SOLID-STATE BIOSENSOR FOR THE DETECTION OF NUCLEIC ACID AMPLIFICATION

Thesis submitted in fulfilment for the requirement of the Degree of

DOCTOR OF PHILOSOPHY

By

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April 2022

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TABLE OF CONTENTS

DECLARATION BY THE SCHOLAR	iv
SUPERVISOR'S CERTIFICATE	v
ACKNOWLEDGEMENT	vi
ABSTRACT	vii
LIST OF ACRONYMS & ABBREVIATIONS	viii
LIST OF FIGURES	xii
LIST OF TABLES	xvii
CHAPTER 1	1
INTRODUCTION	1
1.1 Background	1
1.1.1 Brief introduction to biosensors	1
1.1.2 Transition metal oxide and their applications	2
1.1.3 Nucleic acid amplifications tests: Need and techniques	3
1.2 Current progress in electrochemical NAATs	4
1.3 Targeted nucleic acid for NAATs in this thesis	4
1.4 Research objectives	6
CHAPTER 2	7
DESIGN AND DETECTION PRINCIPLE OF THE FABRICATED SENSORS	7
2.1 Outline	7
2.2 Design of the sensor	7
2.3 Charge transfer kinetics	9
2.4 Detection principle	10
2.4.1 Introduction to the redox probe	10
2.4.2 DNA (or RNA) interaction with methylene blue	11
2.4.3 Sensing principle	12
CHAPTER 3	13
FABRICATION OF OXYGEN VACANCY MODULATED MNO2 ELECTRODE	13
3.1 Introduction	13
3.2 Experimental protocol:	13
3.2.1 Materials and reagents:	13
3.2.2 Methods:	14

3.2.2.1 Fabrication of MnO2 thin films:	14
3.2.2.2 Assembling the device:	16
3.2.2.3 Preparation of methylene blue solutions	16
3.2.2.4 Physical and chemical characterisations:	17
3.2.2.5 Preparation of circular DNA	17
3.2.2.5.1 1 5'-Phosphorylation:	17
3.2.2.5.2 Ligation:	17
3.2.2.5.3 Exonuclease treatment:	
3.2.2.5.4 Real-time (fluorescence) and electrochemical rolling-circle amplif	ication
(RCA) of DNA:	
3.2.2.5.5 Genomic DNA isolation from <i>S. aureus</i> :	
3.2.2.5.6 Real-time and electrochemical q-PCR amplification:	
3.3 Result and Discussion:	20
3.3.1 Underlying Concept:	20
3.3.2 Characterization of as-fabricated sensing electrode:	
3.3.2.1 Morphological analysis and Film-substrate junction electrical analysis	:22
3.3.2.2 Structural and compositional analysis of MnO ₂ sensing layer:	25
3.3.3 Electrode optimization:	
3.3.3.1 Electrochemical impedance measurement:	
3.3.3.2 Current-voltage measurements:	
3.3.4 Validation of Redox Probe and NAAT Detection with Electrochemical Rea	adout:29
3.3.5 Limit of Detection Study for End-point Electrochemical HRCA and q-PCF	8:35
3.4 Conclusion:	
CHAPTER 4	40
FABRICATION OF TiO2:V2O5 NANOCOMPOSITE BASED ELECTRODE	40
4.1 Introduction	40
4.2 Experimental protocol:	41
4.2.1 Chemicals and Instrumentation:	41
4.2.2 Electrode fabrication and assembly:	
4.2.3 Preparation of methylene blue solutions:	43
4.2.4 Electrochemical measurements:	
4.2.5 Primer optimization, fluorescence, and electrochemical LAMP assays	43

4.2.6 In vitro transcription of SARS-CoV-2 RdRp RNA from plasmid and concentration
assessment
4.2.7 Indirect magnetocapture of SARS-CoV-2 RdRp plasmid DNA and RNA44
4.3 Result and discussion
4.3.1 Underlying Concept:
4.3.2 Structural, compositional, and electrical analysis47
4.3.2.1 X-ray diffraction study:47
4.3.2.2 X-ray photoelectron spectroscopy (XPS) analysis:
4.3.2.3 Surface morphological analysis and film-substrate junction electrical analysis: 53
4.3.3 Electrode optimization:
4.3.3.1 Electrochemical impedance spectroscopy (EIS) study:
4.3.3.2 Electroanalytical response (I-V characteristics):
4.3.4 Probe validation:
4.3.5.1 Electrochemical read-out using fabricated TVO electrode
4.3.5.2 Limit of detection (LOD) study:
4.3.6 Selectivity and specificity studies using pre-concentration method and comparison
with a standard detection technique
4.4 Conclusion
CHAPTER 5
CONCLUSION
5.1 Introduction:
5.2 Overview and conclusion
$5.2.1 \ Oxygen \ vacancy \ modulated \ MnO_2 \ electrodes \ \dots \ 72$
$5.2.2 \text{ TiO}_2$: V_2O_5 based binary metal oxide nanocomposite electrodes73
5.3 Limitations and future scope74
PUBLICATION AND PATENTS94
SYNOPSIS1
AUTHOR BIODATAi

DECLARATION BY THE SCHOLAR

I hereby declare that the work reported in the PhD thesis entitled "Solid-State Biosensor for the Detection of Nucleic Acid Amplification" submitted at Bennett University, Greater Noida, India, is an authentic record of my work carried out under the supervision of Dr Ashvani Kumar. I have not submitted this work elsewhere for any other degree or diploma. I am fully responsible for the contents of my PhD. Theses.

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(Signature of the Scholar)

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SUPERVISOR'S CERTIFICATE

This is to certify that the work reported in the PhD thesis entitled **"Solid-State Biosensor** for the Detection of Nucleic Acid Amplification", submitted by Tanvi Agarkar at Bennett University, Greater Noida, India, is a bonafide record of her original work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.

(Signature of Supervisor)

Dr Ashvani Kumar Associate Professor Bennett University Date 28-04-2022

ACKNOWLEDGEMENT

First and foremost, I would like to express my sincere appreciation to my supervisor, Dr Ashvani Kumar for his support in my Ph. D. program. I was very fortunate to have such an understanding and enthusiastic supervisor. He provided me with complete freedom to work and taught me invaluable research aptitude to aid my future endeavours. It has been an incredible learning experience to work under his supervision. I have benefited immensely from his knowledge as an innovator, advisor, and person.

I am highly obliged to my research advisory committee: Prof. Krishna Thyagarajan, Prof. Rama S. Komaragiri, and Associate Prof. Poulomi Sadhukhan, for their insightful lessons, comments, and encouragement. Also, I would like to extend my sincere acknowledgement to all the faculty members of the Physics department for helping me in some or other way and providing me with an affable atmosphere to work and grow.

A profound sense of gratitude binds me to our collaborator Associate Professor Souradyuti Ghosh for his professional knowledge and efforts in resolving my queries. He was a guiding light throughout the Ph. D. journey. I have always been an admirer of his sincerity and diligent work ethic.

Sincere thanks to all the laboratory technicians from the electronics, mechanical, chemistry, biotechnology, and physics department for their cooperation and assistance. As I have worked in various labs and because of their support, it was a wonderful working experience. I am indebted to my colleagues and friends for their constant care and all the fun we had in these last few years. I thank Bennett University for the scholarship provided to me to conduct my research with financial independence.

Finally, I would like to thank my loving family and friends, for their faith and encouragement throughout my studies. I owe a great deal to my parents who are my pillars of strength and enthusiasm in all my highs and lows. They taught me to be persistent and strive through all the challenges I encounter. My father's trait of being a stickler for documentation helped me tremendously throughout. I share my thesis credit with my mother as I feel she has partially completed this degree with me. All thanks to my sister and my best friends who were constantly there to lift my spirit and handle my breakdowns. Warm regards to my nephew and brother-in-law for all the cheerful appreciation. The presence of all the above-mentioned people has truly made this experience worth living.

ABSTRACT

Rapid detection of infectious pathogens has been a priority set by the World Health Organization in terms of their ASSURED agenda for developing biosensors. Developing an affordable, sensitive, selective, user-friendly, and reliable disease diagnostic tool is the need of the hour. In this context, nucleic acid amplification of genomic DNA or plate growth assay and immunocapture by aptamer/antibody are routine tests performed to identify causative pathogens. The readout of nucleic acid amplification is typically done using agarose gel electrophoresis and real-time fluorescence monitoring. However, these processes are usually lengthy and sophisticated involving high-end instrumentations (real-time fluorescence reader). Even with vast progress made in terms of reducing the size and cost of thermal cyclers, the means to get a rapid readout remains unmet.

Approaching the diagnosis from an electrochemistry pathway is a relatively economical, decentralized, and yet highly sensitive route. This work combines the electrochemical sensing approach with isothermal nucleic acid amplification to achieve an efficacious and robust biosensing device with the aid of an electrochemically active redox probe. The work uniquely employs transition metal oxide (TMO) which is a relatively less explored class of material for nucleic acid amplification detection. TMO having a high degree of tunability in terms of electrical, morphological, and electrochemical properties proved to be an ideal candidate for applications. The TMO based electrochemical bi-electrode sensing devices (EBSD) were fabricated by a cost-effective method and further characterized using several techniques such as XRD, XPS, SEM, and electrical measurements. The optimized electrodes were utilized for sensing end-point nucleic acid amplification using voltammetry, electrochemical impedance spectroscopy (EIS), and amperometry techniques. The devices were tested for sensing various viral and bacterial nucleic acid sequences including dengue virus sequence DNA, Staphylococcus aureus genomic DNA, plasmid DNA and in vitro transcribed SARS-CoV-2 RdRp RNA. The performance was observed to be reliable and comparable to the standard techniques (standard quantitative real-time polymerase chain reaction (qPCR) fluorescence and electrochemical detection using screen-printed electrode). This work provided an economical, and straightforward approach for NAATs with the potential of scaling up via batch-processing.

LIST OF ACRONYMS & ABBREVIATIONS

ATP	Adenosine triphosphate
AR	Analytical reagent
AOS	Average oxidation state
B.E.	Binding energy
CA	Chronoamperometry
CE	Counter electrode
I-V	Current-voltage
DENV	Dengue virus
DTT	Dithiothreitol
EDL	Electric double layer
EBSD	Electrochemical bi-electrode sensing device
ECB	Electrochemical biosensor
EIS	Electrochemical impedance spectroscopy
e-e	Electrode-electrolyte
EEC	Equivalent electric circuit
FESEM	Field-Emission Scanning Electron Microscope
G-L peak	Gaussian-Lorentzian peak
AuNPs	Gold nanoparticle
HRCA	Hyperbranched RCA
IUPAC	International Union of Pure and Applied Chemistry
IEP	Isoelectric point
IPA	Isopropyl alcohol
i-NAATs	Isothermal nucleic acid amplification techniques
LMB	Leuco-methylene blue
LOD	Limit of detection
LRCA	Linear RCA
LAMP	Loop-mediated isothermal amplification
MO-NC	Metal oxide nanocomposite
MB	Methylene blue
MEQ-LAMP	Microfluidic electrochemical quantitative loop-mediated
	isothermal amplification

MEA	Monoethanolamine
NTC	Non-target control
NAATs	Nucleic acid amplifications tests
PDMS	Polydimethylsiloxane
PNK	Polynucleotide kinase
q-PCR	Quantitative polymerase chain reaction
QRCE	Quasi-reference counter electrode
RT-LAMP	Reverse transcription LAMP
RT-PCR	Reverse transcription polymerase chain reaction
RE	Reference electrode
RdRp	RNA dependent RNA polymerase
RCA	Rolling circle amplification
RT	Room temperature
SPEs	Screen-printed electrodes
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
Ag	Silver
SMU	Source measurement unit
SWV	Square-wave voltammetry
ТМО	Transition metal oxide
vraR	Vancomycin resistance associated regulator gene
VTM	Viral transport medium
WE	Working electrode
WE XRD	Working electrode X-ray diffraction

LIST OF SYMBOLS

α	Absorption coefficient
Oads	Adsorbed oxygen
ω	Angular frequency
E	Applied electric field
Eg	Bandgap energy
ΔΙ	Change in peak current
R _{ct}	Charge transfer resistance
χ^2	chi-squared
Z _{CPE}	Constant phase element impedance
R_c	Contact resistance
Q_{dl}	Constant phase element representing electric double-layer
C_t	Cycle threshold values
D_e	Diffusion coefficient of electrons
D_i	Diffusion coefficient of ions
q	Electric charge
$arGamma^*$	Electrode surface covered by the analyte
Je	Electronic current density
μ_e	Electronic mobility
Z_f	Faradaic impedance
F	Faraday constant
C_f	Film capacitance
Q_f	Film constant phase element
R_f	Film resistance
<i>e'</i>	Free electrons
Z _{im}	Imaginary part of impedance
Ji	Ionic current density
μ_i	Ionic mobility
O _{lat}	Lattice oxygen
I ₀	Magnitude of reduction peak current for NTC
I ₁	Magnitude of reduction peak current for target samples
n _e	Number of electrons

Number of ions
Numerical value of the admittance
Oxygen gas
Oxygen vacancy
Peak current
Peak height
Peak separation value
Percent portion
Photon energy
Real part of impedance
R-squared value
Scan rate
Sensing signal
Series resistance
Spin-orbit parameter
Surface area of the electrode
Temperature
Time constant
Universal gas constant
Warburg coefficient
Warburg resistance
Warburg impedance

LIST OF FIGURES

Figure 1.1: Elements of a biosensor1
Figure 2.1: Electrochemical Bi-electrode Sensing Device (EBSD) schematic7
Figure 2.2: (a) Schematic, (b) Picture of PDMS microliter cavity layer
Figure 2.3: Schematic of charge transport kinetics (QRCE: quasi-reference counter electrode, SL: sensing layer, CC: current collector)
Figure 2.4: Redox couple for Methylene blue (MB) redox probe10
Figure 2.5: Schematic illustration depicting MB (blue sphere with positive sign) interacting with nucleic acid in the electrolyte
Figure 2.6: Schematic representation of the sensing principle
Figure 3.1: Schematic for spin coating deposition method
Figure 3.2: Picture of the fabricated electrochemical bi-electrode sensing device (EBSD). a) top view, and b) above front view of fabricated electrode, c) complete EBSD setup during measurements, and d) cross-sectional view with PDMS cavity filled with the electrolyte 16
Figure 3.3: Schematic showing increase in oxygen vacancies with temperature20
Figure 3.4: UV-Visible spectroscopy results. (a) Absorbance spectrum of FM350, FM450, and FM550. (b) Tauc plot of αhv^2 versus hv for direct band gap of MnO ₂ films annealed at 350, 450, and 550°C. Inset: Bandgap values of EBSD device presented w.r.t. the annealing temperature
Figure 3.5: FESEM images of FM350-FM550 surfaces, a) cross-section of FM550, b-c)
FM350 images, d-f) FM450 images, and g-i) FM550 images22
Figure 3.6: Electrical measurements, a) DC conductivity of FM350-FM550 device (inset shows electrical connections for the measurement). b) AC conductivity for FM350 and FM550 at 0 V and 0.4 V. 23
Figure 3.7: Equivalent circuit diagram for AC conductivity measurements
Figure 3.8: XPS spectra for (a, d) Mn 2p _{3/2} ; (b, e) Mn 3s; (c, f) O 1s of FM450 and FM550, respectively

Figure 3.9: Electroanalytical results comparing the performance of FM350, FM450, and FM550 electrodes using EIS and I-V characteristics. (a) EIS Nyquist plots of FM350, FM450,

Figure 3.11: Schematic representation of, (a) circularization in RCA using a target DENV RNA and padlock probe, and (b) mechanism of linear and hyperbranched RCA......30

Figure 3.15: Limit of detection study of RCA and RT-PCR reactions comparing EBSD performance with standard detection methods. (a) Plot of signal s, the %-change in the peak current for different target DNA concentrations (0.002 to 20 pM) w.r.t. NTC from the I-V measurement on FM550 electrode for RCA reaction products for n = 6 EBSD. (b) Relative fluorescence of RCA reaction products w.r.t. NTC for n = 5 samples. (c) Plot of signal s, the

Figure 3.16: Peak current curves of the limit of detection study for (a) RCA reaction. (b) RT-Figure 4.1: Pictures of (a) the fabricated electrochemical bi-electrode sensing device (EBSD) with TiO₂:V₂O₅ nanocomposite sensing layer, and (b) Screen printed electrode......42 Figure 4.2: X-ray diffraction pattern of the TVO electrodes. (a) XRD complete scan for bare FTO, and 0-50%-TVO. (b-e) Deconvolution of 26.5° peak into FTO, V, and Ti peaks for bare Figure 4.3: X-ray photoelectron spectroscopy analysis of the TVO electrodes. (a-f) V 2p3/2 Figure 4.4: X-ray photoelectron spectroscopy analysis of the TVO electrodes. (a-f) O 1s for 0 Figure 4.5: X-ray photoelectron spectroscopy analysis of the TVO electrodes. (a-f) Ti 2p for Figure 4.6: FE-SEM images for morphological analysis of the fabricated electrode of bare Figure 4.7: Thickness and electrical measurements. (a) Measured thickness profiles of 0 to 50%- TVO layers. (b) I-V characteristics of FTO-TVO junction for all fabricated electrodes to Figure 4.8: Electrochemical impedance spectroscopy measured (EIS) study using 100μ M MB for 0 to 50%-TVO electrodes. (a) Nyquist plot, (b) Schematic representation of charge transfer at the electrode-electrolyte interface and its equivalent electrical circuit diagram. (c) Bode plot.

recorded on 20%-TVO electrode using MB solutions. (Inset: magnified view of the data in nA

current range). (b) Calibration curve for MB detection constructed from the measurements presented in panel (a) where the current measured after 100 s was plotted as a function of MB Figure 4.11: Electroanalytical data of 100µM-MB for FTO alone and 20%-TVO deposited Figure 4.12: (a) I-V characteristics of 20%-TVO collected for 100µM MB and buffer alone. (b) Current signal comparison of 20%-TVO electrode and MnO₂ (FM550) electrode for Figure 4.13: Measurement of the electrochemical LAMP products and device reproducibility. (a) Schematic representation of LAMP, electrochemical and standard testing of LAMP products, and the detector sensing principle. (b) SWV (baseline corrected) for NTC and TC (DNA, and RNA) for LAMP reaction (MB concentration 50µM, initial nucleic acid concentration 10^3 copies) averaged over n=3 replicates. (c) Statistical data showing % change before and after amplification for 5 different devices for LMAP reactions averaged over n=3 Figure 4.14: Chronoamperometry of electrochemical LAMP experiment on NTC and plasmid Figure 4.15: Limit of detection (LOD) study for electrochemical LAMP on pure 101 - 104copies of SARS-CoV-2 RdRp plasmid DNA and RNA. (a, d) Tested on 20%-TVO, (b, e) Tested on SPE, (c, f) real-time fluorescence measurement for LOD using LAMP and RT-Figure 4.16: Schematics of indirect magnetocapture method followed by fluorescence or electrochemical LAMP. a). Step 1, 5'-biotinylated probe oligonucleotides incubated with a solution containing target nucleic acid as well as host nucleic acid and polymerase inhibitor results in a probe-target complex. Step 2, incubation with streptavidin magnetic bead causes immobilization of the probe-target complex on it. Step 3, magnetic decantation wash rids the assay of polymerase inhibitors and host nucleic acid. Step 4, in situ LAMP or in situ RT-LAMP generates amplicon. b). LAMP amplicon detection using electrochemistry or real-time PCR-Figure 4.17: LAMP performed on magneto-extracted SARS-CoV-2 plasmid DNA tested on (a) 20%-TVO, (b) SPE, respectively. (c) Real-time fluorescence results for the plasmid DNA. LAMP performed on magneto-extracted SARS-CoV-2 RNA tested on (d) 20%-TVO, (e) SPE, respectively. (f) Real-time fluorescence results for the plasmid RNA. The TC sample in each

LIST OF TABLES

Table 3.1: Bandgap and contact resistance for FM350, FM450, and FM550 electrodes.
Table 3.2: Data fitting parameters for AC conductivity measurement of FM350, FM450, and
FM550 electrodes
Table 3.3: Summary of parameters obtained by fitting EIS Nyquist plot
Table 3.4: EIS fitting parameters of NTC/TC samples
Table 4.1: Valuation of the fabricated TVO-electrode based sensing device
Table 4.2: Crystallite size and film thickness values for 0 to 50 mol% TVO
Table 4.3: Binding energy (B.E.) and percent portion (δ (%)) of the V2p _{3/2} , O1s, Ti2p peaks
for the titanium oxide-vanadium oxide (TVO) nanocomposite films with different Ti mol%. (δ
(%) =peak area*100/total curve area; the area was considered after background subtraction).
Table 4.4: Equivalent circuit parameters for 0 to 50%-TVO electrodes according to the EIS
spectra shown in Figure 4.857
Table 4.5: S(I) (signal %-change with respect to NTC) for electrochemical detection on 20%-
TVO and SPE for $10^1 - 10^4$ copies of pure SARS-CoV-2 RdRp plasmid DNA and RNA64
Table 4.6: S(I) (signal %-change with respect to NTC) for electrochemical LAMP mediated
detection of 100 - 1000 copies of magneto-extracted nucleic acid from aqueous, human
genomic DNA spiked, and serum spiked sample on 20%-TVO electrode and SPE. TC sample
implies non-magneto-extracted LAMP for 1000 copies70

CHAPTER 1

INTRODUCTION

1.1 Background

This chapter will briefly introduce the concept of biosensors, the role of transition metal oxide in biosensing applications, and the need for nucleic acid amplification and detection including its current progress. This discussion will also describe the targeted nucleic acid and finally, the research objectives of this thesis will be presented.

1.1.1 Brief introduction to biosensors

The development of a biosensor is a multidisciplinary field involving researchers from Physics, Chemistry, Biotechnology, and Material Science background with the objective of fabricating more reliable, sophisticated, and economical devices for a wide range of applications [1]. The standard definition for electrochemical biosensor accepted by the International Union of Pure and Applied Chemistry (IUPAC) is "An electrochemical biosensor is a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with an electrochemical transduction element" [2]. As demonstrated in Figure 1.1, the device is a combination of two parts a bio-element and a sensor element (transducer). The bio-element (redox probe, in this case) interacts with the specific analyte (nucleic acid) and the sensor element transduces the resultant changes in the interacting bio-element into a detectable electronic signal. The bio-element is sensitive to the analyte specifically and ideally, do not sense other analyte present in the background. This element can be an enzyme, antibody, probe DNA, or redox probe etc. [1]. An ideal biosensor must possess the following qualities for accurate and reliable performance such as sensitivity (lower detection limit), repeatability and reproducibility, linearity (related to accuracy), highly responsive, and stable over time (long shelf life) [3].



Figure 1.1: Elements of a biosensor

Biosensors can be commonly classified based on the sensing element (i.e., measured parameter). The sensing element varies depending on the type of transducer and the corresponding signal generated. The transducer can be optical (depending on the optical properties e.g., photometric, fluorescence or absorbance), physical (piezoelectric, thermometric), electrical, magnetic, or electrochemical [4]–[8]. Among the various categories, electrochemical transducers are most often studied. In an electrochemical biosensor, an electrical signal is generated as a result of the biochemical reactions between the analyte, bioreceptor, and the transducer. The benefits of using electrochemical biosensors (ECB) are that they can be used in a resource-limited setting, hold equivalent instrumental sensitivity (to standard methods), and are suitable for miniaturization. The frequently employed detection techniques for ECB include amperometry, voltammetry, coulometry, or impedimetry [9], [10]. In the present work, the voltammetry technique has primarily been used, where the resultant current from an applied potential (as a function of time) is measured. Other techniques such as impedimetry (measuring the system impedance), and amperometry (measuring current as a function of time for an applied potential) were also used to obtain the sensing signal. This research work is focused on fabricating electrochemical biosensors by cultivating the potential of transition metal oxides for efficient sensing.

1.1.2 Transition metal oxide and their applications

In the fabrication of various electrochemical biosensors, transition metal oxide (TMO) has proven to be an effective class of material to be employed as electrode modifiers as reported by various researchers [11], [12]. The sensor functionality can be tuned by modifying the surface of the working electrode by nano-structuring [13], nanocomposite formation [14], doping [15], surface functionalization [16] etc., for improved electrochemical properties which can be adapted to the targeted biosensing application. TMO based sensors have significantly lowered the limit of detection, improved analytical performance, shown excellent repeatability, and provided a wider linear range of detection. Another benefit of this class of materials is the possibility of various low-cost synthesis routes like hydrothermal [17], [18] co-precipitation [19], [20], chemical etching [21], or sol-gel [22]–[24] that permits the fabrication of different morphology like nanorods [25], nanospheres [26], nanoparticles [27], or flower-like structures [28].

TMOs are actively being used in numerous applications including catalysis [14], fuel cell [29], supercapacitor [30], gas sensing [31], [32] and biosensing. Exploring the domain of biosensing,

TMO has been employed for sensing a wide range of biomolecules like uric acid, glucose [33], [34], dopamine [35], [36], serotonin [37] etc. The commonly employed TMOs are Fe_2O_3 [38], TiO₂ [39], [40], WO₃ [41], ZnO [42], MnO₂ [43], among others due to their sensitive electrochemical activity. Despite their inexpensive nature and popularity in the biosensing domain, the utility of the transition metal oxides (TMO) in nucleic acid amplifications tests (NAATs) has remained surprisingly under-explored.

1.1.3 Nucleic acid amplifications tests: Need and techniques

Nucleic acid amplification tests (NAATs) are routine biological processes with applications in pathogen detection, identifying food and water contamination, diagnosing genetic diseases, and biowarfare prevention [44]. It is an essential step in biomedical analysis as the amount of DNA found in numerous biological samples is at a concentration low enough that direct detection is not possible [38]. It, therefore, necessitates the intervention of NAATs to make millions to billions of copies of the target nucleic acid followed by quantitative detection. Presently, quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) has been employed as the gold standard of detection owing to its accuracy, and specificity [45], [46]. However, this approach is time-intensive involving a sophisticated sequence of thermal steps and precise temperature control [47], [48], demanding a specialized laboratory with high-priced instruments and trained personnel for operations [16], [46], [49]. These downsides of real-time RT-PCR have spurred the advancement of various isothermal nucleic acid amplification techniques (i-NAATs) such as recombinase polymerase amplification (RPA) [50], [51], rolling circle amplification (RCA) [46], [52] and loop-mediated isothermal amplification (LAMP) [53]–[56].

Currently, the quantification of NAATs is usually done by using a fluorescent DNA binding dye or sequence-specific probe technique [57]–[59]. The dye (e.g. SYBR green) binds to the minor grooves of double-stranded DNA only and emits fluorescence in the bound state with a 1000 folds higher intensity as compared to that of its unbound states [60]. The intensity of the fluorescence is directly proportional to the amplified product [57]. In the probe-based technique, an amplicon sequence-complementary nucleic acid probe carrying a reporter molecule (at the 3' end) and a quencher dye (at the 5' end) is utilized. In real-time PCR, Taq DNA polymerase then cleaves the nucleic acid probe to generate fluorescence to quantify the amount of DNA amplified using NAAT [61]. Alternatively, a molecular beacon with complementarity to the amplicon sequence and carrying a fluorescence/quencher pair could

also be utilised [62], [63]. Such fluorometric methods, though accurate and used as a standard practice, are laborious, lengthy, and expensive that demands skilled hands for operations. An attractive alternative to optical detection has been proposed based on the electrochemistry of the DNA-binding redox probes [64]. Advantageously, electrochemical techniques are sensitive to electrochemically responsive species (redox probes) irrespective of background light and sample colour as in the case of fluorometric methods [65]–[67]. At the same time, such measurements could be carried out using a source measurement unit (SMU) or an electrochemical workstation that is significantly inexpensive compared to real-time PCR instruments.

1.2 Current progress in electrochemical NAATs

The electrochemical biosensors (ECB) for NAAT reported to date commonly uses 3-electrode assembly constituting a working (WE), counter (CE), and reference electrode (RE). Recently, the electrochemical PCR based readout has been utilized in the detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in sewage water [68]. To negate the temperature requirement during amplification isothermal amplification techniques are opted [69]. Electrochemical RCA with MB as redox mediator has been carried out for the detection of synthetic DNA [70] and N and S gene of SARS-CoV-2 RNA [46]. Interestingly, the studies based on ECB predominantly utilized gold or carbon as the working electrode. To state a few, Jiong Zhang[71] et al. employed gold (WE), platinum wire (CE) and Ag/AgCl (RE) for the amplified detection of BRCA-1 mutant DNA [NAAT: gold nanoparticle (AuNPs)-mediated amplification]. Similarly, Naoki Nagatani [72] et al. and Kuangwen Hsieh [73] et al. used carbon (WE), carbon (CE) and Ag/AgCl (RE) for electrochemical monitoring of influenza virus RNA [NAAT: RT- LAMP] and gold (WE), platinum (CE) and platinum (RE) for quantitative detection of pathogenic DNA [NAAT: microfluidic electrochemical quantitative loopmediated isothermal amplification (MEQ-LAMP)], respectively [47], [48]. Despite their inexpensive nature and possibility of various tunable properties, the true potential of the TMO in NAATs is yet to be explored. In this work, TMO was used as an electrochemically active sensing layer for efficient and sensitive detection of various targeted nucleic acid amplification.

1.3 Targeted nucleic acid for NAATs in this thesis

The present work was focused on detecting several bio-analytes using the in-house fabricated electrochemical bi-electrode sensing device (EBSD) starting with the dengue virus (DENV)

serotype-2 sequence using rolling circle amplification (RCA). In the year 2021, dengue virus infections have affected 0.12 million in India and 1.5 million globally and there are no approved drugs currently available in the market [74]–[76]. The virus induces fever in the affected individual at the initial stage and manifests into serious internal organ damage without timely detection and proper care. Hence, early detection of DENV is a must to avoid fatalities. Therefore, an electrochemical approach to isothermally amplified DENV sequence will provide an effective, rapid, and cost-efficient alternative.

Secondly, the analytical sensitivity of the device was tested for detecting the vancomycin resistance-associated regulator gene (*vraR*) gene in *Staphylococcus aureus* (*S. aureus*) using PCR [77]. *S. aureus* is an opportunistic pathogen that is responsible for over 1,20,000 infections and 20,000 deaths in 2017 in the United States alone [78]. Moreover, multi-drug resistant *S. aureus* (MRSA, e.g., N315 stain used for genomic DNA isolation in this study) accounted for 25% of all *S. aureus* infections in European countries [79]. Therefore, it was anticipated that a sensitive electrochemical q-PCR (quantitative polymerase chain reaction) enabling the detection of the *vraR* gene from the MRSA N315 strain genomic DNA would provide a valuable molecular diagnosis tool in *S. aureus* induced bacteremia prevention.

Furthermore, the biosensing ability of EBSD was tested for the detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) plasmid DNA and RNA dependent RNA polymerase (RdRp) RNA. SARS-CoV-2 has been declared a pandemic by the WHO on 11th March 2020, within a few months after the reported first case [80]. The virus has an enormous socio-economic impact worldwide. The SARS-CoV-2 is the etiological agent of coronavirus disease (COVID-19), which has infected around 25,69,66,237 people including 51,51,643 deaths, as of 22 November 2021 (according to WHO) [81]. Hence, Covid-19 diagnostics have been a major priority among healthcare agencies facing challenges like assay quantity, ease in data handling, reagents cost, and process time being the key points of concern [80]. Among various isothermal amplification techniques, loop-mediated isothermal amplification (LAMP) has remained one of the most utilized techniques in terms of NCBI PubMed entries. Using 4 -6 primers and a strand displacement polymerase, LAMP could generate a considerable amount of DNA concatemer amplicon within 30 - 45 min with analytical sensitivities in the order of 10 copies [82]. Besides optical methods, the detection of LAMP amplicons can be executed using an ECB as well. In terms of NAAT molecular diagnostics in resource-limited settings, this opens a route for rapid detection and would be an asset in outbreak regions or remote zones

where centralized facilities are unavailable. In terms of electrochemical detection of SARS-CoV-2, i-NAATs such as RCA have been employed that detect the pathogen with a limit of detection (LOD) in the order of $10 - 10^3$ copies [46], [83]. On the other hand, antigen-based techniques have inefficient LOD in real-life settings (10^6 copies/mL) [84]. LAMP and proven amenability to electrochemical NAAT, it has surprisingly not been utilized in the biosensing of SARS-CoV-2 RNA.

1.4 Research objectives

This work aims to fabricate an electrochemical nucleic acid amplification sensor to minimize sample-to-answer turnaround time with an easy data user interface. The work is focused on designing a novel dual-electrode sensor geometry with a low-cost fabrication process based on transition metal oxides (TMO).

The objectives of this research work are stated below:

- 1. Designing and fabrication of Manganese Oxide based nucleic acid amplification sensor.
 - a. Modulation of conducting states of the working electrode
 - b. Optimization of electrochemical characteristics of the working electrode.
- 2. Detection of dengue virus (DENV) serotype-2 sequence and *vancomycin* resistanceassociated regulator gene (vraR) gene in *Staphylococcus aureus* (*S. aureus*) genomic.
- 3. Implementation of binary nanocomposite for nucleic acid amplification detection.
- 4. Detection of plasmid DNA and in-vitro transcribed SARS-CoV-2 RdRp RNA from,
 - a. Aqueous solution,
 - b. Sample spiked with excess human genomic DNA,
 - c. A serum-spiked viral transport medium (VTM)-mimic sample

CHAPTER 2

DESIGN AND DETECTION PRINCIPLE OF THE FABRICATED SENSORS

2.1 Outline

This work is focused on designing, conceptualizing, and testing an electrochemical biosensor for NAATs. Therefore, in this chapter, the overall design and the underlining detection principle of the fabricated sensors including the biochemistry of the analyte and bio-element will be discussed.

2.2 Design of the sensor



Figure 2.1: Electrochemical Bi-electrode Sensing Device (EBSD) schematic.

The electrochemical bi-electrode sensing device (EBSD) (Figure 2.1) was conceived and fabricated in-house using an inexpensive and simple chemical thin film deposition technique known as sol-gel spin coating. An electrochemical sensing device by convention employees 3-electrode geometry involving a working electrode where the redox reaction of interest occurs, a counter (or auxiliary) electrode to counterbalance the redox reaction, and a standard reference electrode to measure the accurate voltage drop across the working electrode [85]. Contrary to this, employing a 2-electrode geometry with a working and pseudo- or quasi-reference counter electrode (QRCE) holds the following advantages, (i) simplification of the sensor structure, (ii) lowering of the ohmic resistance effect, (iii) elimination of liquid junction potential, and (iv) avoiding contamination of the target solution by the solvent molecules (or ions) of a conventional reference electrode [86].

In the present device, the working electrode was comprised of a conducting substrate (Fluorinedoped tin oxide (FTO)), employed as a current collector, coated with transition metal-oxide (TMO) film that acts as a sensing layer and a silver wire as a QRCE.



Figure 2.2: (a) Schematic, (b) Picture of PDMS microliter cavity layer.

The EBSD was additionally engineered with isolated polymer cavities to hold the test samples during measurements by avoiding contamination. A thin, transparent, and flexible layer of Polydimethylsiloxane (PDMS) was fabricated and integrated onto the working electrode. The PDMS patches were fabricated by mixing the base and curing agent in a ratio of 10:1, respectively in a glass petri dish. The dish was desiccated for 5 min to burst any air bubbles inside the mixture and cured for 30 minutes at 70°C. After ambient cooling, the moulded 2 mm thick PDMS was edged in a 2×2.5 cm² rectangular piece and separate circular cavities of diameter 0.25 cm were punched using a hole punch plier. The cavity serves the bi-fold purpose of providing a uniform contact area for every measurement and preventing evaporation of the test sample. An advantage of using the proposed EBSD in comparison to frequently used commercial screen-printed electrodes (SPEs) is that for every test sample to be evaluated a separate SPE unit would be needed. Therefore, a bundle of SPEs would be required to perform any series measurements or to have a statistical dataset for any nucleic acid amplification tests. Whereas the fabricated 2.5×2.5 cm² working electrode in the proposed EBSD supplied around 30 individual PDMS test cavities. This could be utilized to perform 30 measurements on a single fabricated electrode. Overall, the proposed bi-electrode geometry provides a biosensor assay for implementing multiple measurements on a single working electrode. Figure 2.2 shows the schematic and picture of the fabricated PDMS microliter cavity layer.

2.3 Charge transfer kinetics



Figure 2.3: Schematic of charge transport kinetics (QRCE: quasi-reference counter electrode, SL: sensing layer, CC: current collector).

The kinetics governing the charge transfer mechanism at the electrode-electrolyte (e-e) interface of the EBSD relied on the electrode conductivity and electrochemical activity of the surface. When the working electrode was immersed in the electrolyte, depending on the surface isoelectric point (IEP) and solution pH, an electric double layer (EDL) was formed. The reduced and oxidized species of the electrolyte were in equilibrium and no current was passed [87]. On changing the applied potential, charge imbalance was caused across the interface and that triggered the ions inside the electrolyte solution to rearrange themselves to achieve a new equilibrium position, thereby initiating ionic conduction.

Consequently, the high current was rushed through the system (EDL charging current) and an intense current was recorded in the first cycle of every electroanalytical data measurement. During the voltage sweep, when it was thermodynamically or kinetically favourable at a certain voltage, the electron migrates through the localized states across the e-e interface and a strong current peak (Faradaic current) was observed (Figure 2.3) [88], [89]. The rate of electron transfer at the interface was higher than the rate of diffusion in the bulk of the ionic liquid as a result an electric field was established in the bulk electrolyte. Subsequently, the ions drifted to neutralize the field and negate the corresponding variation of their electrochemical potential. Ultimately, the electrolyte would carry out the ionic conduction, and the localised states at the e-e interface would be responsible for the electronic conduction. The proposed device

measured the change in total current density (J_t) which would be the sum of the contribution from ionic (J_i) and electronic $(+J_e)$ current densities. The current density for ionic and electronic charge carriers is described (in 1-D) by the following equations [89],

$$J_{i} = qn_{i}\mu_{i}E - qD_{i}\frac{\partial n_{i}}{\partial x}$$
$$J_{e} = qn_{e}\mu_{e}E + qD_{e}\frac{\partial n_{e}}{\partial x}$$

Where, q, n, μ , E, and D are the electric charge, the number of charge carriers, carrier mobility, applied electric field and diffusion coefficient, respectively.

2.4 Detection principle

2.4.1 Introduction to the redox probe



Figure 2.4: Redox couple for Methylene blue (MB) redox probe

The electrochemical activity of nucleic acid is inadequate for direct detection, therefore a redox indicator is employed for the purpose [90]. Commonly used redox probes are transition metal complexes (e.g. like $Os[(bpy)_2phen]^{2+}$ [76], ruthenium (Ru) complexes $[Ru(NH_3)_6]^{3+}$ [54], [71], [91]) or organic dyes (e.g. methylene blue (MB) [72], [73], [92]–[94]). Jiong Zhang[71] et al. employed ruthenium hexamine for the amplified detection of BRCA-1 mutant DNA, whereas Naoki Nagatani[72] et al. and Kuangwen Hsieh[73] et al. used MB for electrochemical monitoring of influenza virus RNA and quantitative detection of pathogenic DNA, respectively. Among the probes, MB, whose DNA-modulated redox behaviour was first demonstrated by Prof. Jacqueline Barton [95], has remained among the most suitable NAAT redox mediator to date [53]. Hence, MB was chosen as a redox indicator in this study.

The organic dye methylene blue (MB) is a thiazine class probe. Its formal chemical nomenclature is 3,7-bis(dimethylamino)phenothiazine-5-ium chloride with the molecular formula $C_{16}H_{18}N_3SCl$. MB binds to the double-stranded DNA (dsDNA) with high affinity [96]. When dissolved in water it readily loses a chloride counterion and carries an intrinsic positive charge [97]. It undergoes reversible reduction and forms a redox couple with leuco-methylene blue (LMB) in a two-electron and one-proton transfer reaction (Figure 2.4) [98]. The LMB as suggested by its name is ideally a colourless solution, whereas during measurements due to consecutive voltage cycles the solution was not completely colourless but appeared lighter shade of blue suggesting reversible MB reduction. The reduction of MB generates an intense Faradaic current response which is recorded as a sensing signal for amplification detection.





Figure 2.5: Schematic illustration depicting MB (blue sphere with a positive sign) interacting with dsDNA in the electrolyte.

The interaction between DNA and MB is the basis of the EBSD detection principle. DNA is a biomolecule composed of two chains of polynucleotides intertwined together in a helix structure. The nucleotides consist of a ribose sugar, a phosphate group, and an aromatic base (Figure 2.5) [99]. The sugar and phosphate jointly form the "backbone" of DNA which is negatively charged due to the oxygen-phosphorus bond. Therefore, a non-covalent electrostatic interaction occurs between the cationic MB molecule and the anionic DNA. It is a nonspecific type of interaction that occurs along the strands, outside the double helix of DNA. Also, owing to the planar ring system of MB, it intercalates into the DNA ladder [96], [100]. P. O.

Vardevanyan et al. used absorption spectra to study the MB-DNA interactions based on MB absorption of light and fluorescence. They concluded that the binding is multimodal with one strong binding mode (semi-intercalation) and a weak binding mode (electrostatic). Z.R. Liu et al. in their work on dsRNA-protein interactions by MB-intervened photo-crosslinking mentioned the MB-dsRNA (double-stranded RNA) binding as analogous to MB-DNA interaction [101].



2.4.3 Sensing principle

Figure 2.6: Schematic representation of the sensing principle

The working principle of the proposed device relied on the dynamic electrochemistry at the interface of the sensing layer and the electrolyte. The sensor relies on measuring the Faradaic current response of the "free" redox probe MB. The response would be directly proportional to free probe concentration i.e., for higher concentration intense current would be obtained. During amplification, millions of amplicons (i.e., replicated DNA) are generated that sequestered the "free-to-diffuse" MB [73]. Due to this, the charge transfer at the electrode would be reduced and a lower redox current is recorded. Thus, the EBSD is a transducer that converts nucleic acid amplification into a detectable electronic signal by measuring the redox activity of an electroactive species [72], [73], [90].

CHAPTER 3

FABRICATION OF OXYGEN VACANCY MODULATED MNO₂ ELECTRODE

3.1 Introduction

The electrochemical bi-electrode sensing device (EBSD) has been fabricated based on transition metal oxide (TMO) that is employed as a sensing layer on the working electrode. In this work, the manganese oxide thin film was chosen to act as the sensitive layer to pick up electrochemical signals. Several oxides of manganese such as MnO, MnO₂, and Mn₂O₃ are popularly used for electrode materials in electrochemical sensing [35], [102], [103]. MnO₂ is one of the most common manganese oxides with a wide range of applications in molecular adsorption, energy storage, biosensing, and memory devices [35], [104]–[108]. It has excellent biocompatible properties which makes it a popular candidate in biosensing.

It has been postulated that creating oxygen vacancies in the MnO_2 sensing layer will improve its conduction state and thus enhance the sensitivity of the device towards detecting any minute change in the concentration of DNA binding redox probe. Hence, sol-gel spin-coated MnO_2 is used as the sensing electrode and its electronic conduction states are manipulated by annealing at various temperatures. The device (EBSD) was then utilized in detecting dengue virus (DENV) serotype-2 sequence (using rolling circle amplification or RCA) and vancomycin resistance-associated regulator gene (*vraR*) gene in *Staphylococcus aureus* (*S. aureus*) genomic DNA (using PCR). The EBSD results are then compared with a standard fluorescence readout employing real-time PCR (rt-PCR) instrument.

3.2 Experimental protocol:

3.2.1 Materials and reagents:

All chemicals used in this work were analytical reagent (AR) grade and used without further purification. Manganese acetate Mn (CH₃COO)₂ · 4H₂O, ethoxyethanol (C₄H₁₀O₂), spermidine, dithiothreitol (DTT), methylene blue (MB), and monoethanolamine (MEA) (C₂H₇NO) were purchased from SRL chemicals. Polyethylene glycol (PEG) (C_{2n}H_{4n+2}O_{n+1}) was purchased from CDH chemicals. Fluorine-doped tin oxide (FTO) deposited glass, and 99.99% pure silver

wire were purchased from commercial sources and cleaned with isopropyl alcohol (IPA) before use. Silicone Elastomer Kit comprised of Base/Curing Agent was purchased from SYLGARD 184 to formulate Polydimethylsiloxane (PDMS) layer. Oligonucleotides were purchased from either Eurofin or Sigma with "desalting" purity and then used without any further purifications. The phi29 enzyme, T4 polynucleotide kinase (PNK) enzyme, T4 DNA ligase enzyme, adenosine triphosphate (ATP), dNTP, BSA and Phi29 buffer were procured from New England Biolab, USA. SYBR[™] Green I Nucleic Acid Gel Stain 10,000X was purchased from Invitrogen. PCR mix (with and without SYBR Green I) was purchased from HiMedia, India. Ultrapure water dispensed from a Millipore Milli-Q Type I water purification system, which was then double autoclaved was utilized in preparing all solutions and dilutions. Phosphorylation, annealing, and ligation reactions were performed in an Eppendorf Mastercycler Nexus system. Real-time RCA studies were carried out in Bio-Rad CFX96 rt-PCR and analysed using CFX Maestro software.

3.2.2 Methods:

3.2.2.1 Fabrication of MnO₂ thin films:



Figure 3.1: Schematic for spin coating deposition method

MnO₂ sensing layer was coated onto the FTO substrate using the sol-gel spin coating method. The solution was prepared by dissolving Mn(CH₃COO)₂·4H₂O metal salt in a mixture of ethoxyethanol solvent, MEA and PEG, where MEA acts as a stabiliser and PEG is used as a binder [109]. The solution turned from light pink (due to the metal salt) to cola colour when MEA was added to it. A 0.5 M of precursor solution was prepared and stirred at room temperature (RT) for 30 min on a magnetic stirrer. When the salt is completely dissolved, it is filtered using a 0.22-micron pore size syringe filter and kept aside for ageing for at least 12 hrs at room temperature. The obtained solution was spin-coated onto FTO coated glass substrate.

FTO substrate was partially covered with a physical mask to prevent the deposition of the sensing layer onto the substrate to make electrical contact with FTO. The schematic presentation of the deposition method is shown in Figure 3.1. The amount of precursor solution, rotation speed and deposition time were optimised by initially depositing on a glass substrate. To achieve uniform deposition, spin coating was done in two steps i) 2000 RPM for 10 sec with 5-sec acceleration, ii) 6000 RPM for 180 sec with 10-sec acceleration. The deposition was done within 24 hrs of solution preparation. The deposited films were dried at ~170° for 10 min on a hot plate followed by the annealing at the temperatures of 350, 450, and 550°C, respectively for 1 hr. MnO₂ deposited FTO electrodes annealed at 350, 450, and 550°C are referred to as FM350, FM450, and FM550, respectively.



Figure 3.2: Picture of the fabricated electrochemical bi-electrode sensing device (EBSD). a) top view, and b) above front view of the fabricated electrode, c) complete EBSD setup during measurements, and d) cross-sectional view with PDMS cavity filled with the electrolyte

3.2.2.2 Assembling the device:

A fabricated PDMS patch and the as-prepared electrode were cleaned using IPA, the patch was adhered onto the sample by activating the PDMS using a UV ozone activator. The holes were filled with 5μ L of the desired electrolyte using a micropipette. A silver (Ag) QRCE was immersed in the micro-cavity to complete the bi-electrode geometry. The real-time assembled EBSD sensor is shown in Figure 3.2.

3.2.2.3 Preparation of methylene blue solutions

The methylene blue solutions were prepared in 10 mM Tris-HCl buffer pH 7.5. The buffer itself was made in non-DEPC treated ultrapure Milli-Q type I water (double-autoclaved). It was noted that DEPC treated water led to unwanted redox peaks in I-V experiments. The dilutions of methylene blue (e.g., 100, 50, 25, 12.5 μ M) were carried out using serial dilution using the same buffer as diluent.

3.2.2.4 Physical and chemical characterisations:

The various characterisation tools were utilised to probe the structural, electrical, compositional, and optical properties of the fabricated films. The optical band gap was obtained using an Ocean Optics UV-vis spectrophotometer within the wavelength range of 200-900 nm. The oxidation state of as-synthesized MnO₂ was examined by X-ray photoelectron spectroscopy (XPS) (Physical Electronics, PHI 5000 VersaProbe III) in the energy range of 0-1200 eV using the Al monochromatic X-ray source. The sample surface morphology was studied by Field-Emission Scanning Electron Microscope (FESEM) (FEI Quanta 200 F) at 20kV. Electrochemical impedance spectroscopy (EIS) measurements were performed using Metrohm Autolab Potentiostat/Galvanostat (Model Autolab PGSTAT302N). All the current-voltage measurements were recorded using Keysight B2912A source measurement unit (SMU).

3.2.2.5 Preparation of circular DNA

3.2.2.5.1 1 5'-Phosphorylation:

Precursor ssDNA oligonucleotide 5'-GTC GCT TTT AGA GTA GAT GAG TGC AAG CCT TTA GCG ACG TCC AGT CCG CGA ACC ACA TCA TCC CTA AGT CCA CTA CAC CAT GCG TAC CTC AGC TTT GGA CTG GAC-3' was diluted to 8 μ M. The oligonucleotide was designed so that it would fold itself to a dumbbell shape, thus bringing it's 5'- and 3'- termini adjacent to each other and allowing a self-annealing ligation. The oligonucleotide solution was snap-cooled (5 minutes heating at 95°C immediately followed by 5 minutes incubation on ice) to linearize the DNA for improving the phosphorylation efficiency. The 5'- phosphorylation was carried on the oligonucleotides (final concentration 4 μ M) in presence of T4 PNK (0.375 Units μ L⁻¹), ATP (1 mM), ligase buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 1 mM ATP, 10 mM DTT), DTT (5 μ M), and spermidine (1.7 mM). The addition of all the reagents was performed on ice. The solution was incubated at 37°C for 3 hours followed by annealing by slow (1 h) cooling from 95°C to 4°C (this step also inactivated the T4 PNK).

3.2.2.5.2 Ligation:

Circularization of the annealed 5'-phosphorylated DNA (concentration 3.2 μ M for selfannealing precursor, 1.6 μ M for sticky-end precursors and 3.2 μ M for the padlock) was carried out in presence of T4 DNA ligase (8 Cohesive End Units μ L⁻¹), ATP (1 mM), and T4 DNA
ligase buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 1 mM ATP, 10 mM DTT). The addition of all the reagents was done on ice. The ligation mixture was then incubated at 16°C for 16 hours followed by enzyme inactivation at 75°C for 20 min. The ligation efficiency was checked in 13% denaturing PAGE gel. The actual concentration of the circularized oligonucleotide was calculated using the unligated linear oligonucleotide loaded in the same gel as the reference.

3.2.2.5.3 Exonuclease treatment:

The ligated sample was snap-cooled as described in the phosphorylation section. The exonuclease digestion was carried out in 25 μ L reaction volume as described below. Both exonuclease I (2.4 units μ L⁻¹) and III (12 units μ L⁻¹) were added in 1X exonuclease III buffer (10 mM Bis-Tris-Propane-HCl pH 7, 10 mM MgCl₂, 1 mM DTT). The reagents were added while keeping the centrifuge tubes on ice. The solutions were incubated at 37°C for 4 hours, followed by enzyme inactivation at 85°C for 20 minutes and then stepwise annealing from 85°C to 4°C to generate the desired secondary structure.

3.2.2.5.4 Real-time (fluorescence) and electrochemical rolling-circle amplification (RCA) of DNA:

The RCA reactions were carried out in presence of primer (5'-TAC GCA TGG TGT AGT GGA CT-3', a sequence from DENV serotype-2) and hyperbranched RCA (HRCA) primer (5'-GCG AAC CAC ATC ATC-3'). For linear RCA or LRCA reactions, the oligonucleotide substrates (circular DNA and primer) were first incubated at 30°C for 30 min in 50 mM Tris-HCl (pH 8) and 50 mM NaCl. For hyperbranched RCA or HRCA reactions, an additional HRCA primer was also added to the above mix. The real-time-RCA reactions was carried out in 25 μ L volume in presence of circular DNA (0.02 μ M), primer (concentration 2 fM – 0.02 nM), HRCA primer (0.2 µM, present only for HRCA reactions), dNTPs (1.0 mM), BSA (0.2 μg uL⁻¹), phi29 buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 10 mM (NH₄)₂SO₄, 4 mM DTT), 0.4X SYBR Green I, and phi29 DNA polymerase (0.2 units μL^{-1}). All the reagents except circular DNA, primer, HRCA primer, Tris-HCl (50 mM, pH 8.5), and NaCl (50 mM) were added as a mastermix. The reactions were incubated for 2 h at 30°C and fluorescence intensities were recorded in 2 min intervals. For the electrochemical RCA, the SYBR was replaced with MB (100 µM), and the experiment was incubated for 2 hrs at 30°C followed by inactivation at 70°C for 20 min in an Eppendorf Mastercycler^R nexus system. For measurement of the electrochemical endpoint, the completed reaction was transferred to ice and immediately

subjected to measurement on the EBSD. In these experiments, NTC refers to non-target control, where the LRCA or HRCA reaction was carried out only in presence of circular DNA alone (as well as HRCA primer for HRCA conditions) but in the absence of primer. Target refers to the sample where both circular DNA and primer (as well as HRCA primer for HRCA conditions) was present.

3.2.2.5.5 Genomic DNA isolation from S. aureus:

1 mL of an overnight *S. aureus* (N315) culture in tryptic soya broth (HiMedia) was taken into a 1.5 mL microcentrifuge tube and centrifuged for 2 minutes at 5000 × g to pellet the bacteria. The pellet was re-suspended in 500 µL of lysostaphin solution (200 µg/mL lysostaphin; 20 mM Tris-HCl, pH 7.5; 10 mM EDTA). After incubating at 37°C for at least thirty minutes, proteinase K (HiMedia) was added to a final concentration of 100 µg/mL and incubate at 56°C for an hour. Phenol:chloroform:isoamyl alcohol (SRL) was added to the culture at a ratio of 1:1 followed by centrifugation at max speed (~12,000 - 13,000 × g) for 10 minutes. The aqueous top layer was transferred to a new microcentrifuge tube and NaCl was added to a final concentration of 200 mM. 100% isopropanol (Merck) was added to the culture at a ratio of 1:1 and incubated at -20°C for 30 minutes and centrifuged at maximum speed (~12,000 - 13,000 × g) for 10 minutes g) for 10 minutes followed by removal of supernatant carefully. 1 mL of ice-cold 70% ethanol was added and again centrifuged at maximum speed (~12,000 - 13,000 × g) for 10 minutes and the supernatant was discarded. After air drying, the transparent pellet was resuspended in 100 µL TE buffer.

3.2.2.5.6 Real-time and electrochemical q-PCR amplification:

In each case, the assay was performed in 25 μ l solution. The stated concentration of genomic DNA was added to 1X real-time PCR mix containing Taq DNA polymerase, 0.4 mM dNTP, SYBR Green I (or 50 μ M MB for electrochemical PCR), and 1X Taq buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, (pH 8.3 at 25°C)), 0.4 μ M forward and reverse primer against *vraR* gene (sequences 5'-CCATGAGTTGAAGCCAGATTT-3' and 5'-CCTGCATCTAATGCACGATA-3', respectively) and molecular grade water. All reagents except the template DNA (*S. aureus* genomic DNA) were added as a mastermix. PCR was set at the following settings: 95°C for 180 seconds, then 39 cycles of 95°C for 10 seconds, 52°C for 10 seconds, and 72°C for 30 seconds. For real-time PCR, this was followed by melt curve

analysis. For measurement of the electrochemical endpoint, the completed reaction was transferred to ice and immediately subjected to measurement on the EBSD.

3.3 Result and Discussion:



3.3.1 Underlying Concept:

Figure 3.3: Schematic showing increase in oxygen vacancies with temperature.

The design of the device and geometry has been explained thoroughly in chapter-2 of this thesis. In this work, it was hypothesized that the optimal modulation of the electronic conduction states of the sensing layer made of transition metal oxide would result in the sensitive detection of any electrical change at the electrode-electrolyte interface (e-e interface). This in turn would help achieve the objective of the EBSD i.e., to detect the ionic current arising out of concentration variation of the DNA-binding redox dye. Oxygen vacancies (O_{vac}) were thus introduced into the metal-oxide sensing layer to manipulate their conducting states via annealing at various temperatures (Figure 3.3) [110].



Figure 3.4: UV-Visible spectroscopy results. (a) Absorbance spectrum of FM350, FM450, and FM550. (b) Tauc plot of $(\alpha h\nu)^2$ versus $h\nu$ for direct band gap of MnO₂ films annealed at 350, 450, and 550°C. Inset: Bandgap values of EBSD device presented w.r.t. the annealing temperature.

During the formation of O_{vac} , lattice oxygen (O_{lat}) was converted to oxygen gas (O_2) and led to the generation of free electrons (e') as per the equation given below [111]:

$$O_{lat} \leftrightarrow O_{vac} + 2e' + \frac{1}{2}O_2(g)$$

These additional electrons would thus magnify the electronic conductivity of the sensing layer. These newly generated electrons caused disorder in the lattice and produced new localised energy levels near the conduction band minima [112], [113].

Electrode	Bandgap	Contact resistance
FM350	3.68 eV	3.43 ΚΩ
FM450	3.50 eV	1.20 ΚΩ
FM550	3.33 eV	1.16 KΩ

Table 3.1: Bandgap and contact resistance for FM350, FM450, and FM550 electrodes.

The impact of the generation of oxygen vacancies on the sensing layer can be studied using UV-Visible spectroscopy. UV-Visible spectroscopy was performed to obtain the bandgap value of the sensing layer (MnO₂) annealed at varying temperatures. Spectral data were recorded in absorbance mode as shown in Figure 3.4a. The direct bandgap can be calculated from the Tauc plot from the spectral data that satisfies the following equation,

$$(\alpha h\nu)^n = A\big(h\nu - E_g\big)$$

Where α is absorption coefficient, hv is the photon energy, n corresponds to 2 for direct bandgap, A is a constant and E_g is the bandgap energy. Figure 3.4b shows a Tauc plot of $(\alpha hv)^2$ vs photon energy (hv) for FM350, FM450, and FM550. The intersection of linear extrapolation of the plot with the x-axis provides E_g value as shown in Table 3.1. The extended conduction band was directly reflected in the optical absorption onset in the Tauc plot for the MnO₂ electrodes (Figure 3.4b). It demonstrated a reduction in optical band gap (Figure 3.4b (inset)) with increased annealing temperature to the lowest value of 3.33 eV [114], [115] for FM550 which suggests improved conductivity of the sensing layer. The observations were in consonance with the reported results for ZnO, TiO₂, and SnO₂ [112], [113], [116], [117].

3.3.2 Characterization of as-fabricated sensing electrode:

3.3.2.1 Morphological analysis and Film-substrate junction electrical analysis:



Figure 3.5: FESEM images of FM350-FM550 surfaces, a) cross-section of FM550, b-c) FM350 images, d-f) FM450 images, and g-i) FM550 images.

The surface morphology of the electrodes was studied using FESEM, while the current-voltage (I-V) characteristic was recorded using SMU to study the electrical behaviour of the film and substrate interface. Figure 3.5a depicts a cross-sectional image of FM550 film revealing the film thickness to be 300 nm, while Figures 3.5 b-c, d-f, and g-i show the surface morphology of FM350, FM450, and FM550 electrodes at different magnifications, respectively. For FM450 film strewn micro-rods structure with an approximate average length of 5 μ m along with distributed micro-flower structures were observed. On the other hand, a structural transformation from micro-rod to micro-flowers was observed for FM550. This could be due to the crystalline phase transition of MnO₂ to Mn₂O₃ or the co-existence of more than one phase of manganese oxide. In the case of FM550, micro-flowers of ~0.5 μ m diameter were found to be uniformly distributed across the surface.



Figure 3.6: Electrical measurements, a) DC conductivity of FM350-FM550 device (inset shows electrical connections for the measurement). b) AC conductivity for FM350 and FM550 at 0 V and 0.4 V.

The I-V characteristics of FTO/MnO₂ junction were performed for FM350, FM450, and FM550 electrodes between -0.9 to +0.9 V using 100 μ M-MB and found to be demonstrating weak rectifying behaviour (Figure 3.6a) similar behaviour was reported for WO₃ film deposited on FTO coated glass [118]. The current is limited by the contact resistance comprising of the series resistance of metal oxide film and junction resistance. The contact resistance calculated for a linear region above + 0.25V for FM350, FM450, and FM550 is presented in Table 3.1 with FM550 having the lowest contact resistance. Thus, the electrical property of the electrodes improved with increasing annealing temperature due to the presence of higher oxygen vacancy concentration which was independently confirmed using XPS results discussed later in this

work. Next, the AC conductivity of FM350, and FM550 was measured using electrochemical impedance spectroscopy at DC voltages of 0 and +0.4 V over a frequency range of 1Hz to 1MHz, respectively. The corresponding Nyquist plots ($-Z_{im}$ vs. Z_{re}) (Figure 3.6b) were fitted with an equivalent electric circuit (EEC) having resistance in series (R_s) to a parallel combination of film resistance (R_f) and constant phase element (CPE, Q_f) as shown in Figure 3.7. The fitting parameter values of all the components are shown in Table 3.2. The n-value of all the four plots is very close to "1" indicating the capacitive nature of Q_f . A sharp decline in the R_f and rise of C_f value with increased annealing temperature indicated receding solid-state interface layer resistance of FTO/MnO₂ that have originated due to higher concentration of oxygen vacancies.

Element	Element Parameter		FM350 at 0 V +0.4 V		FM550 at +0.4 V	
Series resistance (R _s \Ω)	R	73.096	198.99	319.98	394.85	
Film resistance (Rr\Ω)	R	1.25M	0.535M	0.139M	90.52k	
Film constant	$Y_0(nS * s^n)$	0.307	0.531	0.144	0.122	
(Qdl)	n	0.97	0.99	0.97	0.98	

Table 3.2: Data fitting parameters for AC conductivity measurement of FM350, FM450, and FM550 electrodes.



Figure 3.7: Equivalent circuit diagram for AC conductivity measurements.



3.3.2.2 Structural and compositional analysis of MnO₂ sensing layer:

Figure 3.8: XPS spectra for (a, d) Mn 2p_{3/2} peak; (b, e) Mn 3s peak; (c, f) O 1s of FM450 and FM550, respectively. The oxidation state of Mn was evaluated using a highly-surface-specific XPS technique for FM450 and FM550 films. The XPS pattern results were fitted with the Gaussian-Lorentzian peak (G-L peak) and are presented in Figure 3.8 following subtraction of the Shirley background. The peak of Mn 2p_{3/2} (Figure 3.8 a, d) was deconvoluted into two G-L peaks at the binding energy (B.E.) values of 638.8 and 640.8 eV, corresponding to Mn^{3+} and Mn^{4+} for FM450 and at 639.2 and 640.45 eV for FM550, respectively [108], [119]. The peak separation value denoted by ΔE_s in Mn 3s spectra corresponds to various oxidation state of Mn i.e., 6 eV for Mn^{2+} (MnO), ≥ 5.3 eV for Mn^{3+} (Mn₂O₃), and 4.7 eV for Mn^{4+} (MnO₂) [120], [121]. Here (Figure 3.8 b, e) the ΔE_s for FM450 is 4.77 eV [122], [123] whereas it is 5.35 eV for FM550 revealing the dominance of MnO₂ in FM450 over Mn₂O₃ in FM550. The variation of the oxidation state from +4 to +5 suggests the absence of oxygen atoms. The surface average oxidation state (AOS) of Mn was calculated using the formula: $AOS = 8.956 - 1.126 \Delta E_s$ [124], [125]. The AOS value reduced significantly from 3.58 to 2.94 suggesting the creation of more oxygen vacancies on the sample surface due to the heat treatment of the sample at elevated temperature.

The O1s (Figure 3.8 c and f) core-level spectra are deconvoluted into P1, P2, and P3 peaks at 527.15, 528.65, and 530.75 eV for FM450 and P1', and P2' peaks at 527.12 and 529.26 eV for FM550. The P1 (P1') peaks attributed to Mn-O-Mn, P2 (P2') and P3 peak corresponds to Mn-O-H and H-O-H, respectively. By comparing the relative area of P2 (37.57) and P2' (43.18) peaks for the two films, a greater hydroxide (OH⁻) concentration was observed in FM550. The superior OH⁻ content results in a highly active surface area that ensures efficient conduction across the interface [126]. Thus, the XPS results conclusively assure improved conduction and sensing response of FM550.

3.3.3 Electrode optimization:



3.3.3.1 Electrochemical impedance measurement:

Figure 3.9: Electroanalytical results comparing the performance of FM350, FM450, and FM550 electrodes using EIS and I-V characteristics. (a) EIS Nyquist plots of FM350, FM450, and FM550 devices with 100 μ M concentration of MB in Tris-HCl buffer (10 mM, pH 7.5). (b) Electrode-electrolyte interface with equivalent

electrical circuit used to fit impedance spectra for Nyquist plots (c) I-V characteristics (baseline corrected) comparing the performance of FM350, FM450, and FM550 devices for 100 μ M-MB concentrations (arrows indicate the direction of the voltage scan). (d) I-V characteristics (baseline corrected) show the impact of various MB concentrations on the performance of FM550. [Inset: MB concentration vs. Log₁₀(peak height) with error bar for n=7 cycles]

To understand the conduction mechanism of sensing electrodes, all three electrodes were tested using EIS in the presence of 100 µM-MB electrolyte under the oscillating potential of 10 mV at a DC voltage of -0.45 V across the frequency limit of 1 MHz to 1 Hz (Figure 3.9a). The EIS data were analysed by fitting an EEC to the FTO-MnO₂-electrolyte system (Figure 3.9b). The familiar Randles circuit was modified by introducing an additional time constant and used as an EEC. Here R_s represents the ohmic solution resistance of the electrolyte, the electric doublelayer capacitance is substituted by a constant phase element (Q_{dl}) $(Z_{CPE} = 1/Y_0(j\omega)^n)$ considering film inhomogeneity, R_{ct} is the charge transfer resistance, and $W (Z_w = \sigma/\omega^{1/2} - \omega^{1/2})$ $j\sigma/\omega^{1/2}$; $\sigma = 1/\sqrt{2} \cdot Y_0$ at $\omega = 1 \ rad/s$) is the Warburg resistance that models the linear ionic charge diffusion towards the electrode. The additional time constant is used to model the FTO-MnO₂ interface since charge carriers primarily travel through the film before interacting with the substrate. It consists of a parallel combination of a capacitor (C_f) and a resistance (R_f) [127], [128]. All the values of equivalent circuit elements are compiled in Table 3.3. The average value of R_s for all the devices is obtained to be ~500 Ω . The carrier lifetime (τ) for any interface represented by an RC circuit can be calculated from the well-known relation, $\tau =$ $R_f * C_f$, (Table 3.3). A decrease in τ -value from FM350 to FM550 is correlated with increased conductivity. The *n*-value of the Q_{dl} for FM350 is 0.68 that portrays capacitive nature of the electrode, whereas FM550 is more resistive in nature with an *n*-value of 0.59.

The FM550 device has a higher slope in the linear section of the Nyquist plot and shorter projection on the real axis as compared to other devices, this suggests lower Warburg resistance and thus faster ion diffusion at the e-e interface [129].

Element	Parameter	FM350	FM450	FM550
Solution resistance (R _s \Ω)	R	346.54	504.37	482.9
Film resistance $(\mathbf{R}_f \mid \mathbf{k} \mathbf{\Omega})$	R	11.50	9.7	5.4
Film capacitance (C _f \µF)	С	0.7	0.75	0.88
Time constant (τ\ms)	$R_f * C_f$	8.05	7.03	4.95
Charge-transfer resistance $(\mathbf{R}_{ct} \mathbf{k} \Omega)$	R	66	5.7	2.53
Double-layer constant phase	$\mathbf{Y}_0(\mu S*s^n)$	3.5	0.105	6.68
element (Q _{dl})	n	0.68	0.665	0.595
Warburg impedance (W)	$Y_0(\mu S * s^{1/2})$	6.50	1.28	1.19

Table 3.3: Summary of parameters obtained by fitting EIS Nyquist plot.

3.3.3.2 Current-voltage measurements:

I-V characteristics of the EBSD with sensing electrodes FM350, FM450, and FM550 were compared to optimise the working electrode. The externally applied voltage was swept from an initial voltage (-1 V) to a final voltage (+1 V) and back for multiple cycles and the average value is presented. The baseline correction was applied by subtracting a straight line connecting the two extremes of the reduction peak. The peak height (H_P) values for FM350, FM450, and FM550 are -2.68 μ A, -3.51 μ A and -4.52 μ A, respectively. The I-V measurements for each electrode have been repeated 3-times and are represented in Figure 3.9c. Superior electrocatalytic activity of FM550 device was observed plausibly due to large surface area of micro-flower structure and enhanced electron-transfer ability due to the higher accessible area, and therefore shorter charge diffusion path [129], [130]. Increased electrical conductivity of FM550 results in shifting of reduction peak voltage (-0.35 V) towards more anodic potential compared to FM450 (-0.4 V) and FM350 (-0.54 V) which suggest easier electron transfer

across the interface. After the superiority of the FM550 electrode has been established, it is therefore utilized for further studies in this work.

3.3.4 Validation of Redox Probe and NAAT Detection with Electrochemical Readout:

To understand electrochemical behaviour, the EBSD with FM550 electrode was tested under varying concentrations of MB ranging as 100, 50, 25, 12.5, and 6 μ M (Figure 3.9d). The reduction peak intensity was found to be decreasing with lower MB concentration due to the reduced electron transfer rate at the e-e interface. The concentration versus peak height (H_p) was plotted in the inset of Figure 3.9d, suggesting a linear range from 25-100 μ M that was used further for sensing application. The I-V measurements were also performed at different scan rates on FM550 using 100 μ M-MB solution (Figure 3.10a). For a reversible reaction, the peak current varies linearly with the square root of the scan rate (i.e., $I_p \propto \nu^{1/2}$) in accordance with the Randles–Sevcik equation which does not seem to be the case here. **Error! Reference s ource not found.**a (inset) shows that $I_p \propto \nu$ which agrees with the following equation of current response for surface absorbed analyte [131],

$$I_p = \frac{n^2 F^2}{4RT} \nu A \Gamma^*$$

Where *n* is the number of electrons exchanged in the redox process, *A* is the surface area of the electrode, and Γ^* is the electrode surface covered by the analyte. There is a shift in the ΔE_p with ν can also be observed from the curve which conclusively describes the process as quasi-reversible in nature [94], [132].



Figure 3.10: Electroanalytical results of FM550 device. (a) I-V measurement at different scan rates (40 to 140 mV/s) for 100μ M-MB on FM550 (Inset: Peak height value presented as a function of scan rate). (b) Comparative I-V measurement for Ag/electrolyte/FM550 and Ag/electrolyte/FTO with 100μ M-MB.

Also, to establish the importance of MnO_2 in the device geometry and on the sensing signal, I-V measurements were performed with FTO without MnO_2 coating and with MnO_2 coating as a working electrode and comparatively presented in Figure 3.10b. It is observed that the analytical signal is 10 times more intense due to the presence of MnO_2 . In this case, the isoelectric point (IEP) of MnO_2 (IEP = 4-5) [133] is higher than the pH value of the electrolyte solution (pH = 7.5). As a result, the surface of MnO_2 is negatively charged that enhancing surface adsorption of positively charged MB molecules



Figure 3.11: Schematic representation of, (a) circularization in RCA using a target DENV RNA and padlock probe, and (b) mechanism of linear and hyperbranched RCA.

There exist four different DENV serotypes that are difficult to distinguish except NAAT reactions or ELISA assays involving costly antibodies [134]. Moreover, cross-infection with different serotypes has also been linked with severe dengue [74]. Therefore, an ultrasensitive NAAT assay that can be carried out at near ambient temperature (30 - 40 °C) without using real-time PCR would be invaluable for sequence-specific detection of DENV serotypes in limited-resource settings. A rolling circle amplification (RCA) nucleic acid-sensing assay, typically performable at 30°C is ideal for this objective [135]. In an RCA assay for detecting DENV from the extracted RNA of a clinical sample, the target DENV RNA would first anneal to a 5'-phosphorylated and 3'-hydroxylated padlock probe (Figure 3.11a). A DNA or RNA ligase would then catalyze the formation of a phosphodiester bond between the phosphorylated and hydroxylated 5'- and 3'-termini, joining them to form a circular DNA. Next, a primer complementary to the circular DNA, a strand displacement DNA or RNA polymerase (most commonly \$\$\phi29 DNA polymerase for 30°C (i.e., at ambient temperature) assay condition), dNTP mix and suitable buffer would be added to the reaction [135] (Figure 3.11b). Sometimes, the long target DNA or RNA strand would itself be digested by the polymerase to act as the complementary primer [136], [137]. Together, this would trigger a linear RCA (LRCA) reaction with a limit of detection in the order of 10^9 copies/mL (pM). A second primer bearing complementarity to the amplicon, called hyperbranched RCA or HRCA primer, would cause HRCA reaction with the capability of detecting as low as 100 - 1000 copies/reaction of target nucleic acid (Figure 3.11b) [135].



Figure 3.12: Circular DNA generation, secondary structure, and primer binding sites. (a) Self-assembly and ligation process to generate the circular DNA used in this study. (b) Secondary structure of the circular DNA used in the RCA experiments in this study, its ligation site, primer and HRCA primer binding site.

To simulate a DENV detecting HRCA assay, a circular DNA (synthesized using self-annealing ligation, Figure 3.12a), an LRCA initiating first primer complementary to the circular DNA, and an HRCA primer (one that is complementary to the amplicon) were designed (Figure 3.12b). The primer carried a DENV serotype-2 sequence that would anneal to the complementary region in the circular DNA. Along with HRCA primer (see location in Figure 3.12b) and ϕ 29 DNA polymerase, this would initiate HRCA reaction mimicking a DENV detecting RCA assay doable at 30°C. The redox probe MB would bind to the DNA electrostatically as well as intercalation into the DNA ladder structure (π -stacking) [138]. For non-target control (NTC) samples, a large amount of free MB was available (due to the absence of any amplicon) and responsible for higher charge transfer at the electrolyte/FM550 electrode interface resulting in a higher magnitude of reduced peak current. Post amplification for

samples containing the primer (target), a larger concentration of amplicons (i.e., replicated DNA) were generated. These amplicons then complexed with MB and consequently decreased the effective MB in the electrolyte solution. Hence, the reduction peak current would drop significantly and enable the detection of DNA amplified samples (Figure 3.13 a and b).



Figure 3.13: Electroanalytical results for NTC and target in LRCA reaction and sensing principle. (a, b) Schematic representation of the electrochemical DNA detection approach. (c) I-V characteristics (baseline corrected) for NTC and target in LRCA reaction (MB concentration 100 μ M, primer concentration 20 nM) averaged over n=5 replicates. (d) EIS Nyquist plots for NC and PC for LRCA reaction (MB concentration 100 μ M, primer

concentration 20 nM; Inset shows the equivalent circuit diagram). (e) Statistical data showing % change before and after amplification for 7 different devices for LRCA reactions averaged over n=5 replicates on each device.

Element	Parameter	NTC	ТС
Solution resistance (R _s \Ω)	R	540	476
Film resistance (R _f \kΩ)	R	0.2	100
Film capacitance (C _f \µF)	С	0.805	19.8
Charge-transfer resistance (R _{ct} \kΩ)	R	10.4	31
Double-layer constant	$\mathbf{Y}_{0}(\boldsymbol{\mu}\mathbf{S}\ast\mathbf{s}^{\mathbf{n}})$	0.9	0.78
phase element (Q _{dl})	n	0.806	0.849
Warburg impedance (W)	$Y_0(\mu S * s^{1/2})$	24.8	26

 Table 3.4: EIS fitting parameters of NTC/TC samples.

Figure 3.13c depicts the endpoint (i.e., after the completion) I-V response of RCA reaction products (primer concentration 20 nM, MB concentration 100 µM, LRCA reaction) averaged over n = 5 replicates. Both the experiments (RCA as well as electrochemical measurement) have been performed at ambient temperature. Therefore, the electrochemical RCA, if successful and sensitive enough, would be highly utilitarian in detecting DENV and its serotypes in limited-resource settings. The EBSD test results for electrochemical end-point RCA have been in agreement with the aforementioned explanation, where the peak current magnitude for NTC samples was \sim 3 times higher than that of target samples with a change in peak current (ΔI) of 6.31 μ A (Figure 3.13c). The EIS measurements have been performed on endpoint LRCA reaction products as shown in Figure 3.13d. The inset of Figure 3.13d shows the EEC diagram. The semicircular section in the high-frequency region corresponds to the electron transfer limited process and subsequent charge transfer resistance (R_{ct}) equals the semicircular diameter. The R_{ct}-value for the target sample is observed three times higher than the value for NTC sample (Table 3.4) which implied that the "free" MB has been captured by replicated dsDNA and a very low amount was available for charge transfer. These results are in synchronization with the proposed hypothesis.

Figure 3.13e presents the signal S(I) for 7 different devices with a target having 20 nM primer concentration (10^{10} initial target copies). The signal S(I) values obtained for all the 7-devices

are found to be more than 35% which assured good sensitivity and reproducibility of the devices. The signal S(I) of the device is calculated as $S(I) = [I_0 - I_1]/I_0 \times 100\%$, where I_0 and I_1 are the magnitude of reduction peak current for NTC and target samples, respectively. This method of quantification has also been formerly adapted in DNA amplification detection by M. Tsaloglou et al. in electrochemical detection of RPA amplified product, and by Won et al. in the detection of real-time electrochemical PCR [54], [65], [139].

Also, the storage ability of the fabricated device was tested with a gap of 6 months (fabricated and tested in February 2020 and re-tested in August 2020). The devices were stored in a storage box inside a vacuum desiccator in an ambient atmosphere.

The device was tested using 100μ M methylene blue solution at room temperature and plotted data is an average of 6 consecutive voltage scans (Figure 3.14). The peak voltage values for both the curves are nearly similar but the current peak value drops to 80% of its initial intensity. However, the intensity of the signal is significantly high for NAAT detection.



Figure 3.14: I-V characteristics of FM550 electrode measured with a gap of 6 months (the arrows indicate the direction of voltage scan).

3.3.5 Limit of Detection Study for End-point Electrochemical HRCA and q-PCR:

The sensitivity of the EBSD using the FM550 electrode was evaluated for end-point electrochemical HRCA and compared with those obtained from rt-PCR instrument-based fluorescence endpoint readout. For endpoint HRCA, the primer carrying the DENV sequence was varied between 2 fM to 20 pM with 100 μ M of MB as the redox probe concentration.

Percentage change in peak current is computed with respect to NTC and the signal 's' for all DNA concentrations of EBSD (Figure 3.15a) was compared with relative end-fluorescence with respect to NTC (Figure 3.15b). The data presented in Figure 3.15a was replicated with six separate devices (EBSD 1 - 6) for each initial target (primer) concentration. Also, the peak current for target copies 0, 2 fM, 0.2 pM, and 20 pM were presented in Figure 3.16a.

Approximate 40% current change has been obtained for the lowest concentration of 2 fM, while 55 - 60% change was detected for 20 fM. The results demonstrate a similar degree of change in peak current for all the devices across the tested concentrations, implying a high degree of reproducibility (Figure 3.15a). When compared with end-point fluorescence measurement (Figure 3.15b), the device showed comparable sensing ability. Conclusively, EBSD has achieved a detection limit of 2 fM (1.2 x 10³ copies/µL) of DENV sequence DNA for endpoint RCA reaction products. The analytical sensitivity of our device was similar to published electrochemical RCA platforms employing methylene blue as the redox probe used for sensing DNA methylation (LoD 1 fM, differential pulse voltammetry) [140] or for *Pseudomonas* aeruginosa 16S rRNA detection (LoD 10 fM, differential pulse voltammetry) [141]. In a reallife detection scenario, an extracted RNA from the clinical sample would be annealed to the padlock probe, followed by ligation (circularization) of the padlock, and subsequently HRCA assay in presence of methylene blue (Figure 3.11). If the target is a long genomic DNA or a long viral RNA, it could either be digested by the ϕ 29 enzymes to generate a primer, or an additional primer complementary to circular DNA would be added [137], [142]. Similar to the demonstrated electrochemical RCA experiment, a drop in current (with respect to negative control) could be utilized the detect and quantify the presence of pathogen nucleic acid.

Next, the electrochemical PCR for detecting *S. aureus* genomic DNA was conducted on $10^1 - 10^4$ copies of *vraR* gene and was compared with the change in cycle threshold values (ΔC_t) obtained from real-time qPCR experiments (Figure 3.15 c and d). Also, the peak current for initial target copies $10^1 - 10^4$ copies were presented in Figure 3.16b. Conventionally, C_t value in qPCR is specified as the cycle at which the amplification-derived fluorescence crosses a baseline threshold. It was observed that the EBSD was able to detect as low as 10^3 copies with a ~12% current change compared to NTC (Figure 3.15c). The analytical sensitivity of the method was comparable to published reports of electrochemical PCR nucleic acid-sensing employing methylene blue as the redox mediator such as the detection of Lambda phage DNA using a microfluidic flow-through device (489 bp, LoD 2 x 10^4 copies, measurement using

square wave voltammetry) [143] or *Chlamydia trachomatis* DNA templates (612 bp, LoD 10³ copies, measurement using square wave voltammetry) [139]. Overall, the limit of detection study conducted on isothermal amplification and conventional q-PCR indicated that the device has similar analytical sensitivity as that of the real-time fluorescence measurement.



Figure 3.15: Limit of detection study of RCA and q-PCR reactions comparing EBSD performance with standard detection methods. (a) Plot of signal s, the %-change in the peak current for different target DNA concentrations (0.002 to 20 pM) w.r.t. NTC from the I-V measurement on FM550 electrode for RCA reaction products for n = 6 EBSD. (b) Relative fluorescence of RCA reaction products w.r.t. NTC for n = 5 samples. (c) Plot of signal s, the %-change in the peak current for different target DNA concentrations (0.002 to 20 pM) w.r.t. NTC from the I-V measurement on FM550 electrode for RCA reaction products w.r.t. NTC for n = 5 samples. (c) Plot of signal s, the %-change in the peak current for different target DNA concentrations (0.002 to 20 pM) w.r.t. NTC from the I-V measurement on FM550 electrode for PCR reaction products for n = 6 EBSD. (d) ΔC_t value of qPCR reaction products w.r.t. NTC for n = 3 samples.



Figure 3.16: Peak current curves of the limit of detection study for (a) RCA reaction. (b) q-PCR reaction.

The proposed work is primarily focused on detecting NAATs and isothermal NAATs conducted on extracted (i.e., pure) nucleic acid samples as a template. In real-life scenarios, however, the pathogen nucleic acid typically remains mixed with host nucleic acid, and sometimes, with additional pathogen nucleic acid as well. To differentiate target nucleic acid from non-target DNA or RNA, one would need to design specific primers that would selectively bind and amplify the target nucleic acid. The resulting amplicon generation and subsequent current drop would signify the presence of target nucleic acid. However, this may not be as effective if the host or non-target nucleic acid remains present in large quantity compared to the target, and the former may therefore lead to a background signal. In those scenarios, a sequence selective pre-concentration of the target nucleic acid could be utilized to remove the non-target nucleic acid. In our future device prototypes, we are therefore integrating a sequence selective pre-concentration of target nucleic acid followed by transition metal oxide electrode mediated electrochemical NAAT for detection.

3.4 Conclusion:

Detection of pathogen nucleic acid sequences through NAAT or isothermal NAAT is of utmost importance in molecular diagnosis. However, such bioanalytical assays are typically carried out using qPCR which predominantly remains confined to centralized labs. Adoption of electrochemical methods of analysing NAATs, on other hand, would decentralize the assays due to the low cost of the instruments involved (i.e., a conventional thermal cycler and electrochemical workstation). In this regard, most of the assays concerning electrochemical readout of NAATs have utilized electrodes made of noble metals that are costly and not commonly available for in-house fabrication. Any electrode materials based on transition metal oxides would in turn be inexpensive with a wide range of tunability owing to their versatile electronic states.

The current work shows that the electronic state of MnO_2 can be successfully tailored by creating O_2 vacancies through annealing at various elevated temperatures. This principle was utilized in fabricating an affordable, speedy, and user-friendly DNA amplicon detection device. This device utilizes bi-electrode geometry to read out a change in current signals resulting from the interaction of DNA with cationic dye MB in amplified samples. Finally, the electrodes were utilized in detecting dengue virus DNA sequence (using electrochemical RCA conducted at 30° C) and *S. aureus* genomic DNA (using electrochemical PCR). Along with 4 – 5 orders of dynamic range, the analytical sensitivity of the device was in the order of 10^3 copies/µL for RCA and 10^3 copies for electrochemical PCR and was comparable to real-time fluorescence measurement assays carried out in rt-PCR instruments.

CHAPTER 4

FABRICATION OF TiO₂:V₂O₅ NANOCOMPOSITE BASED ELECTRODE

4.1 Introduction

In the present work, a metal oxide nanocomposites (MO-NC) based working electrode was fabricated that can improve electronic conduction and further benefit assay performance. In addition, It was hypothesized that a magnetic pre-concentration of target nucleic acid from a real-life mimic sample (containing host nucleic acid and complex biofluid) followed by electrochemical i-NAAT (LAMP in this case) would improve assay selectivity and be compatible with these electrode modifications. COVID-19 outbreak, caused by the SARS-CoV-2 RNA virus, elevates the need for a rapid, reliable, inexpensive, and easily accessible disease diagnostic tool. Therefore, a MO-NC based electrochemical bi-electrode sensing device (EBSD) was fabricated for end-point NAAT detection of SARS-CoV-2 nucleic acid (RdRp plasmid DNA and in vitro transcribed RNA). The working electrode was a fluorinedoped tin oxide (FTO) surface modified by titanium oxide-vanadium oxide (TiO₂-V₂O₅) binary metal oxide nanocomposite that forms the sensing layer. V₂O₅ was chosen to tune the surface charge interaction between the MB and electrode. The fabrication was done by a simple and low-cost technique called sol-gel spin coating, where TiO₂:V₂O₅ (TVO) thin films were spincoated on FTO from a stable mixture of vanadium and titanium oxide precursor solution. The Ti concentration in V₂O₅ was optimized in terms of the electrical and electrochemical response of the electrode.

The EBSD was then utilized to electrochemically detect end-point LAMP readout for SARS-CoV-2 RdRp plasmid DNA and in vitro transcribed RNA in an aqueous solution. Additionally, the sensing device was also applied to detect LAMP performed on magneto-extracted SARS-CoV-2 plasmid and RNA from a) an aqueous solution; b) a sample spiked with excess human genomic DNA, and c) a serum-spiked viral transport medium (VTM)-mimic sample. The EBSD results were compared with the gold-standard rt-PCR instrument's fluorescence readout and electrochemical assays performed on commercially available screen-printed electrodes (SPE).

4.2 Experimental protocol:

4.2.1 Chemicals and Instrumentation:

The chemicals vanadyl (acetylacetonate) (VO(acac)2) ($C_{10}H_{14}O_5V$), 1-butanol (C_4H_9OH), and benzyl alcohol ($C_6H_5CH_2OH$) were procured from SRL chemical, India for the preparation of V₂O₅ precursor solution. Titanium tetraisopropoxide (TTIP) (Ti{OCH(CH₃)₂}₄), and isopropyl alcohol (IPA) were purchased from GLR Innovations and SRL chemicals, India, respectively, for the preparation of TiO₂ precursor solution. Silicone Elastomer Kit, SYLGARD 184, comprised of base and curing agent was purchased from Dow Corning Corporation to prepare Polydimethylsiloxane (PDMS) polymer layer. All chemicals were analytical reagent (AR) grade and used as received without further refinement. Fluorine-doped tin oxide (FTO) deposited glass, and 99.99% pure silver wire were procured from commercial sources and further cleaned with IPA prior to use.

The plasmid construct with RNA dependent RNA polymerase (RdRp) gene with T7 RNA polymerase promoter (4538 bp) was procured from Addgene (plasmid #14567, https://www.addgene.org/145671/). The Bst 2.0 polymerase, RTx enzyme, dNTP, and SnaBI were purchased from NEB, USA. The SYBR I (10,000X concentrated) was purchased from Invitrogen, USA. Molecular biology grade water was purchased from HiMedia, India. The RNase inhibitor was purchased from Takara, India. Streptavidin quoted magnetic beads were purchased from Sigma or Invitrogen (Dynabeads M-280). 5'-biotinylated probe b (5'-[BIO]-AAAAAAAAAAAGGAGCAAGAACAAGTGAGGCCATAATTC, HPLC purified) was purchased from Sigma. Primer oligonucleotides (desalting purified) were purchased from Eurofins or Sigma. The electrochemical studies such as current-voltage measurements were performed at room temperature using Keysight technologies b2901a precision source/measure unit (SMU) controlled by Quick IV Measurement software. The electrochemical impedance spectroscopy (EIS), square-wave voltammetry (SWV), and chronoamperometry (CA) were performed using Metrohm Autolab PGSTAT302N electrochemical workstation. The film thickness was evaluated using the Alpha-Step D-300 stylus profiler. The crystal structures were analyzed using Bruker D8-Advanced X-ray diffraction (XRD). Surface morphology was studied using field-emission scanning electron microscope (FESEM) Nova NanoSEM 450 (FEI). To analyze the surface oxidation state X-ray photoelectron spectroscopy (XPS) was performed using a Thermo Fisher NEXSA surface analyzer (supplied with Al monochromatic X-ray source, 1486.6 eV).

4.2.2 Electrode fabrication and assembly:

The TiO₂:V₂O₅ (TVO) nanocomposite thin films were fabricated by the sol-gel spin coating technique. The V₂O₅ precursor solution was prepared by mixing 1-butanol (C₄H₉OH), and benzyl alcohol (C₆H₅CH₂OH) solvents in the volume ratio of 1:9 and gradually adding 1 wt% of VO(acac)₂ salt into the mixture [144]. The solution was kept overnight stirring at 45°C with a rotation of 350 RPM. The solution colour turns a bottle green after the salt was dissolved [145]. For the synthesis of TiO₂ solution, liquid TTIP precursor was dissolved in IPA to achieve an equimolar solution as of V₂O₅ precursor. The TVO precursor was prepared by adding 0, 10, 20, 30, 40, and 50 mol% of TiO₂ to the V₂O₅ solution.

The thin film was deposited on the conductive FTO coated glass. Before deposition, FTO was cleaned using IPA and subsequently treated with UV-Ozone to remove any surface contamination and make the surface more hydrophilic and suitable for deposition.

Spin coating was performed in two steps, initially at 1000 RPM for 30 sec followed by 2000 RPM for 180 sec. During deposition, FTO/glass substrate was partially covered using a physical mask to make electrical contact directly on FTO. The film was then pre-annealed at 110°C for 10 min. This process was repeated thrice to get the optimized film thickness. Further, the dried film was annealed in the air using a muffle furnace at a temperature of 450°C for 1 hour with a heating rate of 8°*C*/minute. The films with 0, 10, 20, 30, 40, and 50 mol% of TiO₂ are addressed as 0%-TVO, 10%-TVO, 20%-TVO, 30%-TVO, 40%-TVO, and 50%-TVO hereafter in this work, respectively.



Figure 4.1: Pictures of (a) the fabricated electrochemical bi-electrode sensing device (EBSD) with $TiO_2:V_2O_5$ nanocomposite sensing layer, and (b) Screen-printed electrode.

Post-annealing the electrodes, electrical contacts were fabricated using freshly peeled copper wire and silver conducting paste. A thin layer of PDMS was integrated onto the electrode that functions as an electrolyte holding cavity. Figure 4.1a shows pictures of the fabricated and assembled EBSD during measurements, along with commercially purchased screen-printed electrode.

4.2.3 Preparation of methylene blue solutions:

The methylene blue solutions were prepared in 10 mM Tris-HCl buffer pH 7.5. The buffer itself was prepared in double-autoclaved non-DEPC treated ultrapure Milli-Q type I water. It was observed that DEPC treated water results in undesirable redox peaks in electroanalytical experiments. The concentrated methylene blue solution was serially diluted to lower concentrations (e.g., 100, 50, 25, 12.5 μ M) using the same buffer as the diluent.

4.2.4 Electrochemical measurements:

The present device has been tested with a modified electrochemically active $TiO_2:V_2O_5$ nanocomposite sensing layer. The electroanalytical signals were recorded using cyclic I-V measurement (-1 to +1 V and back; scan rate= 40 mV/s), and square-wave voltammetry (0 to -1 V; scan rate= 50 mV/s) for multiple cycles.

4.2.5 Primer optimization, fluorescence, and electrochemical LAMP assays

The experiments concerning the LAMP primer optimization and assays have been described in detail in a separate work from our group currently undergoing peer review [146]). Briefly, the LAMP assay involved 0.4 μ M outer primers, 0.332 μ M forward and backward inner primer, 1 μ M forward loop primers and 0.4 μ M back loop primers in their final concentration [147] (sequences, F3: CGA TAA GTA TGT CCG CAA TT, B3: GCT TCA GAC ATA AAA ACA TTG T, FIP: ATG CGT AAA ACT CAT TCA CAA AGT CCA ACA CAGACT TTA TGA GTG TC, BIP: TGA TAC TCT CTG ACG ATG CTG TTT AAA GTT CTTTAT GCT AGC CAC, Loop F: TGT GTC AAC ATC TCT ATT TCT ATA G, Loop B: TCA ATA GCA CTT ATG CAT CTC AAG G). A 25 μ L LAMP assay also contained the following components in their respective final concentrations: 1× Bst 2.0 DNA polymerase reaction buffer [20 mM Tris-HCl, 50 mM KCl, 10 mM (NH4)2SO4, 2 mM MgSO4, 0.1% Tween-20, pH 8.8], dNTPs (1.4 mM), SYBR I (1× diluted from 10,000X stock), 8 U of Bst 2.0 DNA polymerase, MgSO4 (6 mM), template (plasmid #14567, https://www.addgene.org/145671/ or in vitro transcribed RNA, 1 μ L) or magneto-extracted nucleic acid on beads (2 μ L). For electrochemical LAMP, the SYBR I was replaced with MB (final concentration 50 μ M). For reverse transcription LAMP (RT-LAMP), the reaction was also added with 7U (0.25 μ L) of reverse transcriptase RTx. The fluorescence LAMP and electrochemical LAMP were conducted in real-time PCR (64°C for 1 h, with fluorescence monitoring every 1 min, followed by melting curve analysis) or thermal cycler (64°C for 1 h followed by 85°C for 20 min), respectively.

4.2.6 In vitro transcription of SARS-CoV-2 RdRp RNA from plasmid and concentration assessment

The experiments involving the in vitro transcription of SARS-CoV-2 RdRp RNA from plasmid and its concentration assessment is described in detail in a separate work from our groups currently undergoing peer review [146]. Briefly, the RdRp sequence bearing plasmid (#14567, <u>https://www.addgene.org/145671/)</u> was linearized using the restriction enzyme SnaBI. The linearized plasmid was then treated with in vitro transcription kit, resulting in the generation of RdRp RNA. The RNA was subjected to cDNA formation. Simultaneously, the plasmid was utilized in generating a concentration vs C_t (cycle threshold) standard curve generated using real-time PCR assays (forward primer: ACACAATGGCAGACCTCGTC and reverse primer: CAAAGCTTGGCGTACACGTT). The standard curve was then applied to assess the concentration of the cDNA, and in turn, the in vitro transcribed RNA.

4.2.7 Indirect magnetocapture of SARS-CoV-2 RdRp plasmid DNA and RNA

The working scheme of the indirect magnetocapture (optimized in a separate manuscript from our group [146]) has been illustrated in Figure 4.16. Briefly, 100 - 1000 copies of the target nucleic acid present in a 40 µL aqueous solution, an aqueous solution containing 1 ng human genomic DNA (extracted from MCF-7 cells) or 5% (for RNA magnetocapture) – 10% (for plasmid DNA magnetocapture) v/v fetal bovine serum was incubated with a 5'-biotinylated probe nucleic acid. After heating (65°C 2 min) and incubation in ice (15 min, for RNA) or room temperature (15 min, for plasmid DNA), the solution was incubated with 10 µL streptavidin magnetic beads (15 min). After magnetic decantation wash (buffer composition 5 mM Tris-HCl, 0.5 mM EDTA, 1 M NaCl, pH 7.5, 200 µL, 3 washes), 2 µL of the beads were subjected to LAMP assays as described above.

4.3 Result and discussion

4.3.1 Underlying Concept:

The key factors responsible for the electrochemical activity of transition metal oxides (TMOs) are the electrode-electrolyte interface (e-e interface), electronic conductivity, and ionic diffusion. The TMOs can be tailored to achieve the desired electrochemical performance via various approaches one of which is combining two metal oxides to form a nanocomposite. The metal oxide nanocomposite (MO-NC) can effectively provide more active sites on the electrode surface, improve electronic conduction, and enhance the interfacial surface area [148]. In this work, a binary MO-NC TiO₂:V₂O₅ sensing layer was conceptualized for efficacious detection of nucleic acid by sensing the change in the ionic concentration of the reporter probe (methylene blue) as a result of nucleic acid amplification. It was hypothesized that incorporating TiO₂ into the V₂O₅ host matrix will improve the electrical conductivity of V₂O₅. The higher conductivity improves the charge transfer rate in the sensing layer, resulting in better sensitivity. The proposed device, utilizing a MO-NC, would thus measure minute changes in the electric transport through the system which is a combination of electronic and ionic charge transport.

In addition to the nanocomposite, we sought to explore the effect of surface charge on the electron transfer, and by extension, towards NAAT biosensing. When the working electrode was immersed in the electrolyte, depending on the surface isoelectric point (IEP) and solution pH, an electric double layer (EDL) is formed. For V_2O_5 the IEP is reported to be 1.5-2.3 [149]. Since the electrolyte pH was 8.8, it would cause the methylene blue cations to accumulate at the V_2O_5 surface. The reduced and oxidized species of the electrolyte were in equilibrium and no current was passed [87]. On changing the potential, charge imbalance was caused across the interface and therefore, causing the ions inside the electrolyte solution to rearrange themselves to achieve a new equilibrium position, thereby initiating ionic conduction. To validate the effect of interaction between IEP and electrolyte pH as well as the impact of nanocomposite fabrication, a dual-electrode TMO-based electrochemical readout system has been designed for the nucleic acid amplification tests (NAATs) at low cost, minimal processing, and with a handy user interface.

The proposed electrode geometry is a cost-efficient device costing \gtrless 12/- (\$0.15) per device (Table 4.1) where each device can be used for a single test sample. This cost covers the complete dual-electrode geometry, including the QRCE electrode. In comparison, the gold-

based and carbon-based SPEs would cost ₹ 440 (\$5.78) and ₹ 250[150] (\$3.27), respectively. However, while being a fraction of the cost of its commercial counterparts, the proposed TMO bi-electrode should also match their sensitivity in the NAAT assay.

Chemicals	Bulk Price (₹)	Price of quantity per unit device (₹)	
Vanadyl (acetylacetonate)	4372/100 gm	1.89/0.04 gm	
1-butanol	531/500 ml	3.8/3.6 ml	
benzyl alcohol	888/500 ml	0.71/0.4 ml	
Titanium tetraisopropoxide	4284/500 ml	0.28/0.033 ml	
Isopropyl alcohol	1475/2500 ml	1.74/2.96 ml	
Device Components	Bulk Price (₹)	Price of quantity per unit device (₹)	
Precursor solution	1.442/1000 µl	0.057/40 μl	
FTO	250/30 device	8.33/1 device	
Silver wire	365/2.7 gm	2.7/0.02 gm	
Silver paste	5000/10 gm	0.5/0.001 gm	
Total	Cost:	~₹ 12/- per device	

 Table 4.1: Valuation of the fabricated TVO-electrode based sensing device

4.3.2 Structural, compositional, and electrical analysis



4.3.2.1 X-ray diffraction study:

Figure 4.2: X-ray diffraction pattern of the TVO electrodes. (a) XRD complete scan for bare FTO, and 0-50%-TVO. (b-e) Deconvolution of 26.5° peak into FTO, V, and Ti peaks for bare FTO, 0, 20, 50%-TVO, respectively

Ti content (mol %)	Crystallite Size (nm)	Film thickness (nm)
0	0.243	16.9
10	0.200	18.9
20	0.206	19
30	0.183	21.3
40	0.183	20.3
50	0.215	22.9

Table 4.2: Crystallite size and film thickness values for 0 to 50 mol% TVO

The as-fabricated electrodes were further probed with various characterization tools such as XRD, XPS, SEM, electrical and electrochemical techniques to examine and optimize the electrode for the sensing application. The crystallinity of the sensing layer was investigated by X-ray diffraction (XRD) for a 2θ range of 10-90° with 0.02° step size. The analysis was done in grazing incidence mode ($\theta \approx 0.5^{\circ}$) so that the crystal structure of the deposited thin film (~20nm) could be investigated. Figure 4.2a presents XRD patterns for 0% to 50%-TVO along with bare FTO (for reference). The peaks at 26.7°, 33.9°, and 37.95° are characteristic peaks of FTO (JCPDS #077-0452) corresponding to (110), (101), and (200) orientations, respectively [151]. The peaks labelled in Figure 4.2a with their respective indices belong to the orthorhombic V₂O₅ XRD pattern (JCPDS #001-0359) [152] and characteristic peaks corresponding to any other phases are not observed. This suggests the absence of any secondary vanadium oxide phase within the XRD detection limit. The average crystallite size was calculated with the most intense V_2O_5 peak (010) using the Debye Scherrer equation [153] and found to be 0.205 + 0.022 nm (2.05 Å) (Table 4.2). The peak around 26.5° was observed to be a result of multiple peak overlapping, therefore the peak was separately analyzed in Figure 4.2 (b-h) for the case of bare FTO, and 0 to 50%-TVO. The bare FTO consists of a single peak relating to FTO (110) orientation whereas a second peak corresponding to Vanadium (V) (101) orientation was observed for 0%-TVO (Figure 4.2 b, c). For 10%-TVO a shoulder peak was detected relating to the signature peak of the tetragonal (anatase) TiO₂ phase (JCPDS # 021-1276) [154]. The peak becomes more prominent with the increasing Ti concentration and a separate TiO₂ peak can be observed for 40, and 50%-TVO (Figure 4.2 g, h) as indicated by the (*) symbol in Figure 4.2a. This shows the gradual incorporation of TiO₂ in the parent matrix

of V_2O_5 . The presence of two separate crystalline phases for V_2O_5 and TiO_2 for all the TiO_2 concentrations confirms the formation of metal oxide composite.



4.3.2.2 X-ray photoelectron spectroscopy (XPS) analysis:

Figure 4.3: X-ray photoelectron spectroscopy analysis of the TVO electrodes. (a-f) V 2p3/2 for 0 to 50%-TVO electrodes, respectively.



Figure 4.4: X-ray photoelectron spectroscopy analysis of the TVO electrodes. (a-f) O 1s for 0 to 50%-TVO electrodes, respectively.



Figure 4.5: X-ray photoelectron spectroscopy analysis of the TVO electrodes. (a-f) Ti 2p for 0 to 50%-TVO electrodes, respectively.

		V 2	p3/2		O 1s				Ti 2p				
X	V (IV)		V (V)		Olat		Oads		Ti (III)				
(X%- TVO)	BE	δ_1	BE	δ_2	BE	δ_1	BE	δ_2	2p1/2	Δ			
110)	(eV)	(%)	(eV)	(%)	(eV)	(%)	(eV)	(%)	2p _{3/2}	Δ_{2p}			
0%	515.68	46.01	516.98	53.99	530.08	65.68	530.98	15.31					
100/	10% 516.28	11 51	517.28	58 /0	530.08	64.69	530.88	18.78	19 79	464.08	5.6		
10 /0		41.31	41.31 317.20	36.49					458.48	5.0			
20%	516 18	61 15	61 15	61 15	61 15	517.28	7 28 38 85	5 530 18	63 19	530 78	24.87	464.28	57
2070	510.10	01.15	517.20	50.05	550.10	05.17	550.70	24.07	458.58	5.7			
30%	516.28	58.90	517 /8	/1 10	530.18	8 60 83	92 520.99	20.58	464.38	57			
30 /0	510.20	38.90	517.40	41.10	550.18	00.85	550.88		458.68	5.7			
40.9/	515 78	515 70 50 26 516	516.08	41 74	520.08	520.08 60.40	60 40 520 99	22 62	464.18	5.8			
4070 515.78	13.78 38.20 310.98	41./4	529.98	09.49	+9 330.00	22.02	458.38	5.0					
509/	516.08	16 08 61 61 517 10 20 2	28.28	530.08 68	68.83	530.99	21.22	464.18	57				
3070	510.08	01.01	517.10	20.20	550.08	00.05	550.88	21.22	458.48	5.7			

Table 4.3: Binding energy (B.E.) and percent portion (δ (%)) of the V2p_{3/2}, O1s, Ti2p peaks for the titanium oxide-vanadium oxide (TVO) nanocomposite films with different Ti mol%. (δ (%) =peak area*100/total curve area; the area was considered after background subtraction).

To examine the surface composition and oxidation state of the sensing layer X-ray photoelectron spectroscopy (XPS) was performed. The high-resolution XPS spectra for 0 to 50%-TVO are presented in Figures 4.3, 4.4, and 4.5 for vanadium (V) 2p, Oxygen (O) 1s, and Titanium (Ti) 2p peaks respectively. All the spectral data were fitted using Lorentzian-Gaussian curves following Shirley-type background subtraction (indicated in the "grey" coloured curve). The binding energy (B.E.) values and percent proportion (δ (%)) of various peaks are presented in Table 4.3.

Figure 4.3 (a-f) shows the V2p_{3/2} peak deconvoluted into two peaks at ~516.05 and ~517.2 eV corresponding to V⁴⁺ and V⁵⁺ oxidation state [155]–[157], respectively, this confirmed the presence of mixed oxides of vanadium VO₂ and V₂O₅. In the ambient atmosphere, oxygen gets readily absorbed on the surface leading to higher V⁵⁺ contribution (~54%) in 0%-TVO (Table 4.3). After Ti incorporation, oxidation of V was prevented and the contributions of V⁵⁺ component were therefore reduced to ~39% for 20%-TVO and similar for higher Ti concentration (Table 4.3). The presence of the VO₂ phase was not detected in the XRD analysis. Figure 4.4 (a-f) shows O 1s peak deconvoluted into three peaks at ~530.09, ~530.9 and ~532.3 eV corresponding to lattice oxygen (O_{lat}), adsorbed oxygen (O_{ads}), and V-OH, respectively

[155], [157], [158]. It was complex to assign lattice oxygen peak to V-O species or Ti-O species as both peaks have the B.E. values in the same range[156]. Various experimental observations have suggested that the amount of adsorbed oxygen species is directly proportional to the amount of oxygen vacant sites (O_{vac}) [159], [160]. A DFT study predicted that the O_{ads} species coupled with O_{vac} may possibly be modified to O⁻ [161]. Therefore, a higher concentration of O_{ads} species results in higher electronic concentration that causes improved conduction. From Table 4.2, 20%-TVO has the highest O_{ads} component (24.87%) and must therefore possess optimum electrical conduction. Figure 4.5 (a-f) shows Ti 2p peaks (except for 0%-TVO) deconvoluted into three peaks at B.E. of ~458.5, ~464.2, and ~460.12 eV corresponding to $Ti^{4+} 2p_{3/2}$, $Ti^{4+} 2p_{1/2}$, and $Ti^{3+} 2p_{1/2}$, respectively [158]. The spin-orbit parameter (Δ_{2p}) equals to that of TiO₂ ($\Delta_{TiO_2} \sim 5.7$ eV) and not to Ti-metal ($\Delta_{metal} = 6.1$ eV) this confirmed that the Ti is present in its oxide form (Ti⁴⁺, dominantly) [162]. Also, the shoulder peak of Ti³⁺ indicates the presence of Ti₂O₃.

4.3.2.3 Surface morphological analysis and film-substrate junction electrical analysis:



Figure 4.6: FE-SEM images for morphological analysis of the fabricated electrode of bare FTO, and 0 to 50%-TVO, respectively.


Figure 4.7: Thickness and electrical measurements. (a) Measured thickness profiles of 0 to 50% - TVO layers. (b) I-V characteristics of FTO-TVO junction for all fabricated electrodes to calculate contact resistance.

Next, the surface morphology of the electrodes using FE-SEM measurements was investigated. Figure 4.6 shows the top view FE-SEM image of bare FTO, and 0 to 50%-TVO. The surface morphology of FTO appears compact and covers the entire glass surface. Most grains are irregularly shaped and can be predominantly categorized in grain sizes of ~200 and ~450 nm. It should be noted that this is the standard morphology for the FTO layer over a glass substrate [163], [164]. The as-fabricated 0%-TVO film appears featureless, but as the Ti content increased to 10 mol% a random pattern was visible along with a few "nanostrips" like structures. As the content increased to 20 mol%-Ti the "nanostrips" structure became more prominent having an approximate length of 126 nm. The nanostructured electrode improves the electrochemical reaction rate owing to its increased surface-to-volume ratio, thus increasing the electrode surface active sites to the analyte [16]. Further, with an increase in the Ti content from 30 to 50 mol%, the surface morphology appears to be more granular.

The layer thickness was evaluated using the surface profilometer and the corresponding surface profiles and thickness values are presented in Figure 4.7a and Table 4.2, respectively. It was observed that the layer thickness increased very marginally with Ti content and the average thickness was 19.88 nm with a very slight deviation of \pm 2.09 nm from the mean value. This implies that any electrical or electrochemical characteristics of the deposited layer would not be a consequence of its thickness.

To study the impact of increasing Ti content on the electrical character of the sensing layer, current-voltage (I-V) characteristics of TVO/FTO junction were recorded for all the variants using SMU (Figure 4.7b). The voltage was swept in the range of -1 to +1 V and the measured current value presented was averaged over n = 3 replicates. The slope of the I-V curves was observed to be increasing as the electrode composition varied from pristine V_2O_5 (0%-TVO) to 20 mol% Ti-content V₂O₅ (20%-TVO), and again reduced to a lower value for higher Ti mol%. The curves are all nearly linear throughout excluding a small range from -0.35 to +0.35V. The calculated contact resistance (R_c) values on either side of +0.35 V are distinct, with the R_c value reducing at higher voltage (Figure 4.7b (inset plot)). This indicates the presence of an interfacial Schottky barrier at the TVO/FTO junction that required 0.35 V to overcome. The curve for 20%-TVO shows a nearly-ohmic behaviour with a R_c value nearly equal on either side of +0.35 V. This implied that optimal electrical conduction has been achieved with 20 mol% Ti as suggested by the XPS results. This could be the result of an interplay between the Ti acceptor lowering the number of charge carriers and Ovac introducing donors into the lattice matrix. As reported by XPS data 20%-TVO had the highest O_{ads} percent proportion implying the highest content of Ovac (Table 4.3). Overall, the electrical conductivity analysis was in agreement with the XPS analysis.

4.3.3 Electrode optimization:





Figure 4.8: Electrochemical impedance spectroscopy measured (EIS) study using 100μ M MB for 0 to 50%-TVO electrodes. (a) Nyquist plot, (b) Schematic representation of charge transfer at the electrode-electrolyte interface and its equivalent electrical circuit diagram. (c) Bode plot.

Electrochemical impedance spectroscopy (EIS) was performed to electrically map the sensor geometry using passive circuit elements to facilitate the analysis of various phases of charge transfer that respond at different AC frequencies. Considering the entire system, intrinsic resistance (R_s) of the electrolyte, double-layer capacitance (C_{dl}) resulting from the passive ions that alter the surface charge distribution, and Faradaic impedance (Z_f) encountered by the active ions during charge exchange at the e-e interface were anticipated. Figure 4.8a shows EIS data (Nyquist plot) for all the six electrodes fitted to an equivalent electrical circuit (EEC) presented in Figure 4.8b. As shown in the EEC, Faradaic impedance (Z_f) is mapped using a series combination of charge transfer resistance (R_{ct}) and Warburg impedance (W). The EEC is the modified version of the well-known Randles circuit with an additional time constant to model the sensing layer [128], [165]. Since the sensing layer was semiconducting in nature, it contributed a considerable layer resistance. It has been observed that only after incorporating the layer/film impedance, the experimental data and the fit could reach a good agreement. The layer/film impedance consists of a parallel combination of resistance (R_f) and a constant phase element (CPE, Q_f). The Q_f accounted for surface inhomogeneity and roughness of the sensing layer.

Element	Parameter	0%	10%	20%	30%	40%	50%
$R_{s}(\Omega)$	R	440.24	609.15	307.06	619.03	499.51	529.87
$C_{dl}(\mu F)$	С	3.12	7.34	4.24	5.60	6.14	7.15
$R_{ct}(\Omega)$	R	917	858	352	430	677	760
W	$Y_0(\mu S * s^{1/2})$	7.84	21.1	44.4	26.1	16.2	32.6
0	$Y_0(\mu S * s^n)$	39.2	48.3	54.1	34.0	55.3	56.3
∠ _f	n.	0.50	0.52	0.53	0.57	0.49	0.45
$R_{f}(k\Omega)$	R	9.343	1.233	0.59	1.152	1.403	1.243
	χ^2	0.012	0.006	0.016	0.006	0.044	0.008

Table 4.4: Equivalent circuit parameters for 0 to 50%-TVO electrodes according to the EIS spectra shown inFigure 4.8.

All the fitting parameter values have been presented in Table 4.4 for electrodes with various Ti content. The R_{ct}, for all electrode variants, contributes only a small portion to the total resistance, while the layer resistance (R_f) dominantly defines the final resistance to charge exchange. The lowest R_f value for 20%-TVO (590 Ω) indicates the highest conductivity and easier charge transfer at the e-e interface. The admittance (Y₀) value for both W and Q_f are highest for 20%-TVO, supporting good conductivity. The average n-value of Q_f for all the electrodes is 0.51 (± 0.045) which shows an equal resistive and capacitive component in the characteristics of a Q_f supporting its inevitability. The fitting applicability and a lower χ^2 value (Table 4.4) validates the chosen EEC. The Bode plots for all the electrodes are shown in Figure 4.8, demonstrating good agreement between the experimental and fitted data. The impedance value indicated by the curve is consistent with that observed by the Nyquist plot having the lowest value for 20%-TVO.

4.3.3.2 Electroanalytical response (I-V characteristics):



Figure 4.9: (a) I-V characteristics (baseline corrected) evaluating the performance of 0% to 50% TVO devices for 100 μ M MB (arrows indicate the direction of the voltage scan). (b) I-V characteristics (baseline corrected) showing the response of 20%-TVO towards various MB concentrations, each curve is an average of n=5 cycles (inset: calibration curve showing MB concentration vs. peak height (H_P)).

Furthermore, all the fabricated electrodes with varying Ti content were tested for their electroanalytical performance towards MB reduction. The I-V measurements were performed using SMU by sweeping a voltage from -1 V to +1 V and back for 6 consecutive cycles and the average current values for 5 cycles is plotted. The first cycle was not considered because of the high current intensity dominantly due to electric double layer (EDL) charging. Figure 4.9a shows I-V characteristics of 0% to 50% TVO for 100 μ M MB after subtracting a baseline connecting the two extremes of the reduction peak around -0.36 V. The peak intensity was lowest for pristine V₂O₅ (0%-TVO), and it increased linearly to its maximum value for 20%-TVO and again gradually decreased for higher Ti content. This was in continuation of the same trend observed for electrical conductivity and EIS measurements. The superior electrochemical activity of 20%-TVO was plausibly due to the optimum electronic conductivity of the electrode that aided the efficient charge transfer at the e-e interface. Based on the excellent structural, electrical, and electroanalytical properties of 20%-TVO, thus this electrode was selected to be utilized in the sensing application.

4.3.4 Probe validation:



Figure 4.10: Calibration curve for concentrations (100-12.5 μ M) of methylene blue (MB) in 10 mM tris-HCl buffer using chronoamperometry. (a) Chronoamperometry measurements recorded on 20%-TVO electrode using MB solutions. (Inset: magnified view of the data in nA current range). (b) Calibration curve for MB detection constructed from the measurements presented in panel (a) where the current measured after 100 s was plotted as a function of MB concentration.

To evaluate the electrochemical interaction of methylene blue (MB) with 20%-TVO I-V characteristics (Figure 4.9b) and chronoamperometry (CA) (Figure 4.10a) were recorded for various concentrations of MB (100, 50, 25, and 12.5 μ M). For I-V characteristics, the cathodic current peak was considered for analysis and the corresponding voltage value was applied for recording CA for 300 sec. The high probe concentration enhances the ionic conductivity of the system which therefore yields a higher current signal (-10.78 μ A for 100 μ M MB) (Figure 4.9b). The peak current intensity reduces linearly with probe dilution to -1.053 μ A for 12.5 μ M MB. The inset Figure 4.9b is a plot of absolute peak current value versus MB concentration including trendline and R-squared value (R² = 0.99) suggesting linear relation over the complete concentration range. It was also supported by the linearity of the calibration curve (Figure 4.8b) obtained from CA data (R² = 0.98).

Additionally, the I-V characteristics of 20%-TVO and FTO alone as working electrode for MB $(100\mu M)$ were presented (). This affirmatively claim that the Faradaic current peak is originated due to the charge transfer between MB and TVO sensing layer and not due to the current collector FTO.



Figure 4.11: Electroanalytical data of 100µM-MB for FTO alone and 20%-TVO deposited FTO.



Figure 4.12: (a) I-V characteristics of 20%-TVO collected for 100μ M MB and buffer alone. (b) Current signal comparison of 20%-TVO electrode and MnO₂ (FM550) electrode for 100μ M-MB solution (the data presented is an average of n=5 cycles).

Also, analyzing the I-V characteristics of 20%-TVO for MB (100μ M), and buffer alone solution revealed the presence of considerable electrical double layer (EDL) capacitance (non-Faradaic current) as shown in Figure 4.12a [131]. To reduce the contribution of this charging current and to improve Faradaic current sensitivity toward probe concentration, the square wave voltammetry (SWV) technique has opted for NAATs detection [166].

Furthermore, the electroanalytical response of the proposed 20%-TVO electrode was compared with that of our previously reported MnO_2 bi-electrode system [167]. As mentioned earlier, the lower IEP (1.5 – 2.3 [149]) of the TVO layer should attract more positively charged MB from the electrolyte (pH 7.5 for buffer and pH 8.8 for amplification) compared to the MnO₂ system

(IEP 4 – 5 [133]). In principle, this should cause a higher current signal for the former. To validate this hypothesis, the electroanalytical response (I-V characteristics) of 20%-TVO with our previously reported MnO₂ electrode (FM550) was compared for 100 μ M concentration of MB (Figure 4.12b). The experiment demonstrated that the MB reduction peak intensity of FM550 was merely 43% of that of 20%-TVO, thus clearly validating the proposed hypothesis about the enhanced electrochemical activity of the present electrode using the IEP-based electrode charge modulation.

4.3.5 NAATs detection of LAMP products





Figure 4.13: Measurement of the electrochemical LAMP products and device reproducibility. (a) Schematic representation of LAMP, electrochemical and standard testing of LAMP products, and the detector sensing

principle. (b) SWV (baseline corrected) for NTC and TC (DNA, and RNA) for LAMP reaction (MB concentration 50μ M, initial nucleic acid concentration 10^3 copies) averaged over n=3 replicates. (c) Statistical data showing % change before and after amplification for 5 different devices for LMAP reactions averaged over n=3 replicates on each device.

After characterization and optimization, the 20%-TVO electrode was used for electrochemical detection of SARS-CoV-2 RdRp plasmid DNA and in vitro transcribed RNA in an aqueous solution using isothermal amplification (LAMP). In our separate manuscript currently under peer review [146], the LAMP primer optimization, in vitro transcription, and RNA concentration determination were assessed. In that manuscript, the utility and optimization of an indirect magnetocapture of SARS-CoV-2 nucleic acid was demonstrated followed by its real-time fluorescence and electrochemical (SPE-based) LAMP-mediated detection [146]. As stated earlier, this work aims to investigate if the TVO bi-electrode could be utilized to detect electrochemical LAMP and whether it has a similar performance as that of real-time fluorescence and SPE electrodes.

Figure 4.13a schematically represents (a.1) the LAMP amplification, (a.2) the tools used for detecting the amplification, and (a.3) the sensing principle. In the case of no template control (NTC, negative control), the amplicon concentration was very limited due to the absence of a template. The high redox current so obtained was generated due to the rapid diffusion of free MB molecules at the electrode surface. For the case of template control (TC, i.e., positive control) sample, millions of amplicons were produced after amplification that interacts with MB molecules via intercalation into amplicon, hydrogen bonding [168], and electrostatic interactions between the cationic MB molecule and negatively charged phosphate DNA backbone [169]. Thus, the amplicon sequestered the available MB which consequently reduces the current signal. Figure 4.13b presents the sensor response for NTC and TC reaction products with the initial concentration of 10^3 target copies for both DNA and RNA. The electrochemical amplification response was gauged based on absolute current magnitude for the target analyte and NTC sample as shown in Figure 4.13b. A significant change in cathodic peak current (ΔI) was observed for both DNA (1.87 μ A) and RNA (2.56 μ A), the data points and the error bars represent the average of 3 consecutive cycles and the corresponding standard deviation, respectively. For the current change-based detection, a sensing signal S(I) was defined to calculate percent change in the magnitude of current after amplification,

$$S(I) = \frac{I_0 - I_1}{I_0} * 100 \%$$

Where, I_0 and I_1 denotes peak current value for NTC and TC, respectively. The signal S(I) and absolute current response (for a target analyte and NTC) were hence used to evaluate sensor capability for NAATs as well as a qualitative measure of the degree of amplification. The S(I) was expected to normalize the current magnitude difference bi-electrode set-up and the SPE electrode. The NAAT was performed on 5 separate sensing devices for DNA and RNA each (with 20%-TVO as a working electrode) for statistical analysis (Figure 4.13c). For the case of plasmid DNA amplification, the average %-change (S(I)) was observed to be 26.67% (\pm 7.77) whereas, it was 45.71% (\pm 6.04) for RNA amplification. The coefficient of variation was 13.2% and 29.1% for RNA and DNA LAMP detection, respectively. Another approach for NAAT is using chronoamperometry (CA, Figure 4.14), where the Δ I value was calculated at the 100th second after the current reached its saturation value for NTC and TC (with plasmid DNA) was 394 nA. While the Δ I from CA can also in principle be used as a measure for detection, it was not further explored in the present work.



Figure 4.14: Chronoamperometry of electrochemical LAMP experiment on NTC and plasmid DNA with 10³ initial target copies.



Figure 4.15: Limit of detection (LOD) study for electrochemical LAMP on pure $10^1 - 10^4$ copies of SARS-CoV-2 RdRp plasmid DNA and RNA. (a, d) Tested on 20%-TVO, (b, e) Tested on SPE, (c, f) real-time fluorescence measurement for LOD using LAMP and RT-LAMP, respectively. Error bars represent standard deviation.

Table	4.5: S(I) (signal	%-change with	respect to l	NTC) for ϵ	electrochemical	detection on	20%-TVO	and S	SPE for
$10^{1} - 10^{1}$	10 ⁴ copies of pure	e SARS-CoV-2	RdRp plasr	nid DNA a	and RNA.				

Target	S(I) for	r DNA	S(I) for RNA		
Concentration	20%-TVO	SPE	20%-TVO	SPE	
10	9.99 <u>+</u> 2.5	6.98 ±2.3	14.20 ± 4.8	25.25 ± 0.5	
10 ²	14.30 ±4.9	14.39 ±1.5	20.52 ± 1.4	28.97 ± 1.8	
10 ³	25.21 ±2.6	17.31 ±2.4	41.02 ± 6.3	38.01 ±1.6	
104	30.87 <u>+</u> 4.7	18.64 ±1.2	55.10 ±6.5	38.00 ±2.5	

The device sensitivity for the end-point measurement was tested for a range of $10^1 - 10^4$ initial target copy number of SARS-CoV-2 RdRp plasmid DNA and in vitro transcribed RNA. It should be noted that the SPE and real-time fluorescence measurement data in this work is independent of our other manuscript focusing on the magnetocapture aspect [146]. The sensing

response for SWV measurement employing the 20%-TVO electrode was then compared with that of a commercially purchased screen-printed electrode (SPE) and real-time fluorescence (Figure 4.15). At the same time, the S(I) (%-change) of the current signal (with respect to NTC) was evaluated for the 20%-TVO electrode and SPE (Table 4.5). In general, the current response for the TVO electrode was less than that for SPE electrodes for the same concentration of nucleic acid. This could possibly be due to the semiconducting nature of the TVO electrode as opposed to conductive carbon electrodes present in SPE. Expectedly, it was observed that the absolute current decreases with increasing initial target copies while the %-change increases at the same time. As the amplification efficiency would be directly correlated to initial target concentration, increasing initial target copies would increase amplicon concentration that in turn would bind and reduce free MB in solution. This would then decrease the electron transfer and resulting current, increasing S(I) with respect to NTC (Table 4.5). Despite the lesser current signal for the TVO electrode, the S(I) was similar for both TVO and SPE electrodes, indicating comparable performance. The sensing response of 20%-TVO and SPE was comparable for electrochemical LAMP performed on plasmid DNA and in vitro transcribed SARS-CoV-2 RNA-dependent RNA polymerase (RdRp) RNA (Figure 4.14 and Table 4.5). Here the limit of detection (LOD) was defined as the lowest initial target copies for which the %-change in the peak height is at least 10%. Therefore, the LOD of 20%-TVO for SARS-CoV-2 RdRp plasmid DNA was 10^2 copies (2.5 copies/µL) whereas for in-vitro transcribed RNA was 10^1 copies $(0.25 \text{ copies}/\mu\text{L})$. In addition, these experiments also demonstrated that the proposed 20% TVO biosensor has a comparable sensitivity as that of the commercial electrode.

4.3.6 Selectivity and specificity studies using pre-concentration method and comparison with a standard detection technique



Figure 4.16: Schematics of indirect magnetocapture method followed by fluorescence or electrochemical LAMP. a). Step 1, 5'-biotinylated probe oligonucleotides incubated with a solution containing target nucleic acid as well as host nucleic acid and polymerase inhibitor results in a probe-target complex. Step 2, incubation with streptavidin magnetic bead causes immobilization of the probe-target complex on it. Step 3, magnetic decantation wash rids the assay of polymerase inhibitors and host nucleic acid. Step 4, in situ LAMP or in situ RT-LAMP generates amplicon. b). LAMP amplicon detection using electrochemistry or real-time PCR-based fluorescence readout depending on the amplification mastermix composition.

Moreover, the amenability of the 20%-TVO electrodes was investigated with a magnetoextraction mediated pre-concentration of target SARS-CoV-2 nucleic acid. So far TVO device platform has demonstrated analytical sensitivity of 10^2 and 10^1 copies for plasmid DNA and in-vitro transcribed RNA, respectively, using pure nucleic acid as a template and has comparable performance as that of commercial SPE as well as real-time fluorescence. However, a real-life sample is unlikely to contain pure nucleic acid alone and usually remains present with polymerase inhibitor biomolecules (such as heme and immunoglobulin G [170]) and host nucleic acid, affecting the detection sensitivity and specificity. While traditional realtime PCR NAAT assays circumvent these factors using prior nucleic acid extraction and sequence-specific TaqMan probes, both add to the cost of the experiment. To investigate the applicability of 20%-TVO electrodes in biosensing NAAT of real-life mimicking sample containing polymerase inhibitors and host nucleic acid, the device was used in detecting a sequence-specific magnetic preconcentration of clinically relevant 10² and 10³ copies of target nucleic acid followed by in situ electrochemical LAMP. In an indirect magnetocapture (Figure 4.16), the biotinylated probe oligonucleotide anneals to the target nucleic acid which is then immobilized on a streptavidin magnetic bead [171]. A magnetic decantation wash then removes the polymerase inhibitors and host nucleic acid from the solution. The optimization of an indirect magnetocapture has been demonstrated followed by real-time fluorescence and electrochemical (SPE-based) LAMP in a separate manuscript (currently undergoing peer review [146]). In this study, the aim was to investigate the sensitivity of the magneto-extraction and succeeding in situ LAMP on the proposed 20% TVO electrode. If successful, the proposed bi-electrode could in the future be employed for the detection of pathogen nucleic acid from clinical samples potentially having host genomic DNA polymerase inhibitors. It should be noted that the SPE and real-time fluorescence measurement data in this work are independent of our other manuscript focusing on magnetocapture optimization [146].



Figure 4.17: LAMP performed on magneto-extracted SARS-CoV-2 plasmid DNA tested on (a) 20%-TVO, and (b) SPE, respectively. (c) Real-time fluorescence results for the plasmid DNA. LAMP performed on magneto-extracted SARS-CoV-2 RNA tested on (d) 20%-TVO, and (e) SPE, respectively. (f) Real-time fluorescence results for the plasmid RNA. The TC sample in each case comprised 10³ copies of pure plasmid DNA. Error bars represent standard deviation.

In the current study, the end-point SWV measurements were carried out after electrochemical in situ LAMP was performed on magneto-extracted SARS-CoV-2 plasmid and RNA. The

grouped column bar graph for the peak current (shown in Figure 4.17 a-b and Figure 4.17 d-e) illustrated that the electrochemical response of magneto-extracted SARS-CoV-2 plasmid and RNA from i) aqueous solution; ii) sample spiked with excess human genomic DNA; and iii) serum-spiked VTM-mimic sample using 20%-TVO and SPE, respectively. At the same time, the S(I) percentage change of the current signal, with respect to NTC, corresponding to each of these experiments was evaluated for 20%-TVO electrode and SPE (Table 4.5). For each sample type, clinically relevant concentrations of 10^2 and 10^3 target copies were tested along with a non-magneto-extracted LAMP TC sample with 10³ copies. The NTC samples, in this case, consisted of a magneto-extraction performed on a sample but in the absence of a target nucleic acid. As the absence of a template led to the non-existence of amplicon in the NTC samples, the peak current was the highest. Similarly, a significant presence of LAMP amplicon in non-magneto-extracted (i.e., pure) TC samples led to the sequestration of free MB, decreasing the peak current for all cases. The experiments also demonstrated that the current signal, as was in the case of pure nucleic acid-based LAMP assays, was generally lower for 20%-TVO compared to SPE. In addition, the sensitivity of TVO-based LAMP detection was comparable to that of real-time fluorescence LAMP in terms of detecting magnetocaptured SARS-CoV-2 nucleic acid (Figures 4.17c and 4.17f).

Ideally, a test sample with more initial target copies was anticipated to produce a higher amplicon and therefore would have a lower value of peak current. Accordingly, NTC should have the highest current followed by 10^2 and 10^3 copies. The peak current for the magnetocapture detection of 10^2 and 10^3 copies, while consistently lower than NTC, however, were within the error limit of each other for both SPE and 20%-TVO. In addition, the mean current responses for magneto-extracted $10^2 - 10^3$ copies were similar to that of the TC samples consisting of pure 10³ copies of nucleic acid (Figure 4.17 a-b and Figure 4.17 d-e). While SPE's current signal response was consistently higher than that of the 20%-TVO, it was also unable to differentiate between magneto-extracted $10^2 - 10^3$ copies. Also, when compared in terms of signal S(I) (Table 4.6), the performance of 20%-TVO and SPE demonstrated comparable performance for detecting 10^2 and 10^3 copies. The current response measures the total amplicon presence. Usually, the LAMP amplicon level reaches a plateau at the end of a 1 hr run [172]. Therefore, the comparable current response for positive control consisting of pure 10^3 copies and magneto-extracted 10^2 and 10^3 copies may have originated from a similar amount of amplicon generated at the end of the LAMP assay. It implied that a differentiable current response could have been obtained if the reaction could have been stopped earlier [73]. Overall,

the bi-electrode assembly was thus able to detect pure as well as magneto-extracted 100 to 1000 copies of target SARS-CoV-2 RdRp DNA and RNA, therefore conclusively differentiating a positive from a negative sample. The experiments also demonstrated that the sensing response of 20%-TVO and SPE was analogous to each other in detecting clinically relevant 100 copies (2.5 copies/ μ L) and the presence of human genomic DNA or serum did not hinder the sensing response of TVO. As demonstrated in our other study [146] concerning the magnetocapture assay optimization, the sample-to-answer time is 2 hrs (25 min for magneto-extraction, 80 min (1 hr for amplification, and 20 min for inactivation) for LAMP, 10 min for electrochemical measurement. The amplification time can further be reduced to 1 hr if the reaction is terminated at an earlier time, making the final sample-to-answer time total 1 hr. Both are significantly shorter than the sample-to-answer time for real-time PCR (total of 2.5 hrs, 1 hr for nucleic acid extraction, and 1.5 hrs for PCR).

Table 4.6: S(I) (signal %-change with respect to NTC) for electrochemical LAMP mediated detection of 100 – 1000 copies of magneto-extracted nucleic acid from aqueous, human genomic DNA spiked, and serum spiked sample on 20%-TVO electrode and SPE. TC sample implies non-magneto-extracted LAMP for 1000 copies.

	Target Concentration	S(I) fo	r DNA	S(I) for RNA		
Sample		20%-TVO	SPE	20%-TVO	SPE	
	ТС	46.51 ±6.4	46.17 ± 1.8	66.63 ±0.5	44.19 ± 1.7	
Aqueous	100	16.39 ± 7.4	$32.17\pm\!\!1.9$	53.71 ±18.8	$40.18\pm\!\!2.8$	
	1000	37.99 ± 3.8	$41.49\pm\!\!0.7$	63.67 ± 17.2	7.23 ± 0.6	
Human Genomic	ТС	$56.51 \pm \! 6.4$	$46.80\pm\!\!0.0$	36.16 ± 3.6	31.04 ± 1.4	
	100	53.82 ±2.7	$44.62\pm\!\!0.9$	7.63 ± 3.5	22.21 ± 1.9	
	1000	47.26 ± 0.7	34.04 ± 0.9	35.69 ± 2.1	$26.42\pm\!\!0.2$	
	ТС	59.32 ±4.2	$49.19\pm\!\!3.4$	25.18 ±6.5	40.08 ± 2.8	
Serum	100	36.71 ±7.0	14.18 ±0.6	14.23 ± 5.3	7.33 ±2.3	
	1000	49.51 ±7.4	17.87 ± 1.5	17.98 ±6.2	13.29 ±2.2	

4.4 Conclusion

In this study, a highly sensitive dual-electrode electrochemical sensor (DEES) was built suitable for specific detection of nucleic acid amplification and demonstrated its functioning by sensing LAMP for the identification of SARS-CoV-2 nucleic acid. The working electrode of DEES included a transition metal oxide (TMO) sensing layer, which is a rather underexplored class of material for NAAT application. The properties of TMO based sensing layer were modulated by forming a binary metal oxide nanocomposite of titanium dioxide and vanadium oxide (TiO₂:V₂O₅) with various Ti content to facilitate sensitive detection. While choosing TiO₂:V₂O₅ as the sensing layer, the MB electron transfer performance as a function of sensing layer IEP was also probed. Next, the performance of the electrode was assessed in biosensing and compared against commercially available screen-printed electrodes (SPE) as well as standard real-time fluorescence measurements. The highly sensitive LAMP assay combined with the 20%-TVO enabled electrochemical approach helped detect pure 10^2 and 10¹ copies (2.5 and 0.25 copies/µL, respectively) of SARS-CoV-2 RdRp plasmid DNA and invitro transcribed RNA, respectively with at least three orders of dynamic range. Its performance was comparable to that of the SPE, and real-time fluorescence assays were performed in realtime-PCR instruments.

The device possesses excellent repeatability as suggested by the statistical data set and a low (13.2 to 29.1%) coefficient of variation. The DEES demonstrated excellent selectivity and compatibility with in-situ LAMP performed on magneto-extracted SARS-CoV-2 plasmid and RNA with $10^2 - 10^3$ copies of target nucleic acid from a) aqueous solution; b) sample spiked with excess human genomic DNA, and c) a serum-spiked sample. The performance of DEES in detecting LAMP was found to be comparable to that of the standard real-time fluorescence. and SPE carbon electrodes. Despite being a fraction of the cost of the commercial carbon SPEs, the TMO-based DEES demonstrated similar sensitivity as that of commercial SPE carbon electrodes. These experiments demonstrated that nanocomposite TMO-based DEES, a novel class of device, by itself or in combination with a preconcentration technique, was capable of efficacious detection of clinically significant copies of target nucleic acid in pure form as well as from a real-life mimic (human genomic DNA or serum-containing) sample. Such performance and selectivity would be critical for future studies to be conducted on clinical samples.

CHAPTER 5

CONCLUSION

5.1 Introduction:

This chapter will present the conclusion of the study by highlighting the key research findings. It will showcase the credibility of the developed devices and their significance relative to the existing standard techniques. It will also outline the limitations and proposes the scope for future research.

5.2 Overview and conclusion

This study aimed to fabricate an electrochemical biosensing device based on transition metal oxides (TMOs) and employ them in nucleic acid amplification tests (NAATs). The results from various physical, electrical, and electrochemical characterization techniques demonstrated the tunability of TMOs towards synthesizing an electrochemically active electrode for efficacious detection. The as mentioned objectives in the "Introduction", chapter 1, can be broadly categorized into two sections, the primary objective was to fabricate the proposed biosensor and its optimization to obtain a high performing electrode. The secondary objective was to perform NAATs and test the device capability in terms of cross-sensitivity and limit of detection (LOD). During this research work, two variants of sensing electrodes were fabricated: (a) oxygen vacancy modulated MnO₂ electrodes, and (b) TiO₂:V₂O₅ based binary metal oxide nanocomposite electrodes. The outcomes from the individual electrodes are discussed below:

5.2.1 Oxygen vacancy modulated MnO₂ electrodes

The MnO_2 electrodes were used to detect the dengue virus (DENV) DNA sequence and *S. aureus* genomic DNA. The electrodes were modulated by thermal treatment at elevated temperatures. The study revealed the following observations:

i. This work has successfully modulated the electronic conduction state of the fabricated electrodes by introducing oxygen vacancies into the lattice, which was further validated by an x-ray photoelectron spectroscopy (XPS) study.

- ii. Treating the electrode at elevated temperatures results in the morphological transformation from micro-rods to micro-flower like structures that provided more active sensing sites.
- iii. Further electrical (contact resistance) and electrochemical (electrochemical impedance spectroscopy (EIS)) studies aided the electrode optimization process. The electrode with the lowest contact resistance and charge transfer resistance was chosen for NAATs application.
- iv. The preferred electrode then electrochemically detected DENV serotype-2 sequence via electrochemical rolling circle amplification (RCA) and vancomycin resistance associated regulator (*vraR*) gene in *S. aureus* via electrochemical polymerase chain reaction (PCR). The current-voltage (I-V) characteristics were recorded to obtain the amplification signal. When tested for multiple devices the performance was found to be reproducible with a small window of deviation.
- v. The device possessed an analytical sensitivity of 10³ copies/µL for RCA and 10³ copies for PCR similar to that of the standard real-time fluorescence measurement.

5.2.2 TiO₂:V₂O₅ based binary metal oxide nanocomposite electrodes.

The $TiO_2:V_2O_5$ metal oxide nanocomposite (MO-NC) electrodes were utilized to detect plasmid DNA and in vitro transcribed SARS-CoV-2 RdRp RNA. The titanium (Ti) was added to the vanadium oxide (V₂O₅) parent lattice in the interest of fabricating binary metal oxide nanocomposite. The following outcomes were obtained from this work:

- i. The work has successfully fabricated an electrochemically active working electrode by tunning the Ti dopant concentration to form a TiO₂:V₂O₅ nanocomposite. The presence of a separate TiO₂ phase in the x-ray diffraction (XRD) analysis confirmed the formation of the metal oxide composite.
- Various probing tools including XPS, I-V characteristics for contact resistance measurements, and EIS indicated the TiO₂:V₂O₅ nanocomposite with 20% Ti-content (20%-TVO) to be the optimum performing electrode.
- iii. The chosen electrode successfully detected loop-mediated isothermal amplification (LAMP) of SARS-CoV-2 RdRp plasmid DNA and in vitro transcribed RNA in an aqueous

solution for a range of $10^1 - 10^4$ initial target copy number using square-wave voltammetry (SWV) technique.

- iv. The limit of detection (LOD) of 20%-TVO for SARS-CoV-2 RdRp plasmid DNA was 10³ copies (25 copies/μL) while for in-vitro transcribed RNA was 10² copies (2.5 copies/μL). The device performance was found to be equivalent to that of the commercially available screen-printed electrode (SPE) and real-time fluorescence measurement.
- v. The device proved to be highly selective for LAMP performed on magneto-extracted SARS-CoV-2 plasmid and RNA with $10^2 10^3$ copies of target nucleic acid from a) aqueous solution; b) sample spiked with excess human genomic DNA, and c) a serum-spiked viral transport medium (VTM)-mimic sample. The device capability was verified against SPEs and real-time fluorescence.

COVID-19 outbreak, caused by the SARS-CoV-2 RNA virus, elevates the need for a rapid, reliable, inexpensive, and easily accessible disease diagnostic tool. The gold-standard approach of the real-time reverse transcriptase polymerase chain reaction (RT-PCR) test though accurate holds several limitations. This work provided supporting evidence for an alternate and efficient method for NAATs. An isothermal amplification technique was chosen to reduce the cost of an expensive real-time PCR (rt-PCR) machine and to reduce the process complexity due to the requirement of a single constant temperature. The electrochemical readout will decentralize the detection process and simplify data interpretation. The inexpensive and versatile nature of TMOs made them an attractive class of materials for sensing applications.

5.3 Limitations and future scope

The presented work holds the following limitations that provide the opportunity for future work extension,

- It has been understood that even though the fabricated electrochemical bi-electrode sensing device (EBSD) performance was comparable to standard RT-PCR, it may not completely be a replacement for the latter. But it can be a promising substitute, especially in a resourcelimited setting.
- ii. The EBSD in its current state was not equipped for on-chip amplification due to the lack of substrate heating arrangements, therefore the isothermal amplification was performed

separately using a conventional thermal cycler. As this study was at its preliminary stage and due to time constraints, the objective of fabricating an all-in-one device geometry is not accomplished. This offered scope for the extension of this study by providing a heating element for real-time amplification monitoring.

iii. The promising performance of TMO based electrodes in this study has opened a new class of electrode fabrication or modification alternatives in the field of NAATs application.

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International Conference Presentation

 Presented (oral) virtually in an international conference "Sensors 2021" (Location: Milan, Italy) on October 20-22 entitled "Rapid, Cost-Effective, and Intelligible Bi-Electrode Electrochemical Sensing System for Detection of Attomole-Level Dengue Virus Sequence".

SYNOPSIS

of the proposed thesis entitled

SOLID-STATE BIOSENSOR FOR THE DETECTION OF NUCLEIC ACID AMPLIFICATION

To be submitted for the award of

Doctor of Philosophy

by

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Contents

1.	Introduction	3
2.	Motivation and Research Gap	4
3.	Aim and Objective	4
4.	 Research Methodology a. Study Design b. Device Fabrication c. Electrode Characterizations d. Sample preparation e. Data Collection 	5
5.	Organization of the Proposed Thesis	7
6.	List of Publications and Patents	10
	References	11

1. Introduction

Nucleic acid amplification tests (NAATs) are routine biological processes with applications in pathogen detection, identifying food and water contamination, diagnosing infectious diseases, and biowarfare prevention. It is an essential step in biomedical analysis as the amount of DNA found in numerous biological samples is in minute concentration therefore direct detection is not possible[173]. It, therefore, necessitates the intervention of NAATs to make millions to billions of copies of the target nucleic acid followed by quantitative detection.

Presently, real-time reverse transcription-polymerase chain reaction (RT-PCR) has been employed as the gold standard of detection owing to its accuracy, and specificity [45], [46]. However, this approach is time-intensive, requiring a specialized laboratory with overpriced instruments and trained personnel for nasopharyngeal/ oropharyngeal (NP/OP) sample collection and testing [16], [46], [49]. These downsides of real-time rt-PCR have spurred the advancement of various isothermal nucleic acid amplification detection (a-NAAD) techniques such as recombinase polymerase amplification (RPA) [51], [174], rolling circle amplification (RCA)[46], [52] and loop-mediated isothermal amplification (LAMP) [53]–[55] to name a few [56].

Currently, the quantification of NAATs is usually done using a fluorescent DNA binding dye or sequence-specific probe technique [57]–[59]. Such fluorometric methods, though accurate and used as a standard practice, are laborious, lengthy, and expensive.

An attractive alternative to optical detection has been proposed based on the electrochemistry of the DNA-binding redox probes [64]. Advantageously, electrochemical techniques are sensitive to electrochemically responsive species (redox probes) irrespective of background light and sample color as in the case of fluorometric methods [65]–[67]. At the same time, such measurements could be carried out in electrochemical workstations that are significantly inexpensive compared to real-time PCR instruments.

An electrochemical biosensor is a transducer of oxidation (or reduction) of an electroactive species to an electrical current signal. The electrochemical activity of DNA is not adequate for direct detection, therefore a redox indicator is employed for the purpose [90]. The device senses the redox probe concentration and the variation in mass transfer to the working electrode. Since DNA has a strong affinity for redox probes the binding significantly reduces the "free" probe concentration in the electrolyte. After DNA amplification high concentration of amplicons (i.e.,

replicated DNA) are produced that captures a greater number of probe molecules which in turn reduces the electrochemical signal [72], [73], [90].

Commonly used redox probes are transition metal complexes (e.g. ruthenium (Ru) complexes [54], [71], [91]) and organic dyes (e.g. methylene blue (MB) [72], [73], [93]). Jiong Zhang[71] et al. employed ruthenium hexamine for the amplified detection of BRCA-1 mutant DNA, whereas Naoki Nagatani et al. [72] and Kuangwen Hsieh [73] et al. used MB for electrochemical monitoring of influenza virus RNA and quantitative detection of pathogenic DNA, respectively. In the present work, MB is employed as a redox probe that belongs to the thiazine class with the molecular formula $C_{16}H_{18}N_3SC1$ [94]. It binds to the DNA electrostatically as well as intercalates into the DNA structure [138].

2. Motivation and Research Gap

The electrochemical biosensors for NAAT detection reported to date commonly use 3electrode assembly with platinum (Pt) and/or silver/silver chloride (Ag/AgCl) as standard reference and counter electrode, and gold (Au) or commercially available screen-printed carbon electrode as working electrode [73], [93], [175], [176]. These standard reference electrodes made-up of a noble metal elevate the cost of detection. Despite their inexpensive nature and possibility of electronic state modulation, the utility of the transition metal oxides (TMO) in sensing nucleic acids and NAATs has remained surprisingly underexplored. TMO structure involves a central TM atom surrounded by bounded oxygen atoms that form various crystal structures such as monoxide (MnO), di-oxide (TiO₂), spinel (NiCo₂O₄), etc. Due to their partially occupied d-orbit, TMOs possess multiple oxidation states which support rapid redox reactions. Hence, TMOs are widely used as electrode material in energy storage applications such as lithium-ion batteries, supercapacitors, solar cells, etc [177], [178].

3. Aim and Objective

This work aims to fabricate an electrochemical nucleic acid amplification sensor to minimize sample-to-answer turnaround time with an easy data user interface. The work is focused on designing a novel dual-electrode sensor geometry with a low-cost fabrication process based on transition metal oxides (TMO).

The objectives of this research work are stated below:

- 1. Designing and fabrication of Manganese Oxide based nucleic acid amplification sensor.
 - a. Modulation of conducting states of the working electrode

Synopsis-4

- b. Optimization of electrochemical characteristics of the working electrode.
- 2. Detection of dengue virus (DENV) serotype-2 sequence and *vancomycin* resistanceassociated regulator gene (*vraR*) gene in *Staphylococcus aureus* (*S. aureus*) genomic.
- 3. Implementation of binary nanocomposite for nucleic acid amplification detection.
- 4. Detection of plasmid DNA and in-vitro transcribed SARS-CoV-2 RdRp RNA from,
 - a. Aqueous solution,
 - b. Sample spiked with excess human genomic DNA,
 - c. A viral transport medium (VTM)-mimic sample.

4. Research Methodology

There were various processes and instrumentations involved in the fabrication, characterization, and testing of the fabricated biosensor. The details of used methodology are discussed below:

4.1 Device Fabrication

The TMO layer deposition was fabricated using a chemical thin film deposition technique known as sol-gel spin coating (figure 1). The precursor solution was prepared using the required metal salt and organic solvents. The solution was then spin-coated on the cleaned substrate via a spin coater at optimized deposition parameters. A transparent polymer micro-cavity was attached at the top of the sensing layer to hold the test sample during measurement and avoid evaporation.



Figure 1 Schematic representation of device fabrication, assembly, and testing.

4.2 Electrode Characterizations

- 1. Structural and Morphology Analysis:
 - a. X-Ray Diffraction (XRD) using Bruker D8 ADVANCE ECO.
 - b. Layer Thickness measurement using KLA Tencor Alpha-Step D-300 stylus profiler.
 - Field Emission Scanning Electron Microscopy (FE-SEM) using FEI Quanta 200 F
- 2. Compositional Analysis:
 - X-Ray Photoelectron Spectroscopy (XPS) using Physical Electronics, PHI 5000 VersaProbe III
- 3. Optical Analysis:
 - a. UV-vis Spectroscopy using Ocean Optics UV-vis spectrophotometer
- 4. Electrical and Electrochemical Measurements
 - a. Electrochemical Impedance Spectroscopy (EIS) using Metrohm Autolab Potentiostat/Galvanostat (Model Autolab PGSTAT302N).
 - b. Electrical Measurements using Keysight B2912A source measurement unit (SMU).

4.3 Sample Preparation

Nucleic acid amplification was performed to electrochemically detect dengue virus sequence DNA (using rolling circle amplification) and *Staphylococcus aureus* genomic DNA (using polymerase chain reaction) using an optimized MnO₂ based working electrode. Whereas LAMP reaction was performed on plasmid DNA and in vitro transcribed SARS-CoV-2 RdRp RNA in aqueous solution to detect electrochemically by TiO₂:V₂O₅ nanocomposite-based electrode. Additionally, LAMP was performed on magneto-extracted SARS-CoV-2 plasmid and RNA from a) aqueous solution; b) sample spiked with excess human genomic DNA, and c) a viral transport medium (VTM)-mimic sample.

4.4 Data Collection

Electrochemical data measurement was performed using SMU and electrochemical workstation. The I-V measurements were performed using SMU within the voltage of and repeated for multiple consecutive cycles. Square-wave voltammetry was performed by an electrochemical workstation from a voltage range of 0 to -1V for a few consecutive cycles. The recorded data was baseline corrected and averaged over the number of cycles.

5. Organization of the Proposed Thesis

The proposed thesis contains five chapters that include the introduction, literature review, the four research objectives, and conclusions. A brief overview of chapter wise description of the proposed thesis is provided below:

Chapter 1 presents the essential introduction of the properties of transition metal oxide (TMO) and their applications in the field of biosensing, the need for nucleic acid amplification and present methods of amplification detection. It provides a survey encircling the currently preferred amplification methods for electrochemical detection and recent electrochemical setups employed in detection. It will also highlight the motivation and objective of the present work.

Chapter 2 describes the design and concept of the sensor. The working principle of the proposed device relied on the dynamic electrochemistry at the interface of the sensing layer and the electrolyte. The device was composed of three components: a working electrode, an ionic conductor (electrolyte), and a quasi-reference counter silver electrode (QRCE) as shown in figure 2a. The working electrode was comprised of a conducting substrate (FTO), coated with a transition metal-oxide-semiconductor film acting as a sensing layer.



Figure 2 Schematic representation of a) device geometry, b) sensing principle

The proposed device measures any change in the electric transport through the system because of the variation in the ionic concentration of the electrolyte. The electrolyte is a solution of nucleic acid-binding electroactive species that undergoes charge transfer at the interface. Since nucleic acid has a strong affinity for redox probes, the binding significantly reduces the "free" probe concentration in the electrolyte. After amplification high concentration of amplicons are produced that sequestered a higher number of probe molecules which in turn reduces the electrochemical signal (figure 2b). The present device works as a transducer and detects amplification by measuring the change in the current signal.

Chapter 3 encompasses the fabrication and optimization of the sensing layer made of TMO (MnO_2) and its characterization using various techniques. The conducting states of sensing layer were modulated using heat treatment at various elevated temperatures. The work proposes that heating at various temperatures introduces oxygen vacancies into the metal oxide resulted in the generation of new conducting paths. The structural, compositional, electrical, and electrochemical properties were probed by FE-SEM, XPS, EIS etc. characterization techniques. The XPS analysis indicates a higher contribution of Mn^{3+} oxidation state than Mn^{4+} when annealed at elevated temperature, suggesting the formation of oxygen vacancies. As a result, the electrical conduction was improved as evident from the significant reduction in charge transfer resistance (R_{ct}) value from EIS measurements.

The fabricated device with an optimized electrode was then employed to detect dengue virus sequence DNA (using rolling circle amplification) and *Staphylococcus aureus* genomic DNA (using polymerase chain reaction). The amplification test was performed by recording I-V characteristics and EIS of the negative control (NTC) and target samples and analyzed in terms of the %-change in the current. The device was able to achieve the limit of detection in the order of 10³ target DNA copies. The sensitivity was equivalent to the standard RT-PCR results.

Chapter 4 presents another approach for efficient detection of nucleic acid amplification by fabricating a transition metal oxide nanocomposite (TMO-NC) based sensing layer. The TMO-NC can effectively provide more active sites on the electrode surface, improve electronic conduction, and enhance interfacial surface area [148]. The V₂O₅ precursor solution was added with various concentrations of TiO₂ solution to form the TiO₂:V₂O₅ binary nanocomposite. The electrodes were analyzed for optimum performance using XRD, XPS, EIS, etc. characterization tools. In the XRD spectra, two crystalline phases corresponding to V₂O₅ and TiO₂ were detected which confirms the formation of the nanocomposite. The most intense redox peak observed for 20%-Ti content NC in the I-V characteristics with 100-MB suggests the highest electrochemical activity of the electrode. The 20%-Ti content electrode also holds the lowest charge transfer resistance (R_{ct}) value in EIS measurement.

The optimized Ti content electrode was used for the detection of plasmid DNA and in-vitro transcribed SARS-CoV-2 RdRp RNA in an aqueous solution. The device was then tested for cross-sensitivity by detecting magneto-extracted SARS-CoV-2 plasmid and RNA from three various environments a) aqueous solution; b) sample spiked with excess human genomic DNA, and c) a viral transport medium (VTM)-mimic sample. The results were compared with the standard real-time fluorescence and commercially purchased screen-printed electrodes (SPE). The device possesses excellent repeatability and analytical sensitivity of the order of 10³ copies for plasmid DNA and 10² copies for in-vitro transcribed SARS-CoV-2 RdRp RNA and found to be equivalent to that of the SPE and real-time fluorescence data assays performed in RT-PCR instruments.

Chapter 5 concludes the findings of the present research work and the scope for future work. This work demonstrated two distinct approach of fabricating TMO based working electrode a) oxygen vacancies modulation of MnO₂ electrode, and b) formation of TiO₂:V₂O₅ binary nanocomposite. The optimized MnO₂ electrodes were able to detect dengue virus sequence DNA and *Staphylococcus aureus* genomic DNA in the presence of MB redox probe with a detection limit of 10^3 copies/µL. The TiO₂:V₂O₅ nanocomposite electrodes were able to sense plasmid DNA and in-vitro transcribed SARS-CoV-2 RdRp RNA. The electrode showed a sensitivity of 10^3 copies/µL for plasmid DNA and 10^2 copies/µL for RdRp RNA. The electrodes also possess low cross-sensitivity when tested for aqueous solution, excess human genomic DNA, and VTM-mimic samples. The results from the fabricated device were found to be in agreement with the standard RT-PCR results.

This work offers the utilization of TMO based sensing layers for their application is fabricating new class of low-cost biomedical sensing devices via controlling their structural, chemical, electrical, and electrochemical properties.

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