DEVELOPMENT OF A POINT-OF-CARE DIAGNOSTIC PROTOTYPE SYSTEM FOR KIDNEY HEALTH MONITORING

By

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A thesis submitted in fulfilments of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

To

School of Engineering,

Faculty of Science and Engineering,

Macquarie University,

Sydney, Australia.

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MACQUARIE
University
SYDNEY·AUSTRALIA

To my parents

Minal Nitin Prabhu

Nitin Mukund Prabhu

And

To my siblings

Late Goru Nitin Prabhu

Sonal Bhalchandra Samant

Acknowledgements

First, I would like to express my deepest and sincerest gratitude to my principal supervisor ***Professor Subhas Mukhopadhyay***. I would also like to thank my associate supervisors ***Emeritus Associate Professor Andrew Davidson, Dr Mohsen Asadniaye Fard Jahromi***. I am also thankful to my adjunct supervisor, ***Associate Professor Guozhen Liu***, for their excellent support. My all supervisors' guidance and inspiration throughs helped me a lot. Without their extensive expertise and patient guidance, this research work would not have been feasible.

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Words cannot express my gratitude and appreciation to my parents and siblings, late Goru and Sonal, for the constant motivation and support throughout my life.

Finally, yet importantly, I would like to thank my friends and colleagues, who have always stood beside me and helped me in times of need.

Statement of Candidate

I certify that the work in this thesis has not previously been submitted for a degree, nor has it been submitted as part of the requirements for a degree to any other university or institution other than Macquarie University.

I also certify that the thesis is an original piece of research and has been written by me.

In addition, I certify that all information sources and literature used are indicated in the thesis.

Sumedha ..

Sumedha Nitin Prabhu

Candidates Statement About the Impact of COVID-19 Changes on the Thesis

Dear Examiners,

Many Higher Degree Research candidates have had to make changes to their research schedules due to the impact of COVID-19. Below you will find a statement from the candidate, which is approved by their Supervisory Panel. It indicates how their original research plan has been affected by COVID-19 pandemic restrictions. The candidate will also add details about relevant ongoing restrictions in place caused by the COVID-19 pandemic.

Candidate's Statement:

1) Due to restricted research hours at Macquarie University amid the COVID-19 pandemic, the research work took more time to finish, and the thesis submission date was extended.

2) Due to the COVID-19 pandemic restrictions for work hours, the human ethics committee research approval for using real human samples of healthy and kidney patients has not been obtained. Due to this issue, all the experiments were performed using heat-inactivated human serum samples.

Extended Abstract

Early-stage diagnosis of a disease is significantly essential for a healthier lifestyle. Creatinine is a biological metabolic waste that constantly gets diffused in the human blood. It is excreted by the kidneys to reduce levels of blood toxicity, thus maintaining homeostasis. Kidney function can be qualitatively and quantitatively monitored by checking levels of creatinine in serum samples. Therefore, kidney disease patients are prescribed for frequent testing of their serum creatinine levels. Currently, serum creatinine levels testing can be done only at pathological laboratories. It is time-consuming, expensive, requires a pathologist for venepuncture, sample analysis and creatinine concentration data analysis. For understanding the results, a patient requires to visit a general practitioner/nephrologist/oncologist. Therefore, fast-acting, portable, cost and time-effective Point-of-Care (PoC) devices are in demand. In this work, an Internet of Things (IoT)-based creatinine specific, precise, easily operable, accurate, portable, patient-friendly, internet connectable, rechargeable, reusable, cost and time effective PoC diagnostic device prototype for the measurement of creatinine levels from serum, is developed in a stepwise manner.

Micro-Electro-Mechanical-Systems (MEMS)-based interdigital (ID) sensors having 1-1-25 structural dimensions are developed in the initial set of experiments. The sensors are first characterized in the air using the electrochemical impedance spectroscopy (EIS) technique. This characterization helped in understanding the sensitive range of the developed MEMS ID sensor. Then, the complex non-linear least-square (CNLS) software is used for calculating the equivalent circuit. It is also used for checking the component parameters with the help of experimental data fitting with its theory-based predicted response depending on Randle's model. Finally, the model infers the protocols performed within the biochemical cell in its' equivalent circuit. The characterisation of the developed MEMS ID sensor is performed. Different Milli-Q water-based samples carrying varying creatinine concentrations are analysed by dipping the sensing area inside the samples.

In the second set of experiments, creatinine specific Molecularly Imprinted

Polymer (MIP) and Non-Molecularly Imprinted Polymer (NIP) are developed using modified precipitation polymerization methodology. Creatinine powder ($C_4H_7N_3O$) as a template, methacrylic acid (MAA) ($C_4H_6O_2$) as a functional monomer, divinylbenzene (DVB) ($C_{10}H_{10}$) as a cross-linker molecule, 2, 2-azoisobutronitrile (AIBN) ($C_8H_{12}N_4$) is used as a reaction initiator. Acetonitrile (ACN) (C_2H_3N) and toluene (C_7H_8) are used as solvents. Milli-Q water, methanol (MeOH) (CH_4O), and tetrahydrofuran (THF) (C_4H_8O) are used for the process of washing which is necessary for the removal of excess chemicals used during the process of MIP as well as NIP polymerization. Acetic acid (AcOH) (CH_3COOH) reflux is used for the removal of locked template molecules from the cavity of the newly formed MIP molecule after the washing steps. The process of synthesis is done by using chemicals in the following ratio: template (creatinine), monomer (MAA) and cross-linker (DVB) at a ratio of 2:0.510:7.5. The MIP and NIP polymers are characterized using Ultra-High-Performance Liquid Chromatography (UHPLC) technique for sorption studies and selectivity test. During the sorption studies, the developed MIP is found to be capable of determining concentrations of creatinine up to 50 parts per million (ppm). It is three times higher than the normal range of creatinine concentration in the human blood. This high-end adsorption and detection capacity of developed MIP made it ideal for detecting creatinine over a wide range of concentrations. During the selectivity studies, Creatine ($C_4H_9N_3O_2$), 2-pyrrolidone (C_4H_7NO), N-hydroxysuccinimide ($C_4H_5NO_3$), 1-methylhydantoin ($C_4H_6N_2O_2$), and hydantoin ($C_3H_4N_2O_2$) are used for checking the specificity of MIP polymer molecules towards creatinine. It is observed during the analysis that the developed MIP polymer is highly selective towards creatinine. The Field Emission Scanning Electron Microscope (FESEM), Scanning Electron Microscopy (SEM) and Energy Dispersive Analysis X-ray (EDAX) analysis are done to study the structural features of MIP and NIP. In FESEM studies, the MIP and NIP particles show similar polymer associated properties such as spherical, isotropic and monodisperse. The EDAX analysis confirmed the presence of elements such as Carbon (C), Oxygen (O), Nitrogen (N). It also revealed the sample purity and the absence of unexpected contaminations offering pseudo results or absorption interference. Fourier-Transform Infrared (FTIR) analysis is used for confirmation of successful removal of creatinine as a template molecule by breaking the MIP polymer-creatinine bonding. The FTIR analysis confirmed the successful removal

of creatinine from the polymer cavity. After the MIP synthesis, the functionalization of the MEMS ID sensor is done by developing a MIP polymer functionalization suspension solution. It is developed using 1 gm of MIP polymer, 1.5 mL of acetone and 200 µL of acrylic resin. The PTL-MM01 Dip Coater instrument is run at a uniform speed of 200 mm/minute to achieve a uniform thickness of coating layer of MIP polymer over the sensing surface of the MEMS ID sensor. Testing for repeatability and reusability of MIP functionalization is also done. It is noticed that the repeatability measurements of the functionalized MEMS sensor are relatively constant for up to five uses. The MIP functionalized MEMS sensor can also be reused constantly for a total of five experiments. The EIS characterization of the MIP functionalized MEMS ID sensor is performed. It is done by using Milli-Q water and serum solution-based samples carrying varying creatinine concentrations.

In the third set of experiments, the effect of thickness of the acrylic coating and the MIP functionalization layer on the performance associated sensitivity of the MEMS ID sensor is studied. Two types of coating materials, such as acrylic resin coat and the MIP selective functionalization suspension, are applied to the two separate MEMS sensor's sensing areas. The performance-based coated and functionalized sensor analysis is performed by measuring the MEMS sensor's sensitivity with varying thicknesses of the coating and the functionalization layers. The MIP-functionalized selective MEMS ID sensor coating exhibited the highest sensitivity while measuring creatinine levels from the serum samples. The net effect of speed of withdrawal and time of dipping on the coating and functionalization layer thicknesses is investigated. This study concluded that the faster speed of withdrawal would result in a thinner layer of coating and functionalization layers. The coating and functionalization layer thicknesses are increased with an increase in the net time of dipping. However, the findings show that rising the MEMS sensor's coating and functionalization layer thicknesses substantially raise the degree of saturation. The ideal parameters for generating the best MIP functionalization layer thickness are 1 second of dipping time and 200 mm/second of the speed of dipping and withdrawal.

In the fourth set of experiments, after finding out the ideal functionalization thickness of MIP polymer over the MEMS ID sensor for the process of functionalization, a single frequency measurement is done. In this experiment, 1020

Hz frequency is found to be the operating frequency where the MIP functionalized MEMS sensor is able to clearly distinguish between every single Milli-Q and serum creatinine sample having different concentrations. The EIS technique is applied for this set of experiments, and the LCR meter instrument is used. It is observed that the LCR meter is a susceptible instrument, but it is heavy, complicated and does not permit internet connectivity.

In the fifth set of experiments, to overcome the limitations of the LCR meter, a Long Range Wide Area Network (LoRaWAN) based, low power microcontroller associated, 1020 Hz single frequency-based, internet connectable, portable, functionalized MEMS ID sensor connected PoC sensing device has been developed. The PoC diagnostic prototype system attached with functionalized MIP coating for selective creatinine specific adsorption is utilised further for multiple sample analysis. The utilization is done for finding capacitance and resistive properties of the sample under test (SUT). A low power microcontroller-associated diagnostic device prototype system development is done for easing an understanding of complicated test results. This prototype system is for the selectively quantitative measurement of creatinine levels from serum samples for kidney health management. The microcontroller board has an inbuilt LoRaWAN chip, which helped connect the PoC diagnostic prototype device with the aid of a designated gateway for transferring the measured data to an IoT-associated remotely located cloud server. Adafruit is a complimentary IoT associated cloud server, and it is utilised for the storage of collected data. The Adafruit cloud server can be used anywhere and thus aids the healthcare provider in monitoring the data for earlier detection of side-effects of treatment of chemotherapy/other medications/kidney health from a long-distance. The device transfers data, and the collected data can be stored. It is easily accessible to general physicians/oncologists/nephrologists situated over a distance for a long-distance patient healthcare monitoring facility. The developed sensing system covers a wide range of creatinine levels from 6-15.5 ppm (normal) and higher levels up to 50 ppm (upper limit of detection). As the device detection range is over three times the acceptable creatinine range in human serum levels, it is suitable for prophylactic and prognostic PoC care facility for kidney health. Calibration of the PoC device has been successfully done at 1020 Hz frequency for obtaining the simplified form of results. Furthermore, varying serum creatinine sample concentrations are

analysed, and the unknown sample result is also obtained.

In the sixth set of experiments, the results obtained using the developed PoC diagnostic prototype system for creatinine are cross-checked and verified with the standard Creatinine Colorimetric Assay Kit. Thus, it assisted in the validation of the results obtained PoC diagnostic prototype system and helped in the error calculation.

The presented creatinine specific system is easy, fast, lightweight thus portable, but sensitive in its testing using the presented PoC diagnostic device for kidney health monitoring.

Keywords –

Point-of-Care (PoC), Internet of Things (IoT), Micro-Electro-Mechanical-Systems (MEMS), interdigital (ID), electrochemical impedance spectroscopy (EIS), complex non-linear least-square (CNLS), Molecularly Imprinted Polymer (MIP), Non-Molecularly Imprinted Polymer (NIP), Ultra-High-Performance Liquid Chromatography (UHPLC), parts per million (ppm), Field Emission Scanning Electron Microscope (FESEM), Scanning Electron Microscopy (SEM), Energy Dispersive Analysis X-ray (EDAX), Fourier-Transform Infrared (FTIR), Long Range Wide Area Network (LoRaWAN), Creatinine Colorimetric Assay Kit.

Research Outputs

Publications related to the field of this thesis are as follows:

Peer-reviewed journals

1) IoT-Associated Impedimetric Biosensing for Point-of-Care Monitoring of Kidney Health

Details - S. Prabhu, C. Gooneratne, K. A. Hoang and S. Mukhopadhyay, IoT-Associated Impedimetric Biosensing for Point-of-Care Monitoring of Kidney Health, in IEEE Sensors Journal, (Early Access) doi: 10.1109/JSEN.2020.3011848.

2) Molecularly Imprinted Polymer-based detection of creatinine towards smart sensing

Details - Prabhu, S.N., Mukhopadhyay, S.C., Gooneratne, C.P., Davidson, A.S. and Liu, G. (2020), Molecularly Imprinted Polymer-based detection of creatinine towards smart sensing. Med Devices Sens, 3: e10133. doi:10.1002/mds3.10133.

3) Development of MEMS Sensor for Detection of Creatinine using MIP Based Approach - A Tutorial Paper

Details - S. N. Prabhu, C. P. Gooneratne and S. C. Mukhopadhyay, Fellow, IEEE, Development of MEMS Sensor for Detection of Creatinine using MIP Based Approach -A Tutorial Paper, IEEE Sensors Journal Special Issue on Sensors Tutorials: A Vigorous Dive into the Vast Sea of Sensor-Related Knowledge, doi: 10.1109/JSEN.2021.3077060.

4) Functionality Evaluation of Micro-Electro-Mechanical-Systems Sensor for Varied Selective Functionalization Thickness to Determine Creatinine Concentration

Details - S. N. Prabhu, S. C. Mukhopadhyay and R. Morello, "Functionality Evaluation of Micro Electro Mechanical Systems Sensor for Varied Selective Functionalization Thickness to Determine Creatinine Concentration," in IEEE Sensors Journal, doi: 10.1109/JSEN.2021.3081828.

Conference proceedings:

1) Interdigital sensing system for detection of levels of creatinine from the samples

Details - S. N. Prabhu, S. C. Mukhopadhyay, C. Gooneratne, A. S. Davidson and G. Liu, Interdigital sensing system for detection of levels of creatinine from the samples,

2019 13th International Conference on Sensing Technology (ICST), ISBN:978-1-7281-4807-6, 2-4 Dec. 2019, Sydney, Australia, 2019, pp. 1-6, doi: 10.1109/ICST46873.2019.9047672.

2) Highly selective Molecularly Imprinted Polymer for creatinine detection

Details - S. N. Prabhu, S. C. Mukhopadhyay, A. S. Davidson and G. Liu, Highly selective Molecularly Imprinted Polymer for creatinine detection, 2019 13th International Conference on Sensing Technology (ICST), ISBN:978-1-7281-4807-6, 2-4 Dec. 2019, Sydney, Australia, 2019, pp. 1-5, doi: 10.1109/ICST46873.2019.9047696.

3) Development of a Point-of-Care diagnostic smart sensing system to detect creatinine levels

Details - S. Prabhu, C. Gooneratne, K. Anh Hoang and S. Mukhopadhyay, Development of a Point-of-Care diagnostic smart sensing system to detect creatinine levels, 2020 IEEE 63rd International Midwest Symposium on Circuits and Systems (MWSCAS), ISBN:978-1-7281-8058-8, 9-12 Aug. 2020, Springfield, MA, USA, 2020, pp. 77-80, doi: 10.1109/MWSCAS48704.2020.9184441.

4) A Unique Developmental Study in the Design of Point-of-Care Medical Diagnostic Device for Kidney Health Care of Metastatic Brain Cancer Patients to Avoid Chemotherapy Side-Effects

Details - Sumedha N. Prabhu[1]*, Prof. Subhas C. Mukhopadhyay[1], 4th International Conference on Communication Devices and Networking 2020 (ICCDN 2020), Sikkim Manipal Institute of Technology, Sikkim, India. doi: 10.1007/978-981-16-2911-2_38. The outcome of this conference is published in the form of a Springer Nature book chapter. The details are described below in the book chapters section.

5) IoT Enabled PoC Medical Diagnostic MEMS-Based Sensor Device for Kidney Healthcare

Details - Sumedha Nitin Prabhu[1], Subhas Chandra Mukhopadhyay[1], 14th International Conference on Sensing Technology 2022 (The conference paper has been accepted, I will present it online).

Book chapters

1) IoT for Smart Homes

Details - Anindya Nag[1], Eshrat E. Alahi[1], Nasrin Afsarimanesh[1], **Sumedha Prabhu[1]** and Subhas Chandra Mukhopadhyay[1], IoT for smart homes, in the book Sensors in the Age of the Internet of Things Technologies and Applications, The Institution of

Engineering and Technology International Series on Sensors, edited by Octavian Adrian Postolache, Edward Sazonov and Subhas Chandra Mukhopadhyay, published by The Institution of Engineering and Technology, London, United Kingdom, ISBN 978-1-78561-634-1 (hardback), ISBN 978-1-78561-635-8 (PDF), pp 171-199, 2019, doi: 10.1049/PBCE122E_ch7.

2) Interdigital Sensing System for Kidney Health Monitoring

Details - S. N. Prabhu[1]*, C. P. Gooneratne[2], K. A. Hoang[3], S. C. Mukhopadhyay[4], A. S. Davidson[5] and G. Liu[6], Interdigital Sensing System for Kidney Health Monitoring, in the book Interdigital Sensors Progress over the Last Two Decades, Smart Sensors, Measurement and Instrumentation, edited by Subhas Chandra Mukhopadhyay, Boby George, Joyanta Kumar Roy, Tarikul Islam, published by Springer Nature Switzerland AG, Gewerbesstrasse 11, 6330 Cham, Switzerland, ISBN 978-3-030-62683-9 (hardback), ISBN 978-3-030-62684-6 (eBook), pp 267-309, 2021, doi: 10.1007/978-3-030-62684-6_11.

3) A Unique Developmental Study in the Design of Point-of-Care Medical Diagnostic Device for Kidney Health Care of Metastatic Brain Cancer Patients to Avoid Chemotherapy Side-Effects

Details - Sumedha N. Prabhu[1]*, Prof. Subhas C. Mukhopadhyay[1], A Unique Developmental Study in the Design of Point-of-Care Medical Diagnostic Device for Kidney Health Care of Metastatic Brain Cancer Patients to Avoid Chemotherapy Side-Effects, in the book Lecture Notes in Electrical Engineering 776, Advances in Communication, Devices and Networking, Proceedings of ICCDN 2020, edited by S. Dhar et al., published by Springer Nature Singapore Pte Ltd., ISBN 978-981-16-2910-5, 2022 doi: 10.1007/978-981-16-2911-2_38.

Poster presentation

1) Smart sensing system for early detection of side effects of brain cancer chemotherapy by checking levels of creatinine from the samples diagnostics

Details - S. Prabhu*, S. Mukhopadhyay, A. Davidson, G. Liu, IEEE NSW section UNITE 2019, Mercure, Sydney, Australia, 9 August 2019.

2) Molecularly imprinted polymer-coated impedimetric smart creatinine-MEMS sensor for determining levels of creatinine from samples for IoT enabled PoC diagnostics

Details - S. Prabhu*, S. Mukhopadhyay, K.A. Hoang, C. Gooneratne, A. Davidson, G. Liu, 31st Anniversary World Congress on Biosensors, Busan Exhibition and

Conference Centre, Busan, Korea. The poster is virtually presented from Macquarie University, Sydney, Australia on 28 July 2021.

Google Scholar

https://scholar.google.com.au/citations?user=Yni0x64AAAAJ&hl=en

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YouTube work blog

https://www.youtube.com/channel/UCv8mVHPTG8Hqo18oTuC3tdQ

More details about my work are available at

https://researchers.mq.edu.au/en/persons/sumedha-prabhu

Awards and recognitions

1) I was awarded a second prize for oral presentation at the Higher Degree Research Conference held on 20 June 2019 at the School of Engineering, Macquarie University, Sydney, Australia.

2) I have also participated in 3 Minute Thesis (3MT), an Asia Pacific level competition initiated by The University of Queensland, and I won 3 prizes at the Macquarie University level. In the 3MT competition, I won third prize at the School of Engineering level. I also won two People's Choice awards at the Faculty of Science and Engineering and the entire Macquarie University level. During this time, I was honoured twice with cash prizes and once with a research budget. Macquarie University posted my 3MT

People's Choice award win on their official university website (https://www.mq.edu.au/thisweek/2020/09/03/the-future-is-bright-macquaries-3mt-winners-wow-the-judges/?fbclid=IwAR1fOUpJhezbeutozjrexGcnNxcEw4Lpla7QHtYgaRLnKDDgeuANnU5bKTo#.X1d4b3kzaUk), and (https://students.mq.edu.au/study/my-research-program/3-minute-thesis-competition) as well as on the Twitter handle. After my 3MT People's Choice win on Macquarie University level, Macquarie University celebrated my win with my official photoshoot and invited SBS world news presenter for Australia, Mr Anton Enus, for brightening my research presentations.

3) Macquarie University had also audio-visually recorded my 3MT speech in their on-campus video recording studio. Macquarie University posted my audio-visually recorded 3MT speech in MQ video recording studio on their official YouTube channel (https://www.youtube.com/watch?v=pdXO71T9bTQ&feature=youtu.be).

4) My research success was covered by journalist Rhea Nath from an Indo-Australian Media channel, "Indian Link Media", and published on their official press (https://www.indianlink.com.au/features/5-minute-home-kidney-health-test-wins-three-uni-awards/), Twitter handles as well as a Facebook page.

5) I have also received an offer to commercialise my PoC diagnostic prototype system on a larger scale from TiE Sydney.

6) I am an active member of a professional IEEE technical society, New South Wales section, Australia. IEEE Macquarie University Branch has appointed me to the IEEE Student Branch Executive Committee, and currently, I am holding IEEE Women In Engineering Affinity Group Vice-Chair position.

Research grants

1) I have won Macquarie University Postgraduate Research Fund (PGRF) 2020 - Round 2 funding of $5000 for conference paper/poster & travel-related research work.

2) I have won the People's Choice award in 3MT, an Asia Pacific level competition on the Macquarie University level. I was awarded a $250 Higher Degree Research budget, a small grant money token of appreciation from Macquarie University for my research work.

Presentations at conferences workshops/posters/invited talks

1) Presented at the Higher Degree Research Conference, School of Engineering,

Macquarie University, Sydney, Australia, 20 June 2019.

2) Presented at the IEEE IMS NSW Chapter 3rd Annual Workshop, Western Sydney University, Australia, 25 July 2019.

3) Presented poster at the IEEE NSW section UNITE 2019, Mercure, Sydney, Australia, 9 August 2019.

4) Presented at the 13th International Conference on Sensing Technology (ICST) 2019, Sydney, Australia, 2 December 2019.

5) Presented at the 13th International Conference on Sensing Technology (ICST) 2019, Sydney, Australia, 3 December 2019.

6) Presented at the 63rd IEEE International Midwest Symposium on Circuits and Systems Conference (MWSCAS) 2020, Springfield, MA, USA. (Presented virtually from Sydney, Australia, 11 August 2020 + Attended virtually from Sydney, Australia, 9-12 August 2020).

7) Invited talk from the IEEE Macquarie University Student Branch Women in Engineering Affinity, Macquarie University, Sydney, Australia on the topic "Girl's Voice" in "Stay Strong and Build Resilience during Corona Crisis" workshop, (Virtually presented), 13 October 2020.

8) Presented at the 1st Annual Workshop of IEEE Sensors Council NSW Chapter and 4th Annual Workshop of IEEE IMS NSW Chapter, Macquarie University, Sydney, Australia (Virtual presentation), 11 December 2020.

9) Presented at the 4th International Conference on Communication Devices and Networking 2020 (ICCDN 2020), Sikkim, India (Presented virtually from Sydney, Australia, 19 December 2020 + Attended virtually from Sydney, Australia, 19-20 December 2020).

10) Presented at the 2nd Annual Workshop of IEEE Sensors Council NSW Chapter and 5th Annual Workshop of IEEE IMS NSW Chapter, Macquarie University, Sydney, Australia. (Virtual presentation), 16 April 2021.

11) Presented poster at the Elsevier Biosensors 2021's respective conference, the 31st Anniversary World Congress on Biosensors, Busan, South Korea (Presented virtually from Sydney, Australia, 28 July 2021 + Attended virtually from Sydney, Australia, 26-29 July 2021).

Undergraduate thesis guided

1) The development and testing of a portable sensing system for detection creatinine

level

2) The development and testing of sensing system for detecting creatinine level using IoT system

3) Sensing molecular mobility in porous materials

Contents

Acknowledgements ..... **iv**

Statement of Candidate ..... **vi**

Candidates Statement About the Impact of COVID-19 Changes on the Thesis ..... **vii**

Extended Abstract ..... **viii**

Research Outputs ..... **xiii**

Contents ..... **xx**

List of Tables ..... **xxv**

List of Figures ..... **xxvi**

List of Equation ..... **xxx**

1 Introduction **1**

1.1 Introductory Details 2
1.2 Problem Definition 3
1.3 Estimated GFR 5
1.4 Facts and Statistics 7
1.5 Diagnosis 14
1.6 Research Motivation 14
1.7 Research Objectives 15
1.8 Research Contributions 16
1.9 Current Status of Developed PoC Diagnostic Prototype System for Creatinine Detection 17
1.10 Thesis Outline 17

2 Literature Review **20**

2.1 Introduction 21
2.2 Structure and Functioning of Kidneys, Kidney Health 23
2.3 Biochemical Markers of CKD 27
2.3.1 Creatinine 27
2.3.2 BUN 28
2.4 Analytical Methods for the Measurement of Creatinine, a CKD Biochemical Marker 28
2.4.1 UHPLC 29
2.4.2 GC 30

2.4.3 CE 31
2.5 Current Advancements in CKD Biosensing 32
2.5.1 Enzyme Nanoparticles-based Biosensors 32
2.5.2 Nanomaterial-based Electrochemical Creatinine Biosensors 34
2.5.3 Enzymeless/Chemical Creatinine Biosensors 38
2.5.4 Creatinine Immuno Biosensors 42
2.5.5 Conductometric Creatinine Biosensors 43
2.5.6 Electrochemiluminescence Creatinine Biosensors 44
2.5.7 Lossy Mode Resonance-based Fiber Optic Creatinine Biosensors 44
2.5.8 Multiplex Assays 45
2.5.9 EIS-based MIP Biosensors 46
2.6 Chapter Summary 47

3 MEMS ID Sensors and EIS Technique 48
3.1 Operating Principle of Interdigital Sensors 49
3.2 Heavy Permeation Depth Planar ID Sensors 51
3.2.1 MEMS Sensors Fabrication 51
3.2.2 Detail Process of Fabrication 53
3.3 Electrochemical Impedance Spectroscopy 57
3.4 Experiments with the Developed Sensing System 61
3.4.1 Experimental Setup 61
3.4.2 Preparation of Creatinine Spiked Aqueous Samples 63
3.4.3 EIS Measurement 63
3.4.4 Single-Frequency Resistance-based Sensor Response 69
3.4.5 Repeatability and Reusability Testing 70
3.4.6 Calculation of Standard Deviation 72
3.4.7 Unknown Sample Measurement and Error Calculation 72
3.4.8 Current Issues and Future Requirements 73
3.5 Chapter Summary 75

4 MIP Functionalized MEMS ID Sensor for Creatinine Detection using EIS Technique 76
4.1 Introduction 77
4.2 General Principle of MIP 79
4.2.1 Categories of MIP 81
4.2.2 Effects of Monomers, Cross-Linkers, Porogenic Solvents and Reaction Initiators in Synthesis of MIP 82
4.2.3 Synthesis Methods for MIP 85
4.3 Materials and Methods 87
4.3.1 Chemicals 87
4.3.2 Apparatus and Instruments 89
4.3.3 Synthesis of Specifically Selective Polymer by using MIP Technology 90

4.3.4 Preparation of Creatinine Spiked Serum Samples 93
4.3.5 Sorption Studies of Creatinine by MIP as well as NIP 94
4.3.6 Selectivity Test 95
4.3.7 Functionalization of MEMS ID Sensor using MIP and EIS Test 95
4.3.8 Binding Procedure of Creatinine on MIP Functionalized Sensor 98
4.3.9 EIS Experimental Measurements 98
4.3.10 CNLS Impedimetric Curve Fitting 99
4.3.11 Testing for Repeatability and Reusability using EIS Technique 100
4.3.12 Single Frequency Measurement, S_x Curve Equation Development and Measurement of Unknown Sample 100
4.4 Results and Discussions 101
4.4.1 FESEM, SEM and EDAX Characterisation of MIP and NIP Polymers for Structural and Elemental Analysis 101
4.4.2 FTIR Spectroscopy Analysis for Confirmation of Successful Removal of Creatinine as a Template Molecule by Breaking the Polymer Bonding 108
4.4.3 Sorption Studies of Creatinine by MIP and NIP for Calculating Upper Saturation Limit 109
4.4.4 Study of Selectivity of the MIP Towards Structurally Similar Compounds with Creatinine 111
4.4.5 EIS and Analytical Measurement for Creatinine Detection using MIP Functionalized Sensor 114
4.4.6 Analysis of Nyquist Plot Data using CNLS Curve Fitting 118
4.4.7 Testing for Repeatability and Reusability of MIP Functionalization and the MEMS Sensor in Creatinine Measurements using EIS Technique 124
4.4.8 Development of Calibration Curve by using LCR Meter 126
4.4.9 The Comparative Study Within the CNLS Measuring and Single-Frequency Measuring System 127
4.4.10 S_x Curve Equation Development and Measurement of Unknown Sample 127
4.5 Chapter Summary 129

5 Functionality Evaluation of MEMS Sensor for Varied Selective Functionalization Thickness 130
5.1 Introduction 131
5.2 Functionality Evaluation of MEMS Sensor for Varied Selective Functionalization Thickness to Determine Creatinine Concentration 131
5.2.1 Surface Coating of the MEMS Sensor with Acrylic Resin 133
5.2.2 Specifically Selective MIP Functionalization Material for Creatinine on the MEMS Sensing Area 135
5.2.3 Creatinine Level Analysis with the Acrylic Resin Coated and MIP Functionalized MEMS ID Sensor 137
5.2.4 Functionalized MEMS Sensor Sensitivity and the Saturation Level Measurement 140
5.2.5 Thickness of Functionalization Reliance on the Speed of Withdrawal and Time of Dipping 144

5.3 Chapter Summary 146

6 IoT-Enabled Microcontroller-Based MEMS ID Sensor Connected PoC System 147

6.1 Introduction 148

6.2 Electrochemical Impedance Measuring by using the Microcontroller-based MEMS ID Sensor Connected System 149

6.2.1 Flow Process of the PoC System Working 150

6.2.2 Electrical Circuit Drawing of PoC System 150

6.2.3 The Flow of the PoC Diagnostic System Software Process 152

6.3 Results and Discussion 156

6.3.1 Development of Calibration Curve by using the Proposed PoC Diagnostic System at Single-Frequency 156

6.3.2 Reactance Measurement Comparison between the LCR Meter and PoC Diagnostic System 158

6.3.3 IoT Data Collection from the PoC Diagnostic System 159

6.3.4 The Response of Biosensor Dependant on Single-Frequency Reactance Value for PoC System 161

6.4 Chapter Summary 163

7 Creatinine Colorimetric Assay Kit for Validation of PoC Diagnostic Prototype Sensing System 164

7.1 Introduction 165

7.2 Necessity of Experiments using Creatinine Colorimetric Assay Kit 165

7.3 Creatinine Colorimetric Assay Reagent Preparation 166

7.3.1 Creatinine Standard 166

7.3.2 Creatinine Assay Buffer 167

7.3.3 Creatine Probe 167

7.3.4 Creatininase 167

7.3.5 Creatinase 167

7.3.6 Creatinine Enzyme Mix 168

7.4 Experiments with Creatinine Colorimetric Assay Kit 168

7.4.1 Preparation of Working Standards 168

7.4.2 Preparation of Samples 168

7.5 Creatinine Colorimetric Assay Procedure 169

7.5.1 Setting the Reaction Wells 169

7.5.2 Creatinine Reaction Mix 171

7.6 Creatinine Colorimetric Assay Kit Data Analysis 172

7.7 Results and Discussion 173

7.7.1 Measurement of the Unknown Sample by using the Proposed PoC Diagnostic System at Single-Frequency and its Comparison with Standard Reference Method Creatinine Colorimetric Assay Kit 173

7.8 Chapter Summary 176

8 Conclusion and Future Work 177
8.1 Conclusion 178
8.2 Future Work for PoC Diagnostic Prototype System and Development Associated Possibilities 179

References 185

Appendix A: List of Acronyms and Symbols 209

Appendix B: List of Units 214

Appendix C: Code for Data Transmission 216

Appendix D: Copyright Forms of all Publications 218

List of Tables

Table 1.1: An average calculated GFR depending on age. 7
Table 1.2: Pattern of CKD demises (fundamental or related cause), by sex, 1998-2018 [10]. 9
Table 1.3: Pattern of CKD demises (fundamental or related cause), by sex, 1998-2018 [10]. 12
Table 1.4: Pattern of CKD demises (fundamental or related cause), by remoteness and socioeconomic area, for 2016-2018 [10]. 12

Table 3.1: The overview of the specifications of the Hioki 3536 LCR meter. 63
Table 3.2: Identification of creatinine concentrations in serum samples using a calibration curve obtained by the CNLS technology. 73

Table 4.1: A shortlist of various synthesis methods of MIP. 86
Table 4.2: The mean values of reactance and standard deviation for various serum creatinine concentrations. 116
Table 4.3: Equivalent circuit parameters for aqueous and serum samples. 121
Table 4.4: Identification of creatinine concentrations in serum samples using a calibration curve obtained by the CNLS technology. 123

Table 6.1: The mean values of reactance and standard deviation for various serum creatinine concentrations. 158

Table 7.1: Samples dilutions used to make the standard Creatinine Colorimetric Assay plot [217] 169
Table 7.2: Preparation procedure for creatinine reaction mix and background reaction mix [217] 172
Table 7.3: Measurement of an unknown sample, cross-checking and validating single-frequency PoC results with commercially available Creatinine Colorimetric Assay Kit. 174

List of Figures

Figure 1.1: A healthy vs diseased kidney [2].....2
Figure 1.2: Stages of CKD [5].....5
Figure 1.3: Relation between GFR stages and albuminuria categories [5].....6
Figure 1.4: Pattern of CKD demises (fundamental or related cause), by sex, 1998-2018 [10].....9
Figure 1.5: Pattern of CKD demises (fundamental or related cause), by age group and sex, 2018 [10].....11
Figure 1.6: Pattern of CKD demises (fundamental or related cause), by remoteness and socioeconomic area, for 2016-2018 [10].....13

Figure 2.1: An anterior, posterior and superior view of a transverse section of human kidneys [12-13].....24
Figure 2.2: An ultrastructure of the nephron [14].....25
Figure 2.3: Schematic diagram of UHPLC instrument system.....29
Figure 2.4: Schematic diagram of the GC instrument system.....30
Figure 2.5: Schematic diagram of CE instrument system.....32
Figure 2.6: Schematic diagram of fabrication of creatinine amidohydrolase nanoparticles/creatine amidinohydrolase nanoparticles/sarcosine oxidase nanoparticles/glassy carbon electrode [28].....33
Figure 2.7: Graphic diagram of chemical reaction involved in the fabrication of enzymes/carboxylated multi-walled carbon nanotubes/polyaniline/platinum hybrid electrode [44].....37
Figure 2.8: Schematic of a disposable non-enzymatic electrochemical creatinine biosensor [52].....39

Figure 3.1: Electrical field lines of the parallel-plate capacitor as well as the planar ID sensor. The evolution of electric field lines and gradual transition from parallel plate to a planar plate capacitor (a) parallel plate capacitor, (b) intermediate stage and (c) ID sensor.....49
Figure 3.2: Structural pattern of a typical ID sensor.....50
Figure 3.3: The structural pattern of the currently developed MEMS planar ID sensor.....50
Figure 3.4: Electric field having various pitch lengths [80].....51
Figure 3.5: New and improved silicon-based MEMS planar ID sensors on silicon wafer chip [84].....52
Figure 3.6: ID electrode configurations.....52
Figure 3.7: The initial prototype process of the sensor production on the silicon wafer.....54
Figure 3.8: Small-sized thin film MEMS ID sensors.....54
Figure 3.9: Steps involved in MEMS sensor development (a) and (b).....55
Figure 3.10: The shifting of the phase in the current signal concerning the applied voltage.....59
Figure 3.11: Randle's equivalent circuit along with the Nyquist plot for that equivalent circuit.....60
Figure 3.12: The block diagrammatic representation of the measurement system setup.

.....61
Figure 3.13: The process of LCR meter EIS measurement system with data acquisition computer. 62
Figure 3.14: The Hioki 3536 LCR meter [110]. 62
Figure 3.15: (a) Frequency vs resistance for 10 Hz to 5000 Hz and (b) Frequency vs reactance for 10 Hz to 5000 Hz. 64
Figure 3.16: Nyquist plot for 10 Hz to 5000 Hz. 65
Figure 3.17: (a) Frequency vs resistance for 10 Hz to 100 Hz and (b) Nyquist plot for 10 Hz to 100 Hz. 66
Figure 3.18: (a) Frequency vs resistance for 10 Hz to 100 Hz and (b) Nyquist plot for 10 Hz to 100 Hz. 67
Figure 3.19: (a) Frequency vs resistance for 10 Hz to 100 Hz and (b) Nyquist plot for 10 Hz to 100 Hz. 68
Figure 3.20: Single-frequency resistance-based sensor response at 100 Hz. 69
Figure 3.21: Repeatability of results for 6, 10 and 14 ppm creatinine concentrations.70
Figure 3.22: Graph (a), (b) and (c) showing reusability of the sensor for 6, 10 and 14 ppm concentrations. 72

Figure 4.1: The functioning principle of MIP technology (a) Schematic diagram of MIP polymerisation process, (b) Diagrammatic representation difference between MIP and NIP surfaces for target molecule specific binding. 80
Figure 4.2: Chemical structures of various functional monomers utilised in non-covalent MIP synthesis [144]. 83
Figure 4.3: Chemical structures of various cross-linkers utilised in non-covalent MIP synthesis [144]. 84
Figure 4.4: Chemical structures of reaction initiators used in non-covalent MIP synthesis [144]. 85
Figure 4.5: Structure of creatinine and other compounds used in this chemical synthesis of MIP and NIP polymers. 88
Figure 4.6: Complete illustration of MIP polymerisation and creatinine extraction process. 90
Figure 4.7: Soxhlet extraction process for MIP (a) Assembly of soxhlet extraction apparatus, (b) Creatinine molecule extraction from MIP powder kept inside cellulose thimble filter suspended in AcOH solution. 93
Figure 4.8: Nyquist plot obtained after air profiling of MEMS sensor before MIP functionalization. 95
Figure 4.9: (a) MIP functionalization process using PTL-MM01 Dip Coater instrument and (b) MIP functionalized MEMS sensor. 97
Figure 4.10: Diagrammatic illustration of the adsorption process of creatinine molecules (a) Uncoated (non-functionalized) sensing surface, (b) MIP functionalized sensing surface; (c) Addition of sample solution on the MIP functionalized sensing surface, (d) Trapping of creatinine molecules on the MIP functionalized sensing surface. A white marking on (b) and (c) indicates the MIP cavities on the sensing surface. In contrast, a blue marking on (d) indicates the creatinine molecules entrapped inside the MIP functionalized polymer cavities.....97
Figure 4.11: Different parts of the MEMS planar ID sensor having 1-1-25 configuration connected to Au pin connector jig. 99
Figure 4.12: (a) FESEM image of MIP, (b) EDAX analysis showing the atomic percentage of MIP, (c) EDAX analysis showing the weight percentage of MIP, (d) EDAX analysis graph of MIP, (e) FESEM image of NIP, (f) EDAX analysis is showing

the atomic percentage of NIP, (g) EDAX analysis showing the weight percentage of NIP, (h) EDAX analysis graph of NIP. Structural analysis of MIP and NIP. 104
Figure 4.13: (a) Spot SEM image of MIP for EDAX analysis, (b) Spot EDAX analysis graph of MIP, (c) Spot SEM image of NIP for EDAX analysis, (d) Spot EDAX analysis graph of NIP. 105
Figure 4.14: (a) Region SEM image of MIP for EDAX analysis, (b) Region EDAX analysis graph of MIP, (c) Region SEM image of NIP for EDAX analysis, (d) Region EDAX analysis graph of NIP. 107
Figure 4.15: A top view of MIP functionalized MEMS sensor's sensing surface captured using FESEM analysis. 107
Figure 4.16: FTIR spectra of (a) MIP before centrifuge, (b) NIP, (c) MIP after centrifuge and (d) MIP after soxhlet extraction. 109
Figure 4.17: Adsorption study of MIP and NIP in different concentrations. 110
Figure 4.18: UHPLC adsorption peaks value and size comparison between two different samples treated using MIP and NIP are carrying 45 ppm concentration of creatinine at λ 235 nm. 111
Figure 4.19: Adsorption of structurally similar compounds (%) performed for analysing the specificity testing of MIP for creatinine using structurally similar compounds. . 112
Figure 4.20: The spectral responses for UHPLC for all structurally similar compounds at various wavelengths. 114
Figure 4.21: EIS Measurement of creatinine samples (a) Real impedance vs frequency range at different concentrations of aqueous creatinine samples, (b) Real impedance vs frequency range at different concentrations of serum creatinine samples, (c) Imaginary impedance vs frequency at different concentrations of aqueous creatinine samples, (d) Imaginary impedance vs frequency at different concentrations of serum creatinine samples, (e) Nyquist plot for EIS profiling of functionalized sensor at different concentrations of aqueous creatinine samples, (f) Nyquist plot for EIS profiling of functionalized sensor at different concentrations of serum creatinine samples..... 118
Figure 4.22: The Nyquist plots and their corresponding Randle's model estimated by CNLS analysis. Aqueous samples carrying various concentrations of creatinine are (a) 6 ppm, (c) 10 ppm, and (e) 14 ppm. Serum samples carrying various concentrations of creatinine are (b) 6 ppm, (d) 10 ppm, and (f) 14 ppm are displayed. (g) Circuit diagram used for CNLS analysis of all aqueous and serum creatinine samples. 120
Figure 4.23: A CNLS-dependant calibration curve showing the change in reactance (X) concerning the change in the serum creatinine concentrations at 1020 Hz. 123
Figure 4.24: Repeatability and reusability of the sensor done for functionalization testing of the MEMS ID sensor using a 10 ppm serum creatinine sample. 124
Figure 4.25: Repeatability and reusability of the sensor done for functionalization testing of the MEMS ID sensor. 125
Figure 4.26: Repeatability and reusability of the sensor done for functionalization testing of the MEMS ID sensor. 125
Figure 4.27: Standard calibration curve for the LCR meter. 127
Figure 4.28: Concentration of creatinine (ppm) vs sensitivity of reactance (ΔX/Xo (%)) at fixed frequency 1020 Hz. 128

Figure 5.1: Graphics of the process of dip-coating. 132
Figure 5.2: FESEM images of the acrylic resin coated layer. 134
Figure 5.3: FESEM images of the selective MIP-polymer functionalized layer (a) MIP functionalization coat thickness, and (b) MIP functionalized MEMS ID sensors cross-section. 135

Figure 5.4: Serum creatinine analysis with the help of acrylic-coated MEMS sensor having a varying thickness of coats: (a) 55 µm, (b) 120 µm, (c) 180 µm, (d) 240 µm. 137
Figure 5.5: Serum creatinine analysis with the help of MIP functionalized MEMS sensor having a varying thickness of coats: (a) 66 µm, (b) 135 µm, (c) 200 µm, (d) 270 µm. 139
Figure 5.6: Acrylic resin-coated sensors calibration curves for the varying thickness of functionalizations: (a) 55 µm, (b) 120 µm, (c) 180 µm. 141
Figure 5.7: MIP functionalized sensors calibration curves for the varying thickness of functionalizations: (a) 66 µm, (b) 135 µm, (c) 200 µm. 142
Figure 5.8: The acrylic resin and MIP functionalized MEMS sensor sensitivity. 143
Figure 5.9: Acrylic resin and MIP functionalized sensors saturation levels. 143
Figure 5.10: MEMS sensor speed of withdrawal-dependent functionalization layer thickness. 144
Figure 5.11: A time-dependent functionalization layer thickness. 145

Figure 6.1: Flow process of the PoC system. 151
Figure 6.2: Electrical circuit diagram of PoC system. 152
Figure 6.3: The flow of the PoC diagnostic system software process for creatinine concentration calculation and data transferring to the IoT cloud server. 153
Figure 6.4: PoC diagnostic prototype system. 154
Figure 6.5: The laboratory setup of the PoC system and serum sample data measurement using the Adafruit website. 154
Figure 6.6: Functional biosensing scheme of PoC diagnostic system. 155
Figure 6.7: Standard calibration curve for the PoC system. 157
Figure 6.8: The comparison between reactance (X) measurements of the LCR meter and the PoC diagnostic system. 158
Figure 6.9: (a), (b), (c) and (d) The actual data of creatinine from the serum samples. 161
Figure 6.10: A single-frequency reactance-associated calibration curve showing the change of reactance (X) concerning the change in creatinine concentrations at 1020 Hz for the PoC system. 162

Figure 7.1: The flowchart of commercial Creatinine Colorimetric Assay. 170
Figure 7.2: The coloured product of Creatinine Colorimetric Assay Kit after incubation for an hour at 37°C. 171
Figure 7.3: The working standard plot of Creatinine Colorimetric Assay. 172
Figure 7.4: The comparison between the proposed PoC diagnostic system and Creatinine Colorimetric Assay Kit. 174
Figure 7.5: Comparative study between the PoC diagnostic system results and the Creatinine Colorimetric Assay Kit results. 175

Figure 8.1: The future of developing the PoC diagnostic prototype system for using a single drop of blood. 180
Figure 8.2: Graphic drawing of the proposed biosensor array for real-time quantification of creatinine and BUN levels from the blood sample. 181
Figure 8.3: Schematic representation of potential smart kidney health system for continuous home-based IoT-enabled monitoring of kidney health with creatinine and BUN levels as biomarkers. 182

List of Equation

(Equation 3.1)……...60
(Equation 3.2)……...69

(Equation 4.1)……...94
(Equation 4.2)……...94
(Equation 4.3) 122
(Equation 4.4)……123

(Equation 6.1)……157
(Equation 6.2)……161

1

Introduction

1.1 Introductory Details

The kidneys are pair of bean-shaped fist-size organs. Individuals kidneys produce urine by filtering excess water and wastes from their blood and controlling blood pressure. Kidneys maintain the balance of minerals (sodium, potassium, and phosphorous) in an individual's blood. Kidneys also impact the production of red blood cells and the metabolism of vitamin D, essential for bone health and strength. Kidney disease indicates that individuals' kidneys have been damaged somehow and cannot filter blood as effectively as they are supposed to do. Waste products and fluid build-up in patients' bodies are observed if their kidneys are damaged. Inflammation in the ankles, weakness, poor sleep, nausea and shortness of breath are possible side effects. The damage might worsen without therapy, and the patients' kidneys may eventually stop functioning [1]. That is a severe concern because it might put the patient in a life-threatening condition. Figure 1.1 shows the difference between healthy and diseased kidneys.

Figure 1.1: A healthy vs diseased kidney [2].

If a patient has diabetes, high blood pressure (hypertension), they are more likely to develop kidney (renal) disease. When kidney disease continues, it leads to renal failure, which necessitates two options for therapy; dialysis or a kidney transplant, and they help the patient stay alive. Kidney cysts, acute kidney disease (AKD), chronic kidney disease (CKD), kidney stones, diabetic nephropathy, glomerulonephritis, hydronephrosis, interstitial nephritis, kidney

tumour, lupus nephritis, nephrotic syndromes, renal artery stenosis, urethral stricture and kidney infections are some examples of other kidney issues [1].

If a patient's kidney condition worsens, wastes in their blood might build up to dangerously high levels, making them feel sick. Hypertension, anaemia, weak bones, poor nutritional status, blood clotting tendency, and nerve damage are possible problems. Kidney disease also raises patients' chances of heart attack, clotting in blood vessels, and brain stroke issues. These issues may develop gradually over time. Diabetes, hypertension and other illnesses can lead to CKD [3]. Kidney disease patients always feel tired and non-energetic, a poor appetite, inability in concentrating, sleeplessness, night muscular cramps, puffiness surrounding the eyes, particularly in the morning, have itchy dried skin, bad breath, a metallic taste in the mouth, need to urinate more frequently, especially at night and also pass foamy urine. Patients may also have variations in the appearance of their urine, haematuria, oedema, lower-back discomfort (around the kidneys), discomfort or sensation of scorching during micturition, and chronic hypertension. Failing of the kidneys results in accumulation of the waste products and built up excessive fluid in the blood, causing shortness of breath, nausea, vomiting, confusion, seizures, and other complicated symptoms.

1.2 Problem Definition

Kidney diseases, mainly known for one of its subtype called chronic kidney disease or CKD. It is a disorder that is characteristically known for kidneys' gradual function deterioration over time. Thus, chronic renal disease is another name for CKD. CKD denotes a group of diseases that affect the kidneys and decrease their capacity to maintain the patient healthy by carrying out the tasks outlined. If the patient's CKD worsens, the waste contents can build up to dangerously high quantities in their blood, making them unwell. Hypertension, weak bones, anaemia, poor nutritional health, and nerve damage are possible problems for the patient. CKD also raise the hazard of blood vessels and heart damage in individuals. These issues develop progressively over time.

Early identification and treatment can typically prevent the progression of CKD. When CKD worsens, it can lead to renal failure. It requires dialysis or a kidney transplant for staying alive. The strongest indicator of the functioning of kidneys is the glomerular filtration rate (GFR). Persistent proteinuria (protein in the urine) indicates the presence of CKD. Diabetes, hypertension, and a family history of renal failure are all high-risk factors. When a patient's

blood sugar level is too high, it damages several organs in their body, including the kidneys, blood vessels, heart, eyes, and nerves. When a patient's blood pressure over the walls of their blood vessels increases, hypertension develops, in case it is left uncontrolled or inadequately regulated; hypertension can lead to heart attacks, strokes, and CKD. CKD causes hypertension, and hypertension causes CKD. The kidneys can also be damaged by abusing some over-the-counter pain killers and consuming illegal drugs such as heroin. Some of the CKD symptoms can be controlled or cured depending upon patients' response to the drug regimens. In other cases, treatments can help to slow the disease and prolong life.

CKD is prevalent, expensive, and frequently diagnosed too late to be reversed. In addition, various risk factors, such as hypertension, tobacco smoking, poor glucose control, overweight and obesity, and controllability, make it primarily avoidable, as per Kidney Health Australia 2019. Additional conditions which distress the kidneys are listed below:

1) Glomerulonephritis is a collection of disorders that affect the kidney's filtration units, causing inflammation and damage. Glomerulonephritis is the third most prevalent form of kidney disease and may lead to CKD development.

2) Large cysts grow in the kidneys due to inherited disorders such as polycystic kidney disease, causing harm to the surrounding tissue that may lead to CKD development.

3) Malformations that occur during the development of a baby in the mother's womb may cause CKD. A constriction, for example, may occur, preventing normal urine outflow and causing backflow of urine up towards the kidneys. It induces infections and kidney injury, which may lead to CKD.

4) Other disorders that influence the immune system, such as lupus, may lead to CKD development.

5) In men, obstructions are caused by kidney stones, tumours, or an enlarged prostate gland may lead to CKD development.

6) Recurrent urinary tract infections may lead to CKD development.

CKD can be detected using a minimum of four basic tests: blood pressure, urine albumin, serum creatinine, and blood urea nitrogen (BUN) [4]. Following are the stages of CKD.

1) Early stages (1-2) - The kidneys can continue work when they are mildly injured. Thus there are typically no symptoms. However, it is tough to diagnose.

2) Middle stages (3-4) - The content of waste in the blood (creatinine and urea) increases, and the patient becomes sick. In addition, increased urination causes kidney function to slow down.

3) End-stage (5) - A patient needs dialysis or a transplant for staying alive.

1.3 Estimated GFR

An estimated GFR is the estimated glomerular filtration rate, which is the most accurate test for determining a patient's kidney function and kidney disease stage. The doctor can figure out estimated GFR by the creatinine test results, patient's gender, age, and body size. The GFR of a patient indicates the stage of kidney disease and aids in treatment planning. When a patient's GFR is low, it suggests their kidneys are not functioning correctly. The earlier kidney illness is discovered, the more likely it is to be slowed or stopped. Figure 1.2 displays different stages of CKD.

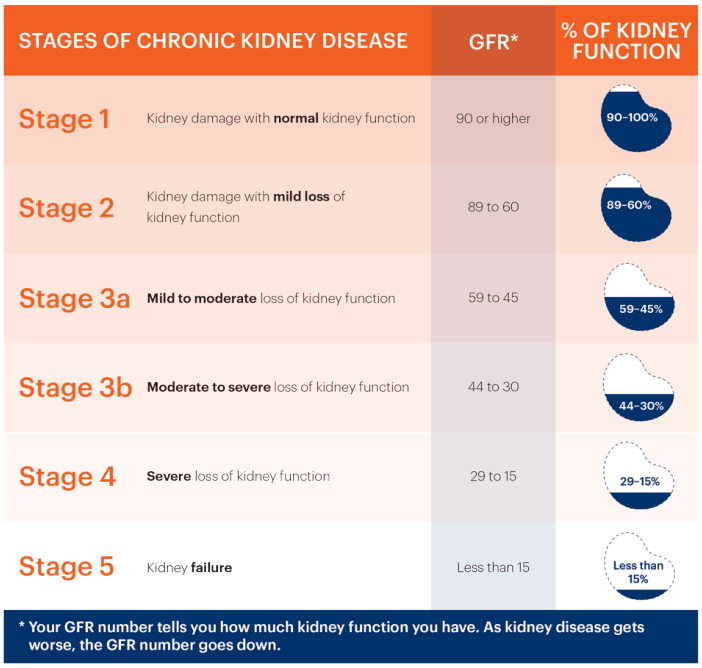


Figure 1.2: Stages of CKD [5].

CKD is diagnosed when patients estimated GFR falls below 60 for three months or when patients estimated GFR falls above 60, but they have kidney impairment (as seen by high

albumin levels in their urine). The doctor looks into the source of the patients' kidney disease and monitors their kidney function to better plan their therapy.

A basic urine test will usually be performed to see any blood or albumin within the excreted urine. Albumin is a type of protein. Albuminuria is a condition in which a patient's urine contains albumin. Kidney disease can be detected early if there is blood or protein in the urine.

People with a high level of albumin in their urine are more likely to have kidney failure as their CKD progresses (estimated using Figure 1.3)

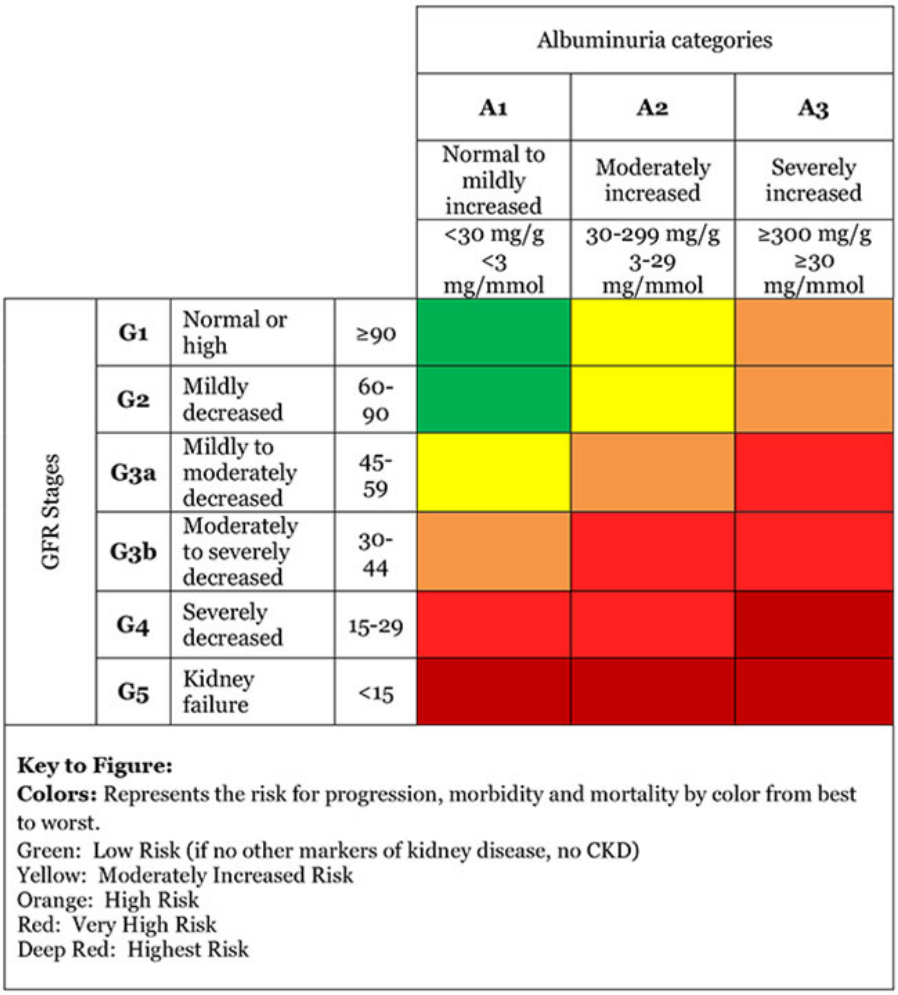


Figure 1.3: Relation between GFR stages and albuminuria categories [5].

The usual estimated GFR in adults is more than 90. However, even in those who do not have kidney disease, the estimated GFR decreases with age. Table 1.1 illustrates an average calculated GFR depending on age.

Table 1.1: An average calculated GFR depending on age.

Age (years)	Average Estimated GFR
22-29	116
30-39	107
40-49	99
50-59	93
60-69	85
70+	75

1.4 Facts and Statistics

CKD is a lesser-known illness that kills a greater number of people than prostate cancer or breast cancer. The World Health Organisation database declares CKD is a silent killer, an underappreciated public health global emergency. Approximately 90% of people with CKD are unaware that they have it. Furthermore, two out of every five persons with severe CKD are unaware of their condition. About one-half of persons with impaired kidney function (not on dialysis) are unaware that they have the condition. CKD affects around one in every three persons with diabetes and one in every five persons with hypertension. For all patients with CKD, heart disease is the principal reason for mortality. COVID-19 puts CKD and kidney transplant patients at an increased risk of developing severe complications. However, CKD is a condition that can be managed. It is probable to slow or stopover CKD advancement towards kidney failure through early diagnosis and precise treatment.

CKD affects 10% of the worldwide population. Millions of people pass away each year because of the lack of inexpensive treatment for CKD [6]. CKD was ranked 27th on the list of causes of total fatalities globally in 1990. It jumped to 18th in 2010, as per the 2010 Global Burden of Disease research. Only HIV and AIDS have seen such rapid progress up the list [7]. Over 2 million individuals worldwide are on dialysis or have had a kidney transplant to keep alive. However, this figure may solitarily reflect 10% of those who require treatment for survival [8]. The 2 million renal failure patients are treated majorly in a few countries, including

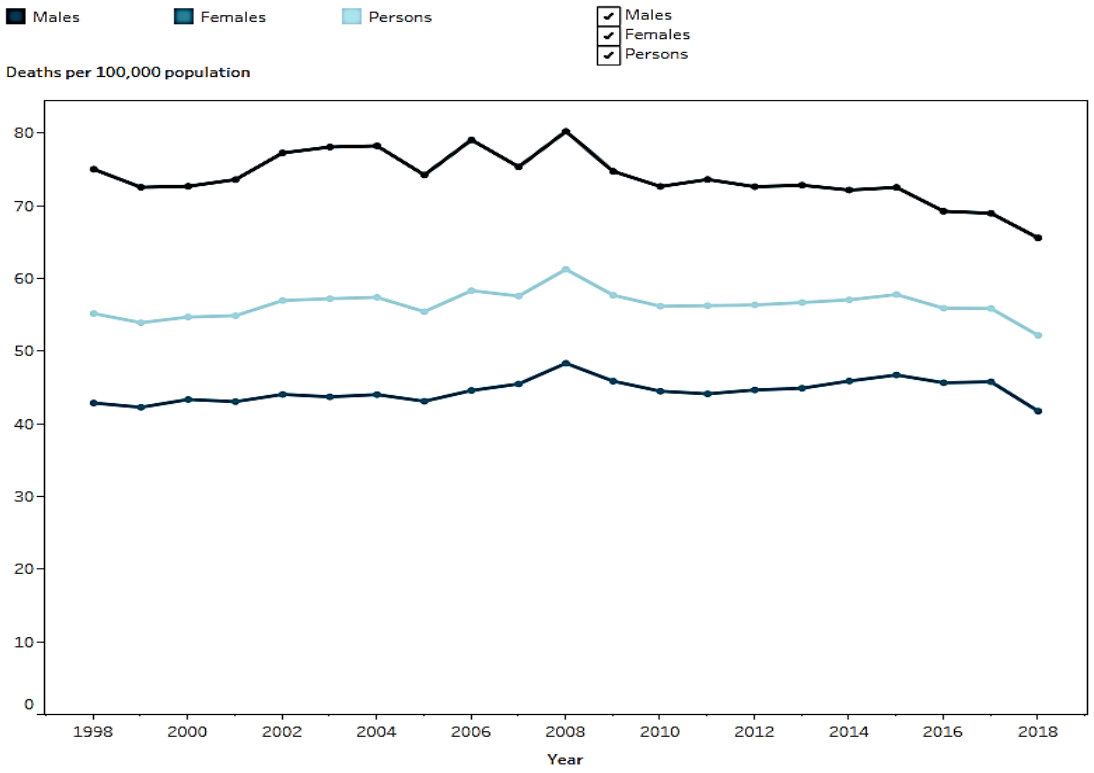
Japan, the United States, Brazil, Germany, and Italy. About 12% of the world's population lives in these five nations. Only 20% of the world's population is treated in the roughly 100 developing nations that make up more than half of the world's population [8]. Renal failure patients over 80% who obtain therapy live in wealthy nations with universal health care and substantial senior populations [7]. The number of instances of renal failure is expected to rise disproportionately in emerging nations, including China and India, where the population of aged people is rising [7].

Most patients who need dialysis or kidney transplantation live in middle-income nations. They have significant financial problems. Many people residing in other 112 nations no longer afford therapy. It results in practically 1 million people dying every year from untreated renal failure [8]. CKD treatment in the United States is expected to cost more than $48 billion per year. For caring fewer than 1% of the total Medicare-covered population, renal failure treatment requires 6.7% of the entire Medicare budget [6]. One in every five males and one in every four women in the globe aged 65 to 74 years old has CKD [6]. Noncommunicable diseases, including diabetes, CKD or heart disease, have surpassed communicable diseases including AIDS, malaria, or influenza as the leading causes of global mortality. Approximately 80% of the current burden befalls in the middle- and low-income nations, with 25% occurring among adults under 60 years [8].

The burden of disease analysis evaluates the impact of different diseases on the population's health. Various circumstances, injuries and associated risk factors play a prominent role in that. For example, the Australian Burden of Disease Study 2015 utilised various resources for quantifying these diseases' lethal and non-lethal effects.

Urinary diseases and kidneys contributed to 1.4% of Australia's disease burden in 2015. CKD signified the common burden from this disease group. It comprised 1.2% of the total burden in the year 2015. Collectively they were 0.8% in 2003 and 0.9% in 2011. From the entire CKD burden, 77% is due to the lethal burden, whereas 23% is the non-lethal burden (data as per the Australian Institute of Health and Welfare (AIHW) in 2019) [9]. In Australia, as per data analysis of the AIHW's National Mortality Database, CKD shared 11% of all demises in 2018. CKD is the fundamental cause of demise in about 3,600 demises (21% of CKD deaths). In addition, it is a linked reason of demise in a further with 13,200 demises (79% of CKD deaths).

Age-related rates for CKD is the fundamental or related cause of demise. It continued comparatively steady from the year 1998 to the year 2015, surveyed by deteriorating to 2018. Typically, CKD is the fundamental or related reason of demise in about 13,700 demises every year from 1998 till 2018. It parallels demise rates of 66-80 per 100,000 population for males. For females, it is 42-48 per 100,000 (Figure 1.4 and Table 1.2).



Trends in CKD deaths (underlying or associated cause), by sex, 1998 to 2018
Note: Age-standardised to the 2001 Australian Standard Population.
Source: AIHW analysis of the National Mortality Database.
http://www.aihw.gov.au/

Figure 1.4: Pattern of CKD demises (fundamental or related cause), by sex, 1998-2018 [10].

Table 1.2: Pattern of CKD demises (fundamental or related cause), by sex, 1998-2018 [10].

Deaths per 100,000 Population			
Years	**Males**	**Females**	**Persons**
1998	75.0	42.8	55.1
1999	72.5	42.3	53.9
2000	72.7	43.3	54.7

2001	73.6	43.0	54.9
2002	77.2	44.0	56.9
2003	78.1	43.7	57.2
2004	78.2	44.0	57.4
2005	74.2	43.1	55.4
2006	79.0	44.6	58.3
2007	75.3	45.6	57.6
2008	80.2	48.3	61.2
2009	74.7	45.8	57.7
2010	72.6	44.5	56.2
2011	73.6	44.1	56.2
2012	72.6	44.6	56.3
2013	72.8	44.9	56.7
2014	72.1	45.9	57.0
2015	72.5	46.7	57.8
2016	69.2	45.6	55.9
2017	68.9	45.8	55.8
2018	65.5	41.7	52.1

In 2018, the Australian CKD demise rates (as the fundamental or related reasons):

1) They are 1.6 times greater amongst males than females. They were 66 and 42 demises per 100,000 population, individually. In addition, age-related rates for males are more significant than for females in all groups of ages.

2) Raised with age, about half (51%) of CKD demises happening in people 85+ and above. In addition, CKD demise rates for males and females are maximum in the 85 and 85+ age groups. They were 2,100 and 1,500 per 100,000, individually. However, they are a minimum of four times more significant for males and females in the age group 75-84 years. It was 516 and 303 per 100,000, individually as per Figure 1.5 and Table 1.3.

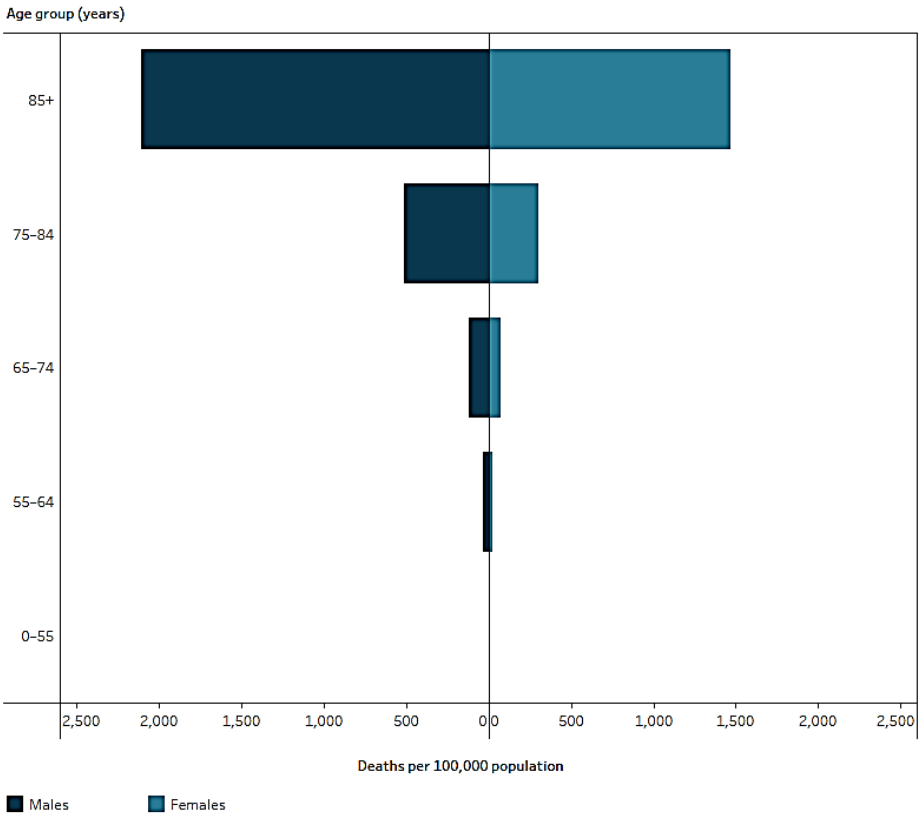


Figure 1.5: Pattern of CKD demises (fundamental or related cause), by age group and sex, 2018 [10].

CKD demise rates (as the fundamental or related reason of demise) are raised with remoteness and socioeconomic difficulties. The rates are as follows:

1) The rates are nearly double as higher in remote and very remote areas than in major Australian cities. It is 99 and 52 per 100,000 population, individually between 2016 and 2018. The variance in these demise rates is extra noteworthy for females over males. It is 2.4 times higher for females (97 and 41 per 100,000). For males, it is 1.6 times higher (103 and 68 per 100,000).

2) It is early double in the lowest socioeconomic group than the highest socioeconomic areas. It is found to be 70 and 38 per 100,000, individually in 2018. This variance is parallel for females and males as per Figure 1.6 and Table 1.4.

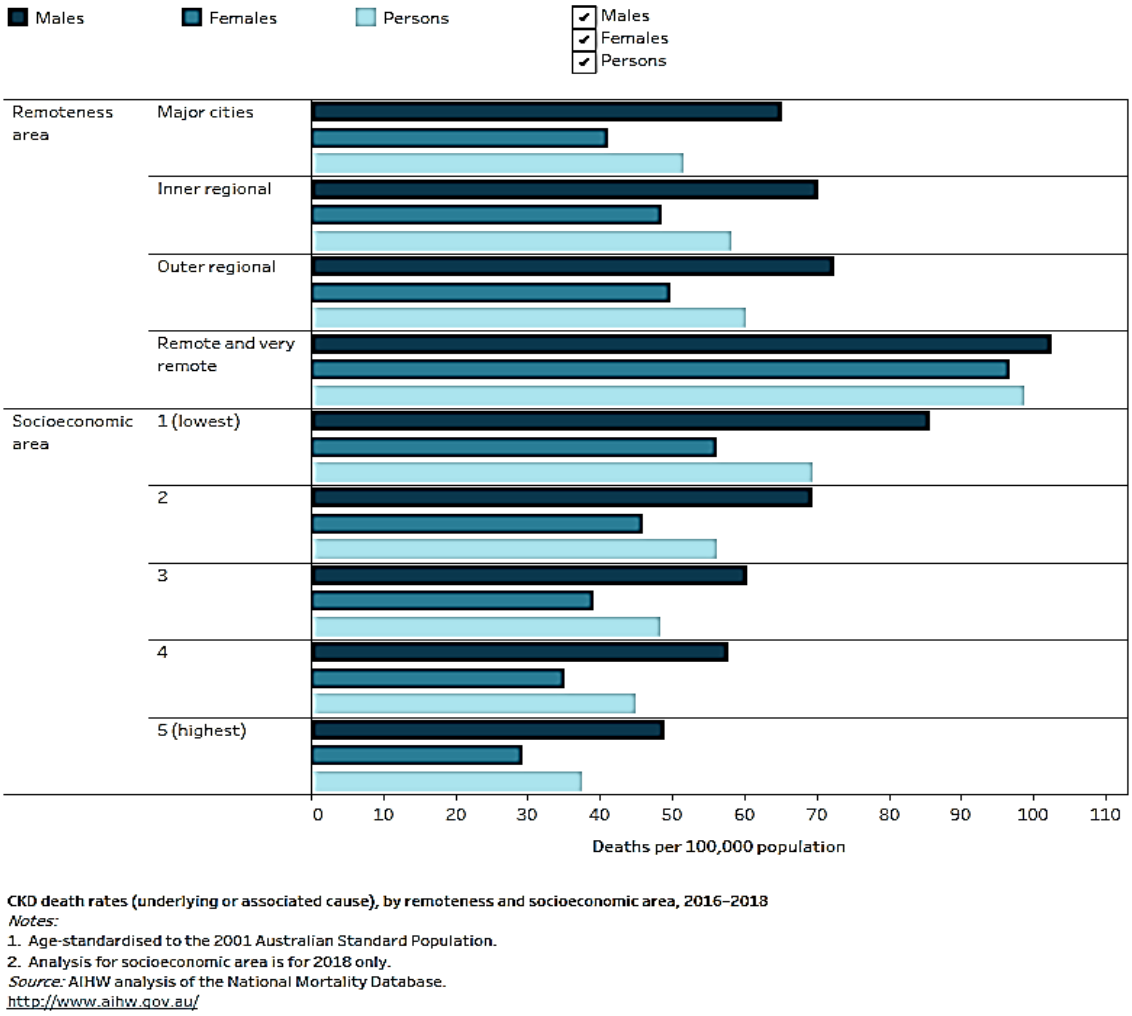
Table 1.3: Pattern of CKD demises (fundamental or related cause), by sex, 1998-2018 [10].

Age Group (years)	Males (per 100,000 Population)	Females (per 100,000 Population)
85+	2,108	1,465
75-84	516	303
65-74	123	73
55-64	36	22
0-55	3	2

Table 1.4: Pattern of CKD demises (fundamental or related cause), by remoteness and socioeconomic area, for 2016-2018 [10].

Areas		Deaths per 100,000 Population		
		Males	Females	Persons
Remoteness area	Major cities	65.3	41.1	51.5
	Inner regional	70.3	48.5	58.2
	Outer regional	72.5	49.8	60.2
	Remote and very remote	102.6	96.7	98.7
Socioeconomic area	1 (lowest)	85.7	56.3	69.5
	2	69.4	45.9	56.3

	3	60.5	39.1	48.4
	4	57.9	35.1	44.9
	5 (highest)	49.0	29.2	37.6



CKD death rates (underlying or associated cause), by remoteness and socioeconomic area, 2016–2018
Notes:
1. Age-standardised to the 2001 Australian Standard Population.
2. Analysis for socioeconomic area is for 2018 only.
Source: AIHW analysis of the National Mortality Database.
http://www.aihw.gov.au/

Figure 1.6: Pattern of CKD demises (fundamental or related cause), by remoteness and socioeconomic area, for 2016-2018 [10].

Amid 2016-2018, 1,500 CKD-associated demises (as the fundamental and/or related reason) amongst Indigenous Australians have 72 per 100,000 (Queensland, New South Wales, South Australia, Northern Territory, and Western Australia only).

Subsequently regulating for alterations in the age groups of the populations:

1) The demise rate amongst Indigenous Australians is nearly four times greater for non-Indigenous Australians. It is 186 and 52 per 100,000, correspondingly.

2) This difference is more significant for females than males. It is above four times more significant for Indigenous females (186 and 41 per 100,000, correspondingly). It is also thrice greater for Indigenous males (189 and 66 per 100,00, correspondingly).

CKD is frequently recorded as a related reason when additional circumstances are the fundamental reason for demise. For example, in 2018, there were 13,200 demises where CKD is documented as a related reason for demise. In Australia, the CKD is greatest frequently recorded as a related reason for demise due to:

1) Cancers (19%).

2) Illnesses of the cardiovascular system is 37%: mainly coronary heart disease as it is 19% and other forms of heart illness is 11%.

3) The respiratory system's illness is 8.8%: mainly chronic obstructive pulmonary disease is 3.9%, and pneumonia is 2.0%.

4) Nutritional, endocrine, and metabolic illnesses is 8.8%. Mainly type 2 diabetes as it is 3.7% and unspecified type of diabetes is 2.7%.

1.5 Diagnosis

CKD is characterised by a lack of symptoms or nonspecific symptoms such as fatigue, headaches, and nausea. A physical examination and a review of the patient's medical history may be the first steps taken by the concerned doctor. Then, the concerned doctor selects the diagnostic tests for CKD based on the patient's symptoms, medical history, age, daily routine, and overall health. Urine tests, blood tests, Normalised Protein Nitrogen Appearance (nPNA) test, Subjective Global Assessment (SGA) test, body weight measurement, blood pressure test, imaging tests, and biopsy are the list of tests used to diagnose CKD.

1.6 Research Motivation

The signs of deteriorating kidney functioning and progress of CKD are not visible early on; therefore, early detection and treatment is vital and can typically stop CKD from worsening

and lower the chance of renal failure. Kidney failure can be avoided or slowed through medication and lifestyle modifications and an early-stage referral to a nephrologist. The precise detection of existing patients' CKD stage identification and stopping further kidney functional deterioration is most important. It helps to increase patients' quality of health and overall lifespan. The early identification can significantly minimise the risk of CKD development by initiating therapy at the earliest stage with the help of prognostic and prophylactic care when there is a chance to stop CKD from worsening [3].

The doctor can figure out estimated GFR by the creatinine test results, patient's gender, age, and body size. Finding out the GFR of a patient is vital as it indicates about kidney disease stage and aids in treatment planning. The creatinine levels aid in understanding levels of GFR; creatinine is of utmost essential biochemical marker for kidney health. The creatinine level analysis, GFR calculation by the concerned nephrologist, early detection of CKD followed by healthier and longer life of the patient are interdependent.

If creatinine level quantification is made possible to the patient from home using a PoC diagnostic device, kidney health will become much more manageable. Therefore, the development of a PoC diagnostic prototype system for kidney health monitoring is essential.

The following are the primary factors that influenced my decision to develop a PoC diagnostic prototype system for kidney health monitoring:

1) The limits of present technologies for early identification of CKD;

2) The lack of a household PoC diagnostic prototype system that everyone can use in the future for a routine check-up of their kidney health; and

3) The high expenses associated with CKD and its healthcare measurement.

1.7 Research Objectives

This research study has the objective to design, develop, and assess a portable smart PoC diagnostic prototype biosensing system for early diagnosis of kidney function loss and/or detection of CKD by analysing and quantifying a biochemical marker (creatinine) from a serum sample. The PoC diagnostic prototype biosensing system needs to be user-friendly, highly selective and rapid than the available techniques. The system needs to be precise, portable,

repeatable, reusable, time and cost-effective, robust, internet connectable, helpful in long-distance healthcare monitoring, which is supportive for home-operable needs.

For fulfilling the research objectives, over the planar MEMS-based ID sensors' sensing area, the selectivity of the analyte creatinine will be added. The EIS approach will be utilised to assess the SUT. The electrochemical characteristics, which are proportional to the concentration of the target, i.e. creatinine molecule, will be detected after pipetting the SUT on the creatinine selective sensing surface.

An IoT-based embedded PoC diagnostic prototype system can measure the serum SUT's creatinine level and send the data to an IoT-enabled complimentary cloud server. The user's data will be received, collected systematically. Further, it will construct a patient's kidney health database using the developed portable PoC diagnostic prototype system.

1.8 Research Contributions

The development of a smart sensing system capable of detecting and quantifying creatinine molecules from the heat-inactivated serum samples is the PoC diagnostic prototype system's key contribution. The smart sensing system relies upon the designing and developing of a smart planar MEMS ID sensor capable of quantifying the target molecules, specifically the creatinine molecules. The developed PoC diagnostic prototype system's low cost, time-efficient, and resilient qualities distinguish it. This study's main contributions are summarised below:

1) To use the utmost appropriate MEMS ID sensor, which permits the fringing electric field to penetrate deep enough to facilitate bulk-sample analysis. Characterisation of the sensor and finding the operating frequency range. The academic collaboration between Saudi Arabia's KAUST and Macquarie University resulted in developing the planar MEMS ID sensor utilised in this study.

2) To develop and customise a strategy for inducing creatinine molecule selectivity in the smart MEMS ID-based sensing system.

3) To develop synthetic polymer for specifically adsorbing and quantifying creatinine molecules from the serum samples carrying various creatinine concentrations.

4) To construct a portable PoC diagnostic prototype system for the quantitative measurement of creatinine. To design and construct an IoT-based microcontroller-connected sensing system.

5) To cross-check and validate the functioning of the developed smart PoC diagnostic prototype system using a reference standard technique of Creatinine Colorimetric Assay Kit.

1.9 Current Status of Developed PoC Diagnostic Prototype System for Creatinine Detection

The developed PoC diagnostic prototype system is a low power sensing system, it works for one week, and later it needs battery charging. The system performance is uniform and measures a single serum sample in 5 minutes. The MIP functionalized MEMS sensor attached to the developed PoC diagnostic prototype system is constantly reusable for five sample measurements. The system provides stable output between the 10°C-40°C temperature range. The output of the PoC system is cross-verified by the standard method of Creatinine Colorimetric Assay Kit. The safe data server is effortlessly accessible to the concerned oncologist/nephrologist or general physician from a random remote destination. They can precisely take prophylactic measures for their patients by monitoring the creatinine serum sample levels from a long distance. The concerned physician can notify their patient in case of necessity. The developed Internet of Things (IoT)-based PoC diagnostic prototype system is creatinine specific, precise, easily operable, accurate, portable, patient-friendly, internet connectable, rechargeable, reusable, cost and time-effective. The developed PoC diagnostic prototype system successfully detects and quantifies creatinine molecules from the heat-inactivated serum samples for kidney health monitoring. Thus all the research objectives are achieved successfully.

1.10 Thesis Outline

The thesis is divided into a total of eight chapters, and they are as follows:

Chapter 1

This chapter introduces human kidney diseases, their diagnosis techniques. It describes the research motivation, objectives, contributions and the current status of the developed PoC diagnostic prototype system for creatinine detection. It also shows the direct relationship

between the precise detection of creatinine in the early CKD diagnosis and its assistance in starting patient therapy.

Chapter 2

This chapter delivers a detailed literature review of the existing biochemical markers of CKD. It emphasises current advances in CKD biosensing techniques for monitoring CKD. It also focuses on the biochemical markers and biomechanical kidney function evaluation.

Chapter 3

In this chapter, the operation of a developed planar MEMS ID sensor is presented. It also explains the fundamental theory of EIS. Finally, the information from the SUT is extracted and converted into an electrical signal for further examination using an experimental setup.

Chapter 4

This chapter explains the process to synthesise synthetic polymer with MIP and NIP in detail. This chapter also covers a revolutionary creatinine quantification approach that combined EIS with MIP technology.

Chapter 5

This chapter explains how the effect of acrylic coating and MIP functionalization (selective coating) thickness over the sensitivity of planar MEMS ID sensors in the detection of heat-inactivated human serum samples carrying varying concentrations of creatinine.

Chapter 6

This chapter explains the design and application of a portable IoT-based microcontroller-connected PoC diagnostic prototype system. The PoC diagnostic prototype system is capable of quantifying creatinine levels from the heat-inactivated human serum samples spiked with creatinine and is capable of sending the processed data to a cloud server powered by IoT.

Chapter 7

This chapter explains the steps involved in cross-checking and validating the results received from the developed PoC diagnostic prototype system via using the standard technique of Creatinine Colorimetric Assay Kit.

Chapter 8

This chapter summarises the study findings in the conclusion section and discusses the developed PoC diagnostic prototype system's future potential.

2

Literature Review

Publications about this chapter:

- **Prabhu S.N.**, Gooneratne C.P., Hoang K.A., Mukhopadhyay S.C., Davidson A.S., Liu G., (**2021**), Interdigital Sensing System for Kidney Health Monitoring. In: Mukhopadhyay S.C., George B., Roy J.K., Islam T. (eds) Interdigital Sensors. Smart Sensors, Measurement and Instrumentation, vol 36. **Springer**, Cham, doi: 10.1007/978-3-030-62684-6_11

2.1 Introduction

The details described in the previous chapter confirm that the CKD is the risk factor if the individuals have hypertension, diabetes, established heart problems (heart attack or heart failure), brain stroke, obesity, mostly 60+ years age, family history of kidney failure, and/or having Aboriginal or Torres Strait Islander origin.

Hypertension is a condition in which the pressure within the arteries carrying blood from patients' hearts towards their whole body rises. If it is not treated, it can harm patients' kidneys permanently. Hypertension can also occur due to kidney disease or stenosis of the renal artery. Patients' kidneys regulate the quantity of water in their blood vessels. It generates renin, a hormone that aids in blood pressure management.

Diabetic nephropathy is a form of kidney disease that affects 20 to 30% of patients with diabetes. It is a severe condition that can exacerbate other diabetes problems, including nerve and eye damage. It raises the circulatory system's risk in kidney disease patients. Diabetic nephropathy is recognized as or end-stage kidney disease (ESKD) as well. It is a well-known reason for kidney failure.

In patients having CKD and circulatory disease is the principal reason for mortality. Patients with chronic renal disease are two to three times additional prone than the overall population to have cardiovascular issues such as heart attack, angina, heart failure and/or brain stroke [3].

Factors common to CKD and cardiovascular disease, such as hypertension, contribute to this elevated risk. However, researchers have investigated that CKD is an issue for developing circulatory disease in and of itself. A history of circulatory system disease is a known issue for the progression of CKD.

The kidneys are responsible for regulating water and salt levels, removing waste, and producing various hormones. CKD raises the issue of heart disease in a variety of ways, such as:

1) High blood pressure - Renin, a hormone produced by the kidneys. It helps in controlling blood pressure. It also aids in the regulation of salt and fluid levels in the body.

2) Heart strain - Excess fluid in the body puts strain on the heart and raises the risk of problems, including left ventricular hypertrophy, leading to heart failure.

3) Stiff arteries - The kidneys produce a calcitriol hormone which aids in regulating calcium use throughout the body. Calcified arteries and heart valves can occur in CKD patients, possibly resulting from inefficient calcitriol hormone production.

4) Hyperlipidaemia - Some patients with CKD have high levels of LDL cholesterol, which may cause hormonal imbalances. LDL cholesterol levels that are too high are a known risk factor for cardiovascular disease.

5) Blood clots – Patients with CKD and/or kidney failure have a higher risk of blood clotting. A clot is also called a thrombus, gets trapped in a blood artery. A thrombus is a rigid solid structure. It can stop the flow of blood. Many problems, such as venous thrombosis, heart attack, and/or brain stroke, are increased due to thrombus. A clot might cause high blood pressure in one of the renal arteries [3].

The diagnosis of CKD is generally made using a list of blood tests, urine tests, SGA tests, general check-ups, imaging tests and biopsies. Blood tests, urine tests, and imaging tests are currently treated as the gold standard for assessing the CKD diagnosis. CKD can be detected using a minimum of four basic tests: blood pressure, urine albumin, serum creatinine, and BUN [4].

However, the blood tests, urine tests, and imaging tests are internationally accepted standard diagnostic techniques; they have some undisputable limitations. These diagnostic analysis techniques are costly, time-consuming. They are not available 24/7 in most of the hospitals. The patient cannot interpret their report at home post receipt of their reports, and they need to visit a concerned nephrologist to understand their CKD status. Although the blood tests and urine tests are susceptible, the imaging test reports require a few months to years to observe the differences between past and present CKD stages. To fulfil this, a real-time assessment of the CKD stage is essential.

Biochemical markers of CKD stages can be utilised to manage and monitor kidney disorders such as CKD since they can offer a real-time diagnosis of the stable/declining stages of the CKD.

This chapter will provide a literature review of the structure and functioning of kidneys, kidney health, creatinine as a biochemical marker for CKD stages diagnosis from blood and urine. It also provides details about current biosensors for monitoring kidney diseases.

2.2 Structure and Functioning of Kidneys, Kidney Health

The human kidneys are two organs located on the right and left sides of the body inside the retroperitoneal space. Figure 2.1 shows an anterior, posterior and superior transverse view of the human kidneys. The kidneys filter blood plasma, thus removing the waste products of metabolism and toxic substances from the body, followed by excreting them from the urine. This process also involves maintaining the constant composition and volume of the blood, called homeostasis [11].

During this process, blood enters the kidney via the renal artery, which further divides into smaller arteries. The arteries further divide into arterioles. The arterioles come in contact with the structural and functional units of the kidneys called nephrons. Figure 2.2 depicts the ultrastructure of the nephron. The nephrons help in the process of filtration of blood and the formation of urine. The filtered blood then passes through venules, followed by several large veins and finally leaves the kidney via the renal vein. The collection of formed urine is done within collecting ducts, and further, it goes out of the kidney with the help of ureters. The single kidney consists of about a million nephrons. A nephron has two parts, i.e. a Bowman's capsule as well as a renal tubule. The renal tubules of numerous nephrons join a shared collecting duct. In total, three steps are involved in urine formation. First, the glomerular ultrafiltration takes place within the Bowman's capsule. Second, the tubular reabsorption and secretion and takes place within the renal tubule. In the third step, the collecting duct helps in the maintenance of the level of water inside the body.

Blood arrives in the Bowman's capsule through the afferent arteriole. Then it passes through a bundle of a network of capillaries called the glomerulus. Later it leaves the Bowman's capsule through the efferent arteriole. The lumen size of the afferent arteriole is noticeably larger than that of the efferent arteriole. This creates a flow of blood with a big size inlet and a smaller size outlet resulting in the hydrostatic blood pressure within a bunch of capillaries. It is significantly higher than normal blood pressure. The osmotic and hydrostatic pressures within the Bowman's capsule allow the passing of water and solutes from blood plasma via an ultrafiltration membrane inside the Bowman's capsule space of nephrons. The filtration membrane performances are similar to a sieve permitting expressly passing of small molecules, including water, glucose, amino acids, inorganic ions, and various metabolic waste products, including BUN and creatinine.

Along with ultrafiltration, the kidney has other essential functions. For example, the kidney converts a precursor of vitamin D in its active form, calcitriol. It also synthesises the hormones erythropoietin and renin. The fluid passed from nephrons is called a glomerular ultrafiltrate. The net quantity of produced filtrate every minute is called a glomerular filtration rate. The glomerular filtration rate is constant via multiple feedback mechanisms within the kidneys called renal autoregulation. The glomerular filtration rate is also under the freedom and control of hormones, and it is usually accomplished with the help of rhythmic constriction and dilation of the afferent arteriole.

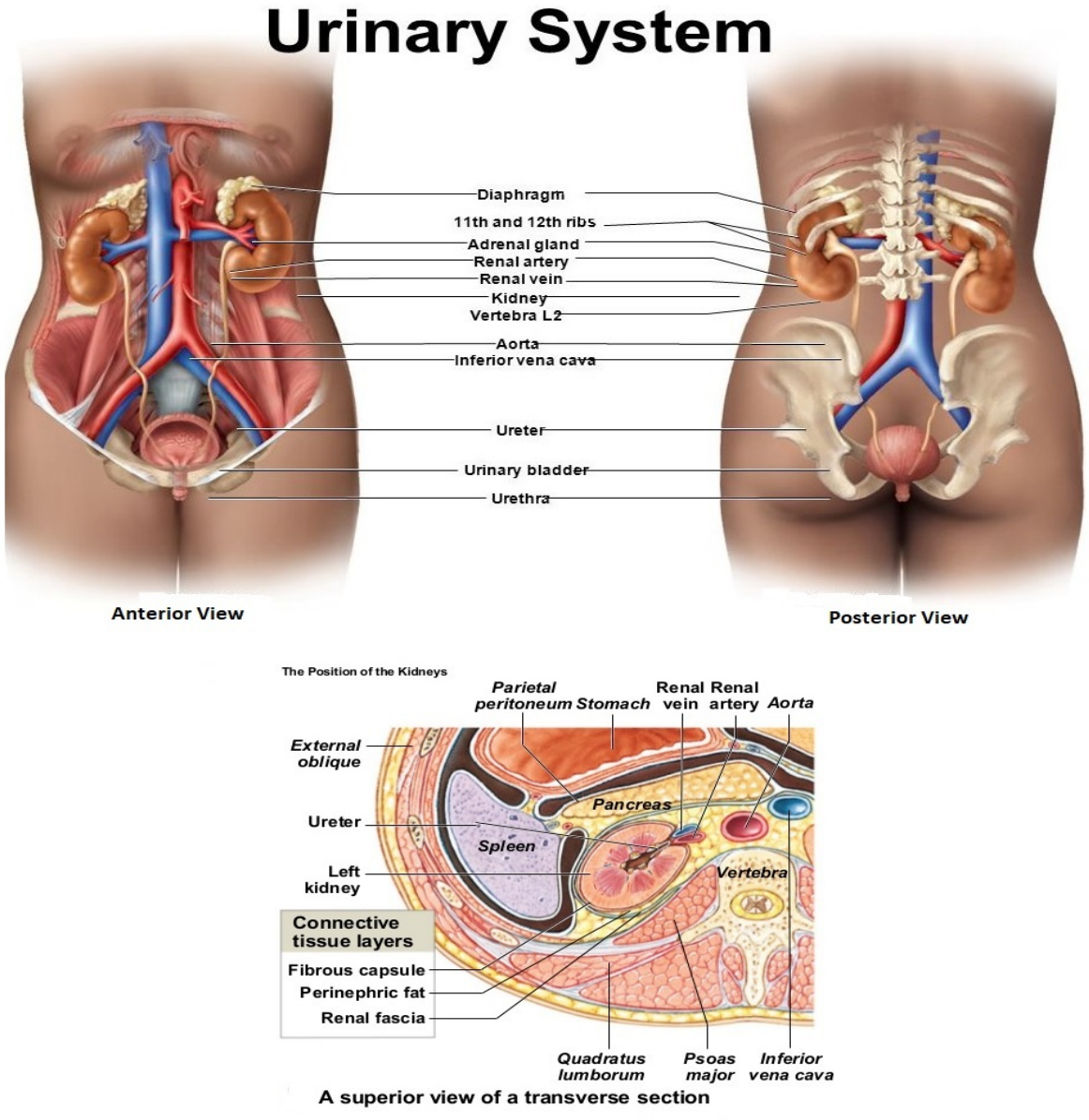


Figure 2.1: An anterior, posterior and superior view of a transverse section of human kidneys [12-13].

It also causes the fall and rise of glomerular blood pressure. A healthy person generates about 150-180 L of total glomerular filtrate. Just 1% of this produced filtrate is considered excreted urine, whereas the remaining 99% is reabsorbed in the blood as the filtrate passes from the lengthy renal tubule. Again, it is due to the efferent arteriole. Just after leaving the Bowman's capsule, the filtrate passes through the branches that protrude out from the capillaries' network, called peritubular capillaries, bordering the renal tubule. The initial part of the renal tubule, called the proximal convoluted tubule, reabsorbs around the two-thirds amount of the filtrate. During this, water and solutes are transferred via the epithelial cells, making the lining of the tubule inside the extra tubular space.

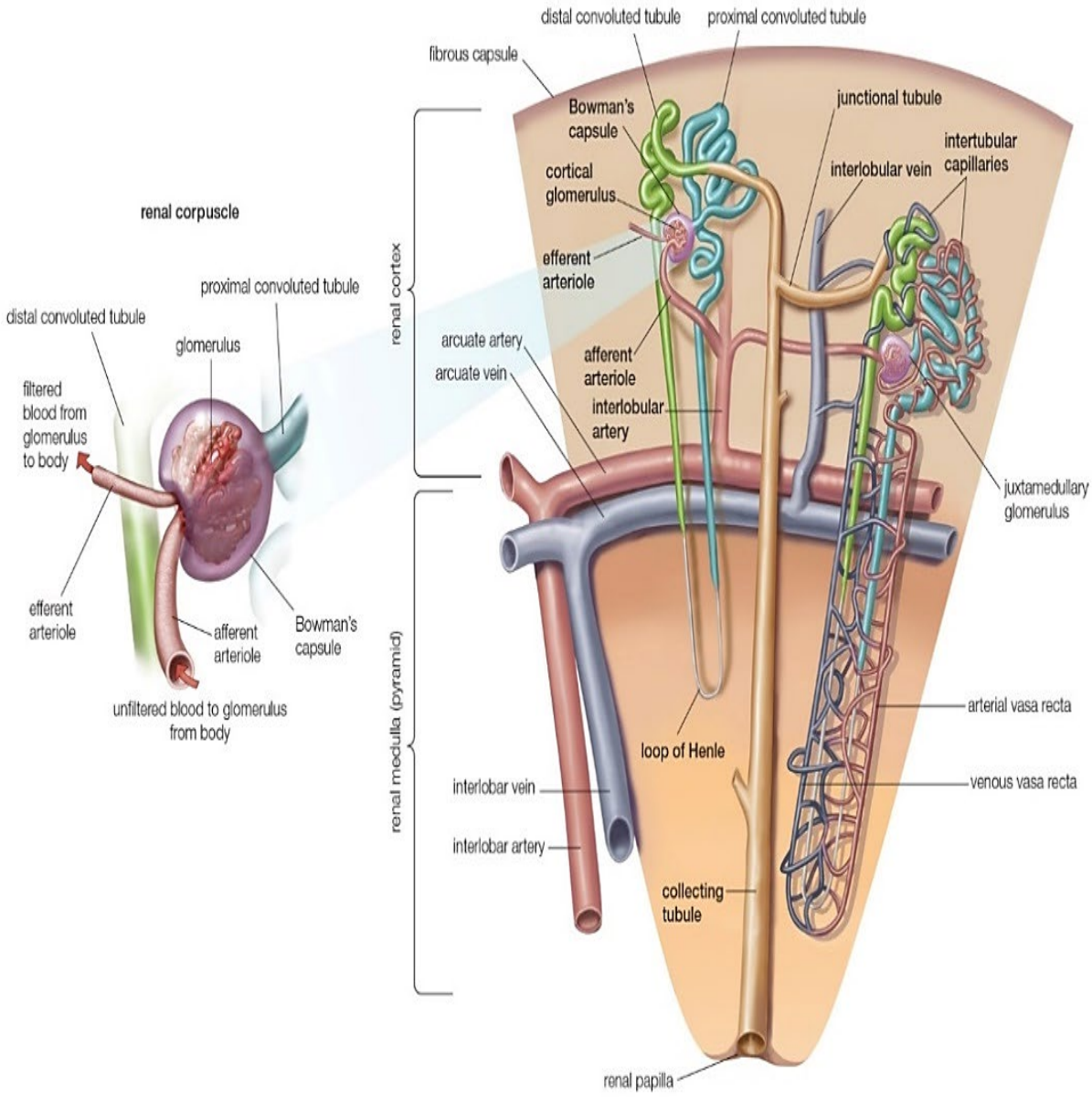


Figure 2.2: An ultrastructure of the nephron [14].

Further, it is taken care of by the peritubular capillaries. The absorption of sodium is of utmost significance because it generates an osmotic pressure that allows water and an electrical gradient that passes negatively charged ions. The levels of sodium within the epithelial cells are maintained at lower levels because of the sodium-potassium pumps, which continually pump sodium ions outside within the extracellular space. This phenomenon generates a concentration gradient that provides sodium diffusion from the tubular fluid inside the cells. The absorption of sodium ions is performed by symport proteins. They also bind glucose molecules and a few other solutes. Due to this, almost all glucose and amino acid molecules are reabsorbed inside the blood. In addition, around half of nitrogenous waste products are reabsorbed into the bloodstream.

The kidneys reduce levels of metabolic wastes inside the blood to a homeostatic level but do not entirely remove their presence from the body. Few reabsorptions are also taken care of by the paracellular route via the tight junctions within the epithelial cells. Simultaneously, the tubular secretion wherein the further waste products such as drugs and other solutes leave the bloodstream to join the tubular fluid also occurs. The reabsorption and secretion processes occur within the Loop of Henle, a nephron loop, and inside the distal convoluted tubule. These parts of the renal tubule have few more vital functions. The critical function of the Loop of Henle is to generate and preserve an osmolarity gradient within the medulla region of the kidney that permits the collecting ducts in the concentration of the urine at the further phase. The ascending limb of the Loop of Henle dynamically pumps sodium ions out, thus creating the medulla region of the kidney into the salty region. The descending limb of the Loop of Henle is permeable for water but significantly not so permeable for sodium ions. Water leaves the tubule by the process of osmosis. Later the remaining filtrate becomes increasingly concentrated as it further moves towards the base level. The ascending limb of the Loop of Henle is permeable to ions but not with the molecules of water. Due to this, the concentrated filtrate loses sodium ions as it moves upward and converts into increasingly and gradually diluted at the topmost region of the Loop of Henle. The medulla region is in balance with the Loop of Henle, and it also has the identical gradient of salinity, i.e. salted at the lowest region. Reabsorption and the process of secretion within the distal convoluted tubule are underneath the regulation of several hormones. This one is the kidneys' response towards the needs of the body and regulations in the urine composition as per necessity. The collecting duct collects tubular fluid from numerous nephrons. The fundamental functioning of the collecting duct is concentrating the urine as well as the conservation of water inside the body. This is made

probable because of the gradient of osmolarity formed through the Loop of Henle. The filtrate gets salty within deeper regions of the medulla. The filtrate lacks the amount of water content as it passes further downwards towards the collecting duct. The hormones also control the collecting duct. Under hormonal control, it can regulate the quantity of reabsorbed water according to the body's hydration status. During the time of dehydration, additional water is reabsorbed into the blood, and a minor amount of water is excreted via the formation of concentrated urine and vice versa [11].

CKD is a steady loss of renal function, characteristically developed over months or years. Several conditions, including internal and external to the kidneys, can be a reason for increasing injury to the kidneys over the period, resulting in CKD. Among these, the utmost known reasons are diabetes as well as hypertension. Both directly injure blood vessels inside the kidney and also destroy the renal tissue. An AKD, if not fully cured, might also develop in CKD. Symptoms of CKD develop gradually over a while, developing from renal insufficiency towards ESKD. Every so often, the primary loss of renal tissue will not result in any noticeable symptoms. It is due to the remaining healthy tissue. They become further active, and by increasing their functionality, they compensate for the loss of tissue, which is also called renal adaptation. Generally, noticeable symptoms are observed when a significant part of kidney function is lost by that time. Therefore, early detection of CKD is essential.

2.3 Biochemical Markers of CKD

Creatinine and BUN are the main biological waste products excreted by kidneys. The build-up of poisonous creatinine and nitrogenous urea wastes causes a series of indications like vomiting, nausea, confusion, and seizures. The CKD results in rising levels of serum creatinine and BUN. Therefore, creatinine and BUN are considered biochemical markers of kidney functioning.

2.3.1 Creatinine

Creatinine is an instinctively formed non-enzymatic degradation by-product of a substance called creatine [15]. It is formed during the daily muscular building and usage process. The kidneys eliminate creatinine from the blood, but as kidney function declines, the creatinine level rises. Kidneys eliminate creatinine by cleaning blood plasma through

glomerular ultrafiltration for maintaining homeostasis within the body. The amount of creatinine in the serum helps measure the GFR and thus acts as the most important biochemical marker of kidney functioning [15]. GFR, determined by a blood test for creatinine; thus, it is the most important biochemical marker than the BUN blood test. It is the most significant indicator of kidney function, indicating how much renal function a patient has. GFR over 90 mL/minute/1.73 m^2 is considered normal. If the GFR is consistently lesser (60 mL/minute/1.73 m^2) for a minimum of three consecutive months, it concludes that the patient has CKD. Patients need to consult a nephrologist if their GFR is less than 30. A GFR of less than 15 suggests that the patient needs to begin renal failure therapy. A nephrologist will advise the patient on various options for the treatments for kidney failure, including dialysis or kidney transplantation [16]. The normal creatinine range in human serum is 0.5-1.1 mg/dL (5-11 ppm) for females whereas it is 0.6-1.2 mg/dL (6-12 ppm) for male. It is due to the gender-based body composition of males having more mass of lean muscles [17]. Levels over 15 ppm in adults need medical attention, and levels over 59 ppm indicate severe damage to one or both kidneys.

2.3.2 BUN

The BUN level in the blood is also checked for the presence of urea (a waste product made by the breakdown of proteins). The BUN test determines how much nitrogen is present in the patient's bloodstream due to urea waste. The liver produces urea disseminates into blood, which is then excreted in the urine, thus helpful in the detection of kidney functioning. Urea nitrogen is a typical waste product in individuals' blood. It is generated due to the protein breakdown from the meal as per individuals' metabolism. It is typically eliminated from individuals' blood with the help of their kidneys. As kidney function declines, the level of BUN increases. BUN levels can also increase if the patient consumes extra proteins. It declines if they consume a lesser quantity of proteins. Normal BUN levels range from 7 to 20 mg/dL (2.5 to 7.1 mmol/L) [16].

2.4 Analytical Methods for the Measurement of Creatinine, a CKD Biochemical Marker

Creatinine levels play an essential role in detecting GFR value. This research discusses creatinine as a CKD biochemical marker specific and sensitive analytical method, including

different segregation and detection techniques available in the scientific world to measure creatinine, a CKD biochemical marker. UHPLC, gas chromatography (GC), and capillary electrophoresis (CE) are practical CKD biochemical markers detection techniques.

2.4.1 UHPLC

The UHPLC is a powerful and accurate technology. It is helpful for qualitative separation and quantitative measurements that are primarily utilised in biological materials and pharmaceutical molecules to identify a single or mixture of particles. The stationary phase is contained in a column. The sample is presented inside the mobile phase by an injector. The mobile phase is driven through the column and detector by a pump, and the particle retention period is shown by a detector. The period at which a specific molecule elutes from the column is called the retention time. It is a distinguishing feature of a particle. UV spectroscopy, electrochemical, fluorescence, and mass spectrometric detectors are the most frequently used detectors in UHPLC instruments [18-19].

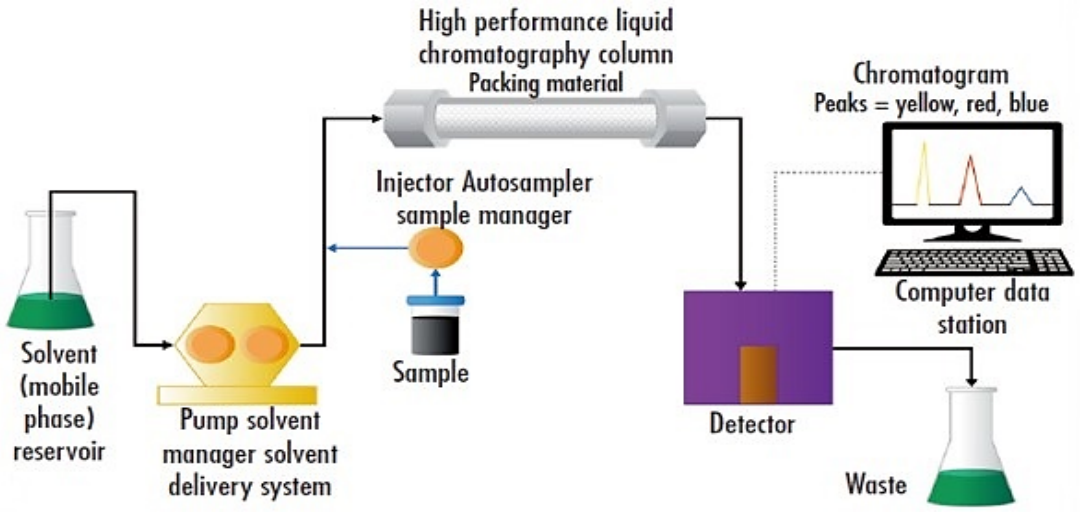


Figure 2.3: Schematic diagram of UHPLC instrument system.

Several UHPLC instruments subtypes are available depending on the stationary phase system: normal phase, size-exclusion, reverse phase, and ion-exchange UHPLC. The schematic diagram of UHPLC instrumentation is shown in Figure 2.3. Pump, injector, column, detector, and data collection system are all included in the UHPLC instrument system. The separation occurs in the UHPLC column. It is regarded as one of the essential components of the UHPLC system.

2.4.2 GC

GC is a form of chromatography mainly used in chemistry. It is utilised for separating and analysing substances. GC is typically used to find out the purity of chemicals. It is also used for separating the various components from a combination of components [20-21]. Gas-liquid partition chromatography or vapour-phase chromatography are other terms for GC [20].

The technique of separating chemicals is based on a mixture. It is performed by injecting a gaseous or liquid sample into a mobile phase. It is commonly referred to as the carrier gas. It is done by passing the carrier gas through a stationary phase in GC. In most cases, the mobile phase is an inert or non-reactive gas like argon, helium, hydrogen or nitrogen [22]. The stationary phase is a tiny layer of viscous liquid on a solid particle surface on an inert solid support. It is inside a glass or metal tubing column [23]. In specific columns, the stationary phase is made of solid particles [23]. The gas-phase goes via a glass or metal column housed in an oven, where the gas temperature may be adjusted. A computerised detector monitors the eluent exiting the column [22]. The schematic diagram of GC instrumentation is shown in Figure 2.4.

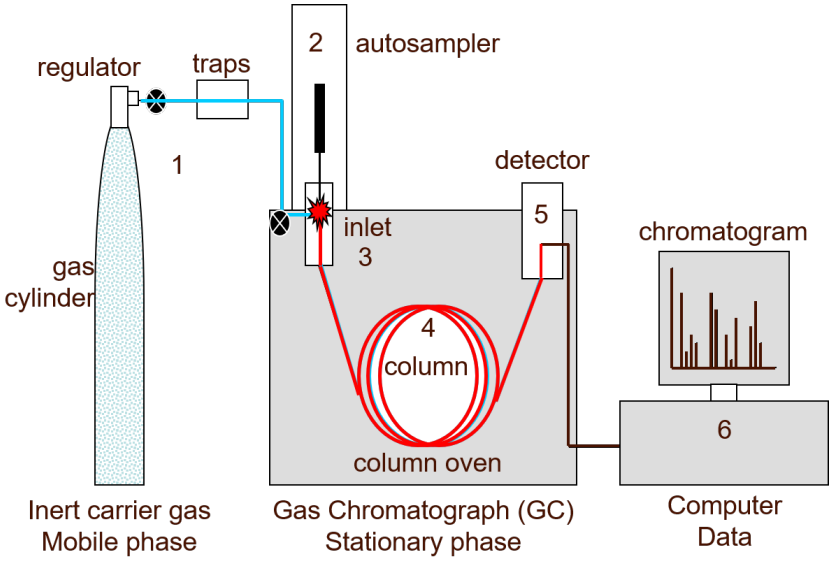


Figure 2.4: Schematic diagram of the GC instrument system.

A gas chromatograph is a chemical analysis tool used to segregate compounds in a complicated sample [23]. It consists of a narrow flow-through tube called the column. The sample flows in a gas stream which is also known as the carrier gas. The sample flows at different rates dependent on individual chemical, physical properties, and interactions with a

specific column lining or filling. It is referred to as the stationary phase. The compounds are recognised electronically as they depart the column's end [22]. The stationary phase's role in the column is to segregate distinct components. It is performed by forcing each to depart at a different time [22].

In a GC analysis, a pre-decided liquid analyte is injected into a temperature-controlled heated port connected to the column through a rubber disc [23]. Adsorption of analyte molecules over packing materials stationary phase or column's walls occurs. It occurs when the carrier gas travels the analyte molecules from the column, resulting in separation [23]. The numerous components of the detection mixture are segregated as they go forward lengthwise in the column and reach the end of the column at different times. It is because each type of molecule has a specific rate of movement, also called retention time. A detector is utilised for tracking each component's time to reach the outlet. It allows the amount of that component to be calculated [22]. The retention duration of the analyte in the column and the sequence in which compounds elute from the column is used to identify substances qualitatively [23].

2.4.3 CE

CE is an electrokinetic segregation technique that uses micro-and nanofluidic channels and capillaries with submillimeter diameters. CE is most commonly associated with capillary zone electrophoresis [24]. Under the influence of an electric field, analytes migrate across electrolyte solutions in CE techniques. Non-covalent interactions can segregate analytes based on their ionic mobility and/or partition them into an alternative phase. Analytes can also be concentrated or "focused" by using pH gradients and conductivity.

Figure 2.5 depicts a basic design of a CE apparatus. Capillary electrodes, a detector, source and destination vials, a sample vial, a high voltage power supply, and an information acquisition computer together make a system of CE. An electrolyte is poured into the capillary, the source vial, and the destination vial. The inlet of a capillary is added into a vial holding the sample. Capillary action, syphoning, pressure, or electrokinetics are used to enter the sample into the capillary; subsequently, return it to the source vial. An electric charge is provided from the source to the destination vials. It is also provided to the electrodes, which causes the analytes to migrate. Electroosmotic flow pulls all positive and negative ions from the capillary in the same direction in the most frequent CE mode. Due to the ion's electrophoretic mobility, the analytes segregate as they travel. They are detected near the capillary's output end. The

detector's output is delivered to a computer. The information is later presented as an electropherogram, which shows the detector reaction over time. In an electropherogram, separated chemical components show peaks with varying migration periods [25].

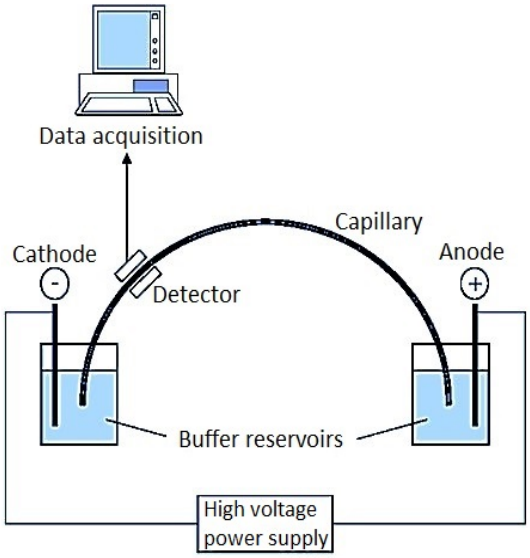


Figure 2.5: Schematic diagram of CE instrument system.

2.5 Current Advancements in CKD Biosensing

Biosensor advances have recently made it possible to measure the functional quality of human kidneys. These studies and enhancements have improved the performance of existing biosensors and resulted in rapid, reliable, and extremely sensitive creatinine detection. The development of cost-effective techniques that can be used outside of laboratories is under investigation. Kidney health biosensors are classified below according to the currently available reviews. Enzyme nanoparticles-based biosensors, nanomaterials-based electrochemical biosensors, enzymeless/chemical creatinine biosensors, creatinine immuno biosensors and conductimetric creatinine biosensors are the different types of creatinine biosensors.

2.5.1 Enzyme Nanoparticles-based Biosensors

The enzymes were immobilised over various nanocomposites in creatinine biosensors to detect creatinine in biological fluids. Direct immobilisation of enzymes over nanocomposites, on the other hand, may result in denaturation, resulting in a loss of activity

and stability. Instead of native enzyme molecules, the problem was solved by using enzyme nanoparticles. Enzyme nanoparticles were nanoscale aggregates of enzyme molecules that exhibit remarkable physiological and chemical characteristics. Enzyme nanoparticles have shown remarkable potential as augmenting electrodes because of their unique catalytic, thermal, electrical, mechanical, optical, and electronic characteristics, in addition to their increased surface area. Due to this, using enzyme nanoparticles instead of native enzymes in the building of an amperometric biosensor has increased the biosensor's analytical performance and simplified the fabrication of the enzyme electrode [26]. For better amperometric detection of creatinine in blood, enzyme nanoparticles of sarcosine oxidase, creatine amidinohydrolase and creatinine amidohydrolase were immobilised onto a glassy carbon electrode and used to make an amperometric creatinine biosensor.

At pH 7.25, 34°C, the biosensor showed an optimal current of 0.4 V within 10 seconds, with a limit of detection (LOD) of 50 µM. It had an operating range of 50 to 1000 µM. When kept at 4°C, the electrode operated for up to 30 days during regular use [27]. In the laboratory studies, Pundir et al. developed a better amperometric creatinine biosensor. They have done it by immobilising sarcosine oxidase, creatine amidinohydrolase and creatinine amidohydrolase enzyme nanoparticles on a glassy carbon electrode. The modified glassy carbon electrode demonstrated the ease of availability, low background current, ultra-low-cost, high conductivity and making it a superior choice over Au, C, and silicalite electrodes. This enzyme nanoparticles/glassy carbon electrode-based biosensor had the best reaction against silver/silver chloride (Ag/AgCl). The reaction was performed at 25°C temperature, 0.1 V, within 2 seconds at a pH 6.0. With a conventional enzymic colorimetric technique, the enzyme nanoparticles/glassy carbon electrode had a reduced LOD of 0.01 µM.

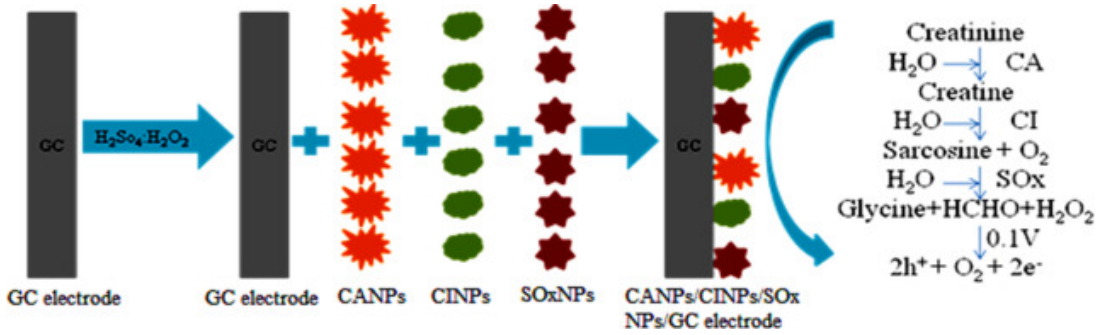


Figure 2.6: Schematic diagram of fabrication of creatinine amidohydrolase nanoparticles/creatine amidinohydrolase nanoparticles/sarcosine oxidase nanoparticles/glassy carbon electrode [28].

It had an excellent correlation coefficient $R^2 = 0.99$. The analytical recovery of added creatine in serum of 0.1 and 0.15 mM was 97.970.1% and 98.760.2%, respectively. It also had 2.06% and 3.09% cyclic voltammetry value differences within and between batches. The biosensor assessed creatinine levels in the blood from fit people and people with kidney problems. Post 240 days of storage and usage at 4°C, the enzyme nanoparticles modified electrode missed just 10% of its initial activity [28], denoted in Figure 2.6.

2.5.2 Nanomaterial-based Electrochemical Creatinine Biosensors

Nanomaterial-based electrochemical creatinine biosensors provide significant benefits over commercial biosensors, such as increased selectivity and detection sensitivity, and have tremendous promise for use in sick patients [26]. Because of the high volume to surface area ratio of nanomaterials, a higher quantity of sites was accessible for molecular exchanges. Nanomaterials, particularly nanoparticles, provide a viable method to expand the bio-recognition area.

Researchers are increasingly incorporating nanoparticles into the materials used to make biosensors to increase the system's performance in present and future sensing applications. The development of enzymatic creatinine biosensors using various nanoparticles is sub-divided into three types, and they are addressed below.

2.5.2.1 Iron-Oxide Nanoparticles-based Biosensor

Sarcosine oxidase, creatine amidinohydrolase, creatinine amidohydrolase were covalently immobilised over an iron-oxide nanoparticle/chitosan graft-polyaniline composite film to create an amperometric creatinine biosensor [29]. The sensor had biocompatibility and solid superparamagnetic activity. It provided more significant contact and reduced lethality. Iron-oxide nanoparticles interfered with the immobilisation of target biomolecules [30]. The magnetic behaviour of these bioconjugates culminated in enhanced distribution and recovery of biomolecules for necessary biosensing applications [31-33].

Immobilisation of such bioactive molecules over a surface charged with superparamagnetic nanoparticles were required. When polarised at 0.4 V against Ag/AgCl, the biosensor demonstrated an optimal response at 30°C and pH 7.5 within 2 seconds. For creatinine in the concentration range of 1-800 μM, the electrocatalytic reaction was linear. The

biosensor has a sensitivity of 3.9 μA/μM/cm^2 and a LOD of 1 μM. Creatinine had an apparent Michaelis-Menten constant value of 0.17 mM. When maintained dry at 4°C for more than 200 days, the biosensor lost 10% of its original sensitivity post 120 usages.

Nanomaterials have desired features in biosensing applications. They can be listed as an excellent adsorption capacity, high surface reaction activity, high catalytic efficiency and high surface-to-volume ratio. Nanomaterials have a one-of-a-kind capability to endorse rapid electron transport amongst the enzyme's active and electrodes. Because of their biocompatibility, high superparamagnetic characteristics, low toxicity and decreased mass transfer barrier, magnetite nanoparticles have gotten much interest. The optimal response, working potential, pH, and enzyme loading for an iron-oxide nanoparticles-based amperometric biosensor for detection of creatine were 0.30 V, 7.0, and 2.0 U (creatine amidinohydrolase), 1.0 U (sarcosine oxidase), respectively.

With a detection limit of 2.0×10^{-7} mol L^{-1}, the biosensor showed a linear response from 2.0×10^{-7} mol L^{-1} to 3.8×10^{-6} mol L^{-1} and 9.0×10^{-6} mol L^{-1} to 1.2×10^{-4} mol L^{-1}. Kaçar et al. [34] employed a biosensor to determine creatinine from commercial creatinine powders. It was found to be a high sensing capability. The stability, biocompatibility, accuracy, response time and sensitivity of these nanoparticles-based amperometric creatinine biosensors have been improved. A non-nanoparticle-based microfabricated creatinine biosensor (5.2 mM) [35] and a CPE-based creatinine biosensor (5.15 mM) both had lower apparent Michaelis-Menten constant than the nanoparticle-based creatinine biosensor [36].

Because of the low apparent Michaelis-Menten constant, enzymes immobilised on carboxylated multiwalled carbon nanotubes/polyaniline, zinc oxide nanoparticles/chitosan/multiwalled carbon nanotubes/polyaniline, and iron oxide nanoparticle-nanoparticles/chitosan grafted polyaniline composite films maintained their activity despite a low diffusion barrier. It significantly benefited other composites since the nanoparticles kept the conducting characteristics and allowed for simple, quick enzyme integration with a low apparent Michaelis-Menten constant and high sensitivity.

These biosensors had a higher sensitivity than non-nanoparticle-based creatinine biosensor based on controlled pore glass (0.0000208 μA/μM/cm^2) [37], poly-2-hydroxyethyl methyacrylate (0.0000139 μA/μM/cm^2) [35], gas-permeable membrane (0.000001 μA/μM/cm^2) [38], poly (carbamoyl) sulfonate-hydrogel with Nafion (0.005 μA/μM/cm^2 [39],

polyvinyl alcohol (0.0001256 μA/μM/cm^2) [40], and poly (carbamoyl) sulfonate-hydrogel matrix (0.00024-0.00046 μA/μM/cm^2) [41].

2.5.2.2 Zinc-Oxide Nanoparticles and Carboxylated Multi-Walled Carbon Nanotubes-based Biosensor

Pundir et al. built a creatinine biosensor using a zinc-oxide nanoparticle/chitosan/carboxylated multi-walled carbon nanotubes/polyaniline composite screen on a platinum electrode (Figure 2.7) [42-44]. Individual carboxylated multi-walled carbon nanotubes in a suspension might be cytotoxic. However, immobilising carboxylated multi-walled carbon nanotubes on a surface or inside a composite prevented cytotoxicity [45]. The addition of nanoparticles to carboxylated multi-walled carbon nanotubes films may result in a novel nanostructure with excellent optical, electrical, and electrocatalytic properties [46]. Electroanalysis using metal nanoparticles-modified electrodes offered exceptional benefits. Such as better electrolysis because of improved electron transport catalysis, a high operative superficial area, and regulation on the microenvironment of the electrode [47-48].

The zinc oxide nanoparticles/chitosan/carboxylated multi-walled carbon nanotubes/polyaniline composite film-modified electrode, the enzymes sarcosine oxidase, creatine amidinohydrolase and creatinine amidohydrolase were immobilised. When polarised at 0.5 V vs Ag/AgCl, this enzyme electrode detected creatinine levels as low as 0.5 μM in 10 seconds at pH 7.5 and 30°C temperature. The creatinine biosensor has a sensitivity of 0.030 μA/μM/cm^2 and an operating range of 10-650 μM for creatinine. When kept dry at 4°C for 120 days, the biosensor lost 15% of its initial activity. The apparent Michaelis-Menten constant was 0.35 mM [49].

2.5.2.3 Carboxylated Multi-Walled Carbon Nanotubes-based Biosensor

The advantages of carbon nanotubes, such as their excellent electrical characteristics, high surface area, and electrocatalytic activity, have lately garnered much interest in developing electrochemical enzyme biosensors. This design ensures an expanded zone for biomolecule immobilisation. When conducting polymers were added to carbon nanotubes, they reduced mass exchange impedance and charge transfer resistance more than other nanomaterials [50].

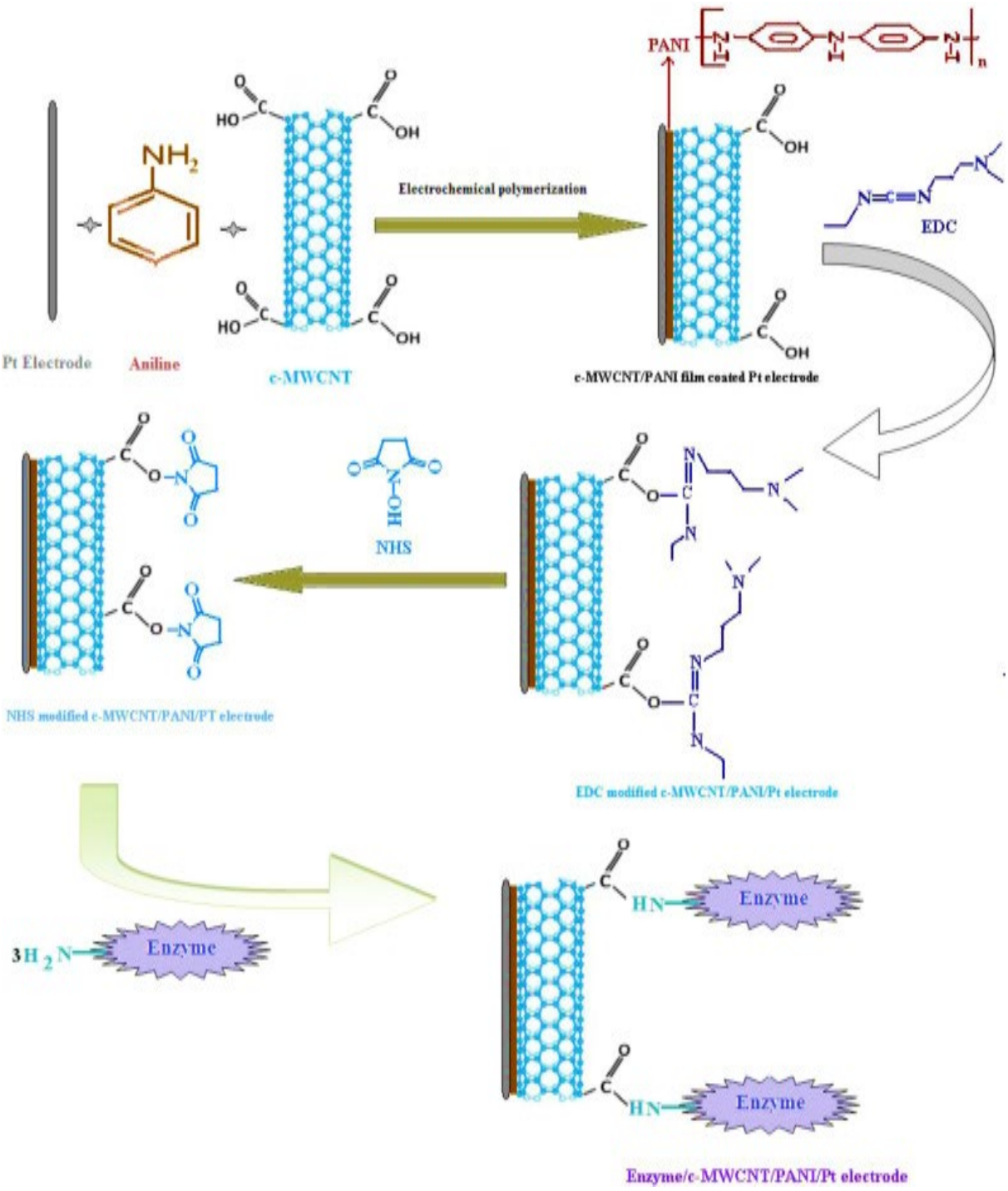


Figure 2.7: Graphic diagram of chemical reaction involved in the fabrication of enzymes/carboxylated multi-walled carbon nanotubes/polyaniline/platinum hybrid electrode [44].

An amperometric creatinine biosensor was created by covalently immobilising sarcosine oxidase, creatine amidinohydrolase and creatinine amidohydrolase over a carboxylated multi-walled carbon nanotubes polyaniline nanocomposite film electrodeposited

over the surface of a platinum electrode. It was performed using N-ethyl-N-(3-dimethylaminopropyl), carbodiimide and N-hydroxy succinimide. It showed a lower detection limit of 0.1 μM. The linearity in the 10-750 μM creatinine concentration range. At 35°C, 0.2 V, pH 7.5, the creatinine biosensor demonstrated optimal response within 5 seconds. The biosensor has a 0.040 μA/μM/cm^2 sensitivity and an apparent Michaelis-Menten constant of 0.26 mM. Even after 180 days of regular usage, the biosensor's storage stability was strong, with 85% of the primary current response remaining [42-44].

2.5.3 Enzymeless/Chemical Creatinine Biosensors

The following are examples of enzymeless electrochemical creatinine biosensors based on various concepts.

2.5.3.1 Silver Nanoparticles/Polyoxometalate/Glassy Carbon Modified Electrode Voltammetric Creatinine Biosensor

Using silver nanoparticles/polyoxometalate functionalized reduced graphene oxide coated glassy carbon electrode was an enzymeless electrochemical sensor based on MIPs. It was developed for the detection of creatinine [51]. With a LOD of 1.51×10^{-11} M, this MIP biosensor demonstrated good sensitivity in the detection of creatinine.

2.5.3.2 Disposable Non-Enzymatic Electrochemical Biosensor

Copper was electrodeposited on screen-printed carbon electrodes to create a disposable non-enzymatic creatinine biosensor. Electrochemical and microscopic methods were used to characterise the biosensor. Creatinine was detected electrochemically in phosphate buffer at pH 7.4. The development of a solvable copper-creatinine composite was utilised for making the determination. The pseudoperoxidase action of the copper-creatinine composite was used to build up the copper-creatinine complex. A detection limit of 0.0746 μM and linear range of 6-378 μM. The sensor responded to creatinine consistently and was devoid of interference from chemicals such as ascorbic acid, glucose, urea and dopamine. Blood serum was used to conduct the real sample analysis [52]. Physical design, enzyme activity, the surface activity of the inner polymer membranes, working electrode used to eliminate interference or immobilise enzymes. The outer membrane presence required to prevent biofouling and/or improve oxygen

dependence contributed to a biosensor's sensitivity. By replacing the functioning electrodes with nanoparticles, the superficial functions of these electrodes were improved. The primary goal of nanomaterials was to increase the surface area of the working electrode. However, due to the vast number of quinoid moieties at the nanotube tips, the nanomaterials also significantly increased electrocatalytic activity. These biosensors also had a faster response time over non-nanoparticle biosensors based on poly-2-hydroxyethyl methyacrylate (300 seconds) [53], polyvinyl alcohol (104 seconds) [40], polypyrrole doped with sulfonated phenoxy resin (100 seconds) [54], CPE containing 10% platinum power (90 seconds) [36], poly(1,3-diaminobenzene) (60 seconds) [35], CPE (30 seconds) [55], cellulose acetate (20 seconds) [56] and poly (carbamoyl) sulfonate-hydrogel matrix (20 seconds) [57]. Nanoparticles improved the performance of electrochemical creatinine biosensors, according to the study (Figure 2.8).

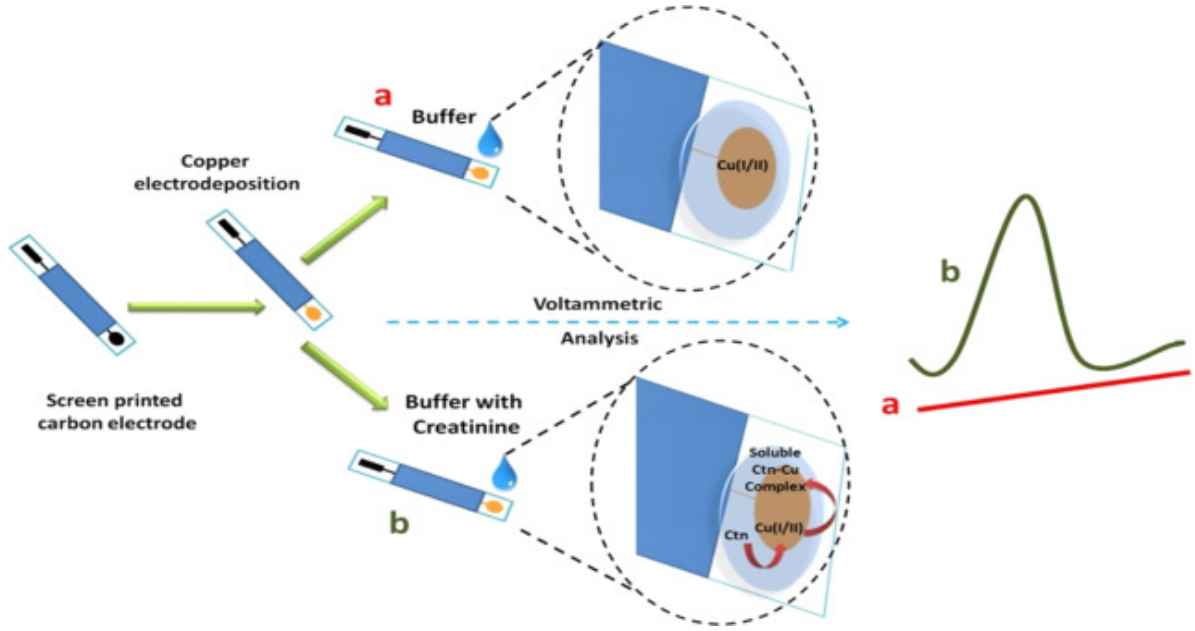


Figure 2.8: Schematic of a disposable non-enzymatic electrochemical creatinine biosensor [52].

2.5.3.3 An Innovative Structure-Specific Creatinine Biosensor

For ensuring creatinine without enzymes, a structure-dependent amperometric method was devised [58]. Creating a dissolvable copper-creatinine combination over the copper electrode surface provided the basis for this new technique. As a result, the oxidative current produced by the surface-oxide layer was relational to the creatinine concentration. The layered copper complex and the chelating capacity of creatinine determines the sensitivity and

selectivity of this new technique. The main problem with these biosensors was that they were suitable for urine analysis and not blood analysis.

2.5.3.4 Enzymeless Creatinine Biosensor based on Poly (3,4-Ethylenedioxythiophene)-β-Cyclodextrin

Enzyme-free methods that use neutral carriers, including crown ethers and cyclodextrin as ionophores for detecting physiologically important compounds, have recently gotten much interest. Cyclodextrins were hydrophobic cyclic glucopyranose oligomer molecules having a hydrophilic outside and a hydrophobic interior cavity. Non-covalent interactions, i.e. Van der Waals forces, electrostatic forces and hydrogen bonding, enable cyclodextrins to form complexes with newly introduced molecules of suitable size. The ability of cyclodextrin to combine with several physiologically important organic and neutral compounds allows the detection of these molecules via host-visitor chemistry.

Other creatinine biosensor utilising poly-3,4-ethylenedioxythiophene-modified glassy carbon electrode with -cyclodextrin was described [59]. The weak non-covalent contacts among the amide hydrogen of creatinine and the glucopyranose oxygen atom in the -cyclodextrin. They were responsible for the molecular recognition contact among -cyclodextrin and creatinine. Consistent changes in the electrode potential vs concentration of creatinine revealed a complex between -cyclodextrin and creatinine. Electrochemical impedance analysis was used to investigate the specific interactions of the -cyclodextrin incorporated poly-3,4-ethylenedioxythiophene film with the neutral Tris buffer solution having creatinine mixed within.

2.5.3.5 Electrochemical Creatinine Biosensor based on MIPs

By the solvent-evaporation dispensation of poly (ethylene-co-vinyl alcohol) for producing the MIPs, an enzymeless electrochemical biosensor based on MIPs was constructed. It was used for the specific detection of creatinine [60]. The ethylene and vinyl alcohol percentage in the poly (ethylene-co-vinyl alcohol) was calculated to improve detection sensitivity. The carbonyl functions were assigned to the verified spectra once the format molecule was removed after washing with 20 mL of ethanol.

2.5.3.6 Electrochemical Creatinine Biosensor for Specific Detection of Creatinine based on Preanodized Carbon Screen-Printed Electrode

By the preanodised carbon screen-printed electrode, an enzymeless electrochemical method based on Jaffe's reaction was devised. It was for specific and quantitative identification of creatinine in human urine [61]. The electrode and the active methylene group in creatinine established a stable presence of chloride ions with a selective carbon-carbon bond. In phosphate buffer saline at pH 6.7, creatinine was quantified using the technique of voltammetry.

2.5.3.7 Creatinine Biosensor based on Voltammetric Performance of Creatinine at Phosphomolybdic Polypyrrole Film Modified Electrode

The electrochemical behaviour of creatinine was investigated in this case. It was performed using Keggin type phosphomolybdate-doped polypyrrole film improved glassy carbon electrode [62]. The redesigned electrode was investigated using 0.5 order differential voltammetry to record the electrochemical behaviour of creatinine. Creatinine inhibited the reduction of the adjusted electrode in 0.5 M H_2SO_4 and 0.5 order differential voltammetry technology. It provided benefits due to its low cost, simplicity, high sensitivity, speed, and capability to monitor inhibitory movement towards the phosphomolybdate-doped polypyrrole film modified glassy carbon electrode process.

2.5.3.8 Creatinine Capacitive Biosensor based on a Photografted MIP

For the production of chemosensors, MIPs are a straightforward approach for embedding recognition sites. It was with the specificity of antibodies and enzymes in synthetic polymers. To produce a counterfeit receptor layer, a capacitive creatinine chemosensor used photopolymerization of the monomer actylamidomethyl propanesulfonic acid. The cross-linker methylenediacrylamide [63] was also used for MIP synthesis. A SAS monolayer was formed by adsorbing the creatinine template, the cross-linker, the monomer and the photoinitiator onto a vision electrode surface. The UV treatment resulted in the formation of an ultrathin polymer film. The electrode surface was selective to creatinine after the template was removed. In MIP synthesis, functional monomers were polymerised in the existence of template molecules and the initiator molecules. The template molecules were ejected after polymerisation, leaving

locations with induced molecular memory that recognised the print molecules. A reduction in electrode capacitance was used to detect creatinine binding. The sensor functioning was reversible and very selective, with no reaction to a rise in creatinine, urea, or glucose.

Another sensor used screen-printed gold (Au) electrodes and was based on a novel MIP for creatinine capturing. On the screen-printed Au electrode surface, a layer of carboxylic polyvinyl chloride was initially placed. Creatinine molecules were linked to the screen-printed Au electrode/carboxylic polyvinyl chloride surface. After that, N, N′ methylenebisacrylamide and acrylamide polymerisation filled up the gaps surrounding them. Following the removal of the templates, the polymer was left with binding sites capable of explicitly recognising creatinine at various concentrations. The same technique was repeated without creatinine on an Au non-imprinted polymer to evaluate the sensor's sensitivity. Three experimental methods were used to examine their retention and molecular recognition properties. They are listed as UV-Visible spectrophotometry, EIS technique, cyclic voltammetry and differential pulse voltammetry. The study discovered that the MIP has a unique detection ability for creatinine but not for other structurally similar substances like urea or glucose. The biosensor was also tested on individuals with various urine creatinine levels, and it appeared to be a viable tool for screening creatinine.

Furthermore, a partial least square analysis was performed. It was performed to find a link between concentrations obtained by Jaffe's reaction as a reference technique and the anticipated creatinine concentrations from voltammetric data. With a linear range of 0.1 ng/mL to 1 g/mL. The EIS and differential pulse voltammetry biosensor responses had a LOD of 0.016 ng/mL and 0.081 ng/mL, respectively [64].

2.5.4 Creatinine Immuno Biosensors

Immunosensors were biosensors that combined immunology with chip-based electrochemistry. These devices, like standard immunoassays, were based on solid-phase immunoassay principles. It was with an antibody or antigen immobilised on the surface of the sensor. The modalities of immunosensors were as follows:

1) A distinct identified species were detected after binding by fluorescence or luminescence in an indirect (heterogeneous) immunosensor.

2) A direct (homologeneous) immunosensor detects the binding. It is done by changing potential or current and was a sensitive and trouble-free method.

Using an indirect competitive assay technique, an electrochemical creatinine sensor was created [65]. The sensing electrode was coated in platinum and consolidated into an electrochemical cell by using a creatinine-revised electrode. A sample blend of anti-IgG (mouse)-graphene oxide and anticreatinine antibody conjugate was mixed in the cell. For the antigen-binding sites of the conjugated antibodies for anticreatinine, the creatinine to be detected competes with membrane immobilised creatinine. Glucose was added after a washing stage, and the amount of H_2O_2 generated was measured amperometrically. The membrane was thought to limit unspecific antibody or redox-active protein binding, keeping any unwanted reactions at the electrode. The sensor had a LOD of 40 nM. It can quantify in the range of 0.09-90 μM. This incredible sensitivity was beneficial for low creatinine levels and severely weakened or limited volume samples, such as blood from newborns or blood obtained from a capillary.

Nevertheless, one measurement cycle takes 30 minutes, and information on adjustment bend for more special focuses was unavailable. A standardized immunosensor based on a size exclusion redox labelled immunoassay having a good detection range of 0.09-900 μM was later described by the group [66]. Engineered redox-labelled creatinine and creatinine from the sample compete for antigen-binding sites of anticreatinine antibodies in this technique. Not adsorbed by antibodies, redox-labelled creatinine pass from the cellulose membrane towards the glassy carbon electrode. If the creatinine concentration in the sample were high, the indication generated by redox-labelled unbound creatinine was substantial. The creatinine immunosensors did not require any recuperation and had a good range and sensitivity, but they bothered many expensive antagonistic creatinine antibodies.

2.5.5 Conductometric Creatinine Biosensors

A new conductometric creatinine biosensor that was extraordinarily delicate and stable had been created [67]. The detection technique was based on the fact that many biological processes in solution cause electrical resistance changes among two parallel electrodes. A sensor sensitive to solid-state ammonium contact was used in the biosensor. The glutaraldehyde covalent attachment technique was used to chemically immobilise creatininase over the superficial area of the solid-state interaction ammonium sensitive membrane. In phosphate

buffer pH 7.20, the biosensor had a LOD of 2×10^{-6} M. The response time was 10 seconds. The biosensor's linear dynamic range in phosphate buffer with pH 7.20 was 1×10^{-19} M creatinine concentration. At 4°C, the biosensor displayed outstanding operating and storage constancy for at least four weeks. Conductometric transducers have a considerable benefit in that. They were built as a single piece having noticeable similarities. The biosensors were rough and relatively inexpensive and did not require a reference electrode.

2.5.6 Electrochemiluminescence Creatinine Biosensors

Cao et al. had developed a self-enhanced solid-state electrochemiluminescence (ECL) platform. It was built using polyethyleneimine (PEI) capped CdS quantum dots (PEI-CdS QDs) as the coreactant and $Au@SiO_2@Ru(bpy)_3^{2+}$ doped silica ($Au@SiO_2@RuDS$) nanocomposite as the ECL emitter in their study. PEI-CdS QDs included a double coreactant of PEI and CdS QDs, resulting in a substantial increase in $Au@SiO_2@RuDS$ of ECL. PEI-CdS/$Au@SiO_2@RuDS$ was immobilised using a polyaniline hydrogel to enhance electron transport and immobilisation. As a recognition element, a creatinine imprinted poly(o-aminophenol) film was electrodeposited on the PEI-CdS/$Au@SiO_2@RuDS$ surface. For creatinine sensing, the developed sensor had shown good sensitivity, stability, repeatability, and selectivity. In the region of 0.05 nM to 5 nM, the change in ECL intensity and logarithm of creatinine concentration exhibited a strong linear relationship, with a detection limit of 0.02 nM. The sensor was used to detect creatinine in human blood and urine samples. The findings were very similar to those obtained using a commercial enzyme-linked immunosorbent assay kit [68].

2.5.7 Lossy Mode Resonance-based Fiber Optic Creatinine Biosensors

Sharma et al. had effectively developed a rapid and simple detection method for monitoring creatinine content in an aqueous solution and an artificial urine sample. The sensor was created on a lossy mode resonance-based optical fibre technology, with molybdenum sulphide (MoS_2) @ tin oxide (SnO_2) nanocomposite serving as the lossy mode resonance supporting material $MoS_2@SnO_2$ nanocomposite serving as artificial antibodies. The sensor's performance was tested across a creatinine concentration range of 0 to 2000 g/mL, within the physiological range of human blood and urine. The sensor's maximum sensitivity and detection

limit, respectively, were determined to be 0.41 nm/(g/mL) and 1.86 g/mL. High selectivity, long-term stability, repeatability, and quick response are just a few of the benefits of the sensor. The sensor probes' near-complete recovery with the artificial urine sample demonstrates their potential utility in biological applications [69].

2.5.8 Multiplex Assays

A few multiplex immunoassays have recently been developed to detect CKD for accurately monitoring the process of kidney functioning. When compared to single tests, the automated multiplex detection assay demonstrated the same analytical accuracy and sensitivity. They are very beneficial when a small sample volume is available for the testing process. M. Anderson et al. developed a “Magnetic Luminex”-based multiplex assay. Cystatin C, Clusterin, CXCL10/IP-10, Lipocalin-2/NGAL, Fetuin A/AHSG, Osteopontin, TFF3, RBP4, and TIM-1/KIM-1/HAVCR are among the nine protein biomarkers measured simultaneously in blood, plasma, and urine samples by the Luminex Performance human kidney biochemical marker assay [70].

Merck Pty. Ltd. has developed a range of multiplex assays that simultaneously detect different markers from human urine samples (Assay 1-4), serum and plasma samples (Assay 5-6).

MILLIPLEX MAP Human Kidney Injury Magnetic Bead Panel 1 - Toxicity Multiplex Assay is a standard 96 well plate assay. It helps detect Collagen IV, Calbindin, GSTα, Fatty Acid Binding Protein 1, Kidney Injury Molecule-1, IP-10, TFF-3, Osteoactivin, TIMP-1, Renin in one detection assay [71]. MILLIPLEX MAP Human Kidney Injury Magnetic Bead Panel 2 - Toxicity Multiplex Assay is a standard 96 well plate assay. It helps detect albumin, α-1-Microglobulin, Clusterin, Cystatin C, EGF, Lipocalin-2/NGAL, and Osteopontin in one detection assay [72]. MILLIPLEX MAP Human Kidney Injury Magnetic Bead Panel 3 - Toxicity Multiplex Assay is a standard 96 well plate assay. It helps detect Uromodulin and β-2-Microglobulin in one detection assay [73]. MILLIPLEX MAP Human Kidney Injury Magnetic Bead Panel 4 - Toxicity Multiplex Assay is a standard 96 well plate assay. It helps detect FABP1, PTH, EGF, KIM-1, IP-10, Osteopontin, and Renin in one detection assay [74].

MILLIPLEX MAP Human Kidney Injury Magnetic Bead Panel 5 - Toxicity Multiplex Assay is a standard 96 well plate assay. It helps detect Collagen IV, α-1-Microglobulin,

Osteoactivin, Lipocalin-2/NGAL, Uromodulin, TIMP-1 in one detection assay [75]. MILLIPLEX MAP Human Kidney Injury Magnetic Bead Panel 6 - Toxicity Multiplex Assay is a standard 96 well plate assay. It helps detect Clusterin, β-2-Microglobulin, RBP4, Cystatin C in one detection assay [76].

2.5.9 EIS-based MIP Biosensors

The MIP is easy to synthesis, and it has a low production cost. MIP also has storage stability, higher level of mechanical strength. It is durable to heat and pressure and also has suitability in harsh and concentrated environments. The MIP is highly specific and selective in the adsorption of creatinine from the samples having a range of analyte concentrations. Every sample's creatinine content will be variable; therefore, a stable, cost and time-effective, highly selective polymer having detection flexibility is required for creatinine sample analysis. It also needs to be safe and non-problem causing for household usage. From the literature review, it was noticed that all the above techniques are quite complicated and not user friendly in the future for the common person household usage. Therefore, we have developed an IoT-based MIP polymer functionalized MEMS ID sensor for creatinine detection and quantification based on the EIS technique to fulfil the above necessities. It has been discussed in detail in chapter 3, 4 and 5.

2.6 Chapter Summary

The use of biochemical markers of CKD can help researchers better understand the CKD stages along with steadiness/decline in kidney functioning. In conjunction with imaging methods for understanding kidney health, biochemical tests play an essential role in assessing and diagnosing kidney functioning, including monitoring various CKD stages. Inside the laboratory, CKD stages can be monitored using various techniques, including Creatinine Colorimetric Assay Kit. The traditional methods are typically laboratory-based, time-consuming, costly, and complicated. Creatinine biosensors are being developed to address these concerns.

On the other hand, biochemical sensors play an essential role in monitoring the activity of the CKD stage by carefully measuring creatinine levels. Several biosensors have been developed to check creatinine levels to understand CKD stages for biochemical assessment of kidney functioning. This chapter has presented an overview of the existing CKD biochemical markers, focusing on current CKD biochemical sensor technologies. In Chapter 3, the EIS method and its usage for our developed MEMS ID sensor characterisation are described in depth.

3

MEMS ID Sensors and EIS Technique

Publications about this chapter:

- **S. N. Prabhu**, S. C. Mukhopadhyay, C. Gooneratne, A. S. Davidson and G. Liu, "Interdigital sensing system for detection of levels of creatinine from the samples," **2019 13th International Conference on Sensing Technology (ICST)**, **2019**, doi: 10.1109/ICST46873.2019.9047672
- **Prabhu S.N.**, Gooneratne C.P., Hoang K.A., Mukhopadhyay S.C., Davidson A.S., Liu G., (**2021**), Interdigital Sensing System for Kidney Health Monitoring. In: Mukhopadhyay S.C., George B., Roy J.K., Islam T. (eds) Interdigital Sensors. Smart Sensors, Measurement and Instrumentation, vol 36. Springer, Cham, doi: 10.1007/978-3-030-62684-6_11

3.1 Operating Principle of Interdigital Sensors

The planar ID sensors are composed of finger alike or comb alike repetitive patterns of parallel electrodes on a substrate. The electrodes are used to build up the capacitance associated with the electric fields, which pass through the SUT and transmit vital information related to the properties of the used sample [77]. One of the significant positive sides of the planar ID sensors is the one-sided access to the SUT. This property aids to pass the sample solution along with acoustic or electromagnetic fields from only a single side. The power of the output signal is monitored by altering the total number of comb-like electrodes, the total sensing area of the ID sensor and the ID electrode spacing in-between them. The non-destructive single-sided sample access is an additional benefit of these sensors; thus, these properties make them useful in testing real biological samples and other applications [78].

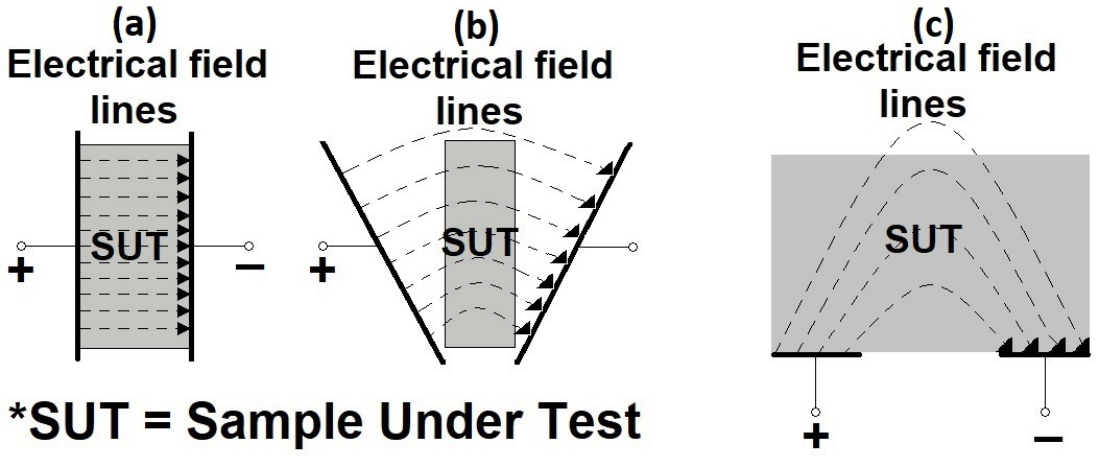


Figure 3.1: Electrical field lines of the parallel-plate capacitor as well as the planar ID sensor. The evolution of electric field lines and gradual transition from parallel plate to a planar plate capacitor (a) parallel plate capacitor, (b) intermediate stage and (c) ID sensor.

The planar ID sensors follow the working principle of parallel plate capacitors. The gradual transition from a parallel plate to a planar plate capacitor is displayed in Figure 3.1. The electrodes allow access to SUT by opening up on only a single side of the sensor. The electrode pattern of the sensor can be kept uniform or repeated multiple times to get a signal and keep the signal to noise ratio in a good range [79]. The structural pattern of an ID sensor is shown in Figure 3.2. An alternating current is supplied as an excitation voltage to the positive terminals. An electric field is generated from the positive and moves towards the negative

terminal. The electric field protrudes out from SUT from the excitation electrode and is collected by a negative sensing electrode. It transmits essential information related to the characteristic properties of SUT, namely density, concentration, impedance and many more. Figure 3.3 displays the structural pattern of the currently developed MEMS planar ID sensor. Figure 3.4 displays the electric field generated in-between excitation positive electrodes and sensing negative electrodes for varying pitch lengths-where the distance in-between two adjacent electrodes has identical polarisation. The varying pitch lengths show varying penetration, as described in Figure 3.4. The depth of penetration increases by increasing the net pitch length, but it results in the weakening of the electric field.

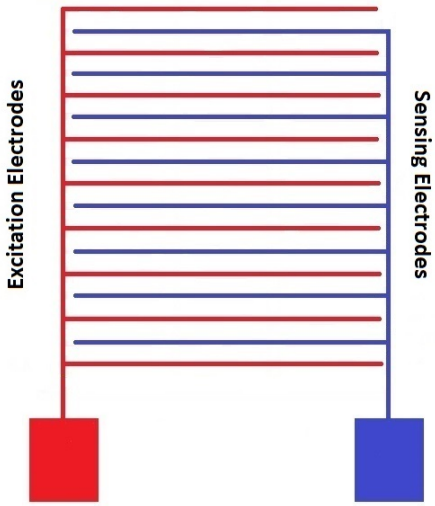


Figure 3.2: Structural pattern of a typical ID sensor.

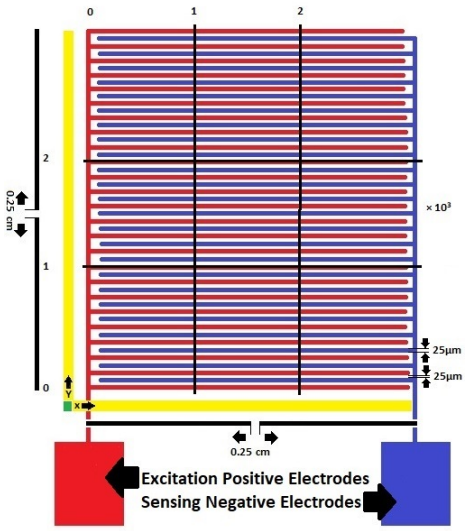


Figure 3.3: The structural pattern of the currently developed MEMS planar ID sensor.

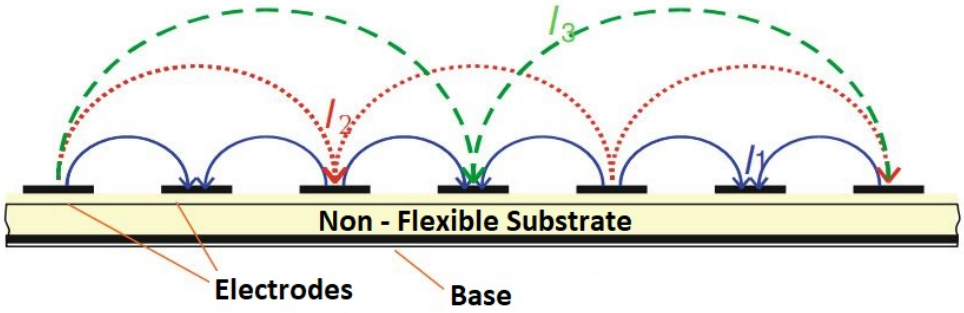


Figure 3.4: Electric field having various pitch lengths [80].

3.2 Heavy Permeation Depth Planar ID Sensors

The presented ID sensors are designed with more repetitions of sensing and excitation electrodes to raise the penetration depth of the electric field. Various structural designs have been studied from available literature [81-83]. Figure 3.4 denotes the excitation pattern for multiple sensing electrode geometry in an ID sensing design. These ID sensors have been manufactured depending on a very simplified geometrical pattern. Figure 3.3 displays the structural configuration of an ID sensor having the configuration of 1-1-25, presenting one sensing electrode between two consecutive excitation electrodes. The spacing between two consecutive electrodes on ID sensors is 25 µm. During ID sensor development, sensors having 25 µm and 50 µm spacing between consecutive electrodes were developed. The impact of spacing between consecutive electrodes of ID sensors having 25 µm and 50 µm were studied on the sensing outcome during the sensor design finalisation step. The ID sensors with 25 µm consecutive electrode spacing offered much improved results. Therefore, they were chosen over the 50 µm consecutive electrode spacing sensor design. A time-dependent sine wave electrical charge is provided to the positive excitation electrodes of the ID sensors. The alternating charge passes through the SUT; from the positive excitation electrode towards the direction of the negative sensing electrode. This phenomenon transmits important information about the properties of SUT when it is in superficial contact with the detecting sensor [77-78].

3.2.1 MEMS Sensors Fabrication

These sensors are fabricated at KAUST, Saudi Arabia. It is resulted due to an academic research tie-up between the School of Engineering, Macquarie University and a former scholar

from KAUST, Saudi Arabia. The sensor fabrication is done by etching and photolithography technologies on a single silicon/silicon dioxide (Si/SiO_2) wafer having a 4-inch diameter and 525 μm thickness. In total, 36 number of working sensors are designed on a single wafer chip. The single sensor is dimensionally 10 mm × 10 mm × 10 mm and has a sensing area of 6.25 mm^2. The MEMS technology is used for the manufacturing process of these ID sensors.

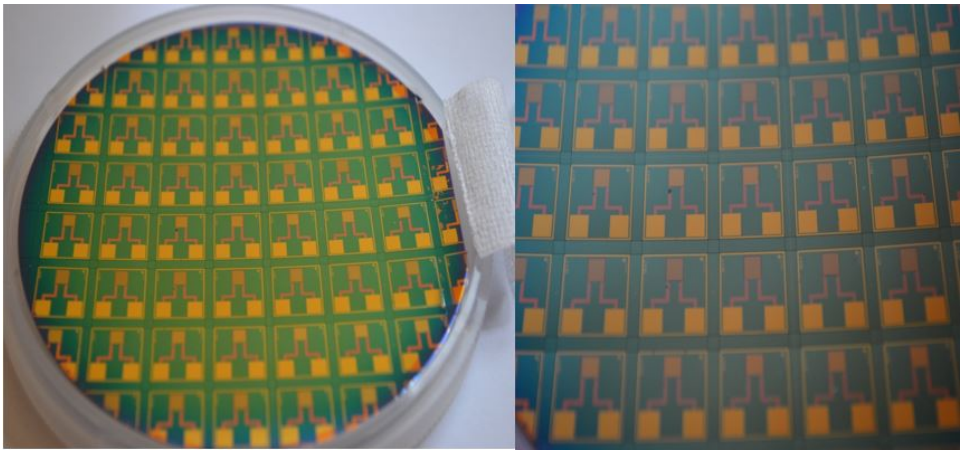


Figure 3.5: New and improved silicon-based MEMS planar ID sensors on silicon wafer chip [84].

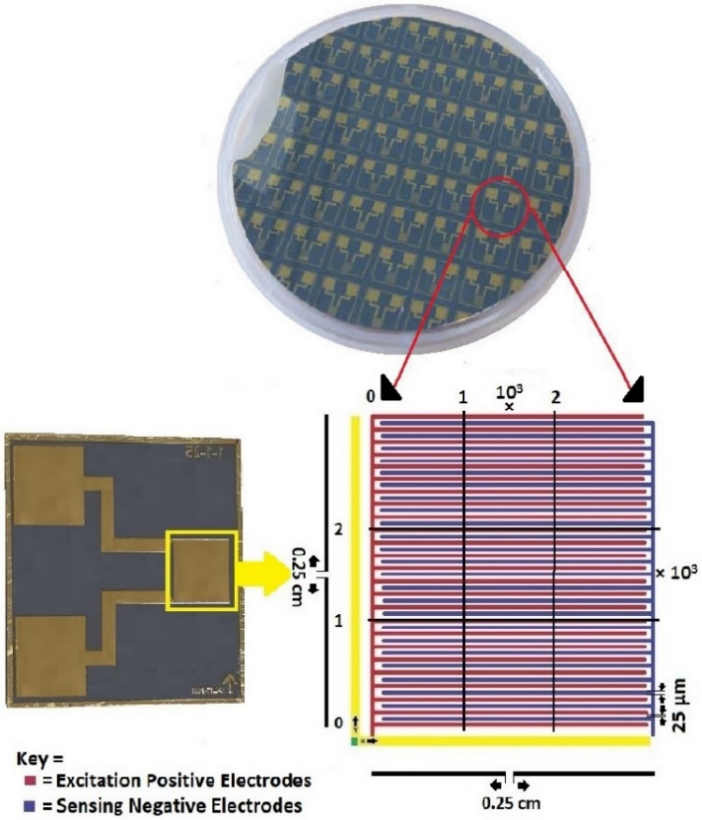


Figure 3.6: ID electrode configurations.

Sensor manufacturing involved multiple steps such as coating of photoresist, ID sensor pattern transfer using UV exposure, plasma etching metal deposition done by DC magnetron sputtering process followed by lifting off. First, the thin-film electrodes are made by sputtering with 500 nm of Au on the surface of chromium (Cr) to provide a firm attachment of ID electrodes over the base substrate. In a further stage, the printed wafer surface is coated with a Silicon Nitride (Si_3N_4) layer of 200 nm layer thickness. The Si_3N_4 acts as a protective covering on the sensing area of ID sensors from damage or cracking associated with close contact of SUT with the sensing area. The coating also prevents the accumulation of moisture inside the Si/SiO_2 base substrate. The moisture interference might hamper the dielectric properties of SUT and result in a change in the performance of the sensor. In the final stage, the bonding pads are allowed to open with the help of the plasma etching process [85]. Figure 3.5 shows MEMS planar ID sensors on a silicon wafer chip. Figure 3.6 displays ID electrode configurations.

The ID sensors have numerous applications in manufacturing lines [86], monitoring of environmental parameters [83, 87-90], moisture as well as humidity detection system [91-92], gas sensing [93], detection of photosensitive materials [94]. It is developed based on the ID pattern for the identification of hazardous pollutants in seafood [81, 90]. One of the ID sensors is made for determining phthalates from the water-based samples [87-89].

3.2.2 Detail Process of Fabrication

Etching plus photolithography techniques over a-Si/SiO_2 (one side polished) 525 μm thick, p-doped, 4-inch diameter wafer developed the current version of the planar structure having ID electrodes. Figure 3.6 displays a graphical representation of electrode configurations.

The manufacturing phase of the initial prototype of the developed silicon wafer sensors is shown in a concise version within Figure 3.7. The sensor is manufactured having a 1-1-25 configuration. The 1-1-25 sensors are designed for improved signal quality, low impedance and increased sensitivity. In non-contact measurements, the dies used for production carry a stiff substrate that is beneficial. It is necessary to retain a distinct geometry of the structure of the electrode to execute model-based parameter valuation algorithms effectively. For scheming the ID structural designs, CoventorWare® software is used. The structural design differed as per the spatial wavelength of the periodic ID structure, whilst continuous 25 μm values for

electrode width and the dimension of the area of sensing (2.5 mm × 2.5 mm) are set. Thus, 36 working sensors are made in each wafer, having a dimension of 10 mm × 10 mm × 10 mm and 6.25 mm^2 as a sensing area of the sensor.

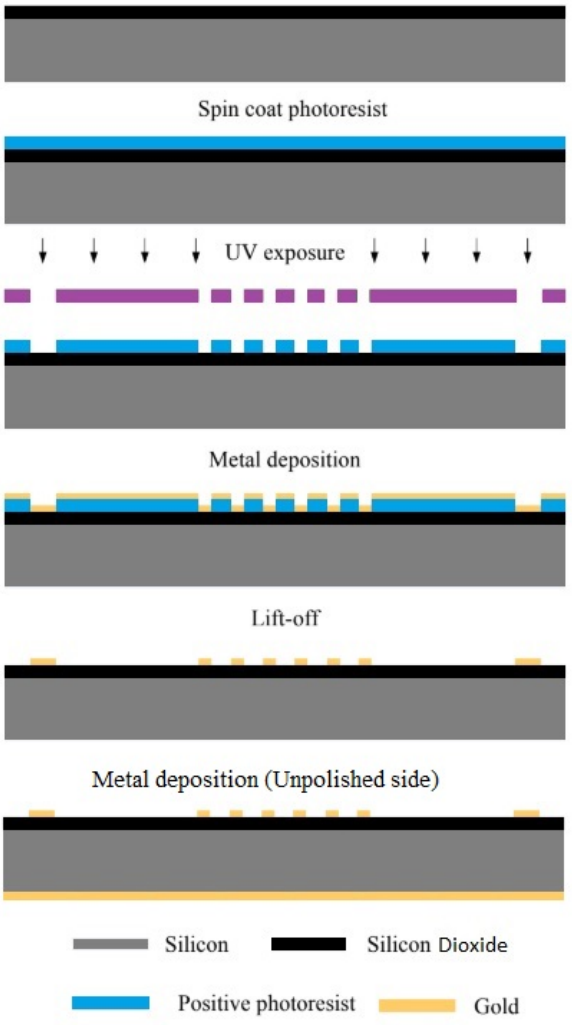


Figure 3.7: The initial prototype process of the sensor production on the silicon wafer.

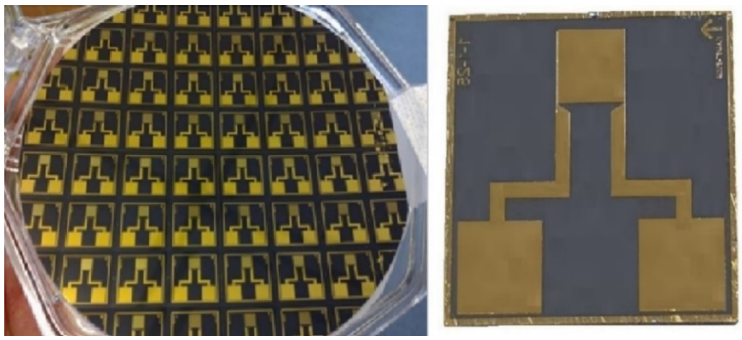
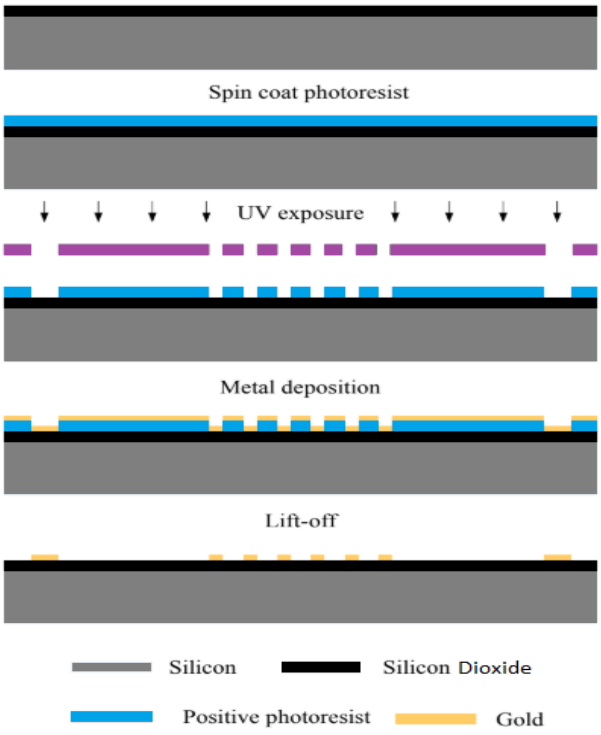
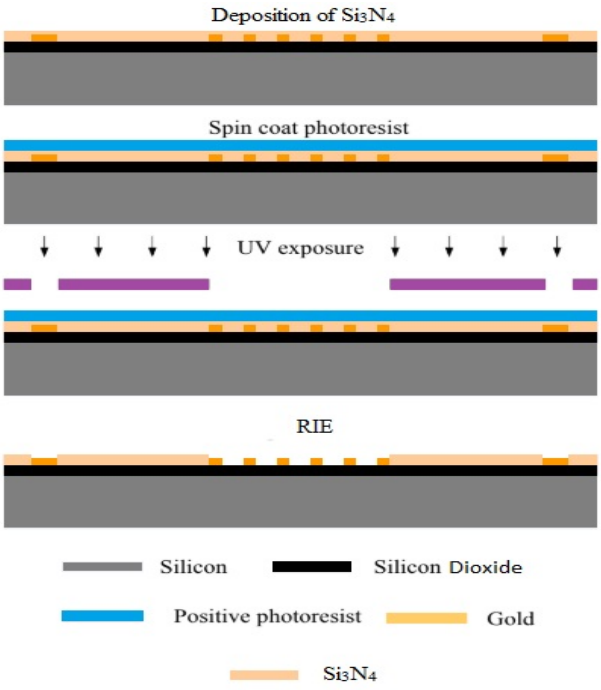


Figure 3.8: Small-sized thin film MEMS ID sensors.



(a) Sensor manufacture process (Mask 1 - Designing a lift-off process for Au electrodes).



(b) Sensor manufacture process (Mask 2 - plasma-enhanced chemical vapour deposition for the deposition of Si_3N_4 and reactive ion etching (RIE)).

Figure 3.9: Steps involved in MEMS sensor development (a) and (b).

Using a mask laser writer, the design is printed over a soda-lime clear mask having a diameter of 5-inch. Before baking (pre-baking) is carried out over the wafer substrate at 150°C. This step is helpful to the photoresist for improved connecting over the surface. A spin coating instrument is utilised on a 4-inch wafer for coating 4 μm of EC13027 (a positive photoresist). An oven is utilised for after baking (post-baking) the wafer for 1 minute at 100°C. To pass the ID designs to the wafer's photoresist, a UV light exposure instrument is utilised for softening the resist on the bonding pad windows. Over a parting of 30 μm between the mask and the wafer, a continuous 200 mJ/cm^3 dose of UV light is exposed. The wafer is then further advanced with a solution (AZ 726) based on Tetra-Methyl Ammonium Hydroxide (TMAH) for 1 minute. Plasma ashing is utilised to conduct a "Descum" procedure for extracting the remaining photoresist in channels at 70°C. Subsequently, Cr 20 nm and Au 500 nm quantity are sputtered onto the wafer substrate by the DC magnetron sputtering process. The Au and Cr both act as a barrier layer for proper adhesion. The electrodes have the benefit that the Au and Cr are inert and versatile. At a pressure of 30 mTorr, Argon gas is used.

The direct current (DC) power is established to 800 W. The process of lift-off is carried out afterwards using an acetone solution. The opposite side of the wafer is also polished by sputtering by using Cr (20 nm) and Au (500 nm) after lift-off. It is going to serve as a guard plane. The guard plane is powered by a follow-up op-amp identical to the sensing voltage, removing any current through the substrate between the guard and sensing electrodes. The capacitance and conductance amongst the guard and the sensing plane are therefore removed, resulting in more straightforward response analysis and enhanced sensitivity of the device. Figure 3.8 displays the fabricated silicon-based ID sensor. The enhanced designing pattern of the silicon sensors has been developed. For Mask 1, the preliminary production process is identical as in Figure 3.7. As shown in Figure 3.9, the enhanced component is used in the manufacturing of Mask 2. Two types of coating materials, parylene C and Si_3N_4, are used to coat the two different wafers of manufactured sensors. Parylene C is a material that protects the sensor against the corrosive materials from the SUT. It is also a comparatively weaker material than that of Si_3N_4.

Therefore, 1 μm coating thickness is used for parylene C whilst Si_3N_4 coating thickness is kept at 200 nm. The SUT is a serum sample containing varying concentrations of creatinine (non-corrosive material); the sensors with Si_3N_4 coating are preferred for further analysis.

The wafer is coated by a specific procedure (plasma-enhanced chemical vapour deposition (PECVD)) with Si_3N_4 of 200 nm layer after lift-off. Si_3N_4 is utilised as a passivation layer in many microdevices. This is because its film properties are effortlessly monitored and adjusted by changing the approaches and settings of its deposition. Besides, for long periods, Si_3N_4 tolerates continuous air exposure at high temperatures. Si_3N_4 deposition uses PECVD technique consequences in films that possess decent adhesion, a lower density of the pinhole, decent coverage of measures and uniformity. The complete area of the film is coated with Si_3N_4, excluding the bonding pads and the electrodes. The wafers fabricated with the 1-1-25 sensor geometry are further coated with a selective creatinine imprinted polymer layer over bonding pads and the electrodes that can attract and adsorb creatinine from SUT. Since the sensing area is kept open during Si_3N_4 coating, a molecular-selective coating can be applied directly on the electrodes and the sensing area. This is beneficial for complementary biomolecule (creatinine). The approach helped target analytes to be attracted and adsorb as close as possible to the sensing (electrode) surface. It leads to high detection signals. Creatinine spiked SUT is directly coated on the sensing surface of the electrodes, as the electrodes are accessible to creatinine and are coated with creatinine imprinted polymer. The uncoated electrodes with the Si_3N_4 approach aided in the firm binding of creatinine imprinted polymer over the sensors sensing area. The creatinine imprinted polymer coating is specifically selective towards creatinine attracting and capturing it from the sample solutions. This way, the target biomolecules are bound to the electrodes as near as possible, leading to strong detection signals. A 4 μm of EC13027 (a positive photoresist) is layered over the wafer after coating Si_3N_4. For softening the resist on the bonding pad windows and the electrodes, a UV light exposure method is used. It is used for opening the space by having electrodes and the bonding pads after the development of RIE. One of the dry etching methods is RIE; it also avoids the undercutting caused by the process of wet etching. An anisotropic etch is produced in the dry etching process. For high-fidelity pattern transfer, an anisotropic etch is essential. The most recent silicon sensors are shown in the following Figure 3.5.

3.3 Electrochemical Impedance Spectroscopy

The EIS technique is a well-known standard technique for measuring the capacitive and resistive properties of sample materials by providing a minor alternative current (AC) signal. The main advantages of using the EIS technique are testing of the sample in a non-destructive manner, one-sided sample access, label-free identification of components for various chemical

and biological investigation purposes. Various applications of EIS has been testified, including the identification of fat percentage inside meat [95], levels of toxins from shellfish [81], levels of bacterial endotoxins in food [96], the content of phthalates in juices and water [88], a study of corrosive behaviour of materials [97-98] and detection of electrical properties for the process of soybean coagulation [99]. The technique has various applications in several research areas including, the mechanism of corrosion [100], detection of coating [101-102], battery optimisation [103], as well as biological sensing [104].

From the list of methods currently available for the process of measurement of impedance, frequency response analyser (FRA) has turned into a practising standard for the quantification of EIS. The FRA is a FastTrack method for the practical evaluation of the change in impedance. The technique analyses the system impedance for a broad range of frequencies and makes a comparison of outcomes with the standard data. The main benefit of the comparative analysis process is noise reduction, but it may result in the weakening of the generated signal. The FRA is a solo sine wave contribution methodology involving a minor amplitude AC signal of a pre-decided frequency. It is put over the DC bias potential, which is provided to the electrode, and it is followed by the measurement of subsequent AC is performed. With lower amplitude AC, the system relics pseudo-linear. The protocol is reanalysed at a pre-decided optimum frequency range. The impedance measurements are taken five times, and their average data are taken for analysis. For finalising the systems' strength, repeatability, and linearity, the current method is considered practical for a reversible and stable system within the equilibrium. Due to the above purpose, immediate impedance calculations are needed for non-stationery systems [105-107].

The study of the impedance of a linear system is quite simple when compared with non-linear systems. During the EIS analysis, a small AC voltage is provided to the electrochemical cell. Because of the small current, the receiving system is non-linear. Inside a non-linear system, the outcome related to current will carry harmonics of the excitation voltage frequency and might get hampered by the harmonics.

The EIS can be measured with the help of a minor AC signal followed by measurement of the phase angle in the current signal concerning the provided signal. In EIS, the impedance is calculated with a low excitation signal for measuring the electrochemical cell response as a pseudo-linear. The charge outcome to the sine wave excitation signal at the same frequency shows a shift in phase angle within a linear system and is displayed in Figure 3.10.

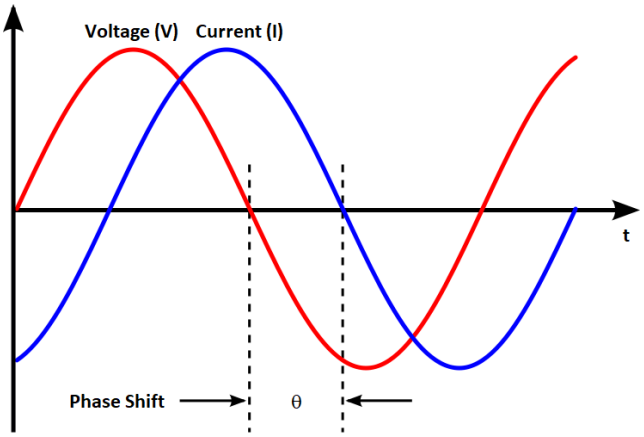


Figure 3.10: The shifting of the phase in the current signal concerning the applied voltage.

The impedance is described as the calculation of the power of the electrical circuit to resist the flowing of electrical charge when the voltage is provided. Within an AC circuit, the impedance is shown as a composite value which includes real resistive as well as imaginary reactance.

The Nyquist plot displays an imaginary impedance part against the real impedance for every single excitation frequency. It offers various advantages. The resistance of the solution can be studied very effortlessly with the Nyquist plot. The Nyquist plot can be used for calculating the EIS parameters, such as solution resistance (R_s), double-layer capacitance (C_{dl}), electrode polarization resistance (R_p). The R_s can be obtained by an inducing arc onto the X-axis. The main disadvantage of the Nyquist plot is related to its' inability to indicate anything about frequency. Thus, making it troublesome in the calculation of the double-layer capacitance [106]. The phase shift and the impedance against frequency can be represented by the Bode plot. The main difference between Nyquist and Bode plots is that the Bode plot represents information about the frequency at one of its axes. Therefore, the Bode plot helps in a better understanding of the relationship between frequency and impedance.

Randle's equivalent circuit is commonly used [108] for the interpretation and analysing of the EIS outcomes in electrical presentation, as described in Figure 3.11. The model is helpful in describing diffusion, as well as kinetics procedures, which take place at the borders between electrodes. It involves the R_s in a sequence connection to the analogous fusion of C_{dl} to the charge transfer resistance (R_{ct}) when the Warburg impedance (W1) is used in series [104]. Randle's equivalent circuit model is named after Randle [109]. The circuit diagram consists of two resistors (R1 and R2), two capacitors (C1 and C2) and W1. It incorporates R_s (R1), and R_{ct}

(R2) are in parallel in combination connected to capacitances C1 and C2 in a series connection with W1 [104].

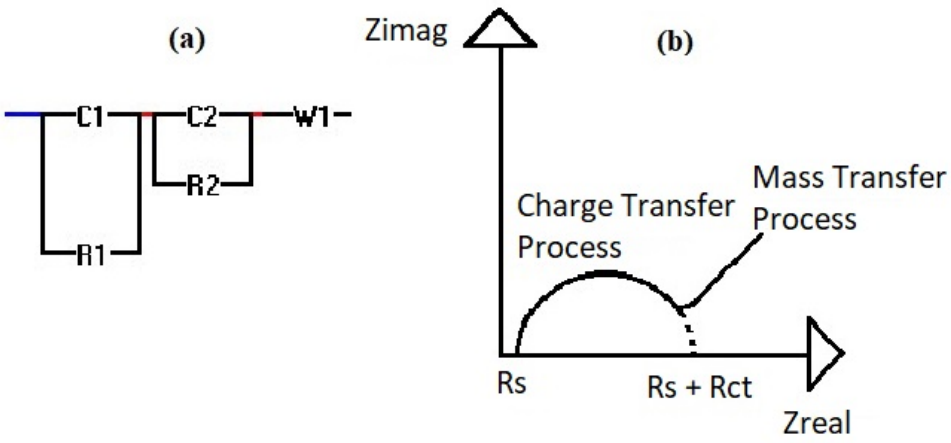


Figure 3.11: Randle's equivalent circuit along with the Nyquist plot for that equivalent circuit.

The EIS experimental results in electrical research as displayed in Figure 3.11 (a). The Nyquist plot has a semi-circular area and a 45° angular line, shown in Figure 3.11 (b). Within this, the impedance of faradic reaction involves an active (R_{ct}) (R2) as well as an electrochemical diffusion component, also called a Warburg element (W1). At the high-frequency range, the semi-circular region displays a slower charge transfer, whilst the strait line indicates a significantly faster mass transfer at a low range of frequency. The charge transfer resistance is measured with the help of inducing the semicircle to the Zreal axis shown in Figure 3.11 (b).

In contrast, the R_s (R1) can be calculated by inducing measurements to the real axis (X real ordinate) value at the higher-frequency intercept, the one that intercepts close to the origin of the Nyquist plot (Figure 3.11 (b)). The R_{ct} (R2) can be calculated by extrapolating the semicircle with the Zreal axis, as shown in Figure 3.11 (b). Both C1 + C2 capacitance can be calculated from the frequency at the highest of the semi-circular region in the Nyquist plot using the following (Equation 3.1).

$$\omega = 1/R_{ct} \times (C_1 + C_2) \qquad \textbf{(Equation 3.1)}$$

To get Randle's equivalent parameters, the adjustment of the model circuit to the experimentally obtained data need to be done with the help of the CNLS curve fitting technology.

3.4 Experiments with the Developed Sensing System

Following experiments are done for detection of creatinine from the aqueous samples using the developed MEMS ID sensor.

3.4.1 Experimental Setup

The experimental setup includes a humidity meter and a digital thermometer, the MEMS ID sensor, SUT's carrying varying concentrations of creatinine, MIP polymer coating suspension, and a high precision Hioki 3536 LCR meter.

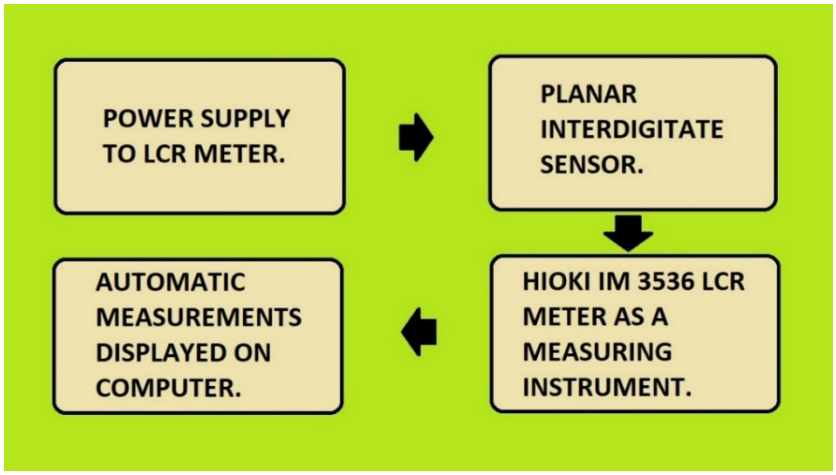


Figure 3.12: The block diagrammatic representation of the measurement system setup.

The LCR meter is connected to the computer through the RS232 connection port for data collection and analysis. The measurement system setup is diagrammatically represented in Figure 3.12. The MEMS sensor is connected to the excitation and sensing electrode with the help of a specifically made jig having Au pin connectors shown in Figure 3.13.

The EIS experiments are done using the Hioki 3536 LCR meter. The experiments are done by use of the slow mode of equipment testing. A 1 V alternative current is applied to the sensor with the help of the Au pin connector, and the frequency is swept between 10 Hz to 100

kHz. All the experiments are repeated thrice, and the average of the results is used to confirm the reproducibility and trustworthiness of the obtained results.

The LCR meter is utilised in performing all the EIS measurements. The LCR meter has the highest speed (fastest time as 1 ms) and accuracy (± 0.05% rdg). The instrument is served for three purposes, the measurement instrument, the power supplier and it is also connected to the data acquisition computer for collecting and saving the obtained data in Microsoft Excel format. Figure 3.13 displays the process of LCR meter EIS measurement system with data acquisition computer. Table 3.1 shows the Hioki 3536 LCR meter technical specifications. Figure 3.14 shows the Hioki 3536 LCR meter instrument.

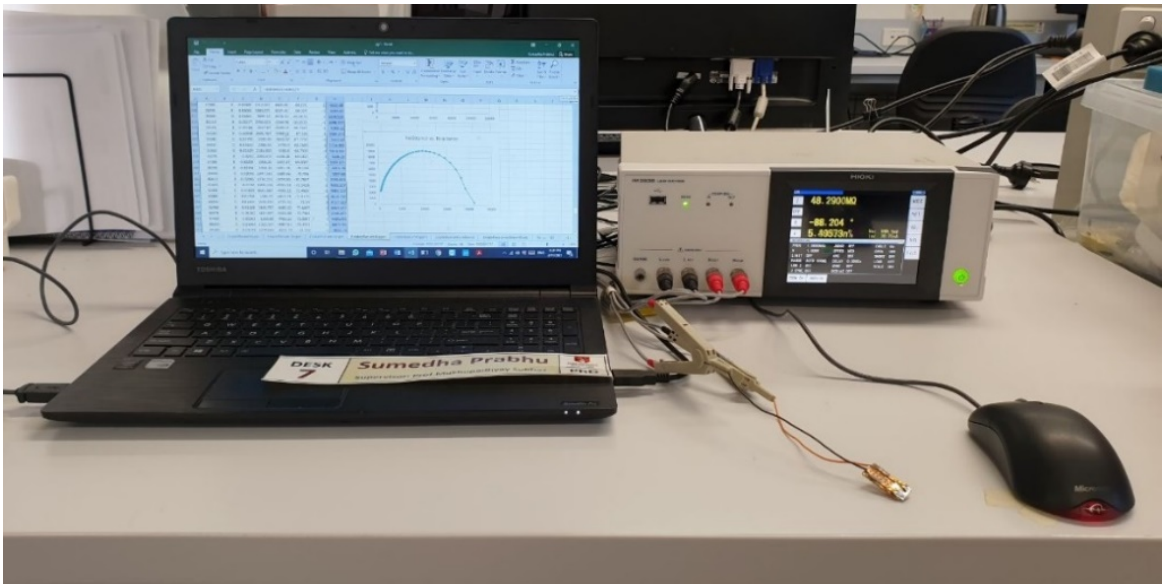


Figure 3.13: The process of LCR meter EIS measurement system with data acquisition computer.

Figure 3.14: The Hioki 3536 LCR meter [110].

Table 3.1: The overview of the specifications of the Hioki 3536 LCR meter.

Hioki 3536 LCR Meter	
Operating frequency range	4 Hz to 8 MHz
Level of measurement (CC mode)	Normal mode: 4 Hz to 1 MHz: 10 µA to 50 mA (max. 5 V) 1.0001 MHz to 8 MHz: 10 µA to 10 mA (max.1 V) Low-impedance high-accuracy mode: 10 µA to 100 mA (max. 1 V)
Level of measurement (V mode and CV mode)	Normal mode: 4 Hz to 1 MHz: 10 mV to 5 V_{rms} (max. 50 mA) 1.0001 MHz to 8 MHz: 10 mV to 1 V_{rms} (max. 10 mA) Low-impedance high-accuracy mode: 10 mV to 1 V (max. 100 mA)
Time of measurement	1 ms (approx.)
Range of accuracy	1 mΩ to 200 MΩ
Parameters of measurement	X, Y, Z, R_S, R_P, θ, G, B, C_S, C_P, L_S, R_{dc}, L_P, Q, D, ε, σ

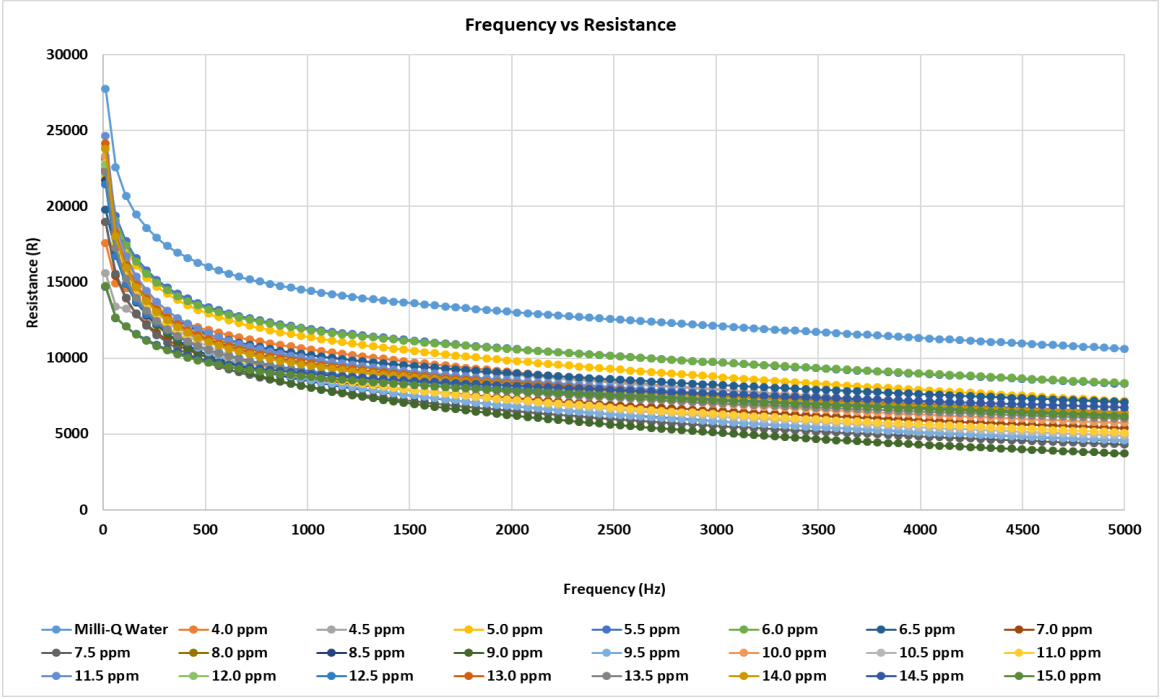
3.4.2 Preparation of Creatinine Spiked Aqueous Samples

A concentrated stock solution of 1,000 ppm creatinine aqueous solution is prepared by mixing 1 gm of creatinine powder in 1,000 mL of Milli-Q water and stored inside the refrigerator for future usage. Serial dilution technique is utilised to prepare the aqueous samples of 0.1 to 50 ppm creatinine concentrations. The Milli-Q water is used as zero levels of creatinine (control) for all other different samples with varying creatinine concentrations.

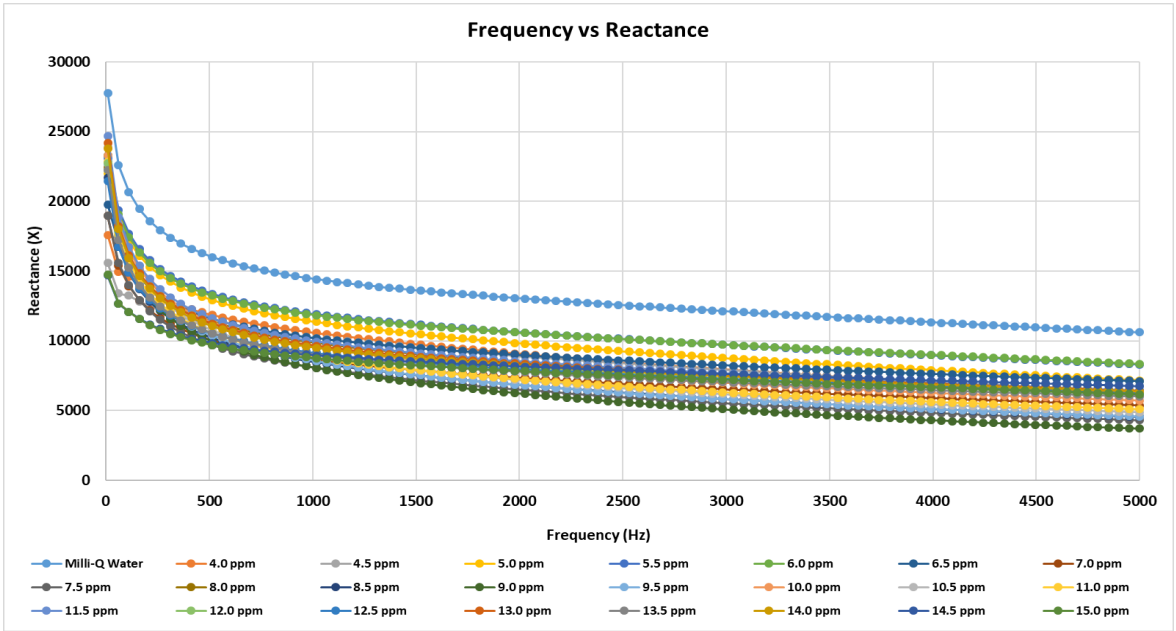
3.4.3 EIS Measurement

The detection of creatinine concentration is done with the fabricated sensor using the EIS technique [89]. The sensing area of the MEMS ID sensor is susceptible in its detection but

not specifically sensitive for adsorption of creatinine. Therefore, all the measurements are done by immersing the sensing area of the plain MEMS ID sensor in the pure standard solutions carrying different concentrations of creatinine.



(a) Frequency vs resistance for 10 Hz to 5000 Hz.



(b) Frequency vs reactance for 10 Hz to 5000 Hz.

Figure 3.15: (a) Frequency vs resistance for 10 Hz to 5000 Hz and (b) Frequency vs reactance for 10 Hz to 5000 Hz.

In order to ensure the reliability of the sensor, every sensing measurement and the sensing results are repeated five times in identical conditions, and the average is plotted in the data. As it is the first set of MEMS ID sensor characterisation experiments, the sensing experiment is performed for a specific range of frequency from 10 to 5000 Hz. Therefore, it helped understand the working frequency zone of the developed MEMS ID sensor without any coating layer.

Figure 3.12 shows a block diagram of the EIS data acquisition setup. Figure 3.15 (a) shows the resistance (R) over 10 to 5000 Hz frequency. Figure 3.15 (b) shows the reactance (X) over 10 to 5000 Hz frequency. The normal creatinine range group has higher concentration levels ranging from 4 ppm to 15 ppm. It also has samples from slightly higher concentrations than the normal range.

Since changes in the resistance (real part of impedance) are more distinctive than reactance (imaginary part of impedance), the resistance is considered for all subsequent investigations (Figure 3.15 (a) and (b)). Figure 3.16 depicts the impedance spectrum of different concentrations of creatinine in the form of a Nyquist plot. The precise results are seen in a lower frequency range from 10 Hz to 100 Hz for 4 ppm to 15 ppm samples, in Figure 3.17 (a) and (b). It showed the visible changes for resistance and its corresponding Nyquist plot.

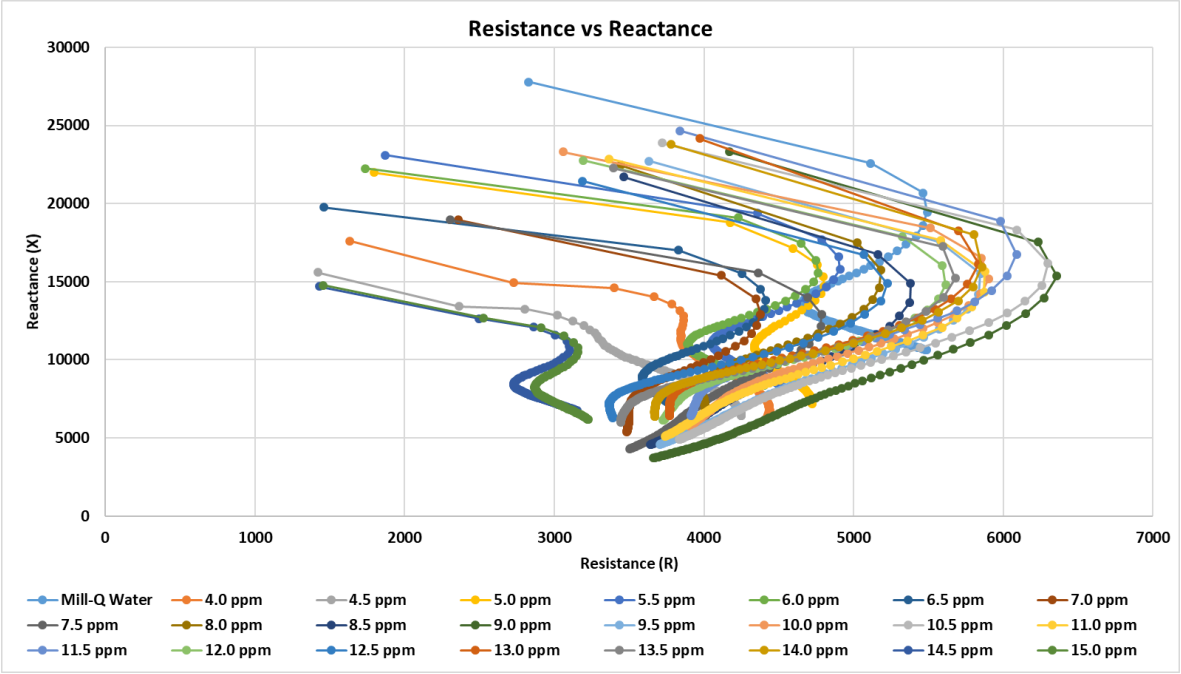
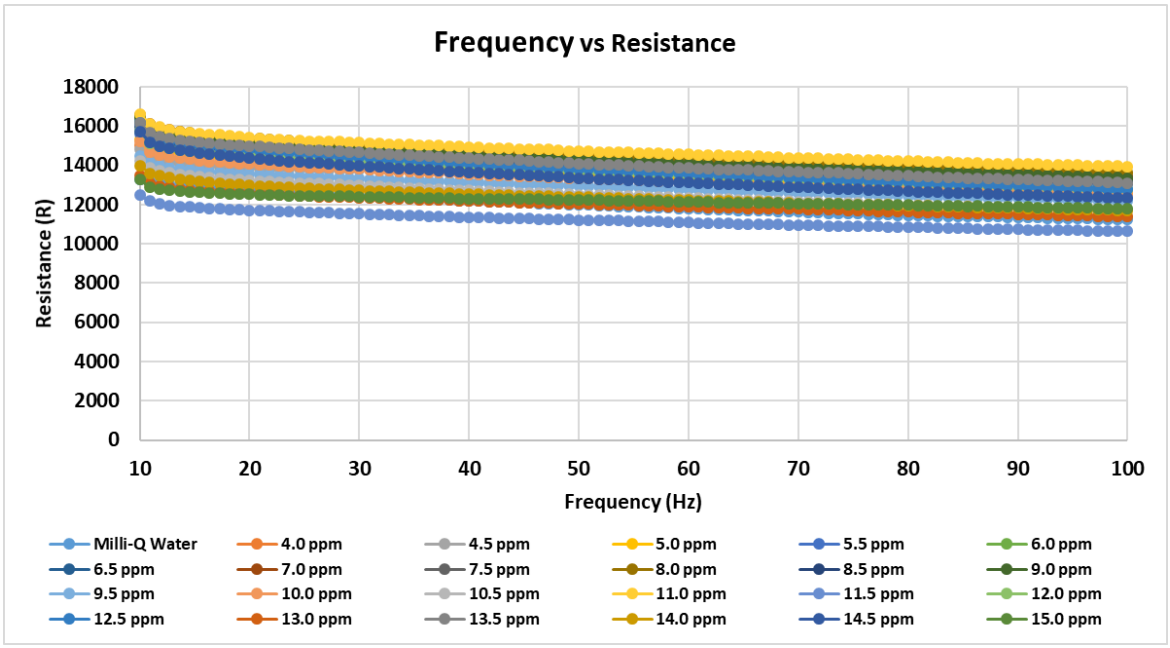
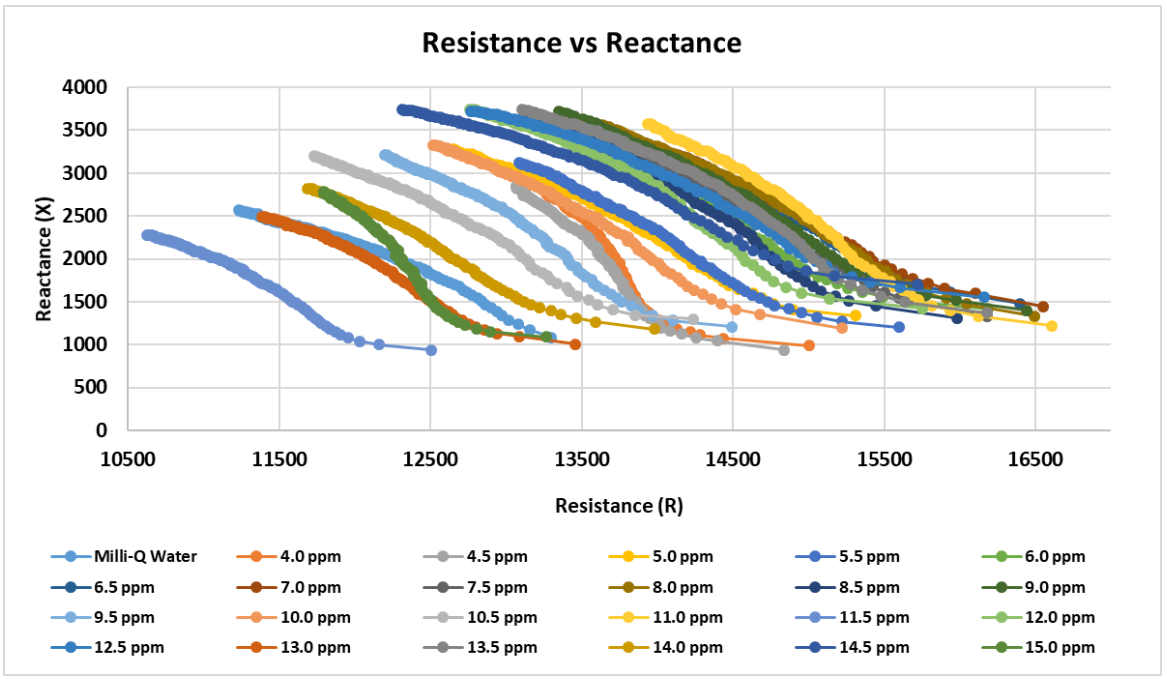


Figure 3.16: Nyquist plot for 10 Hz to 5000 Hz.



(a) Frequency vs resistance for 10 Hz to 100 Hz.

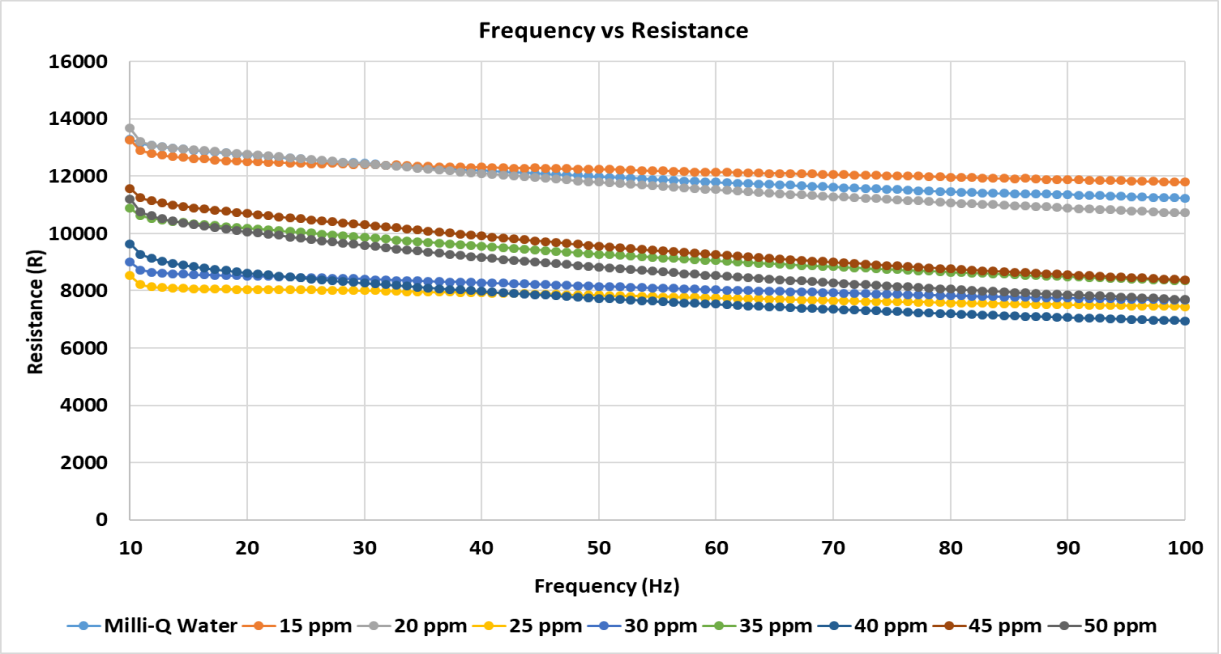


(b) Nyquist plot for 10 Hz to 100 Hz.

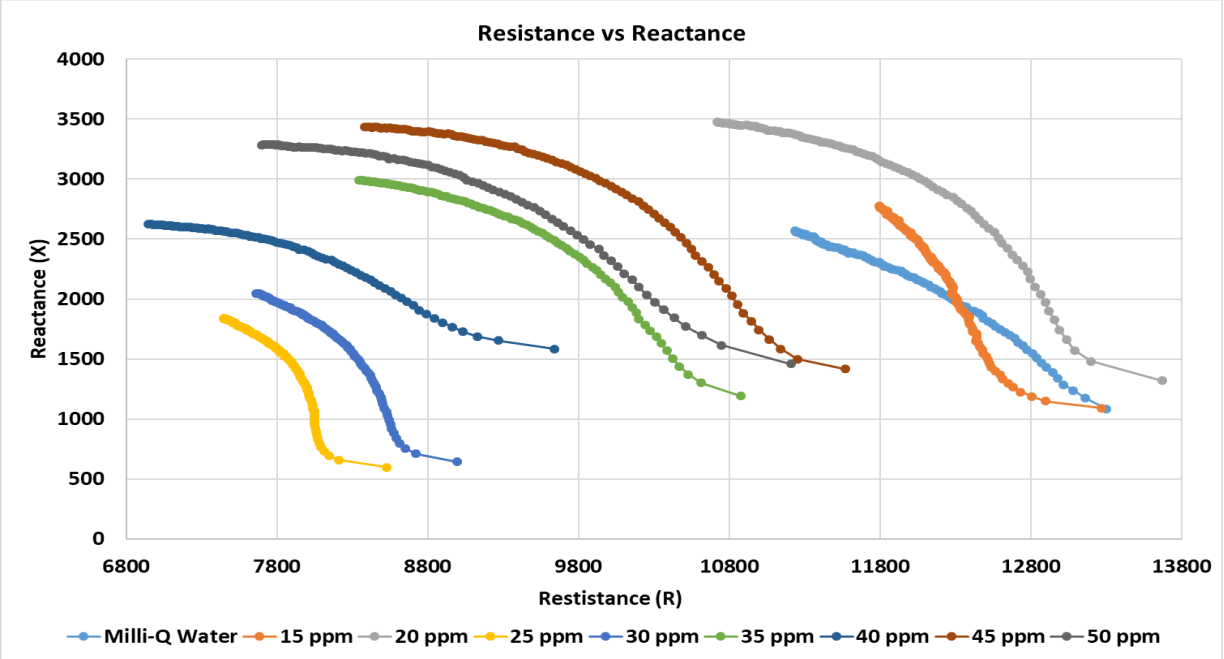
Figure 3.17: (a) Frequency vs resistance for 10 Hz to 100 Hz and (b) Nyquist plot for 10 Hz to 100 Hz.

The higher levels of creatinine are tested from 15 ppm to 50 ppm range, which is three times higher than that of the normal upper limit of creatinine of 12 ppm. Figure 3.18 (a) shows

the relationship between resistance and frequency ranging from 10 Hz to 100 Hz, whereas Figure 3.18 (b) shows a Nyquist plot for higher levels of creatinine concentrations (15 to 50 ppm).



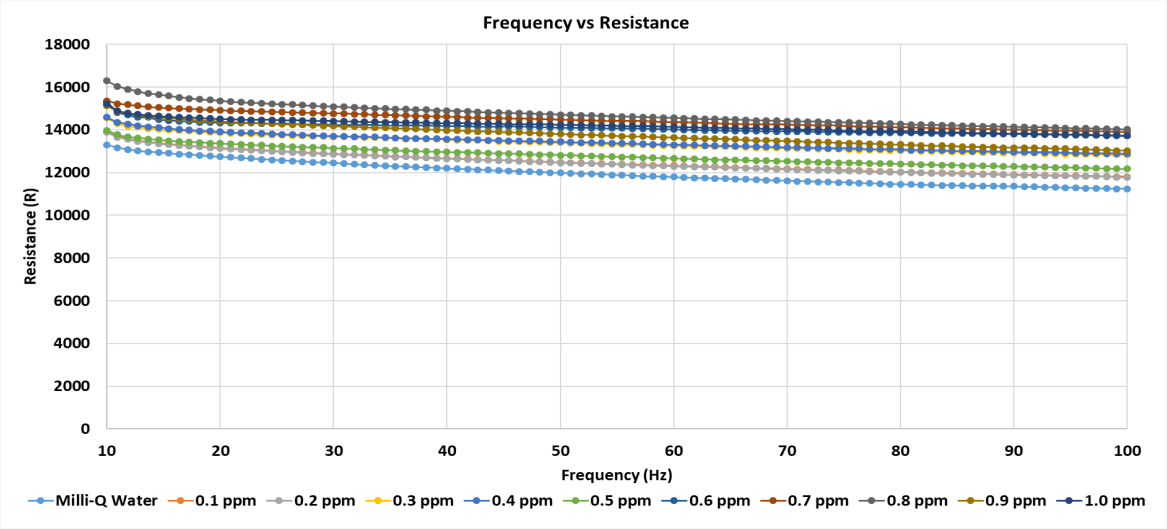
(a) Frequency vs resistance for 10 Hz to 100 Hz.



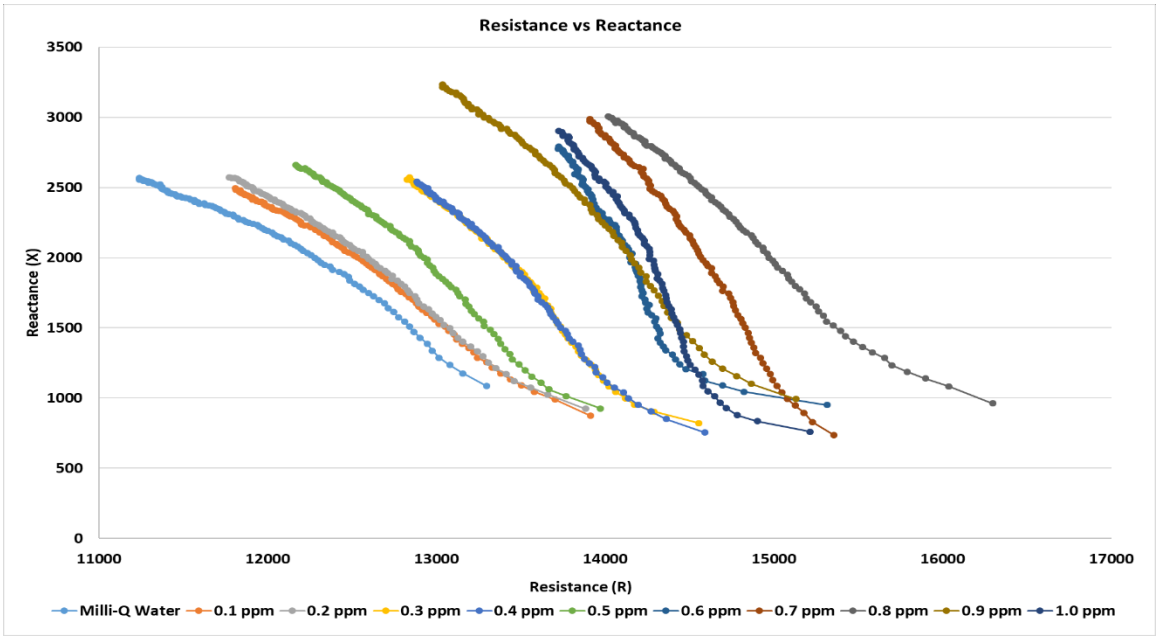
(b) Nyquist plot for 10 Hz to 100 Hz.

Figure 3.18: (a) Frequency vs resistance for 10 Hz to 100 Hz and (b) Nyquist plot for 10 Hz to 100 Hz.

The lower limit of detection (LLD) is also studied for the current sensor, and it is seen from Figure 3.19 (a) and (b) that this sensor is not only able to differentiate the normal and higher creatinine range separately but also the LLD range very clearly. The sensing area of the sensor is tested using creatinine dissolved in the sample solutions with a wide range of concentrations but not specifically functionalized to make it selective only concerning adsorption of creatinine from the mixture of other dissolved substances in the sample solution.



(a) Frequency vs resistance for 10 Hz to 100 Hz.



(b) Nyquist plot for 10 Hz to 100 Hz.

Figure 3.19: (a) Frequency vs resistance for 10 Hz to 100 Hz and (b) Nyquist plot for 10 Hz to 100 Hz.

3.4.4 Single-Frequency Resistance-based Sensor Response

The proposed sensor has good potential to be used as a PoC diagnostic prototype system for the prognosis of rising creatinine levels, which can be used to determine the kidneys healthcare functioning. Narrowing down the frequency range to a single frequency helps in easing sensing as well as data analysis. The process also reduces the overall experimental time. The choice of 100 Hz as an operating frequency is governed by the fact that, at this frequency, the sensor can clearly distinguish between all aqueous samples at all concentrations when tested using a non-functionalized MEMS ID sensor. Therefore, according to the Nyquist plot data analysis, a frequency of 100 Hz is determined as the optimal operating frequency. The changes are observed in resistance values for the non-coated MEMS ID sensor. The sensitivity of resistance (%) to the concentration at 100 Hz frequency is calculated using the following (Equation 3.2).

$$S_R = \frac{R_0(Control) - Rx\,(Sample)}{R_0\,(Control)} \qquad \textbf{(Equation 3.2)}$$

Where S_R is the sensitivity of the resistance at a particular frequency, and Rx (sample) is the resistance value of the sample carrying creatinine concentration at a particular frequency.

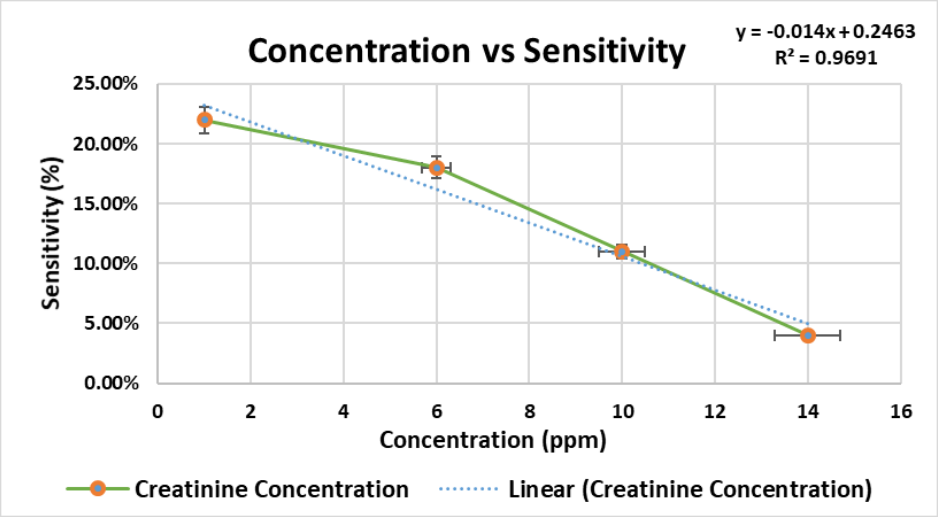


Figure 3.20: Single-frequency resistance-based sensor response at 100 Hz.

The R_0 (control) is the resistance value of control (Milli-Q water) at a particular frequency. The single frequency resistance-based sensor response is shown in Figure 3.20. It displays changes in resistance for various extended normal concentrations of creatinine (1-14 ppm) range at 100 Hz frequency. It is observed that there is a linear relationship between the resistance and the increasing levels of creatinine with a correlation coefficient of 0.9691.

3.4.5 Repeatability and Reusability Testing

To monitor the creatinine measurements in real-time, the user needs to use the sensor for repeatable times with consistent performance. Therefore, repeatability and reusability testing are essential parameters of a sensing experiment performed for the developed MEMS ID sensor. It is observed that the sensing surface requires cleaning with distilled water before its subsequent measurement. In this experiment, the sensing area of a sensor is upside-down immersed in a uniformly mixed sample for allowing maximum attachment of creatinine molecules from the sample on the clean MEMS ID sensors sensing surface.

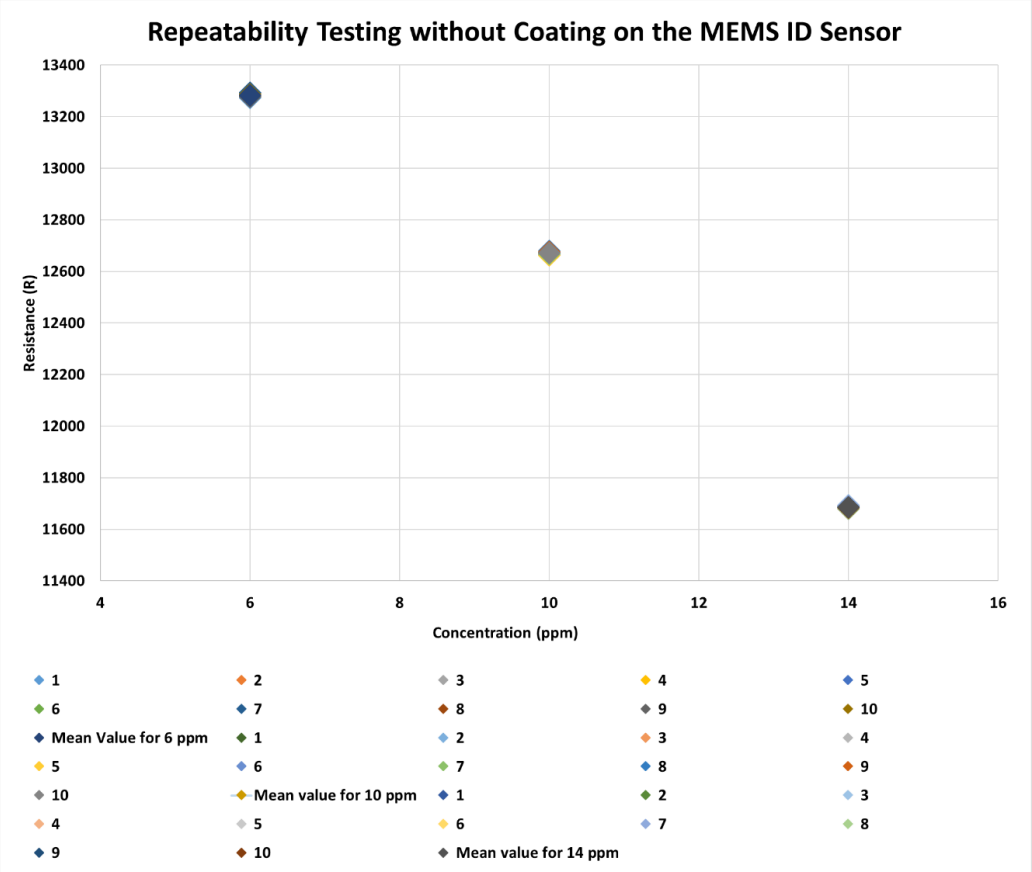
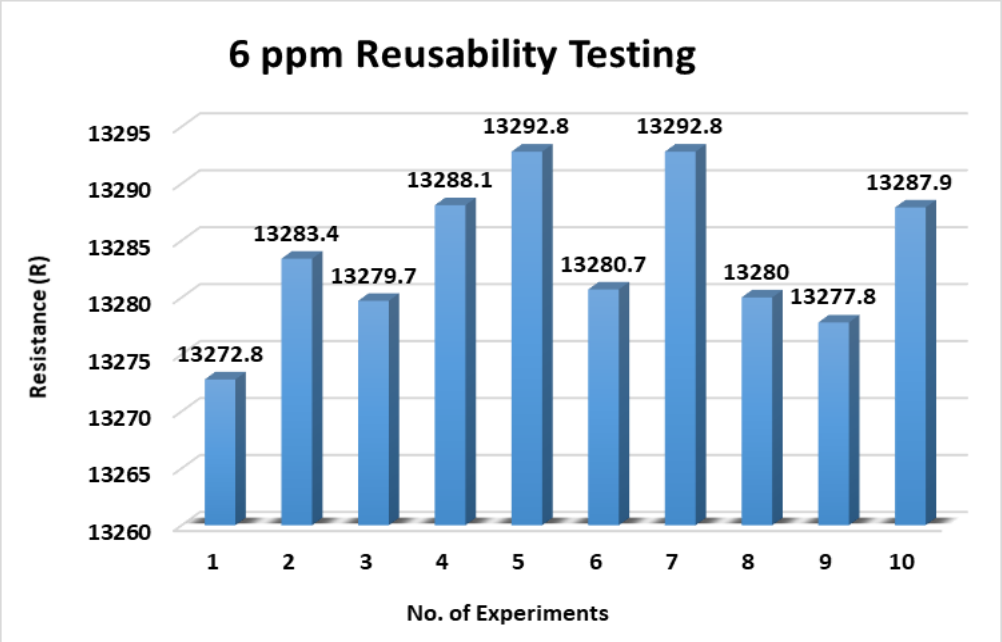


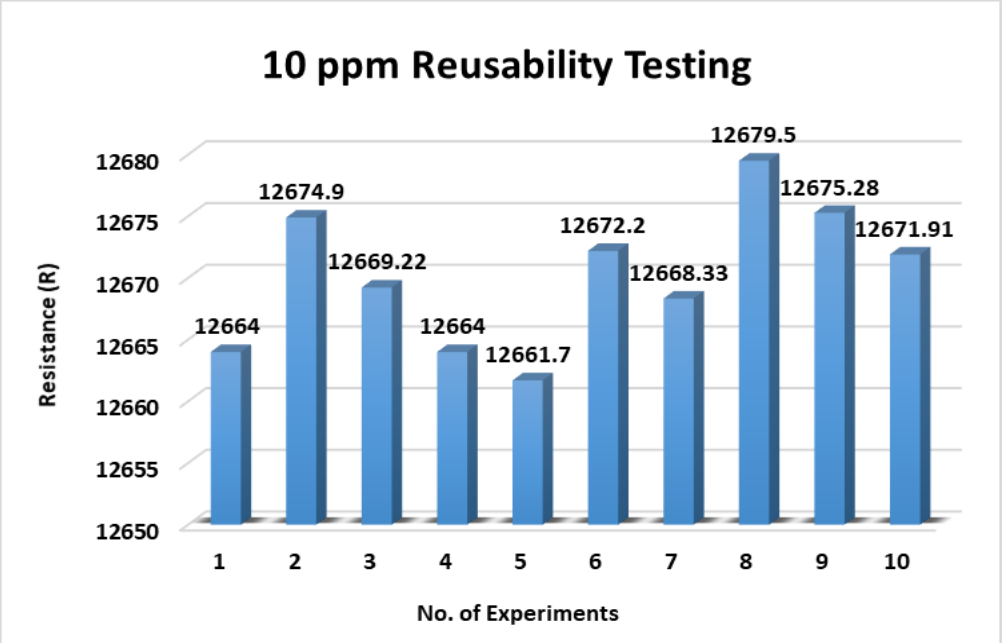
Figure 3.21: Repeatability of results for 6, 10 and 14 ppm creatinine concentrations.

The readings are taken for 6, 10 and 14 ppm samples. The sensing area is washed with distilled water thoroughly before and after each measurement, followed by air-drying before every single measurement. The sensing area results from the repeatability and reusability experiments are performed 10 times for each sample concentration. Figure 3.21 (a) shows repeatability of results for 6, 10 and 14 ppm concentrations. Figure 3.22 (a), (b) and (c) shows the reusability of the sensor for 6, 10 and 14 ppm concentrations.

(a)



(b)



(c)

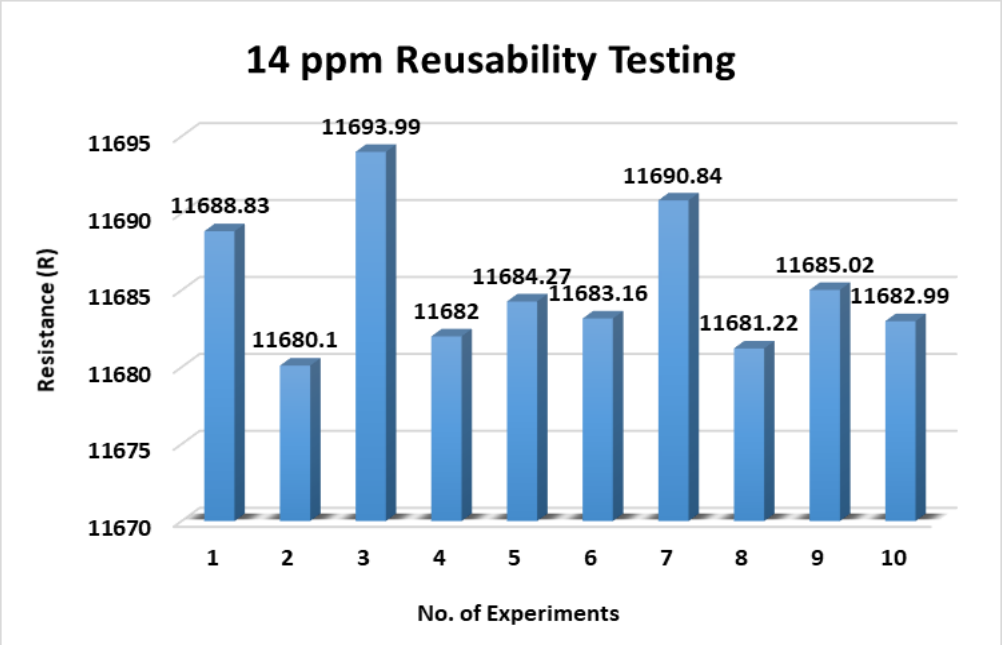


Figure 3.22: Graph (a), (b) and (c) showing reusability of the sensor for 6, 10 and 14 ppm concentrations.

3.4.6 Calculation of Standard Deviation

The standard deviation formula is used to observe how much every result in the MEMS ID sensor repeatability experiment has deviated from the mean value of the results (Figure 3.21). The 6, 10 and 14 ppm showed standard deviation values of 6.62, 5.72 and 4.52. There is a direct relationship observed between the concentration and the value of standard deviation, where the concentrations of the samples increased the value of standard deviation uniformly decreased.

3.4.7 Unknown Sample Measurement and Error Calculation

Using the current sensing system, standard samples of other concentrations are measured by treating them as unknown samples. The unknown sample quantification is done using the S_R based calibration curve equation, as shown in Figure 3.20.

The accuracy of the unknown sample quantification is analysed by comparing the results obtained from the S_R curve and original concentration values measured using the EIS technique and calculating the error%.

The sensor is not coated with any creatinine specific coat; the error% observed is under 30% for all samples given in Table 3.2. Thus, the results obtained from both methods are found to be in good agreement overall.

Table 3.2: Identification of creatinine concentrations in serum samples using a calibration curve obtained by the CNLS technology.

Sample	Actual Concentration (ppm)	Proposed MEMS ID Sensor (S_R) (ppm)	Error (%)
S1	5.5	6.16	21.0
S2	6.0	4.73	12.0
S3	8.5	6.16	27.0
S4	9.5	11.87	24.0
S5	11.5	14.02	17.0

3.4.8 Current Issues and Future Requirements

The MEMS ID sensor needs to be selective only towards creatinine for developing the creatinine selective smart sensing system. It also needs to attract and adsorb only the creatinine molecules for its detection and quantification purpose. Imprinted polymer-based detection technology makes the sensor specifically selective for the analyte, the creatinine. The MIP is a technique capable of synthesising the creatinine specific molecule using multiple chemicals. The MIP is coated on the MEMS sensor for making the sensor's sensing area functionalized. The functionalization means making MEMS ID sensing surface specifically selective towards adsorption of creatinine with the help of the functionalization (coating) process. It is achieved by using the MIP suspension coat solution to create solid functionalization over the MEMS ID

sensors sensing surface. It aids in the adsorption of the creatinine from aqueous and serum samples (explained in chapter 4). The specifically selective nature of the MIP functionalized MEMS sensor helped in the precise detection of creatinine levels from the aqueous and serum samples.

3.5 Chapter Summary

The ID sensors are the most common sensor structures having multiple electrodes. Various advantages have made them ideal for multiple applications, such as one-side entry into the SUT, controlled strength of the signal, highest sensitivity of detection, simplified output and smaller size of the sensor. EIS is a multipurpose method that uses a frequency-dependent small-amplitude AC signal to describe the capacitive and resistive features of SUT. The technique of EIS has been extensively employed in biosensor applications with the help of various methods due to the highest sensitivity and simplicity of the technique [111]. Evaluations of monitoring of environment [112], identification of phthalates in water and juices [88], harmful chemicals found in seafood [90], detection of humidity [113] and detection of the DNA [114] has been stated using ID sensors in combination with the process of EIS measurement. The working principle of the planar ID sensor has been described in this chapter. The fundamental theory of EIS has also been discussed. An experimental setup that is used for EIS has been described. It collects the data from the SUT and transforms it for further study into an electrical signal. The EIS and ID sensor system is beneficial in a detailed study of various concentrations of SUT. After successfully developing a MEMS ID sensor with a 1-1-25 configuration, the sensors are used to detect varying concentrations of creatinine from aqueous samples. The developed MEMS ID sensor is also characterised using the EIS technique, single-frequency resistance-based sensor response, repeatability, and reusability testing analysis. The values of standard deviation for creatinine concentrations are also obtained. Unknown sample measurement and error (%) value calculation is also performed for checking the reliability of functioning of the developed MEMS ID sensor. The MEMS ID sensors are susceptible to detecting many things from SUT, and they need a selective coating of imprinted polymer on the sensing area of the sensor. It helps to make the developed MEMS ID sensor highly specific and selective towards attraction and adsorption of the analyte of interest, i.e. creatinine. Chapter 4 describes the MIP functionalized MEMS ID sensor for creatinine detection from aqueous and serum samples.

4

MIP Functionalized MEMS ID Sensor for Creatinine Detection using EIS Technique

Publications about this chapter:

- **S. N. Prabhu**, S. C. Mukhopadhyay, A. S. Davidson and G. Liu, "Highly selective Molecularly Imprinted Polymer for creatinine detection," **2019 13th International Conference on Sensing Technology (ICST)**, **2019**, doi: 10.1109/ICST46873.2019.9047696
- **S. Prabhu**, C. Gooneratne, K. A. Hoang and S. Mukhopadhyay, "IoT-Associated Impedimetric Biosensing for Point-of-Care Monitoring of Kidney Health," **IEEE Sensors Journal**, **2020**, doi: 10.1109/JSEN.2020.3011848
- **S. Prabhu**, C. Gooneratne, K. Anh Hoang and S. Mukhopadhyay, "Development of a Point-of-Care diagnostic smart sensing system to detect creatinine levels," **2020 IEEE 63rd International Midwest Symposium on Circuits and Systems (MWSCAS)**, **2020**, doi: 10.1109/MWSCAS48704.2020.9184441
- **S. N. Prabhu**, S. C. Mukhopadhyay, C. P. Gooneratne, A. S. Davidson, G. Liu, "Molecularly Imprinted Polymer-based detection of creatinine towards smart sensing," **Medical Devices and Sensors**, **2020**, doi: https://doi.org/10.1002/mds3.10133
- **Prabhu S.N.**, Gooneratne C.P., Hoang K.A., Mukhopadhyay S.C., Davidson A.S., Liu G., (**2021**), Interdigital Sensing System for Kidney Health Monitoring. In: Mukhopadhyay S.C., George B., Roy J.K., Islam T. (eds) Interdigital Sensors. Smart Sensors, Measurement and Instrumentation, vol 36. **Springer**, Cham, doi: 10.1007/978-3-030-62684-6_11
- **S. N. Prabhu**, C. P. Gooneratne and S. C. Mukhopadhyay, "Development of MEMS Sensor for Detection of Creatinine using MIP Based Approach -A Tutorial Paper," **IEEE Sensors Journal**, **2021**, doi: 10.1109/JSEN.2021.3077060

4.1 Introduction

Traditionally, when individuals get diagnosed with kidney disease, they get prescribed for a routine blood check-up, ultrasound, MRI, X-ray and/or CT scan combined with hybrid radionucleotide scans such as SPET/PET. These are commercially available standard techniques. The blood check-up helps in understanding serum creatinine and BUN levels precisely, thus helpful in knowing the waste content levels of the blood. The MRI technique utilises radio waves and magnets. The MRI creates detailed pictures of the soft tissues and body organs. These images can be seen in three dimensions. X-ray and CT scan uses X-rays and computers for creating three-dimensional pictures of patients' renal system. An ultrasound uses sound waves to create a picture of a patients' renal system. These techniques are well developed, pre-standardised and practised everywhere. However, they are time-consuming and costly. Blood testing is performed at pathology laboratories by a pathologist, whereas other scannings are performed at radiology centres by a clinical technician/ radiologist. For these analyses, a kidney disease patient needs to visit a pathology laboratory and/or radiology centre during his/ her sickness multiple times. These pathology/radiology centres are not mostly 24/7 operable, their test results are not immediately available, and they are also uninterpretable to patients. After receiving results, a patient needs to revisit a general practitioner/nephrologist/oncologist to understand their kidney health conditions.

There is a need for a precise, home-based, portable, easily interpretable, rechargeable, reusable, internet and user friendly, cost and time-effective sensing system for long-distance monitoring of kidney patients by doctors over the internet. This kind of system will support kidney patients as it will significantly reduce their visits and money expenditure to the pathology, radiology centres, and kidney healthcare clinics. The home-based system will help them in understanding their creatinine status right at home. This system will be also helpful to medical practitioners as they can easily monitor their patients located over the long-distance with the help of a sensing diagnostic system with internet connectivity and secure data transfer.

Creatinine level analysis using serum samples obtained through pathological blood testing is a universally accepted, widely used standard technique. Although blood testing is a standard technique, it has certain drawbacks, as indicated above in this chapter. The pathology method is invasive as it involves venepuncture. It requires a pathological laboratory setup, a visit to the pathological laboratory/blood sample collection laboratory. It also requires pathology experts in venepuncture and for performing analysis of samples, instrumentation,

chemical reagents, and an expert analyst to obtain sample data. It needs a pathologist for interpretation of the results of the pathological sample report.

Furthermore, sample preparation, storage and sending it to the pathology analysis centre from the blood sample collection laboratory are time-consuming, highly sensitive and difficult. Artificially synthesised creatinine, specifically selective polymers, can be quite useful in overcoming these issues. Molecular imprinting technology, also known as MIP, is a fast and low-cost method of synthesising polymers with selectivity and sensitivity only to a specifically targeted template molecule (i.e. creatinine). MIP technology has been effectively utilised in a variety of applications as solid-phase extraction materials [115], enzyme-mimic catalysts [116-118], and binding assays [119-120]. It is increasingly being utilised in imprinted layers to build chemical sensors [121-122].

MIP's have been used to build electrochemical sensors based on several transduction models, including potentiometric [123], conductometric [124], voltammetric [125], and capacitive sensors [126].

It is described in the above section that there are some difficulties and restrictions related to the existing methodologies used for prognostic as well as prophylactic care of kidney health. It encouraged fabricating creatinine specific selective sensors and developing a LoRaWAN-based complete sensing system to make a patient-friendly kidney health system. Currently, creatinine values cannot be monitored from home. The patient needs to visit the pathology laboratory for the blood test and/or frequently go for an ultrasound/MRI/X-ray/CT scan. The visits depend on their creatinine levels and underlined disease conditions associated with healthcare necessities. The individual results or the difference between consecutive test results are not patient-friendly in understanding. A patient needs to visit a medical practitioner to understand their kidney health status every time post testings. There is a lack of a patient-friendly system that the patient can utilise for their regular check-up.

This study aims to develop a PoC diagnostic prototype system for kidney health monitoring that is simple, affordable, rapid, reusable, repeatable, internet connectable, distantly operable and a system that can be utilised as a long-distance home-based system outside of laboratories. Developing a sensing system that combines the benefits of synthetic MIP-based polymer with a rapid and time and cost-efficient electrochemical biosensor appears to be a highly viable path towards our aim.

To the best of our knowledge, no MIP-based MEMS ID sensor for creatinine detection has been introduced yet. Therefore, this chapter describes the synthesis of an impedimetric creatinine-selective biosensor that combines MIP technology with the EIS methodology and MEMS ID sensor to provide sensitive and rapid serum creatinine measurements.

4.2 General Principle of MIP

The sensing area is functionalized with the MIP particles to introduce creatinine associated specificity and selectivity to the designed MEMS-based planar ID sensors. The technique of molecular imprinting is non-expensive, which permits the synthesis of artificial binding sites inside chemically synthesised synthetic polymer particles. The difference between MIP and NIP is that, while synthesising NIP, the creatinine template molecule is not used. Therefore its structure is not precise for specific attraction and adsorption of the creatinine molecules. Instead, the NIP is used as a control polymer. Figure 4.1 shows the functioning principle of MIP technology (a) schematic diagram of MIP polymerisation process and (b) diagrammatic representation difference between MIP and NIP surfaces for target molecule specific binding.

A template (T) molecule is uniformly mixed with a monomer (M) molecule in MIP technology. In the next step, the cross-linker (CL) molecule assembles a self-generated structural geometry (1). The polymerisation process of all the chemicals (T + M + CL) results in the systematic production of a heavily cross-linked structure. It has imprinted sites (2) for the targeted template (creatinine). In the last step, template molecules are extracted (which are locked within the polymer powder matrix) with the help of the soxhlet extraction process. The process helps in leaving empty three-dimensional cavities in the MIP structure. In the future, the MIP cavities allow specifically selective recognition, adsorption and binding of the targeted creatinine molecule (3) [127].

The MIP have significant applications in the various branches of clinical analysis, medical diagnosis, and the targeted drug delivery process. MIP permits the formation of artificial receptors carrying binding sites similar to natural binding sites. The advantage of using MIP is that the polymer powder can withstand much harsher conditions, including higher levels of temperature, pressure and pH. The polymer can repeat its functions without losing its adsorption activity up to a certain number of times. The MIP is easy to prepare, it has a meagre production cost, and the polymer is also storage stable. The MIP can be synthesised in large

quantities along with having significant reproducibility [128]. This chemically synthesised synthetic MIP powder is a decent alternative to naturally available sensitive biological receptors. A technique for creating artificial recognition sites with a memory of a pre-set target molecule, molecular imprinting has grown in popularity in several domains of biology and chemistry. Sensors [88, 129-131], assays [132], artificial antibodies [133-134] and stationary chromatographic phases [135-136] are some of the most promising MIP uses.

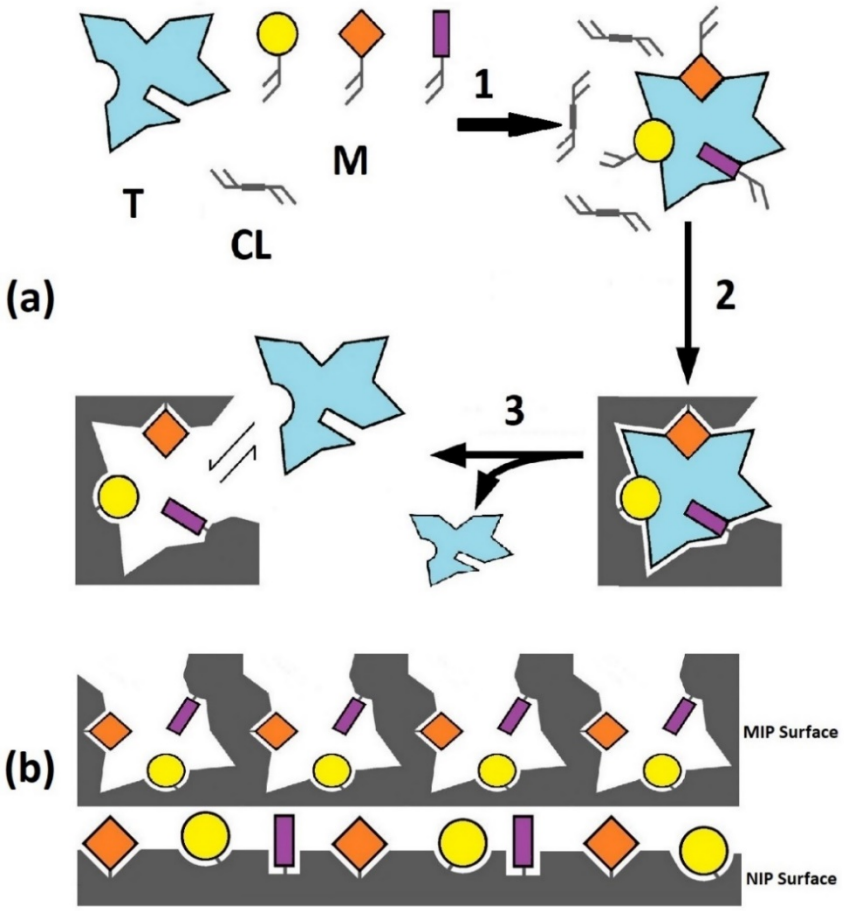


Figure 4.1: The functioning principle of MIP technology (a) Schematic diagram of MIP polymerisation process, (b) Diagrammatic representation difference between MIP and NIP surfaces for target molecule specific binding.

The popular alternative methodologies to synthesise MIP are processes of bulk polymerisation and precipitation polymerisation. The precipitation polymerisation process synthesises the specifically selective polymer powder for creatinine molecule [137]. For the conventional MIP bulk polymerisation process, the polymers are formed in bulk quantity. The polymer needs crushing, grounding, and sieving processes to achieve the desired sizing of

polymer powder. During all these processes significant quantity of template recognition sites are damaged, and overall it is also an inefficient time process. When compared to the process of bulk polymerisation, the sphere-shaped polymer powder is entirely synthesised using precipitation polymerisation. Therefore, in the current creatinine specific MIP synthesis, the precipitation polymerisation process is preferred.

4.2.1 Categories of MIP

Two types of molecular imprinting techniques have been devised founded on the covalent or non-covalent bonds. These bonds are observed between the template and functional monomers. Because of its numerous benefits, such as ease of preparation, quick extraction of the template molecule and rapid rebinding, the non-covalent technique has been widely adopted [138-141].

4.2.1.1 Covalent Imprinting

The template molecule is covalently attached to the functional monomer using reversible covalent bonds in the covalent method. The imprint template is chemically extracted from the polymer matrix after the process of polymerisation. The covalent bond will be created once more when the template is reintroduced into the polymer matrix from the sample solution. Various types of template molecules, including sugars [142] and glyceric acid [143], have been studied using the covalent method. The necessities for covalent imprinting vary from those for non-covalent imprinting, particularly when it comes to the template, monomer and cross-linker ratios.

The fundamental benefit of this method is that it results in a uniform spreading of recognition sites in the matrix of the artificially synthesised polymer [144-145]. However, this approach necessitates an acid hydrolysis step to extract the imprinted molecule from the polymer matrix [144].

4.2.1.2 Non-Covalent Imprinting

Due to its simplicity, non-covalent imprinting is the most often utilised method for synthesising MIP's. The template-monomer complex is generated through non-covalent weak

interactions (ionic, hydrophobic, and hydrogen bonding) between the template molecule and the monomer in the non-covalent imprinting process [146-148]. The non-covalent technique can employ various functional monomers, but MAA has been widely employed as a functional monomer. This is due to its ability to interact with many functional groups [144].

Due to the higher intensity of interactions with MAA, amino acids are commonly employed in the non-covalent technique [149]. With the help of polar chemical environments, the formed bonds between the template and monomers can be easily broken.

4.2.2 Effects of Monomers, Cross-Linkers, Porogenic Solvents and Reaction Initiators in Synthesis of MIP

The significant influence of the effects of monomers, cross-linker, porogenic solvents and initiator is seen on the synthesis of the MIP. It is explained below.

4.2.2.1 Monomers

Monomers are crucial in MIP production because they offer complimentary interactions with the template molecule and substrates. The template commands the number of functional monomers linked covalently in the covalent imprinting procedure. Therefore, altering the template to functional monomer ratio does not affect it. However, in the non-covalent technique, the appropriate template to monomer ratio is determined empirically by experimenting with various template/monomer ratios [150].

To maximise the imprinting effect, the monomer's functionality needs to be matched with the template's functionality in a complimentary manner. The typical functional monomers employed in non-covalent molecular imprinting are shown in Figure 4.2.

4.2.2.2 Cross-Linkers

The amount and kind of cross-linker used in the MIP preparation can have a significant impact on selectivity. It is because the cross-linker controls the shape of the MIP. In addition, they stabilise the imprinted recognition sites and provide mechanical stability to the polymer matrix in the production of the MIP. Figure 4.3 shows the chemical structure of some typical cross-linkers employed in non-covalent molecular imprinting.

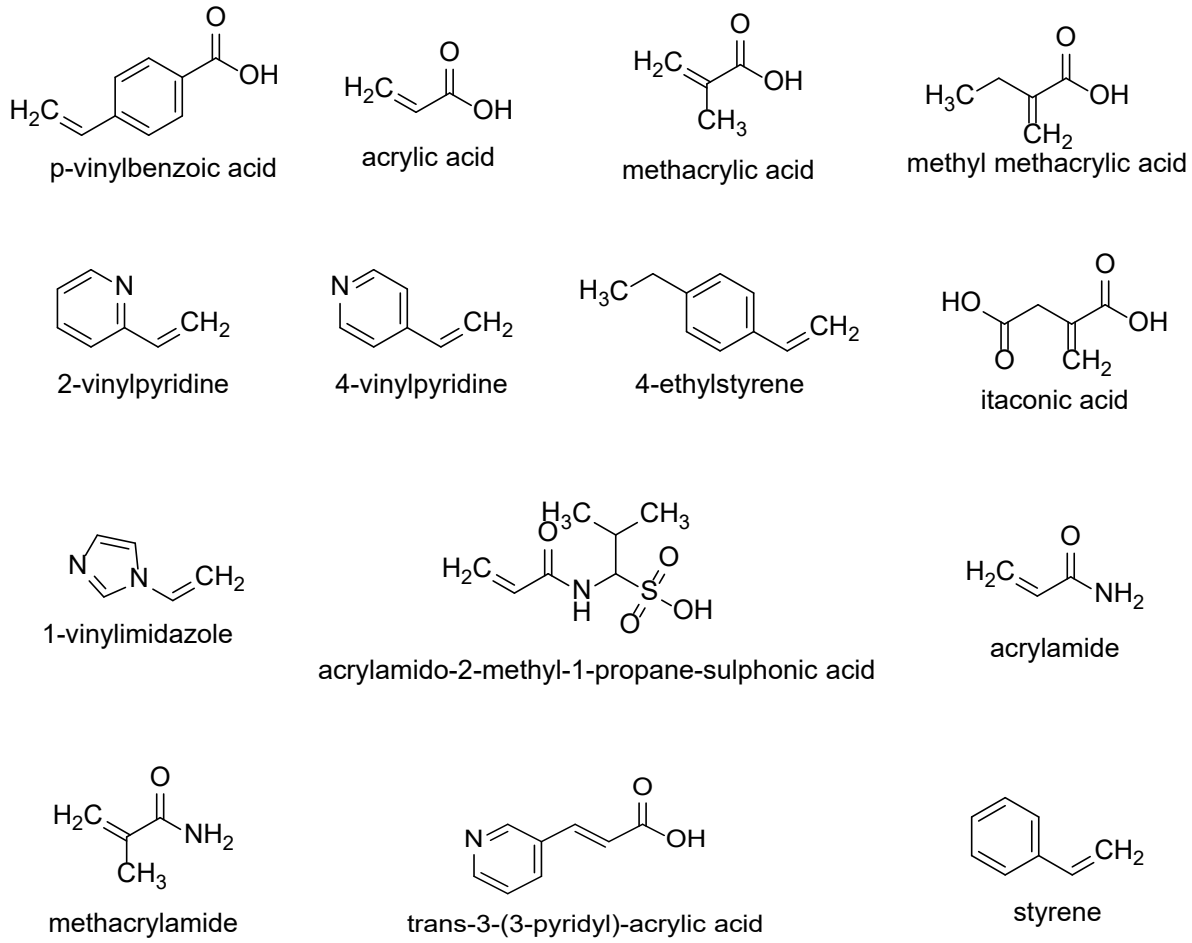
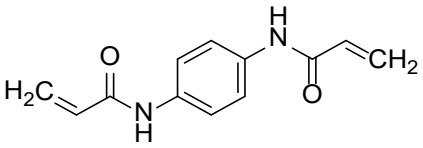


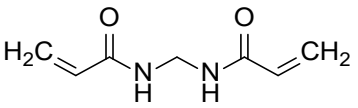
Figure 4.2: Chemical structures of various functional monomers utilised in non-covalent MIP synthesis [144].

4.2.2.3 Porogenic Solvents

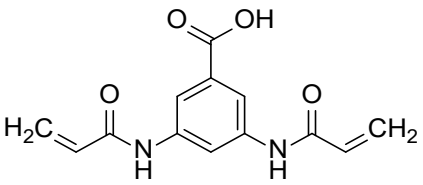
Porogenic solvents are critical in determining the strength of non-covalent bonding and affecting polymer shape, both of which have a direct impact on MIP performance. The porous structure of the polymer matrix can be influenced by porogenic solvents [151]. The porogenic solvents need to be soluble in the template, cross-linker, monomer, and reaction initiator. Furthermore, the porogenic solvents can generate big holes to ensure that the polymer has suitable flow-through qualities. Furthermore, the porogenic solvent also needs to have a low polarity to prevent interference during the template/monomer complex formation, critical for good selectivity [144].



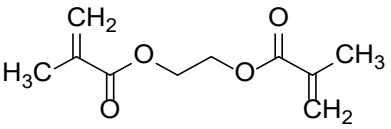
N,N'-1,4-phenylenediacrylamine



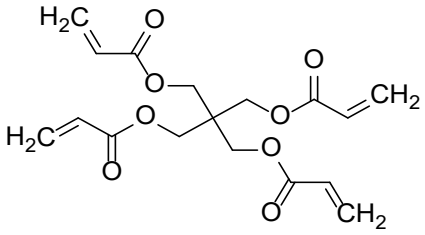
N,N'-methylenediacrylamide



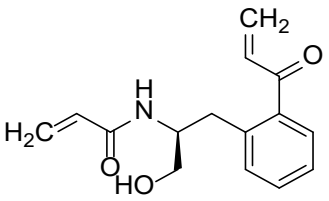
3,5-bis (acrylamido) benzoic acid



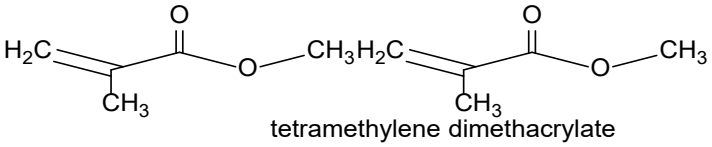
ethylene glycol dimethacrylate



pentaerythritol tetraacrylate

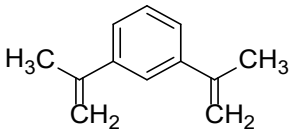


N, O-bisacryloyl-phenylalaninol

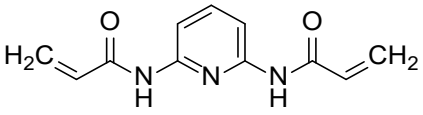


tetramethylene dimethacrylate

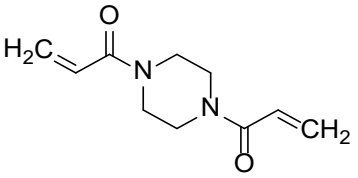
CH_2:
CH_2:



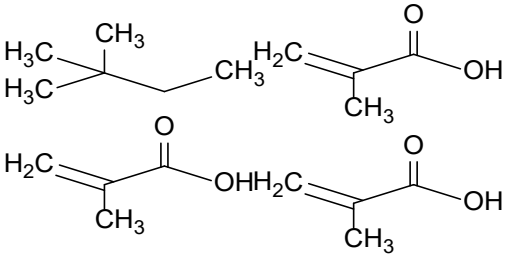
1,3-diisopropenyl benzene



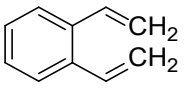
2,6-bisacryl amidopyridine



1,4-diacryloyl piperazine



trimethylpropane trimethacrylate



divinylbenzene

Figure 4.3: Chemical structures of various cross-linkers utilised in non-covalent MIP synthesis [144].

4.2.2.4 Reaction Initiators

To start the polymerisation process, various initiators with diverse chemical structures and characteristics can be utilised. For example, the chemical structure of specific non-covalent MIP initiators is shown in Figure 4.4.

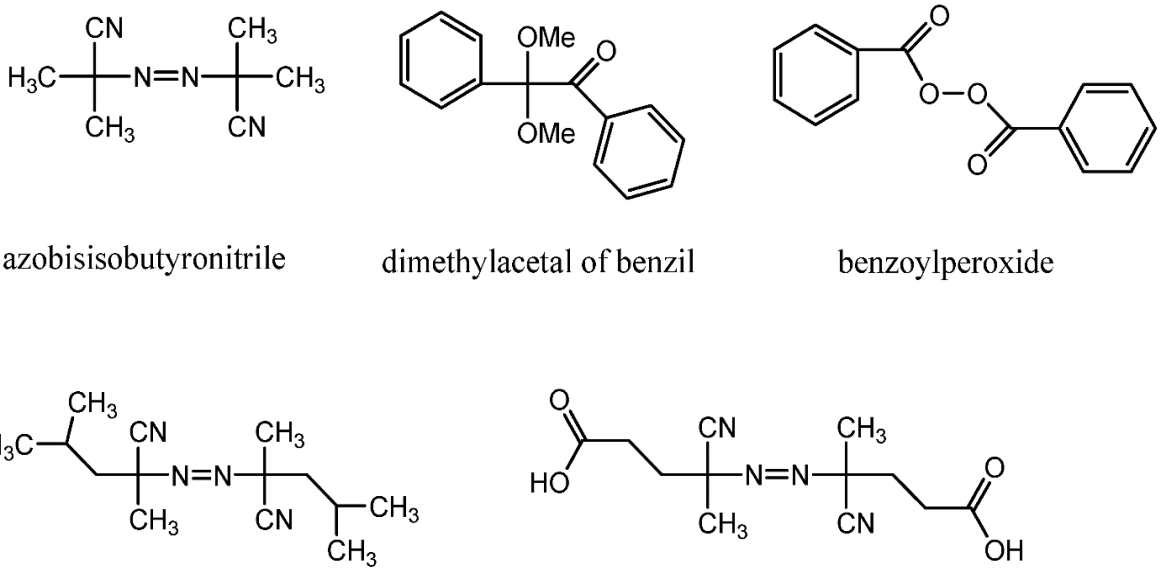


Figure 4.4: Chemical structures of reaction initiators used in non-covalent MIP synthesis [144].

4.2.3 Synthesis Methods for MIP

MIP's can be made in various shapes and sizes, including microspheres, nanospheres, and polymer monoliths, depending on the purpose. Bulk polymerisation is widely employed to synthesise MIP's in the form of a hard monolith that had to be crushed, ground and sieved to get the correct particle size for many years. These procedures take time, and some recognition sites will be destroyed during the crushing and grounding of the MIP. Furthermore, the particle form is irregular, which is inconvenient for their applications [152]. Several unique preparation techniques, such as precipitation [153-154], suspension [140, 155] and emulsion polymerisation [156-157], are later reported.

Within this category, precipitation polymerisation is unusual since the other processes require additives such as stabilisers, which might negatively affect the process of MIP synthesis. Microspheres of uniform size may be made via precipitation polymerisation; they have a greater active surface area, allowing them to include more recognition sites than MIP made by bulk polymerisation. Changing the polymerisation conditions may readily adjust the size and porosity in this process. Table 4.1 provides a review of the benefits and drawbacks of various MIP preparation processes.

Table 4.1: A shortlist of various synthesis methods of MIP.

Synthesis Method	Advantages	Disadvantages
Precipitation polymerisation	• Uniformity in polymer size. • Imprinted microspheres.	• Higher dilution factor. • The hefty volume of the template.
Bulk polymerisation	• There is no need for complex instrumentation. • Simplicity as well as universality.	• Lower performance rate. • Additional protocol for grinding and sieving. • Non-uniform shape and size of polymer.
Surface polymerisation	• Thinner imprinted layers. • Monodispersed polymers.	• Time inefficient. • Complex system.
Multi-step swelling polymerisation	• Monodisperse polymers.	• Need aqueous emulsions.

	• The diameter of the polymer is controllable. • Outstanding for UHPLC.	• Complicated procedure.
In-situ polymerisation	• In-situ process. • Cost-efficient. • Single-step preparation process.	• Every new template requires additional optimisation.
Suspension polymerisation	• Possible to work on a large scale. • Spherical shaped particles. • Exceedingly reproducible.	• Incompatibility to water with various imprinted products.

4.3 Materials and Methods

The chemical reagents, their role during the process of MIP synthesis, apparatus, instruments required for various analysis and their methods are described below.

4.3.1 Chemicals

For making the MEMS ID sensor specifically selective towards creatinine, a creatinine powder ($C_4H_7N_3O$) is used as a template. The functional monomer MAA ($C_4H_6O_2$) is used. A cross-linker molecule is chosen in the form of DVB ($C_{10}H_{10}$), and as a reaction initiator, AIBN ($C_8H_{12}N_4$) is used. ACN (C_2H_3N), as well as toluene (C_7H_8), are utilised as solvents. The Milli-Q water, methanol (MeOH) (CH_4O) and THF (C_4H_8O) are used for the process of washing

which is necessary for the removal of excess chemicals used during the process of MIP as well as NIP polymerisation. AcOH (CH_3COOH) is used for the removal of locked template molecules from the cavity of the newly formed MIP molecule after the washing steps. The Milli-Q water is used for all the experiments.

Creatine ($C_4H_9N_3O_2$), 2-pyrrolidone (C_4H_7NO); N-hydroxysuccinimide ($C_4H_5NO_3$), 1-methylhydantoin ($C_4H_6N_2O_2$); and hydantoin ($C_3H_4N_2O_2$) are used for checking the specificity of MIP molecules towards creatinine. All the chemicals mentioned above are used for experiments and are procured from Sigma-Aldrich, Australia.

Acrylic Resin; and acetone (C_3H_6O) are used to process functionalization purchased from Sigma-Aldrich, Australia. All the commercially ordered chemical reagents are of the purest grades. They did not require pre-distillation before their usage. All the glassware's are purchased from Sigma-Aldrich, Australia. Figure 4.5 displays structures of chemicals used during the creatinine selective MIP and NIP synthesis process.

Human serum male AB plasma, having a sterile-filtered origin from the USA, is ordered from Sigma-Aldrich, Australia. It is used as a base for making various serum creatinine samples of varying concentrations of creatinine.

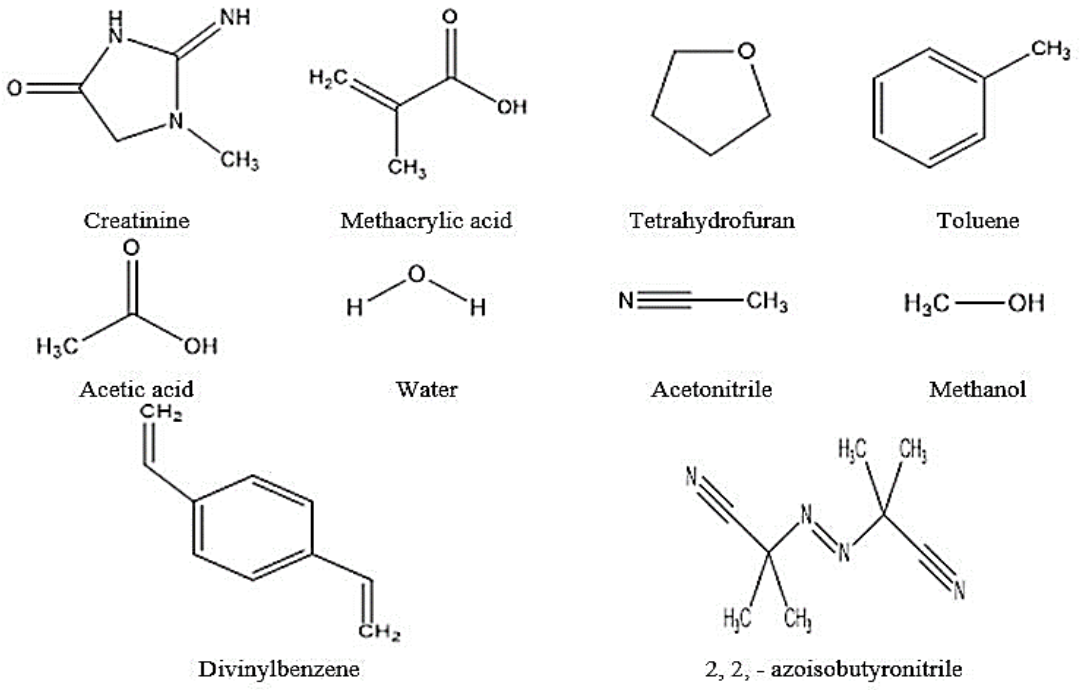


Figure 4.5: Structure of creatinine and other compounds used in this chemical synthesis of MIP and NIP polymers.

4.3.2 Apparatus and Instruments

Advantec® MFS Inc., Japan, manufactured cellulose thimble filter and round qualitative filter paper no. 1 are utilised for the post-synthesis soxhlet extraction and washing processes. The UHPLC column (Luna 5u C18 100A) is ordered from Phenomenex, Australia and used for UHPLC analysis. Thermo Fisher Scientific, USA, a brand UHPLC 2 mL screw neck vials are used for UHPLC analysis. Sartorius® Stedim Biotech GmbH, Germany, brands' Minisart hydrophilic syringe filters (0.2 μm) are used for UHPLC analysis. Elix® Advantage water purification machine, Merck Company, USA, is used for obtaining the Milli-Q water. MIP synthesis is done inside a water bath, and the instrument is purchased from PolyScience, model WB02. MIP washing and centrifuge process are done using Allegra X-30 Centrifuge, Australia. A 1000 mL capacity analogue heating mantle is purchased from Huanghua Faithful Instrument Co. Ltd., China. It is utilised in the process of heating soxhlet extraction. The soxhlet extraction apparatus is used to extract the stuck molecule of creatinine from the polymer matrix. It is procured from Sigma Aldrich, Australia.

SEM with EDAX facility from PHENOM XL Benchtop SEM is preferred for SEM and EDAX analysis. Captured SEM images and EDAX graphs are used to image the sensing surface and characterise polymer surface structure, dimensions, and chemical composition confirmation. The JEOL JSM 7100F FESEM from Thermo Fisher Scientific, USA, is used for the FESEM analysis. They are used for the characterisation of the polymer surface using two-dimensional analysis. It is also used for taking images of the MEMS sensing surface. These analyses are done by functionalizing the MIP and NIP polymers over the MEMS sensors sensing surface by taking images using FESEM, SEM. The graph is obtained using an EDAX analyser. Acrylic resin is also coated over MEMS sensors sensing surface, and images are taken using FESEM instrument. EDAX is for checking the chemical composition of the polymer. Before SEM and EDAX analysis of the acrylic resin, MIP and NIP polymer, the sputtering of the Cr is performed. It is performed by an Emitech K550 Sputter Coater from PerkinElmer, Australia, to get high-quality images by improving the conductivity of the sample under imaging space.

The Nicolet iS5 FTIR spectrophotometer with accessory Attenuated Total Reflectance (ATR) is utilised for both the polymers (MIP and NIP) during FTIR analysis. The FTIR analysis is performed using the FTIR analyser by placing 0.1 gm of a single type of sample

powder under the instrument's detector probe. A vortex mixer from Heidolph Reax top shaker, Germany, is utilised for mixing the samples uniformly.

The detection of the quantity of captured creatinine (adsorption studies) using MIP and NIP polymers is done with Agilent 1260 Infinity UHPLC using a Luna C18 detection UHPLC column. It is from Agilent Technologies Australia Pty. Ltd., Australia and used for all the UHPLC analysis. For finding the MIP selectivity for creatinine and its structurally similar compounds, the UHPLC instrument is used. For the functionalization of MIP over the sensors sensing surface, the Desktop PTL-MM01 Dip Coater having speed adjustability (1-200 mm/minute) is utilised. The dip-coating process is carried out by adjusting the dipping speed at 200 mm/minute at 1 second dipping time.

4.3.3 Synthesis of Specifically Selective Polymer by using MIP Technology

The MIP is developed with the help of the technique of precipitation polymerisation. The technique of precipitation polymerisation has multiple advantages, e.g. easy preparation, low cost of production, storage stability, high level of selectivity, higher toughness, strength for tolerating heat and pressure, chemical stability and usability within strong chemical environments. In precipitation polymerisation, the MIP synthesis is followed by the simple soxhlet extraction polymer washing process. The precipitation polymerisation technique is also preferred because, after washing, it allows the usage of polymer powder with minor crushing. Overall, precipitation polymerisation is considered the easiest technique which helps in the synthesis of MIP [158-160]. Figure 4.6 displays the MIP polymerisation and creatinine extraction process.

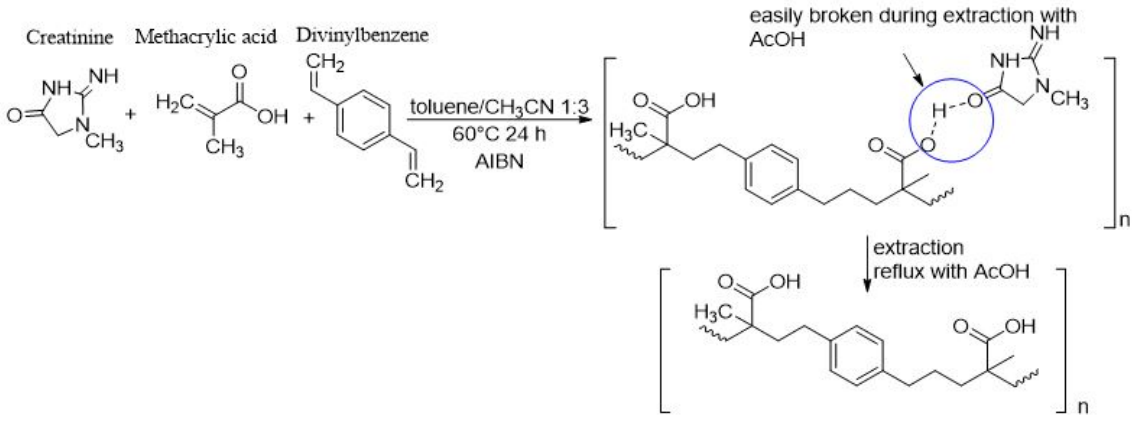


Figure 4.6: Complete illustration of MIP polymerisation and creatinine extraction process.

In this research, an isotropic, spherical, and monodispersed MIP is developed for creatinine with the help of a precipitation polymerisation technique. The currently developed MIP synthesis precipitation polymerisation method for creatinine detection is different from the existing techniques. The MIP synthesis using the developed method is also much simpler than that of the existing techniques. Creatinine is a target molecule in the synthesis of MIP, and it does not get mixed in any non-aqueous solutions. For obtaining the uniform creatinine concentrate, the creatinine (0.10 gm) is completely dissolved inside Milli-Q water (2 mL) [161] which makes this chemical reaction a different than usual process of MIP synthesis.

In the process of MIP synthesis, the first step is started by dissolving creatinine powder (0.10 gm) into 2 mL of Milli-Q water. In the second step, a round bottom flask is taken, and MAA (0.510 mL), DVB (7.50 mL) and AIBN (3.22 mL) are mixed. The process of synthesis is done by using chemicals in the following ratio = creatinine (template): MAA (monomer): DVB (cross-linker) at a proportion of 2: 0.510: 7.5. In the third step, after adding the above chemicals, ACN (96 mL) and toluene (32 mL) are added in 3:1 proportion as solvents. The mixture of chemicals inside a flask (round bottom) is consistently rotated with the help of a vortex mixer. In the fourth step, creatinine concentrate (2 mL) is added to the mixture of chemicals. In the fifth step, after the addition of creatinine concentrate, the reaction mixture is slowly rotated with the help of a vortex mixer [161]. In the sixth step, the resulting mixture is sealed using parafilm, followed by degassing it for 15 minutes using purging of N_2 gas. The process of purging N_2 aids in eliminating the presence of other gasses from the solution, e.g. O_2. The presence of O_2 affects the process of polymerisation negatively [162-163]. In the seventh step, degassing is followed by 10 minutes of rotation of the flask (round bottom) with 2 G-force speed using a vortex mixer. The flask is sealed using the parafilm in the eighth step, followed by keeping it for 24 hours inside preheated water bath at 60°C. The water bath helps in finishing the process of MIP polymerisation.

In the ninth step, the NIP synthesis is also done using the procedure mentioned above. The main difference in NIP synthesis lacks in the addition of creatinine concentrate (template) while performing the process of polymer synthesis. Except for lacking creatinine, all other chemicals, quantities, and synthesis processes remained unchanged during making NIP polymer. In the tenth step, post-process of polymerisation of MIP and NIP, the supernatants obtained from the reactions are discarded. Finally, in the eleventh step, the polymer powders are collected in two separate glass bottles.

In the twelfth step, both the polymer powders are washed thrice, first using MeOH, second using Milli-Q water and third using THF, followed by air-drying inside a fume hood and Petri plate overnight. During the process of washing, the powders are first washed four times using MeOH (50 mL) at 20°C. Every time washing is done at a speed of 1960 G-force for 15 minutes in the first washing step. In the second wash, the powders are washed four times using Milli-Q water (50 mL) at 20°C. The washing using Milli-Q water at each time is done for 15 minutes, and the speed of the centrifuge is maintained at 1960 G-force. In the third wash, the powders have again washed a total of three times using THF (40 mL) at 20°C. During each THF washing, the polymer is washed at the speed of 1960 G-force for 15 minutes. To avoid the unexpected mixing of multiple chemicals, the supernatants are discarded after every single centrifuge step for MIP and NIP. Post third THF washing, the MIP and NIP powders are dried inside the laboratory thoroughly by placing them in a Petri plate (10 hours inside a fume hood).

The synthesis and centrifugal washing of the MIP and NIP polymers are followed by the soxhlet extraction process (thirteenth step). The soxhlet extraction is only required for the MIP and not for the NIP polymer. This is because creatinine is used as a template molecule while synthesising the MIP. Even after multiple washing processes, the creatinine template molecule remains stuck inside the cavity of the MIP polymer, thus needing acid reflux for 24 hours using the soxhlet extraction apparatus. In the thirteenth step, soxhlet extraction is performed. During the soxhlet extraction, the MIP is first placed inside the cellulose thimble filter. The complete process of soxhlet extraction is done for creatinine removal (template molecule) with the help of acid reflux using AcOH (300 mL for 24 hours). At the end of soxhlet extraction, the solvent left inside the round bottom flask (Figure 4.7 (a)) is screened for the presence of extracted creatinine molecules from the MIP powder using the UHPLC analysis solvent run process. It is analysed using the same process, which is used for the detection of creatinine using “Adsorption Studies of Creatinine Specific MIP and NIP Polymers” discussed in the results section. In the fourteenth step, post soxhlet extraction process, the polymer is removed with the help of round qualitative filter paper no. 1. The acquired filtrate is disposed of as chemical waste. In the fifteenth step, for removing the unwanted presence of AcOH inside dried powder, the powder of MIP is transferred to new round qualitative filter paper no. 1. The polymer is washed with Milli-Q water (200 mL) over the filter paper. In the sixteenth step, post drying, the polymer is transferred to a different round qualitative filter paper no. 1. The polymer is again washed with Milli-Q water (200 mL) for removing the leftover traces of AcOH. This is followed by an overnight drying of MIP inside the fume hood. In the seventeenth step, the

MIP (dried form) is gathered inside mortar and pestle, and it is very delicately ground for collecting the polymer particles of very fine size. It is stored under refrigeration inside a glass bottle. In the eighteenth step, the NIP (dried form) is also gathered inside a mortar and pestle, and it is also delicately ground for collecting the perfect sized NIP particles. Finally, the NIP storage is also done under refrigeration inside a glass bottle in the nineteenth step. The process of soxhlet extraction is displayed in Figure 4.7 (a) and (b).

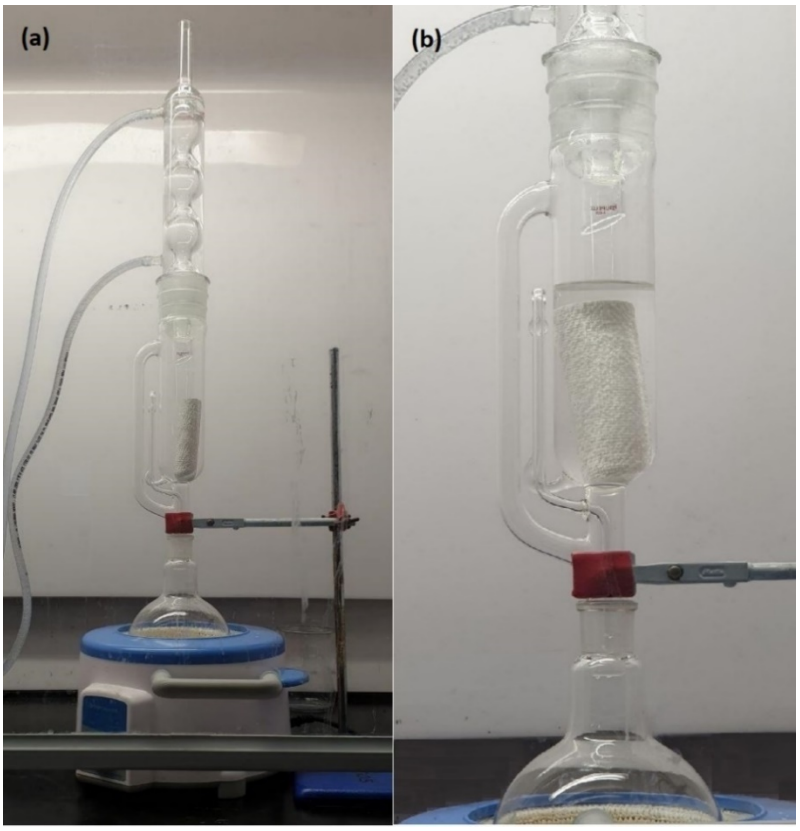


Figure 4.7: Soxhlet extraction process for MIP (a) Assembly of soxhlet extraction apparatus, (b) Creatinine molecule extraction from MIP powder kept inside cellulose thimble filter suspended in AcOH solution.

4.3.4 Preparation of Creatinine Spiked Serum Samples

A concentrated stock solution of 100 ppm creatinine serum solution is prepared by mixing 1 mg of creatinine powder in 10 mL of heat-inactivated human serum. The creatinine spiked serum samples stock is stored inside the refrigerator for preserving its active functionality. The serial dilution technique is used for preparing the samples of lower concentrations of 1 to 15 ppm from the stock. The pure serum carrying zero levels of creatinine

content is used as a control solution for all other different samples with varying creatinine concentrations.

4.3.5 Sorption Studies of Creatinine by MIP as well as NIP

The adsorption quality of the synthesised polymer is analysed by a UHPLC device using a Luna C18 detection UHPLC column for determining the quantity of creatinine molecules captured by the MIP and NIP polymers. The two different solutions are used for the mobile detection phase. Milli-Q water is used as solution A whereas UHPLC grade ACN is used as solution B. The isocratic gradient elution technique is done for 4 minutes for every sample with mobile phase configuring 50% A and 50% B. The solutions flow rate is maintained using 0.2 mL per minute. The injection volume of MIP and NIP processed solutions is set as 1.0 μL, and the detection of creatinine is performed at λ = 235 nm. The UHPLC analysis is performed by maintaining the pressure of 50 bar on the Luna C18 column. The Luna C18 column temperature is set at 40°C. The adsorption capacity of the MIP and NIP polymers is calculated using (Equation 4.1).

$$\boldsymbol{Q} = \frac{\boldsymbol{Volume\,(C_{initial} - C_{final})}}{\boldsymbol{mass}} \qquad \textbf{(Equation 4.1)}$$

where Q (mg/g^{-1}) is the total creatinine mass adsorbed each gm of synthesised polymer, the V (L) is the total volume of the solution used for adsorption. The $C_{initial}$ (mg/L^{-1}) and C_{final} (mg/L^{-1}) are initial and final creatinine concentrations. The imprinting factor α is found as 11.25 (Equation 4.2).

$$\boldsymbol{\alpha} = \frac{\boldsymbol{QMIP}}{\boldsymbol{QNIP}} \qquad \textbf{(Equation 4.2)}$$

where α = imprinting factor, $QMIP$ = the adsorption capacity of the MIP (mg/g^{-1}), $QNIP$ is the adsorption capacity of the NIP (mg/g^{-1}) as displayed in (Equation 4.2).

4.3.6 Selectivity Test

A 10 mg of MIP is uniformly mixed and 10 mL of 50 ppm solutions each of structurally similar compounds to creatinine naming creatine, 2-pyrrolidone, N-hydroxysuccinimide, 1-methylhydantoin and hydantoin dissolved in Milli-Q water. The polymer compound mixtures are incubated at 37°C for 1 hour on a shaker at the speed of 1 G-force for allowing the polymer to get maximum adsorption of structurally similar compounds. Then, the solutions are centrifuged, and aliquots are filtered through hydrophilic syringe filters to obtain samples. This is analysed using UHPLC.

The two solutions are used for the mobile detection phase. The isocratic gradient elution technique is performed for 8 minutes for every sample with mobile phase configuring 50% A and 50% B. The UHPLC solutions flow rate is maintained at 0.2 mL per minute. The sample solutions injection volume is adjusted to 5.0 µL. The detection of the compounds is performed at multiple wavelengths λ = 210, 235, 280, 310, 350, 410, 450 and 500 nm, respectively. The UHPLC analysis is performed by maintaining the pressure of 50 bar on the Luna C18 column. All the samples are analysed by setting the temperature of the Luna C18 column to 40°C.

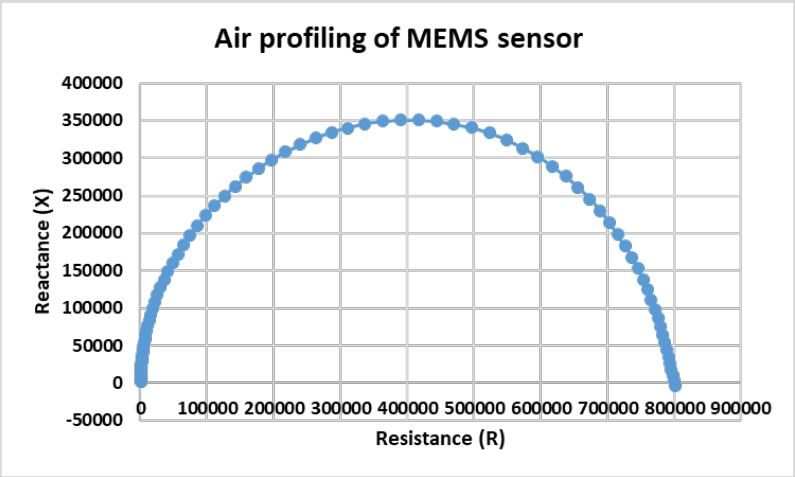


Figure 4.8: Nyquist plot obtained after air profiling of MEMS sensor before MIP functionalization.

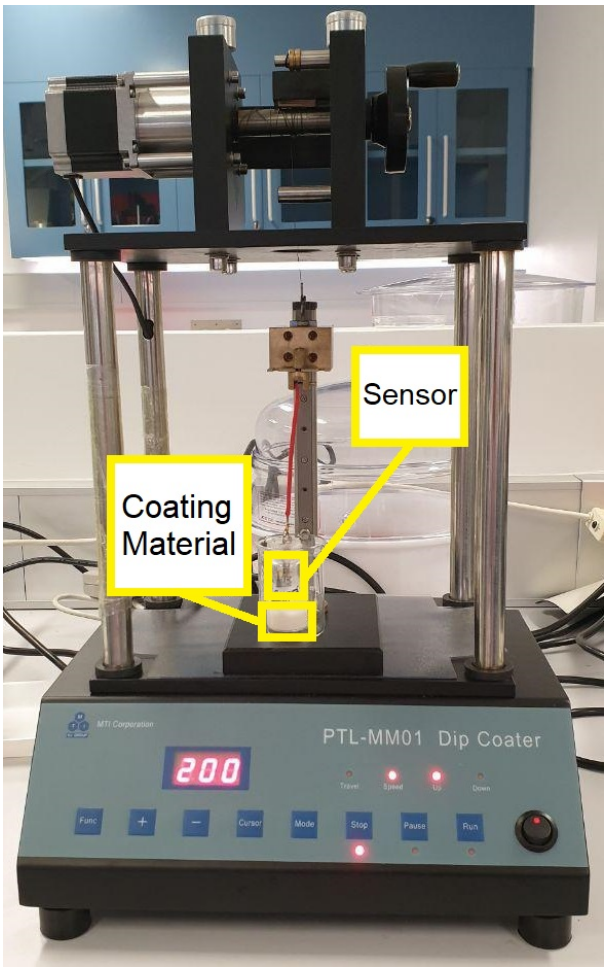
4.3.7 Functionalization of MEMS ID Sensor using MIP and EIS Test

In the first step, the MEMS-based ID sensors' air profiling is done at 1 V AC, and the frequency is swept between 10 Hz to 100 kHz. It helped in the characterisation of the sensor as

well as finalising the optimal operating frequency. In the second step, the MEMS-based sensors' sensing area is functionalized by using a MIP coat. Figure 4.8 shows air profiling of MEMS sensor before MIP functionalization.

The sensing area is precleaned with acetone once before functionalizing. The self-assembled single (SAS) monolayer technology and acrylic resin are used for the MIP functionalization process. 200 µL of acrylic resin, 1 gm of MIP and 1.5 mL of acetone are mixed inside a glass beaker. The functionalization is done using the PTL-MM01 Dip Coater instrument to make the uniform functionalization layer on the sensing area. The dipping and withdrawal of the sensing area of a sensor are made at a uniform speed of 200 mm/minute at 1 second dipping time. Due to the quick evaporation of acetone, the functionalization suspension becomes a solidified coat on the sensing area. Figure 4.9 (a) displays the functionalization process of the MEMS ID sensor and (b) MIP functionalized MEMS sensor. The average cost of the creatinine MIP functionalization suspension solution used for the functionalization of the sensing surface is about 1.0 Australian dollar.

(a)



(b)

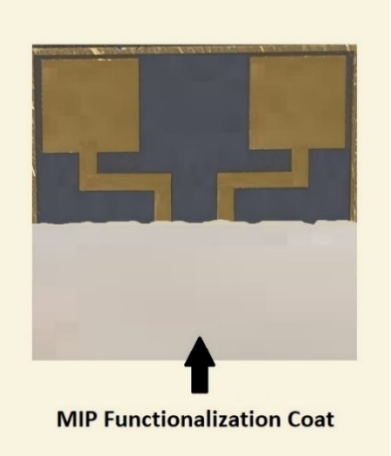


Figure 4.9: (a) MIP functionalization process using PTL-MM01 Dip Coater instrument and (b) MIP functionalized MEMS sensor.

After functionalization of the sensing area, the sensor is again characterised by using the EIS technique. This helped in understanding the changes concerning the non-functionalized sensing area. In the third step, the serum samples carrying the aqueous and serum creatinine concentrations of 6, 10 and 14 ppm are analysed by adding 20 µL of a sample to the sensing area. Finally, the air profiling of the MEMS sensor is done once again using the EIS technique and the Hioki 3536 LCR meter instrument. Figure 4.10 displays the representation of steps necessary in making the creatinine specific MIP functionalized biological sensing surface for specific adsorption of creatinine on the surface of MEMS ID sensors.

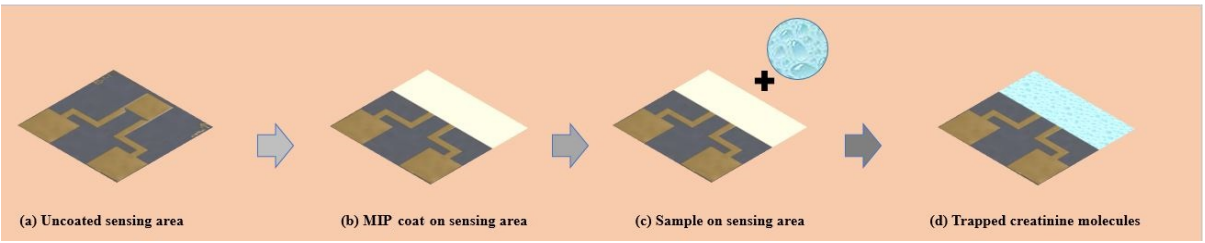


Figure 4.10: Diagrammatic illustration of the adsorption process of creatinine molecules (a) Uncoated (non-functionalized) sensing surface, (b) MIP functionalized sensing surface; (c) Addition of sample solution on the MIP functionalized sensing surface, (d) Trapping of creatinine molecules on the MIP functionalized sensing surface. A white marking on (b) and (c) indicates the MIP cavities on the sensing surface. In contrast, a blue marking on (d) indicates the creatinine molecules entrapped inside the MIP functionalized polymer cavities.

4.3.8 Binding Procedure of Creatinine on MIP Functionalized Sensor

The MIP has specific cavities which selectively adsorb the creatinine biomolecules on the sensors sensing surface from the creatinine aqueous and serum samples. Figure 4.10 illustrates the adsorption process of creatinine molecules. Post pipetting the SUT (20 μL) on the MIP pre-functionalized area of a sensor, the sensor is attached to the shaker for 1 minute for insuring the uniform distribution of the sample in the sensing MIP functionalized region of the used sensor. Because of the hydrogen bonding of the creatinine imprinted polymer, the creatinine molecules are captured by the porous polymer cavities. Then, around 10 minutes delay time is given to MIP for adsorbing the creatinine molecules from the sample. The extra solution is then cleaned by spraying the Milli-Q water. After the washing process, the sensor takes 30 minutes to air-dry the sensing surface. Post drying, the creatinine adsorbed MIP functionalized sensing surface is analysed using the EIS technique to measure creatinine concentration from the sample solution.

4.3.9 EIS Experimental Measurements

The techniques of EIS is utilised for measuring the dielectric properties of the SUT carrying various creatinine concentrations. Despite its potency and popularity, this measurement technique is extremely sensitive to humidity and temperature. The performance of the sensor varies due to the change in temperature and humidity. Therefore, all of the tests are carried out in a controlled environment with the same humidity (31%) and laboratory temperature (25°C) parameters to nullify the effects of temperature and humidity variations. To maintain the uniformity in temperature and humidity levels, the experimental setup included with humidity meter and a digital thermometer, the MEMS ID sensor, SUT's carrying various creatinine concentrations, MIP functionalization suspension and a high precision Hioki 3536 LCR meter. The LCR meter is connected to the computer through the RS232 connection port, series of experiments are carried out for data collection and analysis. The MEMS sensor is connected to excitation and sensing electrode with the help of a specifically made jig having Au pin connectors shown in Figure 4.11.

The experiments are done by use of the slow mode of equipment testing. First, a 1 V AC supply is applied to the sensor with the help of the Au pin connector, and the frequency is swept between 10 Hz to 100 kHz (Figure 4.8). Next, the first characterisation is performed in

the air. Then, all the experiments are repeated five times, and the average of the results is used to confirm the reproducibility and reliability of the obtained results.

The MIP functionalized sensor is electrically attached to the LCR meter with the same metallic Au pin connectors in the second step. An input voltage of 1 V is supplied to the sensor to perform a frequency sweep of 10 Hz to 100 kHz. The sample solution is allowed to bind, as illustrated in Figure 4.10. Finally, the EIS measurement technique measures the altering property of the impedance after an additional 30 minutes, when the MIP functionalized sensor surface is air-dried thoroughly. The reactance X (imaginary impedance) parameter offers acceptable results over Resistance R (real impedance).

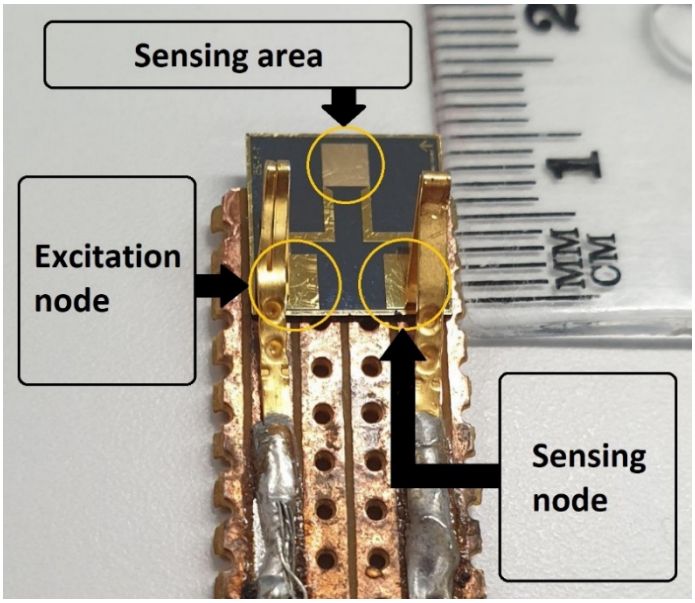


Figure 4.11: Different parts of the MEMS planar ID sensor having 1-1-25 configuration connected to Au pin connector jig.

4.3.10 CNLS Impedimetric Curve Fitting

The EIS Spectra Analyser software is implemented for analysing the obtained impedance spectrum data for 6, 10 and 14 ppm creatinine samples made using Milli-Q and serum creatinine solutions. The mathematical algorithm of electrochemical spectrum analyser software is utilised for the theoretical calculations and for obtaining the equivalent circuit measured parameters. For curve fitting, calculated impedance values from the 6, 10 and 14 ppm (Milli-Q and serum) sample solutions. Milli-Q water and pure serum carrying zero creatinine content are used as control. The theoretically obtained values are found to be in an

acceptable range after analysis with a complicated algorithm, which is also called the CNLS impedimetric curve fitting technique. It is used for measuring the identical circuit parameters for all samples and control.

4.3.11 Testing for Repeatability and Reusability using EIS Technique

To determine the creatinine levels in real samples and reduce e-waste, the sensor is supposed to function for repeatable times with constant performance. Therefore, repeatability and reusability testing are essential parameters of a sensing experiment. Both the testing is done for the developed sensor after functionalizing the sensing surface with MIP functional coat. It is observed that the sensing surface requires cleaning before its subsequent measurement. The readings are taken for a 10 ppm serum creatinine sample. In the repeatability experiment, 20 µL of serum samples of 10 ppm creatinine concentration are pipetted on the sensing surface in individual experiments. For the reusability experiment, 1% AcOH solution is made using Milli-Q water. The functionalized sensor is dipped inside 1% AcOH made using Milli-Q water for about 20 minutes to remove the captured creatinine molecules. When a once used functionalized sensor is inserted into the 1% AcOH solution, it helps remove creatinine by breaking polymer bonding formed between the MIP functionalization coat and creatinine molecules. It aids in cleansing the previously used functionalized creatinine MIP functionalization before every single measurement. The functionalized sensing area repeatability and reusability experiments are done five times using the EIS technique.

4.3.12 Single Frequency Measurement, S_x Curve Equation Development and Measurement of Unknown Sample

This analysis is performed with various serum creatinine samples by using the developed functionalized sensor at 1020 Hz operating frequency (decided with the help of the EIS technique and CNLS analysis results). The choice of 1020 Hz as an operating frequency is governed by the fact that, at this frequency, the sensor can clearly distinguish between aqueous and serum creatinine samples at all concentrations. The SUT concentration measurement is done using a MIP functionalized MEMS sensor with the EIS technique. The sensor measurement of creatinine is taken five times, and the average of the results for reactance is analysed.

4.4 Results and Discussions

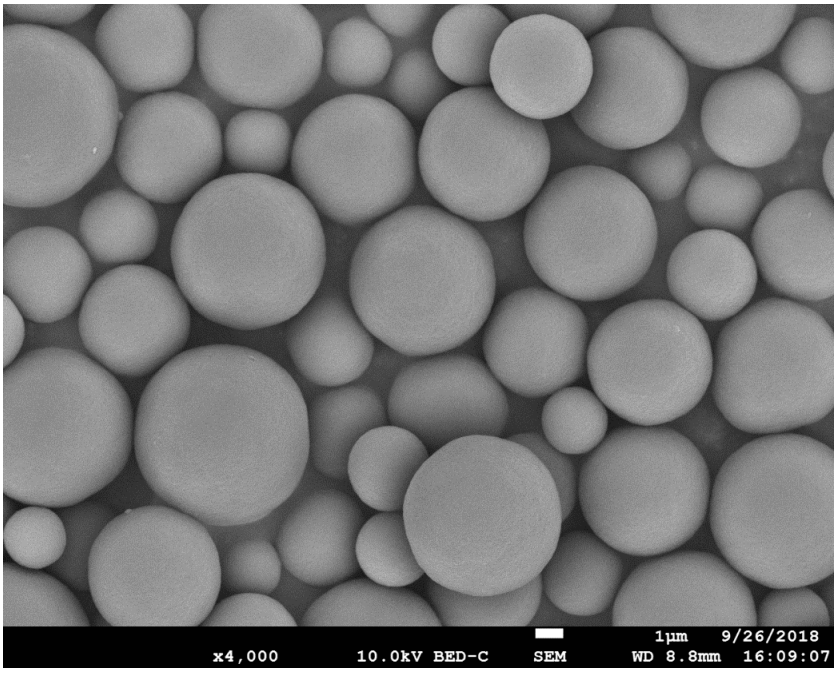
The precipitation polymerisation method is used to synthesise the creatinine specific MIP, which has various advantages. They are listed as high selectivity, robustness and physicochemical stability. In the pre-polymerisation process, the creatinine molecules interacted with the functional monomers through the non-covalent binding. In contrast, during the polymerisation procedure, the creatinine molecules are properly imprinted in the matrix of the polymer. The non-covalent method is famous for MIP synthesis. It is due to the less involvement of effort to extract the creatinine template from the matrix of the polymer. At the same time, covalent bonding requires a higher amount of energy in extracting the creatinine template from the matrix of the polymer. Furthermore, MIP synthesised by bulk polymerisation needs to be crushed and ground before use, whereas MIP made via template extraction can be used immediately.

4.4.1 FESEM, SEM and EDAX Characterisation of MIP and NIP Polymers for Structural and Elemental Analysis

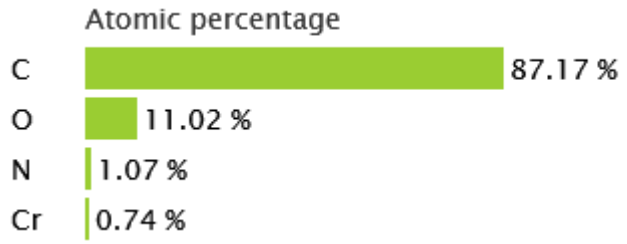
After synthesising the powdered MIP and NIP polymers and functionalizing the synthesised polymers on the sensors sensing area, FESEM and EDAX analyses are carried out to study the polymer superficial structural features. The FESEM imaging for MIP and NIP is shown in Figure 4.12 (a) to (e), respectively. It is observed in Figure 4.12 (a) that all the MIP particles are spherical, isotropic and monodispersed. The sizes of the particles are between 2 μm to 6 μm with an average size of 4.0 μm. It is observed in Figure 4.12 (e) that all the NIP particles show similar polymer-associated properties such as spherical, isotropic and monodispersed in nature. Their sizes also vary from 5 μm to 7.5 μm with an average size of 5.25 μm. While synthesising MIP polymer, the creatinine as a template is used; therefore, the average size of the MIP is found to be much compact, and it is 4.0 μm. Whereas, whilst synthesising NIP polymer, the creatinine as a template molecule is not used, therefore the average polymer size in the case of NIP is slightly larger, and it is 5.25 μm. Figure 4.12 (b), (c), (d) show EDAX analysis of MIP whereas Figure 4.12 (f), (g), (h) show EDAX analysis of NIP. Figure 4.12 (b), (f) show the atomic percentage of C, O, N and Cr in MIP and NIP. Figure 4.12 (c), (g) show the weight percentage of C, O, N and Cr in MIP and NIP. The EDAX graphical spectra of MIP (Figure 4.12 (d)) confirm the presence of elements such as C, O, N.

It also reveals the sample purity and absence of unexpected contaminations offering pseudo results or absorption interference. The minor peak of Cr in EDAX graphical analysis confirms the chromium is used as a coating material to improve the conductivity of the sample under imaging space. The same results are observed for NIP (Figure 4.12 (h)), which depicts polymer purity with the presence of elements such as C, O, N and Cr. Figure 4.13 (a), (b), (c), and (d) show spot SEM and EDAX analysis of MIP and NIP. Whereas Figure 4.14 (a), (b), (c) and (d) show regional SEM and EDAX analysis of MIP and NIP. They confirmed the purity of the sample not only at randomly selected polymer but also within the entire space of scanned area all together lengthen 50 × 50 µm, hence successfully validating the complete absence of other unexpected impurities on an elemental level. The FESEM, SEM and EDAX analysis helped in understanding MIP and NIP structural analysis. It plays an important role in understanding the elemental presence and confirming the absence of unexpected elements in the sample space. Figure 4.15 shows a top view of the MIP functionalized MEMS sensor's sensing surface captured using FESEM analysis.

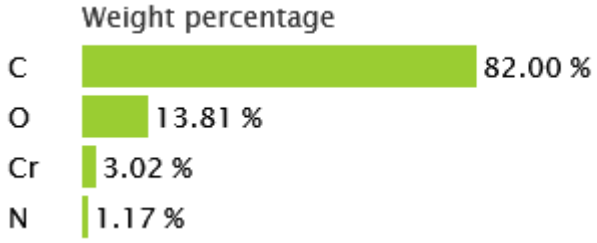
(a)



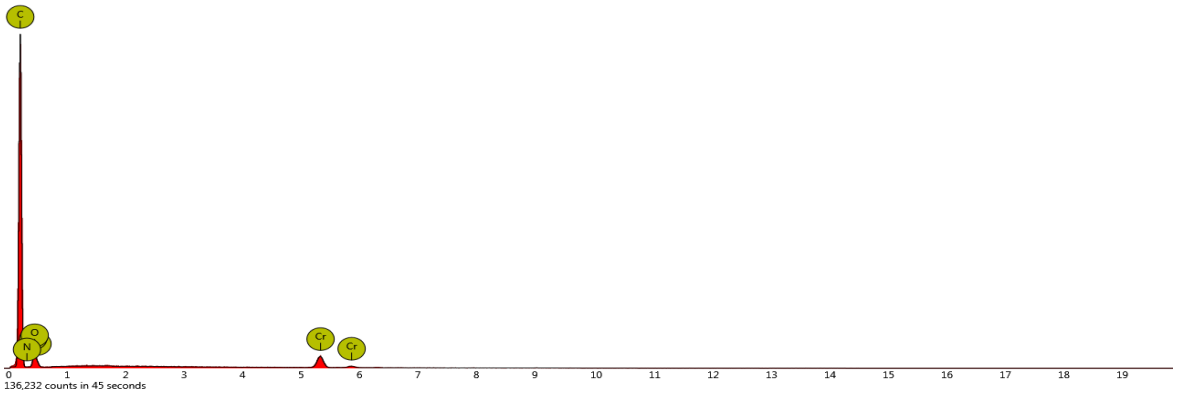
(b)



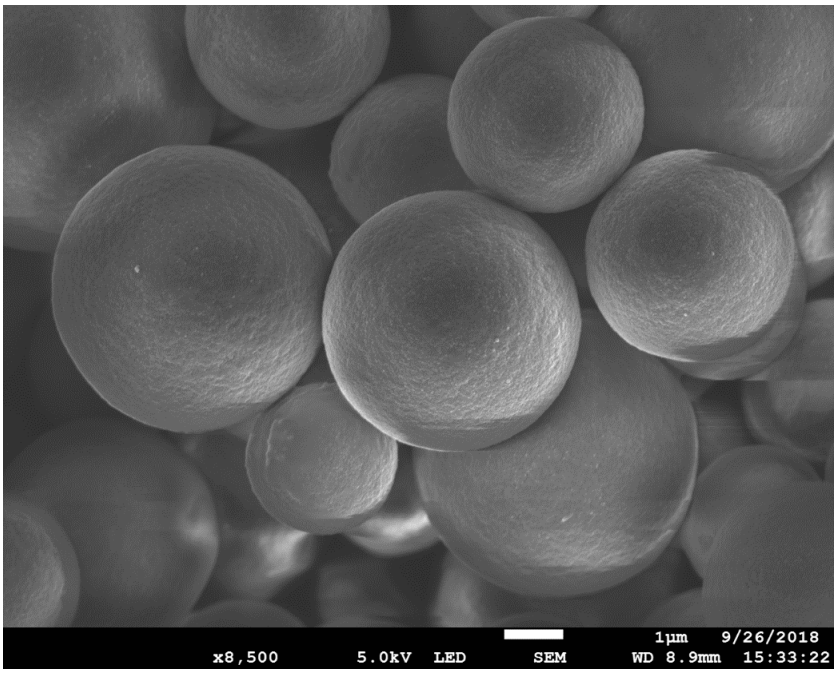
(c)


Weight percentage
C
82.00 %
O
13.81 %
Cr
3.02 %
N
1.17 %

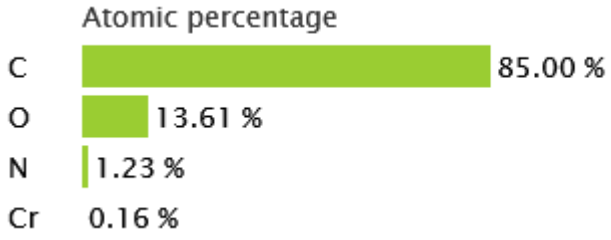
(d)


C
O
N
Cr
Cr
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
136,232 counts in 45 seconds

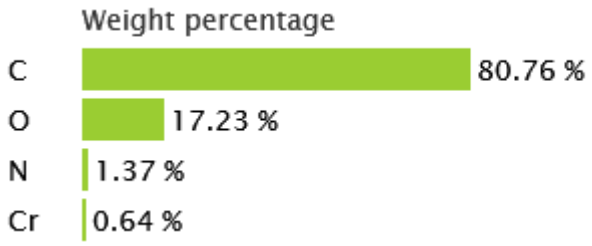
(e)


x8,500 5.0kV LED SEM
1µm 9/26/2018
WD 8.9mm 15:33:22

(f)


Atomic percentage
C
85.00 %
O
13.61 %
N
1.23 %
Cr
0.16 %

(g)



(h)

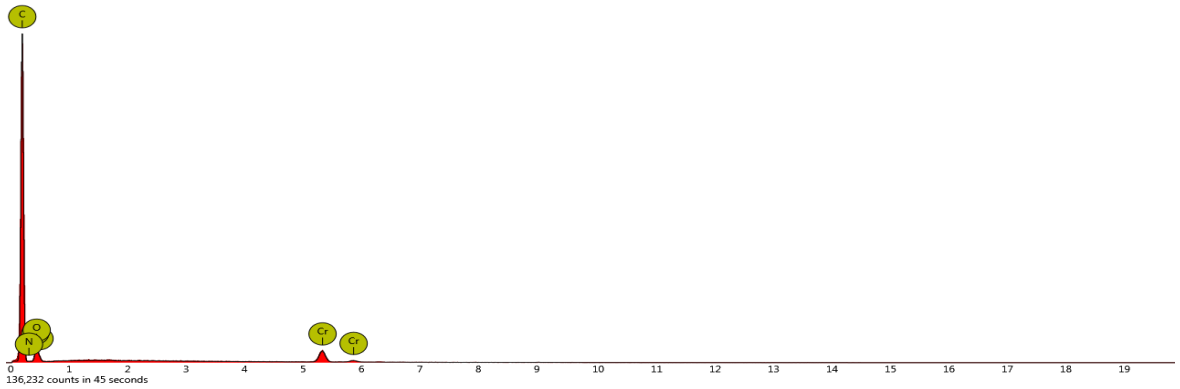
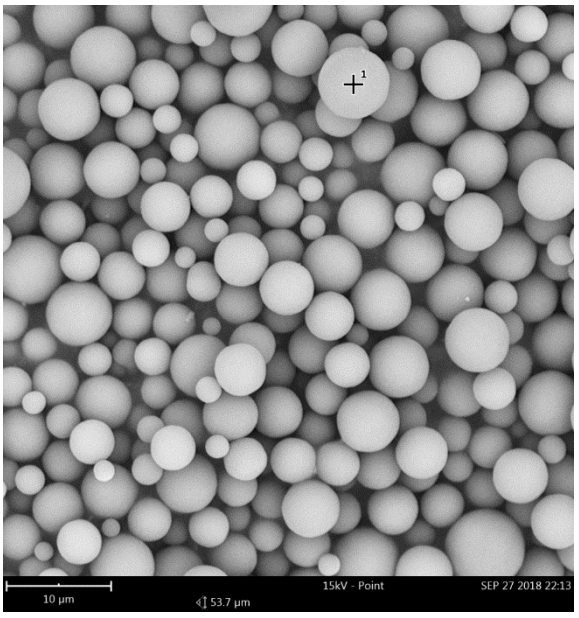
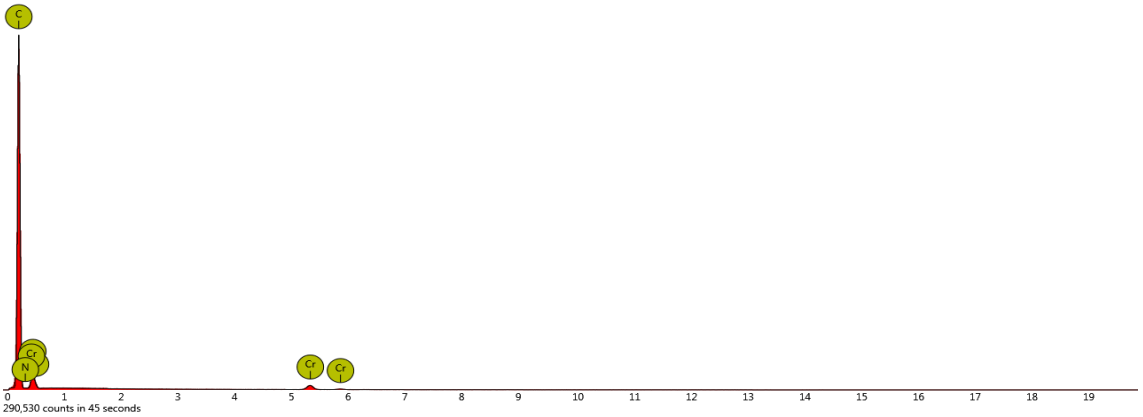


Figure 4.12: (a) FESEM image of MIP, (b) EDAX analysis showing the atomic percentage of MIP, (c) EDAX analysis showing the weight percentage of MIP, (d) EDAX analysis graph of MIP, (e) FESEM image of NIP, (f) EDAX analysis is showing the atomic percentage of NIP, (g) EDAX analysis showing the weight percentage of NIP, (h) EDAX analysis graph of NIP. Structural analysis of MIP and NIP.

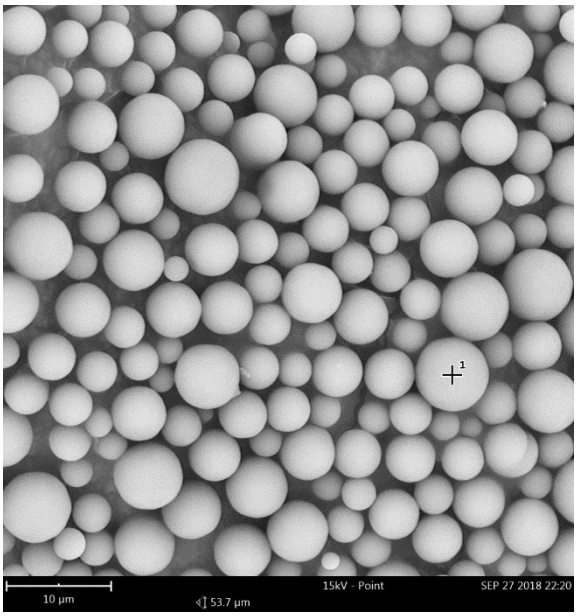
(a)



(b)



(c)



(d)

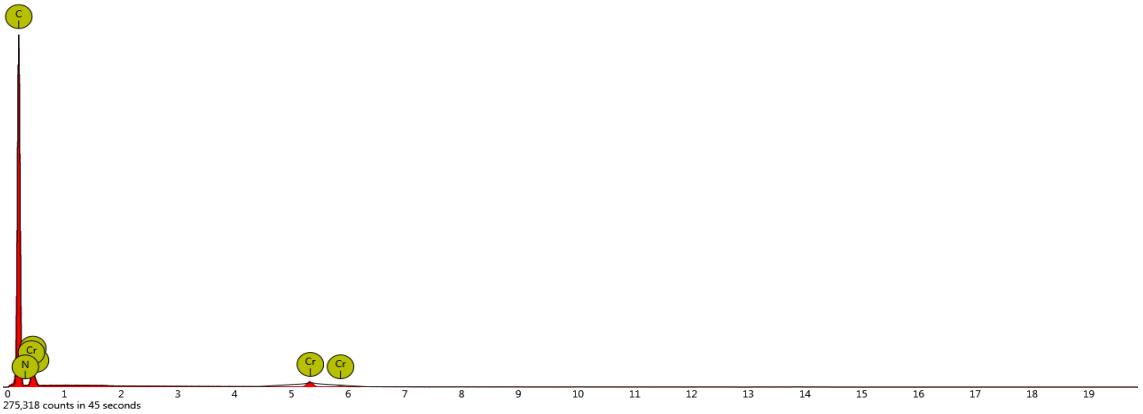
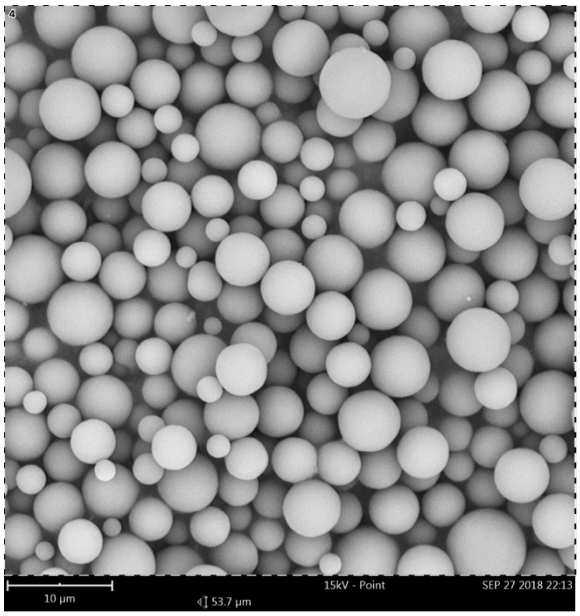
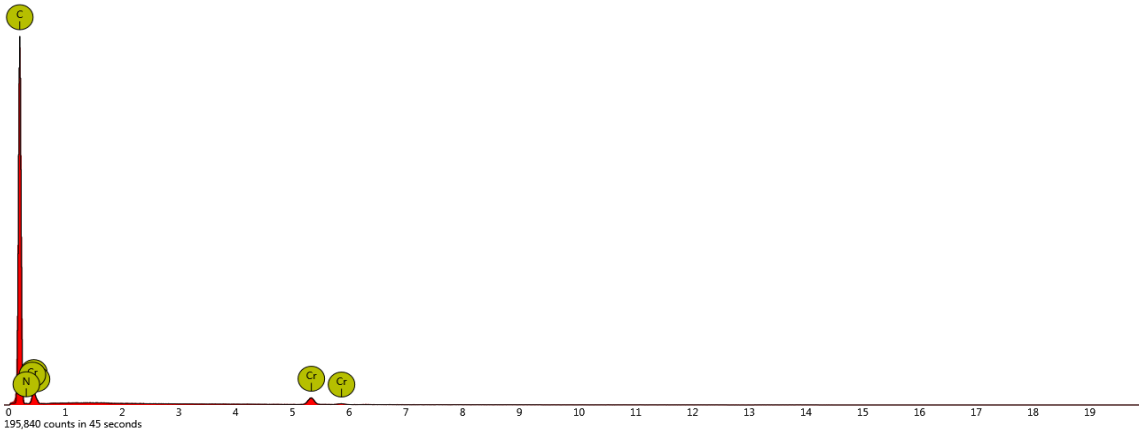


Figure 4.13: (a) Spot SEM image of MIP for EDAX analysis, (b) Spot EDAX analysis graph of MIP, (c) Spot SEM image of NIP for EDAX analysis, (d) Spot EDAX analysis graph of NIP.

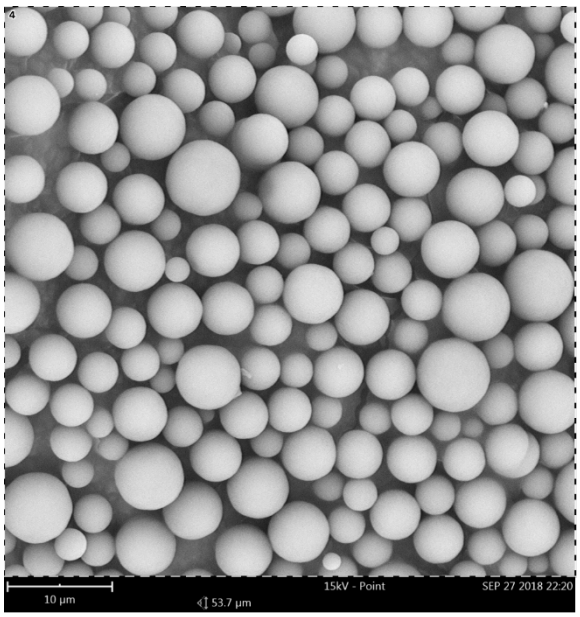
(a)


10 µm
53.7 µm
15kV - Point
SEP 27 2018 22:13

(b)


C
N
Cr
Cr
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
195,840 counts in 45 seconds

(c)


10 µm
53.7 µm
15kV - Point
SEP 27 2018 22:20

(d)

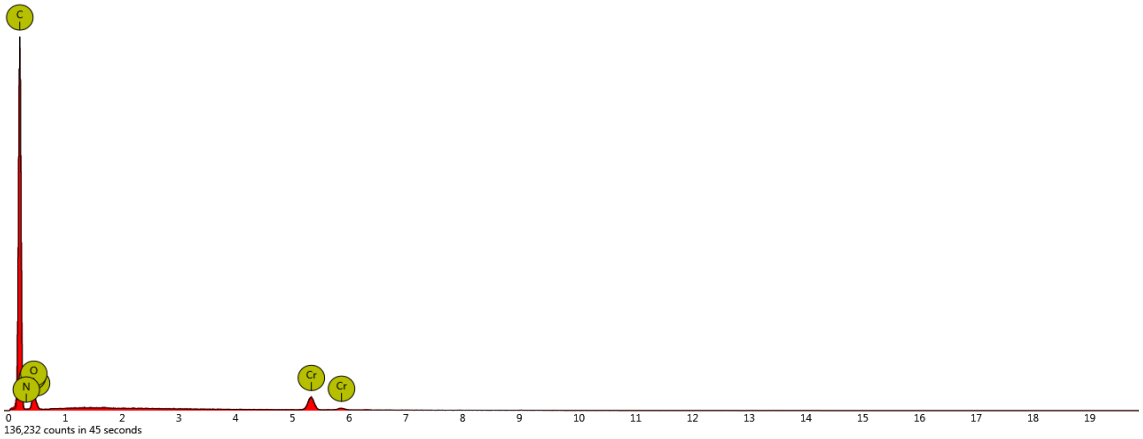


Figure 4.14: (a) Region SEM image of MIP for EDAX analysis, (b) Region EDAX analysis graph of MIP, (c) Region SEM image of NIP for EDAX analysis, (d) Region EDAX analysis graph of NIP.

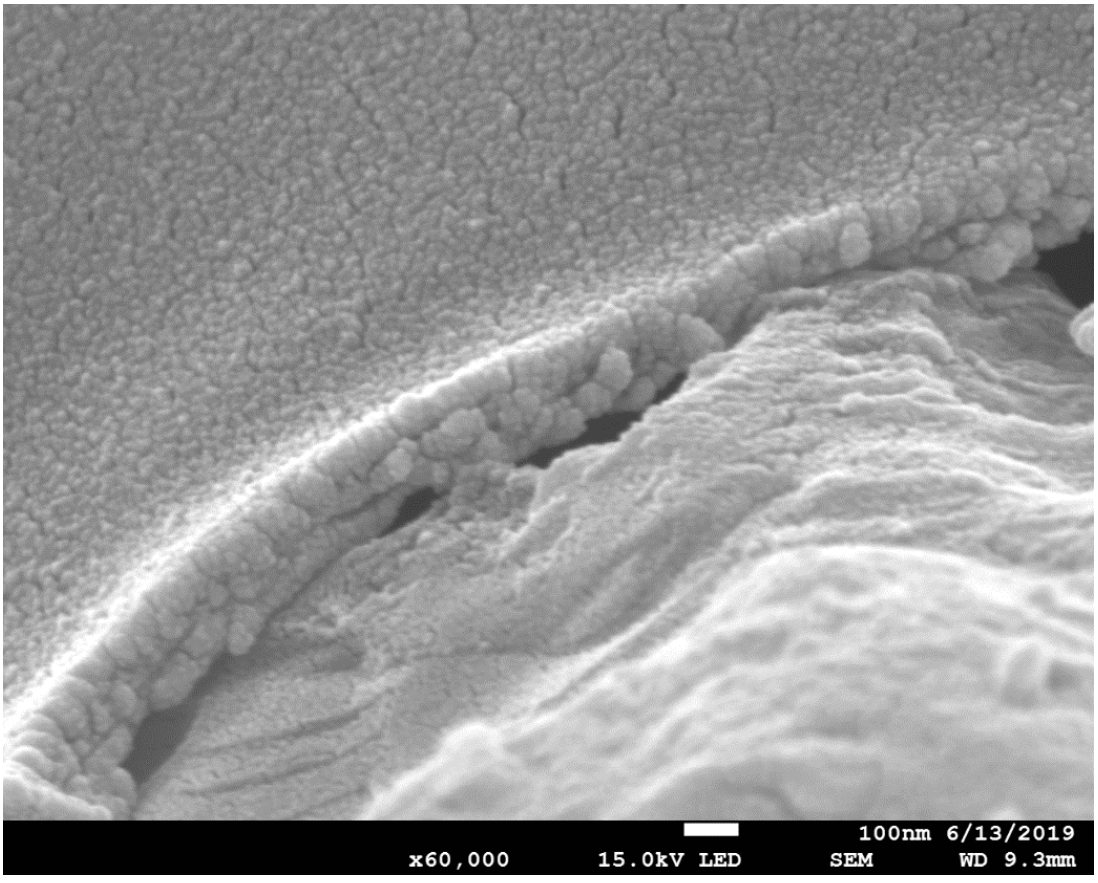


Figure 4.15: A top view of MIP functionalized MEMS sensor's sensing surface captured using FESEM analysis.

4.4.2 FTIR Spectroscopy Analysis for Confirmation of Successful Removal of Creatinine as a Template Molecule by Breaking the Polymer Bonding

The FTIR analysis is done on MIP and NIP obtained from the polymerisation to find a functional group in synthetic polymer matrices. The FTIR spectrum of Figure 4.16 (a) represents the MIP before the centrifuge process, and Figure 4.16 (c) represents MIP after the centrifuge process. It is seen from the spectra that both Figure 4.16 (a), (c) are characterised by the presence of absorption for the intra-polymer matrix bonding at the wavelength range of 1,630-1,660 cm^{-1} regions.

MIP is showing absorption around wavelength 1,630 cm^{-1} region. This proves the occurrence of intra-polymer bonding whilst forming a complex polymer structure and confirms the successful synthesis of the creatinine-specific MIP by bonding between creatinine as a template molecule and MAA as a functional monomer.

Figure 4.16 (b) shows a complete absence of absorption at the 1,630-1,660 cm^{-1} wavelength regions, confirming that creatinine is not added as a template molecule during the NIP polymerisation process and hence proved the absence of bonding between creatinine and MAA during NIP synthesis.

However, the absence of absorption in Figure 4.16 (d) at the 1.630-1,660 cm^{-1} wavelength region confirms successful extraction of creatinine template molecule from the MIP matrix after the soxhlet extraction process. It is done by boiling AcOH using a soxhlet extraction process that breaks the bonding between creatinine and MAA. Due to the absence of bonding the Figure 4.16 (d), absorption is absent at the 1,630-1,660 cm^{-1} wavelength regions.

The FTIR analysis also helps to justify that the creatinine molecule is present in MIP in before (Figure 4.16 (a)) and after centrifuge (Figure 4.16 (c)) samples. The polymer bonding breaks after using AcOH in soxhlet extraction of MIP (Figure 4.16 (d)). Due to this, the creatinine in the polymer matrix becomes absent. This proves that the soxhlet extraction process is done successfully. Therefore, the successful removal of the creatinine template molecule from the MIP matrix is confirmed from the FTIR analysis.

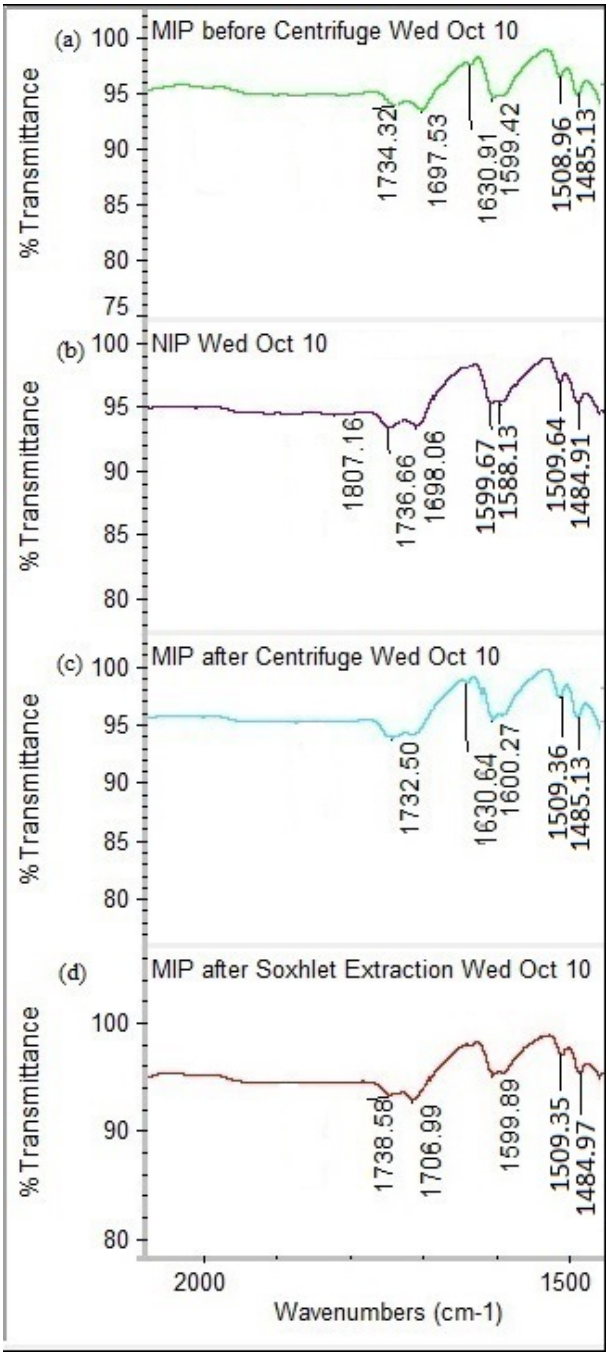


Figure 4.16: FTIR spectra of (a) MIP before centrifuge, (b) NIP, (c) MIP after centrifuge and (d) MIP after soxhlet extraction.

4.4.3 Sorption Studies of Creatinine by MIP and NIP for Calculating Upper Saturation Limit

The static adsorption studies are essential to calculate the upper saturation limit of creatinine concentration, after which the MIP and NIP get fully saturated. This analysis is done

by separately mixing 10 mg of creatinine MIP and NIP in two different falcon tubes containing each 10 mL of the creatinine sample with the concentrations ranging from 1 to 50 ppm at 37°C for 1 hour. Then, all the aliquots are filtered through hydrophilic syringe filters to obtain UHPLC grade pure samples. Finally, the samples are analysed using UHPLC.

Figure 4.17 represents the result of the static adsorption studies of creatinine by using MIP and NIP. The results show that the amount of total creatinine bound to MIP constantly increased with increasing levels of concentration up to 10 ppm and then rose steadily till 50 ppm. Whereas for NIP polymer, the amount of creatinine bound rose to 5 ppm and then, it remained almost unchanged till 50 ppm. MIP did not get saturated until the 50 ppm concentration range. The MIP showed significantly more adsorption of creatinine than NIP because it has a higher number of surface selective specific binding sites for creatinine.

The sorption studies using UHPLC prove that the MIP has a higher saturation limit in adsorption of creatinine molecules from the sample solutions, thus confirming that the use of creatinine as a template plays a significant role in making precise capturing sites for target molecule in MIP over NIP polymer.

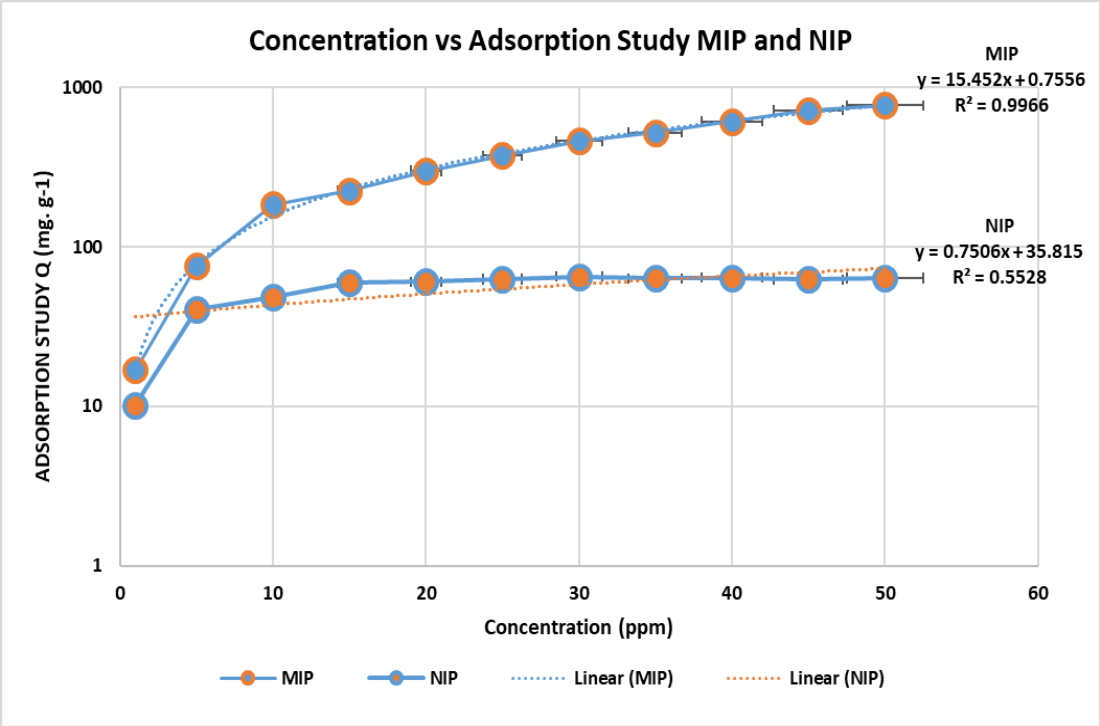


Figure 4.17: Adsorption study of MIP and NIP in different concentrations.

Figure 4.18 shows the UHPLC adsorption peaks value and size comparison between two different samples carrying concentration of creatinine as 45 ppm. The samples are treated

using MIP and NIP in different falcon tubes, and their results are displayed in the same plotting region. MIP has surface selective specific binding sites over NIP, which allows more adsorption of creatinine molecules. It results in the presence of a lesser number of free creatinine molecules in the sample solution treated using MIP when checked with the help of UHPLC analysis. This explains why the sample treated using MIP has a smaller peak when compared with the sample treated using NIP and its peak.

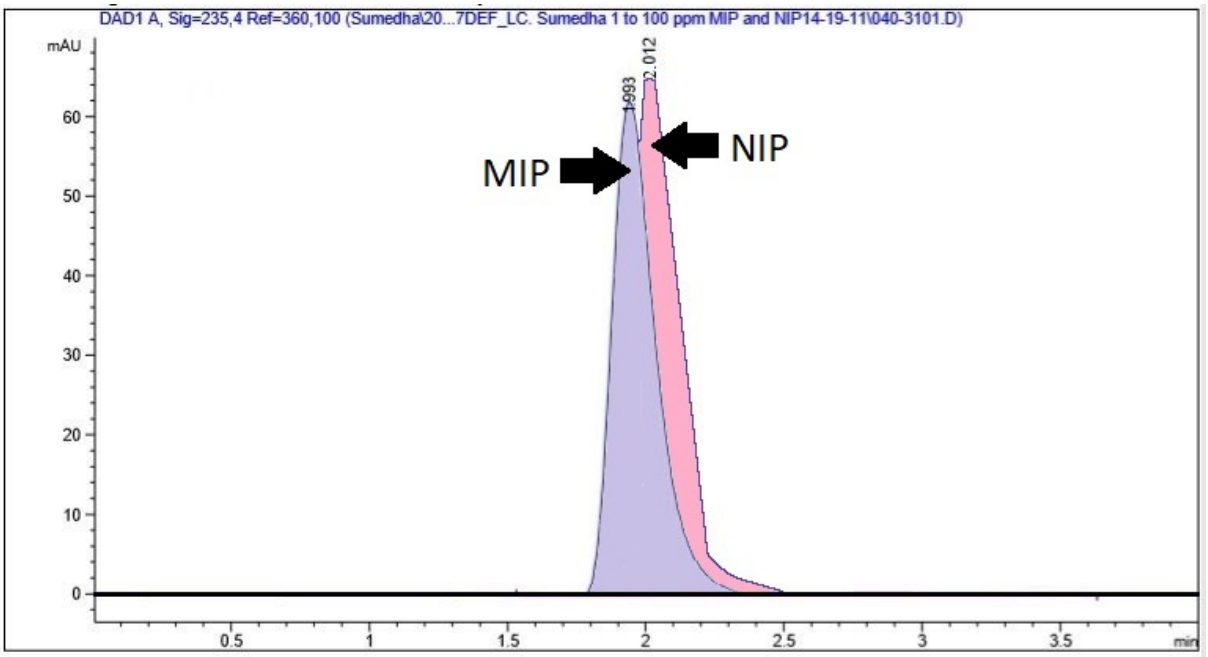


Figure 4.18: UHPLC adsorption peaks value and size comparison between two different samples treated using MIP and NIP are carrying 45 ppm concentration of creatinine at λ 235 nm.

4.4.4 Study of Selectivity of the MIP Towards Structurally Similar Compounds with Creatinine

Figure 4.19 illustrates the selectivity of the MIP towards structurally similar compounds of creatinine such as creatine, 2-pyrrolidone; N-hydroxysuccinimide, 1-methylhydantoin; and hydantoin. It is observed that creatinine (retention time 2.014 minutes) and N-hydroxysuccinimide (retention time 1.426 minutes) are detected at wavelength λ 235 nm. Creatine (retention time 1.892 minutes), 2-pyrrolidone (retention time 2.247 minutes); 1-methylhydantoin (retention time 2.115 minutes) and hydantoin (retention time 1.240 minutes)

are detected at wavelength λ 210 nm. The creatinine imprinted polymer has a high attraction for creatinine in aqueous solutions. The binding attractions of other structurally similar compounds are found to be relatively negligible for compounds such as creatine and 1-methylhydantoin.

In contrast, it is found to be zero for other compounds. Compounds such as creatine and 1-methylhydantoin are structurally highly similar because minor attraction is observed when tested using developed MIP. For other molecules, MIP showed a complete absence of attraction. This proves that the developed MIP is highly selective to creatinine. The spectral adsorption responses of MIP using UHPLC for all structurally similar compounds to creatinine (%) are shown in Figure 4.20.

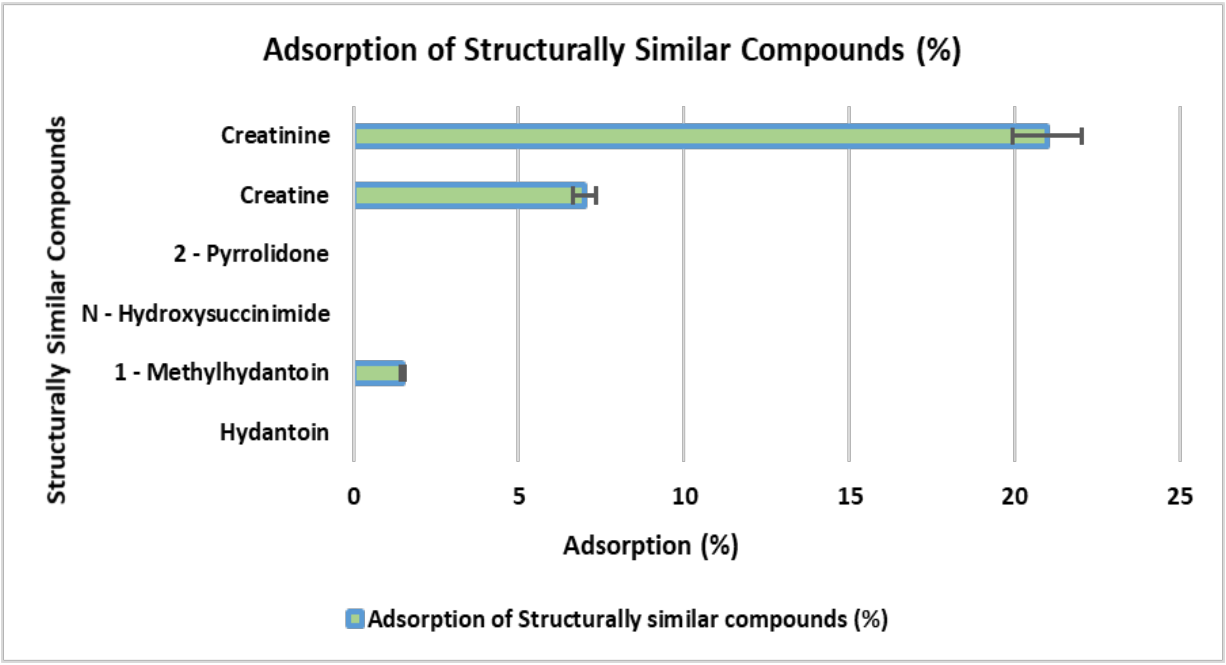
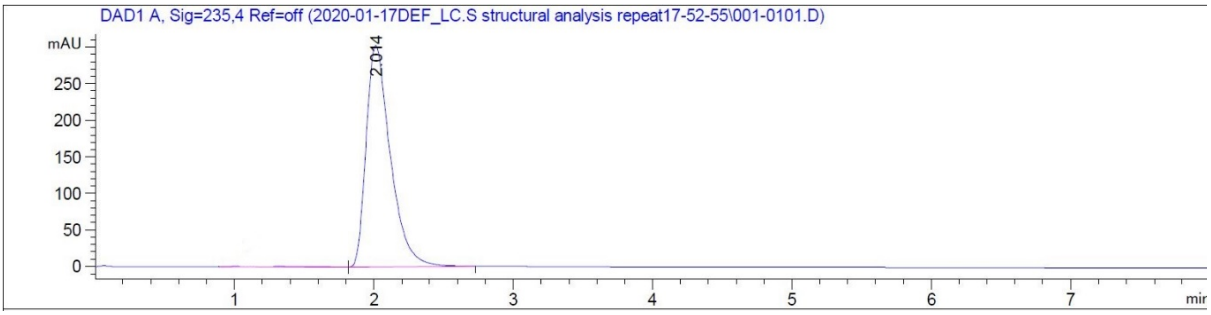
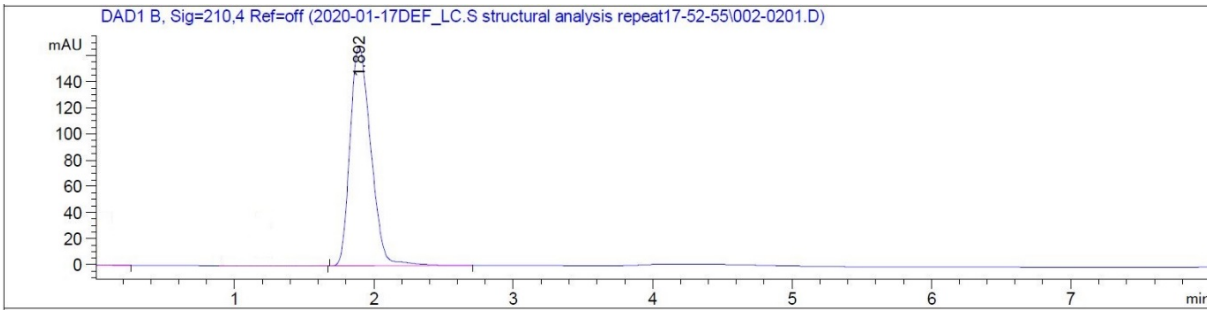


Figure 4.19: Adsorption of structurally similar compounds (%) performed for analysing the specificity testing of MIP for creatinine using structurally similar compounds.

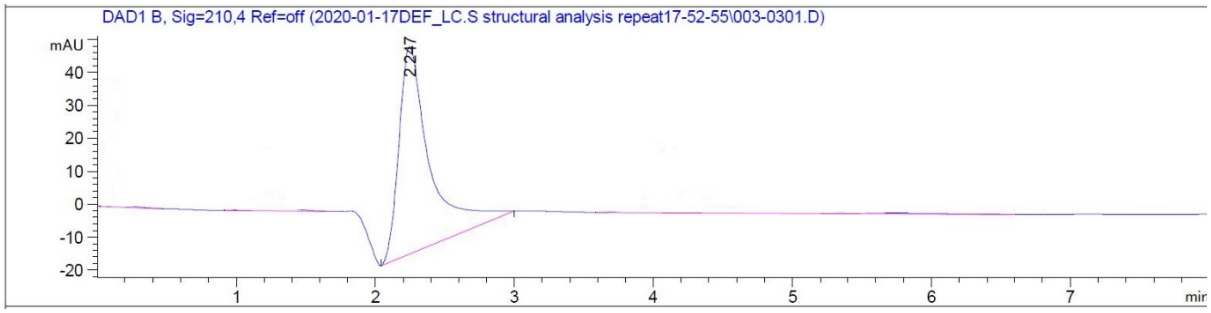
Creatinine



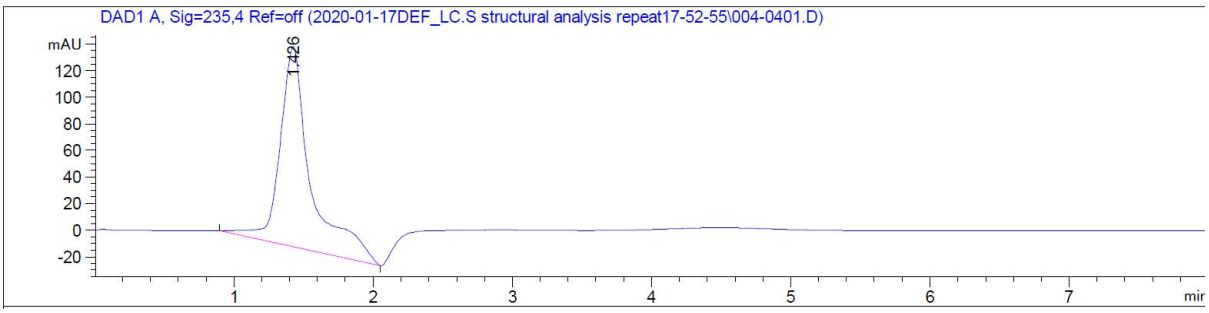
Creatine



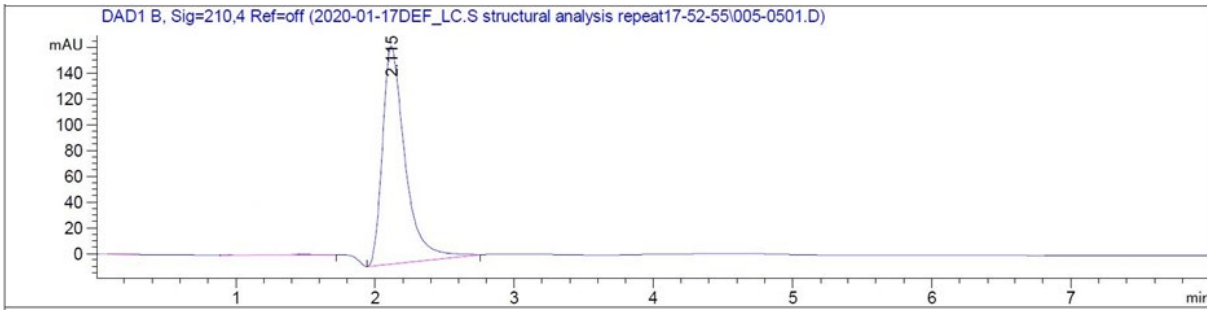
2-Pyrrolidone



N-Hydroxysuccinimide



1-Methylhydantoin



Hydantoin

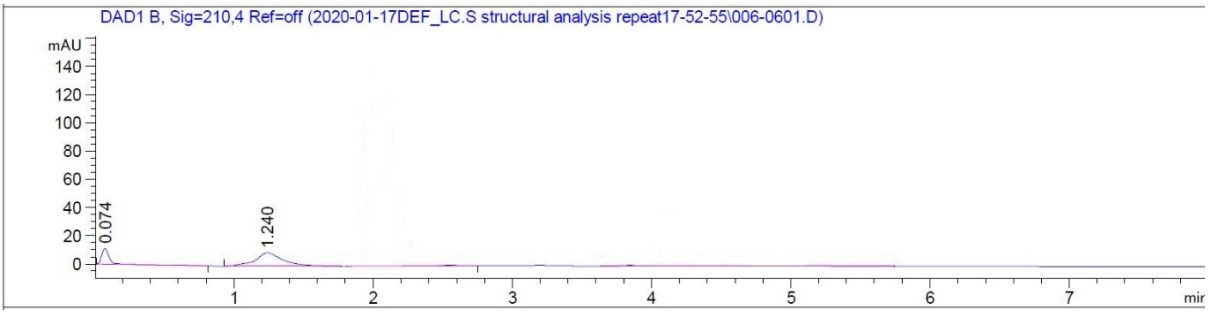


Figure 4.20: The spectral responses for UHPLC for all structurally similar compounds at various wavelengths.

4.4.5 EIS and Analytical Measurement for Creatinine Detection using MIP Functionalized Sensor

The finding of creatinine adsorbed to the chemically synthesised polymer-based detection sites is recognised by using the EIS detection technique. The EIS study for the non-functionalized sensor is studied in this work. The sensor can detect different levels of creatinine content precisely, having an LLD of 0.1 ppm and a higher limit of detection as 50 ppm. The application of creatinine-specific selective functionalization on the sensors sensing area enables it to be highly selective towards creatinine adsorption. While early detection of an increase in creatinine and monitoring of kidney health to prevent further health-related complications are the goal of this research, results of up to 50 ppm are accessed using UHPLC and EIS techniques. Until 50 ppm of MIP functionality is checked to confirm the MIP adsorption of creatinine, the results are shown in Figure 4.17. Serum creatinine levels over 15 ppm need medical attention. For selective creatinine detection, the MIP coated MEMS ID sensors detection limit is 50 ppm. It is three times higher than the expected level of creatinine concentration in human serum [160]. The ability of the MIP coated MEMS ID sensor to detect up to 50 ppm is helpful in case if the coated sensor is used for serum samples carrying very high creatinine levels. It helps establish the sensing technology even if used for a patient with a high creatinine level. Although the results up to 50 ppm creatinine concentration are checked using the EIS technique, only a vital range of creatinine (6-14 ppm) is shown in EIS analysis graphs to fulfil the research aim, the early detection of creatinine rise and also to minimise the complexity of the results in the plotting area.

Figure 4.21 (a) and (b) shows the real impedance, that is, resistance (R) for 10 Hz to 100 kHz range of frequency, from the aqueous and serum samples for different creatinine concentrations. Figure 4.21 (c) and (d) shows the imaginary impedance, that is, reactance (X) for 10 Hz to 100 kHz range of frequency, from the aqueous and serum samples for various creatinine concentrations. Since the change in the imaginary impedance (reactance) is more noticeable than the real impedance (resistance), the reactance (X) is used for future detections. Figure 4.21 (e) and (f) shows the impedance spectrum for different concentrations of aqueous and serum creatinine samples in the Nyquist plot. A noticeable increase in the overall diameter of the semicircle is observed by increasing the creatinine concentration in both aqueous and serum samples. It proved that the creatinine and MIP interactions on the electrode surface cause a gain in the charge transfer resistance process because of the higher amount of creatinine attached to the functionalized sensing surface. The EIS analysis has helped decide the reactance (X) as the most suitable measuring parameter for the detection of creatinine levels from samples.

In chapter 3, when a non-functionalized MEMS ID sensor is used to detect creatinine levels from the aqueous samples, the sensor's sensing area is immersed inside the SUT. In that case, the resistance (R) is found to be the most suitable measuring parameter. However, in the present and the following chapters (chapters 5 and 6), the reactance (X) is the most suitable measuring parameter for the detection of creatinine. This difference is observed due to the MEMS ID sensors sensing area functionalization with the help of the MIP coat. In chapter 3, before the functionalization step, the sensing area is not immersed inside the creatinine solutions.

In contrast to the previous experiments in the present and the following chapter (chapters 5 and 6), the sample is added to the functionalized sensing area, and creatinine molecules are adsorbed and allowed to bind to the MIP cavities. Next, the sensing area is washed for removing excess sample with Milli-Q water, and then it is measured after drying the added sample using the EIS technique. Therefore, in the current and the following chapters (chapters 5 and 6), reactance (X) is the most suitable parameter for all the EIS experiments.

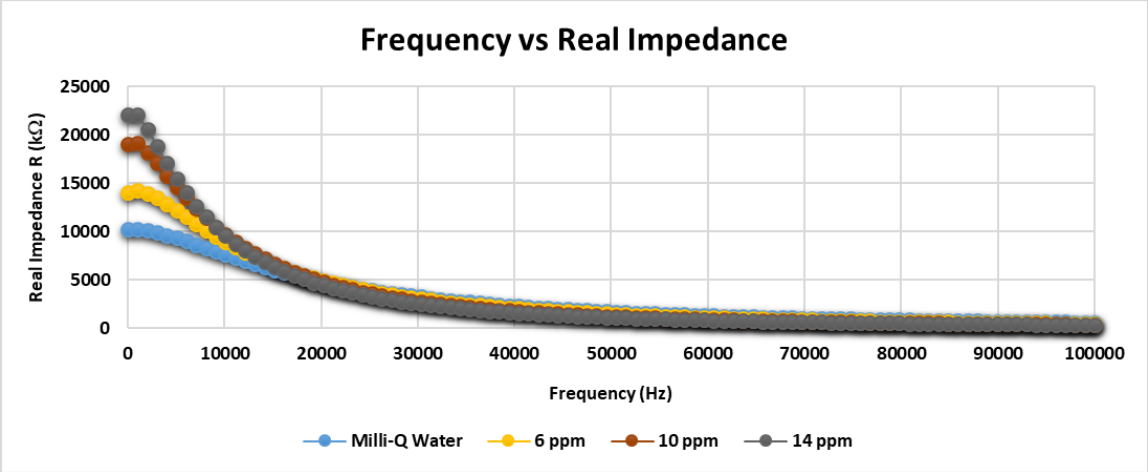
All the measurements are repeated five times to obtain the mean values, and further, they are used for plotting the functionalized MEMS ID sensor associated calibration of results. The mean values of reactance and standard deviation for various serum creatinine concentrations are denoted in Table 4.2.

Table 4.2: The mean values of reactance and standard deviation for various serum creatinine concentrations.

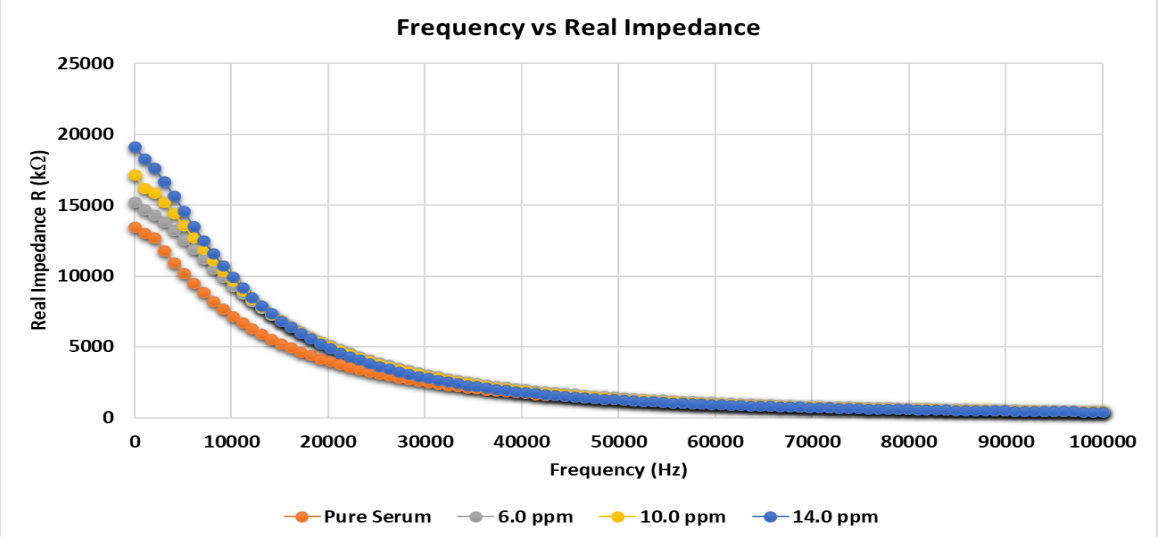
Serum Creatinine Concentration Sample (ppm)	Reactance Value (kΩ)*	Standard Deviation (Ω)
6	1311.89	438.59
10	2107.71	1001.32
14	2773.72	1472.26

* The mean values are obtained after an average of five times measurements.

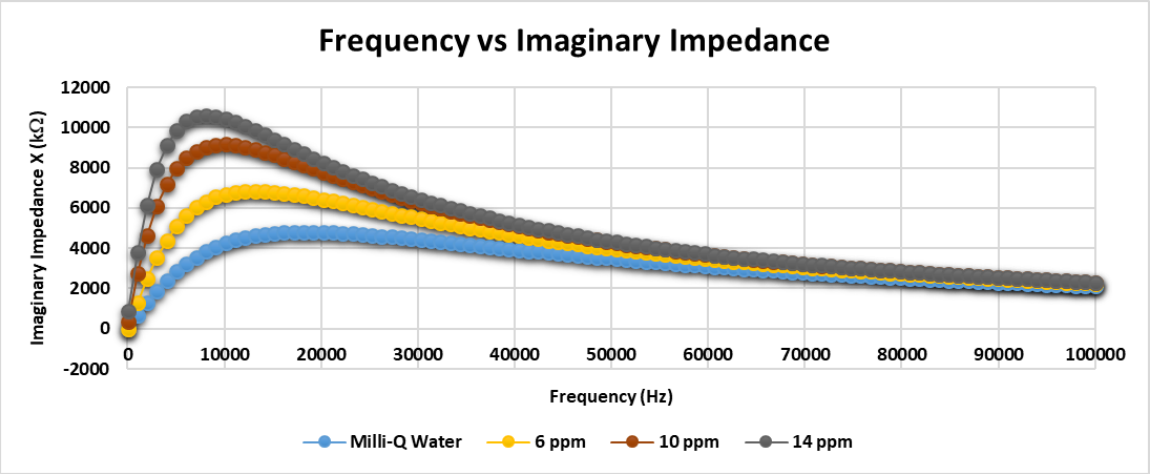
(a) Aqueous Creatinine Samples



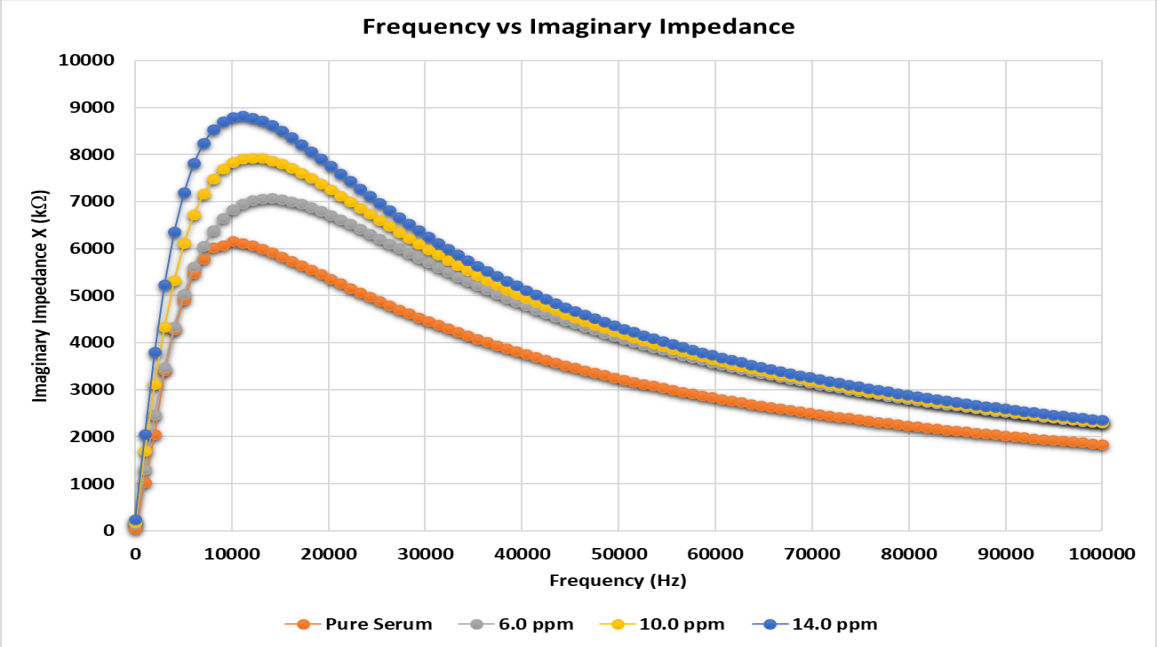
(b) Serum Creatinine Samples



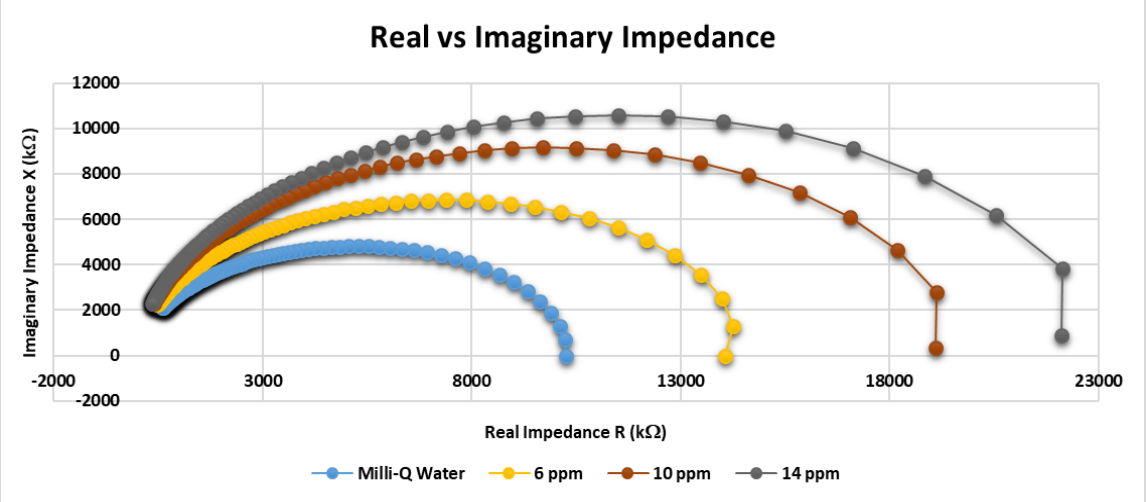
(c) Aqueous Creatinine Samples



(d) Serum Creatinine Samples



(e) Aqueous Creatinine Samples



(f) Serum Creatinine Samples

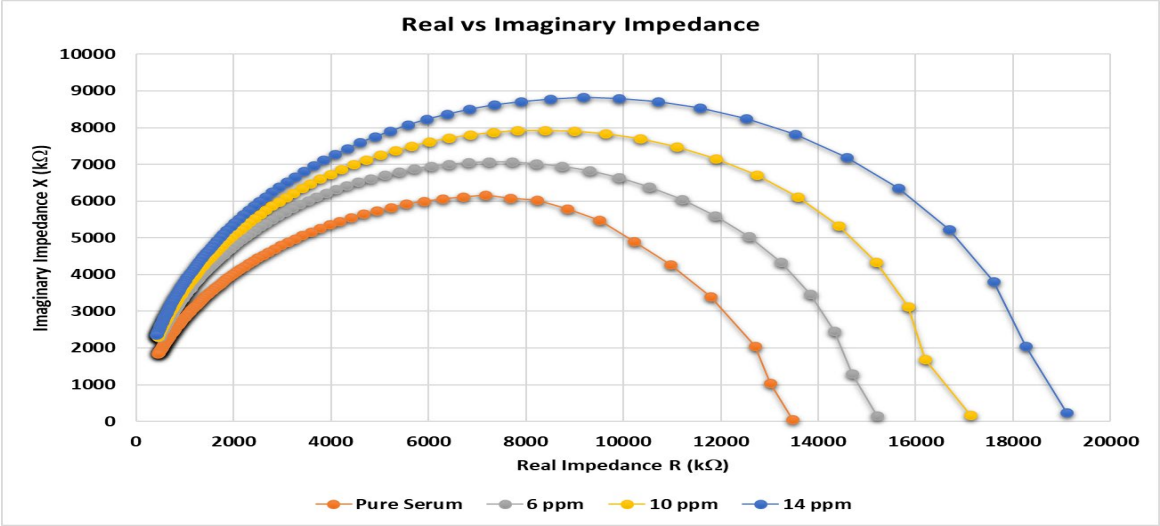


Figure 4.21: EIS Measurement of creatinine samples (a) Real impedance vs frequency range at different concentrations of aqueous creatinine samples, (b) Real impedance vs frequency range at different concentrations of serum creatinine samples, (c) Imaginary impedance vs frequency at different concentrations of aqueous creatinine samples, (d) Imaginary impedance vs frequency at different concentrations of serum creatinine samples, (e) Nyquist plot for EIS profiling of functionalized sensor at different concentrations of aqueous creatinine samples, (f) Nyquist plot for EIS profiling of functionalized sensor at different concentrations of serum creatinine samples.

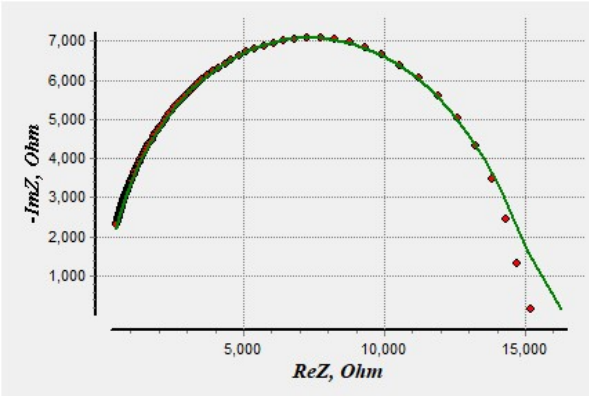
4.4.6 Analysis of Nyquist Plot Data using CNLS Curve Fitting

Randle's equivalent electrical circuit is frequently used to explain and analyse the EIS data in the electrical presentation. The model is helpful in describing the diffusion of sample solutions and kinetics procedures that take place at the borders between electrodes. The circuit involves the R_s in a sequence connection to the analogous fusion of C_{dl} to the R_{ct} when the W1 is used in series. The circuit diagram consists of two resistors (R1 and R2), two capacitors (C1 and C2) and W1. It incorporates R_s (R1), and R_{ct} (R2) are parallel in combination connected to capacitances C1 and C2 in a series connection with W1. The Nyquist plot shows an imaginary impedance versus the real impedance for all excitation frequencies. The Nyquist plot offers numerous advantages.

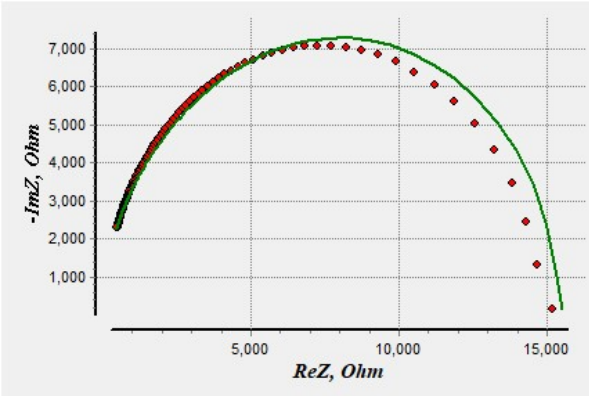
Figure 4.22 shows Nyquist plots and their Randles circuit diagram received with the CNLS software technique for all analysed aqueous and serum samples carrying various concentrations of creatinine. The CNLS is used for calculating the equivalent circuit. It is also used for checking the component parameters with the help of experimental data fitting with its theory-based predicted response depending on Randle's model. Finally, the model infers the protocols performed within the biochemical cell in its' equivalent circuit.

The red coloured dotted markers on the graphs depict the results obtained by the experimental procedure. In contrast, the line markers of green colour illustrate the CNLS software fitted graphical curve figures. The obtained semicircular shaped Nyquist plot is drafted, making the parallel sketching of C1 (Capacitor 1)-R1 (Resistor 1) and C2 (Capacitor 2)-R2 (Resistor 2) and W1. Table 4.3 shows the results from the MIP functionalized sensor equivalent circuit parameters and calculations of the error rate percentage (%) for aqueous and serum samples carrying various concentrations of creatinine. The MIP functionalized MEMS sensor combined with the developed EIS sensing system is used to measure aqueous and serum samples carrying various creatinine concentrations. The calculated error rate percentage of aqueous creatinine samples is less than 16%, and it is less than 11% for serum creatinine samples. The CNLS software helps understand how the change in sample concentrations relates to changes in values of various circuit parameters. The circuit parameter R2 is found to be uniformly increasing as the concentration is increasing for both aqueous as well as serum-based creatinine samples. The CNLS software calculates values for R1, R2, C1, C2, W1, $r^2_{amplitude}$ and error rate (%) when an appropriate circuit of interest is chosen from the list of circuits available within the software database for the analysis. The circuit in Figure 4.22 is used to find out the most sensitive parameter (R2 in this case) within the circuit having 5 different parameters in its structure.

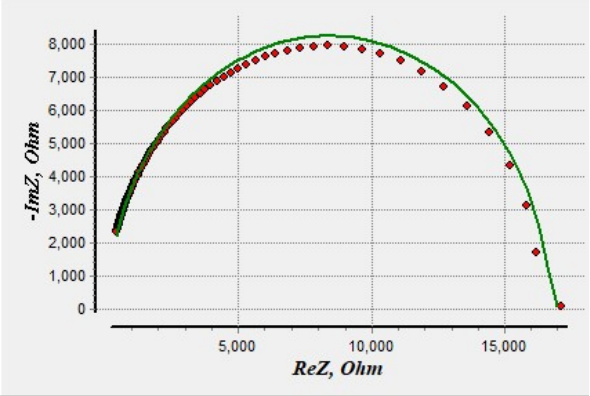
(a) Aqueous Creatinine Sample 6 ppm



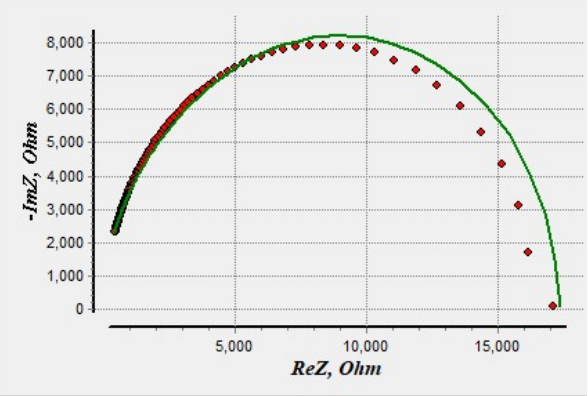
(b) Serum Creatinine Sample 6 ppm



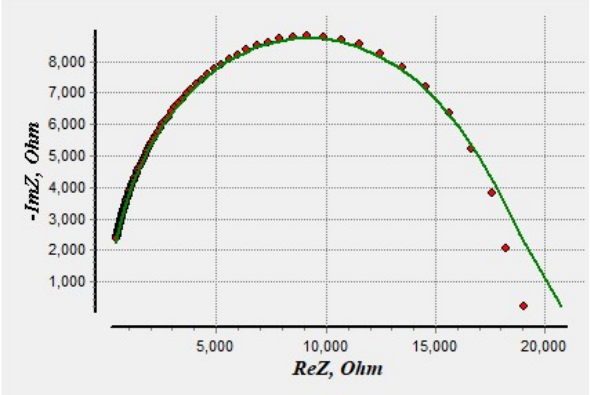
(c) Aqueous Creatinine Sample 10 ppm



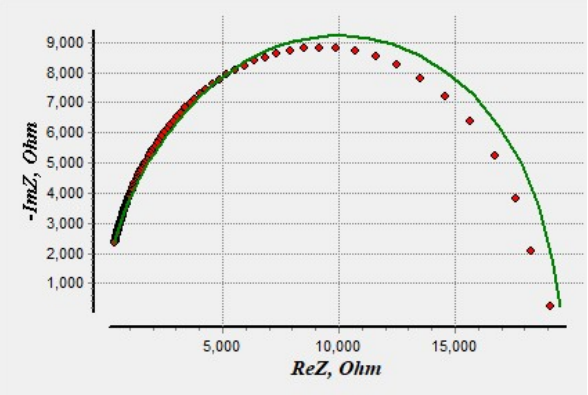
(d) Serum Creatinine Sample 10 ppm



(e) Aqueous Creatinine Sample 14 ppm



(f) Serum Creatinine Sample 14 ppm



(g) Circuit Diagram

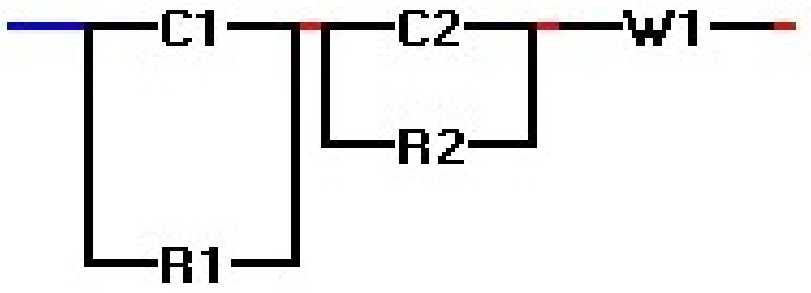


Figure 4.22: The Nyquist plots and their corresponding Randle's model estimated by CNLS analysis. Aqueous samples carrying various concentrations of creatinine are (a) 6 ppm, (c) 10 ppm, and (e) 14 ppm. Serum samples carrying various concentrations of creatinine are (b) 6 ppm, (d) 10 ppm, and (f) 14 ppm are displayed. (g) Circuit diagram used for CNLS analysis of all aqueous and serum creatinine samples.

Table 4.3: Equivalent circuit parameters for aqueous and serum samples.

Aqueous Samples Carrying Various Concentrations of Creatinine						
Component Parameters	**Values of the Parameters 6 ppm**	**Errors (%)**	**Values of the Parameters 10 ppm**	**Errors (%)**	**Values of the Parameters 14 ppm**	**Errors (%)**
C1	7.0565 x 10^{-10}	0.6971	7.0935 x 10^{-10}	1.0445	6.8984 x 10^{-10}	0.66216
C2	1.0118 x 10^{-10}	0.76026	2.6134 x 10^{-10}	0.8663	3.1366 x 10^{-10}	0.9891
R1	115.79	9.1755	161.2	9.058	107.16	8.6117
R2	16304	1.3158	16907	1.8938	20901	1.3795
W1	7.1092 x 10^{6}	15.154	7.571 x 10^{7}	15.693	7.818 x 10^{6}	13.557
$r^2_{amplitude}$	0.0011275	-	0.0020328	-	0.001087	-

Serum Samples Carrying Various Concentrations of Creatinine						
Component Parameters	**Values of the Parameters 6 ppm**	**Errors (%)**	**Values of the Parameters 10 ppm**	**Errors (%)**	**Values of the Parameters 14 ppm**	**Errors (%)**
C1	2.0221 x 10^{-09}	1.1768	2.1459 x 10^{-09}	1.2887	2.4703 x 10^{-09}	1.4816
C2	1.0018 x 10^{-09}	0.75096	9.6199 x 10^{-10}	0.79369	8.9769 x 10^{-10}	0.783

R1	2123.9	2.707	2029.9	3.0561	1714.9	3.6422
R2	13267	1.1411	15308	1.2659	17560	1.318
W1	1123	8.5397	485.28	10.705	1734.1	9.8568
$r^2_{amplitude}$	0.00059504	-	0.00065366	-	0.0007508	-

The mathematical algorithm practices statistical analysis for calculating the mean square r^2_c value for experimentally noticed values inside the obtained spectrum using CNLS with the help of the (Equation 4.3) given below [164, 96]:

$$\boldsymbol{r_c^2 = \sum_{i=1}^{N}\left[\frac{\left(Z'_{i_{observed}} - Z'_{i_{calculated}}\right)^2}{Z'^{\,2}_{i_{observed}}} + \frac{\left(Z''_{i_{observed}} - Z''_{i\ calculated}\right)^2}{Z''^{\,2}_{i_{observed}}}\right]} \qquad \textbf{(Equation 4.3)}$$

where,

$Z'_{i_{observed}}$ The observed value of real impedance.

$Z'_{i\,calculated}$ The calculated value of real impedance.

$Z''_{i_{observed}}$ The observed value of imaginary impedance.

$Z''_{i\ calculated}$ The calculated value of imaginary impedance.

r_c^2 Displays the deflection between the data values obtained with experimental results and the optimal solution in the above equation.

The research aims to use human serum samples in future experiments; aqueous creatinine samples are discontinued. Therefore, from this step onwards, only serum samples are used for all the remaining experiments carrying varying creatinine concentrations. R2 is the most influenced parameter and its value increases/decreases with an increase/decrease in the aqueous and serum samples carrying various creatinine concentrations. The following mathematical formula can be derived and utilised for computing the change in R2 values is as follows (Equation 4.4):

$$\frac{\Delta R2}{R2_0}\ (\%) = \frac{R2\ (Control) - R2\ (Sample)}{R2\ (Control)} \times 100 \qquad \textbf{(Equation 4.4)}$$

The results obtained using the two methods are in an acceptable range. The serum samples carrying creatinine concentrations of 6, 10, 14 ppm and 15.5 ppm (treated as unknown samples) are analysed with the help of a developed biosensor. The Creatinine Colorimetric Assay Kit (explained in detail in chapter 7) is a standard technique. It is used for the verification purpose of the presented technical method. The obtained results are displayed in Table 4.4.

Table 4.4: Identification of creatinine concentrations in serum samples using a calibration curve obtained by the CNLS technology.

Sample	Proposed Biosensor	Standard Reference Method Creatinine Colorimetric Assay Kit	Error (%)
1	6.0025	6.0010	0.02
2	10.4312	10.0109	4.19
3	14.0233	14.0026	0.14
4	15.5043	15.5011	0.02

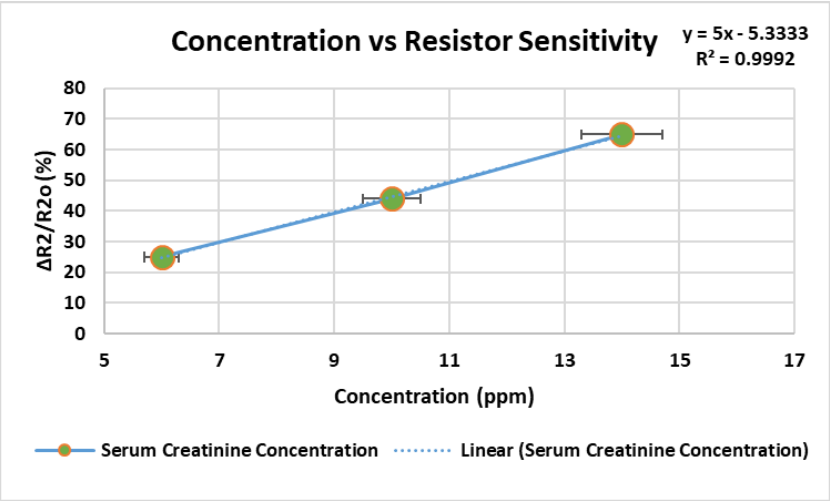


Figure 4.23: A CNLS-dependant calibration curve showing the change in reactance (X) concerning the change in the serum creatinine concentrations at 1020 Hz.

Figure 4.23 represents the calibration curve obtained from CNLS analysis for 6, 10, 14 ppm serum creatinine concentrations at 1020 Hz (single operating frequency). A linear correlation is seen between the R2, and the levels of creatinine spiked serum samples using (Equation 4.3) is depicted in Figure 4.23 ($R^2 = 0.9992$). Creatinine concentrations spiked serum samples are performed with the help of a calibration curve based on the CNLS technique seen in Figure 4.23.

4.4.7 Testing for Repeatability and Reusability of MIP Functionalization and the MEMS Sensor in Creatinine Measurements using EIS Technique

The amount of creatinine biomolecule attached to the functionalized MEMS sensing surface is measured using serum creatinine samples with the EIS technique, and it is organised in Figure 4.24. This presented that the MEMS sensor can be reused five times before any significantly noticeable measurement error is seen post functionalization during the recording of readings. The sensor showed its repeatability five times. During reusability testing after five measurements, the sensor showed an error value of >10%. More than 90-95% of the creatinine biomolecules can be re-attached by the MIP functionalized surface for the initial five experimental results.

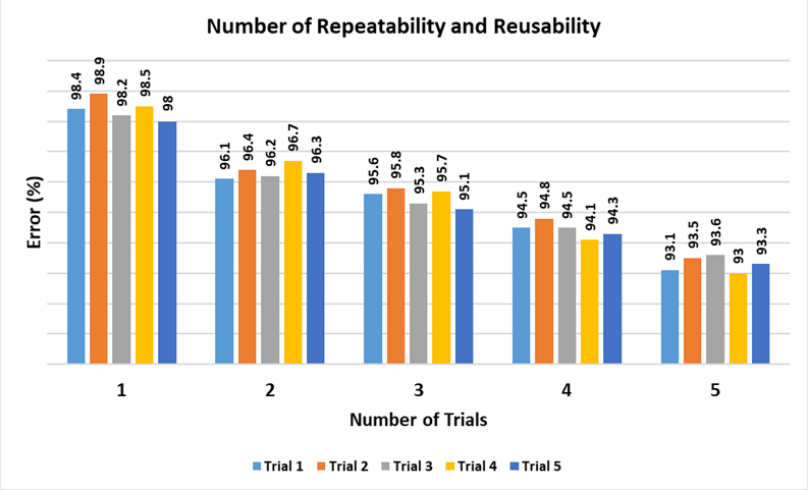


Figure 4.24: Repeatability and reusability of the sensor done for functionalization testing of the MEMS ID sensor using a 10 ppm serum creatinine sample.

After five measurements, the sensor and the MIP functional coat do not work as significantly as before. Due to the damage caused to some of the recognition sites of creatinine-

specific MIP after the multiple usages. It is also because of the repetitive cleaning processes of sensing the surface of a sensor. Due to repetitive usage, some biomolecules do not bind to the creatinine capturing complementary sites of the MIP polymer, permitting creatinine adsorption earlier, resulting in improper output from the sensing system. It is noticed that the repeatability measurements of the functionalized MEMS sensor are relatively constant for up to five uses.

The repeatability and reusability of the MIP functionalized MEMS ID sensor for creatinine measurements is done using EIS Technique (Figure 4.25).

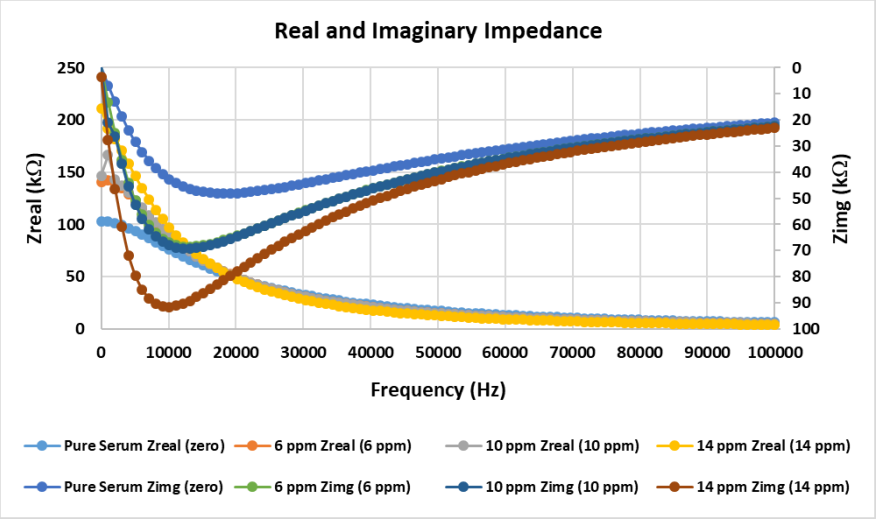


Figure 4.25: Repeatability and reusability of the sensor done for functionalization testing of the MEMS ID sensor.

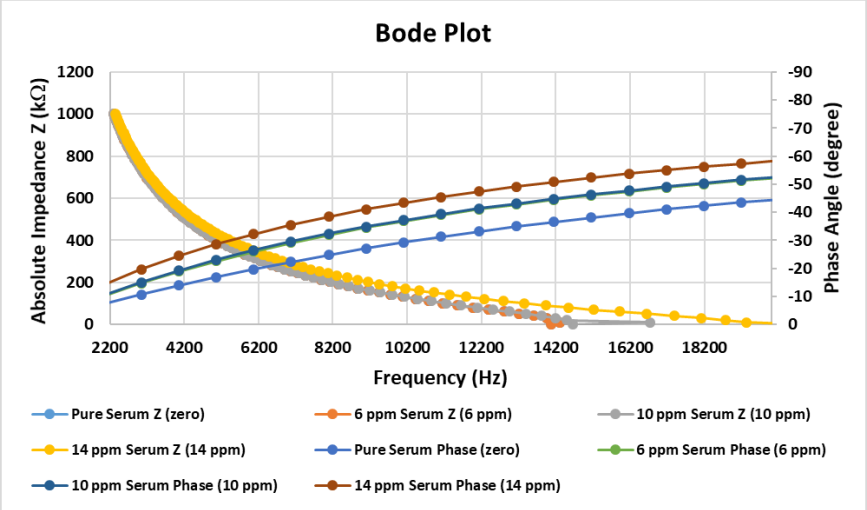


Figure 4.26: Repeatability and reusability of the sensor done for functionalization testing of the MEMS ID sensor.

Figure 4.25 displays the characterisation of the real (resistance) and imaginary (reactance) impedance against frequency for various serum creatinine concentration levels. At low frequencies, the capacitance/imaginary impedance (reactance) ($Z_{imgaginary}$) presents significantly noticeable changes alongside changes in concentration, though, at high frequencies, the change in imaginary impedimetric values are significantly minor. Figure 4.26 shows the behaviour of the phase angle and an absolute impedance, plotted against frequency in the Bode plotting form.

4.4.8 Development of Calibration Curve by using LCR Meter

Three samples carrying different levels of creatinine spiked serum concentrations (6, 10, 14 ppm) are tested by use of the MIP functionalized biosensor by using the LCR meter. From Figure 4.21, the diameter of the semicircular region of the Nyquist plot rises with rising creatinine concentrations. The reactance (X) showed variations distinctively along with the change in creatinine concentrations, which is related to the conducting characteristics of that testing solution (Figure 4.27). The variations are noticeable in the frequency range of 10 Hz to 2800 Hz, as shown in Figure 4.21; it is considered a “sensitive area”. Whereas change in the resistance (R) is seen in a smaller frequency range of 10 Hz to 1000 Hz, they are also considered as a “sensitive area” (Figure 4.21). That is why the reactance (X) from the impedance is utilised to plot the impedance. The reduction in frequency range makes the process of measurement much faster, easier and more time-effective; a single frequency is decided for calibration curve plotting. Therefore, depending upon the change in reactance and the review obtained from the Nyquist plot, the 1020 Hz frequency is finalised as an optimal operating frequency.

The reactance (X) is presented in a graph showing various creatinine spiked serum samples at 1020 Hz. The single frequency dependant calibration graph, which is received from the measurements done using the LCR meter, is depicted in Figure 4.27. From Figure 4.27, the LCR meter-based MIP functionalized biosensor attached sensing systems sensitivity is 182.73 Ω/ppm. It can also be noted that by raising the creatinine concentration, the reactance value is found to be rising. The functionalized MEMS ID sensor showed its reusability five times. After five measurements, the sensor and the MIP functionalization do not work as consistently as earlier. It is observed that the repeatability measurements of the MIP functionalized ID sensor are consistent until five readings when tested using serum samples.

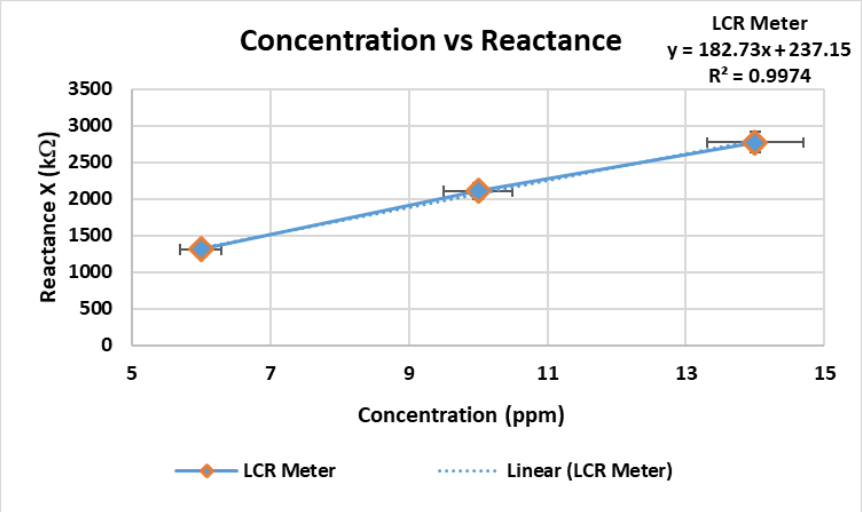


Figure 4.27: Standard calibration curve for the LCR meter.

4.4.9 The Comparative Study Within the CNLS Measuring and Single-Frequency Measuring System

Concerning the results obtained from Table 4.4 of the two techniques, the CNLS measurement gives more precise results due to the calculations done using a broad frequency range at a time. The R2 parameter is obtained by using the theoretical fitting method. Although there are some noticeable advantages in using the single-frequency-based measuring system, they are practical in developing a PoC diagnostic system. The main advantage of the use of single-frequency dependant calculation is that the CNLS technique is not necessary to obtain the value of R2. This will significantly reduce the actual experimental timing for the biosensor and ease up the test protocol. That is why the single-frequency measurement is highly selective for the development of a PoC diagnostic system, as it offers a straightforward measuring method with the much-reduced time of response, which is highlighted characteristic in the development of a fast and straightforward processing creatinine specific PoC system.

4.4.10 S_x Curve Equation Development and Measurement of Unknown Sample

The ΔX is the reactance value of the known serum samples carrying pre-decided creatinine levels (ppm). In contrast, the X_o is the reactance value of pure serum sample without creatinine (control). The S_x (sensitivity of reactance ($\Delta X/X_o$ (%)) curve is developed for

measuring the unknown sample concentrations by merely replacing the reactance values in the equation to obtain the unknown sample concentrations for serum creatinine samples. The precision of the unknown sample quantification is analysed by comparing the obtained results from the Sx curve and original concentration values measured using the EIS technique. The error (%) is calculated. The results obtained from both methods are presented in Figure 4.28 are found to be in good agreement overall.

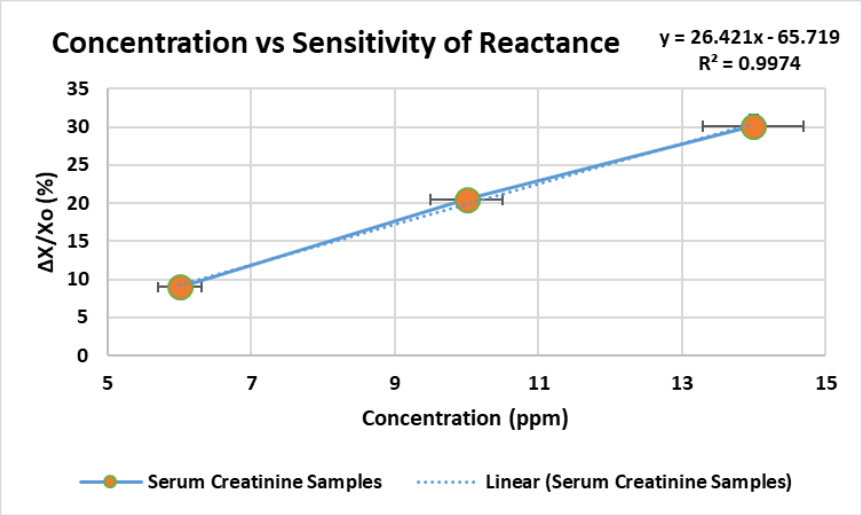


Figure 4.28: Concentration of creatinine (ppm) vs sensitivity of reactance (ΔX/Xo (%)) at fixed frequency 1020 Hz.

4.5 Chapter Summary

This chapter explains how to use the EIS technique and MIP technology to create a revolutionary creatinine level detection sensing approach using aqueous and serum samples. The structural and functional analysis of developed MIP and NIP polymers is performed. The MIP polymers specific selectivity towards creatinine and its structurally similar compounds are also analysed using the UHPLC technique. The tests are carried out using heat-inactivated human serum samples due to human ethics issues. The suggested biosensor has a high specificity, selectivity and rebinding capability for the target creatinine molecule. The designed biosensor detects a concentration as low as 0.1 ppm and as high as 50 ppm (LOD). The 50 ppm value is three times higher than the normal level of human serum creatinine value. The LOD was achieved using the developed MIP methodology. However, the MEMS ID sensor development highlighted that the proposed sensing approach is a simpler, faster, and less expensive creatinine level detection technology. Furthermore, in chapter 5, the employment of the MIP polymer and its functionalization over the MEMS ID sensor is studied for significantly increasing the system's functionality and greatly improving the precision of detection of creatinine levels.

5

Functionality Evaluation of MEMS Sensor for Varied Selective Functionalization Thickness

Publications about this chapter:

- **S. N. Prabhu**, S. C. Mukhopadhyay and R. Morello, "Functionality Evaluation of Micro Electro Mechanical Systems Sensor for Varied Selective Functionalization Thickness to Determine Creatinine Concentration," **IEEE Sensors Journal**, **2021**, doi: 10.1109/JSEN.2021.3081828

5.1 Introduction

Eliminating the natural and synthetic contaminants from the sensing surface is one of the most critical tasks in sensing research to get precise detection of the targeted molecules. The addition of a protective layer of surface functionalization is known as the most efficient protective methodology that has been practised everywhere in controlling the natural and synthetic contaminant impact. Different studies on the functionalization layer have been undertaken in controlling the process of corrosion and improving the corrosive resistance [165], [166], for detecting natural contaminants [167] and also in avoiding biological interventions [168-170]. Furthermore, the protection of a sensing surface is done by functionalizing the membrane using natural and synthetic reactions to act like a precisely selective layer. The incorporation of a specifically selective layer of functionalization raises the sensor selectivity in detecting the target molecule. The specifically selective layer of functionalization has been stated in the detection of various molecules including, a marker of ovarian cancer [171], the presence of phthalate in beverages [88] and cholesterol [172]. After successfully developing the MIP polymer and its selective functionalization coat suspension in the previous chapter, it is necessary to study the functionalization thickness effect on the MEMS ID sensor to determine the creatinine concentrations. Chapter 5 explains the effects of various factors on the MIP functionalization during the determination of varying creatinine concentrations.

5.2 Functionality Evaluation of MEMS Sensor for Varied Selective Functionalization Thickness to Determine Creatinine Concentration

In the presented studies, the method of dip coating with the help of the PTL-MM01 Dip Coater instrument is utilised in generating the layer of functionalization on the surface of the MEMS ID sensor. From the list of simplified and most used techniques, including the spin coating [173-174] as well as the spray coating [175-176], the process of dip coating is frequently used. This technique is well-known, and it is used because of its notable features such as cost-effectiveness, reproducibility, and operational easiness. In the present work, the sensing area of the MEMS sensor is immersed inside the functionalization solution and then removed at a pre-decided speed and time of immersion. The functionalization solution uniformly coated the sensing surface area of the MEMS sensor. Post functionalization of the sensor sensing area gets dried up due to the natural process of evaporation. A hard layer of

functionalization gets generated over the MEMS sensing area [168, 177-179]. The thickness and consistency of the functionalization solution, speed of withdrawal, dipping time, the temperature of the functionalization solution, and the evaporation speed of the chemical components used while making the process of the functionalization solution play a notable role in deciding the functionalization layer characteristics of the MEMS sensor [165, 177, 180]. The graphics of the process of dip coating is displayed in Figure 5.1.

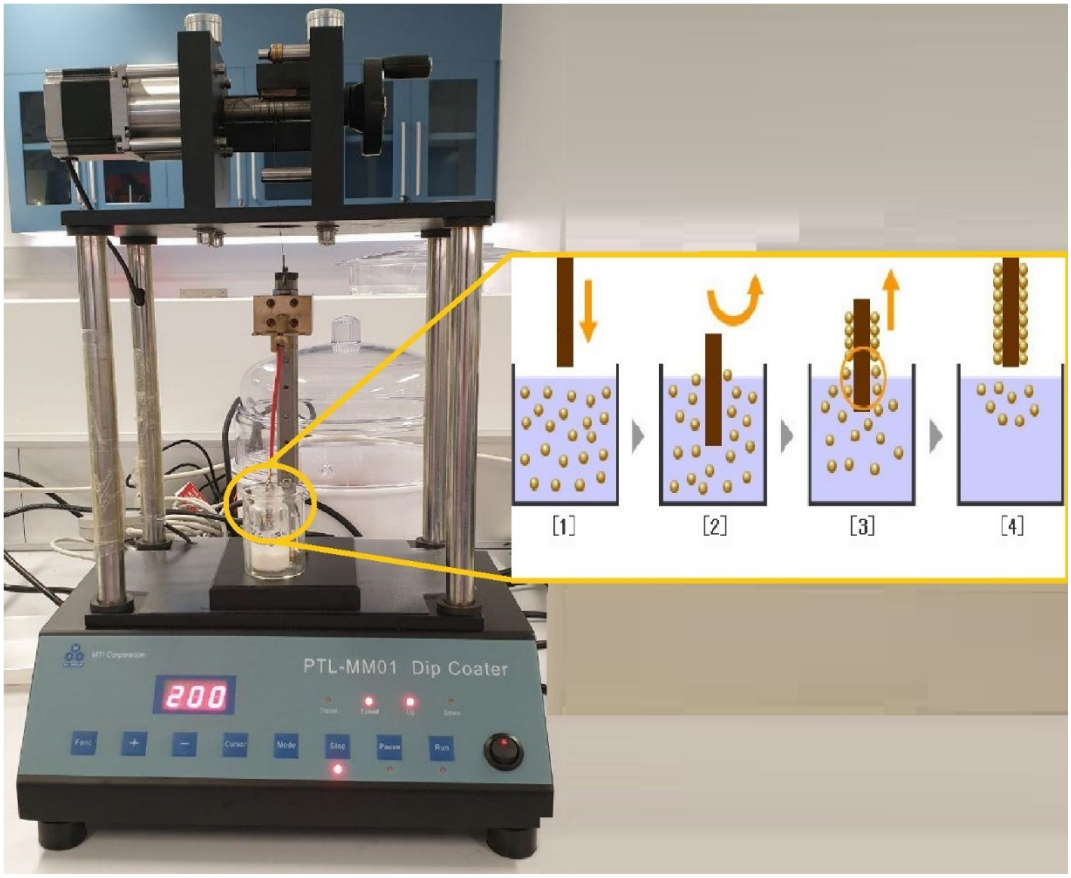


Figure 5.1: Graphics of the process of dip-coating.

It is seen from Figure 5.1 graphics; section [1] shows mounted MEMS sensor before initiating the process of functionalization along with uniform spreading of creatinine specific MIP suspension functionalization solution kept inside a glass beaker. Section [2] shows uniform upside-down dipping of MEMS sensor within the uniformly spread creatinine specific MIP suspension functionalization solution kept inside a glass beaker. Section [3] shows uniform downside-up removal of functionalized MEMS sensor from the uniformly spread creatinine specific MIP suspension functionalization solution kept inside a glass beaker. Section [3] also shows the attraction and binding of MIP from the suspension functionalization solution to the MEMS sensor sensing area. Section [4] shows the complete withdrawal of

functionalized MEMS sensor sensing area from the MIP suspension functionalization solution kept inside a glass beaker. Section [4] also shows a reduced quantity of MIP from the suspension functionalization solution. In the present work, the effect of withdrawal speed and the time of dipping during the dip-coating process is assessed.

The MEMS sensor's functional sensitivity is significantly dependent on the functionalization layer thickness. Hence, the functionalization layer's role in detecting and measuring creatinine is examined in the presented study. The effect of functionalization layer thickness on the performance of the MEMS planar ID sensor on the creatinine measurement is also studied. The acrylic coating and the creatinine-specific selective MIP functionalization layers are analysed for calculating the MEMS ID sensor functional performances associated with the sensitivity.

5.2.1 Surface Coating of the MEMS Sensor with Acrylic Resin

The acrylic resin is used to coat the planar MEMS ID sensor. The sensing surface is washed with acetone to remove any invisible surface debris or contaminations in the first step. The acrylic resin coat is made in the second step by mixing acetone as a liquefier (1.5 mL) and acrylic resin (200 µL) as a protective layer and adhesive agent. It is layered on the MEMS sensors sensing area as a protecting layer of functionalization and acts as an adhesive agent. The layering of acrylic resin is completed with the PTL-MM01 Dip Coater instrument for obtaining a functionalization layer uniformity. In the subsequent stage, the MEMS sensor is dried for 30 minutes at standard laboratory temperature (25°C) and humidity level (31%) to achieve uniformity in the drying process and achieve the sensor functional stability. The effects of interfering parameters associated with the functionalization process, including time, speed and their effect on the thickness of the functionalization, are examined in the presented study. A MEMS sensor having a varying functionalization thickness is prepared for finding out the influence of the thickness of the functionalization layer on the sensitivity and the output of the MEMS sensor. The increasing functionalization thickness is achieved by changing the speed of withdrawal and time of dipping. The minimum coating layer thickness obtained is 55 µm, whereas the maximum thickness is 240 µm for the acrylic resin coating suspension. The FESEM instrument is used for obtaining high-definition images of the MEMS sensor functionalized surfaces. The FESEM images of the acrylic resin coated layer and the MIP-polymer functionalized selective layer are displayed in Figure 5.2 and Figure 5.3.

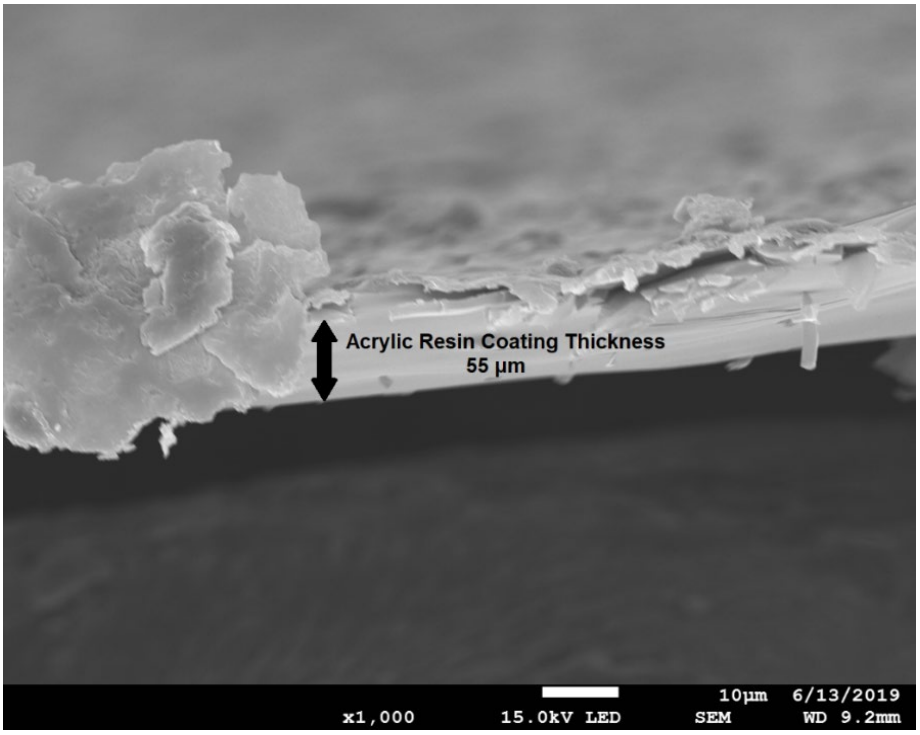
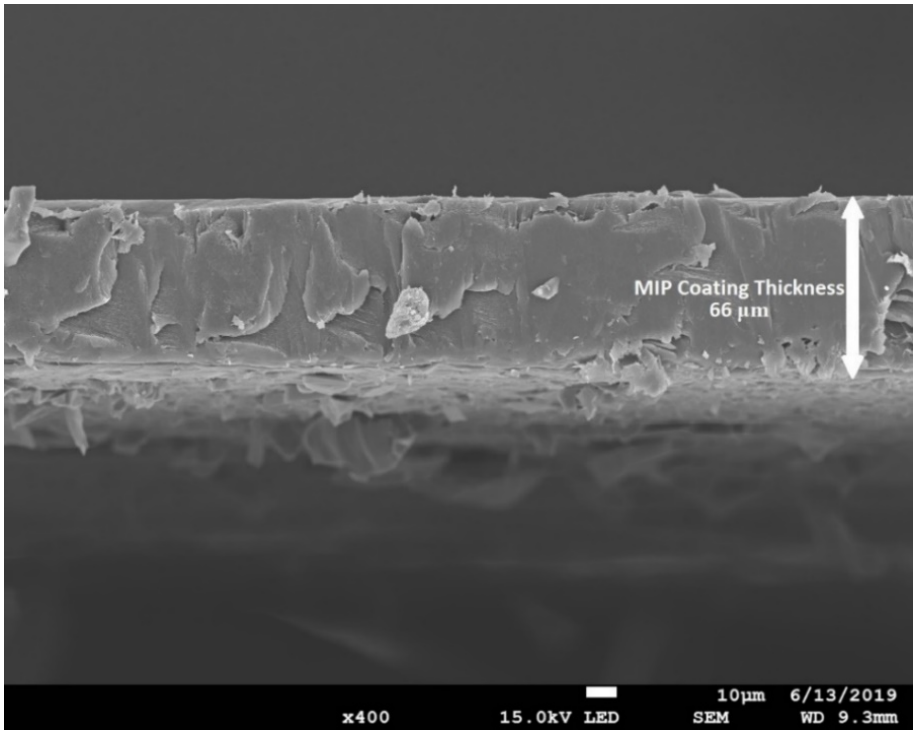


Figure 5.2: FESEM images of the acrylic resin coated layer.

(a)



(b)

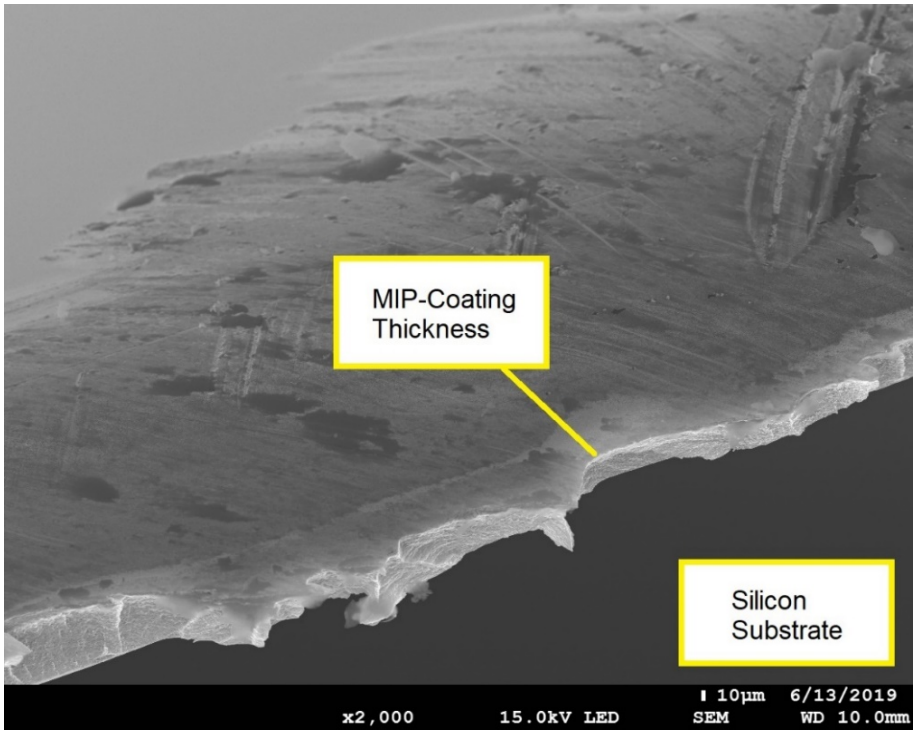


Figure 5.3: FESEM images of the selective MIP-polymer functionalized layer (a) MIP functionalization coat thickness, and (b) MIP functionalized MEMS ID sensors cross-section.

5.2.2 Specifically Selective MIP Functionalization Material for Creatinine on the MEMS Sensing Area

The acrylic resin-coated sensor is not strictly selective against the target biomolecule, e.g. creatinine. The coated MEMS sensing surface cannot specifically and selectively analyse creatinine levels from the serum samples. It is due to the presence of various other biomolecules inside the serum samples. To avoid this issue, the sensing surface of the MEMS sensor is selectively functionalized with a specifically selective synthetic functionalization material. A chemically synthesised MIP methodology is introduced while synthesising a creatinine specific selective functionalization material. MIP is a robust and convenient methodology to synthesise synthetic-detection sites for the target molecule [181-182].

In the first step, the MEMS sensor surface is cleaned using acetone for removing all kinds of surface impurities. In the subsequent stage, a MIP functionalization material suspension solution is made by uniformly mixing acetone as a liquefier (1.5 mL), MIP as a

selective polymer (1 g), and acrylic resin (200 µL) as a protective layer of the functionalization as well as adhesive agent in a small beaker at laboratory temperature.

The functionalization layer's saturation of thickness depends on functionalization material properties and the dip-coating process (speed of withdrawal and dipping time). To avoid rapid evaporation of acetone at laboratory temperature, drying out of both acrylic coating solution and the MIP suspension functionalization solution, instant cracking/peeling off of the functionalization layers from the MEMS ID sensing area, the following essential factors need to be taken into consideration.

First, the liquid suspension bottles containing acrylic coating and MIP functionalization solutions needs to be strictly stored inside the freezer at -4°C and taken out for only 15-20 minutes before the process of functionalization. It helps in slowly increasing the temperature of bottles to laboratory temperature. Second, the lid of the glass storage bottles needs to be opened only for the time of the dip-coating procedure. It is necessary to be closed right after finishing the dipping experiment, followed by restoring the solutions at a -4°C freezer. The individual solution temperatures are maintained at laboratory temperature and humidity level to avoid rapid evaporation of acetone and consecutive instant cracking of acrylic coating solution and the MIP functionalization layer suspension. A PTL-MM01 Dip Coater instrument is used in the process of functionalization, as shown in the previous chapter Figure 4.9 (a).

Post functionalization, the sensor is slowly and uniformly withdrawn from the functionalization suspension solutions to achieve uniformity in both the acrylic coating and the MIP selective functionalization layers. The coated and the functionalized MEMS sensors are air-dried for 1 hour at average laboratory temperature in the final stage.

The increasing functionalization thickness is achieved by changing the speed of withdrawal and time of dipping. The minimum functionalization layer thickness obtained is 66 µm, whereas the maximum thickness is 270 µm for the MIP functionalization layer suspension. The previous chapter, Figure 4.9 (b), displays the functionalized MEMS sensor with a MIP functionalization. The creatinine binding procedure on MIP functionalized MEMS sensor is studied [183-185], and experiments are done with the EIS technique using the LCR meter instrument.

Streptavidin is also used in literature for the biosensor functionalization process. However, in our application, the acrylic resin acted as the optimum coating agent. It created a protective layer of the MIP functionalization and worked as an excellent adhesive agent.

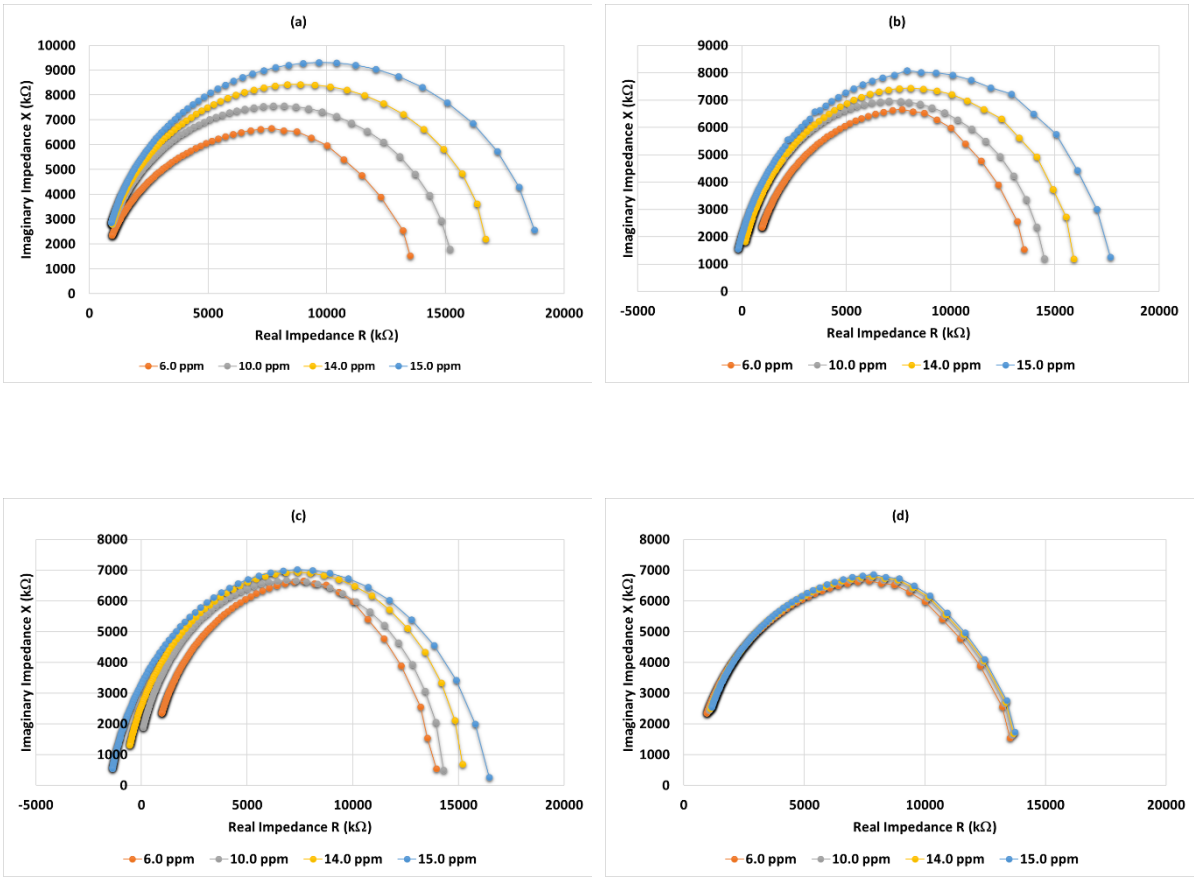


Figure 5.4: Serum creatinine analysis with the help of acrylic-coated MEMS sensor having a varying thickness of coats: (a) 55 µm, (b) 120 µm, (c) 180 µm, (d) 240 µm.

5.2.3 Creatinine Level Analysis with the Acrylic Resin Coated and MIP Functionalized MEMS ID Sensor

The creatinine measurement is done in the initial step using an acrylic resin coated MEMS sensor prepared with varying thicknesses of the coating's (55, 120, 180, 240 µm) and 6, 10, 14 and 15 ppm serum creatinine samples are measured. The MEMS sensor gets saturated at 240 µm thickness of the acrylic resin coating.

Varying thicknesses of coating and functionalization layers are attained by repeating the coating and functionalization process. Post coating of the MEMS sensor with acrylic resin, profiling of the coated sensor has been done in the air. It is tested with the serum samples solutions of varying creatinine levels. The same set of experiments are performed with the 55,

120, 180, 240 μm. Figure 5.4 denotes experimental results in the representation style of the Nyquist plotting technique.

It is noticed that the bidirectional changes happened for the real as well as the imaginary impedance for thickness levels ranging from 55 μm to 240 μm. Furthermore, it is observed that as coating layer thickness decreases, the differences displayed within every Nyquist plot for various creatinine concentrations are significantly distinct. Nevertheless, the acrylic resin coated MEMS sensor having 240 μm thickness of coating did not show significant changes in varying creatinine concentrations.

In the initial step of MIP functionalized MEMS sensor creatinine concentration analysis, the non-functionalized MEMS sensor is characterised using the EIS technique with an LCR meter to determine the MEMS sensor's sensitive frequency range. Then, the MIP functionalization process is completed, and thickness levels of MIP functionalization's (66, 135, 200, 270 μm) is achieved. Post functionalization process, the MEMS sensor with MIP is re-characterised in the air for comparing the profiles of varying MIP functionalization thicknesses. It is noticed that the size of the diameter of the semicircle of the Nyquist plot changes with varying thicknesses. It is also observed that the Nyquist plot semicircle comparatively gets saturated at 270 μm when functionalized using MIP. It is because of a heavier functionalization thickness level over the penetration capacity of the protruding electrical field.

In the next set of experiments, the MIP functionalized MEMS sensor is utilised for testing the creatinine serum samples. Varying thickness levels of MIP functionalization's (66, 135, 200, 270 μm) are achieved using the PTL-MM01 Dip Coater instrument by performing a repetitive dipping technique. The same procedure is used whilst performing experiments with the help of the MIP functionalized MEMS sensor. Four samples having varying creatinine concentrations are analysed by using MIP functionalized MEMS sensor.

The Nyquist plot for the MEMS sensor functionalized with MIP having varying functionalization thickness (66, 135, 200, 270 μm) is displayed in Figure 5.5. Concerning MIP functionalization thickness levels ranging from 66 μm to 270 μm, a rise in the Nyquist plot semicircular region diameter indicates a clear rise in creatinine concentrations in serum samples. The increase in the Nyquist plot diameter is due to the rise in the number of creatinine molecules [183-184].

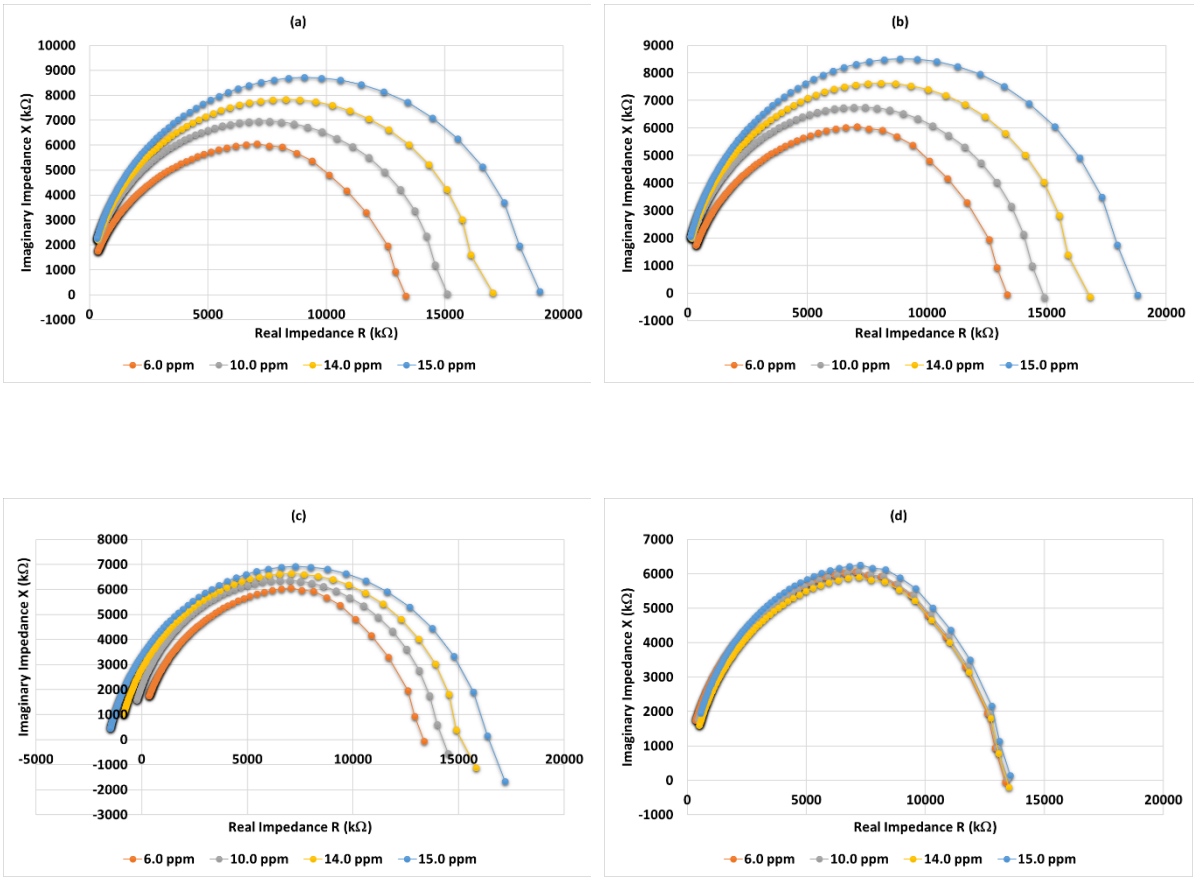


Figure 5.5: Serum creatinine analysis with the help of MIP functionalized MEMS sensor having a varying thickness of coats: (a) 66 µm, (b) 135 µm, (c) 200 µm, (d) 270 µm.

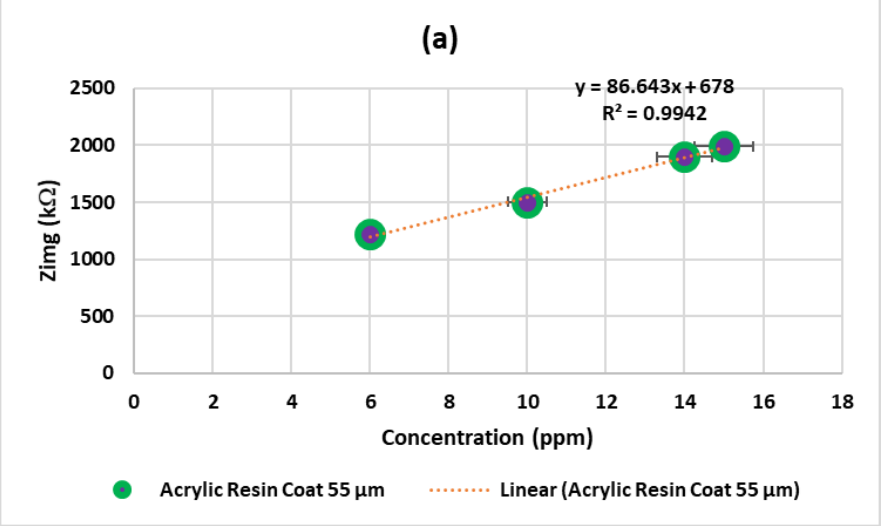
Nonetheless, it was found that the MEMS sensor was saturated at 270 µm and did not display significant changes in the varying amounts of creatinine. The MIP is selectively synthesised for adsorbing the creatinine molecules from the sample solution [183-185]. Therefore, the MIP functionalized MEMS ID sensor showed much better performance than the acrylic-coated one.

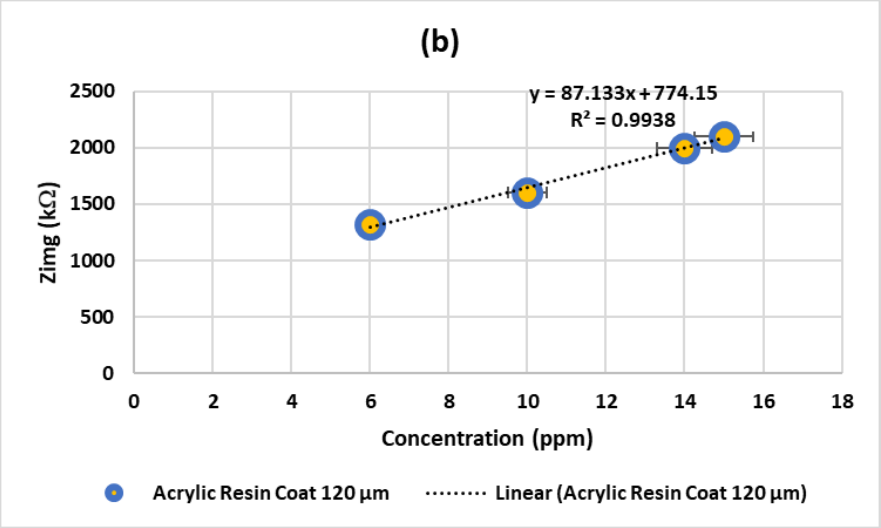
The two different coating and functionalization materials (acrylic and MIP) are saturated at two different thicknesses. The acrylic coating solution is made by mixing acetone as a liquefier and acrylic resin. The MIP functionalization suspension is composed of acetone as a liquefier, MIP as a selective polymer, acrylic resin as a protective layer of the functionalization and an adhesive agent. The acetone gets evaporated, but the combination of MIP and acrylic resin shows an effect on the thickness of the MIP suspension functionalization, making it thicker after drying the coat.

Although the MIP suspension functionalization has shown higher thickness (66, 135, 200, 270 µm) for all the functionalization over acrylic coating solution (55, 120, 180, 240 µm), it is found to be displaying the higher sensitivity. It is due to the usage of creatinine (template molecule), MAA (functional monomer), AIBN (reaction initiator), and DVB (cross-linker) in the synthesis of MIP. Their cross-linking made the MIP highly selective, sensitive, and specific towards adsorption of the creatinine from the spiked serum creatinine samples.

5.2.4 Functionalized MEMS Sensor Sensitivity and the Saturation Level Measurement

A coated and functionalized sensor with varying thicknesses of the coating and functionalization is used to obtain the calibration curve derived from investigating the MEMS sensor output-related sensitivity. The calibration curves are derived by plotting the imaginary impedance against creatinine concentration at 1020 Hz (operating frequency). The maximum changes are noticed with the imaginary impedance at 1020 Hz; it is chosen over the real impedance [183-184]. The acrylic resin coated MEMS sensor calibration curves with three varying coating thicknesses are shown in Figure 5.6. Figure 5.7 shows the MIP functionalized MEMS sensor having three varying functionalization thicknesses calibration curves. By using the slope of the calibration curves, the MEMS sensor sensitivity is indicated. It is found that with the increase in coating and functionalization layer thickness, the curve slope gets affected. This phenomenon occurs because of the passing of fewer electric lines from the sample when the MEMS functionalization thickness level is increased. It is also noticed that the thinnest coating and functionalization layer is most beneficial for achieving the highest sensitivity.





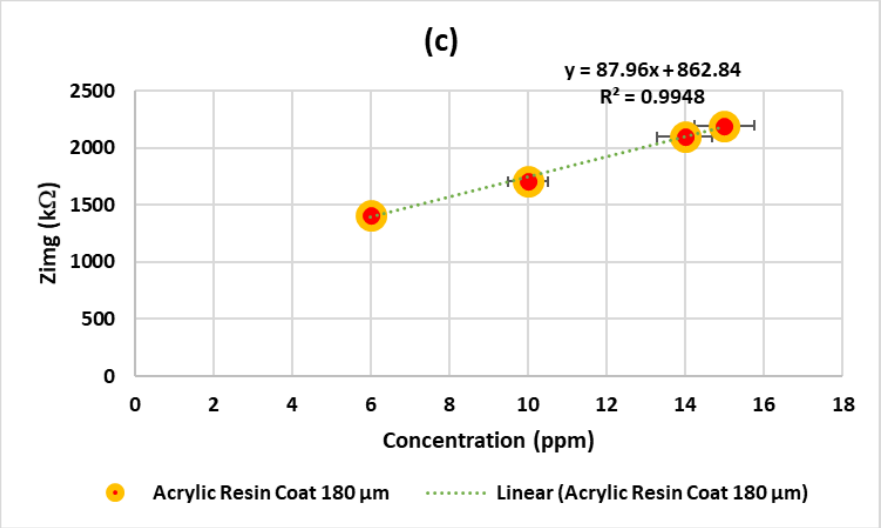
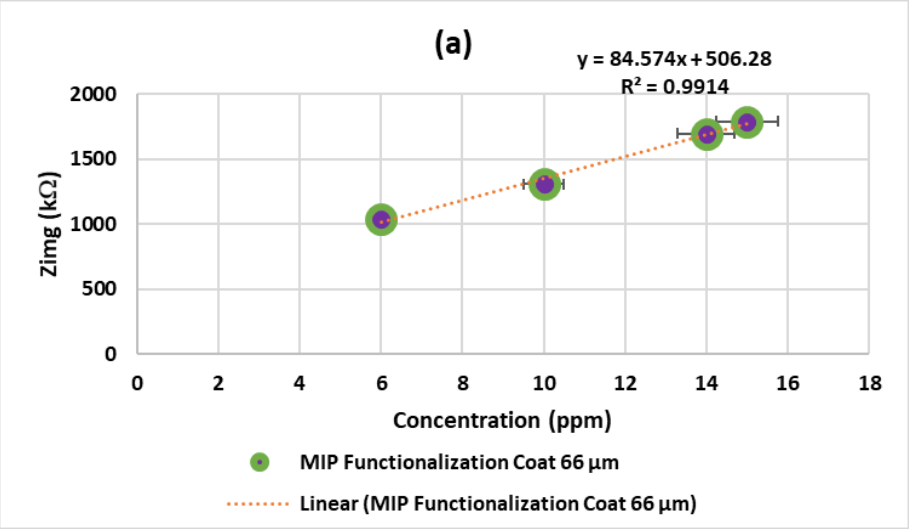
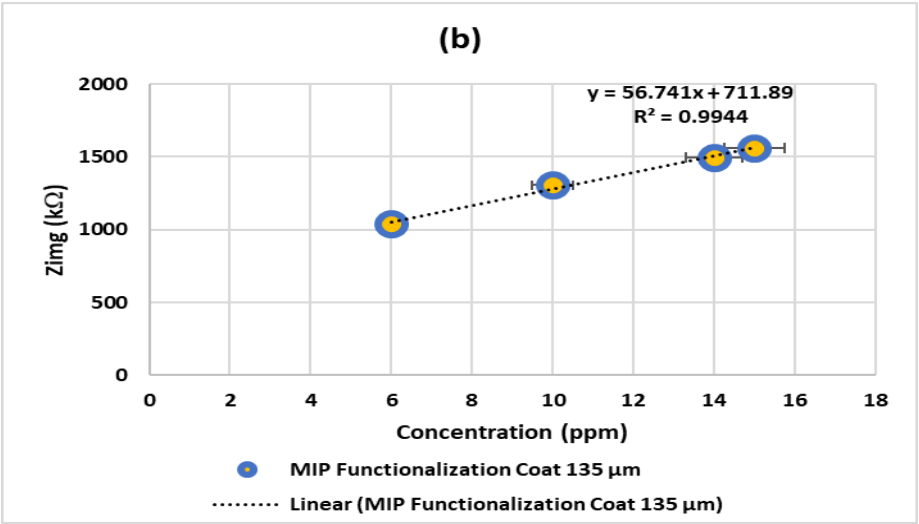


Figure 5.6: Acrylic resin-coated sensors calibration curves for the varying thickness of functionalizations: (a) 55 µm, (b) 120 µm, (c) 180 µm.





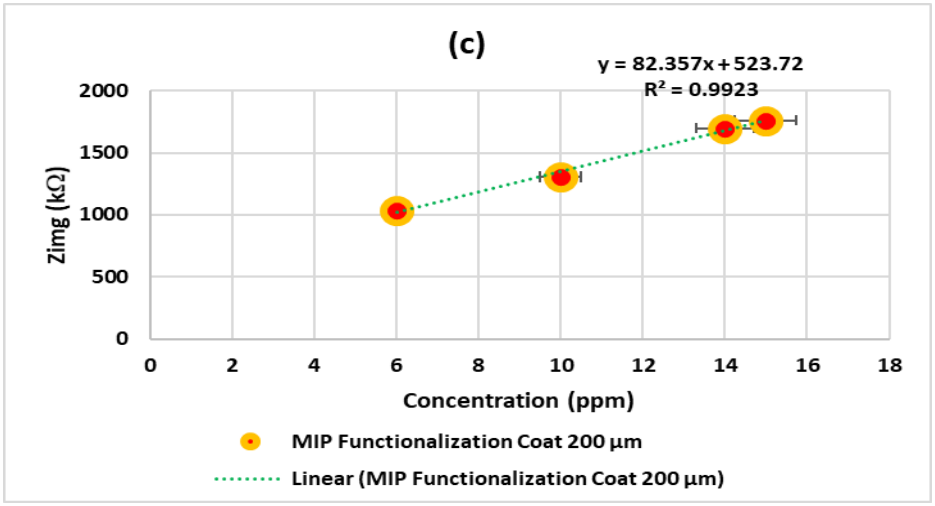


Figure 5.7: MIP functionalized sensors calibration curves for the varying thickness of functionalizations: (a) 66 µm, (b) 135 µm, (c) 200 µm.

Figure 5.8 displays both coating and functionalization sensitivities (acrylic resin coated and MIP functionalized) at varying coating and functionalization thicknesses. From Figure 5.8, the MIP functionalized MEMS ID biosensors sensitivity is 0.15 Ω/ppm (calculated using the equation of the graph). It is noticed that in both the coating and the functionalization, a linear decrease in sensitivity is found. It is linked with a rise in the thickness of the coating and the functionalization. The MIP functionalized MEMS sensor displayed higher sensitivity and selectivity towards capturing creatinine from serum samples over the acrylic resin coated MEMS sensor. The MIP is specifically synthesised by using the creatinine molecule as a template. Therefore, the MIP suspension solution functionalization on the MEMS sensor displayed higher sensitivity [183-185].

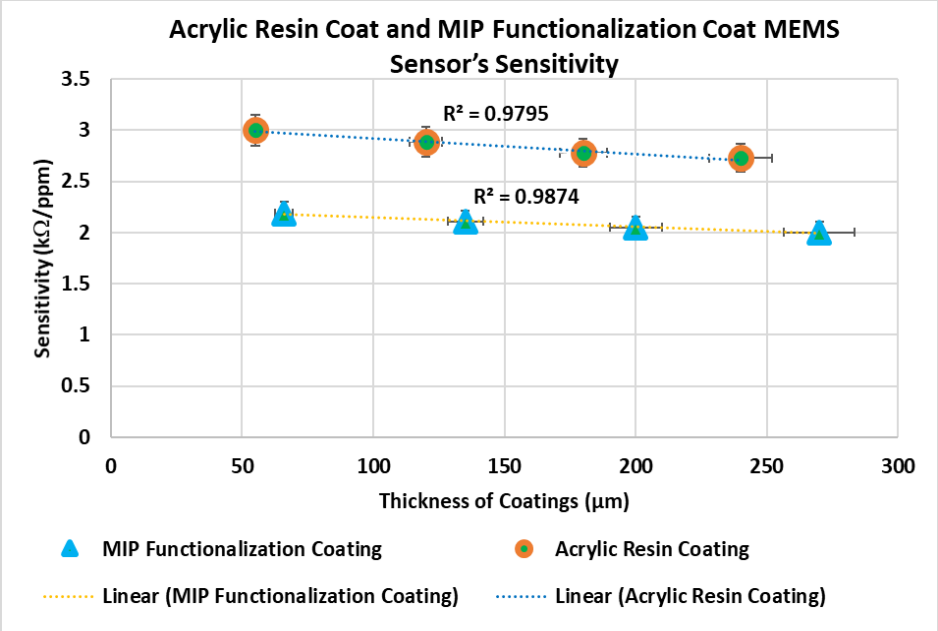


Figure 5.8: The acrylic resin and MIP functionalized MEMS sensor sensitivity.

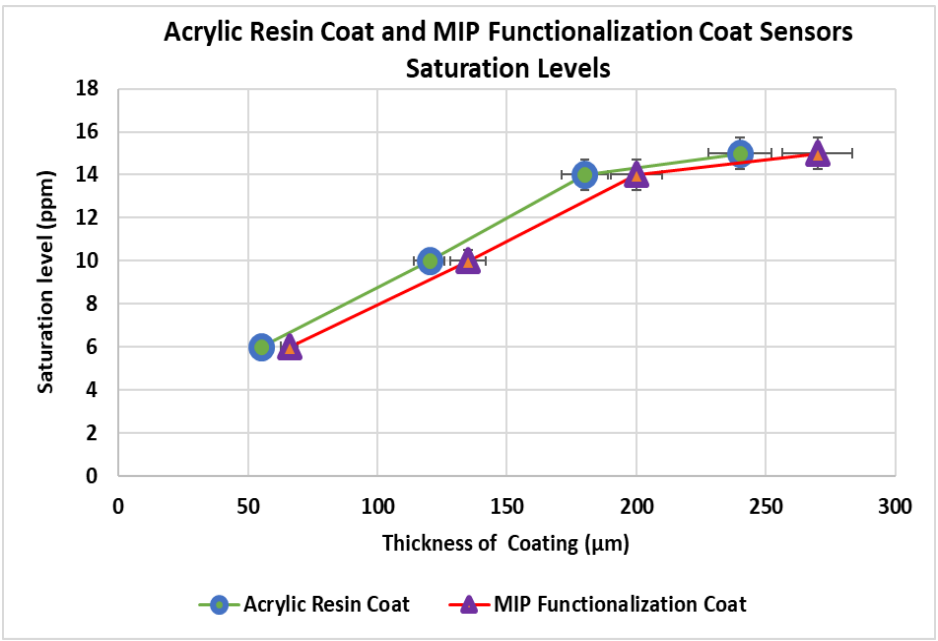


Figure 5.9: Acrylic resin and MIP functionalized sensors saturation levels.

Serum creatinine samples with different creatinine levels have been analysed with coated and functionalized sensors to verify the MEMS sensor saturation level, and the results are shown in Figure 5.9. A direct relation is observed between the increase of the functionalization layer thickness and the enhancement in the level of saturation. This is related to the availability of more creatinine specific capturing sites and the increasing thickness of the

functionalization. Nevertheless, it also reaches the saturation level when the MEMS sensor sensing surface is overloaded with capturing sites. However, the increasing thickness of the functionalization also hampers the passing of the electric field. Thus, a compromise is chosen for the sensitivity and saturation level after finalising the functionalization thickness.

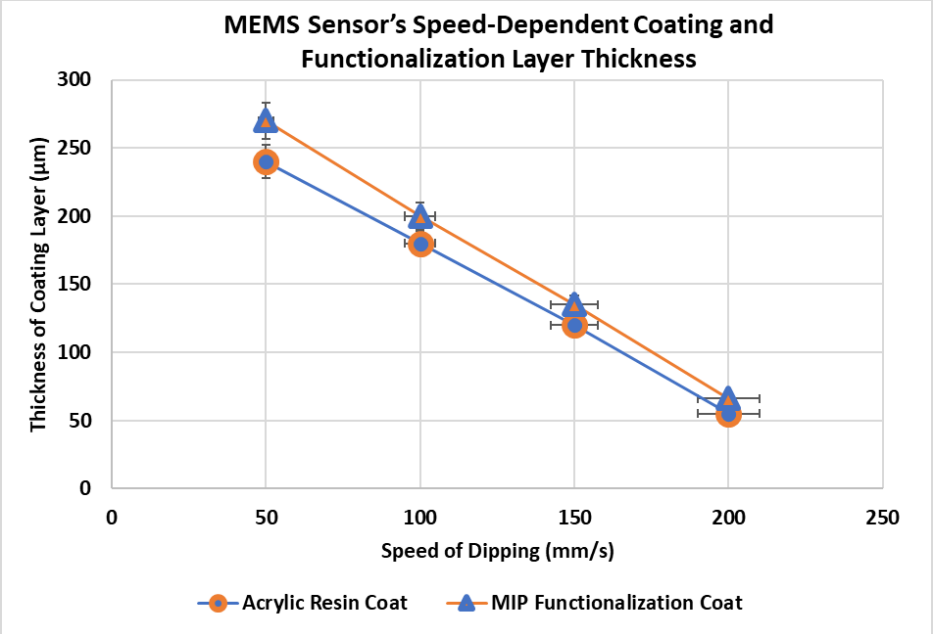


Figure 5.10: MEMS sensor speed of withdrawal-dependent functionalization layer thickness.

5.2.5 Thickness of Functionalization Reliance on the Speed of Withdrawal and Time of Dipping

The PTL-MM01 Dip Coater instrument is utilised for the process of dip-coating. The functionalization layer thickness is analysed by studying the speed of withdrawal and time of dipping for the MEMS sensor. The acrylic coating solution and the MIP suspension functionalization solution are used to conduct this analysis.

The dipping time is pre-set for 10 seconds, and the withdrawal speed is changed from 50 mm/s (minimum speed available) to 200 mm/s (maximum speed available). It is done to investigate the effect of the withdrawal speed on the coating and the functionalization thickness. The reliance of the coating and the functionalization thickness on the withdrawal speed is seen (Figure 5.10). The coating layer thickness is raised from 55 µm to 240 µm for the acrylic resin coating suspension.

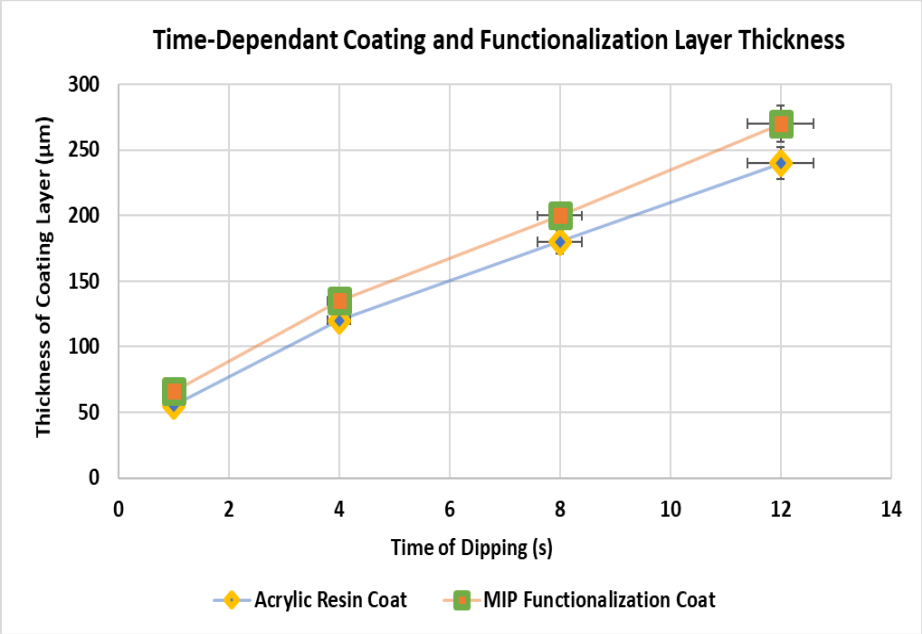


Figure 5.11: A time-dependent functionalization layer thickness.

For MIP functionalization material suspension, the functionalization layer thickness is raised from 66 µm to 270 µm. The maximum thickness is observed at 50 mm/s withdrawal speed.

To identify the time-dependent coating and functionalization thickness, the speed of withdrawal is maintained at 200 mm/s. The time of dipping inside the coating and the functionalization suspension solution is altered from 1 second to 12 seconds. Figure 5.11 shows the outcome of the coating and the functionalization layer thickness concerned with the time of dipping. It is detected that the sufficient thickness of the coating and the functionalization layer is obtained in 1 second. In contrast, the highest level of thickness is obtained in 12 seconds for acrylic resin coating and the MIP functionalization material suspension solutions.

5.3 Chapter Summary

This chapter explains how the effect of coating thickness on the sensitivity of planar MEMS ID sensors, as selectivity and sensitivity are two of the most significant characteristics of a MIP biosensor. The sensor is covered with two distinct types of materials: a protective layer and excellent adhesive coating agent, i.e. acrylic resin, and a highly selective layer of MIP. Estimation of the sensitivity of the coated sensor using varied coating thicknesses is used to investigate its performance level. For this investigation, serum samples carrying various creatinine concentrations are employed. For creatinine measurement, the MIP functionalized MEMS ID sensor is highly sensitive than the acrylic-coated one. The sensors are functionalized with MIP and coated with acrylic resin using the dip-coating process. The thickness and consistency of the functionalization solution, speed of withdrawal, dipping time, the temperature of the functionalization solution, and the evaporation speed of the chemical components used while making the process of the functionalization solution play a notable role in deciding the functionalization layer characteristics of the MEMS sensor. The functionalization layer thickness is increased with an increase in the net time of dipping. However, the findings have effectively shown that rising the sensor functionalization thickness substantially raises the saturation level. The balance between the degree of saturation and the sensitivity is acquired with pre-adjustments to the coating layer's thickness. The adjustments are required to be performed as per the requirement of the application. The findings of chapters 4 and 5 helped show that the proposed MIP biosensing system can be used as a PoC system for prognostic and prophylactic applications and a user-friendly and frequent evaluation of kidney health.

The developed MIP functionalized MEMS ID biosensing is an LCR meter instrument-based creatinine detection system. It is highly sensitive, precise, and a standard technique in sensing, but the LCR meter instrument is heavy, computer-dependent, laboratory-based, non-connectable to the internet, and complicated for household usage. Therefore, in chapter 6, a PoC diagnostic prototype system for replacing the LCR meter instrument is described. PoC device is advantageous over the traditional LCR meter instrument concerning cost, power, time-effectiveness and portability. In addition, it offers MIP functionalized impedimetric PoC diagnostic device with an IoT enabled cloud server connecting by LoRaWAN connection features for smart kidney health monitoring.

6

IoT-Enabled Microcontroller-Based MEMS ID Sensor Connected PoC System

Publications about this chapter:

- Anindya Nag[1], Eshrat E. Alahi[1], Nasrin Afsarimanesh[1], **Sumedha Prabhu[1]** and Subhas Chandra Mukhopadhyay[1], (**2019**), IoT for smart homes, in the book Sensors in the Age of the Internet of Things Technologies and Applications, In The Institution of Engineering and Technology International Series on Sensors, Octavian Adrian Postolache, Edward Sazonov and Subhas Chandra Mukhopadhyay (eds), published by **The Institution of Engineering and Technology**, London, United Kingdom, doi: 10.1049/PBCE122E_ch7
- **S. Prabhu**, C. Gooneratne, K. A. Hoang and S. Mukhopadhyay, "IoT-Associated Impedimetric Biosensing for Point-of-Care Monitoring of Kidney Health," **IEEE Sensors Journal**, **2020**, doi: 10.1109/JSEN.2020.3011848
- **S. Prabhu**, C. Gooneratne, K. Anh Hoang and S. Mukhopadhyay, "Development of a Point-of-Care diagnostic smart sensing system to detect creatinine levels," **2020 IEEE 63rd International Midwest Symposium on Circuits and Systems (MWSCAS)**, **2020**, doi: 10.1109/MWSCAS48704.2020.9184441
- **Prabhu S.N.**, Gooneratne C.P., Hoang K.A., Mukhopadhyay S.C., Davidson A.S., Liu G., (**2021**), Interdigital Sensing System for Kidney Health Monitoring. In: Mukhopadhyay S.C., George B., Roy J.K., Islam T. (eds) Interdigital Sensors. Smart Sensors, Measurement and Instrumentation, vol 36. **Springer**, Cham, doi: 10.1007/978-3-030-62684-6_11

6.1 Introduction

The PoC test method is known for long-distance health monitoring of the patient by the doctor as prognostic and prophylaxis for various diseases using careful nursing of disease-associated biomarkers [186-188]. The aim of the PoC test is to obtain results faster for immediate treatment availability. This significantly improves patient care at a low cost.

The compact, fast, portable, cost-effective and handy system model is always a good option for PoC tests, notably home-based usage [188-190]. The PoC test for the quantification of various biomarkers has stated the diagnosis of multiple diseases, including tuberculosis, diabetes, HIV, CTx-1 [191-194]. This work is based on the development of a PoC diagnostic system for monitoring creatinine levels.

The electrochemistry-based biosensors are broadly incorporated for the development of lab-on-chip diagnostic portable systems. It is crucial in transitioning from expensive and complex laboratory equipment to simple-to-use home-testing systems [195-198]. Because of their low cost, user-friendliness, portability, and reliability, electrochemical biosensors have been widely utilised in various applications, exclusively environmental monitoring and clinical diagnostic [112, 199-203]. A sensing system with IoT capabilities aids in the direct transfer and storage of calculated sensing data to a cloud server. The IoT is a term that denotes the linked objects which surround us being connected to the internet in some way. Smart hospitals, smart houses, smart businesses, smart waste management systems, and smart cities are only a few applications for IoT-connected systems.

Health and healthcare applications, such as health screening, early diagnosis, and elderly care, are among the most appealing IoT technology fields for providing a healthier life. Smart systems and their associated healthcare services allowed by the IoT reduce medical costs, provide early information to the general physician, and potentially reduce the risk of death by increasing life expectancy and improving quality of life [204]. It may also assist in providing vital health information to patients who are bedridden or unable to visit pathological laboratories, pharmacy or healthcare providers due to potential reasons.

Since the previous few years, traditional blood tests and the development of Creatinine Colorimetric Assay-based sensing systems have been practised for creatinine levels management. The Creatinine Colorimetric Assay Kit is used for quantifying the creatinine molecule. The kit is costly, requires laboratory-controlled conditions, experts to perform the

test samples analysis, and is a time-consuming procedure. Synthesising artificial polymers specific to creatinine using MIP technology is a cost-effective and time-effective approach to overcome traditional methods. MIP-coated electrochemical biosensors are extensively utilised while making portable lab-on-chip diagnostic systems as a sophisticated electronic system offering a handy-health testing facility from home. The enabling of wireless data transfer and cloud server empowers the chance of transferring data irrespective of time and location for improved patient long-distance healthcare management, empowered using the IoT. IoT-associated diagnostic systems provide prognostic and prophylactic measures against disease conditions.

In this work, a portable, creatinine MIP-based, IoT enabled impedimetric biosensing system for kidney health monitoring is presented using heat-inactivated human serum because of human ethics issues. The system precisely quantifies creatinine levels and transfers data to an IoT-enabled cloud server from where it can be easily stored and provided to a concerned general practitioner/nephrologist/oncologist.

A MIP-based EIS dependent biosensor is developed for kidney health monitoring from the details reported in the previous chapter. For that development, the high-precision impedance analyser in the form of an LCR meter is used. It is found to be a perfect instrument for detecting the dielectric properties of the SUT by the use of the EIS techniques. It is highly sensitive, precise, repeatable in its output and a reliable EIS instrument, but it is heavy, complicated for house usage, and not internet connectable.

In the current chapter, for replacing LCR meter biosensing system with cost, power, time-effective, portable, single-frequency, a MIP-functionalized impedimetric PoC diagnostic prototype system is developed. It is an IoT-enabled cloud server connecting by use of a LoRaWAN connection. It is beneficial for smart kidney health monitoring using heat-inactivated creatinine spiked serum samples.

6.2 Electrochemical Impedance Measuring by using the Microcontroller-based MEMS ID Sensor Connected System

The complete development of the PoC diagnostic prototype sensing system is mentioned below.

6.2.1 Flow Process of the PoC System Working

This work is based on the electrochemical impedance measurements using the microcontroller-based MEMS ID sensor and further developing it as a PoC diagnostic system for monitoring the data for creatinine levels. Figure 6.1 shows the flow process of the proposed PoC system. This system is a power-efficient microcontroller-connected monitoring system. An impedance analyser is utilised for the collection of the information about impedance, which is formed because of MIP-coated biosensor contact with the various creatinine spiked serum sample concentrations. The microcontroller performed converter work from impedance data to real creatinine levels and related mandatory calculator work. The electrical power supply is given to the system microcontroller from a battery connection.

At last, the measured creatinine spiked serum samples concentration is sent to an IoT connected cloud server system for getting access to the measured data from a long distance to the general practitioner/nephrologist/oncologist. The microcontroller board has an inbuilt LoRaWAN chip. That helped in connecting the PoC diagnostic system with the aid of some gateway for transferring the measured data to the remotely located server. Adafruit is a complimentary IoT-associated cloud server, and it is utilised for the storage of collected data. The Adafruit cloud server can be used anywhere and thus aids the healthcare provider in monitoring the data for earlier detection of side-effects of treatment of chemotherapy on the kidney. The Cayenne Low Power Payload (LPP) library is incorporated for calculated data transfer to the desired personalised channel within Adafruit. CayenneLPP is a library, and it uses LoRaWAN protocol for sending the measured creatinine spiked serum sample level to the Adafruit cloud server.

6.2.2 Electrical Circuit Drawing of PoC System

Figure 6.2 shows the circuit diagram of the PoC system. The electrical circuit drawing of the PoC system constitutes of Arduino MKR WAN 1300 using ATMEL SAMD21 and Murata CMWX1ZZABZ with LoRaWAN chip module [205] as a microcontroller, and AD5933 [206] is used as an impedance analyser for calculation of the impedance of the MIP-coated biosensor. The impedance analyser supplies the excitation electrode of the MIP-coated biosensor with the peak to peak (p-p) AC voltage of 1.98 V. The DC voltage of 1.48 V and the Direct Digital Synthesis (DDS) [194, 207] technology is utilised to form an AC voltage signal

having particular optimal operative frequency 1020 Hz. The Inter-Integrated Circuit protocol [208] is incorporated for procuring the MIP-coated biosensors' impedance data.

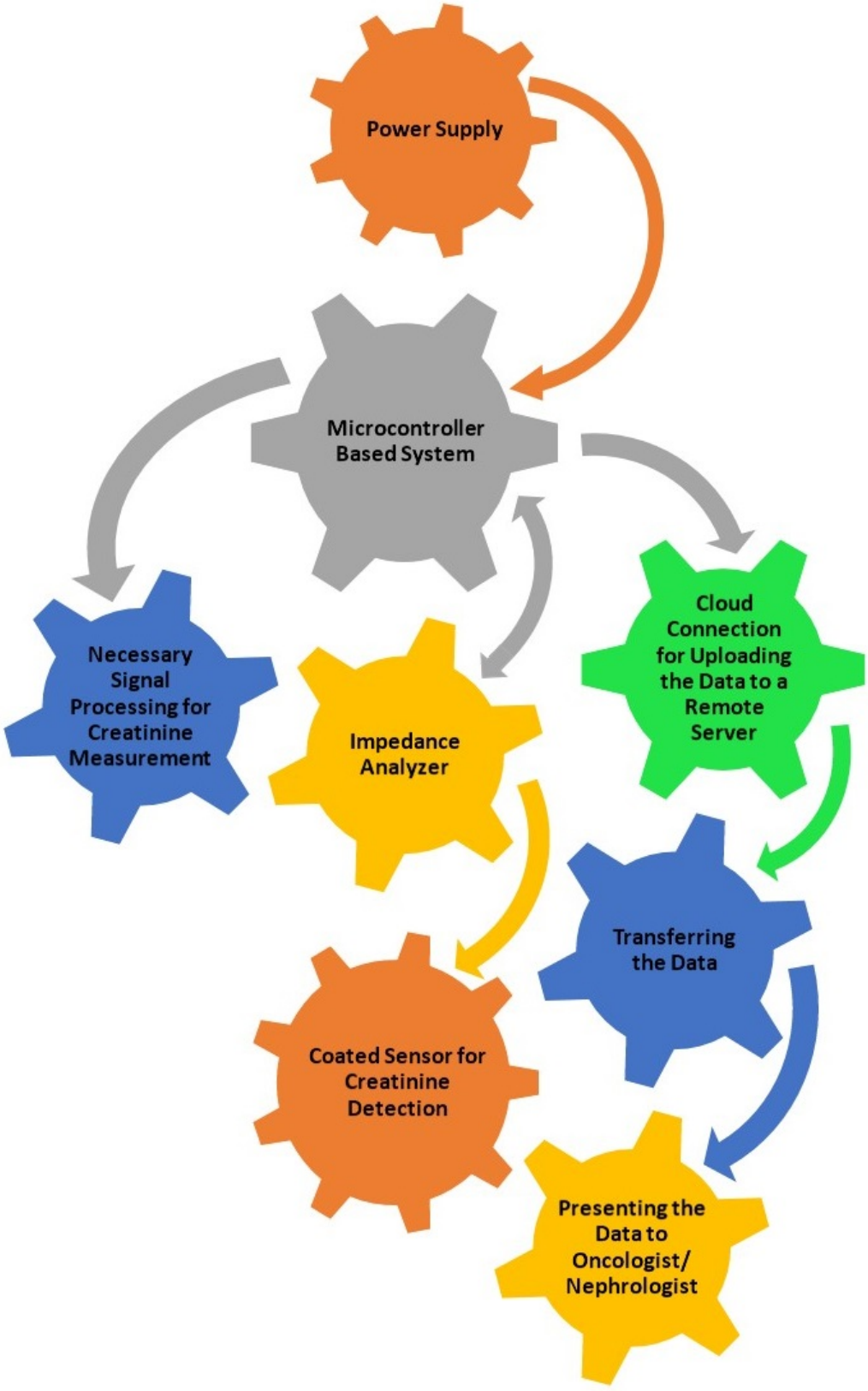


Figure 6.1: Flow process of the PoC system.

An external feedback resistor of 2.2 kΩ is used in the standardisation of the impedance analyser to measure the net gain, as it is required for the measurement of the impedance of the MIP-coated biosensor. 1.3 V supply is provided from Arduino MKR WAN 1300 using ATMEL SAMD21 and Murata CMWX1ZZABZ with LoRaWAN chip module to the AD5933. A rechargeable lithium battery, 3.7 V, is used for powering the main microcontroller. The used microcontroller acquires systems' impedance with the help of an impedance analyser. It uses the impedance data and utilises it for the standardised calibration graphical measurement of creatinine spiked serum samples. The error correction is done to get the real creatinine concentration from samples. The frequency range used to transmit data to the cloud server is 915 MHz.

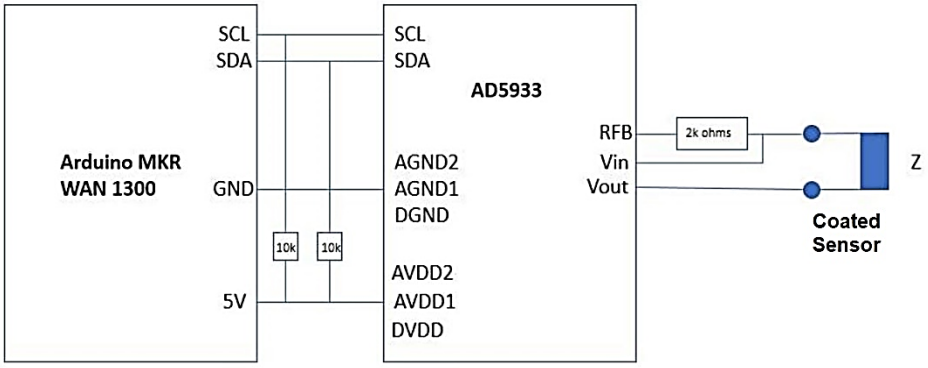


Figure 6.2: Electrical circuit diagram of PoC system.

6.2.3 The Flow of the PoC Diagnostic System Software Process

Figure 6.3 displays the flow of the PoC diagnostic system software process for creatinine concentration calculation and data transferring to the IoT cloud server. For writing all the scripts, the Arduino Integrated Development Environment (IDE) is incorporated, which contains an editing facility for writing code, a text console and a message area along the toolbar, having multiple keys for simple functions [209-211].

The PoC diagnostic system starts the connector, addresses the server, and the microcontroller post the power supply to the diagnostic system. The developed functional system is also utilised for the calculation of creatinine spiked serum sample concentration. The impedance analyser initiated with the pre-set optimal operating frequency for measuring the phase angle and net gain for the MIP-coated biosensor.

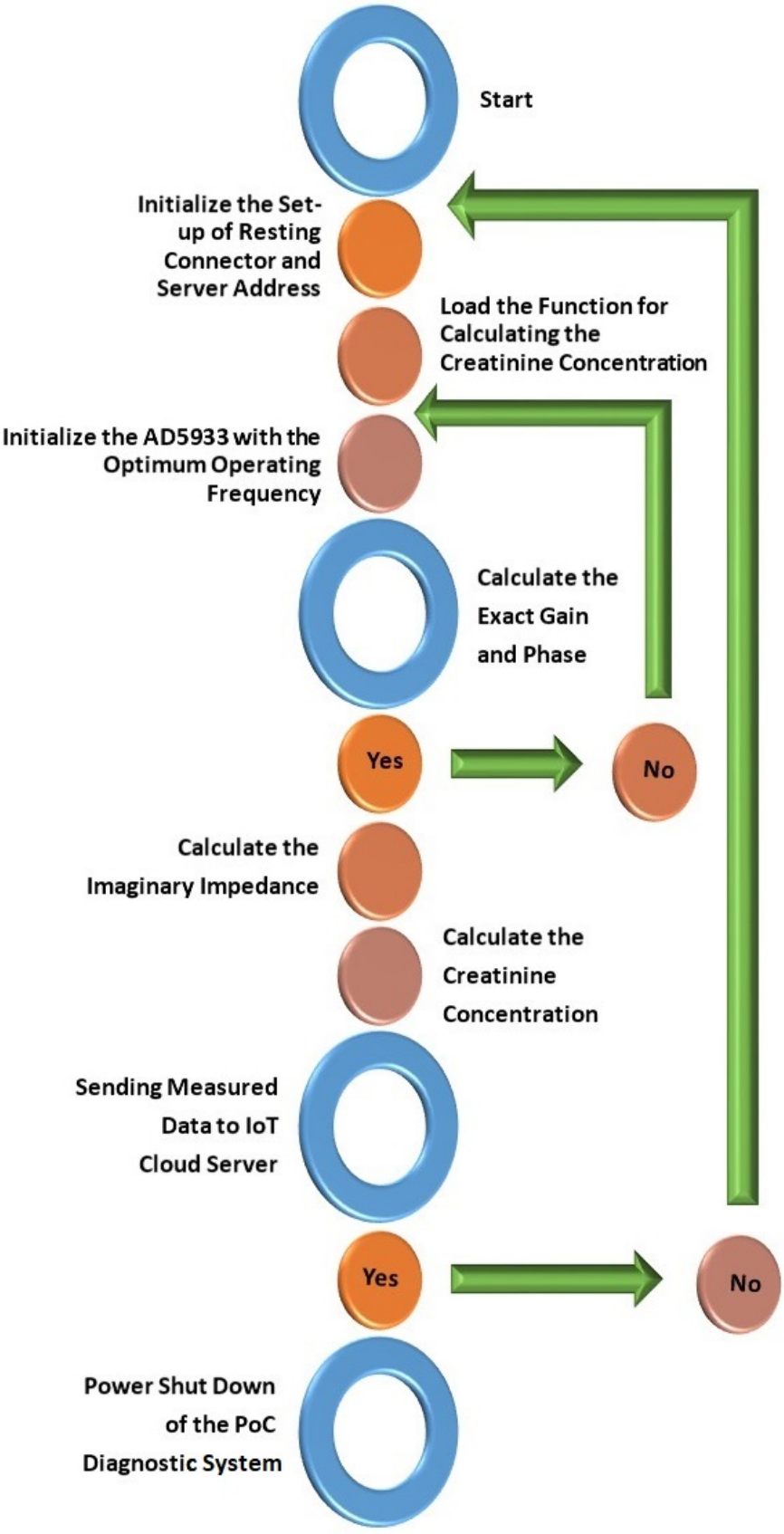


Figure 6.3: The flow of the PoC diagnostic system software process for creatinine concentration calculation and data transferring to the IoT cloud server.

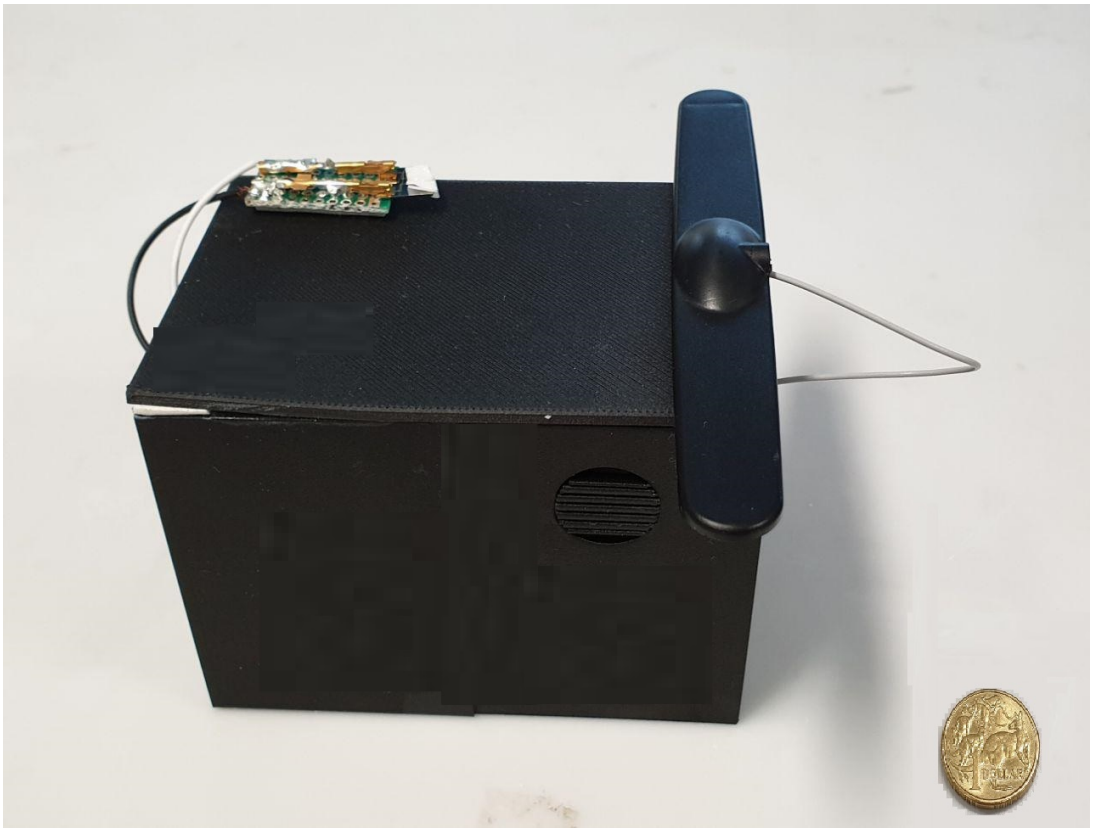


Figure 6.4: PoC diagnostic prototype system.

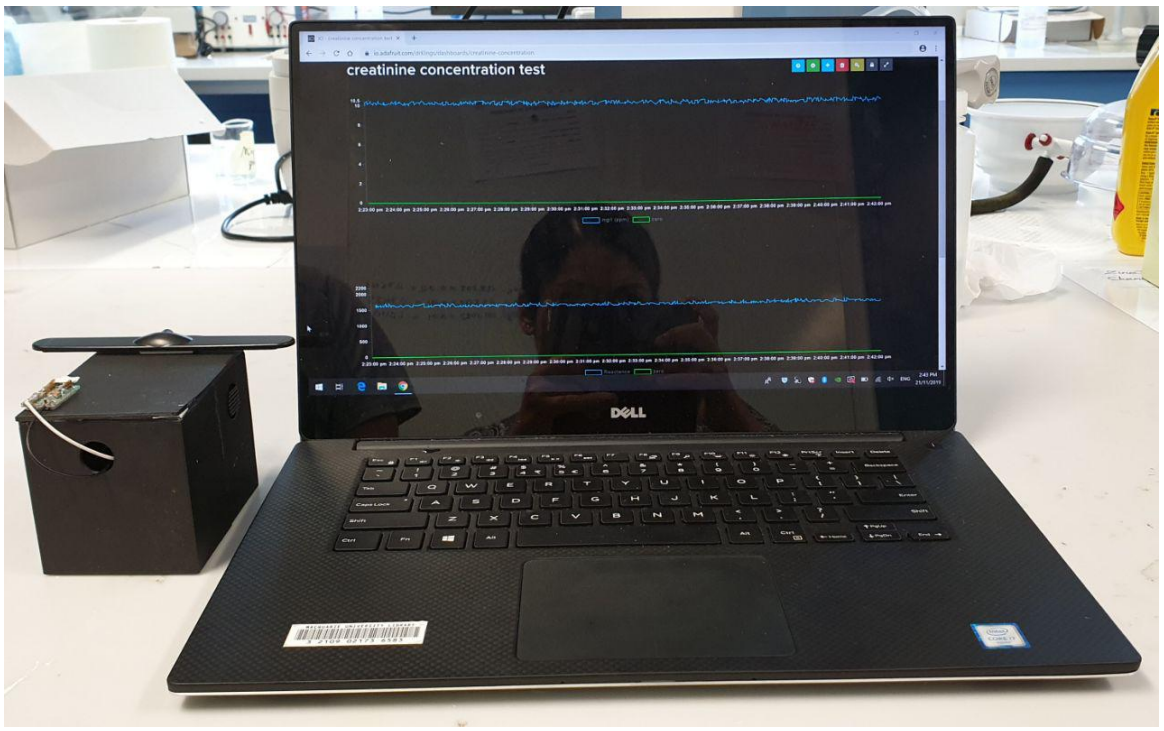


Figure 6.5: The laboratory setup of the PoC system and serum sample data measurement using the Adafruit website.

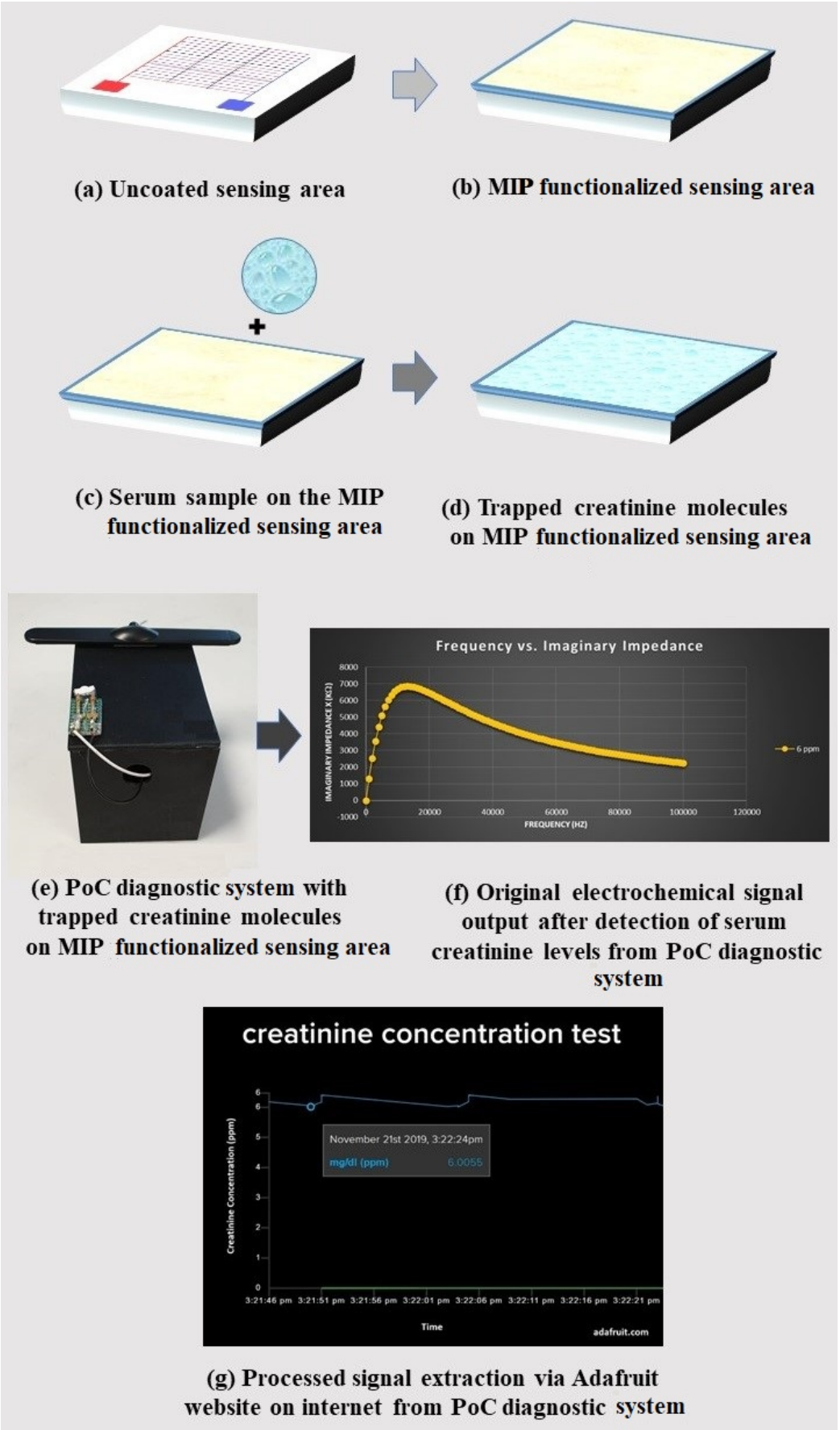


Figure 6.6: Functional biosensing scheme of PoC diagnostic system.

If the starting is not usual, the system again redoes all the steps until the last step process. The phase angle is found to be necessary for the calculation of the imaginary impedance (reactance (X)), as it is utilised to find out the corresponding creatinine concentration level from the calibrated serum sample standards. The error fit process is performed by this PoC system in case of necessity. The measured creatinine concentration is matched and adjusted to fit with the standardised calibration curve, the one obtained with the help of LCR meter measuring experiments. Finally, the data transmission is performed to the IoT-associated cloud server. If in case the PoC diagnostic system is unable to transmit the measured data in ppm format to the cloud server, the whole process restarts from the first step, as shown in Figure 6.3.

The microcontroller can be connected using a gateway, and the key for a safe Application Processing Interface is given within the coding for the safe data transfer. Post transferring the data, the PoC diagnostic system automatically shut down. Thus, the last obtained data will be visible instantly in the Adafruit cloud system server. Further, the data can be monitored by a general practitioner/nephrologist/oncologist for the kidney health of a cancer patient under chemotherapy treatment or a kidney disease patient. The dimensions of the initial prototype system are 10 cm × 7 cm × 7.2 cm, which is further developed and used as a PoC prototype diagnostic system (Figure 6.4). Figure 6.5 shows the laboratory setup of the PoC system and serum sample data measurement using the Adafruit website. A combination of alphabets and numbers are used for granting access from the PoC diagnostic system. Finally, the functional scheme of the current work is displayed in Figure 6.6.

6.3 Results and Discussion

The results associated with the development and creatinine sample measurements taken with the help of the PoC diagnostic prototype sensing system are discussed below.

6.3.1 Development of Calibration Curve by using the Proposed PoC Diagnostic System at Single-Frequency

For measuring the unknown samples, the PoC diagnostic system is also calibrated similarly. The calibration testing is conducted using the same creatinine spiked solutions (6, 10, 14 ppm). Again, 1020 Hz is used as an optimal operating frequency as pre-decided from

LCR meter experiments. At last, the standardisation curve is obtained by marking the creatinine concentration in serum on X-axis and the reactance (X) on the Y-axis (Figure 6.7). It can be noted that the concentrations of creatinine serum samples have an acceptable correlation (R^2 = 0.99) which is almost similar to the earlier one.

The sensibility of the PoC diagnostic system calibration graph is 93.584 Ω/ppm. It is found lesser than that of the calibration graph obtained from the LCR meter experiments. It is noted that the reactance (X) value rises with the rise in the creatinine concentrations. The below Equation 6.1 is derived from the analysis of linear regression.

$$C = \frac{X + 794.61}{93.584} \qquad \textbf{(Equation 6.1)}$$

where C (ppm) is the creatinine concentration, and reactance are denoted as X (Ω).

A sensitivity contrast within the standard calibration derived from the LCR meter and the PoC diagnostic system is seen. The impedance analyser is used by the PoC diagnostic system to calculate the unknown sample impedance value. While calculating the impedance of an unknown sample, the system net gain has been counted and utilised for the calculation of the unknown sample impedance value. Therefore, for improving the impedance measurement of the unknown sample, enhanced system gain is needed.

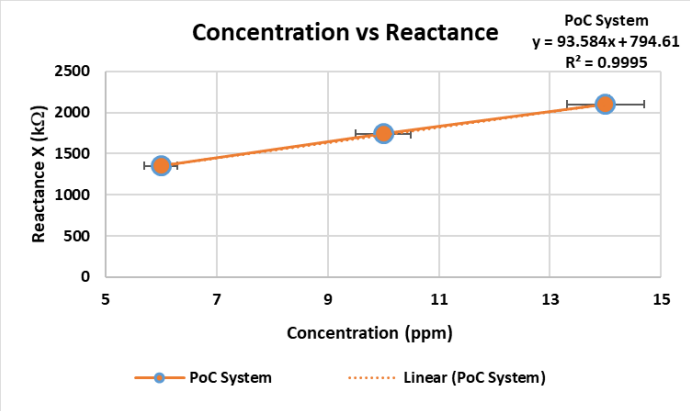


Figure 6.7: Standard calibration curve for the PoC system.

All the measurements of the PoC system are repeated five times to obtain the mean values, and further, the values are used for plotting the PoC associated calibration graph (Table 6.1).

Table 6.1: The mean values of reactance and standard deviation for various serum creatinine concentrations.

Serum Creatinine Concentration (ppm)	Reactance (kΩ)*	Standard Deviation (Ω)
6	1351.33	466.48
10	1740	1001.32
14	2100	1472.27

*The mean values are obtained after an average of five times measurements.

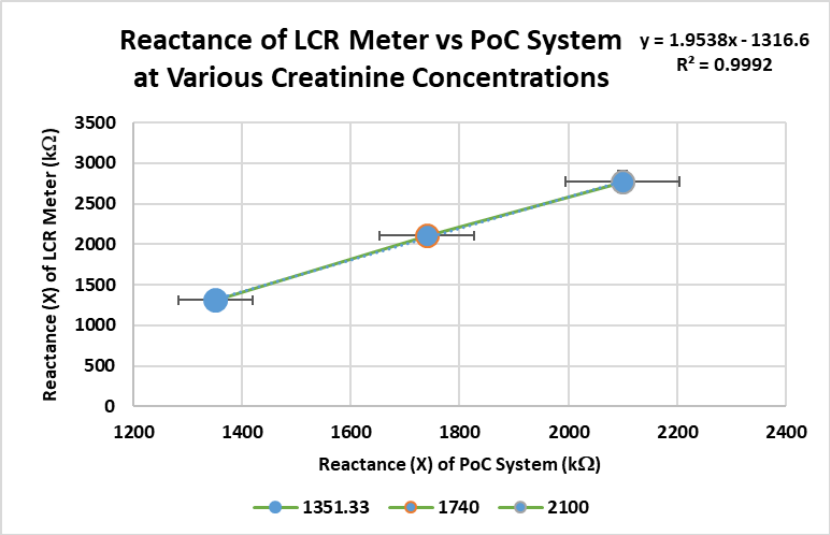


Figure 6.8: The comparison between reactance (X) measurements of the LCR meter and the PoC diagnostic system.

6.3.2 Reactance Measurement Comparison between the LCR Meter and PoC Diagnostic System

The LCR meter instrument is utilised for measuring the reactance (X) value of the MIP-coated biosensor, along with the development of the standard calibration for measuring unknown sample. In comparison, the PoC diagnostic system measures the value of the impedance of the MIP-coated biosensor and incorporates the standard calibration for measuring

the serum sample concentration. That is why it is necessary to have an acceptable correlation between the correlation standard values (Figure 6.7).

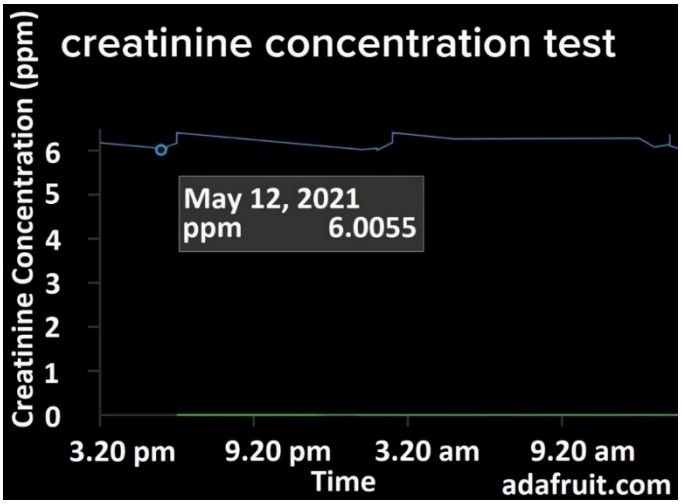
The reactance (X) values of the PoC diagnostic system on the X-axis, whereas the reactance (X) values of the LCR meter plotted on the Y-axis are displayed in Figure 6.8. It can be observed that the values are strongly related along with the correlation coefficient value; R^2 = 0.99. Therefore, the equation of the regression line is utilised for obtaining the reactance value of the LCR meter as well as, in due course, utilising the reactance (X) value for measuring the creatinine from an unknown creatinine spiked serum sample.

6.3.3 IoT Data Collection from the PoC Diagnostic System

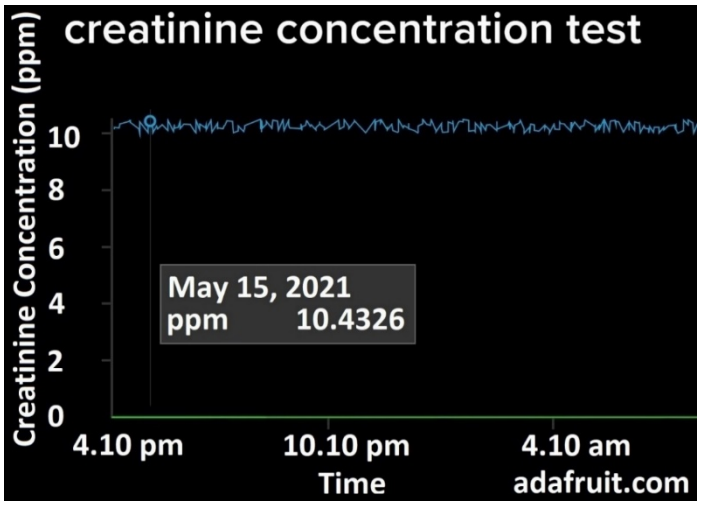
Figure 6.9 (a), (b), (c) and (d) show the actual data of creatinine from the serum samples. The measured concentrations are 6, 10, 14 ppm, and the unknown sample concentration is found to be 15.5 ppm, respectively. All the concentrations are cross-verified by the standard method of Creatinine Colorimetric Assay Kit. The system is utilised for a week steadily to monitor the performance uniformity of the system. The safe data server is accessible from a random remote destination by the oncologist/nephrologist or any general physician effortlessly and precisely take prophylactic measures by monitoring the creatinine serum sample levels and notifying their patient in case of necessity.

The system is lightweight, handy, portable, cost and time effective thus can be very easily adopted for day to day usage for the patient at home. The interference and stability studies are also done for the presented PoC diagnostic system. It is observed that this low power sensing system works for a one-week time, and later it needs battery charging. A single test can be carried out in a 5 minutes' timeslot.

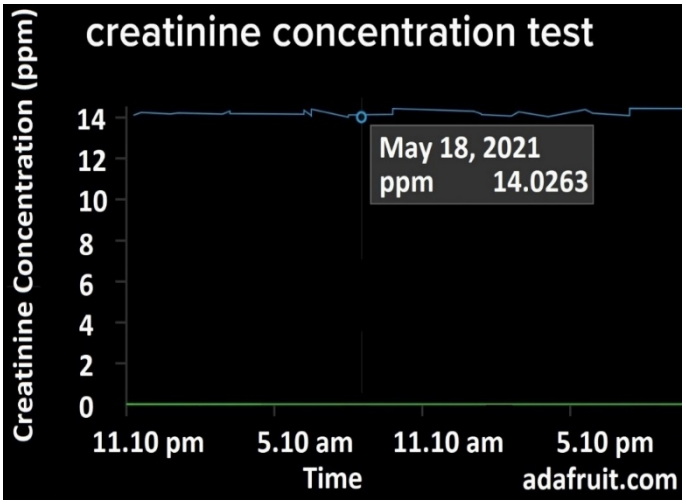
The system provides stable output between the 10°C-40°C temperature range. High humidity levels above 70% significantly interfere with MEMS ID sensor output, and it leads to an uncertain outcome and results in measurement error from the PoC system. The measurements are currently taken within the temperature range of 10°C-40°C, and humidity levels are less than 70%. The shelf-life of unused MIP-coated ID sensor is one year at 4°C.



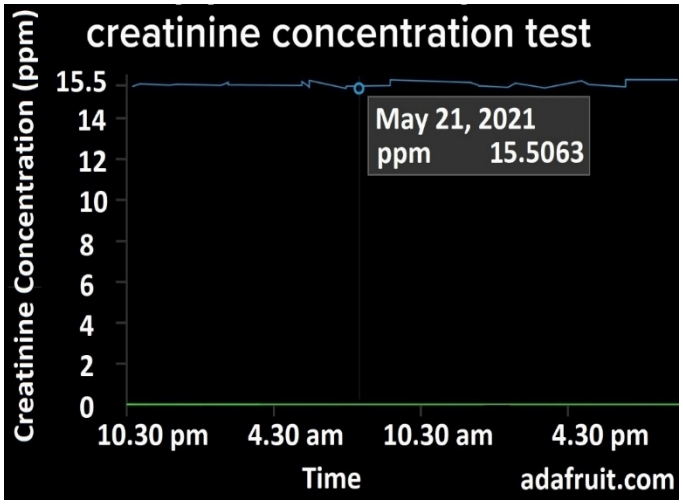
(a) Online data from the developed PoC diagnostic system for 6 ppm.



(b) Online data from the developed PoC diagnostic system for 10 ppm.



(c) Online data from the developed PoC diagnostic system for 14 ppm.



(d) Online data from the developed PoC diagnostic system for an unknown sample carrying 15.5 ppm serum creatinine concentration.

Figure 6.9: (a), (b), (c) and (d) The actual data of creatinine from the serum samples.

6.3.4 The Response of Biosensor Dependant on Single-Frequency Reactance Value for PoC System

The developed biosensor has a great perspective for use as a PoC diagnostic system for kidney health. The single-frequency restrictions used to analyse data are more efficient and straightforward, along with the better time efficiency for experiments. Therefore, the frequency of 1020 Hz is decided as an optimal operating frequency as per the Nyquist plot analysis and the change in values of reactance (X). The percentage of changes in reactance values (Sensitivity %) are measured at 1020 Hz with the following Equation 6.2:

$$\frac{\Delta X}{X_0}\,(\%) = \frac{X\,(Control) - X\,(Sample)}{X\,(Control)} \times 100 \qquad \textbf{(Equation 6.2)}$$

The single-frequency PoC diagnostic prototype system's connected biosensor response is based on reactance and is shown in Figure 6.10. It shows the changes in reactance as per the change in creatinine concentration in serum (6-14 ppm) at 1020 Hz. The linear correlations are

observed between the reactance and the rising levels of serum creatinine with the correlation coefficient value of 0.9974.

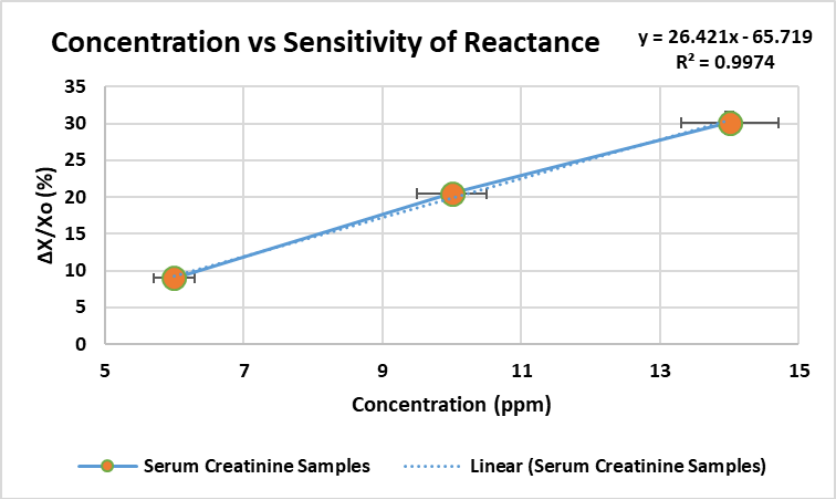


Figure 6.10: A single-frequency reactance-associated calibration curve showing the change of reactance (X) concerning the change in creatinine concentrations at 1020 Hz for the PoC system.

6.4 Chapter Summary

In summary, an IoT-based creatinine selective biosensor is designed to detect and quantify the kidney biomarker in heat-inactivated human serum samples for PoC monitoring of kidney health management. Creatinine can be detected in concentrations as low as 5 ppm (adults) using the existing biosensing PoC diagnostic prototype system. Initially, the EIS technique is used to measure the behaviour of the creatinine functionalized biosensor using an LCR meter. To overcome the issues of the LCR meter, the PoC system is developed and further advanced for precise creatinine level detection from serum samples. It is shifted to IoT based single 1020 Hz frequency to make a user-friendly system to use as a long-distance creatinine measuring prototype system. The creatinine calculation is then performed, and the data is transferred to an IoT-enabled cloud server using a microcontroller-based system.

The featured IoT-based biosensor offers a simple, fast, portable, cost and time effective system for direct creatinine measurement using the heat-inactivated human serum. For example, the standard Creatinine Colorimetric Assay Kit requires at least 4-5 hours to finish the assay procedure. However, using the developed PoC prototype system, each measurement can be performed in 5 minutes.

It is essential to validate the results obtained using the PoC diagnostic prototype system with the help of the existing standard technique. To cross-check the reliability of functioning and also to validate the detection performance of the PoC diagnostic prototype system, a standard technique of Creatinine Colorimetric Assay Kit is used. Chapter 7 explains the cross-checking process in detail with the help of the Creatinine Colorimetric Assay Kit and discuss the reliability of results performed.

7

Creatinine Colorimetric Assay Kit for Validation of PoC Diagnostic Prototype Sensing System

Publications about this chapter:

- **S. Prabhu**, C. Gooneratne, K. A. Hoang and S. Mukhopadhyay, "IoT-Associated Impedimetric Biosensing for Point-of-Care Monitoring of Kidney Health," **IEEE Sensors Journal**, **2020**, doi: 10.1109/JSEN.2020.3011848
- **S. Prabhu**, C. Gooneratne, K. Anh Hoang and S. Mukhopadhyay, "Development of a Point-of-Care diagnostic smart sensing system to detect creatinine levels," **2020 IEEE 63rd International Midwest Symposium on Circuits and Systems (MWSCAS)**, **2020**, doi: 10.1109/MWSCAS48704.2020.9184441
- **Prabhu S.N.**, Gooneratne C.P., Hoang K.A., Mukhopadhyay S.C., Davidson A.S., Liu G., (**2021**), Interdigital Sensing System for Kidney Health Monitoring. In: Mukhopadhyay S.C., George B., Roy J.K., Islam T. (eds) Interdigital Sensors. Smart Sensors, Measurement and Instrumentation, vol 36. Springer, Cham, doi: 10.1007/978-3-030-62684-6_11

7.1 Introduction

A PoC diagnostic prototype system, along with its MIP functionalized biosensor, detects the level of creatinine from the heat-inactivated human serum samples carrying various creatinine concentrations. The functionalization of the MIP polymer using the PTL-MM01 Dip Coater instrument helps generate a uniform coating layer over the sensing area of the MEMS ID sensor. The functionalization of MIP helps in selective and specific adsorption of the creatinine molecules from the serum SUT. It helps to decide the creatinine associated sensitivity as well as specificity of an electrochemical biosensor-based PoC system. To design and build an effective and precise biosensor, the target molecule, i.e. creatinine, is necessary to get electively and specifically captured with the help of the synthetically designed and developed MIP cavities. The design of an efficient biosensor is heavily reliant on the use of a selective MIP functionalization material to advance the recognition of the biosensor.

Enzymes [212], membranes [213], antibodies [214], carbon nanotubes [213], magnetic nanoparticles, and supramolecular assemblies are used to create biosensors that serve as recognition elements for chemical and biochemical molecules. In the literature, various antigen-antibody-based biosensors have been developed [215-216]. An impedimetric MIP-based biosensor PoC diagnostic prototype system has been developed in our research for monitoring kidney health by measuring levels of creatinine in heat-inactivated human serum. It is essential to validate the PoC diagnostic prototype system functioning for real-time cross-verification of the creatinine values obtained from the varying serum samples. Chapter 7 explains how cross-checking is done with the help of the Creatinine Colorimetric Assay Kit and also about how the reliability of results is performed.

7.2 Necessity of Experiments using Creatinine Colorimetric Assay Kit

The developed LoRaWAN-based PoC diagnostic system is connected to the MIP-functionalized biosensor. The MIP-functionalized biosensor helps to measure the creatinine concentration from the serum samples. The measured data is sent to the PoC diagnostic system via an Au pin connecting jig, and further, it is processed by the in-build system within the LoRaWAN-based PoC diagnostic system. The standard plot of the Creatinine Colorimetric Assay Kit helps to validate the results obtained using the LoRaWAN-based PoC diagnostic system.

The human serum male AB plasma, USA origin, sterile-filtered, is locally purchased from Sigma-Aldrich, Australia. The Creatinine Assay Kit (Colorimetric/Fluorometric) is used in this protocol. It is suitable for faster, precise and sensitive measurement of levels of creatinine from biological samples, which includes urine, serum/plasma, cell culture supernatant, as well as cerebrospinal fluid (CSF). It is locally purchased from Abcam. The Creatinine Colorimetric Assay Kit is an enzyme-probe coloured product based on standardised testing to measure the creatinine concentration from real samples. The kit has creatininase (lyophilised), creatinine standard (10 µmol) (lyophilised), creatinase (lyophilised), creatinine enzyme mix (lyophilised), creatinine assay buffer and creatinine probe in DMSO (200 µl). The experiments are done using the Creatinine Colorimetric Assay Kit to obtain the standard plot using standard serum samples with pre-calibrated creatinine concentrations. The standard plot results of the Creatinine Colorimetric Assay Kit are further used to validate the detected values of the LoRaWAN-based PoC diagnostic system. The MIP-functionalized sensor helps to measure the creatinine concentration from the serum samples. The measured data is sent to the PoC diagnostic system via an Au pin connecting jig, and further, it is processed by the in-build system within the LoRaWAN-based PoC diagnostic system. The standard plot of the Creatinine Colorimetric Assay Kit helps to validate the results obtained using the LoRaWAN-based PoC diagnostic system.

7.3 Creatinine Colorimetric Assay Reagent Preparation

The Creatinine Colorimetric Assay Kit contained multiple small vials. All the vials are carefully centrifuged at low speed before opening them in a lab environment. All the reagents are taken out from the freezer (-20°C) and thawed at 4°C using an ice-cold water bath for 12 minutes before making their aliquots. Once opened, all the opened aliquot vials are not restored inside the freezer at -20°C temperature to avoid the possibility of cross-contamination associated interferences or false-positive results. All the unused aliquot vials are stored inside the freezer at -20°C temperature to preserve their functional stability and protect them from light.

7.3.1 Creatinine Standard

The small vial which contains creatinine standard in a powdered form is reconstituted using 100 µl of Milli-Q water to generate 100 mM creatinine standard. The 100 mM standard

is aliquot in 10 vials so that a sufficient volume of solution will be available for use during every single assay, and multiple freeze/thaw cycles can be avoided to a single cycle. Only one vial is used, and the rest of all 9 aliquot vials are stored inside the freezer at -20°C.

7.3.2 Creatinine Assay Buffer

The creatinine assay buffer is used as supplied without any further reconstitution. The assay buffer is equilibrated at room temperature before its use. It is stored inside the freezer at -20°C post usage.

7.3.3 Creatine Probe

The creatine probe is also used as supplied in the kit. It is warmed by placing it inside a 37°C water bath for 1-5 minutes for thawing one of its integral content, the DMSO solution, before its use. The DMSO acquired solidity whilst stored inside the freezer at -20°C as well as at lab standard room temperature. It requires melting for a few minutes at 37°C before use. The melted probe is also aliquot in vials for having sufficient volume for performing the desired number of assays. All extra vials are stored inside the freezer at -20°C. The thawed probe is used immediately.

7.3.4 Creatininase

Creatininase is an enzyme that works on creatinine and converts it into creatine. The creatininase is reconstituted with 220 µl of creatinine assay buffer to generate the 3 aliquots. The aliquot in use is kept in an ice-cold water bath to keep enzyme functionality intact. Rest 2 aliquots are stored inside the freezer at -20°C for further use.

7.3.5 Creatinase

Creatinase is an enzyme that works on creatine and converts it into sarcosine. The creatinase is reconstituted with 220 µl of creatinine assay buffer to generate the 3 aliquots. The aliquot in use is kept in an ice-cold water bath to keep enzyme functionality intact. Rest 2 aliquots are stored inside the freezer at -20°C for further use.

7.3.6 Creatinine Enzyme Mix

The sarcosine is specifically oxidised to produce a reaction product that reacts with a creatinine probe to generate the reddish-pink coloured end product. The creatinase enzyme mix is reconstituted with 220 µl of creatinine assay buffer to generate the 3 aliquots. The aliquot in use is necessary to be kept in an ice-cold water bath to keep enzyme functionality intact. Rest 2 aliquots are stored inside the freezer at -20°C for further use.

7.4 Experiments with Creatinine Colorimetric Assay Kit

The experiments are done with a Creatinine Colorimetric Assay Kit to perform cross-validation of the developed PoC diagnostic system, as explained below.

7.4.1 Preparation of Working Standards

The set of working standards are always prepared freshly just before their every use. The set of working standard dilutions are discarded immediately after the experiments are finished due to their inability to store well over an hour.

7.4.1.1 Procedure

1) Creatinine working standard of 1nmol/µl concentration (1000 µl quantity) is made by diluting 10 µl of 100 mM creatinine working standard in 990 µl of creatinine assay buffer.

2) The 1 nmol/µl creatinine working standard is used to prepare standard samples. These samples dilutions are used to make the standard plot, as shown in Table 7.1 in a 96 well clear microtiter plate.

3) Each dilution reading is repeated twice, and the average colorimetric reading value is taken to plot a creatinine working standard graphical plot.

7.4.2 Preparation of Samples

The set of test samples are in the ppm range. The Creatinine Colorimetric Assay Kit working range is in ppb; all the test serum samples are diluted 1000 times by using a pure serum

before the samples are analysed with the help of this kit. The dilution of test samples helps to ensure that the readings will be in the standard value range. Freshly prepared test samples are used every time for analysis.

Table 7.1: Samples dilutions used to make the standard Creatinine Colorimetric Assay plot [217].

Creatinine Working Standard	Volume of Creatinine Working Standard (µl)	Creatinine Assay Buffer (µl)	Final Volume of Creatinine Working Standard in Well (µl)	End Concentration of Creatinine in Well (nmol/well)
1	0	150	50	0
2	6	144	50	2
3	12	138	50	4
4	18	132	50	6
5	24	126	50	8
6	30	120	50	10

7.5 Creatinine Colorimetric Assay Procedure

The procedure for creatinine colorimetric assay is explained below.

7.5.1 Setting the Reaction Wells

1) Standard wells = Standard working dilutions of 50 µl.

2) Test sample wells = samples of 50 µl.

3) Sample background control wells = 50 µl pure serum samples with zero creatinine content as a blank (control).

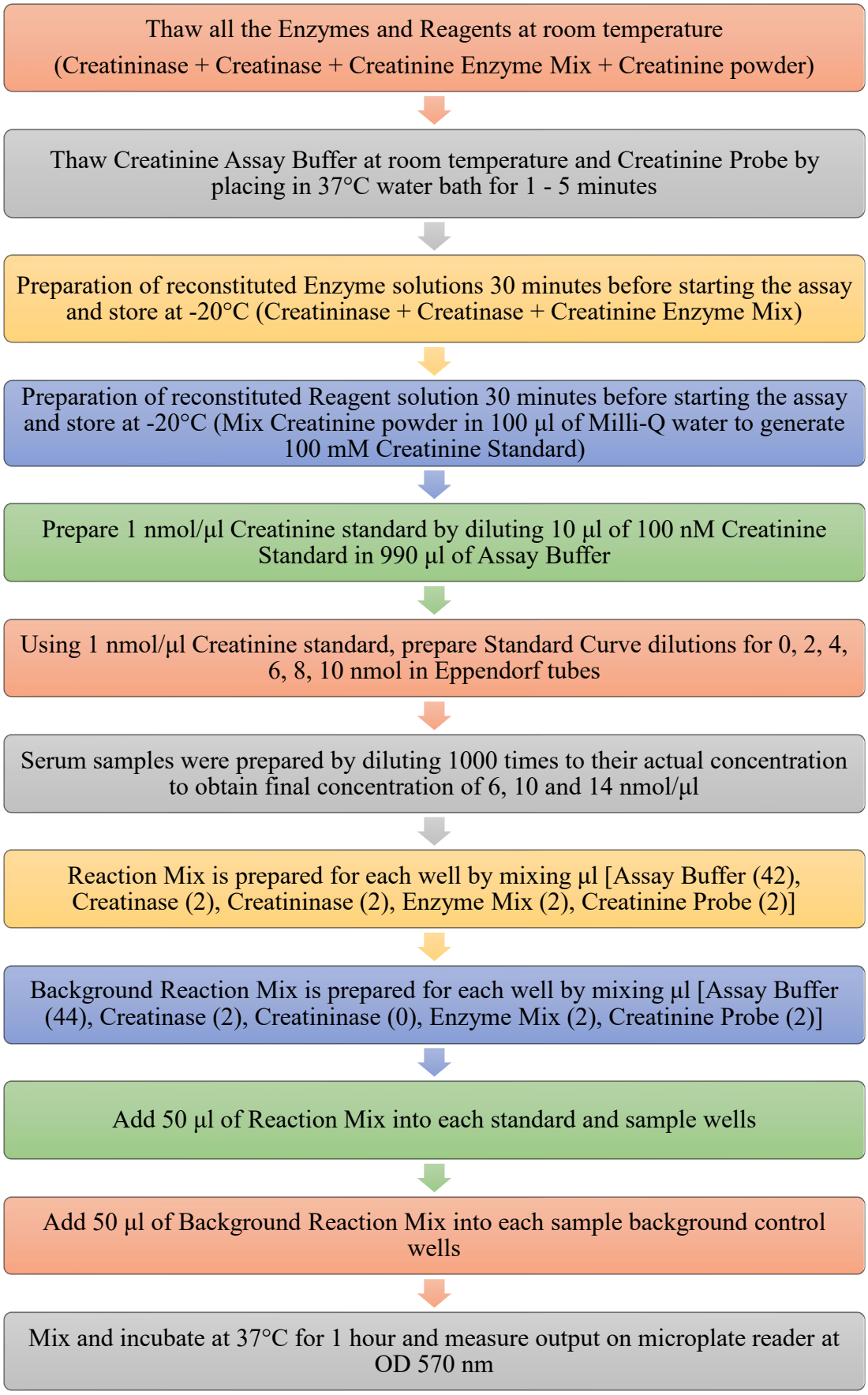


Figure 7.1: The flowchart of commercial Creatinine Colorimetric Assay.

7.5.2 Creatinine Reaction Mix

1) In each 96 well microtiter well, 50 µl of reaction mix is added for the reaction. Total 1500 µl of reaction mix is prepared and used for all creatinine working standard and creatinine test sample wells.

2) A 100 µl of background reaction mix is also prepared and used for pure serum samples with zero creatinine content. Table 7.2 shows the preparation procedure for creatinine reaction mix as well as background reaction mix.

3) All the contents from each well from the 96 microtiter plate are mixed using a 1 G-force at 37°C for 1 hour.

4) The coloured reaction output is read using a SPECTROstar Nano microplate reader at an optical density (OD) of 570 nm. The Creatinine Colorimetric Assay Kit procedure has been illustrated in the flow chart pattern in Figure 7.1.

A total of 30 reaction wells are used for every single analysis. 6 working standards are made and analysed in duplicates ranging from well no. A1 to A12. 8 samples are analysed in duplicates at laboratory temperature ranging from well no. B1 to C4. 1 pure serum sample with zero creatinine concentration is used as blank (control) samples in duplicates from well no. C5 to C6. The Colorimetric Assay Kit results in the form of a coloured product as displayed in Figure 7.2, and the working standard plot is shown in Figure 7.3.

Figure 7.2: The coloured product of Creatinine Colorimetric Assay Kit after incubation for an hour at 37°C.

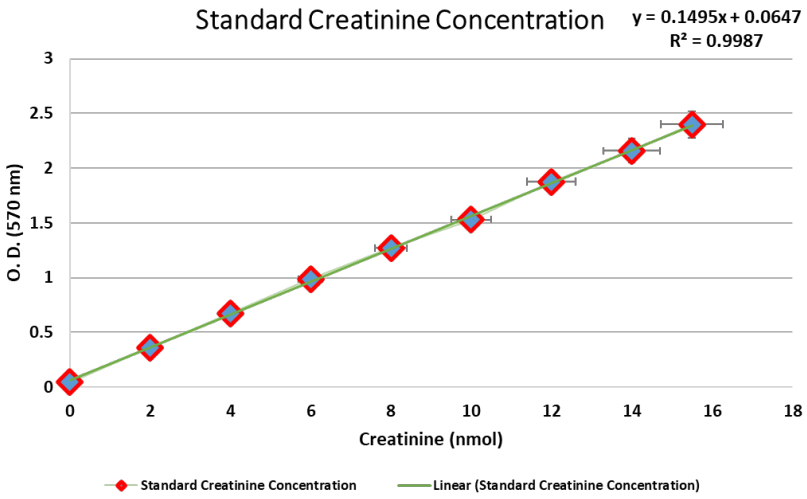


Figure 7.3: The working standard plot of Creatinine Colorimetric Assay.

Table 7.2: Preparation procedure for creatinine reaction mix and background reaction mix [217].

Components	Reaction Mix (µl)		Background Reaction Mix (µl)	
Quantity Needed →	Each Well (50 µl)	All 30 Wells (1500 µl)	Each Well (50 µl)	All 2 Wells (100 µl)
Assay Buffer	42	1260	44	88
Creatinase	2	60	2	4
Creatininase	2	60	0	0
Enzyme Mix	2	60	2	4
Creatinine Probe	2	60	2	4

7.6 Creatinine Colorimetric Assay Kit Data Analysis

1) All the values are taken in duplicates; the average is taken for all 6 creatinine working standards, 8 test samples and a set of pure serum samples used as a blank (control).

2) All the value of background control, pure serum (blank), is significant. Therefore, it is subtracted from all the test sample readings. The blank value (creatinine working standard #1)

is subtracted from all the other creatinine working standards and test sample readings. It helps to get the corrected absorbance.

3) To get the final concentration of creatinine, the values of corrected absorbance of each working standard are plotted.

4) The creatinine working standard plot is drawn for concentration 0 ppb to 10 ppb range.

5) The values for creatinine concentrations for 6, 10, 14 and 15.5 ppm serum test samples are obtained by multiplying the results 1000 times (ppb to ppm conversion factor). The Creatinine Colorimetric Assay is used as a standard diagnostic method in the pathology laboratory; it helps to standardise and validate the values obtained from the MIP functionalized MEMS biosensor of the LoRaWAN-based PoC diagnostic prototype system.

7.7 Results and Discussion

The Creatinine Colorimetric Assay Kit is used to obtain the standard plot by testing the six standards. The concentration of provided known standards is 0 (control), 2, 4, 6, 8 and 10 nmol. The working standard plot obtained from Creatinine Colorimetric Assay Kit is shown in Figure 7.2. All the provided working standard samples are tested in duplicates. The working standard assay is performed at a standard laboratory temperature of 25°C and 31% humidity to maintain uniformity in parameters influencing the sample analysis and data management of both the PoC diagnostic prototype system and Creatinine Colorimetric Assay Kit. After getting the working standard curve for provided samples, the experiments are performed for 6, 10, 14 and 15.5 ppm serum samples by considering them as unknown samples. The unknown samples are run in duplicates. The concentration of creatinine is found out to be 7.0010, 10.0109, 14.0026 and 15.5011. Table 7.3 shows a comparison between the actual and measured serum creatinine concentrations.

7.7.1 Measurement of the Unknown Sample by using the Proposed PoC Diagnostic System at Single-Frequency and its Comparison with Standard Reference Method Creatinine Colorimetric Assay Kit

A single creatinine spiked serum sample that carries a creatinine concentration of more than 14 ppm is made. The unknown creatinine spiked serum sample is tested with the help of

a developed PoC diagnostic system at 1020 Hz single frequency to measure the concentration of creatinine levels. 20 µL of SUT is pipetted on the MIP pre-functionalized area of a MEMS ID sensor attached to the PoC diagnostic system. Then, around 4 minutes delay time is given to MIP for adsorbing the creatinine molecules from the unknown sample. The extra solution is then cleaned by spraying the Milli-Q water, and the sensing area is quickly air-dried using an air drier. Post drying, the creatinine adsorbed MIP functionalized sensing surface is analysed by the PoC diagnostic system to measure creatinine concentration from the unknown sample solution.

Table 7.3: Measurement of an unknown sample, cross-checking and validating single-frequency PoC results with commercially available Creatinine Colorimetric Assay Kit.

Sample	Proposed PoC Diagnostic System	Standard Reference Method Creatinine Colorimetric Assay Kit	Error (%)
1	6.0055	6.0010	0.07
2	10.4326	10.0109	4.21
3	14.0263	14.0026	0.16
4	15.5063	15.5011	0.03

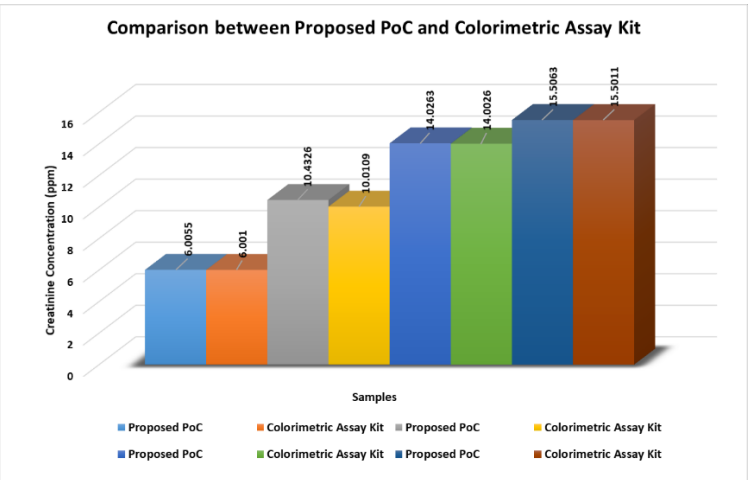


Figure 7.4: The comparison between the proposed PoC diagnostic system and Creatinine Colorimetric Assay Kit.

The obtained results are cross-checked and validated with the help of a commercially available Creatinine Colorimetric Assay Kit. The obtained results with the help of the two above methodologies are tabulated in Table 7.3, whereas their differentiation is depicted in Figure 7.4. It is observed that the obtained PoC diagnostic system data is adjacent with the standardised Creatinine Colorimetric Assay Kit detection method that has an error value of ≤5%. Comparative study between the PoC diagnostic system results and the Creatinine Colorimetric Assay Kit results as displayed in Figure 7.5.

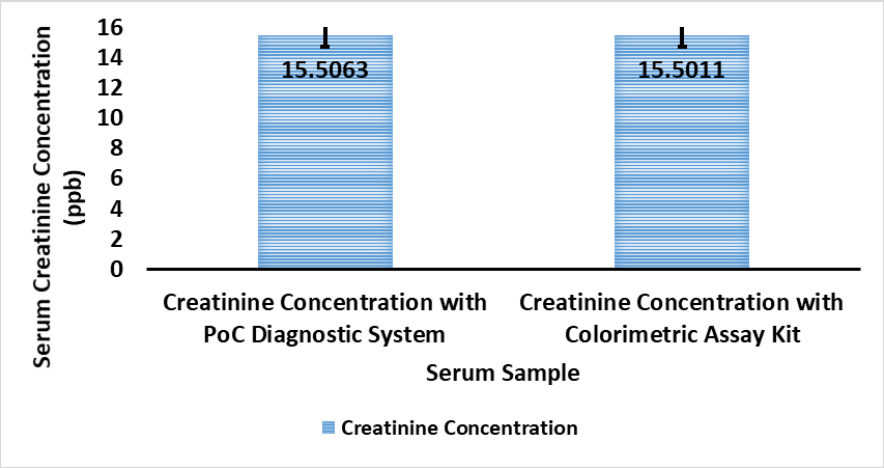


Figure 7.5: Comparative study between the PoC diagnostic system results and the Creatinine Colorimetric Assay Kit results.

7.8 Chapter Summary

A real-time, non-invasive, precise, portable, user-friendly, label-free, internet connectable, low-cost, low-power, PoC diagnostic prototype system that has specificity and selectivity to creatinine has been developed. The most exciting features of the PoC diagnostic prototype system are repeatability, reusability, precision, long-distance healthcare and portability. The system is an IoT and microcontroller-based PoC diagnostic prototype sensing system. It is essential to validate the preciseness of the functionality and the quantitative detection of the PoC sensing system to prove its functioning. A Creatinine Colorimetric Assay Kit analysis is performed by analysing 6 working standard samples in duplicates. 8 samples of known concentration are treated as unknowns, and a set of pure serum as control are analysed in duplicates. Cross-checking and validation of the single-frequency PoC diagnostic prototype system is successfully done in the present chapter. The results are compared with the commercially available standard calibration system, i.e. Creatinine Colorimetric Assay Kit analysis. The outcome is promising and encouraging, with an error minimum of 0.03% and a maximum of 4.21%. It is noticed that the results obtained using PoC diagnostic prototype system data is within an acceptable range, with the standardised Creatinine Colorimetric Assay Kit detection method that holds an error value of $\leq 5\%$ for all analysed samples. Chapter 8 explains the conclusion and future work of the developed PoC diagnostic prototype system.

8

Conclusion and Future Work

Publications about this chapter:

- **S. Prabhu**, C. Gooneratne, K. Anh Hoang and S. Mukhopadhyay, "Development of a Point-of-Care diagnostic smart sensing system to detect creatinine levels," **2020 IEEE 63rd International Midwest Symposium on Circuits and Systems (MWSCAS), 2020**, doi: 10.1109/MWSCAS48704.2020.9184441

8.1 Conclusion

Kidney diseases are still a serious concern in most nations. Although kidney diseases, specifically CKD, are seen through all ages, they are more prevalent in the old age population. Early identification of kidney function decline or loss is critical for successful disease management. Early detection, diagnosis, monitoring of kidney disease problems and choosing drug regimens and appropriate treatment can be supported by tracking kidney functioning biomarkers. Kidney functioning decline or loss can be diagnosed and tracked by regularly measuring the creatinine and BUN in the blood or urine. PoC technologies that are rapid, portable, time and cost-effective are highly valued.

This research develops a functional, specific, repeatable, reusable, accurate, low-cost, low-power, long-distance, IoT-based, and portable system to detect and quantify creatinine levels in the heat-inactivated serum. A capacitive planar MEMS ID sensor is functionalized with artificially synthesised MIP technology. MIP is used to improve the system's stability and reduce the total cost of detection of the creatinine from serum samples. EIS is used to evaluate the resistive and capacitive properties of the SUTs.

A microcontroller-based system is developed to measure creatinine levels in heat-inactivated serum samples and transfer data to an IoT-based Adafruit cloud server. The information from the Adafruit website can be sent/used by a general practitioner/nephrologist/oncologist. A thorough examination for early diagnosis and treatment can begin, and it will help the patient for better revival. With a LOD from 0.1-50 ppm, the developed MEMS ID PoC diagnostic prototype sensing system worked linearly within the range of 6 ppm to 15.5 ppm. It covered the normal range and also a slightly higher creatinine range in the blood. It will be helpful in the case of early identification of kidney disease.

The findings showed that the suggested portable PoC diagnostic prototype biosensing system offers a speedy, user-friendly, and precise method to quantify creatinine levels in the heat-inactivated serum samples carrying various creatinine levels. The suggested technique tests an unknown serum sample carrying creatinine concentration. The concentration of the unknown sample is found out to be 15.5 ppm, and the results are cross-checked and validated using a Creatinine Colorimetric Assay Kit.

8.2 Future Work for PoC Diagnostic Prototype System and Development Associated Possibilities

The heat-inactivated human serum spiked with various creatinine levels is used as the SUT in the developed PoC diagnostic prototype system. The future work involves reducing the total testing time taken by the PoC system and replacing the heat-inactivated serum with a fresh drop of blood samples. In the future, the presented system with some more fine modifications can be used for practical purposes to detect minor differences in creatinine levels for listing the developed PoC system into modern diagnostic tools and make it a successful PoC diagnostic system.

Despite the suggested PoC system's exceptional performance, blood collection for continuous monitoring is exceedingly unpopular. An invasive blood sample is not practicable or acceptable for most individuals in the future who want to use this device at their home for kidney disease healthcare management. Developing a PoC device that can use a single drop of blood as a SUT is highly beneficial and acceptable. By adding a single drop of a freshly withdrawn blood sample by the user, a PoC diagnostic system for a smart kidney health system may be created. The monitoring of kidney functioning loss and/or rising stage of kidney disease may be done automatically by performing testing from home and without the need for frequent visits to the pathology laboratories/imaging centres/nephrology care hospitals. The information gathered from each measurement will be wirelessly forwarded to the concerned nephrology care hospital for further data analysis and long-distance patient healthcare maintenance.

The normal creatinine range in human serum for females is 0.5-1.1 mg/dL (5-11 ppm), whereas, for men, it is 0.6-1.2 mg/dL (6-12 ppm). Levels over 15 ppm need medical attention, and levels over 59 ppm indicate severe kidney damage. The normal range for urine creatinine ranges about 282 ppm-2,600 ppm [218]. The levels of creatinine are also used as a biomarker for the measurement of various therapy drugs and urine testing for the detection of illegal drugs [219]. The upper limit of detection is 50 ppm for creatinine using creatinine selective MIP functionalized MEMS ID sensor. Detecting 282 ppm-2,600 ppm urine creatinine levels will be too much overload for the EIS-based currently developed creatinine selective functionalized PoC diagnostic prototype system.

The normal BUN range in human serum for adults is 7-20 mg/dL (70-200 ppm), whereas, for >60 years, it is 8-23 mg/dL (80-230 ppm). Levels over 600 ppm in serum need

medical attention and indicate severe kidney damage. In addition, the planar MEMS ID sensor will function differently with the BUN selective functionalized MIP coat. Therefore, the detection range will be different while developing the PoC diagnostic prototype system and detecting BUN levels using a BUN-specific MIP coat.

Overall, urine levels of creatinine and BUN will always be high as urine is considered an excretory fluid. The extensive high-level detection of creatinine and BUN will hamper the planar MEMS ID sensor's functioning, having selective coats for creatinine and BUN when connected to the PoC diagnostic system. Therefore, the serum will be considered a choice for the creatinine and BUN level measurements for future detection and developmental purpose by advancing the system with a single drop of blood detection technology. Figure 8.1 shows the future of developing the PoC diagnostic prototype system for using a single drop of blood to measure kidney biomarkers.

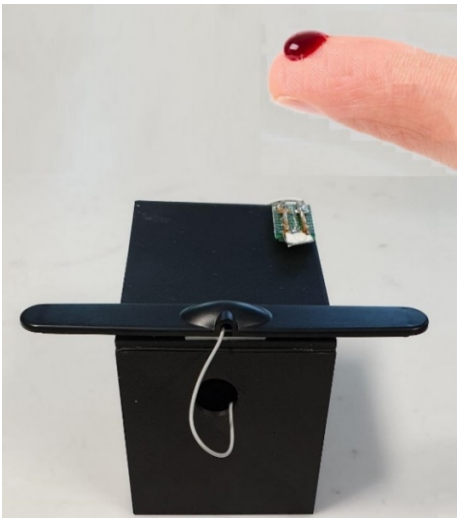


Figure 8.1: The future of developing the PoC diagnostic prototype system for using a single drop of blood.

Recent literature has shown that measuring a single biomarker is insufficient for detecting kidney functioning associated decline/diseases/monitoring a treatment procedure/effects of drug regimens. Simultaneous assessment of many kidney disease biomarkers will be helpful in the kidney function decline/disease-associated prognostic and prophylactic measures. Multiplex PoC devices for the detection of several illnesses have recently been developed [220-222]. Another biomarker of kidney disease is BUN. The rate of kidney functioning loss is accurately and reliably determined by measuring levels of creatinine and BUN in the blood at the same time.

The MIP and NIP for detection of BUN (additional kidney disease biomarker) are just developed using the precipitation polymerisation process. The structural, functional, electrochemical, and stability associated properties of BUN selective MIP and NIP are yet to be studied. Their detailed analysis will be done in the future to continue our currently developed creatinine selective PoC diagnostic prototype system advancement plan.

In the future, a sensor array can be used to create a multiplex assay for better and faster assessment of creatinine and BUN as kidney disease health biomarkers. In addition, the designed system could be taken anywhere as a portable PoC diagnostic prototype system for the regular checking of creatinine and BUN levels by directly pouring one drop of fresh blood samples manually on the MIP functionalized sensing areas.

On a larger scale in the future, the system could be helpful in pathology laboratories/nephrology care hospitals/aged care centres for multiple blood sample analyses. The system could be further developed to obtain a fresh drop of blood samples from the patients using replaceable tiny robotic pricks, and the multiple samples can be tested automatically. The data of the individual or multiple patients could be uploaded and stored under their private account by cross-verifying user name and date of birth details to maintain individual privacy and precision. In case of urgency, the simultaneously tested creatinine and BUN results could be sent to a concerned medical practitioner by highlighting the need for emergency treatment of the patient in case of severe kidney disease painful attack. Figure 8.2 depicts a graphic drawing of the proposed biosensor array for a blood sample's real-time quantification of creatinine and BUN.

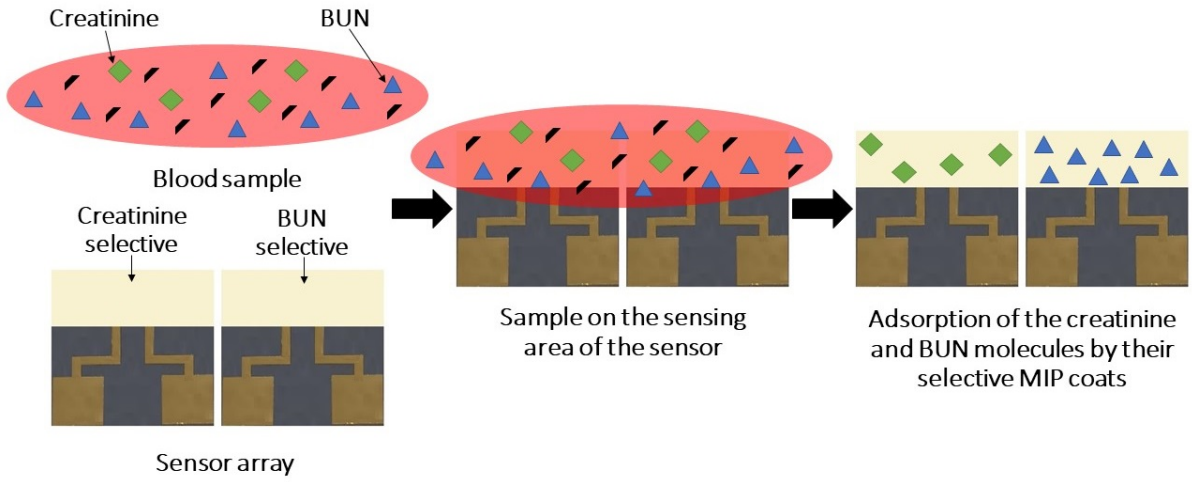


Figure 8.2: Graphic drawing of the proposed biosensor array for real-time quantification of creatinine and BUN levels from the blood sample.

In the future, the smart PoC diagnostic system will allow the daily real-time monitoring of creatinine and BUN, biomarkers of kidney disease function from a fresh drop of a blood sample without the need for frequent pathology laboratory/imaging centre/nephrology care hospital visits.

This technology can establish itself as a breakthrough idea in detecting kidney health decline/kidney disease worsening in its early stages, allowing therapeutic regimens to begin at the earliest when it is most beneficial. Thus, the patient can get appropriate treatment when a developing kidney disease has a higher chance of revival than waiting for a lengthy recovery period or getting a much later diagnosed with an incurable condition of CKD. Furthermore, the prognostic and prophylactic kidney health measurements can be taken in future with the PoC diagnostic sensing system once it is suitable for using a fresh drop of blood sample and quantifying the creatinine and BUN levels simultaneously. Figure 8.3 depicts a schematic representation of potential smart kidney health for continuous monitoring of creatinine and BUN levels.

There are several obstacles to overcome in order to make this concept viable for real-world use. One issue is the influence of the complex composition of the human blood (such as structurally similar compounds and/or interference of 4229 blood chemicals) on the sensor's life, functioning and precise performance.

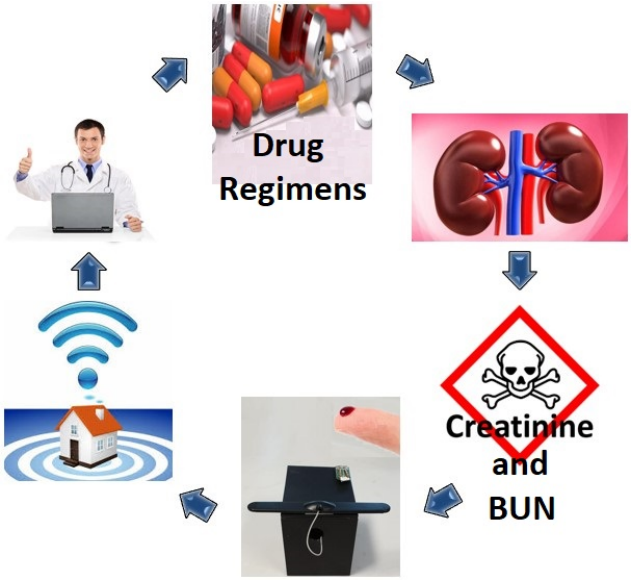


Figure 8.3: Schematic representation of potential smart kidney health system for continuous home-based IoT-enabled monitoring of kidney health with creatinine and BUN levels as biomarkers.

The currently developed MIP functionalized MEMS ID sensor is used to create an IoT-enabled portable PoC diagnostic prototype system for quantifying the heat-inactivated serum creatinine levels. Creatinine selectivity is introduced to a planar MEMS ID sensor using an artificially synthesised MIP technique. The developed PoC prototype system can precisely assess creatinine concentrations from heat-inactivated serum samples spiked with creatinine and send the results to an IoT-based Adafruit website, a complimentary cloud server. The PoC system's transferred data will be given to a general practitioner, specifically to a nephrologist/oncologist, for additional investigation and analysis. One unknown spiked serum creatinine concentration from heat-inactivated serum samples is analysed using the proposed PoC diagnostic prototype system after the calibration curve is developed using three known concentration samples. The serum sample creatinine content is found to be 15.5 ppm. The results are compared to standard Creatinine Colorimetric Assay Kit-based measurements.

The suggested PoC diagnostic prototype system has a linear behaviour in the range of 6 ppm to 14 ppm. This range is excellent enough for detecting normal kidney health and a slight decline in kidney health followed by a rise in serum creatinine concentrations (due to temporary reasons) in AKD, which is helpful for early detection. It is also an excellent choice for detecting a significant loss of kidney functioning in CKD conditions, which is helpful for the understanding of the kidney disease stage and its response to treatment regimens. The results obtained using the developed PoC diagnostic prototype system are in the ppm range. However, they are in decent agreement with the results received from a standard technique of Creatinine Colorimetric Assay Kit, which offers results in the ppb range.

A PoC diagnostic prototype system currently quantifies only the levels of creatinine; designing and developing a smart PoC diagnostic system capable of simultaneously quantifying the levels of creatinine and BUN will be more valuable. The early research and obtained conclusions are purely based on laboratory data. All the known and unknown test samples are from the spiked heat-inactivated human serum samples discussed in the results. The planar MEMS ID sensor can also quantify the creatinine levels from the aqueous spiked creatinine samples. It can also help reveal the functioning of the developed creatinine-based biosensing to the biology/pharmacy/medical students. However, the ultimate goal of the PoC diagnostic prototype system is detecting creatinine from one drop of fresh blood sample is not entirely fulfilled yet due to human ethics approval associated issue. However, the currently obtained results using heat-inactivated creatinine spiked serum samples are very encouraging. Thus, the development of a complete PoC diagnostic system simultaneously quantifying

creatinine and BUN levels from one drop of fresh blood samples is in the mid-process of investigation and can be developed as a future continuation of this research work.

For overcoming issues of specifically elderly patients, a portable smart PoC diagnostic system can turn user friendly, and the data can be transferred to Adafruit, a complementary cloud server. The Adafruit website can be easily used over smartphones. It further eases the usage of the PoC diagnostic system for the home-based management of kidney health biomarkers levels. In future, a complimentary PoC system app can also be developed for Google Play and App Store to make PoC diagnostic system the handiest one. It will be exclusively helpful for remote monitoring of prognostic and prophylactic monitoring of kidney health, which is vital for a smarter modern world e-diagnostic approach.

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Appendix A: List of Acronyms and Symbols

AC	Alternating current
Ag	Silver
Au	Gold
ACN	Acetonitrile
AKD	Acute Kidney Disease
ATR	Attenuated reflectance
AcOH	Acetic acid
AgCl	Silver chloride
AHSG	Alpha 2-HS glycoprotein
AIBN	Azoisobutronitrile
AIDS	Acquired immunodeficiency syndrome
AIHW	Australian Institute of Health and Welfare
B	susceptance
BUN	Blood urea nitrogen
C	Carbon
C1	Capacitor 1
C2	Capacitor 2
C_p	Parallel capacitance
C_s	Series capacitance
C_{dl}	Double-layer capacitance
C (ppm)	Creatinine concentration (ppm)
CC	Constant current
CE	Capillary electrophoresis
CL	Cross-linker
CT	Computed tomography
Cr	Chromium
CV	Constant voltage
CdS	Cadmium sulphide
CKD	Chronic kidney disease

CSF	Cerebrospinal fluid
CTx-1	C-telopeptide of type I collagen
CNLS	Complex non-linear least-square
Cayenne LPP	Cayenne Low Power Payload
D	Tan δ
DC	Direct current
DDS	Direct digital synthesis
DVB	Divinylbenzene
DMSO	Dimethyl sulfoxide
ECL	Electrochemiluminescence
EGF	Epidermal growth factor
EIS	Electrochemical impedance spectroscopy
EDAX	Energy Dispersive Analysis X-ray
ESKD	End-stage-kidney disease
FRA	Frequency Response Analysers
FTIR	Fourier-Transform Infrared
FABP1	Fatty Acid-Binding Protein 1
FESEM	Field Emission Scanning Electron Microscope
G	Conductance
GC	Gas Chromatography
G-force	Gravitational force
GFR	Glomerular filtration rate
HIV	Human immunodeficiency virus
ID	Interdigital
IDE	Integrated development environment
IIC	Inter-integrated circuit
IoT	Internet of Things
KIM-1	Kidney injury molecule-1
KAUST	King Abdullah University of Science and Technology
L_p	Parallel inductance
L_s	Series inductance
LCR	inductance (L), capacitance (C), and resistance (R)
LLD	Lower limit of detection

LOD	Limit of detection
LoRaWAN	Long Range Wide Area Network
M	Monomer
MIP	Molecularly Imprinted Polymer
MoS_2	Molybdenum sulphide
MRI	Magnetic resonance imaging
MEMS	Micro-Electro-Mechanical-Systems
MeOH	Methanol
N	Nitrogen
N_2	Nitrogen gas
NIP	Non-Molecularly Imprinted Polymer
NGAL	Neutrophil gelatinase-associated lipocalin
nPNA	Normalised Protein Nitrogen Appearance
O	Oxygen
O_2	Oxygen gas
OD	Optical density
p-p	Peak to peak
PEI	Polyethyleneimine
PET	Positron emission tomography
PoC	Point-of-Care
PTH	Parathyroid hormone
PECVD	Plasma-enhanced chemical vapour deposition
PEI-CdS QDs	Polyethyleneimine capped CdS quantum dots
Q	Q factor
R	Resistance
R1	Resistor 1
R2	Resistor 2
R^2	Correlation coefficient
$R2_0$	Resistor 2 control solution
R_p	Electrode polarisation resistance
R_p	Parallel resistance
R_s	Solution Resistance
R_s	Series resistance

R_{ct}	Charge transfer resistance
R_{dc}	Direct current resistance
RIE	Reactive-ion etching
RBP4	Retinol binding protein 4
RuDS	$Ru(bpy)_3^{2+}$-containing silica layer
$Ru(bpy)_3^{2+}$	Tris(bipyridine)ruthenium(II)
S_R	Sensitivity of resistance
SAS	Self-assembled single
SEM	Scanning Electron Microscopy
SGA	Subjective Global Assessment
SUT	Sample under test
SiO_2	Silicon dioxide
SnO_2	Tin oxide
Si/SiO_2	Silicon/Silicon dioxide
Si_3N_4	Silicon nitride
SPET	Single-photon emission computed tomography
T	Template
THF	Tetrahydrofuran
TFF3	Trefoil factor 3
TIMP	Tissue inhibitor of metalloproteinases
TMAH	Tetramethylammonium hydroxide
UV	Ultraviolet
UHPLC	Ultra-High-Performance Liquid Chromatography
V_{rms}	Voltage root mean square
vs	Versus
W1	Warburg impedance
X	Reactance
ΔX	Delta Reactance
X_0	Reactance of control solution
$\Delta X/X_0$	Delta Reactance per reactance of control solution
Z	Impedance
$Z_{imaginary}$	Imaginary impedance
Z_{real}	Real impedance

%	Per cent
Δ	Delta
α	Alpha
θ	Theta
σ	Conductivity
ε	Dielectric constant
λ	Lambda
<	Less than
≤	Less than or equal to
>	Greater than
≥	Greater than or equal to

Appendix B: List of Units

°C	Degree Celsius
cm	Centimetre
cm^{-1}	Centimetre inverse
gm	Gram
gm/mL	Gram per millilitre
Hz	Hertz
kΩ	Kilo omega
kHz	Kilohertz
L	Litre
M	Molar
mg	Milligram
mL	Millilitre
mm	Millimetre
$mL/minute/m^2$	Millilitre per minute per Mitre square
mm^2	Millimetre square
mM	Millimolar
mΩ	Milli Omega
MΩ	Mega Omega
ms	Millisecond
mV	millivolt
MHz	Megahertz
mg/g^{-1}	Milligram per gram inverse
mg/L^{-1}	Milligram per litre inverse
mg/dL	Milligrams per decilitre
mJ/cm^3	Millijoule per centimetre cube
mTorr	Millitorr
mol/L^{-1}	Mol per litre inverse
mmol/L	Millimole per litre
mm/minute	Millimetre per minute

mm/second	Millimetre per second
nm	Nanometre
nM	Nanomolar
ng/mL	Nanogram per millilitre
nmol/µL	Nanomole per microlitre
nm/(µg/mL)	Nanometre per microgram per millilitre
Ω	Omega
Ω/ppm	Omega per parts per million
ppb	Parts per billion
ppm	Parts per million
s	second
U	Units
µA	Microampere
µL	Microlitre
µm	Micrometre
µM	Micromolar
µmol	Micromole (10^{-6})
µg/mL	Microgram per millilitre
µA/µM/cm^3	Microampere per Micromolar per Centimetre cube
V	Volt
W	Watt

Appendix C: Code for Data Transmission

Screenshots of reactance measurement from human serum samples carrying different ppm of creatinine concentrations, and data transmission to TTN using LoRaWAN

TTN_Transfer_code_Creatnine | Arduino 1.8.13

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TTN_Transfer_code_Creatnine

```
/*
 * Reactance measurement for varying ppm concentrations transmitted to TTN using LoRa
 */

#include <MKRWAN.h>  // to initialise the LoRa module
#include <CayenneLPP.h>  //payload library initialisation

#include <Wire.h>
#include "AD5933.h"   //Reads impedance values from the AD5933


String appEui = "70B3D57ED003E35F";   // App EUI details from TTN console application
String appKey = "D3D5E7B16AB5FC7E7DF19D90EC4F6B00"; // App Key details from TTN console application


_lora_band region = AU915;     //Transmission frequency band for Australia
LoRaModem modem(Serial1);

CayenneLPP lpp(51);

void setup() {

  Serial.begin(115200);

  if (!modem.begin(region)) {
    Serial.println("Failed to start module");
    while (1) {}
  };
  Serial.print("Your device EUI is: ");
  Serial.println(modem.deviceEUI());

  int connected = modem.joinOTAA(appEui, appKey);
  if (!connected) {
    Serial.println("Something went wrong; are you indoor? Move near a window and retry");
    while (1) {}
  }
```

```
  Serial.println("Successfully joined the network!");

  Serial.println("Enabling ADR and setting low spreading factor");
  modem.setADR(true);
  modem.dataRate(5);

}

void loop(){
  // Prepare Cayenne LPP payload
  lpp.reset();
  lpp.addAnalogOutput(1, reactance);  // for reactance measurement for varying ppm measurements of creatinine

  // Send the data
  modem.beginPacket();
  modem.write(lpp.getBuffer(), lpp.getSize());
  int err = modem.endPacket(true);
  if (err > 0) {
    Serial.println("Message sent.");
  } else {
    Serial.println("Error sending data.");
  }

  // Wait 10 SECONDS between transmissions
  delay(10000);
}
```

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List of Publication Titles and their Copyright Forms

1) Highly selective Molecularly Imprinted Polymer for creatinine detection

2) Interdigital sensing system for detection of levels of creatinine from the samples

3) Development of a Point-of-Care Diagnostic Smart Sensing System to Detect Creatinine Levels

4) IoT-Associated Impedimetric Biosensing for Point-of-Care Monitoring of Kidney Health

5) Molecularly Imprinted Polymer-based detection of creatinine towards smart sensing

6) Interdigital Sensing System for Kidney Health Monitoring

7) A unique developmental study in the design of Point-of-Care medical diagnostic device for kidney healthcare of metastatic brain cancer patients to avoid chemotherapy side-effects

8) Development of MEMS Sensor for Detection of Creatinine using MIP Based Approach -A Tutorial Paper

9) Functionality Evaluation of Micro Electro Mechanical Systems Sensor for Varied Selective Functionalization Thickness to Determine Creatinine Concentration

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Ms. Sumedha N. Prabhu
2019 13th International Conference on Sensing Technology (ICST)

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3.1 The Author hereby grants to the Publisher the perpetual, sole and exclusive, world-wide, transferable, sub-licensable and unlimited right to publish, produce, copy, distribute, communicate, display publicly, sell, rent and/or otherwise make available the Contribution in any language, in any versions or editions in any and all forms and/or media of expression (including without limitation in connection with any and all end-user devices), whether now known or developed in the future, in each case with the right to grant further time-limited or permanent rights. The above rights are granted in relation to the Contribution as a whole or any part and with or in relation to any other works.
Without limitation, the above grant includes: (a) the right to edit, alter, adapt, adjust and prepare derivative works; (b) all advertising and marketing rights including without limitation in relation to social media; (c) rights for any training, educational and/or instructional purposes; and (d) the right to add and/or remove links or combinations with other media/works.
The Author hereby grants to the Publisher the right to create, use and/or license and/or sub-license content data or metadata of any kind in relation to the Contribution or parts thereof (including abstracts and summaries) without restriction.
The Publisher also has the right to commission completion of the Contribution in accordance with the Clause "**Author's Responsibilities – Delivery and Acceptance of the Manuscript**" and of an updated version of the Contribution for new editions of the Work in accordance with the Clause "**New Editions**".

3.2 The copyright in the Contribution shall be vested in the name of the **Author**. The Author has asserted their right(s) to be identified as the originator of the Contribution in all editions and versions, published in all forms and media. The Author agrees that all editing, alterations or amendments to the Contribution made by or on behalf of the Publisher or its licensees for the purpose of fulfilling this Agreement or as otherwise allowed by the above rights shall not require the approval of the Author and will not infringe the Author's "moral rights" (or any equivalent rights). This includes changes made in the course of dealing with retractions or other legal issues.

§ 4 Self-Archiving and Reuse

4.1 **Self-Archiving:** The Publisher permits the Rights Holder to archive the Contribution in accordance with the Publisher's guidelines, the current version of which is set out in the **Appendix "Author's Self-Archiving Guidelines"**.

4.2 **Reuse:** The Publisher permits the Author to copy, distribute or otherwise reuse the Contribution, without the requirement to seek specific prior written permission from the Publisher, in accordance with the Publisher's guidelines, the current version of which is set out in the **Appendix "Author's Reuse Rights"**.

§ 5 The Publisher's Responsibilities

5.1 Subject always to the other provisions of this Clause below, the Publisher will undertake the production, publication and distribution of the Contribution and the Work in print and/or electronic form at its own expense and risk within a reasonable time after acceptance of the Work unless the Publisher is prevented from or delayed in doing so due to any circumstances beyond its reasonable control. The Publisher shall have the

entire control of such production, publication and distribution determined in its sole discretion in relation to any and all editions and versions of the Contribution and the Work, including in respect of all the following matters:
(a) distribution channels, including determination of markets;
(b) determination of the range and functions of electronic formats and/or the number of print copies produced;
(c) publication and distribution of the Contribution, the Work, or parts thereof as individual content elements, in accordance with market demand or other factors;
(d) determination of layout and style as well as the standards for production;
(e) setting or altering the list-price, and allowing for deviations from the list-price (if permitted under applicable jurisdiction);
(f) promotion and marketing as the Publisher considers most appropriate.

5.2 All rights, title and interest, including all intellectual property or related rights in the typography, design and/or look-and-feel of the Contribution shall remain the exclusive property of and are reserved to the Publisher. All illustrations and any other material or tangible or intangible property prepared at the expense of the Publisher including any marketing materials remain, as between the Parties, the exclusive property of the Publisher. The provisions of this sub-clause shall continue to apply notwithstanding any termination of, and/or any reversion of rights in the Contribution to the Author, under this Agreement.

5.3 Without prejudice to the Publisher's termination and other rights hereunder including under the Clause "**The Author's Responsibilities**", it is agreed and acknowledged by the Parties that nothing in this Agreement shall constitute an undertaking on the part of the Publisher to publish the Contribution unless and until: (i) any and all issues in relation to the Work (including all necessary revisions, consents and permissions) raised by the Publisher have been resolved to the Publisher's satisfaction, and (ii) the Publisher has given written notice of acceptance in writing of the final manuscript of the entire Work to the Editor. If following (i) and (ii) above the Publisher has not published the Contribution in any form within a reasonable period and the Author has given written notice to the Publisher requiring it to publish within a further reasonable period and the Publisher has failed to publish in any form, then the Author may terminate this Agreement by one month's written notice to the Publisher and all rights granted by the Author to the Publisher under this Agreement shall revert to the Author (subject to the provisions regarding any third party rights under any subsisting licence or sub-licence in accordance with the Clause "**Termination**").
The Author may also give such written notice requiring publication on the same terms as above if the Publisher has published the Contribution but subsequently ceases publishing the Contribution in all forms so that it is no longer available.
This shall be the Author's sole right and remedy in relation to such non-publication and is subject always to the Author's continuing obligations hereunder including the Clause "**Warranty**".

§ 6 The Author's Responsibilities

6.1 *Delivery and Acceptance of the Manuscript*

6.1.1 The Author shall deliver the Contribution to the Editor (or, if requested by the Publisher, to the Publisher) on or before **[30-08-2020]** (the "**Delivery Date**") electronically in the Publisher's standard requested format or in such other form as may be agreed in writing with the Publisher. The Author shall retain a duplicate copy of the Contribution. The Contribution shall be in a form acceptable to the Publisher (acting reasonably) and in line with the instructions contained in the Publisher's guidelines as provided to the Author by the Publisher. The Author shall provide at the same time, or earlier if the Publisher reasonably requests, any editorial, publicity or other information (and in such form or format) reasonably required by the Publisher. The Publisher may exercise such additional quality control of the manuscript as it may decide at its sole discretion including through the use of plagiarism checking systems and/or peer-review by internal or external reviewers of its choice. If the Publisher decides at its sole discretion that the final manuscript does not conform in quality, content, structure, level or form to the stated requirements of the Publisher, the Publisher shall be entitled to terminate this Agreement in accordance with the provisions of this Clause.

6.1.2 The Author must inform the Publisher at the latest on the Delivery Date if the sequence of the naming of any co-authors entering into this Agreement shall be changed. If there are any changes in the authorship (e.g. a co-author joining or leaving), then the Publisher must be notified by the Author in writing immediately and the Parties will amend this Agreement accordingly. The Publisher shall have no obligation to consider publication under this Agreement in the absence of such agreed amendment.

6.1.3 If the Author fails to deliver the Contribution in accordance with the provisions of this Clause above by the Delivery Date (or within any extension period given by the Publisher at its sole discretion) or if the Author (or any co-author) dies or becomes incapacitated or otherwise incapable of performing the Author's obligations under this Agreement, the Publisher shall be entitled to either:
(a) elect to continue to perform this Agreement in accordance with its terms and the Publisher may commission an appropriate and competent person (who, in the case of co-authors having entered into this Agreement, may be a co-author) to complete the Contribution; or
(b) terminate this Agreement with immediate effect by written notice to the Author or the Author's successors, in which case all rights granted by the Author to the Publisher under this Agreement shall revert to the Author/Author's successors (subject to the provisions of the Clause "**Termination**").

6.1.4 The Author agrees, at the request of the Publisher, to execute all documents and do all things reasonably required by the Publisher in order to confer to the Publisher all rights intended to be granted under this Agreement.

6.1.5 The Author warrants that the Contribution is original except for any excerpts from other works including pre-published illustrations, tables, animations, text quotations, photographs, diagrams, graphs or maps, and whether reproduced from print or electronic or other sources ("**Third Party Material**") and that any such Third Party Material is in the public domain (or otherwise unprotected by copyright/other rights) or has been included with written permission from or on behalf of the rights holder (and if requested in a form prescribed or approved by the Publisher) at the Author's expense unless otherwise agreed in writing, or is otherwise used in accordance with applicable law. On request from the Publisher, the Author shall in writing indicate the precise sources of these excerpts and their location in the manuscript. The Author shall also retain the written permissions and make them available to the Publisher on request.

6.2 Approval for Publishing

6.2.1 The Author shall proofread the page proofs for the Contribution provided by or on behalf of the Publisher, including checking the illustrations as well as any media, social or functional enhancements and give approval for publishing, if and when requested by the Publisher. The Author's approval for publishing is deemed to have been given if the Author does not respond within a reasonable period of time (as determined by the Publisher) after receiving the proofs. The Publisher shall not be required to send a second set of corrected proofs unless specifically requested by the Author in writing but in any event no further amendments may be made or requested by the Author.
In the event of co-authors having entered into this Agreement the Publisher shall send the page proofs to the Corresponding Author only and all persons entering into this Agreement as Author agree that the Corresponding Author shall correct and approve the page proofs on their behalf.

6.2.2 If the Author makes changes other than correcting typographical errors, the Author shall bear all the Publisher's costs of such alterations to proofs including without limitation to alterations to pictorial illustrations. The Publisher shall have the right to charge and invoice these costs plus value added or similar taxes (if applicable) through its affiliated company Springer Nature Customer Service Center GmbH or Springer Nature Customer Service Center LLC, respectively, to the Author, payable within 14 days of receipt of the invoice.

§ 7 Co-operation

Without prejudice to the warranties and representations given by the Author in this Agreement, the Author shall cooperate fully with the Editor and the Publisher in relation to any legal action that might arise from the publication or intended publication of the Contribution and the Author shall give the Publisher access at reasonable times to any relevant accounts, documents and records within the power or control of the Author.

§ 8 Warranty

8.1 The Author warrants and represents that:
(a) the Author has full right, power and authority to enter into and perform its obligations under this Agreement; and
(b) the Author is the sole legal owner of (and/or has been fully authorised by any additional rights owner to grant) the rights licensed in the Clause "**Rights Granted**" and use of the Contribution shall in no way whatever infringe or violate any intellectual property or related rights (including any copyright, database right, moral right or trademark right) or any other right or interest of any third party subject only to the provisions in the Clause "**The Author's Responsibilities**" regarding Third Party Material (as defined above); and
(c) the Contribution shall not contain anything that may cause religious or racial hatred or encourage terrorism or unlawful acts or be defamatory (or contain malicious falsehoods), or be otherwise actionable, including, but not limited to, any action related to any injury resulting from the use of any practice or formula disclosed in the Contribution and all of the purported facts contained in the Contribution are according to the current body of science and understanding true and accurate; and

(d) there is no obligation of confidentiality owed in respect of any contents of the Contribution to any third party and the Contribution shall not contain anything which infringes or violates any trade secret, right of privacy or publicity or any other personal or human right or the processing or publication of which could breach applicable data protection law and that informed consent to publish has been obtained for all research or other featured participants; and
(e) the Contribution has not been previously licensed, published or exploited and use of the Contribution shall not infringe or violate any contract, express or implied, to which the Author, or any co-author, who had entered into this Agreement, is a party and any academic institution, employer or other body in which work recorded in the Contribution was created or carried out has authorised and approved such work and its publication.

8.2 The Author warrants and represents that the Author, and each co-author who has entered into this Agreement, shall at all times comply in full with:
(a) all applicable anti-bribery and corruption laws; and
(b) all applicable data protection and electronic privacy and marketing laws and regulations; and
(c) the Publisher's ethic rules (available at https://www.springernature.com/gp/authors), as may be updated by the Publisher at any time in its sole discretion. The Publisher shall notify the Author in the event of material changes by email or other written means
(the "**Applicable Laws**").
If the Author is in material breach of any of the Applicable Laws or otherwise in material breach of accepted ethical standards in research and scholarship, or becomes the subject of any comprehensive or selective sanctions issued in any applicable jurisdiction (e.g. being subject to the OFAC sanctions list) or if, in the opinion of the Publisher, at any time any act, allegation or conduct of or about the Author prejudices the production or successful exploitation of the Contribution and the Work or brings the name and/or reputation of the Publisher or the Work into disrepute, or is likely to do so, then the Publisher may terminate this Agreement in accordance with the Clause "**Termination**".

8.3 The Publisher reserves the right to amend and/or require the Author to amend the Contribution at any time to remove any actual or potential breach of the above warranties and representations or otherwise unlawful part(s) which the Publisher or its internal or external legal advisers identify at any time. Any such amendment or removal shall not affect the warranties and representations given by the Author in this Agreement.

§ 9 Author's Discount and Electronic Access

9.1 The Author, or each co-author, is entitled to purchase for their personal use the Work and other books published by the Publisher at a discount of 40% off the list price, for as long as there is a contractual arrangement between the Author and the Publisher and subject to any applicable book price law or regulation. The copies must be ordered from the affiliated entity of the Publisher (Springer Nature Customer Service Center GmbH or Springer Nature Customer Service Center LLC, respectively). Resale of such copies is not permitted.

9.2 The Publisher shall provide the electronic final published version of the Work to the Author, provided that the Author has included their e-mail address in the manuscript of the Contribution.

§ 10 Consideration

10.1 The Parties agree that the Publisher's agreement to its contractual obligations in this Agreement in respect of its efforts in considering publishing and promoting the Contribution and the Work is good and valuable consideration for the rights granted and obligations undertaken by the Author under this Agreement, the receipt, validity and sufficiency of which is hereby acknowledged by the Author.
The Parties expressly agree that no royalty, remuneration, licence fee, costs or other moneys whatsoever shall be payable to the Author, subject to the following provisions of this Clause.

10.2 The Publisher and the Author each have the right to authorise collective management organisations ("**CMOs**") of their choice to manage some of their rights. Reprographic and other collectively managed rights in the Contribution ("**Collective Rights**") have been or may be licensed on a non-exclusive basis by each of the Publisher and the Author to their respective CMOs to administer the Collective Rights under their reprographic and other collective licensing schemes ("**Collective Licences**"). Notwithstanding the other provisions of this Clause, the Publisher and the Author shall each receive and retain their share of revenue from use of the Contribution under Collective Licences from, and in accordance with the distribution terms of their respective CMOs. To the fullest extent permitted by law, any such revenue is the sole property of the Publisher and the Author respectively and, if applicable, the registration and taxation of that revenue is the sole responsibility of the respective recipient party. The Publisher and the Author shall cooperate as necessary in the event of any change to the licensing arrangements set out in this Clause.

§ 11 New Editions

11.1 The Publisher has the sole right to determine whether to publish any subsequent edition of the Work containing an updated version of the Contribution, but only after reasonable consultation with the Author. Once notified by the Publisher that an update of the Contribution is deemed necessary, the Author agrees to deliver an updated manuscript in accordance with the terms of the Clause "**The Author's Responsibilities**" and the other relevant provisions of this Agreement, together with the material for any new illustrations and any other supporting content including media enhancements, within a reasonable period of time (as determined by the Publisher) after such notification. Substantial changes in the nature or size of the Contribution require the written approval of the Publisher at its sole discretion. The terms of this Agreement shall apply to any new edition of the Work that is published under this "**New Editions**" Clause.

11.2 If the Author, for whatever reason, is unwilling, unable or fails (including as a result of death or incapacity) to submit an updated manuscript that meets the terms of this Agreement within the above stated period, then the Publisher is entitled to revise, update and publish the content of the existing edition or to designate one or more individuals (which, where co-authors have entered into this Agreement, may be one or more of the co-authors) to prepare this and any future editions provided that the new editions shall not contain anything that is a derogatory use of the Author's work that demonstrably damages the Author's scientific reputation. In such case, the Author shall not participate in preparing any subsequent editions. The Author agrees that the Publisher shall be entitled but not obliged to continue to use the name of Author on any new editions of the Work together with the names of the person or persons who contributed to the new editions. Should the Author or the Author's successors object to such continuing use then they must notify the Publisher in writing when first contacted by the Publisher in connection with any new edition.

§12 Termination

12.1 In addition to the specific rights of termination set out in the Clause "**The Publisher's Responsibilities**" and the Clause "**The Author's Responsibilities**", either Party shall be entitled to terminate this Agreement forthwith by notice in writing to the other Party if the other Party commits a material breach of the terms of the Agreement which cannot be remedied or, if such breach can be remedied, fails to remedy such breach within 45 days of being given written notice to do so.

12.2 Termination of this Agreement, howsoever caused, shall not affect:
(a) any subsisting rights of any third party under any licence or sub-licence validly granted by the Publisher prior to termination and the Publisher shall be entitled to retain its share of any sum payable by any third party under any such licence or sub-licence;
(b) except where stated otherwise in this Agreement, any claim which either Party may have against the other for damages or otherwise in respect of any rights or liabilities arising prior to the date of termination;
(c) the Publisher's right to continue to sell any copies of the Work which are in its power, possession or control as at the date of expiry or termination of this Agreement for a period of 6 months on a non-exclusive basis.

§13 General Provisions

13.1 This Agreement, and the documents referred to within it, constitute the entire agreement between the Parties with respect to the subject matter hereof and supersede any previous agreements, warranties, representations, undertakings or understandings. Each Party acknowledges that it is not relying on, and shall have no remedies in respect of, any undertakings, representations, warranties, promises or assurances that are not set forth in this Agreement. Nothing in this Agreement shall exclude any liability for or remedy in respect of fraud, including fraudulent misrepresentation. This Agreement may be modified or amended only by agreement of the Parties in writing. For the purposes of modifying or amending this Agreement, "in writing" requires either a document written and signed by both the Parties or an electronic confirmation by both the Parties with DocuSign or a similar e-signature solution. Any notice of termination and/or reversion and, where applicable, any preceding notices (including any requesting remediable action under the Clause "**Termination**") must be provided in writing and delivered by post, courier or personal delivery addressed to the physical address of the relevant Party as set out at the beginning of this Agreement or any replacement address notified to the other Party for this purpose. All such notices shall become effective upon receipt by the other Party. Receipt is deemed to have taken place five working days after the respective notice was sent by post or left at the address by courier or personal delivery. If the Publisher is the terminating Party the notice need only be provided to the address of the Corresponding Author. If the Author is the terminating Party a copy of the notice must also be sent to the Publisher's Legal Department located at Heidelberger Platz 3, 14197 Berlin, Germany.

13.2 Nothing contained in this Agreement shall constitute or shall be construed as constituting a partnership, joint venture or contract of employment between the Publisher and the Author. No Party may assign this Agreement to third parties but the Publisher may assign this Agreement or the rights received hereunder to its affiliated companies. In this Agreement, any words following the terms "include", "including",

"in particular", "for example", "e.g." or any similar expression shall be construed as illustrative and shall not limit the sense of the words preceding those terms.

13.3 If any difference shall arise between the Author and the Publisher concerning the meaning of this Agreement or the rights and liabilities of the Parties, the Parties shall engage in good faith discussions to attempt to seek a mutually satisfactory resolution of the dispute. This Agreement shall be governed by, and shall be construed in accordance with, the laws of *Switzerland*. The courts of *Zug, Switzerland* shall have the exclusive jurisdiction.

13.4 A person who is not a party to this Agreement (other than an affiliate of the Publisher) has no right to enforce any terms or conditions of this Agreement. This Agreement shall be binding upon and inure to the benefit of the successors and assigns of the Publisher. If one or more provisions of this Agreement are held to be unenforceable (in whole or in part) under applicable law, each such provision shall be deemed excluded from this Agreement and the balance of the Agreement shall remain valid and enforceable but shall be interpreted as if that provision were so excluded. If one or more provisions are so excluded under this Clause then the Parties shall negotiate in good faith to agree an enforceable replacement provision that, to the greatest extent possible under applicable law, achieves the Parties' original commercial intention.

The Corresponding Author signs this Agreement on behalf of any and all co-authors.

Signature of Corresponding Author: **Date:**

Sumedha Prabhu

…… 23-09-2020

……..

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Appendix “Author’s Self-Archiving Rights”

The Publisher acknowledges that the Author retains rights to archive the Contribution but only subject to and in accordance with the following provisions:

1. **Preprint:**
   A “**Preprint**” is defined as the Author´s version of the Contribution submitted to the Publisher but before any peer-review or any other editorial work by or on behalf of the Publisher has taken place.
   The Author may make available the Preprint of the Contribution for personal and private reading purposes only on any of:
   (a) the Author's own personal, self-maintained website over which the Author has sole operational control; and/or
   (b) a legally compliant, non-commercial preprint server, such as but not limited to arXiv, bioRxiv and RePEc; provided always that once the “Version of Record” (as defined below) of the Contribution has been published by or on behalf of the Publisher, the Author shall immediately ensure that any Preprint made available above shall contain a link to the Version of Record and the following acknowledgement:
   “This is a preprint of the following chapter: [author of the chapter], [chapter title], published in [book title], edited by [editor of the book], [year of publication], [publisher (as it appears on the cover of the book)] reproduced with permission of [publisher (as it appears on the copyright page of the book)]. The final authenticated version is available online at: http://dx.doi.org/[insert DOI]”.

2. **Author’s Accepted Manuscript:**
   The “Author’s Accepted Manuscript” (“**AAM**”) is defined as the version of the Contribution following any peer-review and acceptance, but prior to copyediting and typesetting, by or on behalf of the Publisher.
   The Author may make available the AAM of the Contribution on any of: (a) the Author's own, personal, self-maintained website over which the Author has sole operational control; and/or (b) the Author's employer’s internal website or their academic institution or funder´s repository; provided that in each case the respective part of the AAM is not made publicly available until after the Embargo Period.
   The "**Embargo Period**" is a period ending twenty-four (24) months from the first publication of the “Version of Record” (as defined below) of the Contribution by or on behalf of the Publisher.
   The Author must ensure that any part of the AAM made available contains the following:
   “Users may only view, print, copy, download and text- and data-mine the content, for the purposes of academic research. The content may not be (re-)published verbatim in whole or in part or used for commercial purposes. Users must ensure that the author’s moral rights as well as any third parties’ rights to the content or parts of the content are not compromised.”
   These terms shall also be applicable to the Author.
   Once the Version of Record (as defined below) of the Contribution has been published by or on behalf of the Publisher the Author shall immediately ensure that any part of the AAM made available shall contain a link to the Version of Record and the following acknowledgement:
   “This is an Author Accepted Manuscript version of the following chapter: [author of the chapter], [chapter title], published in [book title], edited by [editor of the book], [year of publication], [publisher (as it appears on the cover of the book)] reproduced with permission of [publisher (as it appears on the copyright page of the book)]. The final authenticated version is available online at: http://dx.doi.org/[insert DOI]”.

3. **Version of Record:**
   The “**Version of Record**” is defined as the final version of the Contribution as originally published, and as may be subsequently amended following publication in a contractually compliant manner, by or on behalf of the Publisher.

4. Any linking, collection or aggregation of self-archived Contributions from the same Work is strictly prohibited.

Appendix “Author's Reuse Rights”

1. The Publisher acknowledges that the Author retains the ability to copy, distribute or otherwise reuse the Contribution, without the requirement to seek specific prior written permission from the Publisher, (“**Reuse**”) subject to and in accordance with the following provisions:
   (a) Reuse of the Contribution or any part of it is permitted in a new edition of the Work or in a new monograph or new textbook written by the same Author provided that in each case the new work is published by the Publisher under a publishing agreement with the Publisher; and
   (b) Reuse of the Version of Record (as defined below) of the Contribution or any part of it is permitted in a thesis written by the same Author, and the Author is entitled to make a copy of the thesis containing content of the Contribution available in a repository of the Author’s academic institution; and
   (c) any other Reuse of the Contribution in a new book, book chapter, proceedings or journal article, whether published by the Publisher or by any third party, is limited to three figures (including tables) or single text extracts of less than 400 words; and
   (d) any further Reuse of the Contribution is permitted only to the extent and in so far as is reasonably necessary: (i) to share the Contribution as a whole to no more than 10 research colleagues engaged by the same institution or employer as the Author for each colleague's personal and private use only; (ii) for classroom teaching use by the Author in their respective academic institution provided that this does not permit inclusion of any of the Contribution in course packs for sale or wider distribution to any students, institutions or other persons nor any other form of commercial or systematic exploitation; or (iii) for the Author to use all or parts of the Contribution in the further development of the Author's scientific and/or academic career, for private use and research or within a strictly limited circulation which does not allow the Contribution to become publicly accessible nor prejudice sales of, or the exploitation of the Publisher's rights in, the Contribution (e.g. attaching a copy of the Contribution to a job or grant application).

2. Any Reuse must be based on the Version of Record only, and provided the original source of publication is cited according to current citation standards. The "**Version of Record**" is defined as the final version of the Contribution as originally published, and as may be subsequently amended following publication in a contractually compliant manner, by or on behalf of the Publisher.

3. In each case where the Author has Reuse rights or the Publisher grants specific use rights to the Author according to the above provisions, this shall be subject always to the Author obtaining at the Author's sole responsibility, cost and expense the prior consent of any co-author(s) and/or any relevant third party.

4. Any linking, collection or aggregation of reused Contributions from the same Work is strictly prohibited.

Publishing Agreement

for Contributions in Collected Works

SPRINGER NATURE

This Publishing Agreement (this "**Agreement**") has been approved by and entered into between
[Sumedha Nitin Prabhu + School of Engineering Level 2, 221 (Data Acquisition Room), 9 Wally's Walk (E6A), Macquarie University, NSW 2109, Australia, (https://orcid.org/0000-0001-7782-7514)]
(the "**Author**")
whereas, in the event that the Author is more than one person, ***[Sumedha Nitin Prabhu]*** serves as corresponding author (the "**Corresponding Author**")

on the one part and

Springer Nature Singapore Pte Ltd.
152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore
(the "**Publisher**")

on the other part;
together hereinafter referred to as the "**Parties**".

The Publisher intends to publish the Author's contribution in a collected work provisionally entitled:
Advances in Communication, Devices and Networking - Proceedings of ICCDN 2020
(the "**Work**")
edited by:
Prof. Dr. Sourav Dhar, Prof. Subhas Chandra Mukhopadhyay, Samarendra Nath Sur,Prof. Chuan-Ming Liu
(the "**Editor**")

The Publisher intends to publish the Work under the imprint **Springer**.
The Work may be published in the book series **Lecture Notes in Electrical Engineering.**

§1 Contracting authors

When the Author is more than one person then, unless otherwise indicated in this Agreement or agreed in writing by the Publisher: (a) the expression "Author" as used in this Agreement will apply collectively for all such persons (each a "**co-author**"); (b) the Corresponding Author hereby warrants and represents that all co-authors of the contribution have expressly agreed that the Corresponding Author has full right, power and authority to sign this Agreement on their behalf, that the Corresponding Author is entitled to act on their behalf, and that they shall be bound by the Corresponding Author, with respect to all matters, responsibilities, notices and communications related to this Agreement; the Corresponding Author shall obtain authorisations and make them available to the Publisher on request; and (c) each co-author is jointly and severally responsible for the Author's obligations under this Agreement which apply to each co-author individually and to the co-authors collectively and the Publisher shall not be bound by any separate agreement or legal relationship as between the co-authors.

§2 Subject of the Agreement

2.1 The Author will prepare a contribution provisionally entitled:
[A unique developmental study in the design of Point-of-Care medical diagnostic device for kidney healthcare of metastatic brain cancer patients to avoid chemotherapy side-effects]

The expression "**Contribution**" as used in this Agreement means the contribution as identified above, and includes without limitation all related material delivered to the Publisher by or on behalf of the Author whatever its media and form (including text, graphical elements, tables, videos and/or links) in all versions and editions in whole or in part.

2.2 The Contribution may contain links (e.g. frames or in-line links) to media enhancements (e.g. additional documents, tables, diagrams, charts, graphics, illustrations, animations, pictures, videos and/or software) or to social or functional enhancements, complementing the Contribution, which are provided on the Author's own website or on a third party website or repository (e.g. maintained by an institution) subject always to the Author providing to the Editor, at the latest at the delivery date of the manuscript for the Contribution, an accurate description of each media enhancement and its respective website or repository, including its/their owner, nature and the URL. The Publisher is entitled to reject the inclusion of, or suspend, or delete links to all or any individual media enhancements.

2.3 In the event that an index is deemed necessary, the Author shall assist the Editor in its preparation (e.g. by suggesting index terms), if requested by the Editor.

§ 3 Rights Granted

3.1 The Author hereby grants to the Publisher the perpetual, sole and exclusive, world-wide, transferable, sub-licensable and unlimited right to publish, produce, copy, distribute, communicate, display publicly, sell, rent and/or otherwise make available the Contribution in any language, in any versions or editions in any and all forms and/or media of expression (including without limitation in connection with any and all end-user devices), whether now known or developed in the future, in each case with the right to grant further time-limited or permanent rights. The above

rights are granted in relation to the Contribution as a whole or any part and with or in relation to any other works.
Without limitation, the above grant includes: (a) the right to edit, alter, adapt, adjust and prepare derivative works; (b) all advertising and marketing rights including without limitation in relation to social media; (c) rights for any training, educational and/or instructional purposes; and (d) the right to add and/or remove links or combinations with other media/works.
The Author hereby grants to the Publisher the right to create, use and/or license and/or sub-license content data or metadata of any kind in relation to the Contribution or parts thereof (including abstracts and summaries) without restriction.
The Publisher also has the right to commission completion of the Contribution in accordance with the Clause "**Author's Responsibilities – Delivery and Acceptance of the Manuscript**" and of an updated version of the Contribution for new editions of the Work in accordance with the Clause "**New Editions**".

3.2 The copyright in the Contribution shall be vested in the name of the **Author**. The Author has asserted their right(s) to be identified as the originator of the Contribution in all editions and versions, published in all forms and media. The Author agrees that all editing, alterations or amendments to the Contribution made by or on behalf of the Publisher or its licensees for the purpose of fulfilling this Agreement or as otherwise allowed by the above rights shall not require the approval of the Author and will not infringe the Author's "moral rights" (or any equivalent rights). This includes changes made in the course of dealing with retractions or other legal issues.

§ 4 Self-Archiving and Reuse

4.1 **Self-Archiving:** The Publisher permits the Rights Holder to archive the Contribution in accordance with the Publisher's guidelines, the current version of which is set out in the **Appendix "Author's Self-Archiving Guidelines"**.

4.2 **Reuse:** The Publisher permits the Author to copy, distribute or otherwise reuse the Contribution, without the requirement to seek specific prior written permission from the Publisher, in accordance with the Publisher's guidelines, the current version of which is set out in the **Appendix "Author's Reuse Rights"**.

§ 5 The Publisher's Responsibilities

5.1 Subject always to the other provisions of this Clause below, the Publisher will undertake the production, publication and distribution of the Contribution and the Work in print and/or electronic form at its own expense and risk within a reasonable time after acceptance of the Work unless the Publisher is prevented from or delayed in doing so due to any circumstances beyond its reasonable control. The Publisher shall have the entire control of such production, publication and distribution determined in its sole discretion in relation to any and all editions and versions of the Contribution and the Work, including in respect of all the following matters:
(a) distribution channels, including determination of markets;
(b) determination of the range and functions of electronic formats and/or the number of print copies produced;
(c) publication and distribution of the Contribution, the Work, or parts thereof as individual content elements, in accordance with market demand or other factors;
(d) determination of layout and style as well as the standards for production;
(e) setting or altering the list-price, and allowing for deviations from the list-price (if permitted under applicable jurisdiction);
(f) promotion and marketing as the Publisher considers most appropriate.

5.2 All rights, title and interest, including all intellectual property or related rights in the typography, design and/or look-and-feel of the Contribution shall remain the exclusive property of and are reserved to the Publisher. All illustrations and any other material or tangible or intangible property prepared at the expense of the Publisher including any marketing materials remain, as between the Parties, the exclusive property of the Publisher. The provisions of this sub-clause shall continue to apply notwithstanding any termination of, and/or any reversion of rights in the Contribution to the Author, under this Agreement.

5.3 Without prejudice to the Publisher's termination and other rights hereunder including under the Clause "**The Author's Responsibilities**", it is agreed and acknowledged by the Parties that nothing in this Agreement shall constitute an undertaking on the part of the Publisher to publish the Contribution unless and until: (i) any and all issues in relation to the Work (including all necessary revisions, consents and permissions) raised by the Publisher have been resolved to the Publisher's satisfaction, and (ii) the Publisher has given written notice of acceptance in writing of the final manuscript of the entire Work to the Editor. If following (i) and (ii) above the Publisher has not published the Contribution in any form within a reasonable period and the Author has given written notice to the Publisher requiring it to publish within a further reasonable period and the Publisher has failed to publish in any form, then the Author may terminate this Agreement by one month's written notice to the Publisher and all rights granted by the Author to the Publisher under this Agreement shall revert to the Author (subject to the provisions regarding any third party rights under any subsisting licence or sub-licence in accordance with the Clause "**Termination**").
The Author may also give such written notice requiring publication on the same terms as above if the Publisher has published the Contribution but subsequently ceases publishing the Contribution in all forms so that it is no longer available.
This shall be the Author's sole right and remedy in relation to such non-publication and is subject always to the Author's continuing obligations hereunder including the Clause "**Warranty**".

§ 6 The Author's Responsibilities

6.1 *Delivery and Acceptance of the Manuscript*

6.1.1 The Author shall deliver the Contribution to the Editor (or, if requested by the Publisher, to the Publisher) on or before **[30-11-2020]** (the "**Delivery Date**") electronically in the Publisher's standard requested format or in such other form as may be agreed in writing with the Publisher. The Author shall retain a duplicate copy of the Contribution. The Contribution shall be in a form acceptable to the Publisher (acting reasonably) and in line with the instructions contained in the Publisher's guidelines as provided to the Author by the Publisher. The Author shall provide at the same time, or earlier if the Publisher reasonably requests, any editorial, publicity or other information (and in such form or format) reasonably required by the Publisher. The Publisher may exercise such additional quality control of the manuscript as it may decide at its sole discretion including through the use of plagiarism checking systems and/or peer-review by internal or external reviewers of its choice. If the Publisher decides

at its sole discretion that the final manuscript does not conform in quality, content, structure, level or form to the stated requirements of the Publisher, the Publisher shall be entitled to terminate this Agreement in accordance with the provisions of this Clause.

6.1.2 The Author must inform the Publisher at the latest on the Delivery Date if the sequence of the naming of any co-authors entering into this Agreement shall be changed. If there are any changes in the authorship (e.g. a co-author joining or leaving), then the Publisher must be notified by the Author in writing immediately and the Parties will amend this Agreement accordingly. The Publisher shall have no obligation to consider publication under this Agreement in the absence of such agreed amendment.

6.1.3 If the Author fails to deliver the Contribution in accordance with the provisions of this Clause above by the Delivery Date (or within any extension period given by the Publisher at its sole discretion) or if the Author (or any co-author) dies or becomes incapacitated or otherwise incapable of performing the Author's obligations under this Agreement, the Publisher shall be entitled to either:
(a) elect to continue to perform this Agreement in accordance with its terms and the Publisher may commission an appropriate and competent person (who, in the case of co-authors having entered into this Agreement, may be a co-author) to complete the Contribution; or
(b) terminate this Agreement with immediate effect by written notice to the Author or the Author's successors, in which case all rights granted by the Author to the Publisher under this Agreement shall revert to the Author/Author's successors (subject to the provisions of the Clause "**Termination**").

6.1.4 The Author agrees, at the request of the Publisher, to execute all documents and do all things reasonably required by the Publisher in order to confer to the Publisher all rights intended to be granted under this Agreement.

6.1.5 The Author warrants that the Contribution is original except for any excerpts from other works including pre-published illustrations, tables, animations, text quotations, photographs, diagrams, graphs or maps, and whether reproduced from print or electronic or other sources ("**Third Party Material**") and that any such Third Party Material is in the public domain (or otherwise unprotected by copyright/other rights) or has been included with written permission from or on behalf of the rights holder (and if requested in a form prescribed or approved by the Publisher) at the Author's expense unless otherwise agreed in writing, or is otherwise used in accordance with applicable law. On request from the Publisher, the Author shall in writing indicate the precise sources of these excerpts and their location in the manuscript. The Author shall also retain the written permissions and make them available to the Publisher on request.

6.2 Approval for Publishing

6.2.1 The Author shall proofread the page proofs for the Contribution provided by or on behalf of the Publisher, including checking the illustrations as well as any media, social or functional enhancements and give approval for publishing, if and when requested by the Publisher. The Author's approval for publishing is deemed to have been given if the Author does not respond within a reasonable period of time (as determined by the Publisher) after receiving the proofs. The Publisher shall not be required to send a second set of corrected proofs unless specifically requested by the Author in writing but in any event no further amendments may be made or requested by the Author.
In the event of co-authors having entered into this Agreement the Publisher shall send the page proofs to the Corresponding Author only and all persons entering into this Agreement as Author agree that the Corresponding Author shall correct and approve the page proofs on their behalf.

6.2.2 If the Author makes changes other than correcting typographical errors, the Author shall bear all the Publisher's costs of such alterations to proofs including without limitation to alterations to pictorial illustrations. The Publisher shall have the right to charge and invoice these costs plus value added or similar taxes (if applicable) through its affiliated company Springer Nature Customer Service Center GmbH or Springer Nature Customer Service Center LLC, respectively, to the Author, payable within 14 days of receipt of the invoice.

§ 7 Co-operation

Without prejudice to the warranties and representations given by the Author in this Agreement, the Author shall cooperate fully with the Editor and the Publisher in relation to any legal action that might arise from the publication or intended publication of the Contribution and the Author shall give the Publisher access at reasonable times to any relevant accounts, documents and records within the power or control of the Author.

§ 8 Warranty

8.1 The Author warrants and represents that:
(a) the Author has full right, power and authority to enter into and perform its obligations under this Agreement; and
(b) the Author is the sole legal owner of (and/or has been fully authorised by any additional rights owner to grant) the rights licensed in the Clause "**Rights Granted**" and use of the Contribution shall in no way whatever infringe or violate any intellectual property or related rights (including any copyright, database right, moral right or trademark right) or any other right or interest of any third party subject only to the provisions in the Clause "**The Author's Responsibilities**" regarding Third Party Material (as defined above); and
(c) the Contribution shall not contain anything that may cause religious or racial hatred or encourage terrorism or unlawful acts or be defamatory (or contain malicious falsehoods), or be otherwise actionable, including, but not limited to, any action related to any injury resulting from the use of any practice or formula disclosed in the Contribution and all of the purported facts contained in the Contribution are according to the current body of science and understanding true and accurate; and

(d) there is no obligation of confidentiality owed in respect of any contents of the Contribution to any third party and the Contribution shall not contain anything which infringes or violates any trade secret, right of privacy or publicity or any other personal or human right or the processing or publication of which could breach applicable data protection law and that informed consent to publish has been obtained for all research or other featured participants; and
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8.2 The Author warrants and represents that the Author, and each co-author who has entered into this Agreement, shall at all times comply in full with:
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8.3 The Publisher reserves the right to amend and/or require the Author to amend the Contribution at any time to remove any actual or potential breach of the above warranties and representations or otherwise unlawful part(s) which the Publisher or its internal or external legal advisers identify at any time. Any such amendment or removal shall not affect the warranties and representations given by the Author in this Agreement.

§ 9 Author's Discount and Electronic Access

9.1 The Author, or each co-author, is entitled to purchase for their personal use the Work and other books published by the Publisher at a discount of 40% off the list price, for as long as there is a contractual arrangement between the Author and the Publisher and subject to any applicable book price law or regulation. The copies must be ordered from the affiliated entity of the Publisher (Springer Nature Customer Service Center GmbH or Springer Nature Customer Service Center LLC, respectively). Resale of such copies is not permitted.

9.2 The Publisher shall provide the electronic final published version of the Work to the Author, provided that the Author has included their e-mail address in the manuscript of the Contribution.

§ 10 Consideration

10.1 The Parties agree that the Publisher's agreement to its contractual obligations in this Agreement in respect of its efforts in considering publishing and promoting the Contribution and the Work is good and valuable consideration for the rights granted and obligations undertaken by the Author under this Agreement, the receipt, validity and sufficiency of which is hereby acknowledged by the Author.
The Parties expressly agree that no royalty, remuneration, licence fee, costs or other moneys whatsoever shall be payable to the Author, subject to the following provisions of this Clause.

10.2 The Publisher and the Author each have the right to authorise collective management organisations ("**CMOs**") of their choice to manage some of their rights. Reprographic and other collectively managed rights in the Contribution ("**Collective Rights**") have been or may be licensed on a non-exclusive basis by each of the Publisher and the Author to their respective CMOs to administer the Collective Rights under their reprographic and other collective licensing schemes ("**Collective Licences**"). Notwithstanding the other provisions of this Clause, the Publisher and the Author shall each receive and retain their share of revenue from use of the Contribution under Collective Licences from, and in accordance with the distribution terms of their respective CMOs. To the fullest extent permitted by law, any such revenue is the sole property of the Publisher and the Author respectively and, if applicable, the registration and taxation of that revenue is the sole responsibility of the respective recipient party. The Publisher and the Author shall cooperate as necessary in the event of any change to the licensing arrangements set out in this Clause.

§ 11 New Editions

11.1 The Publisher has the sole right to determine whether to publish any subsequent edition of the Work containing an updated version of the Contribution, but only after reasonable consultation with the Author. Once notified by the Publisher that an update of the Contribution is deemed necessary, the Author agrees to deliver an updated manuscript in accordance with the terms of the Clause "**The Author's Responsibilities**" and the other relevant provisions of this Agreement, together with the material for any new illustrations and any other supporting content including media enhancements, within a reasonable period of time (as determined by the Publisher) after such notification. Substantial changes in the nature or size of the Contribution require the written approval of the Publisher at its sole discretion. The terms of this Agreement shall apply to any new edition of the Work that is published under this "**New Editions**" Clause.

11.2 If the Author, for whatever reason, is unwilling, unable or fails (including as a result of death or incapacity) to submit an updated manuscript that meets the terms of this Agreement within the above stated period, then the Publisher is entitled to revise, update and publish the content of the existing edition or to designate one or more individuals (which, where co-authors have entered into this Agreement, may be one or more of the co-authors) to prepare this and any future editions provided that the new editions shall not contain anything that is a derogatory use of the Author's work that demonstrably damages the Author's scientific reputation. In such case, the Author shall not participate in preparing any subsequent editions. The Author agrees that the Publisher shall be entitled but not obliged to continue to use the name of Author on any new editions of the Work together with the names of the person or persons who contributed to the new editions. Should the Author or the Author's successors object to such continuing use then they must notify the Publisher in writing when first contacted by the Publisher in connection with any new edition.

§12 Termination

12.1 In addition to the specific rights of termination set out in the Clause "**The Publisher's Responsibilities**" and the Clause "**The Author's Responsibilities**", either Party shall be entitled to terminate this Agreement forthwith by notice in writing to the other Party if the other Party commits a material breach of the terms of the Agreement which cannot be remedied or, if such breach can be remedied, fails to remedy such breach within 45 days of being given written notice to do so.

12.2 Termination of this Agreement, howsoever caused, shall not affect:
(a) any subsisting rights of any third party under any licence or sub-licence validly granted by the Publisher prior to termination and the Publisher shall be entitled to retain its share of any sum payable by any third party under any such licence or sub-licence;

(b) except where stated otherwise in this Agreement, any claim which either Party may have against the other for damages or otherwise in respect of any rights or liabilities arising prior to the date of termination;
(c) the Publisher's right to continue to sell any copies of the Work which are in its power, possession or control as at the date of expiry or termination of this Agreement for a period of 6 months on a non-exclusive basis.

§13 General Provisions

13.1 This Agreement, and the documents referred to within it, constitute the entire agreement between the Parties with respect to the subject matter hereof and supersede any previous agreements, warranties, representations, undertakings or understandings. Each Party acknowledges that it is not relying on, and shall have no remedies in respect of, any undertakings, representations, warranties, promises or assurances that are not set forth in this Agreement. Nothing in this Agreement shall exclude any liability for or remedy in respect of fraud, including fraudulent misrepresentation. This Agreement may be modified or amended only by agreement of the Parties in writing. For the purposes of modifying or amending this Agreement, "in writing" requires either a document written and signed by both the Parties or an electronic confirmation by both the Parties with DocuSign or a similar e-signature solution. Any notice of termination and/or reversion and, where applicable, any preceding notices (including any requesting remediable action under the Clause "**Termination**") must be provided in writing and delivered by post, courier or personal delivery addressed to the physical address of the relevant Party as set out at the beginning of this Agreement or any replacement address notified to the other Party for this purpose. All such notices shall become effective upon receipt by the other Party. Receipt is deemed to have taken place five working days after the respective notice was sent by post or left at the address by courier or personal delivery. If the Publisher is the terminating Party the notice need only be provided to the address of the Corresponding Author. If the Author is the terminating Party a copy of the notice must also be sent to the Publisher's Legal Department located at Heidelberger Platz 3, 14197 Berlin, Germany.

13.2 Nothing contained in this Agreement shall constitute or shall be construed as constituting a partnership, joint venture or contract of employment between the Publisher and the Author. No Party may assign this Agreement to third parties but the Publisher may assign this Agreement or the rights received hereunder to its affiliated companies. In this Agreement, any words following the terms "include", "including", "in particular", "for example", "e.g." or any similar expression shall be construed as illustrative and shall not limit the sense of the words preceding those terms.

13.3 If any difference shall arise between the Author and the Publisher concerning the meaning of this Agreement or the rights and liabilities of the Parties, the Parties shall engage in good faith discussions to attempt to seek a mutually satisfactory resolution of the dispute. This Agreement shall be governed by, and shall be construed in accordance with, the laws of *the Republic of Singapore*. The courts of *Singapore, Singapore* shall have the exclusive jurisdiction.

13.4 A person who is not a party to this Agreement (other than an affiliate of the Publisher) has no right to enforce any terms or conditions of this Agreement. This Agreement shall be binding upon and inure to the benefit of the successors and assigns of the Publisher. If one or more provisions of this Agreement are held to be unenforceable (in whole or in part) under applicable law, each such provision shall be deemed excluded from this Agreement and the balance of the Agreement shall remain valid and enforceable but shall be interpreted as if that provision were so excluded. If one or more provisions are so excluded under this Clause then the Parties shall negotiate in good faith to agree an enforceable replacement provision that, to the greatest extent possible under applicable law, achieves the Parties' original commercial intention.

The Corresponding Author signs this Agreement on behalf of any and all co-authors.

Signature of Corresponding Author: **Date:**

…… 23-01-2021

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1. **Preprint:**
   A “**Preprint**” is defined as the Author´s version of the Contribution submitted to the Publisher but before any peer-review or any other editorial work by or on behalf of the Publisher has taken place.
   The Author may make available the Preprint of the Contribution for personal and private reading purposes only on any of:
   (a) the Author's own personal, self-maintained website over which the Author has sole operational control; and/or
   (b) a legally compliant, non-commercial preprint server, such as but not limited to arXiv, bioRxiv and RePEc; provided always that once the “Version of Record” (as defined below) of the Contribution has been published by or on behalf of the Publisher, the Author shall immediately ensure that any Preprint made available above shall contain a link to the Version of Record and the following acknowledgement:
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2. **Author’s Accepted Manuscript:**
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4. Any linking, collection or aggregation of reused Contributions from the same Work is strictly prohibited.

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Development of MEMS Sensor for Detection of Creatinine using MIP Based Approach -A Tutorial Paper
Prabhu, Sumedha; Gooneratne, Chinthaka; Mukhopadhyay, Subhas
IEEE Sensors Journal

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