrane, as well as interference between ions present in a sample and the membrane, result in changes in the output voltage readings of an ISE system over multiple measurements. The sensitivity, ideally Nernstian response, is often preserved and the changes are viewed as DC baseline variations between measurements. Regular calibration of the ISE system with known buffers, similar to the buffers used in the sample, regularly help alleviate this problem.

Continuous measurements of the output of an ISE system when in contact with a sample show gradual changes in ISE system output, known as drift. The drift is present because of membrane swelling or hydration, the transfer of interfering ions across the membrane, and the leakage of membrane components [101, 102] that results in slow changes in the output of ISE systems. In the next section, we show how the processing unit in our system compensates for these non-idealities and how their effects are minimized in our system performance.

In order to miniaturize solid-contact ISE systems and integrate them in microelectronic fabrication processes, ion-selective field-effect transistors (ISFET) are implemented. Our selected platform for integrating our transducer (ISFET) and the analog front-end is CMOS because of its monolithic integration and low cost. Design principles for the ISFET and analog front-end in our system are provided in chapter 4. For the rest of this chapter, we model our transducer as a system that translates the concentration of a specific ion (i.e. potassium) in the sample to an output voltage using an ISE system.

3.4 Analog Front-End and Processing Unit

The analog front-end reads the ISFET output and provides a buffered/amplifier output of the ISFET circuitry. The design of this block will be explained in more detail in the next chapter.

The processing unit compensates for ISE system non-idealities and improves the preci-
sion of the bacterial sensor output. It is well known that ISE systems (both liquid-contact and solid-contact ISEs including ISFETs) are subject to DC baseline variations and drift [103]. Output DC mismatch between identically designed ISE systems, as well as ISE system output DC variations because of long-term drift can cause discrepancies among multiple absolute measurements of ion concentration.

In the presented detection system, the absolute value of potassium concentration in the sample is not needed for detection. The ISE system output voltage changes ($\Delta V$) as a result of changes in potassium ion concentration after phage/bacteriocin exposure provide a sufficient statistic for detection. Output DC baseline can simply be removed. Output DC cancellation also makes the detection system insensitive to different potassium ion concentrations of the input sample; providing insensitivity to the sample media.

The drift of an ISE system depends on how long the transducer has been used cumulatively (in terms of hours) and how well the membrane contacts the transducer surface (for solid-contact ISE systems). Drift can be quite high in freshly wetted ISE systems, and decreases to a stable value until it increases again at the end of the ISE system life span [103]. The experiments proposed for bacterial detection and identification take less than 10 minutes for detection and the whole infection process is completed within 30 minutes. The drift of the ISE system does not change significantly in this time frame [103]. To a first-order approximation, the drift is constant during the measurement time frame and can be estimated. However, the drift needs to be considered and compensated for in each experiment separately.

The observed output ISE system drift in the bacteria detection system contains two distinct components. The first component is the intrinsic ISE system drift, as discussed before, that is dependent on the ISE system’s stage of life as well as the sample medium. Since the volume of biological recognition element (phage or bacteriocin) added are very small compared to the total sample volume, the medium does not change significantly after BRE addition; thus, drift estimated before BRE addition is quite accurate in capturing the first drift component. The second component of the observed drift results from the metabolism of the bacteria in the sample. The natural metabolism of live bacterial cells includes several ion pumps that result in an output drift as measured at the ISE system output. The cumulative effect of both drift components are observed at the output both before and after BRE addition, thus the drift can be estimated before the BRE addition, and compensated for at the output after BRE addition, for accurate detection and estimation.

The following experimental procedure is performed to remove baseline variations, and to estimate and compensate for the effect of the drift at the ISE system output. The
sample containing the possible bacteria to be detected is added to the ISE system and the recording of the ISE system output starts. The drift is estimated using the output rate of change in the last 30 seconds before the addition of the biological recognition element (BRE) (phages/bacteriocins in this thesis). The BRE is then added, mixed while the output is continuously recorded. The drift-free output signal, the signal after BRE addition, is determined using the earlier measured drift. Figure 3.5 shows the experimental procedure as well as the algorithm followed by the processing unit to cancel the effect of DC variations and drift. The output DC offset is removed to provide zero voltage at the time where BRE is added. If we assume that the drift is $d_k (V/s)$ initially, then the first-order approximation of the actual drift-compensated $\Delta V$ is given by equation (3.3), where “$t$” is the time elapsed from the addition of the BRE to the sample and $\Delta V_k(t)$ is the measured ISE system voltage changes without drift compensation.

$$\Delta V(t) = \Delta V_k(t) - d_k \times t$$  \hspace{1cm} (3.3)

This experimental procedure does not affect the total measurement interval significantly and provides efficient drift-estimation and cancellation for the system. Figure 3.6 shows an example of ISE system output for two different experiments before and after the processing (DC baseline removal and drift compensation).

In our system the outputs are recorded and transferred to a PC where Matlab code...
Figure 3.6: (a) Typical raw ISE output signal for 2 experiments, (b) ISE output signal after processing by the PU that removes DC baseline variations and compensates for drift.

processes the raw ISE output signal to provide DC- and drift-compensated output.

3.5 Complete Bacterial Sensor

Figure 3.7 summarizes how our system using biological detection elements (BREs)—phages or bacteriocins in this thesis—are used for bacterial identification.

Figure 3.7: Conceptual block diagram of the system (a) The bacteria in the sample are sensitive to the selected BRE. After the attachment of BRE, K⁺ ions leak out of the sensitive cells. The increased [K⁺] in the sample yields an increased CMOS ISFET signal (b) The bacteria in the sample are not sensitive to the selected BRE. After the BRE is added, no changes in [K⁺] occur and, hence, no changes in the CMOS ISFET signal are expected.

The first step for bacteria identification and detection is to identify the target bacteria and the spectrum of detection. An effective bacteriophage or bacteriocin is selected,
depending on the target bacteria, whether detection of a wide spectrum of bacteria species is required or detection of a specific strain of bacteria. The sample to be tested either (a) contains target bacteria sensitive to the BRE, or (b) does not contain any of the target bacteria sensitive to the BRE. First the sample alone is applied to the biosensor surface and monitored for potassium concentration over some time. After this period, the BRE is applied to the biosensor. In case (a), the BRE infects the sensitive bacteria in the sample resulting in an efflux of potassium ions for about 30 minutes, thus changing the ISE system voltage; providing positive outcome. In case (b) the same procedure is followed, but since the sample is not contaminated with the target bacteria, the added BRE does not infect any cells, resulting in no change in potassium concentration in the sample and no observed voltage changes at the output of the ISE system. This case provides negative outcome.

Figure 3.7 shows an ideal system where the possible concentration of the bacteria tested for is above our detection limit. In the figure, we have ignored the effect of DC mismatch and drift non-idealities of the ISE system on the observed output signal. In reality, these non-idealities are cancelled properly in the processing unit before making a final decision or before data visualization.

### 3.6 Modelling the System Behaviour

According to the system shown in Fig. 3.7, the processed output signal from the ISE system provides an indication of detection and identification for the sample bacteria. We are interested in creating a model that describes how different blocks and parameters in our system affect the shape and magnitude of the observed output signal, and how the output is related to the bacterial concentration in the sample. In this section, we provide a behavioural macromodel that describes the behaviour of the crucial blocks of the bacterial sensor system that affect the output signal. We also provide insights into how different physical input parameters, like temperature, affect the system performance. This model is only applicable to positive output results, since the system ideally predicts no output voltage changes in the case of negative outcomes.

We start by looking at how the concentration of bacteria and the characteristics of the ISE system affect the magnitude of the maximum observable sensor output signal assuming positive outputs. We then move into the detailed time domain characteristics of the output signal, and propose a behavioural model that captures the effect of input parameters on the sensor system output signal. In Chapters 5 and 6, the system model parameters are extracted from several measurement results and the overall system out-
put are compared with the measurements. After introducing the system model in this section, the algorithm used for system parameter extraction in Chapters 5 and 6 will be presented. The extracted system model parameters provide system model predictive capabilities, when the system is used under various input parameters like temperature. Specific discussion on how the predictive model can be used to estimate the sensor output signal over different temperatures will be presented at the end of this section.

### 3.6.1 Ion-Selective Electrode System Output Voltage versus Potassium Concentration

Figure 3.8 shows the potassium ISE system output voltage versus potassium concentration in a sample. This is often called a calibration curve, though in a typical calibration, often only the linear region is calibrated. Figure 3.8 shows multiple regions of operation of an ISE system output.

![Graph showing ISE system output voltage versus potassium concentration](image)

Figure 3.8: The output voltage of a potassium-selective probe versus the potassium concentration in a buffered sample.

The linear region of the output is the region where there is a Nernstian or close to Nernstian response, that is, a semi-logarithmic linearity. In this chapter we refer to
this region as the linear region of operation. At potassium concentrations below the linear region, the sensitivity decreases and deviates from the Nernst equation, ultimately reaching a saturation region. The potassium concentration at which the ion-selective probe reaches the saturation region, below which it does not provide potassium sensitivity is called the detection limit of the ISE system.

The buffer solution used for calibration needs to have a constant ionic strength (ion concentration) for all measurements that is also higher than the ionic concentration being measured. This buffer solution should be chosen to be similar to the actual buffer solution utilized in the measurements. In the output curve of an ion-selective system, the minimum detection limit is the most important factor that heavily depends on the potassium-selective membrane and also on the buffer solution used. Lower than normal concentrations can be measured using a buffer with lower ionic strength, but a calibration buffer needs to be similar to the buffers used in the actual measurements. The ion concentration numbers shown in Figure 3.8 are typical numbers for most commercial PVC-based potassium-selective membranes, such as the one used in our ion-selective electrode systems (both commercial ISE and ISFET). The sensitivity of the electrode in the linear region has the maximum Nernstian response, but it can be lower depending on the ISE system and the readout circuit, as well as the quality and life span of the membrane. The baseline output voltage of the ion-selective system varies between different ISE systems.

3.6.2 Maximum Ion-Selective Electrode Output Voltage Signal Variations with Bacterial Cell Concentration

According to Fig. 3.7a, in an experiment providing positive outputs, the biological recognition element (BRE) is exposed to the target BRE-sensitive bacteria, resulting in the gradual release of potassium ions from inside the cells to the sample solution. In our experiments, we measure the potassium concentration in the sample using the ISE system for a small time period, then add the BRE, and record the voltage changes as a result of possible potassium concentration variations in the sample.

Assuming the concentration of the sensitive cells in the sample, the ones that will be leaking potassium upon addition of the BRE, are \([C_s]\) cfu/ml, then the total potassium released during the whole infection cycle is linearly proportional to the \([C_s]\). If we assume the total number of potassium ions in a single target bacterium is \(K_{in}\), and the fraction of the internal potassium ions released after exposure to the BRE is \(\alpha\), then the total number of potassium ions released per target bacterium is \(\alpha K_{in}\). \(K_{in}\) is a function of the type of
target bacterium in the sample and $\alpha$ depends on the BRE. The total concentration of potassium $[K_r]$ that is released from target sensitive cells with concentration $[C_s]$ in the sample is calculated according to the following equation:

$$[K_r] = \frac{\alpha K_{in}([C_s] \times 1000)}{N_A} \quad (3.4)$$

where square brackets [ ] around a symbol indicates concentration, and $N_A$ is the Avogadro constant equal to $6.02 \times 10^{23}$. Assuming $[C_s]$ is in cfu/ml, the numbers in parentheses in (3.4) convert $[C_s]$, the concentration of cells, from cfu/ml to moles/litre (M). The total released concentration of potassium ions $[K_r]$ in moles/litre, depends on cell concentration, total potassium content of the cell and $\alpha$. The constant $\alpha$ is the fraction of the internal potassium ions that leak outside the cells and its value is between 0 and 1. In systems using phages, the value never reaches 1 since the pore formed by the phage is repaired after DNA injection [104] when some potassium ions still remain inside the cells. In systems using bacteriocins, the value can reach 1, since the cells ultimately lyse, releasing all the ions. The value of $\alpha$ needs to be experimentally found for each BRE.

In the next section, where we look at the output signal in time domain, $[K_r](t)$ is the time-dependent concentration of the released potassium ions from the sensitive cells in the sample liquid. Figure 3.9 overlays the output curve of a potassium-selective electrode

![Figure 3.9: The output voltage of a potassium-selective electrode system versus the potassium concentration in a buffered sample with overlay of the released potassium in the sample.](image-url)
with the released potassium computed above. If the released potassium is below the minimum detection limit of the probe, no signal (voltage changes as a result of potassium leakage) occurs. If the potassium concentration is above the minimum detection limit ($DL$), then a signal is observed. The total voltage changes as a result of infection-induced potassium efflux depends on the difference between the total potassium ion concentration after infection and the potassium ion concentration in the sample before infection.

Bacterial cells contain a variety of different ions used in their life processes. The small potassium concentration in the sample, as well as the constant voltage as a result of interference from ions present in sample buffer like Sodium ($Na^+$), result in a baseline voltage $V_b$ at the ion-selective probe, corresponding to a potassium concentration $[C_b]$ in the calibration curve. The observed $\Delta V$ at the output of the electrode, after the potassium leakage is complete, compared to the time when the BRE is added, is approximated using the following equation where $S$ is the sensitivity of the ion-selective electrode in the linear region.

$$\Delta V = \begin{cases} 
0, & \text{if } [K_r] < DL \\
S \log \left( \frac{[K_r]}{[C_b]} \right), & \text{if } [K_r] > DL \land C_b > DL \\
S \log \left( \frac{[K_r]}{DL} \right), & \text{if } [K_r] > DL \land C_b \leq DL 
\end{cases}$$

(3.5)

If the complete calibration curve is obtained, it can be used to derive $\Delta V$, the output voltage changes of the ion-selective electrode due to possible BRE exposure, from cell concentration and vice versa without using this equation. This equation provides both qualitative and quantitative insights into the biosensor output. In systems where only detecting of the presence of the target is desirable, output changes above the baseline provide positive detection and the magnitude provides an estimate of the sensitive bacterial cell concentration present.

The value of $\Delta V$ defined by (3.5) is the voltage obtained after the potassium efflux has been completed. In systems using bacteriophages, the potassium efflux is transitory and is completed in approximately less than 30 minutes. In systems using bacteriocins, the efflux continues until complete cell lysis.

### 3.6.3 ISE System Output Signal Over Time

The output signal voltage of the ISE versus time, assuming no signal conversion or amplification is performed, depends on a number of parameters in the system. In the previous section, we computed the ideal final output voltage in steady-state after the potassium efflux is completed. In this section, we provide a behavioural system model
that describes how the sensor output voltage changes over time, and the parameters that affect it. In Chapters 5 and 6, this model is used to compare system model output with the measurement results to test its predictive capability when physical input parameters are modified.

Figure 3.10 shows the functional blocks and physical input parameters (e.g. temperature T and BRE) to the system that affect the ISE time domain output. In this figure, the proposed system model is shown and system model parameters (e.g. delay d and time constant \( \tau \)) are introduced.

![Proposed system model](image)

During exposure of the sample to the BRE (phages, bacteriocins or other agents that breach the bacterial cell walls), the BRE attaches to the surface of sensitive cells and results in the efflux of ions. All the phages and bacteriocins discussed here produce a single-step potassium efflux. There are reports of two-step potassium efflux in T₅ phage and T₇ phage [72]; thus we generalize the discussion to multi-step potassium efflux. We model the behaviour of each step of potassium efflux as a step function that triggers the system. The proposed system model, as shown in Fig. 3.10, that generates a time domain output signal, consists of two blocks. The first block, called the potassium efflux generator in Fig. 3.10, defines the shape of the potassium efflux from the bacterial cells. The output of this block produces the time domain potassium concentration in the sample, that is the result of the potassium released from the BRE-sensitive bacterial cells. The output signal of the potassium efflux generator is converted to a voltage at the ISE system output according to the ISE system transfer function. The ISE system transfer function...
function converts the input potassium concentration to an output voltage according to the ISE system calibration curve. This block (second block in Fig. 3.10) is called the 
ISE voltage converter.

Potassium Efflux Generator

The shape of the potassium efflux from the BRE exposure is dependent upon the BRE utilized in the experiment. If we assume one to multi-step potassium efflux upon BRE exposure to the sample, each having BRE-dependent mutually independent system model parameters, we can start modelling the BRE exposure process as series of potassium efflux steps. We modelled the BRE exposure to sensitive bacterial cells as ideal unit-sized BRE steps that provide triggering inputs to the potassium efflux generator in Fig. 3.10. For each BRE step, there is a leakage of potassium from sensitive cells to the sample, that increases the potassium concentration \([K^+]\) in the sample.

We have modelled the potassium efflux generator block as a system with a first-order low-pass step response, in the logarithmic domain, with a final output \([K_r]\), time constant \(\tau\) and a delay \(d\) associated with the potassium efflux process. The input parameters, that affect the system model parameters \([K_r]\), \(\tau\) and delay \(d\), are the combination of BRE and bacteria species and temperature. The approximate value of \([K_r]\) in Fig. 3.10 was derived in the previous section for single-step transfer systems from the total potassium ions, released from the BRE-sensitive cells. The value of \([K_r]\) depends on the concentration of the BRE-sensitive cells in the sample and the total potassium leakage from each sensitive cell. The value of \([K_r]\) also depends on the BRE-bacteria combination, but is independent of temperature. Both other system model parameters, \(d\) and \(\tau\), depend on BRE, bacteria species and temperature.

For BREs like \(T_5\) and \(T_7\) that trigger multiple steps of potassium efflux, the value of \([K_r]_i\) for each step is a fraction of the total computed value and depends on the cell concentration (cfu/ml). The fraction is mainly dependent on the BRE and needs to be empirically assessed.

ISE Voltage Converter

The ISE voltage converter has a defined voltage versus concentration transfer function explained in section 3.6.2. It converts the total potassium concentration in a sample to a voltage according to its transfer function. The total potassium concentration at any time \((t)\) after the BRE exposure, is the previous potassium concentration in the sample \([C_b]\) plus the potassium concentration as a result of potassium efflux from the sensitive
cells at any time $[K_r](t)$.

The voltage converter transfer function depends on the sample buffer used. The buffer might have different concentrations of interfering ions than the calibration buffer. In our biosensor the sample is always transferred to a buffer that stays constant, is very close to the buffer used during the calibration and does not vary from experiment to experiment, hence the buffer interferences do not cause experiment to experiment variations. In a general case, the buffer interference from ions like Na$^+$ affect the slope of the transfer function and the minimum detection limit. The method to characterize interference is by defining the relative concentration of the interfering ion to the potassium ion that causes 10% error at the sensor output. Table 3.2 shows some of the interference numbers for the commercial ISE used in our experiments. In Table 3.2, the interference numbers for Na$^+$ are particularly important since most of the biological media include high concentration of sodium ions.

Table 3.2: Relative concentration of interfering ions to potassium ion in sample buffer that result in 10% error at the potassium ISE output [11].

<table>
<thead>
<tr>
<th>Interfering ion (I)</th>
<th>$[I]/[K^+]$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$^+$</td>
<td>2000</td>
</tr>
<tr>
<td>Li$^+$</td>
<td>2000</td>
</tr>
<tr>
<td>H$^+$</td>
<td>10</td>
</tr>
<tr>
<td>NH$_4^+$</td>
<td>6</td>
</tr>
</tbody>
</table>

The only other variable affecting the transfer curve is temperature, that according to the Nernstian equation, directly influences the slope of the curve in the linear region. Since we know the sample buffer, the ISE system calibration curve at a given temperature, and the temperature during the experiments, the ISE voltage converter static transfer characteristics are known for each experiment. Upon changes of potassium concentration, the output voltage of the ISE system does not change instantaneously, although the step response of the systems used in our experiments were always below 1 second. As we will discuss later, the time constants associated with potassium efflux from different BREs are in excess of seconds, thus we have assumed the ISE voltage converter provides instantaneous response with no delay or time constant associated with it.

### 3.6.4 Biosensor Calibration Curve

Biosensor calibration curve is the transfer function of the biosensor output versus the input concentration of bacteria for a defined set of physical parameters, including tem-
perature, buffer, bacteria and BRE types. This calibration curve looks very similar to the ISE system calibration curve, where the x-axis is replaced by the bacterial cell concentration. The biosensor calibration curve can be defined by any point in time during the experiment after BRE addition. For example, if the biosensor output is taken at 10 minutes after BRE addition, the calibration curve can be defined for that point. The important parameters in the calibration curve are sensor detection limit/sensitivity and the slope of the calibration curve.

The sensor detection limit/sensitivity depends on the sample buffer interferences and the minimum potassium concentration detectable by the ISE system. The following equation defines the biosensor detection limit, where $C_{min}$ defines the minimum detectable concentration of bacteria in cfu/ml, DL is the minimum detectable potassium concentration by the ISE system, and $[C_B]$ the baseline equivalent concentration of potassium in the sample from interfering ions in the buffer.

$$C_{min} = \frac{N_A \times \max([C_B], DL)}{1000\alpha K_m}$$  \hspace{1cm} (3.6)

The slope of the calibration curve between two different bacterial cell concentrations is defined by the following equation, where $S_c$ is the slope of the calibration curve in V/dec, S is the slope of the ISE calibration curve in the region and $[C_{B1}]$ and $[C_{B2}]$ are the potassium concentrations in the buffers prior to experiment. If $[C_{B1}]$ and $[C_{B2}]$ are the same, the slope of the biosensor calibration curve is equal to the slope of the ISE system calibration curve in the specific region of operation. IF the released potassium from the cells makes the sensor operate in its linear region, then $S_c$ is close to Nernstian slope and if it is operating in the non-linear region, $S_c$ is smaller than Nernstian.

$$S_c = S(1 + \log\left(\frac{[C_{B1}]}{[C_{B2}]}\right))$$  \hspace{1cm} (3.7)

### 3.6.5 Extracting System Model Parameters from Measurement Results

In Chapters 5 and 6, several sensor measurements are performed and provide the measurement data to determine the system model parameters of our behavioural model. The first step involves extracting the above system parameters, including the total concentration of potassium released $[K_r]$, delay $d$ and time constant of the potassium efflux $\tau$, from each measured sensor output. Finding the correlation between the physical input parameters (e.g. BRE, temperature T, bacteria species and sample buffer) and the sys-
tem model parameters allows for the system model to estimate the sensor system output, when each of these input physical parameters changes, allowing for the system model to predict the sensor output.

In this section, we review the algorithm used in Chapters 5 and 6 to extract the system model parameters from the measurement results, as shown in Fig. 3.11. For each measurement, several known inputs and parameters need to be defined in the extraction algorithm. The time domain measurement results for each experiment, $V_{\text{meas}}(t)$, the known calibration curve of the ISE system at the specific temperature of the experiment, the value of the baseline ISE system voltage at the time of BRE addition $V_b$ are the parameters required by the extraction algorithm for specific physical input parameters. The extraction algorithm extracts the system model parameters, $[K_r]$, $d$, and $\tau$ using the code presented in Fig. 3.11. Figure 3.11 shows the pseudo-code that was implemented in “R” [105] for extraction.
Define ISE transfer function.
For each input of \( V_b \) (baseline voltage) and \([K_r](t_i)\) (Potassium released up to time \( t_i \) from BRE exposure), find the ISE voltage output.

**Function ISE-Transfer**
Inputs: \([K_r](t_i)\), \( V_b \)
Output: ISE voltage output

\[ [C_b] = \text{ISE-Cal}(V_b) \]
\[ \text{Total}_K = [C_b] + [K_r](t_i) \]
\[ \text{out} = \text{ISE-Cal}(\text{Total}_K) \]
return out

Find the LMS 3rd order polynomial fit of ISE calibration curve (ISE output voltage (ISE\_out) vs. Input potassium concentration ([K+\_cal]))
The inputs are the calibration vectors at a certain temperature.

**Function ISE-Cal**
Inputs: \([K+\_cal]\) vector, ISE\_out vector
Output: Third-order polynomial function ISE-Cal

\[ \text{ISE-Cal} = \text{Polyfit3}([K+_\text{cal}],\text{ISE}\_\text{out}) \]
/* ISE-Cal is a best fit third order polynomial to calibration data from ISE */

Define iSE transfer function.
For each input of \( V_b \) (baseline voltage) and \([K_r](t_i)\) (Potassium released up to time \( t_i \) from BRE exposure), find the ISE voltage output.

**Function Potassium-Efflux**
Inputs: time, \( d \), \([K_r]\), \( \tau \), initial
Output: Potassium concentration \([K_r](t)\)

\[
\text{if}(\text{time} < d)\{
\text{out} = \text{initial}
\}\text{else}\{
\text{out} = \text{initial} + (([K_r]-\text{initial})*(1-\exp(-\text{(time-d)/}\tau)))
\}\text{return(out)}
\]

Define a semi-logarithmic first-order exponential curve that produces an output potassium concentration \([K_r](t)\) with parameters delay, \( \tau \) and initial (initial K+ at time = 0)

Find least-squares estimates of the parameters of the nonlinear system model using "nls" function in "R", to fit \( V_{model}(t) \) to \( V_{meas}(t) \) by varying the model system parameters (delay, tau and \([K_r]\))

time-vector = time for both measured and system Model (Vector)
\( V_{meas}(t) = \) Time domain measured output voltage (vector)
\( V_{model}(t) = \) Time domain system model output voltage (vector)

**Main Extraction**

**Parameter-LMS-Fit algorithm** uses the \( V_{model}(t) \) at each time stamp \( t \) for comparison between system model and measurement outputs.
\( V_{model}(t) \) is derived by assuming a first-order potassium efflux resulting in an output ISE voltage according to the system model.

**Inputs:** \( V_{meas}(t) \), ISE Calibration Data Points
**Outputs:** \([K_r]\), \( d \), \( \tau \) /* delay is parameter \( d \) */

Do {
\( ([Kr], d, \tau) = \) Parameter-LMS-Fit (time-vector, V-model(t) versus V-meas(t)) /* by varying \([Kr]\), delay and \( \tau \) */
for each time stamp \( t \) {
\( K_{\text{model}} = \text{Potassium-Efflux}(t,d,[Kr],\tau,\text{initial}) \)
\( V_{\text{model}}(t) = \text{ISE-Transfer}(K_{\text{model}}, V_b) \)
}
Stop when relative convergence criterion < 1e-5
}

Find least-squares estimates of the parameters of the nonlinear system model using "nls" function in "R", to fit \( V_{model}(t) \) to \( V_{meas}(t) \) by varying the model system parameters (delay, tau and \([K_r]\))

**Inputs: V meas(t) , ISE Calibration Data Points**
**Outputs: [K_r], d, \( \tau \) /* delay is parameter d */

Do {
\( ([Kr], d, \tau) = \) Parameter-LMS-Fit (time-vector, V-model(t) versus V-meas(t)) /* by varying \([Kr]\), delay and \( \tau \) */
for each time stamp \( t \) {
\( K_{\text{model}} = \text{Potassium-Efflux}(t,d,[Kr],\tau,\text{initial}) \)
\( V_{\text{model}}(t) = \text{ISE-Transfer}(K_{\text{model}}, V_b) \)
}
Stop when relative convergence criterion < 1e-5
}

Figure 3.11: Pseudo-code for extraction of the model parameters from measurement results.
3.6.6 System Model Predictability

The proposed system model presented in section 3.6.3 provides an estimate of the bacterial sensor output when a sample containing BRE-sensitive bacterial cells is exposed to BRE. In order to predict the sensor output signal using the system model, the system model parameters ($K_r$, $d$ and $\tau$) are required. As explained earlier, the system model parameters are dependent on physical input parameters of the sensor system (BRE, bacteria, buffer and temperature) according to Fig. 3.10. In order to identify the appropriate system model parameters for any experiment, the following 3 steps need to be performed:

1) perform several experiments at various input parameters, 2) extract the system parameters using the algorithm presented in section 3.6.5 for each experiment, 3) estimate best function that describes the dependence of each of the system parameters on the input physical parameters.

The temperature and the concentration of BRE-sensitive bacterial cells are the only physical input parameters affecting the system, for a fixed combination of BRE and the BRE-sensitive bacterial cells and sample buffer, . The total potassium concentration from the potassium efflux of BRE-sensitive cells is directly proportional to the concentration of BRE-sensitive cells and the value of system model $K_r$ is estimated using equation (3.4). The next important continuous parameter affecting the system model parameters is temperature.

In the section below, we are interested in estimating how the system model parameters are affected by the temperature, for a fixed BRE, bacteria species and buffer. Previous research on bacteriocins and bacteriophages have provided some guidelines on the characteristics of phage- or bacteriocin-induced potassium efflux. These guidelines will be discussed below. In Chapters 5 and 6, experimental results using different combinations of BREs and bacteria species are provided. We extract the system parameters from each experiment in Chapters 6 and 7, and compare the dependence of the system model parameters on temperature, with the available data from the literature, to show our system model predictability. We also compare the predicted model parameters with the value of the extracted parameters from new independent sets of experiments to show how well the model predicts the system output when changing temperature.

Temperature Dependence of the System Output Signal

Both of the system parameters, delay $d$ and the time constant $\tau$, are dependent on the BRE, the bacteria species, the sample buffer, and the temperature as explained in (3.8). Temperature is an input system physical parameter, though it is almost always kept
constant during each experiment. Here, we ignore the effect of the buffer assuming we use a single buffer in all experiments when working with a specific sample of bacteria. Also the rate of efflux is dependent on the number of BRE agents per sensitive bacterial cell linearly up to a saturation point (which is about 3 to 5 agents per cell [72]). In our experiments we always use a concentration of BRE higher than the saturation point.

\[ d, \tau \propto f(BRE, bacteria) + g(T) \]  

(3.8)

Research has been done on the temperature sensitivity of the potassium efflux from the BRE exposure to sensitive bacterial cells. For a number of bacteriocins and bacteriophages studied, the initial rate of potassium efflux, and the delay before start of the efflux, have been measured and plotted over temperature [72, 88, 87, 106, 92, 93]. The initial rate of efflux used in the references above, is proportional to the time constant of the potassium efflux generator block in our system model in Fig. 3.10. Assays using the same BRE and bacteria, but varying the temperature, have identified multiple regions of temperature dependence. In temperatures below certain \(T_{\text{off}}\), no efflux is observed, presumably because no membrane depolarization is activated, or in the case of phages, no DNA injection is initialized. Above \(T_{\text{off}}\), the temperature dependence of the rate of efflux goes through a region that follows approximately an Arrhenius plot [107]. The Arrhenius equation models the temperature dependence of reaction rates in nature. It can explain many of the natural chemical and biological rates and it has been shown to provide accurate results in phage- and bacteriocin-activated rates of potassium efflux [72, 88, 87, 106, 92, 93].

\[ \ln(V_i) = -\frac{E_a}{R} \frac{1}{T} + \text{constant} \]  

(3.9)

In the Arrhenius equation above, \(V_i\) is the chemical reaction rate, \(E_a\) is the activation energy, \(R\) is the gas constant, \(T\) is the temperature in Kelvin. It shows the natural logarithm of reaction rate is linearly dependent on the inverse of the temperature. Above a certain temperature \(T_{\text{sat}}\), the rate of efflux stays constant. The temperature break-points in the graph and the constants depend on the BRE and bacteria used. We utilize this model in our system and test its applicability depending on the number of temperature data points in our experiments. Figure 3.12 shows the general form of a temperature dependence model proposed previously in [72] and used in our model.

Results in [72, 88, 87, 106, 92, 93] show that the delay before start of potassium efflux decreases as the temperature increases, but the shape of its temperature dependence has not always been quantified, and is variable depending on the combination of BRE and bacterial species used. We have used our extracted model parameter \(d\) over multiple
temperatures in Chapters 5 and 6 to find the best function that describes the dependence of delay on temperature, paying attention to the data from the literature, when available.

The transfer function of the ISE voltage converter is also dependent on temperature as explained before in equation (3.2) and its temperature dependence should be considered in the overall observed output voltage. In our experiments, the ISE system calibration curve is obtained at the target temperature of the experiment, and the appropriate calibration curve is used for extracting system model parameters.

### 3.6.7 System Model Summary

In this section, we proposed a behavioural model that describes the output signal generated based on system parameters. These system parameters (including $d$, $\tau$, $[Kr]$) depend on the sensor system biological and physical parameters (BRE, target bacteria, temperature). In chapter 5 and 6, the time domain measurement results, obtained by experiments performed at different temperatures and concentrations, are fitted with a system model explained in this section and unknown system parameters are extracted. The value of the known measurement parameters are compared against the extracted parameters. Also, the predictive capability of the proposed model is tested by comparing the extracted parameters with the expected results described in this section (temperature and bacterial cell concentration dependence). Also, the output of the proposed system with the extracted parameters will be compared against independent experimental measurement results to see how well the shape of the output signal matches the system model output. The model presented in this section provides a powerful tool to predict the output signal.
and its behaviour over time for accurate detection and estimation.

### 3.7 Experimental Design and Errors

In order to test the bacteria sensing system functionality and performance, some experimental design and testing considerations need to be examined. Here we introduce some of these considerations.

#### 3.7.1 Calibration

There are multiple kinds of calibration used in biosensors. Some of them are only used to confirm system functionality, while others can be repeated periodically during each experiment for better noise immunity and easier post-processing.

**Calibration with buffers** is the first calibration test to perform in a biosensor. The biosensor output is often calibrated to be zero using predefined buffers consistent with the functionality of the biosensor. In our sensor, calibration is performed to check system functionality using the same buffer that is used for bacterial detection measurements, with different known potassium concentrations. This calibration should be repeated at the start of each experiment.

**Calibration using multiple electrodes** is important in systems that have multiple measurement sites, each with their respective electrodes. In systems where sample compartments can be divided, like microfluidic chambers on top of glass or ICs, relevant buffers in contact with multiple electrodes provide calibration for the system, while performing actual measurements with samples. We do not have a multi-compartment sensor, hence this method has not been applied here.

**Calibration with the initial sample** using a buffer, or the sample before the actual measurement is another way to calibrate the biosensor output and minimizes to interferences from the sample. In our system, we perform this calibration at the start of the measurement using the sample that may contain bacteria without added phage or bacteriocin. This calibration provides a baseline for our measurements, helps with processing the biosensor output.

#### 3.7.2 Control Experiments

In this work, all experiments are called control experiments. The system functionality of the biosensor is tested using the control experiments where the contents of the sample are known and prepared under a controlled environment. In other words, none of these
experiments were performed using unknown samples containing unknown bacteria from real patients in a hospital (i.e. clinical isolates). All biosensors have to be tested using control experiments before large studies using clinical samples comparing the biosensor outputs to conventional techniques. We have introduced two sets of control experiments: positive control experiments, where the expected output of the biosensor is positive, target bacteria are present in the sample, and the other are negative control experiment, where the expected output of the biosensor is negative, there are no target bacteria in the sample).

Positive control experiments should provide positive results. Design of these experiments provide an estimate of false negatives in the biosensor system. Negative control experiments need to be designed and performed before positive control experiments to provide insight into the variability and robustness of the biosensor output. Negative control experiment need to be performed exactly the same as the positive control experiment substituting one or more components. The strongest negative control experiments are the ones providing the highest probability of false positives in the system. We repeatedly use positive and negative control experiments through-out this thesis when providing measurement results.

Further information about the design of experiments and the statistical analysis of experimental data can be found in [108].

3.7.3 False Positive and False Negatives

A bacterial sensing system provides a detection output decision where a positive outcome indicates the presence and a negative outcome indicates the absence of the target bacteria in the test sample. False positive and false negative outcomes occur due to both systematic and random system errors. They can occur due to human error as well as system hardware failures. To capture the number of false positives and false negatives in a bacterial sensing system, many blind measurements must be performed to compare the system output decision with the expected outcomes. We have not performed false positive and false negative analyses in our measurements because none of the experiments were blind and the number of experimental measurements were limited. More information on this topic can be found in [109].
3.8 Summary

This chapter provided a thorough discussion of the design of our system components, including an introduction into the biological components used. A behavioural system model was proposed and presented that models the system output signal and the system behaviour over time according to its various physical input parameters. The algorithm to extract the system model parameters from the measurement results were also presented. This model will be tested against experimental results in Chapter 5 and 6 to extract system parameters and predict system behaviour. Finally, a discussion about some important experimental design and measurement considerations—calibration, control experiments and false positives/false negatives— was provided to facilitate our discussion of experimental results in chapters 5, 6 and 7.
Chapter 4

ISFET and Analog Front-End Design

This chapter provides the principles of operation and the design of the analog front-end (AFE) components in the system. A typical AFE in this application consists of an integrated ion-selective electrode system followed by a readout circuit that reads the output of the ISE system. The output of the readout circuit is then fed to an analog-to-digital converter (ADC) whose output is later used for signal processing. Figure 4.1 shows the block diagram of the front-end. In this chapter, a brief introduction into working principle of ISFETs, as the integrated ISE system of choice, will be provided. The design specifications of the ISFET circuits used in the bacterial detection system will be reviewed. Various readout circuit architectures will be introduced and the important parameters for the selection and design of readout circuits in our system will be discussed. The design considerations for design of an ADC for this system will also be discussed.

Figure 4.1: Block diagram of the analog front-end.

We have fabricated two prototype CMOS ICs that integrate an analog front-end for examining the system functionality for use in bacteria sensors. Circuit design details of both of the prototype chips will be provided.
4.1 Introduction to Ion-Selective Field Effect Transistors (ISFETs)

Commercial ISEs that measure ion concentration across a membrane touching two liquids are bulky and expensive and were introduced in section 3.3.1. Integration of ISEs into silicon technologies have been achieved by introduction of the ISFETs. Ion-selective FETs (ISFETs) have been first described in [110]. They are low-cost integrated alternatives for ion-selective electrodes (ISEs) and have been extensively used and reviewed in several papers [4]. Conventional ISFETs are fabricated much like regular MOSFETs except that no gate metal is deposited and an ion-sensitive membrane is deposited as the gate dielectric. The gate is not biased directly on the chip, and an aqueous sample touches the gate, where a reference electrode is inserted in the sample that defines a stable non-changing sample potential. Most ISFETs are used as pH sensors because of the hydrogen (H\(^+\)) sensitivity of SiO\(_2\) used in the MOS gate oxide allows for an easily manufacturable device. In MOSFETs, the threshold voltage of the transistors is defined by the process parameters. ISFETs use the same process technology as MOSFETs, and hence have the same constant part of the threshold voltage \(V_t\).

In addition to MOSFET \(V_t\), an ISFET threshold voltage has a constant potential of the reference electrode, \(E_{ref}\), and the interfacial potential at the solution/oxide interface [4]. Figure 4.2 shows the structure of a CMOS ISFET and the molecular interface between the SiO\(_2\) oxide and the sample liquid. The surface of all oxides contain hydroxyl groups that donate or accept protons from the sample liquid. The site binding model describes the equilibrium between the oxide surface and the H\(^+\) ions on the liquid-oxide interface as shown in [4, 5].

The interfacial potential consists of a constant surface dipole potential plus a pH-dependent term that changes with the concentration of H\(^+\) ions as described in (4.1), where \(\psi\) denotes the pH-dependent surface potential and

\[
V_t = E_{ref} - \psi + \chi^{sod} - \frac{\Phi_{si}}{q} - \frac{Q_{ox} + Q_{ss} + Q_B}{C_{ox}} + 2\phi_f \quad (4.1)
\]

\[
\Delta \psi = -2.3\beta \frac{RT}{F} \Delta \text{pH} \quad (4.2)
\]

\(\chi^{sod}\) is the constant dipole potential of the solvent, \(\Phi_{si}\) is the silicon work function, \(Q_{ox}\) is the fixed oxide charge, \(Q_{ss}\) is the surface state density at the silicon interface, \(Q_B\) is the depletion charge in silicon, \(C_{ox}\) is the gate capacitance per unit area, and \(\phi_f\) is the Fermi-potential [4]. The variations of surface potential with respect to pH are illustrated...
in (4.2) where \( R \) is the gas constant, \( T \) is the temperature in Kelvin and \( F \) is the Faraday constant. Depending on the quality of the oxide and its surface buffer capacity, \( \beta \) can vary from 0 to 1. With the value 1, the ISFET has full Nernstian sensitivity of 58.2 mV/dec at 20°C.

In [103] pH-sensitive ISFETs were enhanced to detect potassium ions by applying a valinomycin-plasticizer-polyvinyl chloride membrane on top of the gate. This was a significant step forward in using ISFETs as a sensor for multiple types of ions other than \( \text{H}^+ \). CMOS-compatible ISFETs were introduced, as in [111], that have stacked vias connecting the gate terminal of a traditional CMOS transistor to a top metal electrode. The ion-sensitive ISFET implementation in a conventional CMOS process is depicted in Fig. 4.3.

CMOS ISFETs can be viewed as the series combination of an ion-sensitive membrane and a conventional MOSFET transistor. An ion-sensitive membrane is deposited on top of the passivation layer that covers the metal electrode to provide the sensitivity to an ion other than \( \text{H}^+ \). In this thesis our focus is on detection of potassium ions; hence potassium-sensitive membrane on top of the passivation layer that results in potassium sensitive ISFETs.

Inclusion of a potassium-sensitive membrane in an ISFET implementation results in a modification of (4.1) that determines the threshold voltage \( V_t \) of an ISFET transistor. Additional oxide-membrane and membrane-sample interfacial voltages need to be added to (4.1) to determine \( V_t \). The interfacial potential of the oxide-membrane is constant.
while the membrane-sample potential consists of a constant term and a potassium ion $K^+$-sensitive voltage $\psi'$. In a $K^+$-selective ISFET system we only consider the $K^+$ dependent voltage changes $\Delta \psi'$ as the voltage of interest, since other voltages are constant and contribute only to a constant DC offset in $V_t$. The potassium-sensitive voltage $\Delta \psi'$ is generated at the membrane-sample interface. Careful design of the membrane can provide a Nernstian response as shown in (4.3).

$$\Delta \psi' = 2.3 \frac{RT}{F} \log(K^+)$$  \hspace{1cm} (4.3)

CMOS ISFETs suffer from two major non-idealities that limit their utilization in practical applications that require the accurate measurement of the concentration of a specific ion in the sample. The first problem is the statistical variation of the threshold voltage of the CMOS ISFET because of trapped charges within the passivation oxide, as well as, at interfaces between the metal-oxide and membrane-oxide [112]. This results in

Figure 4.3: Conceptual development of ISFET shown in cross-section. (a) a pH-sensitive ISFET where the gate oxide is the pH-sensitive membrane (b) Ion-sensitive membrane is added on top of ISFET in (a) to make the ISFET ion-sensitive to ions other than $H^+$ as determined by the chemical composition of the membrane. (c) A cross-section of a MOSFET (d) ISFET implemented in CMOS by depositing an ion-selective membrane on top of the passivation layer.
sometimes large threshold voltages and variations between threshold voltages of ISFETs integrated in the same process. There are techniques to reduce the trapped charges by using UV irradiation and hot-electron injection [113], though these techniques can potentially damage the ISFET surface. Variations in ISFET threshold voltages result in DC baseline variations at the outputs of different ISFET readout circuits. As discussed in Chapter 3, DC baseline variations exist in all ISE systems. Frequent calibration of the ISE system with known buffers eliminates the errors caused by this non-ideality.

The second problem that exists in both liquid-contact ISEs as well as solid-contact ISFETs is drift as explained in Chapter 3. Drift is more severe in ISFETs because of the solid-contact between the membrane and the ISFET surface, passivation layer or any other ISFET surface electrode. Because of liquid exposure and membrane changes, the interface between the membrane and the ISFET surface also changes, resulting in variations in the interface capacitors and voltages. Gradual peeling of the membrane from the ISFET surface also results in high drift in systems when liquid has penetrated through the membrane. The high drift values of ISFETs have limited their use as ion-selective electrodes for accurate measurement of absolute ion concentrations in samples. As explained in Chapter 3, our system does not rely on absolute measurements of ion concentrations, but instead measures changes in ion concentration, and the effect of drift and baseline variations can effectively be removed by employing appropriate measurement procedures as well as effective signal processing by a post-processing unit.

4.1.1 Ion-Selective Membrane

The membrane deposited on top of an ISFET to make it ion-sensitive should have the highest possible permeability for the specific target ion. Since the invention of the ISE, research on the composition of ion-specific membranes with Nernstian responses, long life spans and lower detection limits has been extensive [114, 115, 116]. Since we are using potassium as the ion of interest in this work, we mainly focused on potassium membranes suitable for ISFET applications. Every ion-selective membrane consists of an ionophore that is chemically selective to the ion of choice. The ionophore needs to be embedded in a matrix for mechanical support. There has been research on embedding the ionophore directly inside the oxide on top of the ISFET [117], but in most publications, the ionophore is embedded inside a polymeric matrix membrane. The adhesion of polymeric membranes to solid contacts like metal and oxides is still a challenge, resulting in short lifespans and drift in ISFETs and solid-contact ISEs. There has been extensive research into different types of polymers and protocols to use for good detection limits,
adhesion to solid contacts, and performance capabilities [118, 119]. Polyvinyl chloride (PVC), polyurethane, photo-curable polyurethane [120], Urashi matrix and many more matrixes have been proposed. Membranes based on PVC matrix are still the most popular membranes, though the recipe for the mixture as well as the component optimization in the mixture is still attracting research attention [119].

Considerable research has been done to manipulate membrane parameters such as detection limit and shelf life. Detection limits have been reported to be around $10^{-10}$ M [119] where a $10^{-6}$ M detection limit still remains typical of the widely used traditional PVC-based membranes. Since lower detection limits of an ISE membrane directly results in improved biosensor sensitivity, they are important parameters to be optimized. the membrane to be considered for this sensor needs to be compatible with solid-contact fabrication, be able to function with interfering ions commonly found in biological reagents like sodium. Membranes reported in [121] are polymeric membranes with detection limit of $5 \times 10^{-9}$, but their lower detection limit is achieved by careful manipulation of the internal reference liquid and interfering ions in the sample; hence not compatible with solid-contact sensors or biological reagents. Techniques to improve the detection limits of solid-contact ISEs have been presented in [122, 123, 124] where lower detection limits are achieved by optimizing membrane composition as well as membrane adhesion to the solid surface.

A potassium-sensitive membrane utilizes a macrocyclic substance from a group of depsipeptides, valinomycin, as the ion exchanger. For potassium-selective membranes Valinomycin is the most popular choice [125] and is routinely used to determine potassium concentration in different samples. This antibiotic forms complexes with alkali metal ions and is a member of a group of natural neutral ion-exchangers. It is highly selective to potassium $K^+$ ions because the $1.33\text{Å}$ diameter pores inherent in this macrocyclic molecule allow the $K^+$ ion to fit perfectly inside. Figure 4.4 shows the chemical structure of valinomycin with a $K^+$ ion fitting inside the macrocyclic structure.

Valinomycin is dissolved in an organic solvent such as tetrahydrofuran (THF) along with PVC and a plasticizer dioctylphthalate that together build a porous membrane with embedded valinomycin molecule. Figure 4.5 shows how an ionophore like valinomycin traps the potassium ion from the membrane-liquid sample interface and lets the potassium ion diffuse through the membrane. The charge separation across the membrane results in the ion-selective membrane voltage that is measured by the ISE system.

The membrane was cast from tetrahydrofuran (THF) solution with the composition of components found in [103]. In short, 0.33g high molecular weight PVC (Sigma Aldrich 81387), 0.89ml dioctylphthalate (Sigma Aldrich D201154) and 10 mg valinomycin (Sigma
Figure 4.4: Chemical structure of valinomycin [6] with a K⁺ ion fitting inside the structure.

Figure 4.5: Ionophore valinomycin traps the potassium ion from the membrane-sample liquid interface and provides a charge separation across the interface.

Aldrich 4675) were dissolved in approximately 5ml THF (Sigma Aldrich 401757) as described in Appendix A. The membrane thickness used needs to be between at least 100 – 300µm to avoid pinholes. Pinholes in the membrane result in a loss of sensitivity in the first hours of exposure to the sample liquid [103].

**Biosensor Detection Limit and Interference Dependence on the Membrane**

In this thesis, we have used traditional PVC-based membranes; hence detection limits in the range of 10⁻⁶ M are obtained, where the buffer contains about 0.1M NaCl. This corresponds to minimum detection limit of 6.6 × 10⁶ cfu/ml of bacteria assuming 9 × 10⁷ K⁺ ions/cell are released using *E.coli* as a prototype example (Our experiments support detection limits of around 10⁷ cfu/ml, as shown in Chapter 6 for *E.coli*). To obtain lower biosensor sensitivity/detection limits, other types of membranes should be utilized and/or buffers with lower concentrations of interfering ions, like NaCl, should be used.

As explained in section 3.6.3, interfering ions like NaCl result in errors at the ISE system output. The error is because of the membrane is not ideal and has some physical permeability to ions other than potassium. The presence of interfering ions in the sample
buffer result in increasing the baseline voltage of the ISE because of the movements of these ions inside the membrane, hence higher detection limits. In our experiments the raw sample containing bacteria is removed and the bacteria is suspended in a constant SM medium with a known concentration of ions, see raw sample preparation in Appendix A, before applying the sample on the biosensor. In our experiments SM has 0.15M NaCl that results in detection limits of $10^{-6}$M potassium ion and around $10^{7}$ cfu/ml bacteria in the sample.

### 4.2 ISFET Design and Characterization and ISFET Readout Circuitry

In order to characterize the behaviour of an ISFET compared to a MOSFET, Fig. 4.6 provides a detailed description of the equivalent circuit elements involved [126, 127] in a single-ended ISFET in contact with a liquid sample. Figure 4.6 depicts the generalized equivalent circuit model of an ISFET system where a reference electrode provides a stable biasing voltage to the liquid in contact with the ISFET system. The interface between the ISFET system and the liquid is an ion-selective membrane deposited on top of passivation layer. The passivation layer covers the ISFET electrode that is directly connected to the floating gate of a MOS transistor (the ISFET transistor is a PMOS in Fig. 4.6) and a readout circuit that follows the ISFET transistor. In this figure, we

![Figure 4.6: ISFET and ISFET electrode equivalent circuit.](image-url)
assume an ideal reference electrode (e.g. Ag/AgCl) provides a constant, stable reference voltage \( V_R \) to the solution. \( R_s \) is the small resistance of the solution in contact with the reference electrode and the ISFET system. \( R_{dl} \) is the ion-transfer resistance between the membrane interface and liquid, and \( C_{dl} \) is the double-layer capacitance between the membrane and liquid. The voltage \( \psi' \) generated at the membrane-liquid interface is the generated membrane voltage that changes according to the Nernstian equation and provides the ion-sensitivity response to a specific ion (in our case \( K^+ \)). \( R_m \) and \( C_m \) are the membrane bulk resistance and capacitance. \( R_L \) is the contact resistance between the membrane and the ISFET surface. If the ISFET surface is covered by passivation oxide, this resistance is between the passivation oxide and the membrane. The passivation oxide is modelled as a purely capacitive \( C_{pass} \) layer of oxides (typically \( \text{SiO}_2 \) and \( \text{Si}_3\text{N}_4 \)). The total input-referred capacitance at the gate of the ISFET is \( C_g \). The voltages at the gate \( V_g \), source \( V_s \) and drain \( V_d \) of the ISFET are shown for clarity. In Fig. 4.6, all the voltage parameters stay constant and the only voltage that is changing throughout an experiment is the voltage across the potassium-sensitive membrane and liquid interface \( (\psi') \) that is in turn dependent on the changes in the potassium concentration in the sample.

**ISFET Sensitivity**

The first important specification of an ISFET is its sensitivity towards the target ion. In Chapter 3, the sensitivity of the ion-selective electrode system was introduced, and it was shown how the output voltage changes when the input concentration of a specific ion in a liquid sample is modified. As discussed in Section 3.3.1, the maximum voltage sensitivity of the interface between membrane and liquid \( (\psi') \) is given by the Nernst equation that is proportional to the ISFET system ion sensitivity.

The voltage generated at the gate of the ISFET transistor \( (V_g) \) as a result of the membrane voltage \( (\psi') \) is an important parameter in ISFET systems. Hence the sensitivity depends on the voltage division that occurs because of the circuit elements involved between the membrane and the ISFET transistor gate. In steady state and, assuming the membrane resistance \( R_m \) is not huge, only the passivation capacitor \( C_{pass} \) and the equivalent capacitance seen at the gate of the ISFET transistor \( C_g \) affect this voltage division according to the following equation [126, 127]:

\[
\Delta V_g = \Delta \psi' \frac{C_{pass}}{C_{pass} + C_g}
\]

Since the thickness of the passivation layer is large compared to the thickness of the gate
oxide, the values of passivation layer capacitance and the capacitance seen at the input gate of the ISFET should be considered in the design. The ISFET readout circuits also affect the gate capacitance seen at the input of the ISFET [128] affecting the sensitivity. More discussion on this topic will be provided in Section 4.2.2 on ISFET readout circuitry.

**ISFET Noise Sources**

The main sources of noise in ISFET systems are the 1/f noise from the input ISFET transistor and ISFET readout input transistors, and the white noise from $R_L$ that results from attaching the membrane to the ISFET interface. In practice, noise contributions of ISFETs in CMOS are mainly governed by chemical noise rather than electrical noise due to drift and random fluctuations that arise from ion diffusion inside the membrane as well as membrane attachment onto the solid surface [126, 129, 130]. Though conventional liquid-contact ISEs, with a membrane contacting two liquids, also experience drift as a result of ion diffusion, the problem is amplified in ISFETs, as in other solid-contact ISEs, because of the solid-membrane interface. Drift compensation algorithms are utilized to perform drift prediction and cancellation [131, 132]. The chemical noise contribution from the membrane-liquid interface as well as membrane attachment to the ISFET solid surface need to be optimized for low-noise applications.

### 4.2.1 Single-Ended vs. Differential ISFET System

So far in this chapter, single-ended ISFET system architectures were shown. In a single-ended ISFET system, a single ISFET transistor is biased by a reference electrode in contact with a liquid. An ISFET readout circuit follows the ISFET transistor to buffer and/or amplify its output.

Differential ISFET architectures provide several advantages over single-ended architectures despite their increased area and power consumption. From an ISFET transistor and ISFET readout circuit perspective, differential systems provide common-mode noise immunity and supply noise rejection. There are two common methods of implementing differential ISFET architectures in terms of the ISFET membrane and reference electrode design as described below.

**Differential ISFETs Employing a Single Membrane in Contact with Two Liquids (Sample and Reference)**

Figure 4.7 shows the structure of this differential system implementation [133]. In this architecture, two ISFETs with their individual ISFET readout circuits are identical with
the same membrane deposited on both of their electrodes. The sample liquid is in contact with one of the ISFETs and a reference liquid, with a known ion concentration, is in contact with the second ISFET. Both liquids are in contact with ideal reference electrodes (Ag/AgCl as an example) to provide their individual bias voltages. The variations of the ion concentration in the sample can be monitored with respect to the reference sample.

Since both ISFETs are the same, the differential output cancels the first-order drift and DC offset voltages associated with the ISFET transistors. Since the two liquids, in contact with the two ISFETs, have different compositions, the DC voltages generated at the reference electrode-liquid and sample liquid-membrane are not the same and cannot be cancelled.

The drawbacks of this differential architecture is that still an ideal reference electrode needs to be in contact with both liquids to provide constant bias voltage to both ISFETs. Ideal Ag/AgCl reference electrodes are bulky and cannot be integrated in a conventional CMOS process. Several research groups have fabricated miniaturized reference electrodes on top of electronic ICs [134, 135]; still true single-chip performance can not be achieved using these approaches, because the reference electrode cannot be integrated on the chip. Also, at least two sample compartments, each touching one of the ISFET electrodes, need to be fabricated (e.g., microfluidic chambers) [133] to separate the sample and reference liquids.
Differential ISFETs Employing Two Membranes in Contact with a Single Sample Liquid

In the second type of differential ISFET architecture, two ISFETs with their individual ISFET readout circuits are identical except that two different membranes are deposited on each of them. The same liquid is in contact with both ISFETs and one reference electrode is in contact with the liquid to bias both ISFETs. In this architecture, one ISFET is in contact with an ion-sensitive membrane (potassium-sensitive membrane in our case) and the other ISFET, called the REFET, is in contact with an ion-insensitive membrane [136].

In this architecture, the two membranes should be designed as identical as possible, except for the ion sensitivity, to provide an equal paths between the reference electrode and each ISFET, for maximum common noise immunity and offset cancellation. Figure 4.8 shows the proposed implementation of this architecture for our potassium-sensitive ISFET system where one ISFET is covered by a potassium-sensitive membrane and the other ISFET is covered by a potassium-insensitive membrane. The best scenario would be to have the same membrane composition for both ISFETs, that is, PVC plus plasticizer, with only adding the ion-selective ionophore (valinomycin) to one membrane that provides the potassium sensitivity. The membrane deposited on REFET does not contain the potassium-sensitive ionophore.

The important advantage of this architecture is that a non-ideal reference electrode (pseudo-reference electrode) like a noble metal can be utilized [136], replacing the ideal bulky Ag/AgCl electrode. Noble metals like gold and platinum also provide a stable liquid voltage but the liquid voltage will vary according to the composition of the liquid sample and its ion concentration. For example, replacing the reference Ag/AgCl electrode with a gold metal electrode results in a reference voltage that is also dependent on the potassium concentration in the sample. These electrodes are called pseudo-reference electrodes [137]. Since the variable DC offset voltage generated at the reference electrode is common to both of the ISFETs, it is cancelled out using the differential architecture.

This approach provides a true single-chip design without the need for a separate bulky reference electrode. The noble metal used for the reference electrode could be a gold-plated pad on top of the CMOS IC or one of the gold bond wires used for packaging, to simplify the implementation. This approach also decreases the volume of the sample usage because the bulky Ag/AgCl electrode is now replaced by an integrated small noble metal electrode on the chip.

The fabrication issue to consider with this approach is depositing two independent membranes on two different regions of the small chip. The potassium membranes embed-
ded in PVC in this thesis are between 100-300 $\mu$m thick to ensure pinhole-free operation. This results in challenges with membrane deposition using conventional dispensing methods. Although differential ISFETs were implemented on the chip, because of membrane deposition problems, we performed all experiments using a single-ended approach utilizing an Ag/AgCl reference electrode.

![Diagram of ISFET system](image)

Figure 4.8: (a) Representation of single-ended ISFET system using an ideal Ag/AgCl electrode, (b) A differential ISFET approach using a noble metal (e.g. gold-bonded pad) as a reference electrode, having two separate ISFETs and readout circuitries. The final output is the difference between the outputs of the two ISFETs.

### 4.2.2 ISFET Readout Circuitry

The ISFET readout circuitry provides buffering, amplification and possibly current conversion of the membrane-liquid interface $\psi'$. Two prominent categories of ISFET read-out topologies are voltage-mode and current-mode configurations [133]. Both topologies can be used in single-ended as well as differential architectures.

#### Voltage-Mode ISFET Readout Circuits

In voltage-mode configurations, the ISFET threshold voltage variations are monitored directly, buffered and possibly amplified. The output voltage is directly translated from the ISFET ion-sensitivity (ideally a Nernstian response) to a voltage through the usage of opamps in different feedback configurations [138]. The most popular ISFET readout
circuit is a voltage-mode circuit where the current and the drain-source voltage of the ISFET are kept constant to track the ISFET threshold voltage, measuring the source voltage of the ISFET as the readout output. This configuration has been extensively used in the literature because of its simplicity and good precision [103, 4, 139]. Figure 4.9 shows this configuration. The output is the source voltage of the ISFET $V_s$ that is buffered and follows the $V_g$ of the ISFET.

$$|V_{ds}| = I_b \times R$$

Figure 4.9: Voltage-mode drain-source follower readout circuit connected to the input PMOS ISFET. $I_b$ is the bias current that generates $I_b \times R$ that is equal to ISFET $V_{ds}$ using the drain-source follower configuration.

Both drain and source follow the $V_g$ variations, and the capacitive contributions from the ISFET transistor gate-source capacitance ($C_{gs}$) to the total input capacitance $C_g$ seen at the gate of the ISFET are attenuated by the $g_m R_{out}$ of the ISFET source follower architecture, where $g_m$ is the transconductance of the ISFET transistor and $R_{out}$ is the equivalent resistance seen at the source of the ISFET transistor. Since the gain from the ion-selective membrane to the readout circuit is the most important parameter in designing the readout circuit, this configuration provides very good design parameters for ensuring the sensitivity is not compromised by the introduction of the ISFET transistor capacitors according to (4.4).
Current-Mode ISFET Readout Circuits

In current-mode topologies, the threshold voltage variation of the ISFET, in other words, the voltage appearing at the gate of the ISFET transistor $\Delta V_g$ as a result of membrane-liquid voltage variations $\Delta \psi'$ is directly converted to current and most commonly the current is amplified to provide enough resolution at the output. Current-mode techniques especially in differential mode have been used extensively [136, 140, 141, 142] because of their compact structures and low power consumption. In applications where output linearity is important, readout circuits that convert input voltage to a current result in more complicated transconductance systems. In many systems, such as pH sensors for DNA identification, linearity is not an issue as long as there is enough signal-to-noise ratio (SNR) for detection of the pH changes and prior calibration can provide a one-to-one relationship between pH and the output. The same is true for our bacteria detection sensor. The general circuit for a fixed drain and source voltage, current-mode circuit is shown in Fig. 4.10a with an example implementation from [7] described in Fig. 4.10b.

![Figure 4.10: (a) Structure of an example current-mode fixed drain and source voltage readout circuit connected to the input NMOS ISFET, (b) An example of a simple implementation from [7].](image)

As shown in Fig. 4.10, both drain and source voltages are fixed, so that the threshold voltage variations of the ISFET result in changes in the ISFET current that is mirrored and amplified in later current-mode circuits. The current vs. threshold voltage dependence in this circuit depends on the region of operation in the ISFET. As an example, a square-law dependence exists in the saturation region, where the gain of the transconductance depends on the process parameters. Also in Fig. 4.10, the input $C_{gs}$ of the transistor directly affects the total gate capacitance $C_g$ seen at the input of the ISFET,
resulting in a voltage division between \( C_{\text{pass}} \) and \( C_g \) according to (4.4).

A popular technique in ISFET readout systems is the use of sub-threshold ISFET transistors and readout circuits [142, 143] to save power, since the required bandwidth for the ISFET systems are quite low.

In particular sub-threshold current-mode readout circuits have been popular to save power and linearize the relationship between ISFET current and ion concentration in the sample. The exponential dependence of \( I_d \) current vs. gate source voltage in the sub-threshold voltage domain and logarithmic dependence of \( \Delta \psi' \) versus potassium ion concentration in the sample results in a linear dependence of \( I_d \) vs. \([K^+]\)(concentration of K\(^+\) ion in the sample). The sub-threshold current with linear relationship to H\(^+\) ion concentration in the sample was utilized in [143, 18]. In practice, sub-threshold current-mode circuits provide advantages for systems that require adjustable dynamic range, when sensitivity without particular constraints on the output linearity and accuracy are considered. Various techniques are used in combination with this method to provide simple analog processing on the signal in the current domain, using a Gilbert cell as an example [133] published recently for differential sensing. Truly differential sensing circuits like the one explained in [133] should be used cautiously in ISFET systems since threshold and drift variations between the reference ISFET and the ion-selective ISFET are poorly controlled and result in large DC offsets and output saturation.

In our systems we have used voltage-mode classical ISFET readout circuits paying special attention to preserving the sensitivity at the ISFET input while utilizing simple opamp topologies with low power consumption.

### 4.2.3 Analog to Digital Conversion

The requirements for signal quantization and digital conversion of the signals using the ISFET readout output depend on the dynamic range requirements of the ISFET sensor output as well as the detection limit of the membrane used.

The detection of bacteria that cause urinary tract infection (UTI) is one application area for bacterial sensors. In Chapter 6, we utilize our system in a set of experiments aimed at the detection of bacteria commonly encountered in patients with UTI. For UTI applications where the range of the bacteria concentration causing symptoms is between \(10^5 \text{ to } 10^8\) cfu/ml, assuming the membrane is designed to accommodate the minimum detection limit and the slope is Nernstian (58 mV per 10-fold change in the potassium concentration), then we need about 200mV of dynamic range.

If no compensation of threshold voltage variations is performed in the ISFET readout
circuitry, then the DC baseline of the ISFET potentially ranges from 0V to the supply voltage. Here we assume the worst-case scenario, where the output does change from zero to supply voltage (in our process 3.3V). Assuming we need approximately 5mV of resolution per 10-fold change in the concentration, the number of bits required for the ADC would be approximately \( \log_2(3.3/5mV) \), which is 10 bits. Using differential circuits that cancel the effect of most baseline variations and drift among ISFETs [144] or, using circuits with active DC and drift compensation in the analog domain [132, 145, 132] decreases the resolution requirements of the ADC significantly, to 6 bits, and increases the available dynamic range of the system.

Here the system resolution as well as the dynamic range are solely dependent on the application. The most important parameter is the system sensitivity or the detection threshold, defined by the detection limit of the membrane and specific sample buffer, specifically interfering ions in the sample buffer, chosen during the experiments.

The dynamics of the events happening in our bacterial sensing systems are very slow, hence, very low bandwidth requirements for the ADC. A low-power ADC with extremely low sample rates (less than 100 S/s) with resolutions of 10 bits and lower can therefor be implemented for efficient detection in our system. Examples of such low-power systems meeting these specifications are successive approximation ADCs [146] and dual-slope integrating ADCs.

In our system, the ADC is not integrated. The outputs of the ISFETs are connected to an external 16-bit ADC through a National Instruments data acquisition system where the outputs are digitized and transferred to a PC for processing and visualization.

### 4.3 Design of the First Test IC

The first prototype was fabricated to test the efficacy of using potassium-sensitive ISFETs in a sample containing bacteria. The chip was used for bacterial detection and identification using bacteriophages and the results are presented in Chapter 5 of this thesis. The potassium-sensitive ISFET in a TSMC 0.18\( \mu \)m CMOS process was implemented as depicted in Fig. 4.11 with a 20\( \mu \)m x 20\( \mu \)m top-level metal electrode. The top-metal electrode was covered by a passivation layer, which is a combination of \( SiO_2 \) and \( Si_3N_4 \) oxides used in the CMOS process. A potassium-sensitive membrane, as described in Section 4.4.1, was deposited on top of the passivation layer that covers the metal electrode. The protocol for membrane preparation, chip surface preparation and membrane deposition is described in Appendix A.

The readout circuits were implemented outside the chip on the test PCB using
AD8606 opamps and a TI REF200 as the constant current source in Fig. 4.12. The sensor front-end that reads the threshold voltage variations of the ISFET, as shown in Fig. 4.12, uses the popular source and drain follower presented in [103]. Both \( V_{ds} \) and

\[
\begin{align*}
I_d &= 100 \mu A \\
R &= 5k \Omega \\
R &= 10k \Omega \\
C &= 10nF
\end{align*}
\]

Figure 4.12: Schematic of the ISFET readout circuit. The ISFET transistor implemented on the 0.18\( \mu m \) CMOS was connected to off-chip drain-source follower circuit implemented on a PCB.

the drain current of the ISFET are fixed to 0.5 V and 100 \( \mu A \), respectively. A fixed DC voltage (\( V_{ref} \)) is applied to the solution using an Ag/AgCl reference electrode. The reference electrode that provides constant reference voltage to the liquid in this thesis was RE-5B Ag/AgCl electrode from Bioanalytical Systems [147]. The source voltage (\( V_s \))
of the ISFET is measured using the buffered readout circuit. The ISFET output voltage \( \Delta V_s \) is equal to \( \Delta V_t \) of the ISFET transistor used in a drain-source follower circuit. The output of each of the ISFET readout circuit \( \Delta V_s \) is measured and converted to a digital representation and is referred to as output \( \Delta V \) in the rest of this thesis.

This chip contains seven measurement channels with different sized electrodes as well as other test circuitry. Outputs of the ISFET readout circuits were converted to a 16b digital representation, transferred to a PC using a National Instruments data acquisition board, and visualized in real time using Labview. Figure 4.13 shows the micrograph of the chip. Only 2 ISFETs with the same sized electrodes were used because of their easier exposure during encapsulation. PMOS transistors were used as ISFETs since the bulk-source connection of the n-well removes any distortion caused by bulk-source voltage variations. The PMOS ISFETs were biased in saturation and the current consumption was 100\( \mu \)A per ISFET channel.

![Die photo of the first chip](image)

**Figure 4.13: Die photo of the first chip**

**ISFET Sensitivity**

In Section 4.2, it was mentioned that ISFET sensitivity depends on the voltage division from the membrane voltage \( \psi' \) to the ISFET transistor gate voltage \( V_g \) as defined by (4.4). Figure 4.14 shows the equivalent capacitors and their values resulting from 3 layers of passivation used in the TSMC 0.18\( \mu \)m CMOS process. The circuit parameters for the PMOS ISFET used are also shown. According to (4.4) signal attenuation at the input of the PMOS ISFET gate \( V_g \), is

\[
\frac{C_{pass}}{C_{pass} + C_g} \quad \text{where} \quad C_g = \frac{C_{gs}}{g_m R_{out}} + C_{parasitic}
\]

\( C_{gs} \) is the ISFET gate-source capacitance, \( C_{parasitic} \) is the parasitic capacitance at the gate of the ISFET transistor, \( C_{pass} \) is the total passivation capacitance resulting from 3 layers of passivation (pass1, pass2 and pass 3 in Fig. 4.14), \( g_m \) is the ISFET transistor transconductance and \( R_{out} \) is the output resistance seen from the source of the ISFET to the ground. The value
of $g_m R_{out}$ is quite large because of the large output resistance of the off-chip current source. In integrated designs, this value can affect the contribution of $C_{gs}$ to the total $C_g$, hence affecting the sensitivity.

In our design, parasitic capacitors from interconnects affect the total $C_g$ value since test circuits were connected to this point that ultimately degraded the sensitivity.

![Passivation Layer Diagram]

Figure 4.14: The 3-layer passivation parameters and the ISFET parameters implemented in the first prototype in 0.18µm CMOS.

We tested the ISFET sensitivity for pH with the passivation layer acting as the membrane as well as for pK (potassium sensitivity) by adding the potassium-sensitive membrane on top of the passivation layer. Figure 4.15 shows the calibration curves for both pH and pK measurements. The pH measurements were performed by changing the pH of the SM buffer (see Appendix A for composition) around the base pH of 7.3. The SM buffer was the buffer used in the bacterial detection experiments found in Chapters 5, 6 and 7. For potassium sensitivity measurements, the base buffer was 0.1M NaCl and the KCl concentration was modified to get the appropriate concentration of K$^+$ in the sample.

The experiments were performed at a room temperature of 24°C. During the pH measurements, since the passivation layer is exposed to the liquid, ions diffuse into the passivation layer resulting in increasing the passivation layer capacitance (decreasing equivalent thickness). This results in sensitivity of close to 46mV/dec for pH measurements.

In pK experiments, the membrane deposited on top of the passivation layer and all the capacitances associated with the passivation layer are preserved. According to Fig. 4.15, the sensitivity of 22.1mV/dec is achieved at room temperature which corresponds to estimated parasitic capacitance $C_{parasitic}$ of 17fF. In ISFET systems where a passivation
layer covers the top metal electrode, the value of the parasitic capacitors, the gate-source capacitance of the ISFET transistor and the size of the electrode need to be considered. In this design, the size of the electrode had to increase or the parasitic capacitors had to be minimized for improved sensitivity. Another approach is to remove the passivation layer to eliminate the capacitive division at the input. In our next test design, we paid special attention to the parasitics and connections to the gate of the ISFET transistor to preserve sensitivity. We also tested the possibility of removing the passivation layer to enhance sensitivity.

Figure 4.15: (a) pH calibration curve for the implemented ISFET (24°C), (b) pK calibration curve for the implemented ISFET 24°C
ISFET Noise Contribution

The 1/f noise of the input ISFET transistor is the main contributor of electronic noise in ISFETs. The noise contribution of the input ISFET was simulated using Spectre and determined to be $1.1 \, \mu V$. The chemical noise contribution from the long-term drift and short-term ion-transfer across the membrane was orders of magnitude higher resulting in output changes of 0.1mV to 0.5mV per minute at the output. The conclusion from experiments involving this chip was that electronic noise is not an important parameter in ISFET circuit design. For the next design, we focus on designing the ISFET for better sensitivity.

4.4 Design of the Second IC

The second CMOS prototype was designed to integrate the ISFET and ISFET readout circuitry based on the experience gained with the first prototype chip. The prototype was implemented in an IBM $0.13 \, \mu m$ CMOS process with 8 metal layers. Figure 4.16 shows the die micrograph of the implemented chip.

![Die micrograph of the second implemented chip illustrating Group 1 and Group 2 voltage-mode ISFETs and current-mode ISFETs with the electrode area.](image)

Each side of the chip is connected to a separate ground and power supply and includes independent circuitry. This configuration helps with the encapsulation and testing of separate circuitries on the chip, with all electrodes located at the centre for exposure to the liquid sample.
4.4.1 ISFETs Connected to a Voltage-Mode Drain-Source Follower Readout

Two groups of ISFETs with their respective voltage-mode ISFET readout circuits are implemented on this chip as shown Fig. 4.16 (Group 1 and Group 2). The main difference between these two groups of ISFETs are the layers above the metal electrodes. The aluminum metal 8 electrodes are exposed with no passivation layer covering in Group 1 ISFETs, and the metal electrodes are covered by passivation layers in Group 2 ISFETs. The ISFET transistors and ISFET readout circuits are identical in both groups.

The main difference between the IBM 0.13µm CMOS compared to the TSMC 0.18µm technology used in the first prototype, in terms of ISFET design, is their passivation layers. By default, the IBM process covers the wafer with a thick polyimide layer on top of the passivation layer. This layer is used for additional thermal and humidity protection. There is an option of eliminating polyimide in the process flow, however, this needs special approval from all the designs on the same wafer, which was not granted to our design at the time of design submission. The polyimide layer is not pH sensitive and has an average thickness of 2.5µm (1µm to 7µm) in this process. The structure of the ISFETs covered by passivation layers (Group 2) is identical to the one implemented in the first prototype.

Fig. 4.17 shows the structure of the potassium-sensitive ISFETs implemented with exposed aluminum electrodes (Group 1). In Figure 4.17 two single-ended channels are shown where two electrodes, each connected to their respective ISFET transistors are shown. The ISFET transistors are connected to their ISFET readout circuits. All the metal electrodes are covered by a potassium-sensitive membrane for K⁺ sensitivity.

The PVC-based potassium-sensitive membrane has good adhesion to SiO₂ surfaces and are usually deposited on top of the passivation layer in CMOS ISFETs. In this prototype we investigated the possibility of removing the passivation layer and applying the membrane directly on top of the metal electrodes for improved sensitivity. We followed the same protocol for membrane preparation and deposition, as the first test chip (explained Appendix A), and we obtained good results in terms of adhesion and sensitivity by depositing the membrane directly on top of the aluminum top metal layer.

A combination of 3 different-sized electrodes and 2 different-sized PMOS ISFET transistors were used with the same single-ended readout circuit connected to each one of them, producing total of 6 single-ended channels on each side (Group 1 and Group 2). The electrodes were square with widths of 150µm, 75µm and 37µm. ISFET transistor sizes were 50µm/400nm and 10µm/400nm, respectively. Figure 4.18 shows the structure
Figure 4.17: (a) Block diagram of the implemented Group 1 ISFETs on the second test prototype. Notice the membrane is deposited on top of the metal electrode not the passivation layer. The metal electrodes are exposed using the conventional mask for opening pads.

of one channel (Group 1) consisting of an ISFET connected to a potassium-sensitive membrane-covered electrode and the ISFET readout circuit. The structure of Group 2 is the same with passivation layers and polyimide layer covering the electrodes. An always on switch consisting of transistors Ms1 and Ms2 in Fig. 4.18 is used per channel to provide ESD immunity and pass the process DRC rules. Since noise and bandwidth are not of great concern, to conserve power, the circuits were designed to work in the sub-threshold region. The drain-source follower circuit used in each channel provides an ISFET current of $200\text{nA}$ with an ISFET $V_{ds}$ of $100\text{mV}$. Opamp1 is a two-stage opamp with an output buffer. This buffer is directly connected to the output PADs on the chip and in a differential configuration it drives the input resistor of a difference amplifier. The second opamp is a two-stage opamp as shown in Fig. 4.20. No buffer is required since it only drives the ISFET transistor. The second stage uses the ISFET transistor as a current source that affect the gain of the second stage.

The bias voltages for all the biasing transistors in all the channels are provided by one constant-$g_m$ circuit [148]. The current consumption for each channel is $2.5\mu\text{A}$. 
Figure 4.18: ISFET and drain-source follower ISFET readout circuit. Six channels are implemented by the combination of 2 different-sized ISFETs and 3 different-sized electrodes. The opamp1 (Op1) and opamp2 (Op2) designs are described in Fig. 4.19 and Fig. 4.20.

ISFET Sensitivity

**Group 1 ISFETs:** Group 1 ISFETs are the ISFET transistors and their respective readout circuits connected to exposed metal electrodes. Group 1 circuits were tested for pK sensitivity using a potassium-selective membrane directly deposited on top of the exposed metal electrodes. Figure 4.21 shows the pK sensitivity across different channels calibrated by a buffer solution of 0.1M NaCl and varying concentration of KCl at room
temperature of $24^\circ C$. All the channels provide near Nernstian sensitivity of about 54 mV/dec at room temperature.

![Graph showing ISFET signal vs. -pK (log10(Potassium Ion Concentration in moles/litre))]  

Figure 4.21: Potassium sensitivity of the Group 1 ISFETs across 6 different channels at room temperature ($24^\circ C$).

Figure 4.22 shows the equivalent capacitors that affect the ISFET sensitivity using group 1 ISFETs and their metal electrodes. The passivation layer capacitance does not exist and an aluminum oxide capacitance is replaced between the membrane and the ISFET transistor gate. Aluminum metal forms a thin protective aluminum oxide layer as soon as it is exposed to the air. The thickness of this layer is extremely low (several Angstroms to a few nanometers) [149] with a dielectric constant $\epsilon_r$ of 9.5. The aluminum
oxide results in a 114pF to 1.8nF capacitance in in 37µm × 37µm metal electrodes to 150µm × 150µm metal electrodes, respectively. These capacitance values are very large compared to parasitic capacitors and the input equivalent $C_g$ of the ISFET transistor; hence the gain of 1 in (4.4) and good sensitivity as shown in Fig. 4.21.

![ISFET schematic diagram](image)

Figure 4.22: The aluminum oxide capacitance and the ISFET parameters implemented in Group 1 of the ISFETs showing the equivalent capacitors that affect the ISFET sensitivity.

As expected the channel sensitivity did not change with electrode size and ISFET transistor sizes because the membrane was directly attached onto the surface of the electrodes and the aluminum oxide capacitance provided very high capacitance values. Thus all the ISFETs had the same (very close to Nernstian) sensitivity. Our attempt to eliminate the passivation layer from the potassium-sensitive ISFET provided very promising results with good sensitivity and good adhesion properties. This technique eliminates the sensitivity design constraints on the size of the electrode, that was the result of capacitive division happening at the passivation layer in ISFETs with passivation layer between the membrane and the ISFET electrode.

As explained in the previous sections, the ISFET output signal of different ISFETs (even with the same electrode and transistor size) have different DC baseline because of the threshold voltage variations between them. In Fig. 4.21 we have removed the DC baseline between the multiple channels to show the sensitivity and its variations over multiple electrodes. According to the calibration curve in Fig. 4.21 the detection limit of the implemented ISFETs is $10^{-6}$M or pK of 6. This result is very similar to the characteristics of our commercial ISE (Orion IONPLUS 9719BNWP), where a PVC-based potassium-sensitive membrane is also utilized.

**Group 2 ISFETs:** Group 2 ISFETs are the ISFET transistors and their respective readout circuits connected to metal electrodes that are covered by passivation layer and
polyimide layer. Initially the electrodes were designed for the case of only passivation
layers (no polyimide) assuming the polyimide layer could be excluded. Since this option
was not provided, the sensitivity was low and experiments using bacteria in chapters 5, 6
and 7 were all performed using Group 1 ISFETs.

We tested the functionality this group for pH sensitivity (no membrane deposited)
and pK sensitivity (potassium-sensitive membrane covering the chip). No pH sensitivity
was measured, as expected, since polyimide layer is not pH sensitive. We observed very
little pK sensitivity because of the high thickness (7µm on top of metal 8 electrode) of
the polyimide layer. Also poor adhesion of the membrane to the polyimide layer could
also have contributed to weak sensitivity results.

ISFET Noise Contribution

From the experiments performed using the first test prototype, the chemical noise contri-
bution was high compared to the electronics noise. In this second prototype, the ISFET
and its readout were designed to have maximum of 200µVrms noise to decrease their
size and power requirements. The simulated input-referred noise from each channel is
110µVrms for 50µm/400nm ISFET and 140µVrms for the 10µm/400nm sized ISFET.
The noise is contributed by the ISFET transistor and the input transistors of opamp1
and opamp2 (Op1 and Op2 in Fig. 4.18).

Besides the 6 single-ended channels, we have two extra channels on each side of
the chip with the same electrode size and ISFET transistor. These two channels are for
testing purposes to show how the location of the ISFET electrode affects the performance.
Averaging between multiple channels of ISFET on the chip provides superior performance
and noise immunity. The above 6+2 channels were utilized in the experiments for bacteria
identification. The measurement results are provided in Chapters 5, 6 and 7.

Linearity

The drain-source follower circuit provides good linearity. The simulated slope of the
output voltage ($V_{out}$ in Fig. 4.18) versus the ISFET input when the input voltage of the
ISFET ($V_{in}$ in Fig. 4.18) was swept from 0 to 2.7V changed 0.1% ($\frac{\partial V_{out}}{\partial V_{in}}$ over $v_{in} = 0.5 \sim 2.5 = 0.1%$). As was discussed in reference to current-mode circuits in section 4.2.2, the
linearity of the voltage-mode drain-source follower is superior to current-mode circuits.
4.4.2 ISFETs Connected to Current-Mode Fixed Source and Drain

Two channels of current-mode ISFET readouts were implemented, such that two PMOS ISFETs were connected to current-mode fixed drain and source voltage circuits, as shown in Fig. 4.23. Both drain and source voltages were applied externally and the reference voltage, $V_{Ref}$, was applied to bias the circuits using the Ag/AgCl reference electrode. The opamps were the same as the voltage-mode circuit described earlier (Fig. 4.19 and Fig. 4.20).

![Circuit diagram of the current-mode circuit (one channel) in the second prototype IC.](image)

Figure 4.23: Circuit diagram of the current-mode circuit (one channel) in the second prototype IC.

ISFET Sensitivity and Linearity

As described in Group 1 circuits in the previous section, the voltage division between the aluminum oxide and the internal capacitance at the gate of the ISFET transistor does not impose any gain degradation. If the passivation layer existed, then the voltage division between the passivation capacitance and the gate source capacitance of the ISFET transistor would degrade the sensitivity greatly.

In this test circuit, the current sensitivity and linearity of the output current $I_{out} = I_d$ depends on the region of operation of the ISFET transistor. The output current is not linearly dependent on the input gate voltage and threshold voltage of the ISFET transistor. The problem with using current-mode circuits in ISFET systems where the threshold voltage is variable, is that the sensitivity, the linearity of the output, and the power consumption depend on the threshold voltage and the applied reference voltage. The circuit was simulated with $V_g$ changing from 0.5V to 2.5V. The slope of the changes in the output current was 95\% ($\frac{\partial I_{out}}{\partial V_{in}}$ over $v_{in} = 0.5 \sim 2.5V = 95\%$) showing high...
non-linearity. In applications where non-linearity is not an issue, compact current-mode circuits based on the implementation shown in Fig. 4.23 would provide adequate results.

In our test setup, the ISFET was biased in the triode region (for linear dependence of $I_D$ over $V_g$) with $V_d = 3.1V$ and $V_s = 3V$. Using the above value for $V_d$ and $V_s$ and $V_{Ref}$ of 1V, the pK sensitivity for the two channels were tested where the potassium-selective membrane was deposited on top of the ISFET electrodes. The designs of both channels were identical. Figure 4.24 shows the results for a 3-point calibration obtained by applying a buffer solution of 0.1M NaCl with varying concentration of KCl. In measurements in Chapters 5, 6 and 7, current-mode ISFETs were not utilized because of their non-linearity and channel-to-channel variations in power consumption. Still, for low-power sub-threshold application were extra post-processing of the signal is helpful, current-mode techniques are useful, and should be considered.

![Figure 4.24: Potassium sensitivity of two current-mode ISFETs (24°C)](image_url)
4.4.3 Differential ISFET plus Drain-Source Follower Readout

Two channels, each with a differential ISFET configuration, were implemented on the chip. The experiments were not performed using a differential configuration because of the difficulty of depositing two separate PVC-based membranes on the chip. Figure 4.25 shows the differential architecture and the component parameters. A difference amplifier provides a gain of 20. Because of DC baseline variations between ISFETs, the initial DC baseline difference between the ISFET outputs need to be cancelled using a DC voltage applied to the difference amplifier, as shown in Fig. 4.25. In the differential system,

![Figure 4.25: Structure of a proposed differential ISFET readout and difference circuit.](image)

the reference voltage, $V_{Ref}$, is applied to a gold bond wire or a gold plated pad on the chip to bias the two ISFETs in the differential architecture, as to provide a miniaturized integrated system eliminating the need for a bulky Ag/AgCl reference electrode. Each ISFET channel was designed as the voltage-mode ISFET channel in the previous section with the same Op1 and Op2 architectures and the same $I_d$ current of 200nA. The ISFET transistor size was $10\mu m/400nm$ and the selected metal electrode size was $37\mu m \times 37\mu m$; both the smallest available sizes since the noise and sensitivity are not affected by the electrode size. The electrodes were exposed, no passivation or polyimide layers covered the electrodes.
4.5 Discussion and Summary

This chapter provided an in-depth background and design of a sensor front-end, including the ISFET, and ISFET readout circuits. Two CMOS prototypes, one in TSMC 0.18µm and one in IBM 0.13µm, were designed, implemented and tested. The most important parameters in the design of ISFETs and ISFET readout circuitry were the sensitivity and the capacitance of the passivation layer, the ISFET capacitance and the parasitic capacitors affecting this parameter. The passivation layer in both prototypes degraded the sensitivity performance of the potassium-sensitive ISFETs. In 0.13µm prototype, where a thick polyimide layer was included in the passivation layer, the sensitivity was significantly reduced. On the other hand, deposition of the potassium-selective membrane on top of exposed electrodes, with no passivation or polyimide layers, provided excellent sensitivity performance without need for optimizing electrode sizes. The 1/f noise from the ISFET and readout circuits were small compared to the chemical noise induced by drift, ion-transfer across the membrane, and poor membrane adhesion to the ISFET surface. Both voltage-mode and current-mode circuits were implemented and voltage-mode drain-source follower circuits were chosen because of their better linearity and precision. The required bandwidth for the front-end is very low, so low-power ISFET readout as well as the ADC can be implemented using sub-threshold circuits. A particular type of differential ISFET structure provides the advantage of removing the bulky reference electrode and replacing it with integrated pseudo-reference electrode, significantly reducing the sample volume. The differential structure also cancels the common-mode DC variations, relaxing the requirements on the ADC.
Chapter 5

Detection of \textit{E. coli} Using Bacteriophages

This chapter focuses on the experimental results using bacteriophages (phages) as described in Chapter 3, for the detection and identification of bacteria. Phages can be very specific to a single strain of bacteria or have a broad range of targets. When they attack their target, they attach to the cell membrane and form a pore in the cell membrane. This event is accompanied by transitory potassium efflux from inside the cells to the outside. Figure 5.1 presents the system design and individual building blocks in our bacterial detection system. The input to the system is an unknown sample with possible bacterial contents. The goal of the system is to determine if a specific type of bacteria (bacteria “X” in Fig. 5.1) is present in the sample or not. Figure 5.1 also shows the output variable of each building block to clarify its role in the biosensor system.

Figure 5.1: Overall system diagram using bacteriophages

In this chapter, the protocols used to prepare the bacterial samples for experiments
are introduced. A commercial ISE is first utilized to confirm how the bacteriophages can be utilized for bacterial identification. A brief description of the protocols used for bacteria and phage preparations will be provided in each section with additional details provided in Appendix A. The data obtained using the commercial ISE is then employed to extract the proposed system parameters previously explained in Chapter 3 and to provide a predictive model for system behaviour. Leveraging the data obtained using the commercial ISE, experimental results of using the first test prototype for the detection of different strains of \textit{E. coli} using 2 different phages are provided.

\section*{5.1 Sample preparation and Processing}

Raw samples with defined concentrations of bacteria for the experiments are prepared as explained in Appendix A. The samples are bacterial cells suspended in LB medium. LB contains high concentrations of potassium, sodium and other interfering ions and the potassium-selective ISE system cannot use raw samples directly. To process the raw samples, the bacterial pellets are collected using centrifugation at the speed of 6900g for 1 minute. LB is then removed and the suspension medium, usually SM, is added and the bacterial pellets are mixed so as to be fully suspended in the suspension medium. This sample processing takes less than 5 minutes considering all the centrifugation and mixing and is a simple one-step procedure. The process is also briefly described in Appendix A.

\section*{5.2 Initial Experiments using Commercial ISE}

An example of a commercial ion-selective electrode (ISE) system used in our experiments is shown in Fig. 5.2. The ISE consists of a probe head that is inserted inside a sample solution to take measurements. The output voltage of the ISE is read and displayed in the meter module. The potassium-sensitive probe used in our experiment is a Thermo Scientific Orion IONPLUS 9719BNWP potassium-selective electrode connected to a Thermo Scientific Orion Dual Star pH/ISE bench-top meter. The probe is calibrated using 0.1M KCl standard solution (Thermo Scientific Orion 921906).

Researchers in [86, 87, 72] have quantified the potassium ion efflux resulting from phage infection by using commercial ion-selective electrodes to measure the external potassium ion concentration in the sample. They have shown the dynamics of three different phages—\textit{T}4, \textit{T}5 and \textit{T}7—infected \textit{E. coli} bacteria. As shown in [86, 72, 88], the dynamics of the ion efflux as well as the rate of phage adsorption, depends on the type of phage and temperature. The total efflux of ions from inside the cell to the outside takes
between 30 seconds and 30 minutes depending on the type of phage and temperature.

We have used potassium-selective electrodes to successfully quantify potassium efflux resulting from phage adsorption using other phages including $\lambda$ phage [89], HK97 phage [90] and $T_6$ infecting $E. coli$ cells. Other bacteria such as $Pseudomonas aeruginosa$ and $Salmonella$ cells have also been successfully tested with multiple phage combinations in our group. In this thesis, we have optimized the buffer and assay for experiments involving $\lambda$ phage as well as $T_6$ phage, which, to the best of our knowledge, has been done for the first time in the published literature [150, 151].

The experiments involving the commercial ISE and the prototype IC both used $\lambda cI_{857}$ phage and $T_6$ phage. Both phages were prepared with a titre of $10^{11}$ pfu/ml (plaque-forming units per ml) from low-concentration phage lysate stocks. Each phage stock was then dialyzed in clean SM buffer (composition described in Appendix A), stored at 4°C and used in our experiments as needed for several months without a noticeable titre drop. The detailed description of the protocols utilized to prepare the phage stocks are provided in Appendix A.

The first set of experiments was performed using $E. coli$ K12 BL21 (DE3-Δtail) (referred to as BL21) and $E. coli$ K12 BW25113 with TSX- (referred to as TSX-). The $E. coli$ K12 BL21 strain is a wild type strain while the strain $E. coli$ K12 BW25113 with TSX- is a knock-out strain missing TSX receptors on the outer membrane surface. As explained in [152], TSX is the receptor for the $T_6$ phage; hence $T_6$ does not infect strains missing the TSX. The $E. coli$ strains were prepared and grown in LB media (see Appendix A) and suspended in SM buffer. For the experiments using $T_6$ phage, 10% L-tryptophan is added to the SM as it is required for effective $T_6$ infection [152]. The prepared cells were immediately used for measurements. The two experiments provide
a positive control experiment using the combination of BL21 cells and $T_6$ phage and a negative control experiment using the combination of TSX- and $T_6$. Figure 5.3 shows the protocol for the positive and negative control experiments and their respective expected outcomes.

![Diagram](image)

Figure 5.3: Experimental protocol using the combination of $T_6$ phage and two strains of *E. coli*, that provide positive and negative control experiments.

Figure 5.4 shows the results of these experiments performed at room temperature ($23.7^\circ C$). For each test 100$\mu$l of $T_6$ is used. In Fig. 5.4, both the ISE raw output and the ISE output with the DC baseline removal and drift compensation are presented (Algorithm for DC baseline removal and drift-compensation was presented in Chapter 3). As can be seen from Fig. 5.4, as $T_6$ phage is added, the voltage of the ISE increases as a result of potassium efflux whereas in TSX- experiments, no significant voltage increase is observed. The reason for using room temperature for these experiments is that no significant change in signal was observed when performing experiments at $37^\circ C$ compared to $23.7^\circ C$. The effect of DC offset and drift compensation post-processing are also very clear in Fig. 5.4.

New sets of experiments were performed using phage $\lambda cI_857$ (referred to $\lambda$ here) and *E. coli* K12 BW25113 ΔfluA and *E. coli* K12 BW25113 ΔlamB bacterial strains. The receptor protein for $\lambda$ phage is lamB protein [153] which is lacking in *E. coli* K12 BW25113 ΔlamB strain, making it suitable as a negative control. The *E. coli* strains were prepared and grown in LB media (Appendix A) and suspended in SM buffer.

The results of the experiments performed at multiple temperatures using $\lambda$ phage and the *E. coli* strains mentioned above are shown in Fig. 5.5. The ISE outputs for all measurements are shown after DC baseline removal and drift compensation. As can be seen in Fig. 5.5, the rate of $\lambda$ phage infection and the efflux of potassium ions are both very temperature-dependent.

The experiments performed using ISE and briefly shown in Fig. 5.4 and Fig. 5.5
Figure 5.4: ISE measurements using T6 phage with BL21 and TSX- bacterial cells at 23.7°C. The OD of BL21 cells was 1.1 and for TSX- cells was 1.2. (a) The raw ISE outputs without DC baseline removal and no drift compensation, (b) The ISE output with DC baseline removal and drift compensation.

Figure 5.5: ISE Measurements using λcI857 phage and E. coli. Each experiment used a bacterial concentration corresponding to an OD = 1.2.
confirm how the system using the efflux of potassium ions as a signal can be utilized for bacterial identification. The system model parameters are extracted first using the results of multiple measurements with the commercial ISE. The first CMOS prototype, as shown in Chapter 4, is then used for bacterial identification.

5.3 System Model Parameter Extraction and Predictive Model Estimation

In this section, the modelling parameters introduced in Chapter 3, are extracted from the experimental data presented in Section 5.1 using the techniques and algorithms presented in Sections 3.6. The system model output are also compared against measurement results using the extracted parameters and the predictive capabilities of the system model are tested.

5.3.1 Estimation of ISE Output Voltage Signal versus Bacterial Cell Concentration

The output voltage of ISE after phage infection depends on the concentration of the infected cells and the per-cell amount of potassium efflux into the surrounding media after infection as well as the ISE calibration curve that maps its input potassium concentration to the output voltage. The value obtained also depends on the potassium present in the sample or the interference from other ions in the sample that results in a baseline voltage $V_b$ as introduced in Chapter 3. The concentration of bacteria cells used in section 5.2, with optical densities from 1.1 to 1.2 is in the range of $10^9$ cfu/ml, but the exact value was not confirmed using separate plating technique. The value of $\alpha$, which is the percentage of the potassium efflux from inside the cells, and the exact number of potassium ions inside the cells are unknown. Since we have performed all experiments that provide a positive outcome using one specific concentration of bacteria, we can estimate the value of $[K_r] \times N_A = \alpha K_{in} ([C_s] \times 1000)$ from the output voltages obtained.

According to Fig. 3.10, the output voltage after the infection is complete is the difference between $V_b$ and the output voltage of the ISE corresponding to the total potassium present in the media after infection. Since, for $T_6$ infections, the efflux seems not to be completed, we estimate the total efflux after 15 minutes after the infection is initiated (22 minutes in total time). Table 5.3.1 shows the extracted parameter $\alpha K_{in}$, that shows the total number of potassium ions released per cell. The values in Table 5.3.1 are obtained
using the ISE calibration curve shown in Fig. 5.6. used by the extraction algorithm presented in section 3.6.5.

![ISE Calibration Response Points and Fitted ISE Response](image)

**Figure 5.6: Calibration response of ISE at room temperature (24°C).**

Since each experiment had a different temperatures and the ISE calibration curve is temperature dependent, different calibration curves have been used for each experiment and Fig. 5.6 shows only one example. Based on plating experiments the approximate concentration of BL21 cells at OD of 1.1 was $10^9$ cfu/ml and for the BW25113 cells at OD of 1.2 was $2 \times 10^9$ cfu/ml, and these numbers were used as the bacterial cell concentration $[C_s]$.

<table>
<thead>
<tr>
<th>Cell</th>
<th>OD</th>
<th>$V_b$ (mV)</th>
<th>Phage</th>
<th>Measured $\Delta V$ (Time Post Infection) (mV)</th>
<th>Total $[K^+] = [K_r] \times N_A$ (M)</th>
<th>$\alpha K_{in}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL21</td>
<td>1.1</td>
<td>-148.1</td>
<td>$T_6$</td>
<td>16.43 (17 Mins 24°C)</td>
<td>$2 \times 10^{-4}$</td>
<td>$12 \times 10^7$</td>
</tr>
<tr>
<td>BW25113</td>
<td>1.2</td>
<td>-140.4</td>
<td>$\lambda$</td>
<td>17.1 (15 Mins 37°C)</td>
<td>$3 \times 10^{-4}$</td>
<td>$18 \times 10^7$</td>
</tr>
</tbody>
</table>

In this section, we showed how the value of system model parameter $[K_r]$ can be extracted from measurements. From the value of $[K_r]$ we can estimate the total number of potassium leakage per cell ($\alpha K_{in}$) for each fixed combination of phage and sensitive bacteria (positive controls). The value of of $\alpha K_{in}$ is then used in (3.4) to predict $[K_r]$ as the concentration of sensitive cells $[C_s]$ change in the sample. Alternatively, the value of $[C_s]$ can be estimated using the ISE system output voltage, the extracted $[K_r]$ and $\alpha K_{in}$, obtained here.
5.3.2 ISE System Output Signal Over Time

In order to see how the shape of the ISE measured output signal can be compared to the output of the system model presented in Section 3.6, Fig. 5.7 shows the fitted curve of the positive control BL21 experiment in Fig. 5.4 with the system model output. The system model parameters were first extracted from the measurement results using the algorithm presented in Section 3.6.5; then the system model output was generated and compared to the measurement results, as shown in Fig. 5.7.

![Graph](image)

Figure 5.7: Fitted response of the ISE to the system model in Fig. 3.10 using the algorithm presented in Section 3.6.5. The experiment was performed using an ISE and combination of *E. coli* BL21 OD of 1.1 with added phage *T*<sub>6</sub>. The figure shows the response after phage *T*<sub>6</sub> addition at 23.7°C.

Using the algorithm presented in Section 3.6.5, and multiple measurements obtained using the ISE, the system model parameters were extracted. The values of the delay d and time constant τ of the potassium efflux as introduced in Fig. 3.10 were determined for each experiment. The extracted parameters from Fig. 5.7 as well as other ISE measurements are provided in Table 5.2. From Table 5.2 and the values of d and τ for different combinations of cells and λ phage, the speed of infections can be compared. Also from these experiments, the temperature sensitivity of the potassium efflux can be extracted.
Table 5.2: Parameters extracted from the ISE response according to Fig. 3.10 of the system model.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Phage</th>
<th>Cell OD</th>
<th>Temp. °C</th>
<th>d (Min.)</th>
<th>τ (Min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL21</td>
<td>$T_6$</td>
<td>1.1</td>
<td>23.7</td>
<td>0.25</td>
<td>4.7</td>
</tr>
<tr>
<td>BW25113</td>
<td>$\lambda$</td>
<td>1.2</td>
<td>37</td>
<td>0</td>
<td>1.98</td>
</tr>
<tr>
<td>BW25113</td>
<td>$\lambda$</td>
<td>1.2</td>
<td>30</td>
<td>1.16</td>
<td>2.91</td>
</tr>
<tr>
<td>BW25113</td>
<td>$\lambda$</td>
<td>1.2</td>
<td>24</td>
<td>2</td>
<td>6.5</td>
</tr>
</tbody>
</table>

System Model Predictability

In section 5.3.1, we showed how the system model parameter $[K_c]$ can be extracted from the measurements. For a fixed combination of phage and sensitive bacteria, the only other input parameter that affects the values of system parameters $\tau$ and $d$, is temperature. The predictability of the system model to capture the effect of temperature on the output sensor signal, is dependent on how well the system parameters $\tau$ and $d$ are modelled over temperature. In section 3.6.6, we introduced previous research that has characterized the temperature sensitivity of the phage-induced potassium efflux from sensitive cells. In this section, we compare the extracted temperature sensitivity of the parameters $\tau$ and $d$ with the available literature to show our system predictive capability over different temperatures.

From [86, 72, 88], we expect to see that the initial rate of potassium efflux follows Arrhenius plot for temperatures where the infection occurs. Figure 5.8 shows the data points as well as the fitted Arrhenius line of $ln(1/\tau)$ which is proportional to the logarithm of rate of efflux over the inverse of temperature for the experiments involving $E. coli$ BW25113 and phage $\lambda$, as shown in Table 5.2. What can be observed from this graph as well as Table 5.2 is the decrease in $\tau$ when the temperature increases from room temperature to 37°C according to an Arrhenius plot, confirming the results in [86, 72, 88]. Temperatures below room temperature and temperatures above 37°C were not experimentally tested. Figure 5.8 shows the system model capability to predict the system model parameter $\tau$ at different temperatures using experimental measurement results.

Figure 5.9 shows the value of the extracted system model parameter $d$, as shown in Table 5.2, versus temperature. As shown in Fig. 5.9 the delay of the potassium efflux for a specific combination of phage and bacteria decreases as the temperature is increased toward 37°C confirming the results in [86, 72, 88]. The shape of the graph of delay versus
Figure 5.8: Extracted $\ln(1/\tau)$ versus inverse of temperature and a fitted Arrhenius plot. $\tau$ was extracted from experiments involving phage $\lambda$ and *E. coli* BW25113 $\Delta fhuA$ in Table 5.2.

Figure 5.9: Extracted delay versus temperature and fitted line in temperature range from $24^\circ C$ to $37^\circ C$. Delay $d$ was extracted from experiments involving phage $\lambda$ and *E. coli* BW25113 $\Delta fhuA$ in Table 5.2.
temperature has not been consistently described across different combinations of phage and bacteria. In Fig. 5.9, we have used the least mean square first-order fit for modelling $d$ versus temperature from 24 °C to 37 °C for the combination of *E. coli* BW25113 cells and $\lambda$ phage.

From the parameters extracted in Table 5.2 and the fitted curves for temperature dependence, as well as the algorithm for estimating the final $\alpha K_{in}$, the system output signal can be estimated at various concentrations of sensitive bacterial cells and multiple temperatures. The same measurement, extraction and modelling technique can be used using other phage and bacteria combination to estimate the bacterial sensor output.

To show the system model predictability at multiple temperatures, new sets of independent experiments were performed using the combination of *E. coli* BW25113 cells and $\lambda$ phage at the fixed OD of 1.2 at temperatures 37°C and 30°C. The extraction algorithm, presented in section 3.6.5, was used to extract the system parameters $d$ and $\tau$. The extracted parameters were compared against the estimated values of $d$ and $\tau$ using the graphs in Fig. 5.8 and Fig. 5.9. Figure 5.10 shows $\tau$ and $d$ in one graph, extracted from measurement results at 37 °C and 30 °C, and the estimated $\tau$ and $d$ at these two temperatures. This figure shows the system model can reliably predict the system behaviour at multiple temperatures.

![Figure 5.10: Estimated values of system parameters, delay $d$ and time constant $\tau$ and the extracted parameters from independent measurements at 37 °C and 30 °C. The independent experiments were performed using the *E. coli* BW25113 and $\lambda$ phage.](attachment:image.png)
5.4 Test Chip Experimental Setup and Procedures

5.4.1 Chip Implementation and Preparation

The test chips used in this chapter are the first generation CMOS prototypes as explained in section 4.3. Figure 5.11 shows a micrograph of the chip and the test setup used during the experiments.

To construct the K\textsuperscript{+}-sensitive ISFET, the chips containing the ISFET transistors were first encapsulated using commercial epoxy. All bond wires, bonding pads, and chip scribe lines were covered, leaving only the electrodes exposed as shown in Fig. 5.11 (left). The chemical solution of the membrane components in THF solvent was prepared under a fumehood and mixed. The membrane mixture solution was stored for 10 days and repeatedly used during this time as needed.

Chip surface cleaning and functionalization was performed before each experiment as explained in the Appendix A before deposition of the membrane.

The measurements were performed inside a dark chamber since ISFETs are sensitive to light, and light variations affect their output. The bacterial cells as well as the phages were deposited on the top surface of the chip via autoclave-able tubing connected to their respective glass syringes outside the dark chamber. The syringes were manipulated manually for the transfer and mixing of the cells and phages. Electric heaters were installed under the PCB board to adjust the temperature to the required constant value as needed. Figure 5.11 shows the PCB board and the heater inside the dark chamber. In addition, a temperature sensor with 0.5°C resolution was installed on the chip package.

Figure 5.11: Left: chip micrograph, specifications and encapsulated chip with exposed electrodes; right: test board plus other components of the measurement setup inside the dark chamber.
that reads the temperature continuously during the experiments, verifying that the chip temperature was stable.

5.5 Experimental Results

Three groups of experiments were performed using the CMOS chip. In this section, we first provide the experimental protocols applicable to all groups, and then present the details of the experiments, and provide results.

5.5.1 Experimental Protocol

Each group of tests includes two control experiments to show the system specificity. Each group utilizes a specific phage sensitive to our target bacteria. The two control experiments were performed with two samples each containing differing bacteria strains. In a positive control experiment, the sample contains bacterial cells that are sensitive to the chosen detector phage. As a result of phage infection of the sensitive cells, we expect a release of potassium (K⁺) ions from inside the bacterial cells to the sample outside the cells where ISFET measurements are performed. An increased potassium concentration in the sample and an increased ΔV of the ISFET are expected. In a negative control experiment, the same detector (phage) is used, but the sample does not contain any sensitive cells. Instead, the sample contains bacterial cells at the same concentration as in the positive control experiment that are resistant to the selected phage. Since there should be no infection as phages are added, no potassium concentration changes in the sample and no ΔV changes are expected. Figure 5.3 showed the protocol for positive and negative control experiments in the case of T₆ phage with different strains of E. coli used in experiment Group A. The same protocol but with a different set of sensitive and insensitive cells and a different set of phages are used in experiments Groups B and C.

5.5.2 Group A Experiments using T₆ Phage as the biological recognition element

The positive control experiments were performed using E. coli K12 BL21 (DE3 – Δtail), called BL21 here for simplicity, which are sensitive to T₆ phage. The negative control experiments were performed using bacterial cells E. coli K12 BW25113 with TSX-, called TSX- here for simplicity, which are insensitive to T₆. The phages used throughout
the Group A experiments were $T_6$ phages with a concentration of $10^{11}$ pfu/ml. These experiments were performed at a room temperature of 23.7°C. Bacteria cultures were grown to OD=1.2 that corresponds to a concentration of $10^9$ cfu/ml (see Appendix A for bacteria sample preparation protocol). After washing and resuspension in SM, 0.5ml of the cells were added to the encapsulated die. Recording of the ISFET output started when the sample containing cells was transferred to the top surface of the die and continued until the end of the experiment. After 5 minutes, 20µL of $T_6$ phage were added to the sample and mixed. The mixture was left on the chip for 30 minutes. Figure 5.12a shows the recorded output voltage changes $\Delta V$ in the

![Graph](attachment:image.png)

Figure 5.12: Output $\Delta V$ voltage recordings for Experiment Group A: (a) raw $\Delta V$ output with drift, and estimated drift line (dotted) before the addition of the $T_6$ phage. (b) Estimated output $\Delta V$ after drift compensation.
ISFET. The results shown in Fig. 5.12 were processed in Matlab with a 0.3 Hz low-pass filter. The results show that $\Delta V$ changes with the removal of any DC baseline differences between the experiments ($\Delta V = 0$ at time = 5 minutes). The transient signal recorded in the first 2 minutes after phage addition (time = 5 to 7 minutes) are due to fluid perturbations resulting from the slow mixing of the sample. This time period is greyed out in figures 8 to 11. As is evident in Fig. 5.12a, the drift for positive and negative control experiments before the addition of the phage is very different, since drift components for each experiment are different as explained in Chapter 3. Figure 5.12 draws the drift line evaluated using the last 30-seconds before the addition of phage in each experiment. In Fig. 5.12b, the output of the positive and negative control experiments are shown after drift compensation using drift lines for each experiment in Fig. 5.12a subtracted from the initial raw output. Fig. 5.12b shows the drift-compensated outputs in both positive and negative control experiments, clearly showing the system detection capability.

The results clearly show how the output rises because of $K^+$ concentration changes after the infection and how the output of the negative control experiences the same drift as before the addition of phages.

The goal of the bacterial detection system is to determine if the target bacteria is present in the sample or not. We have explored another approach to process the output data. Looking at the signal development shown in Fig. 5.12 for the whole infection cycle is not an optimal scheme for detection. Using the results of Fig. 5.13 instead illustrates the slope of the $\Delta V$ over time for the first 10 minutes after the addition of $T_6$ phage in the experiment Group A.

![Graph showing the slope of ΔV curve with respect to time for experiment Group A.](image-url)
After adding and mixing the phage with the sample, the potassium ions start to flow out of the infected cells. The slope of the drift-compensated output data for the first couple of minutes is enough for detection purposes. After infection, the slope of the positive control experiments are positive where the slope of negative control experiments are near zero or negative. Fig. 5.13 shows the slope after infection for experiment Group A calculated over a 2 minute interval every 15 seconds. The slope of the outputs are taken after DC baseline removal and drift compensation. The positive output slope for several minutes after infection is the indication (sufficient statistic) of the presence of sensitive cells.

5.5.3 Group B Experiments using \(\lambda\) Phage at 37°C

Another group of experiments were performed using phage \(\lambda cI_{857}\), called \(\lambda\) phage for simplicity here, using two strains of bacteria. The bacteria strain for positive control is \(E. \text{coli} K12 \text{ BW25113}\; \Delta fhuA\) and the negative control strain is \(E. \text{coli} K12 \text{ BW25113}\; \Delta lamB\). The concentration of cells (\(10^9\; \text{cfu/ml}\)) and phages (\(10^{11}\; \text{pfu/ml}\)) are identical to experiment Group A. The rate of infection for \(\lambda\) phage is very temperature dependent, as shown in ISE experiments in Section 5.1, where the highest infection rate happens at 37°C corresponding to the optimal temperature for most of the living bacteria of interest. Experiment Group B measurements were performed at 37°C by adjusting the electric heater in the experimental setup.

For these experiments, we look at the slope of \(\Delta V\) vs. time for the first 10 minutes for both positive and negative control experiments as shown in fig. 5.14a.

5.5.4 Group C Experiments using \(\lambda\) Phage at 23.7°C

Experiment Group C was performed using the same \(\lambda\) phage and the positive and negative control samples as in Group B; except that the measurements were performed at room temperature (23.7°C). As explained earlier, \(\lambda\) phages still infect sensitive cells at this lower temperature, though the initial ion efflux resulting from infection is delayed compared to 37°C [89]. Also the rate of phage adsorption [88] and ion efflux [89] are slower than of 37°C. For this experiment, Fig. 5.14b shows the slope of \(\Delta V\) over time for the first 10 minutes. If the goal is simply detection, the output of measurements at room temperature can be used to provide correct detection decisions. Comparing Fig. 5.14a and b reveals the temperature-dependant dynamics of infection. Infections at 37°C start faster (higher positive slopes right after phage mixture) compared to room temperature infections. The infection rates (slope lines) are also higher at 37°C measurements.
Chapter 5. Detection of E. coli Using Bacteriophages

The experiments Group A, Group B and Group C show how the system can be used for detection as well as for studying infection dynamics by using the slope of ISFET output after proper drift compensation.

In this chapter, we explored two types of output processing and visualization. In the first type, the DC and drift compensated outputs $\Delta V$ are visualized, and in the second type, the outputs are processed to take the slope of $\Delta V$ vs. time, and visualize the data. For both outputs, detection is achieved within 10 minutes of infection. Taking the slope of the ISE system output is a lossy process that discards some of the system information that can be extracted as explained in Section 3.6.5; though it provides advantages in terms of fast visualization of the rate of infection and detection. Either outputs can be used, depending on the system requirements. We have chosen $\Delta V$ outputs as our preferred method of presenting the output for the rest of this thesis because this type of output preserves all system information.

5.6 Summary

This chapter provided experimental results of the use of bacteriophages for identification of different strain of E. coli. Initial experiments were performed to confirm system functionality for detection and identification, using the potassium efflux from phage-infected bacteria cells. Since these experiments were performed at multiple concentrations and temperatures, the results of the ISE experiments were used to extract the system parameters and fit the ISE output to the proposed system model in Chapter 3. System model parameters were extracted using the measurement results and their value were estimate.
using the system predictive model for new sets of experiments. Leveraging the results obtained using a commercial ISE, a subset of those experiments were then performed using the first CMOS prototype. The system using the CMOS prototype successfully provided identification results within 10 minutes. Utilization of the slope of the ISFET output data of the prototype IC was also presented as a mechanism for detection.
Chapter 6
Detection of Bacteria Using Bacteriocins

In this chapter, we utilize the activity of bacteriocins, a heterogeneous group of antimicrobial peptides and proteins produced by bacteria, for the sensitive and specific detection of bacteria. The use of bacteriophages, viruses that infect and kill bacteria, in biosensors for bacterial detection was previously discussed in Chapter 5. In Chapter 5, we used a CMOS ISFET connected to an external data acquisition board for the specific and rapid identification of *E. coli*, to detect the efflux of potassium ions that accompanies the infection of a susceptible cell by a bacteriophage. In this chapter, we significantly advance the technology, leveraging integration of the sensor platform in CMOS as explained in Chapter 4, as well as utilizing the sensitivity of the bacteriocins for detection. The sensor platform integrates an array of sensing electrodes that contact the sample, as well as the respective electronic circuitry required to read and process the data in real time. The sensing electrodes are connected to potassium-sensitive ion-selective field-effect Transistors (ISFETs) able to detect the small variations in the potassium ion concentration in the sample that results when live bacteria are killed by the activity of the bacteriocins, as shown in Fig. 6.1.

This technique, which combines the inherent specificity of bacteriocins with the sensitivity of the integrated CMOS sensor, provides a low-cost, portable biosensor that is able to accurately identify bacteria in less than 10 minutes.

In this chapter, we first plate different bacteria with different bacteriocins to confirm the killing of the cells by the specific bacteriocins. We then use the commercial potassium-selective electrode (Orion IONPLUS 9719BNWP) in a set of experiments to confirm the suitability of using bacteriocins for bacterial identification by detecting the potassium efflux from the sensitive cells. We then utilize the data obtained to extract the system
model parameters introduced in Chapter 3, and predict system model parameters for new sets of measurements, showing system predictability. Leveraging the data obtained using the ISE, we provide experimental results using our second generation of CMOS IC for the detection and identification of three different species of bacteria using two different bacteriocins.

### 6.1 Initial Plating Experiments of the Bacteriocin

Two different categories of bacteriocins are utilized in this work. The first are colicins that target *E. coli* species and lysostaphin that infect *Staphylococcus* species (see Appendix A for protocols used for the preparation of the bacteriocins). Three different strains of bacteria are also used: *E. coli* BW25113, *Staphylococcus aureus* 8325 and *Pseudomonas aeruginosa* PA01.

To test the killing activity of each bacteriocin, a specific strain from the list above was prepared (see Appendix A) and suspended in 0.7% molten top agar and poured on top of an LB-agar plate. 3 µl of 100-fold serial dilutions of each bacteriocin were added in spots on top of the cells, and the plates were incubated overnight at 37°C. The following day, the lowest dilution at which zones of clearing were observed was noted. Figure 6.2 shows the spotting results for the bacteriocins utilized in this work. Each cleared area indicates a killing zone where the bacterial lawn did not grow. When there is no clear zone, the bacteriocin is not killing the bacteria grown on the plate. As evident from Fig. 6.2, all colicins (col A9, col B, col E1 and col K) kill the *E. coli* strain without killing *S. aureus* or *P. aeruginosa* PA01. Lysostaphin only kills *S. aureus* without killing *E. coli*. None of the colicins or the lysostaphin kill the *P. aeruginosa* (PA01).
6.2 Selection of Biological Probes Using a Commercial ISE

Bacteriocins have many distinct mechanisms of action that can be broadly divided into two classes: one acts primarily at the cell membrane and the other acts on processes within the cell. We focus on the first class, specifically on members of this class that kill the target cells through pore formation in the cell membrane. We targeted Gram-negative \textit{E. coli} and Gram-positive \textit{Staphylococcus aureus} 8325 for detection in these studies. These bacteria cause a variety of infections, including skin lesions, food poisoning, as well as urinary tract and bloodstream infections.

Using a commercially available ISE, we examined the ability of four pore-forming members of this group of colicins, A9, E1, K, and B [92, 154, 93, 155] to elicit an efflux of potassium from \textit{E. coli}. The bacteria in the sample were prepared as explained in Appendix A, and resuspended in SM buffer at 4ml volume. For each experiment, 150
µl of the prepared colicin was added to the sample. The potassium concentration in the sample was monitored for approximately five minutes to ensure a stable baseline, then colicin was added and the resulting potassium efflux was recorded, as shown in Fig. 6.3. All the figures in this chapter are shown after DC baseline removal and drift compensation for clarity, as explained in Chapter 3. The sample containing E. coli K12 BW25113 was grown to an OD of 1.2+/− 0.1 in LB and prepared as explained in Appendix A. The temperature in all these experiments was maintained at 37°C by keeping the sample tube immersed in a water bath.

Figure 6.3: Commercial ISE output when different colicins are added to E. coli K12 BW25113 at 37°C.

We observed a very rapid and robust efflux from E. coli with the addition of colicins K and A9, and less intense signal with colicins B and E1. To ensure that the efflux we observed was due to pore formation in the cell membrane, we repeated the efflux experiments with E. coli knockouts lacking the specific outer cell membrane receptors needed for the activity of colicins A9 and B. For example, colicin A9 requires the outer membrane protein ompF for activity [156], and when it was mixed with cells lacking this protein, no efflux was observed. Subsequent addition of colicin E1, which does not require this bacterial host factor, led to detectable potassium efflux, as shown in Fig. 6.4, illustrating that the potassium efflux we observed was linked to the ability of the colicin to form a pore in the bacterial cell membrane. As shown in Fig. 6.4, E. coli K12 BW25113 FepA- is insensitive to colicin B and sensitive to colicin A9 [74]. The OD600 for all bacteria strains was 1.2 and the temperature during the experiments was
Chapter 6. Detection of Bacteria Using Bacteriocins

37°C. These experiments also shows how multiple bacteriocins can be used to identify the bacterial strain in a sample through a process of elimination.

To detect *S. aureus* using an ion-selective electrode, we repeated the potassium efflux experiments using lysostaphin, a bacteriocin derived from *Staphylococcus simulans* that has activity directed specifically against *S. aureus* [157]. The addition of lysostaphin to a sample of *S. aureus* resulted in robust potassium efflux from the cells as shown in Fig. 6.5. We tested the activities of lysostaphin and colicin A9 against *Pseudomonas aeruginosa* PA01, another common Gram-negative human pathogen. As expected, neither elicited potassium efflux from the PA01 cells. In Fig. 6.5, the OD of all bacterial cells were 0.8 in LB and prepared as explained in Appendix A, though the experiments were performed at 26°C to prove system functionality at low temperatures. Taken together, these experiments show that bacteriocins act as simple and specific probes for bacteria when used in combination with a potassium-selective electrode.

One of the applications of our system is in the detection of urinary tract infection (UTI) bacteria. Urinary tract infections are the most common type of bacterial infections [14] and are one of the top five most burdensome infectious diseases [15, 16]. *E. coli* is the most common cause of UTIs, followed by *Staphylococcus, Proteus mirabilis, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* [14].

In order to confirm the capability of our technique for the detection of bacteria in urine, we performed a set of experiments using artificial urine (AU) purchased from
Figure 6.5: Initial experiments with commercial ISE at 26°C with cells having OD = 0.8.

Figure 6.6: Experimental results utilizing ISE for bacterial identification in artificial urine (AU) at 30°C. In the case of E. coli K12, the addition of colicin a9 results in a positive outcome, whereas in PA01, negative results occur.

Dyna-tek Surine, as shown in Fig. 6.6. E. coli K12 BW25113 cells were grown to an OD of 0.8 in LB, washed twice, and resuspended in AU. The samples were left in AU for 5 minutes to ensure any impact the AU is having on the sample is taken into account. That bacterial cells do not grow significantly in AU was confirmed by our previous experiments.
The samples were then washed twice and resuspended in SM (the buffer of choice for all experiments in this chapter). The experiments were performed using colicin A9 (\textit{E. coli} is sensitive to colicin A9) and lysostaphin (\textit{E. coli} is insensitive to lysostaphin) and the results shown in Fig. 6.6 show positive and negative outcomes are achieved respectively. The temperature for these experiments was 30°C.

### 6.3 System Model Parameter Extraction and Predictive Model Estimation

In this section, the modelling parameters introduced in Chapter 3, are extracted from the experimental data presented in Section 6.2 using the techniques and algorithms presented in Sections 3.6.5. The system model output are also compared against measurement results using the extracted parameters and the predictive capabilities of the system model are tested using new sets of measurement results. The system model only predicts the outputs of positive control experiments, expecting zero output signal for negative controls.

#### 6.3.1 Estimation of the ISE Output Voltage Signal versus Bacterial Cell Concentration

In order to accurately model the system response to different concentration of bacteria, three experiments were performed using the commercial ORION ISE system. All these experiments are positive control experiments in which an increase in the potassium concentration in the sample is expected after the addition of bacteriocin. In these experiments, a single batch of \textit{E. coli} K12 BW25113 (\textit{E. coli} K12) sample was grown to an OD of 0.8 in LB and a concentration of $3 \times 10^8$ cfu/ml was confirmed by plating a dilution series of the sample bacteria. The cells were washed and resuspended in SM, as explained in Appendix A. The cells did not multiply in SM, so the concentration of the sample bacteria stayed the same. From the initial batch of cells in SM, two 10-fold dilutions of the sample were prepared, providing 3 samples of bacteria in SM with concentrations of $3 \times 10^8$ cfu/ml (OD =0.8 no dilution), $3 \times 10^7$ cfu/ml (OD =0.8 with 10X dilution) and $3 \times 10^6$ cfu/ml (OD =0.8 with 100X dilution). The experiments were performed one by one using the available ISE, starting with the sample with the lowest concentration, and following the same protocols, as outlined in the previous section. Colicin A9 was added at 5 minutes.

Figure 6.7 shows the output results from the ISE as well as the ISE calibration curve for reference. In panel (a), the $V_b$ is the baseline voltage read at 5 minutes before the
addition of colicin A9 and that corresponds to a potassium concentration $[C_b]$ baseline in the ISE.

Figure 6.7: (a) ISE output positive control results using 3 concentrations of E. coli K12 at room temperature (23.7°C), (b) calibration curve of the ISE showing the raw (without DC baseline removal) output voltage of ISE versus potassium concentration in the sample.

The total potassium efflux from E. coli cells, according to equation 3.4, is approximately $[K_r] = 4.48 \times 10^{-5} \text{M}$, assuming an average number of $9 \times 10^7$ potassium ions per cell and 100% efflux of all potassium ions. This value is not accurate since the total potassium concentration in an E. coli is not a good approximation for all E. coli strains used in our experiments.

To accurately estimate the ISE output voltage $\Delta V$, we extracted the parameter $[K_r]$ from the first set of measurements using the bacterial cells with concentration of $3 \times 10^8$ cfu/ml and the algorithm presented in section 3.6.5. Then using (3.4), we estimated
the value of $\alpha K_{in}$, that is the total potassium efflux per each cell for the combination of colicin A9 and *E. coli* K12. We found the $\alpha K_{in}$ to be 1.9 times the average value. The measurements were performed until 20 minutes after infection. Since the infection was taking place at room temperature, the value obtained after 20 minutes might not be the final value and all potassium concentration values are only valid at 20 minutes. Using the above value for $\alpha K_{in}$, we used (3.4) to estimate the system model parameter $[K_r]$ for the two experiments performed at bacterial concentrations of $3 \times 10^7$ cfu/ml and $3 \times 10^6$ cfu/ml, as shown in Table 6.1. Also, using the extraction algorithm presented in section 3.6.5 and the ISE recalibration curve provided, we estimated the value of ISE voltage output $\Delta V$ at 20 minutes post colicin A9 exposure.

The initial baseline voltage $V_b$ depends on the cells and how much potassium leakage occurs during their suspension in SM, as well as the time gap between suspension and performance of the experiments. The sample with the highest concentration will have a higher $V_b$ baseline because of more potassium leakage. In these experiments, the cells with highest concentration were kept on ice for about an hour before the start of their experiment, so the $V_b$ is higher than normal. Fresh cells produce higher $\Delta V$ because of lower $V_b$ values.

Table 6.1: Comparison of estimated ISE output voltage vs. measurements using *E. coli* K12 with added colicin A9 at room temperature.

<table>
<thead>
<tr>
<th>Cell concentration (cfu/ml)</th>
<th>Total released $K^+$ $[K_r]$ moles/litre (M)</th>
<th>Estimated ISE output (mV)</th>
<th>Measured ISE Output (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$3 \times 10^8$</td>
<td>$8.51 \times 10^{-5}$ (estimated)</td>
<td>23.17(extracted data)</td>
<td>23.17</td>
</tr>
<tr>
<td>$3 \times 10^7$</td>
<td>$8.51 \times 10^{-6}$</td>
<td>14.62</td>
<td>13.5</td>
</tr>
<tr>
<td>$3 \times 10^6$</td>
<td>$8.51 \times 10^{-7}$</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

**Minimum Detection Limit and Prediction Accuracy**

According to Fig. 6.7 and Table 6.1, the minimum detection limit in our system using our membrane and the SM buffer with high NaCl concentration is approximately $3 \times 10^7$ cfu/ml at room temperature. The accuracy of system prediction of the sensor output is 8.2% at $3 \times 10^7$ cfu/ml at 25 minutes after colicin addition, when system model parameters are fitted for $3 \times 10^8$ cfu/ml concentration. The potassium-selective membrane used in these experiments is not new. Calibration of the ISE system has been performed before each experiment. The age of the membrane affects the accuracy of the system in the
In this section, we showed how the value of system model parameter $[K_r]$ can be extracted from measurements. From the value of $[K_r]$ we can estimate the total number of potassium leakage per cell ($\alpha K_{in}$) for a specific combination of bacteriocin and sensitive bacteria (positive controls). The value of of $\alpha K_{in}$ is then used in (3.4) to predict $[K_r]$ and $\Delta V$ as the concentration of sensitive cells $[C_s]$ change in the sample. Alternatively, the value of $[C_s]$ can be estimated using the ISE system output voltage, the extracted $[K_r]$ and $\alpha K_{in}$, obtained here using the extraction algorithm presented in section 3.6.5.

### 6.3.2 Calibration Curve Using Commercial ISE

Assuming the commercial ISE is used as our ISE system, and the biosensor output at 10 minutes post BRE-addition is used for detection, we can obtain the system calibration curve using the measurements provided in Fig. 6.7. In these experiments the amount of $[C_B]$ is not constant and the commercial ISE is working close to its detection limit, hence non-linear region. Before each experiment, the ISE electrodes was calibrated using reference solutions, but the membrane is not new and it was used repeatedly. Fig. 6.8 shows the calibration curve for this system when using *E.coli* K12 cells and colicin A9.

![Calibration Curve](image)

Figure 6.8: Calibration curve of the sensor using commercial ISE as ISE system. Experiments were performed with 3 different concentrations of *E.coli* K12 cells and colicin A9 as BRE at 23.7°C.
The concentration of released potassium ions from the BRE-infected cells increases with time, and the ISE works closer to its linear region of the operation. As we approach the ISE linear region (Nernstian response), the slope of the calibration curve increases. If the concentrations of the released potassium ions are very much larger than the ISE detection limit, the slope of the calibration curve becomes close to Nernstian, which means the sensor output voltage changes by about 58 mV as the concentration of infected bacterial cells are multiplied by 10.

The ISE sensor with the PVC-based potassium-selective membrane has the detection limit of \(10^{-6}\)M potassium concentration [125, 115]. As it can be seen from Fig. 6.8, the minimum detectable concentration of bacterial cells providing a sensor output above zero is \(10^7\) cfu/ml.

6.3.3 ISE system Output Signal Over Time

In order to see how the shape of the ISE system output signal versus time can be approximated by the system model presented in Section 3.6, the system model parameters were first extracted from the measurement results using the algorithm presented in Section 3.6.5; then the system model output was generated and compared to the measurement results. An example of comparing the time domain measurement results with the system model output using the extracted system model parameters is shown in Fig. 6.9. The measurements shown in Fig. 6.9 were performed using the combination of \(E. coli\) K12 bacterial cells and colicin A9 at 23.7°C.

The values of the system model parameters, delay \(d\) and time constant \(\tau\), as introduced in Fig. 3.10 were determined for each experiment, to find the system time-domain dynamics. The extracted parameters are provided in Table 6.2. From Table 6.2 and the values of \(d\) and \(\tau\), for different combinations of bacterial cells and bacteriocins, the speed of infections can be compared. The potassium efflux rate of the combination of colicin A9 and \(E. coli\) K12 is the highest among all colicins tested, producing the lowest time constant, and the rate of efflux of colicin B and \(E. coli\) K12 combination is the slowest at 37°C.

System Model Predictability

In section 6.3.1, we showed how the system model parameter \([K_r]\) can be extracted from the measurements. For a fixed combination of bacteriocin and sensitive bacteria, the only other input parameter that affects the values of system parameters \(\tau\) and \(d\), is temperature. The predictability of the system model to capture the effect of temperature
Figure 6.9: Fitted response of the ISE to the system model in Fig. 3.10 using the algorithm presented in Section 3.6.5. The experiment was performed using an ISE system and a combination of E. coli K12 at a concentration of $3 \times 10^8$ cfu/ml with added colicin A9 at 23.7°C. The figure shows the response after the colicin A9 was added.

Table 6.2: Parameters extracted from the ISE response according to the system model presented in Fig. 3.10 using the algorithm presented in Section 3.6.5.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Bacteriocin</th>
<th>Cell OD</th>
<th>Temp. ($^\circ C$)</th>
<th>d (Min.)</th>
<th>$\tau$ (Min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli K12</td>
<td>colicin A9</td>
<td>0.8</td>
<td>23.7</td>
<td>2.63</td>
<td>7.44</td>
</tr>
<tr>
<td>E. coli K12</td>
<td>colicin A9</td>
<td>10X dilution of cells with OD of 0.8</td>
<td>23.7</td>
<td>2.44</td>
<td>9</td>
</tr>
<tr>
<td>E. coli K12</td>
<td>colicin A9</td>
<td>0.8</td>
<td>26.7</td>
<td>1.61</td>
<td>3.7</td>
</tr>
<tr>
<td>E. coli K12</td>
<td>colicin A9</td>
<td>0.7</td>
<td>30.0</td>
<td>1.54</td>
<td>3.57</td>
</tr>
<tr>
<td>E. coli K12</td>
<td>colicin A9</td>
<td>1.3</td>
<td>37.0</td>
<td>1.55</td>
<td>2.01</td>
</tr>
<tr>
<td>E. coli K12</td>
<td>colicin E1</td>
<td>1.29</td>
<td>37</td>
<td>1.18</td>
<td>2.38</td>
</tr>
<tr>
<td>E. coli K12</td>
<td>colicin B</td>
<td>1.29</td>
<td>37</td>
<td>1.77</td>
<td>2.9</td>
</tr>
<tr>
<td>E. coli K12</td>
<td>colicin K</td>
<td>1.1</td>
<td>37</td>
<td>0.53</td>
<td>1.47</td>
</tr>
<tr>
<td>S. aureus</td>
<td>lysostaphin</td>
<td>0.8</td>
<td>26.7</td>
<td>3.3</td>
<td>5.3</td>
</tr>
</tbody>
</table>

on the output sensor signal, is dependent on how well the system parameters $\tau$ and $d$ are modelled over temperature. In section 3.6.6, we introduced previous research that
has characterized the temperature sensitivity of the bacteriocin-induced potassium efflux from sensitive cells. In this section, we compare the extracted temperature sensitivity of the parameters $\tau$ and $d$ with the available literature to show our system predictive capability over different temperatures.

The temperature-sensitivity of the potassium efflux can be extracted from the data presented in Table 6.2. We expect to see that the initial rate of potassium efflux follows an Arrhenius plot for various temperatures when sensitive bacterial strains are exposed to a bacteriocin, as shown in [92] for colicin A9 and *E. coli* combination. Figure 6.10 shows the data points as well as the fitted Arrhenius line of $\ln(1/\tau)$, which is directly proportional to the logarithm of rate of efflux over the inverse of temperature, for the experiments involving *E. coli* K12 and colicin A9, according to the data shown in Table 6.2.

What can be observed from Fig. 6.10 as well as Table 6.2 is the decrease in system model time constant $\tau$, when the temperature increases, confirming the results in [92]. Temperatures below 23.7°C and temperatures above 37°C were not experimentally tested.

![Figure 6.10: Extracted $\ln(1/\tau)$ versus inverse of temperature and a least squares fitted Arrhenius plot. The values of $\tau$ were extracted from experiments involving colicin A9 and *E. coli* K12 in Table 6.2.](image)

Figure 6.11 shows the value of the extracted system model parameter $d$ vs. temperature. As shown in Fig. 6.11 the delay of the potassium efflux for a specific combination of bacteriocin and bacteria decreases as the temperature is increased toward 37°C, confirming the results in [92].
Chapter 6. Detection of Bacteria Using Bacteriocins

The shape of the graph of delay versus temperature is described as a piece-wise linear curve in [92] for the combination of colicin A9 and *E. coli*. In Fig. 6.11 we have fitted the delay vs. temperature curve to a piece-wise linear curve with a break point at 26.5°C, confirming the results in [92].

To show the system model predictability, two new sets of independent experiments were performed using the combination of *E. coli* BW25113 cells and colicin A9. Experiment 1 was performed at 37°C, and at the cell OD of 1.14, using the commercial ISE. Experiment 2 was performed at 27.3°C, and the cell OD of 0.9. The experimental protocols were the same as the ones used before in this chapter. The extraction algorithm, presented in section 3.6.5, was used to extract the system parameters $d$ and $\tau$. The extracted parameters were compared with the estimated values of $d$ and $\tau$ using the system model predicted values for $\tau$ and $d$ (see Fig. 6.10 and Fig. 6.11). Figure 6.12 shows $\tau$ and $d$ in one graph, extracted from measurement results of Experiment 1 and Experiment 2, and the estimated $\tau$ and $d$ using the system model. This figure shows the system model can reliably predict the system behaviour at multiple temperatures.

From the parameters extracted in Table 6.2 and the estimated system parameters, as well as the algorithm for estimating the final $\alpha K_m$, the system output signal can be estimated at various concentrations of sensitive bacterial cells and multiple temperatures. The same measurement, extraction and modelling technique can be used for other bacteriocin and bacteria combinations to estimate the bacterial sensor output signal.

Figure 6.11: Extracted delay versus temperature. The delay is extracted from experiments involving colicin A9 and *E. coli* K12 according to Table 6.2.
Chapter 6. Detection of Bacteria Using Bacteriocins

Figure 6.12: Estimated values of system parameters, delay $d$ and time constant $\tau$ and the extracted parameters from independent measurements at $37 \, ^\circ C$ and $23.7 \, ^\circ C$ (Experiment 1 and Experiment 2). The independent experiments were performed using the *E. coli* BW25113 and colicin A9. The OD of the cells were 1.14 and 0.9 in Experiment 1 and Experiment 2 respectively.

6.4 CMOS Chip Preparation

The second CMOS prototype IC, as explained in Chapter 4, was used for all the experiments in this chapter. We encapsulated the packaged CMOS integrated circuits with epoxy to cover the bond wires and to provide electrical insulation between the sample solution and the surface of the CMOS integrated circuit. A printed circuit board, which connects to a computer through a data acquisition board and USB port (Fig. 4c), was used to create an electronic platform to configure the microchip and provide sample readout. The biosensor integrated circuit was mounted through a socket that connected the electrical components of the CMOS integrated circuit with the switches and buffers on the printed circuit board. The data acquisition board digitizes the signal outputs from the configurable ISFETs on the CMOS integrated circuit and sends the digitized signals to a PC through a USB cable for real-time visualization. Fig. 6.13 shows the experimental setup using the encapsulated chip. The packaged CMOS IC that is encapsulated has top aluminum metal electrodes that are exposed on top and are internally connected to floating gates of the ISFET transistors in the CMOS IC. In order to build potassium-sensitive electrodes, a potassium sensitive PVC mixture dissolved in THF was prepared.
Figure 6.13: System components from fabricated CMOS IC to the test setup connection to PC. (a) Micrograph of the fabricated CMOS IC in 0.13µm IBM CMOS technology. CMOS IC electrodes are shown in the middle of the die, where they are connected to ISFET readers and controllers. (b) The packaged CMOS IC in a 69-pin PGA package. The bond wires are encapsulated with epoxy, leaving the CMOS IC electrode area exposed. A potassium-selective membrane is deposited on the exposed CMOS IC area. The sample liquid is in contact with the potassium-selective membrane deposited on top of the CMOS IC. (c) The system setup that mounts the packaged CMOS IC on a test PCB which connects to a PC.
Chapter 6. Detection of Bacteria Using Bacteriocins

first as explained in Appendix A. The encapsulated chip was cleaned and prepared using the protocol explained in Appendix A.

6.5 Experimental Results Using the CMOS Chip

We used a combination of two bacteriocins to differentiate between pathogens frequently identified in urinary tract infections. Fresh cultures of *E. coli*, *S. aureus* and *P. aeruginosa* were each prepared, as explained in Appendix A, and resuspended in SM buffer at an OD of 0.8 (corresponding to a density of $3 \times 10^8$ cfu/mL for *E. coli* K12 BW25113 and *Staphylococcus aureus* 8325. The concentration of *Pseudomonas aeruginosa* (PA01) was found to be $4 \times 10^8$ cfu/ml at an OD of 0.8). For each experiment, 100µl was applied to the biosensor sample chamber. The sample was allowed to equilibrate for a period of 5 minutes to allow us to characterize the ISFET baseline variations and drift. Subsequently, 10µL of colicin A9 or lysostaphin was mixed with the sample, and potassium efflux was monitored for 20 minutes. Figure 6.14 shows the experimental setup and the expected output for each combination.

![Figure 6.14: Representation of the experiments performed on CMOS IC. Each experiment consists of a combination of one bacteriocin and one bacterial strain. Two bacteriocins (colicin A9 and lysostaphin) and three different bacterial samples are employed for a total of 6 experiments. The outcome of the experiment is either positive (increase in the potassium concentration in the sample), or negative (no potassium variations).](image)

The measurement outputs were provided by multiple channels on the chip and were processed to remove DC baselines and perform drift compensation. As can be seen in Fig. 6.15, the output voltages of the ISFETs began to rise when the bacterial strain in the sample was sensitive to the bacteriocin. For example, approximately 3 minutes after the
Figure 6.15: CMOS IC detection of 3 different bacteria strains using bacteriocins. The panels show the CMOS IC output signal from one of the available electrodes through a complete cycle of potassium efflux at 26.7°C. *S. aureus* + lysostaphin and *E. coli* + colicin A9 are expected positive results.
addition of lysostaphin to the \textit{S. aureus} sample, we observed a steady increase in output voltage that peaked 20 minutes after mixture. Similarly, when colicin A9 was mixed with \textit{E. coli}, there was an increase in potassium detected after approximately 5 minutes, and the signal continued to increase over 25 minutes. The \textit{P. aeruginosa} control sample, which is insensitive to both lysostaphin and colicin A9, did not provide any significant signal in this assay. The bacteriocin-mediated efflux of potassium ions was monitored by multiple channels on the CMOS microchip, and the ISFETs each converted the changes in potassium concentration to an output voltage. These experiments were performed at room temperature and provide conclusive outcomes without the need for heating to 37°C.

While the bacteriocin-mediated potassium efflux could be detected for up to 25 minutes when the experiment was performed at room temperature, the positive and negative outcomes could be read within 10 minutes without biasing the result, to increase the detection speed. Figure 6.16 shows the CMOS ISFET outputs of an array of electrodes after DC baseline removal and drift compensation. The bars show the average of the outputs measured from multiple electrodes (a minimum of 4 of four from the 6 available channels as described in Chapter 4) on the IC for 10 minutes after mixing the bacteriocin with the sample containing bacteria. Error bars show the standard deviation among the electrodes. We previously showed that bacteriocin-mediated potassium efflux begins more quickly and exhibits higher potassium efflux rates at 37°C than room temperature, thus, the integration of temperature control in the biosensor will allow for faster detection of bacteria; still the system capability of providing outcomes in 10 minutes at room temperature without complicating the design and power requirements for on-chip or external heating demonstrates the versatility of the system.
Figure 6.16: CMOS IC detection and identification capability in 10 minutes using the outputs of the array of electrodes after DC baseline removal and drift compensation at 26.7°C. The baseline and drift calibration has been performed using the IC signal for 1 minute prior to the addition of bacteriocin. The compensated and processed outputs are depicted for 10 minutes post-infection to provide positive/negative outcome. Error bars are computed from measurements taken from multiple electrodes (minimum of 4 out of 6 available electrodes).
6.5.1 CMOS Biosensor Specifications at 10-Minute Detection Time

Table 7.1 shows the sensor output specifications when detecting *E. coli* and *S. aureus* at 10 minutes after bacteriocin addition. Maximum standard deviation of the sensor output across multiple electrodes on each chip and the total mean and standard deviation of the sensor output across all tested chips are shown in Table 7.1. All experimental protocols and parameters are the same as section 6.5.

<table>
<thead>
<tr>
<th>Bacteria and Bacteriocin types</th>
<th><em>E. coli</em> and colicin A9</th>
<th><em>S. aureus</em> and lysostaphin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial concentration (cfu/ml)</td>
<td>$3 \times 10^8$</td>
<td>$3 \times 10^8$</td>
</tr>
<tr>
<td>Detection time (Min.)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Sensor output average (mV)</td>
<td>9.60</td>
<td>12.2</td>
</tr>
<tr>
<td>Standard deviation across multiple sensors (mV)</td>
<td>5.11 [8]</td>
<td>4.54 [10]</td>
</tr>
</tbody>
</table>

6.5.2 Comparison to State-of-the-Art

We designed a new type of bacterial biosensor using a new combination of biological detection elements and ISFETs as transducers. The bacterial biosensor was not specifically optimized for low sensitivity/detection limit, but the detection time, specificity, ease of sample preparation, cost were considered the main objectives of the design. Further optimization of the membrane can enhance the sensor detection limit. Table 6.4 shows the comparison of this work as bacterial biosensor with available state-of-the-art bacterial biosensors that use electrochemical transducers.

In Table 6.4, reference [62] uses phages for bacterial detection where the recognition event transducer is the lysis of the cell after multiple bacterial cell division and phage production. This technique needs actively growing bacterial cells. It is also slow even at high concentration of bacteria because of the bacterial growth and multiplication required.
<table>
<thead>
<tr>
<th>Bacteria type</th>
<th>BRE</th>
<th>Integration</th>
<th>Transducer</th>
<th>Power</th>
<th>50µW</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli, S. aureus</td>
<td>Phage, Bacteria</td>
<td>PDMS-microfluidic</td>
<td>PDMS-microfluidic</td>
<td>CMOS</td>
<td>(ISFET)</td>
</tr>
<tr>
<td>E. coli, S. aureus</td>
<td>Phage, Bacteria</td>
<td>Impedance-based</td>
<td>Impedance-based</td>
<td>VersaSTAT 250W</td>
<td>Ext. potentiostat</td>
</tr>
<tr>
<td>E. coli, S. aureus</td>
<td>Phage, Bacteria</td>
<td>Antibody on DNA</td>
<td>Antibody on DNA</td>
<td>CH700 potentiostat</td>
<td>VersaSTAT 250W</td>
</tr>
<tr>
<td>E. coli, S. aureus</td>
<td>Phage, Bacteria</td>
<td>Multiplication, membrane</td>
<td>Multiplication, membrane</td>
<td>External potentiostat</td>
<td>Ext. potentiostat</td>
</tr>
<tr>
<td>E. coli, S. aureus</td>
<td>Phage, Bacteria</td>
<td>Antibody on polystyrene tubes</td>
<td>Antibody on polystyrene tubes</td>
<td>VersaSTAT 250W</td>
<td>Ext. potentiostat</td>
</tr>
<tr>
<td>E. coli, S. aureus</td>
<td>Phage, Bacteria</td>
<td>External potentiostat</td>
<td>External potentiostat</td>
<td>External potentiostat</td>
<td>External potentiostat</td>
</tr>
<tr>
<td>E. coli, S. aureus</td>
<td>Phage, Bacteria</td>
<td>Square-Wave</td>
<td>Square-Wave</td>
<td>VersaSTAT 250W</td>
<td>Ext. potentiostat</td>
</tr>
<tr>
<td>E. coli, S. aureus</td>
<td>Phage, Bacteria</td>
<td>Impedance-based</td>
<td>Impedance-based</td>
<td>VersaSTAT 250W</td>
<td>Ext. potentiostat</td>
</tr>
<tr>
<td>E. coli, S. aureus</td>
<td>Phage, Bacteria</td>
<td>Antibody on DNA</td>
<td>Antibody on DNA</td>
<td>VersaSTAT 250W</td>
<td>Ext. potentiostat</td>
</tr>
<tr>
<td>E. coli, S. aureus</td>
<td>Phage, Bacteria</td>
<td>Multiplication, membrane</td>
<td>Multiplication, membrane</td>
<td>VersaSTAT 250W</td>
<td>Ext. potentiostat</td>
</tr>
<tr>
<td>E. coli, S. aureus</td>
<td>Phage, Bacteria</td>
<td>Antibody on polystyrene tubes</td>
<td>Antibody on polystyrene tubes</td>
<td>VersaSTAT 250W</td>
<td>Ext. potentiostat</td>
</tr>
<tr>
<td>E. coli, S. aureus</td>
<td>Phage, Bacteria</td>
<td>External potentiostat</td>
<td>External potentiostat</td>
<td>VersaSTAT 250W</td>
<td>Ext. potentiostat</td>
</tr>
<tr>
<td>E. coli, S. aureus</td>
<td>Phage, Bacteria</td>
<td>Square-Wave</td>
<td>Square-Wave</td>
<td>VersaSTAT 250W</td>
<td>Ext. potentiostat</td>
</tr>
<tr>
<td>E. coli, S. aureus</td>
<td>Phage, Bacteria</td>
<td>Impedance-based</td>
<td>Impedance-based</td>
<td>VersaSTAT 250W</td>
<td>Ext. potentiostat</td>
</tr>
<tr>
<td>E. coli, S. aureus</td>
<td>Phage, Bacteria</td>
<td>Antibody on DNA</td>
<td>Antibody on DNA</td>
<td>VersaSTAT 250W</td>
<td>Ext. potentiostat</td>
</tr>
</tbody>
</table>

Table 6.4: Electrochemical Bacterial Biosensor Performance Summary and Comparison
6.6 Summary

This chapter demonstrates a rapid, accurate and reproducible assay that can be modified to detect any bacterial strain. The selection of bacteriocins as the detection element provides a simple biological probe, yet also provides a broad spectrum of reagents for use in detecting bacteria commonly found in human infections.

This chapter summarized the experimental results obtained using bacteriocins for bacterial detection and identification. We provided initial results using a commercial ISE, followed by results provided by the ISFETs realized on our second CMOS test chip. We extracted model parameters from the ISE measurement results according to the system model parameters described in Chapter 3, and showed system model predictive capability by comparing estimated system model parameters with measurement results.
Chapter 7

Biosensor Application for Antibiotic Susceptibility Testing

Antibiotics are agents that kill or inhibit the growth of bacteria and fungi [22]. In this thesis, they are exclusively referred to as antibacterial agents. Antibiotics are either natural, semi-synthetic or synthetic products [21]. They have varying selectivity ranges towards bacterial species. Antibiotic susceptibility testing (AST) refers to testing the sensitivity of certain bacteria to a target antibiotic. AST is particularly important in battling the emerging antibiotic resistance of pathogens by allowing physicians to prescribe the appropriate antibiotics to patients. Furthermore, AST helps researchers test the efficacy of their antibiotics towards various target bacteria and development of new types of antibiotics.

In this chapter, a brief introduction to the categories of antibiotic and their distinct modes of operation will be discussed. We then discuss our target class of antibiotics used in this work and will provide background on experimental designs using these antibiotics. The biological protocols and techniques used in our system are discussed followed by experimental results using a commercial ion-selective electrode system and our CMOS test chip.

7.1 Antibiotic Categories and Resistance

Antibiotics are divided into two broad categories: bactericidal and bacteriostatic [22]. Bactericidal antibiotics kill their target bacteria while bacteriostatic antibiotics slow down or stop the growth of their targets. Another way of classifying the antibiotics is based on their specific mode of action, which can include disruption of the cell membrane, protein synthesis, etc. [21].
Antibiotic resistance is the ability of a bacterial strain to survive exposure to one or more categories of antibiotics to which the strain has shown sensitivity before or in other environmental conditions. There are two general mechanisms of antibiotic resistance. Inherited resistance results from mutation of the genes of the existing bacterial population after exposure to antibiotics or after the acquisition of external resistance-encoding genes [159]. When a population of sensitive strains plus resistant strains of bacteria are exposed to an antibiotic, the sensitive cells are killed leaving the resistant bacteria with abundant nutrition to reproduce rapidly, resulting in rapid growth of resistant bacteria [159]. Inherited resistance is very common and rapid in hospital settings where a variety of bacteria and antibiotics are present. Non-inherited resistance occurs when the bacteria are unresponsive to an antibiotic in certain situations (e.g. low nutrients) even if they are genetically susceptible to it.

Antibiotics are usually designed and further tested (AST) against fast-growing bacteria where both bactericidal and bacteriostatic categories have the most effect on treatment [160]. Current AST techniques rely on culturing techniques where bacteria are grown on a nutritious medium and antibiotics are added to find their efficacy [23]. In the fast phase (log-phase) of bacterial growth, there are abundant nutrients for growth of the bacteria and both categories of antibiotics have the optimal chances of disrupting the functionality of the target bacteria. Many persistent infections, however, are caused by bacteria that are slow-growing or non-growing [161], such as where bacteria are not in the fast growth phase or there are not enough nutrients for growth, and the bacteria have gained non-inherited resistance toward the antibiotic. Testing antibiotics for non-inherited resistance can only be achieved by examining their potential to kill slow-growing and non-growing bacteria where antibiotics kill their target without relying on bacteria growth.

Historically, the most widely used antibiotics are bacteriostatic [21]. However, the global problem of advancing antimicrobial resistance has led to a renewed interest in the use of bactericidal antibiotics as the last resort antibiotics because of their potential to kill slow-growing and non-growing bacteria. In this thesis, we focus on bactericidal antibiotics that have the potential to kill both growing, slow-growing and non-growing bacteria targets.

### 7.2 Model Antibiotics

Examples of currently-available antibiotics that can be used in our sensor system are polymyxins, gramicidin, colistins, vancomycin and neomycin. These are bactericidal antibiotics that induce cell lysis by membrane disruption, an effect that can be mea-
sured by the measuring the release of internal ions of the target bacteria. We have utilized this method in the previous chapters for bacterial detection and identification. Efforts on membrane-active bactericidal antibiotics have gained new attention recently with examples including daptomycin (2003), telavancin (2009) and other antibiotics under development like DCAP [161] and HT61 [162].

In this thesis, we used polymyxin B (PMB) as the model antibiotic for our experiments. Polymyxins are a group of bactericidal antibiotics with a cyclic peptide structure [163]. They generally act against Gram-negative bacteria by binding to the outer membranes of their targets and disrupting both the outer membrane and inner membrane, resulting in an efflux of the internal contents of the target cells and, finally, lysis and death [164]. PMB has been used clinically to treat urinary tract infections, meningitis and blood stream infections caused by susceptible strains of *Pseudomonas aeruginosa.*

We used a commercial ISE system, as well as a test CMOS IC, to determine the efficacy of PMB on two different bacterial strains suspended in different media. These media keep the target bacteria alive without providing nutrition for growth. This test structure ensures an environment for testing antibiotic susceptibility that does not rely on growth of the bacteria.

### 7.3 Experimental Design using Polymyxin B (PMB) Antibiotics

In this section, the initial plating assays were performed to test the susceptibility of different *E. coli* strains. Based on these initial assays, control experiments for PMB AST were designed. Initial measurements of the potassium efflux from control experiments were performed using a commercial ISE.

Polymyxin B sulfate was purchased from Sigma Aldrich, P1004, and was dissolved at 1mg/ml in deionized water, filter-sterilized and kept at $-20^\circ C$ for long-term storage. For regular usage within 1 month, the solution was stored at $4^\circ C$ and used repeatedly. Only very small volumes ($1 - 10\mu l$) of this solution were used in the experiments to provide the final concentration of PMB as specified for each experiment. The bacteria used in each experiment were prepared as explained in Appendix A.

PMB is active against a broad spectrum of Gram-negative bacterial cells. As described in previous chapters, the experiments were designed to have both positive and negative control experiments to provide conclusive evidence of system functionality using at least two different strains of bacteria. Positive control experiments used *E. coli* K12 BW25113
For negative control experiments, the goal was to find species resistant to PMB that could be used in a level 1 laboratory. Since PMB is active against most Gram-negative bacteria and most of the available Gram-positive bacteria are level 2 and above, another approach was used. As was mentioned in the previous section, testing the efficacy of antibiotics should not be solely performed in a medium where all nutrients are present. Antibiotic efficacy against different bacteria strains depends on the media where the bacteria cells are residing. A combination of available *E. coli* strains and several different media, including SM, PBS, N-minimal and LB were tested to see the efficacy of PMB. See Appendix A for experimental protocols and the compositions of these media. In these experiments, each bacterial strain was grown in LB, and the cells were suspended in the chosen media (LB, SM, PBS, etc). PMB, with a total final concentration of 20µg/ml, was added to one batch of suspended cells, while the other batch was incubated in the suspension media without PMB. The mixtures were incubated at 37°C for 20 minutes. Each batch was washed and resuspended again in suspension media to get rid of the residual PMB, then small aliquots of dilutions of each batch were plated using LB plates and incubated overnight at 37°C. The viable bacterial colonies on each plate were counted the next morning. The number of colonies of bacteria that survived PMB in the PMB-added batch were compared with the number of present bacteria in the equivalent batch without PMB. The resulting ratio shows the survival rate of the specific strain in the specific media to the PMB.

PMB exposure resulted in a 10⁶-fold reduction in the number of viable *E. coli* K12 BW25113 Δ*fhuA* bacteria cells suspended in SM compared to the batch without PMB. As for *E. coli* K12 BL21 (DE3 Δtail) bacteria cells, half the cells exposed to 20µg/ml of PMB survived when the cells were suspended in SM medium. This confirms the susceptibility of BW25113 cells and resistance of BL21 cells to PMB. In additional experiments, the same conclusion was not obtained for other suspension media including LB and PBS. In fact BL21 cells were very susceptible to PMB in LB (LB is a nutritious medium) and N-minimal media and they showed some degree of resistance in PBS.

BL21 was the most resistant to PMB in SM with 50% survival rate, so the combination of *E. coli* K12 BL21 (DE3 Δtail) bacteria suspended in SM plus PMB, was chosen as the negative control. Figure 7.1 shows the cultured plates of both our positive and negative control bacteria, with and without added PMB, after 100 times dilution of the sample. As can be seen on the right side both of the plates are full of colonies of bacteria. On the left side where PMB was added, the positive control cells (Keio WT is *E. coli* K12 BW25113 Δ*fhuA*) are completely killed and no colonies can be seen, whereas for negative control
cells (BL21 is *E. coli* K12 BL21 (DE3 Δ*tail*) colonies are present.

![Image](image1.png)

Figure 7.1: Results of plating of both (a) *E. coli* K12 BW25113 Δ*fhuA* (Keio WT) and (b) *E. coli* K12 BL21 (DE3 Δ*tail* (BL21). For each panel the right side is the plating of the sample bacteria alone. On the left side, PMB has been added to the sample suspended in SM. The panel (a) on the left side shows no bacteria is present because of bacterial sensitivity to PMB. On the left side of panel (b), there are many colonies of BL21 bacteria that have survived PMB treatment.

Figure 7.1: Results of plating of both (a) *E. coli* K12 BW25113 Δ*fhuA* (Keio WT) and (b) *E. coli* K12 BL21 (DE3 Δ*tail* (BL21). For each panel the right side is the plating of the sample bacteria alone. On the left side, PMB has been added to the sample suspended in SM. The panel (a) on the left side shows no bacteria is present because of bacterial sensitivity to PMB. On the left side of panel (b), there are many colonies of BL21 bacteria that have survived PMB treatment.

After confirming the results of positive and negative control experiments using the procedure described above, we performed the experiments using a commercial ion-selective electrode (ISE) system. Researchers [164] have shown the efflux of potassium ions followed by the addition of different concentrations of PMB to *E. coli* cells to find different stages of interaction of PMB with the cell envelope. In this thesis, experiments were performed using the above positive and negative control cells suspended in SM. Figure 7.2 shows the experimental procedure and expected output results of each experiment.

Figure 7.3 shows the output results of one positive control and one negative control experiment using the commercial ISE system. Bacteria cells had OD of 1.1 when they
Figure 7.2: Experimental procedure and expected output for mixing 2 different strains of *E. coli* with PMB.

![Diagram showing experimental procedure](image)

Figure 7.3: Experimental results using ISE with both combinations of positive and negative control at 37°C. PMB is added to the mixture at t= 10 minutes.

![Graph showing ISE output](image)

were grown in LB and then were further resuspended in SM for measurements. The outputs shown are drift-compensated and the DC baseline was removed as explained in the previous chapters. Since both bacteria cells and the commercial ISE probe need to reach the water bath temperature of 37°C, they are left in the chamber for 10 minutes. The results are shown after the first 5 minutes. PMB was added at the 10 minute
mark as shown in Fig. 7.3. When performing our experiments, no significant potassium efflux was observed at temperatures below 37°C from sensitive positive control bacteria suspended in SM. The temperature experiments were performed at both 30°C and a room temperature of 24°C and none of them showed any significant leakage. Temperatures above 37°C were not tested. Experiments involving Gram-positive bacteria were also not performed, thus we do not have experimental results showing a negative outcome when utilizing Gram-positive bacteria with PMB since the available Gram-positive cells needed level 2 lab clearance.

After confirming the capability of the system for antibiotic testing using both plating and ISE methods, we then utilized the second CMOS IC, as explained in chapter 4, to perform the tests.

7.4 Measurement Results Using CMOS Chip

The CMOS chip (test chip 2) used in these experiments were the same as the ones used for the colicin experiments described in Chapter 6. The experiments in Chapter 6 were performed at room temperature (with no extra heating required). Since PMB did not produce any significant positive potassium efflux at temperatures below 37°C, the temperature control setup used in Chapter 5 for bacteriophage experiments was utilized: a heater was fixed under the PCB board and the setup was contained in a closed container with a temperature sensor to make sure the temperature stayed constant at 37°C.

The chips were cleaned and the potassium-sensitive membrane was deposited on top of the chips using the protocol in Appendix A. The chips have multiple electrodes exposed. In order to confirm the chip capability to detect positive and negative control experiments and to provide conclusive results of PMB sensitivity, the same experiments were repeated using the commercial ISE. The experimental procedure was the same except the sample volume of bacteria cells in each experiment was 100 µl in the chip experiments (compared to 4ml sample volume in ISE measurements). The final concentration of the PMB added to the sample was also the same, i.e. 20µg/ml. Figure 7.4 shows the results of both positive and negative controls for one electrode from the addition of PMB until 25 minutes later. The results are again drift-compensated and DC baselines were removed as explained in Chapters 3, 5 and 6. As can be seen from the results in Fig. 7.4, the output of the positive control experiment starts to rise after the addition of PMB, while the negative control output stays almost the same (and even falls). The initial signal decrease after PMB exposure are due to slow mixing of the sample with the PMB.

For detection purposes, the first 10 minutes after the addition of PMB is enough to
Figure 7.4: Experimental results using the CMOS test chip with both combinations of positive and negative control at 37°C. PMB is added to the mixture at the 10 minute mark.

provide conclusive positive or negative results. Figure 7.5 shows the chip voltage output for the first 10 minutes after the addition of PMB across multiple electrodes that were exposed to the mixture. The rectangular bars show the average among the electrodes (4 to 6 electrodes and their respective ISFETs and ISFET readout circuits) while the error bars show the standard deviation across the electrodes. Figure 7.5 shows the detection capability of the system using the CMOS chip over less than 10 minutes.

Figures 7.4 and 7.5 show how this system can be used to identify the antibiotic efficacy for a specific pore-forming antibiotic in a very short time. The goal of the system is not identification of the bacteria present in the sample, but identifying antibiotics that provide a strong killing ratio for the bacteria present in the sample. Since the volume needed for experiments is small (100 µl), the bacteria in the sample can be concentrated using centrifugation and be resuspended in clean media to increase the concentration of the released K+ form bacteria, above the system detection threshold. Utilizing this system for antibiotic discovery and efficacy testing can be inexpensively
Figure 7.5: Detection capability of the CMOS chip that provides conclusive results in less than 10 minutes after the addition of PMB across multiple electrodes. Error bars are determined from measurements from 4 to 6 exposed electrodes.

and easily achieved using small samples of known bacteria and antibiotics.

7.4.1 CMOS Biosensor Specifications at 10-Minute Testing Time

Table 7.1 shows the sensor output specifications when the sensor output is used for detection at 10 minutes after antibiotic addition. Maximum standard deviation of the sensor output across multiple electrodes on each chip and the total mean and standard deviation of the sensor output across all tested chips are shown in Table 7.1. All experimental
protocols and parameters are the same as section 6.5.

Table 7.1: CMOS biosensor specifications at 10-minute detection time for antibiotic testing

<table>
<thead>
<tr>
<th>Bacteria and Antibiotic</th>
<th><em>E. coli</em> K12 BW25113 and Polymyxin B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial concentration (OD600)</td>
<td>1.1</td>
</tr>
<tr>
<td>Detection time (Mins.)</td>
<td>10</td>
</tr>
<tr>
<td>Sensor output average (mV)</td>
<td>19.49</td>
</tr>
<tr>
<td>Maximum standard deviation across multiple electrodes on a single chip (mV)</td>
<td>4.7 [4]</td>
</tr>
<tr>
<td>[No. of observations]</td>
<td></td>
</tr>
<tr>
<td>Standard distribution across multiple sensors (mV)</td>
<td>8.5 [8]</td>
</tr>
<tr>
<td>[No. of observations]</td>
<td></td>
</tr>
</tbody>
</table>

7.5 Summary

Our motivation to provide a fast and reliable antibiotic susceptibility testing platform is for correct antibiotic prescription to combat emerging antibiotic resistance as well as to aid the development of new antibiotics. In this chapter, the technique that was utilized in previous chapters for bacteria detection and identification, was modified to provide fast reliable antibiotic susceptibility test (AST) using PMB. This technique can be utilized for other membrane-active bactericidal antibiotics, such as gramicidin, colistins, vancomycin, etc.

To provide proof of system capabilities the following experiments were designed and performed and measurement results were presented. First, traditional culturing techniques were used to find the experimental bacterial samples for our chosen model antibiotic (PMB). Further testing with a commercial ISE system confirmed the efficacy of our technique for the chosen model antibiotic. Finally, we used the second prototype CMOS IC and electronic platform to perform the AST test on two different strains of *E. coli*. The experimental results provide conclusive susceptibility results in less than 10 minutes.

This technique provides a new approach for rapid AST testing for diagnostics to help with antibiotics stewardship and the development of next generation antibiotics.
Chapter 8

Contributions and Future Work

This thesis presented a novel design for a bacterial detection and identification system. The emphasis of the design was to develop a fast, reliable technique with a selection of biological components, and the optimization of protocols to allow for detection and identification of bacteria with high specificity and sensitivity. The system design was based on harnessing the low-cost, low-power and manufacturability offered by standard CMOS fabrication technology.

8.1 Contributions

The following are the contributions of this thesis:

1. Development of a Biological Technique for Rapid and Specific Detection and Identification of Bacteria

We investigated the selection of biological recognition elements (BREs) for bacterial identification and their specific recognition events enabling use of low-cost, specific BREs that were shown to provide rapid transducing events, detectable by CMOS electrochemical sensors. The following BREs were selected and tested:

- Bacteriophages were chosen as biological recognition elements because they are low-cost, naturally occurring and easy to produce. They infect their targets specifically and produce a rapid efflux of potassium ions detectable by a potassium-selective ISFET electrical transducer.

  Bacteriophages λ and T6 were tested with different strains of bacteriophage-sensitive and bacteriophage-insensitive E. coli, allowing for accurate identification of specific strains in less than 10 minutes.
To the best of our knowledge, we are the first to combine the usage of bacteriophages with CMOS ISFETs to create a rapid, integrated system for the detection and identification of bacteria.

- Membrane-active bacteriocins were also chosen as biological recognition elements. They are simple, naturally occurring proteinaceous structures that are extremely low-cost and easy to produce, and specific to their targets. They also produce a rapid efflux of potassium ions detectable by a potassium-selective ISFET electrical transducer.

Multiple *E. coli*-active bacteriocins, called colicins (colicins A9, B, E1 and K) as well as the bacteriocin lysostaphin, were used. The bacteriocins were tested against strains of Gram-negative *E. coli* and *Pseudomonas aeruginosa*, and Gram-positive *Staphylococcus aureus*.

To the best of our knowledge, we are the first to combine the usage of bacteriophages with CMOS ISFETs for the production of a rapid, integrated system for the detection and identification of bacteria.

We designed experimental protocols, including positive and negative control experiments, to test the capabilities of these techniques for bacterial detection and identification using both traditional culturing techniques and the presented bacteria sensor system.

Associated with the experimental design, we designed and optimized the buffers, biological protocols and sample handling assays for the fast and reliable usage of the above BREs for bacterial detection and identification in our system.

2. CMOS Circuit Design and Membrane Preparation

Two CMOS prototypes were designed, implemented and tested for bacterial detection and identification. They included potassium-sensitive ISFETs and their readout circuits. CMOS implementation was chosen because of the small sample volume realized as well as the low power, monolithic integration potential in CMOS fabrication. The two prototypes (one in TSMC 0.18µm CMOS and one in IBM 0.13µm CMOS) were successfully tested utilizing the technique developed for the identification of bacteria using the above BREs.

We investigated the circuit design parameters that affect bacterial sensor performance by testing the prototypes. We successfully investigated the direct attachment of a potassium-sensitive membrane on top of CMOS aluminum electrodes to enhance ISFET potassium sensitivity, relative to the membrane-on-passivation attachment in conventional CMOS ISFETs. Optimizing the membrane composition as well as membrane
attachment to the ISFET electrodes affect the minimum detection limit of the bacterial sensor and improves its noise performance.

3. Signal Processing Technique for Accurate Detection of Bacteria and Estimation of Sensor Output

Measurement protocols were developed and a processing technique was designed to compensate for ISFET DC offset and drift and to provide accurate system output. The proposed detection technique relies on detection of changes in sample potassium ion concentration ($\Delta pK$) in the sample as opposed to the absolute potassium concentrations; hence use of ISFETs without detrimental effects on the system output accuracy, are possible using the proposed simple processing technique.

4. Development of a Behavioural System Model

We developed a behavioural system model for the developed bacterial identification system. We investigated the biological, chemical and sample-specific parameters that affect the output of the bacteria sensor and proposed a predictive model that captures the effect of these parameters including temperature, BRE, bacteria species, sample buffer and ISE system calibration curve.

We extracted the system model parameters from experimental measurement results, providing an accurate predictive model for system design and characterization. The system model output was successfully compared against the measurement results for both systems using bacteriophages and bacteriocins.

5. Application: Development of a System for Antibiotic Susceptibility Testing (AST)

We extended the system used for bacterial detection and identification to test for the susceptibility of bacteria to membrane-active (pore-forming) antibiotics. To the best of our knowledge, this is the first time ASTs are performed using CMOS ISFETs to detect the rapid potassium efflux from bacteria upon exposure to pore-forming antibiotics. No active growth of sample bacteria is required for this test to work; hence the AST can be utilized successfully in samples that contain dormant bacteria.

We have designed and optimized buffers and biological protocols as well as experimental procedures to perform AST, providing rapid, reliable results using low sample volumes. The system was successfully tested using the antibiotic polymyxin B (PMB)
with two strains of Gram-negative *E. coli*. The implemented system provides conclusive evidence of the effectiveness of PMB against sensitive strains in less than 10 minutes.

### 8.2 Publications

The following peer-reviewed publications have been published during the course of this research:


Chapter 8. Contributions and Future Work

8.3 Future Work

The research presented in this thesis provides multiple avenues of investigation for design of improved bacterial sensor systems as well as systems performing AST. The systematic approach for design of bacterial sensors, introduced in this thesis, provides opportunities for further investigation and optimization of the performance specifications of each of the system blocks for better performance and applicability.

8.3.1 Biological Recognition Elements

We have investigated two categories of BREs in this thesis, but only a few from each category were investigated and tested. An immediate extension of this work is to systematically select and test available bacteriophages and bacteriocins against bacterial strains of interest in medical diagnostics.

8.3.2 Multiple Detection and Identification on a Single CMOS Chip

In this thesis, we have focused on the detection of a single strain of bacteria by mixing the sample with the appropriate BRE that infects it. In order to detect and identify multiple strains at once, a selection of BREs can be immobilized on a CMOS chip, each on a separate electrode, to provide detectable signals specific to multiple strains of bacteria simultaneously. This configuration would provide a single platform capable of identifying multiple strains of bacteria of interest very rapidly, at low lost.

The simple protein architecture of bacteriocins allows them to be recombinantly produced and easily engineered in bacterial systems [81]. These features permit bacteriocins with desirable modifications to be engineered. Modifications could include the addition of immobilization domains or the creation of multifunctional domain fusions [85]. This, in turn, will allow the development of ultra-small bacterial typing systems in which a number of bacteriocins with differing specificities are immobilized on independent electrodes of the CMOS biosensor, providing an all-in-one bacterial detection device.

8.3.3 Integration of ADC and Processing Unit

An immediate extension of the implemented IC is the addition of an ADC. As explained in Chapter 4, the system requirements for the ADC are very modest, and extremely low-power ADCs can be integrated on chip.
An important requirement for the system is a processing unit that cancels the effect of the DC baseline and the drift of the ISFET system. The integration of an analog processing unit before the ADC should be considered for this purpose. The integration of a simple processing unit before the ADC offers an opportunity to significantly reduce the dynamic range requirements on the ADC.

8.3.4 Membrane Optimization

The minimum concentration of bacteria detectable by the bacterial sensor depends on the minimum potassium concentration detectable limit of the potassium-selective membrane, as well as the number of interfering ions in the sample. Research into the design and use of potassium-selective membranes with low detection limits can enhance system sensitivity, and enhance the bacterial concentration detection limit. Research into the design of optimized buffers with low concentrations of interfering ions, that provide enough nutrients for bacteria to survive will also improve the detection limit.

8.4 Summary

We have proposed and implemented a system for bacterial detection and identification that provides fast, reliable identification results while consuming low sample volumes. The developed techniques presented in this thesis could significantly advance bacterial detection systems, enabling selective, sensitive, low-cost and portable systems that can be used for fast diagnosis of bacterial infections. Future research in this field can further improve infectious disease control and treatment.
Appendix A

Protocols

A.1 Buffers and Media

A.1.1 Lennox Broth (LB)

Lennox Broth (LB) was prepared from a pre made Lennox Broth mixture (10g/L Tryptone, 5 g/L Yeast Extract, 5 g/L NaCl) that was purchased from Sigma Aldrich (product number: L3022). 20g of the powder was added to 1L of distilled water and mixed. The mixture was autoclaved and stored at room temperature.

A.1.2 SM Buffer

The following mixture of components was added to 1.5L of distilled water and mixed thoroughly: 50mM Tris at a pH of 7.5, 8mM of MgSO\textsubscript{4}.7H\textsubscript{2}O and 0.1M NaCl. All the chemicals were purchased from Sigma Aldrich. The mixture was autoclaved and stored at room temperature.

A.1.3 N-Minimal Medium

The following chemicals were added to distilled water and mixed with the final concentration specified: 0.1 M Tris-HCl at of pH 7.4, 5 mM KCl, 7.5 mM $(NH_4)_2SO_4$, 0.5 mM $K_2SO_4$, 1 mM $KH_2PO_4$, 10 $\mu$M $MgCl_2$ and 0.2% glucose. The stock was then filter-sterilized and stored at room temperature.
Appendix A. Protocols

A.1.4 Phosphate Buffer Saline

The following chemicals were added to distilled water and mixed with the final concentration specified: NaCl 137mM, KCl 2.7mM, $Na_2HPO_4$ 10mM and $KH_2PO_4$ 1.8mM. The stock was then filter-sterilized and stored at room temperature.

A.2 Bacteriophage Preparation

The two phages used in the experiments are $\lambda cI_{857}$ phage and $T_6$ phage. Both phages were prepared with a titre of $10^{11}$ pfu/ml from low-concentration phage lysate stocks. Each phage stock was then dialyzed in clean SM media, stored at 4°C and used in our experiments as needed over several months without noticeable titre drop.

A.2.1 $T_6$ Phage

Below is the protocol modified from an original protocol developed by Kelly Reimer [89] to produce high titre $T_6$ phage using the following steps:

Day 1:

An overnight culture of *E.coli* K12 WT was prepared. Small aliquots of frozen stock of the bacterial cell was added to 3ml of LB Broth, and left to grow in the shaking incubator overnight (about 15 hours) at 37°C.

Day 2:

Step 1: Added about 3ml of the overnight culture prepared overnight to a 200ml of LB to grow in a 37°C shaking incubator until it reached an OD600 of 0.08-0.1.

Step 2: Added the available low-titre $T_6$ phage stock to the culture such that the initial multiplicity of infection (moi : number of phage per bacterial cell present) was 0.001 to 0.01.

Step 3: Waited until the cells began to lyse (OD decreasing), generally 2 hours post-infection. Added 3.2ml of EGTA per 200ml of the culture stock to prevent reabsorption of the phages into the cells.

Step 4: Continued to incubate in the shaker until the decrease in OD stopped.

Step 5: Centrifuged the sample at 4°C at 5000rpm for 30 minutes.

Step 6: Added 8g of PEG per 100ml LB plus 2.5g of NaCl per 100ml LB. Mixed well and moved the culture tube to the cold room overnight.
Day 3 :

Step 1: Centrifuged the tube at 4°C at 5000 rpm for 30 minutes. The phage pellet rested at the bottom and the sides of the tube.

Step 2: Poured off remaining LB and added about 3ml of SM to the tube to dissolve the phage pellets. Vortexed to mix properly.

Step 3: Under the fumehood, added approximately the same amount of chloroform as SM, vortexed and centrifuged the mixer at 5000 rpm for 20 minutes.

Step 4: The liquid on the top was the dissolved phage, the middle contained the PEG and the bottom was the added chloroform. Carefully removed the phage solution without disturbing the PEG band.

Step 5: Performed the spotting assay to find the titre of the phage.

Step 6: Dialyzed the phage in SM to purify the stock.

A.2.2 λ Phage

The following is a modified protocol from Kelly Reimer [89] to produce high titre λ cI857 Sam7 phage stock. The initial stock is a lysogen of λ phage embedded in a bacteria. The following steps were followed:

Day 1 :

Step 1: 2L of λcI$_{857}$ at 30°C in LB were grown until OD600 reached 0.6.

Step 2: Heat shocked the stock in a 65°C water bath until the temperature of the culture reached 45°C (1L took about 10 min with periodic shaking).

Step 3: Switched the culture to a 45°C shaking water bath for 15 minutes.

Step 4: Cooled down the culture to 37°C on ice water to cool(1L took about 2.5 minutes with shaking).

Step 5: Continued growing of the stock at 37°C for 2 hours.

Step 6: After 2 to 2.5 hours of growth, added 10 ml CHCl$_3$ per 1L.

Step 7: Returned the stock to 37°C for 15 minutes while shaking. Then cooled the lysed cultures to room temperature and added pancreatic DNAase I and RNAase, each to a final concentration of 1µg/ml.
Step 8: Incubated the stock for 30 minutes at room temperature.  

Step 9: Added solid NaCl to a final concentration of 1M (29.2 g per 500 ml of culture). Dissolved by swirling. Let it stand for 1 hour on ice.

Step 10: Removed debris by centrifugation at 11000g for 10 minutes at 4C. Pooled the supernatants in a clean flask.

Step 11: Added solid polyethylene glycol (PEG 8000) to a final concentration of 10% w/v (i.e., 50 g per 500 ml of supernatant). Dissolved by slow stirring with a magnetic stirrer at room temperature. Cooled in ice water and let stand for at least 1 hour (or overnight in cold room) on ice to allow the bacteriophage particles to form a precipitate.

Day 2:

Step 1: Recovered the precipitated bacteriophage particles by centrifugation at 11000g for 10 minutes at 4C.

Step 2: Discarded the supernatant, and let the centrifuge bottle stand in a tilted position for 5 minutes to allow the remaining fluid to drain away from the pellet.

Step 3: Removed the fluid with a pipette. Using a wide-bore pipette equipped with a rubber bulb, gently resuspended the bacteriophage pellet in SM (multiples of 12-13 ml per L of supernatant).

Step 4: Washed the walls of the centrifuge bottle thoroughly, since the precipitate of bacteriophages sticks to them, especially if the bottle is old.

Step 5: Extracted the PEG and cell debris from the bacteriophage suspension by adding an equal volume of chloroform and vortexing for 30 seconds.

Step 6: Separated the organic and aqueous phases by centrifugation at 3000g for 15 minutes at 4C.

Step 7: Recovered the aqueous phase, which contained the bacteriophage particles. Dialyzed the phage in SM to purify the stock.

---

1Crude commercial preparations of both enzymes are adequate to digest the nucleic acids liberated from the lysed bacteria. Without digestion, a significant number of bacteriophage particles become entrapped in the viscous solution of nucleic acids.

2The addition of NaCl promotes dissociation of bacteriophage particles from bacterial debris and is required for efficient precipitation of bacteriophage particles from polyethylene glycol.
A.3 Bacteriocin Preparation

Lysostaphin (lysostaphin from *Staphylococcus simulans* recombinant, expressed in *E. coli*) powder was purchased from Sigma-Aldrich. The powder was dissolved in SM at 100µg/ml and stored at 4°C for several months and used repeatedly for experiments.

Colicin B was a gift from Dr. Kathleen Postle Department of Biochemistry and Molecular Biology, Pennsylvania State University. Colicins A9, E1 and K were gifts from Dr Roland Lloubes, Laboratoire d’Ingenierie des Systems Macromoleculaires CNRS-Aix-Marseille University. The following bacterial strains with pore-forming colicin-expressing plasmids were obtained: KS474 pColA9 (Colicin A9), W3110 pES3 (Colicin B), KS272 pERE1 (Colicin E1), and K235 pColK (Colicin K).

The preparation of the bacteriocins was performed by Nichole Cumby from the Department of Molecular genetics, University of Toronto, as follows:

Step 1: Each strain was grown up in 50 mL of Lennox Broth (LB) at 37°C in a shaking incubator to an OD600 of 0.8.

Step 2: Mitomycin C was added to a final concentration of 1µg/ml and the cells were incubated at 37°C for a further three hours and 30 minutes.

Step 3: Cells were then pelleted via centrifugation and the supernatant was discarded. The pellet was resuspended in 5 mL of SM and the cell suspension was lysed open via sonication.

Step 4: The cellular debris was spun down and the supernatant, which contains the colicins, was isolated and dialyzed two times into SM media for 4 hours each time.

Step 5: The sample was dialyzed in SM, and filter-sterilized and stored at 4°C.

A.4 Raw Bacteria Sample Preparation

The measurements were performed using different strains of *E.coli*. On the day before each experiment, overnight cultures of *E.coli* were prepared as follows:

Small aliquots of frozen stock of each bacterial cell was added to 3ml of Lennox Broth, and left to grow in the shaking incubator overnight (about 15 hours) at 37°C.

On the day of each experiment, 100µl of each of the selected overnight cultures were added to 50ml of LB and allowed to grow inside the incubator. The Optical Density (OD) of the cells was measured at 600nm wavelength using a spectrophotometer (Thermo Scientific Genesys 20) and monitored as the cells divided in log-phase until the desired OD was reached.

Independent plating experiments were performed to map the measured OD values for
each cell type to the actual concentration in colony-forming units per ml (cfu/ml). After reaching the desired OD, the samples were ready to be processed as explained in the next section to be used on the biosensor.

A.5 Bacetria Sample Processing

Bacterial cultures were collected by centrifugation at 6900g, washed once in suspension medium and resuspended in the suspension medium. The suspension medium is a medium without potassium concentration, usually SM in this theis, were bacterial samples are kept alive without increasing their concentration.

A.6 Potassium-Sensitive Membrane Preparation, Chip Preparation and Membrane Deposition

A.6.1 Reagents

- Valinomycin: Sigma Aldrich Product number: 94675
- Polyvinyl chloride: Sigma Aldrich Product number: 81387
- Dioctyphthalate: Sigma: Aldrich Product number: D201154
- Tetrahydrofuran: Sigma: Aldrich Product number: 401757
- P20: Shin-Etsu MicroSi Product number: MP-P20
- Acetone
- Isopropanol

A.6.2 Protocol

To create the potassium-sensitive electrodes on the surface of the CMOS ICs, a K$^+$ ion-selective membrane was created using the following protocol:

Step 1: Dissolved 0.33g high molecular weight polyvinyl chloride matrix supplemented with 0.89ml dioctyphthalate and 10 mg valinomycin in approximately 5ml tetrahydrofuran (THF). The membrane mixture solution was stored for 10 days and repeatedly used during this time as needed.
Step 2: The encapsulated CMOS dies were washed with acetone, followed by isopropanol, and finally deionized water.

Step 3: The CMOS die were dried in a stream of dry nitrogen gas and placed in a 110°C drying oven for 15 minutes. They were removed from the oven and left at room temperature under the fume hood for 5 minutes.

Step 4: 5µl droplets of MicroSi primer P20 were deposited on the surface of the chips as an adhesion promoter.

Step 5: The primer was allowed to dry for 5 minutes under the fume hood.

Step 6: 40µl droplets of the membrane solution were dispensed on top of the electrodes 2 to 3 times, with 5-minute intervals in between applications, to create a smooth membrane layer without any pinholes, and with a thickness in the range of 100 to 300µm.

A.6.3 Suppliers


Appendix B

Bacteria Sensing Using Ion-Induced Voltage Fluctuations

This appendix introduces the usage of another type of recognition-event transducer and electrical transducer to detect the potassium efflux from the exposure of sensitive bacterial cells in a sample, by phages and bacteriocins. The results were published in [165, 166].

A technology called sensing of phage-triggered ion cascade has been introduced in [167], that uses bacteriophages (phages) as biological detecting elements, and employs electrical noise analysis to identify bacteria in less than 10 minutes. As discussed in in Chapter 3, during the phage or bacteriocin interaction with the bacterial cells, there is a transitory efflux of ions from the cell. This event creates electric-field fluctuations in the sample for several minutes. We implemented the first silicon-based implementation of the method described in [167], using standard TSMC 0.18µm CMOS technology. Figure B.1 shows the cross-section of the nanowell used to sense the aforementioned voltage fluctuations.

![Nanowell construction in CMOS process](image)

Figure B.1: (a) Nanowell construction in CMOS process, (b) dimensions of the electrodes.

The nanowell consists of two electrodes, made from the top-layer metal (Aluminum)
Appendix B. Bacteria Sensing Using Ion-Induced Voltage Fluctuations

in the CMOS process, and a trench, that is made by removing the top passivation layer using a passivation mask. Figure B.1 also shows the dimensions and separation of the electrodes used in the measurements. Figure B.2 shows the overall block diagram of the sensor system. The outputs of the integrated amplifiers are connected to an off-chip data acquisition system that digitizes the amplified signal and transfers the results to a PC for analysis.

Figure B.2: System block diagram of the sensor.

The sensitivity of the sensor is determined primarily by the noise level of the front-end amplifier. In addition, the overall power consumption needs to be low enough so as to generate a minimal temperature increase (relative to ambient) of the chip surface in contact with the sample. Figure B.3 shows the schematic diagram of the amplifier [168]. The input capacitors are used to reject DC, while the MOS transistors in the amplifier work as pseudo-resistors [168]. This resistance is nonlinear, depending on the voltage difference across the transistors. The core of the amplifier is the operational

Figure B.3: (a) Schematic of the amplifier, (b) schematic of the OTA.
transconductance amplifier (OTA) also shown in Fig. B.3. Input PMOS devices with large gate areas are used in the OTA to reduce 1/f noise. To minimize the thermal noise, M1 and M2 are designed to operate in the sub-threshold region, while M3-M8 operate in strong inversion [168]. The overall gain of the amplifier is 40dB with a measured bandwidth of 0.35 to 70Hz and input-referred noise of $0.3pV^2/Hz$ at 1Hz.

The chip was fabricated in 0.18µm CMOS technology using thick oxide transistors and consumes 122µW with a 3.3V supply for two recording channels. Figure B.4 shows a micrograph of the chip. The two channels, each consisting of an electrode pair and an amplifier, are integrated on-chip along with controlling switches and circuits. The chips were encapsulated to isolate the bond wires from the sample, leaving only the electrodes exposed. Figure B.5 shows the test setup with the encapsulated chip and the supporting printed circuit board.

<table>
<thead>
<tr>
<th>Process</th>
<th>CMOS 0.18µm 1P6M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Core Area</td>
<td>724 x 666 µm²</td>
</tr>
<tr>
<td>Die Area</td>
<td>996 x 1034 µm²</td>
</tr>
<tr>
<td>Supply Voltage</td>
<td>3.3V</td>
</tr>
<tr>
<td>Power Consumption</td>
<td>122µW @ 3.3V</td>
</tr>
</tbody>
</table>

Figure B.4: Micrograph (a)(b) of the chip and the electrodes, and (c) chip specifications.

Figure B.5: (a) Integrated circuit with encapsulated bonding wires, (b) test board.
B.0.4 Experimental Results Using λ Phage

The first set of experiments were performed using $\lambda cI_{857}Sam_7$ phage and two bacterial strains: *E. coli* w3110 $\Delta fhuA$ which is sensitive to the $\lambda$ phage (positive control), and *E. coli* w3110 $\Delta lamB$ which is resistant (negative control). Both positive and negative control bacteria have an optical density (OD) of approximately 0.7 (i.e., concentration of approximately $10^8$ cfu/ml).

The experiment started by mixing 10$\mu$l of bacteria with 5$\mu$l of the $\lambda$ phage. The mixture was then applied to the chip surface and the output of the amplifier was digitized from one to seven minutes. The ambient temperature in the experiments was 23.1°C. Figure B.6 shows a time window of the measured sensor output signal for both sensitive and resistant bacteria when mixed with $\lambda$ phage. The large spikes on the sensitive graph correspond to the time when a bacterium is attacked by the phage. These spikes occur throughout the measurement interval, mainly in the first 5 minutes after mixing the sensitive cells with phage. The noise on the resistant cell sample is mainly due to amplifier noise and random noise introduced from the fluid via the electrodes.

An alternative method, for easier assessment of the time domain measurements, is to compute the power spectral density (PSD) of the observed signal [167]. Figure B.7 shows the PSD of the measured voltage fluctuations over 1 to 10Hz for both sensitive and resistant bacteria. The open-loop PSD plot corresponds to the experiment where no sample was applied to the chip. The noise level after applying the sample (negative control) to the electrodes is slightly higher than the open-loop test results, while the PSD
Appendix B. Bacteria Sensing Using Ion-Induced Voltage Fluctuations

of the positive control signal shows large frequency components in the 1 to 10 Hz band. As computational speed is not an issue in this application, a low-speed ultra-low-power FFT block can be used to compute the FFT, with less than 155 nJ/FFT as reported in [146]. A sampling frequency of 200 Hz with a frequency precision of 0.5 Hz would need a 512-point FFT for this application. For reference, shown in Fig. B.7 is the PSD of the recorded signals obtained by averaging over 512-point FFT blocks that have been computed with 16-bit fixed-point precision.

Figure B.7: The power spectral density of the input-referred signal for positive and negative controls and the open loop test results. Also shown (for reference) is the PSD computed using 512-point FFT.

B.0.5 Experimental Results Using Pyocin

Pyocins are bacteriocins produced by many different Pseudomonas strains that are lethal to other strains of the same or related species. R-type pyocins are bacteriophage tail-like bacteriocins as shown in Fig. B.8. The pyocins used in these experiments were isolated from the Pseudomonas strain PA01, which is known to produce both F- and R-type pyocins. The two different strains of P. aeruginosa that were used in the measurements, PAC64 and PAC10, were clinical isolates from patients in a local Toronto hospital. These strains were grown overnight, then subcultured and grown to mid-log phase. The PAC64 and PAC10 cells are sensitive to the pyocin; PA01 is a separate strain from PAC64 and PAC10. Each chip was used only once in order to avoid any signal modifications due to contamination.
Appendix B. Bacteria Sensing Using Ion-Induced Voltage Fluctuations

Figure B.8: (a) Electron micrograph of R-type pyocin [8], (b) pyocin structure [8], (c) *Pseudomonas aeruginosa* [9].

The first set of experiments was performed by mixing PAC10 cells with PA01 pyocin. The PAC10 cells in this experiment had OD of approximately 0.7 which corresponds to a cell concentration of approximately $10^8$ cfu/ml. Figure B.9 shows a 90-second window of the total 6 minute recording interval of the measured input voltage over time.

Figure B.9: A 90-second window of the time-domain input voltage fluctuations when PAC10 cells with OD=0.7 are mixed with PA01 pyocin (Time origin is from the start of recording time)

When the sensitive *Pseudomonas* strain is mixed with the pyocin, the input voltage fluctuations occur during the time interval of the killing event. Unlike the infection
mechanism of bacteriophages against bacteria as used in the previous section, the ion leakage from inside the bacteria due to pyocin attachment likely continues until the cells die. This results in continuous, long-lived spikes at the output even beyond 10 minutes. The number of the spikes and the amplitude of the fluctuations are also dependent on the concentration of the Pseudomonas cells in the sample. Figure B.9 shows that the presence of the specific *Pseudomonas* strain can be detected by observing the time-domain input voltage when sensitive Pseudomonas cells are mixed with specific pyocin.

The PSD of the input voltage was computed for two experiments, as shown in Fig. B.10. It is clear that increased PSD level in the this band can be used as an indication of the presence of the pyocin targeted host cell.

![Figure B.10](image)

Figure B.10: Power spectral density of the input voltage in dry test and when mixture PAC10 OD=0.7 and PA01 are applied.

A second set of experiments was performed by mixing another strain of *P. aeruginosa*, PAC64, with the PA01 pyocins. Two different concentrations of PAC64 were used in two experiments to show the dependency of the input-referred PSD in a 1-to-10Hz band to the concentration of the cells. In the first experiment, the PAC64 cells with OD=2 were mixed with PAC01 pyocins and data was collected for 6 minutes. The input voltage fluctuations in this experiment were larger with an increased number of spikes and a longer duration of noise activity. Figure B.11 shows the PSD of the input voltage fluctuations for this measurement. In another experiment, the PAC64 cells with OD=0.7, which corresponds
Figure B.11: PSD for a large number of sample points and also for a 512-point FFT for the mixture of two different concentrations of PAC64 with PA01 pyocin.

to a concentration of approximately $10^8$ cfu/ml, were mixed with pyocins and data was collected as mentioned before. Figure B.11 also shows the input PSD for this experiment. From Fig. B.11, it can be observed that there is an increased level of noise when mixing either of PAC64 and PAC10 *Pseudomonas* cells with PA01. Thus, the presence of both sensitive strains can be confirmed by observing the input-referred PSD alone. Figure B.11 also shows that the energy of the fluctuations in a 1 to 10Hz frequency band are much stronger in the higher concentration experiment (OD=2) than the lower concentration (OD=0.7). This is due to an increased number of spikes in the 6-minute recording interval as well as a higher amplitude of the fluctuations.

## B.1 Summary

This appendix provided the early work on bacterial sensor design that detected the electrical fluctuations generated as a result of ion efflux from the bacteria cells exposed to phages and bacteriocins. The research was later expanded to measure the efflux of ions directly using potassium-sensitive ISE system, as opposed to the noise fluctuations that happen as a result of the ion efflux. The use of ISE systems provided more reliable and robust outputs compared to the method presented in this section, hence the research was pursued in that direction.
Bibliography

[1] Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences - University of Copenhagen, Denmark. Phage Typing on an Agar Plate. [Online]. Available: http://pictures.life.ku.dk/atlas/microatlas/food/typing_methods/phage_typing/


