Synopsis of the thesis on

Development of biosensors for the detection of pathogenic bacteria, proteins and other molecules

To be submitted to

The Maharaja Sayajirao University of Baroda

For the Degree of

Doctor of Philosophy in Biochemistry

By

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Under the

Supervision of

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To
The Registrar (Academic Section),
The M. S. University of Baroda,
Vadodara 390002

Date:

Subject: Submission of synopsis of the Ph.D. work entitled "**Development of biosensors for the detection of pathogenic bacteria, proteins and other molecules"**

Respected Sir/Madam,

Kindly accept the synopsis of my Ph. D. work entitled "**Development of biosensors for the detection of pathogenic bacteria, proteins and other molecules"**. My date of registration is 16/09/2016 and registration no. is FOS/2024.

Thanking you,

Yours sincerely,

Jinal Bharatbhai Thakkar

Prof. C. Ratna Prabha
Guide

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INTRODUCTION:

Biosensor is a device which tends to recognize and perform qualitative or quantitative analysis of a particular analyte. Biosensor can monitor a biologically relevant analyte rapidly and precisely in the sample, preparing us to take necessary and appropriate action towards it. Biosensors offer convenient monitoring and are considered to be increasingly unavoidable aspect of modern life in terms of economic (i.e., cheap) and utility aspect such as reusability/disposability, simplicity, portability, sensitivity and selectivity. Biosensors comprise of three basic components: Analyte, Receptor and Transducer and are classified based on them. Receptor recognizes the target analyte and binds to it, the recognition signals are then delivered to the transducer which converts them to the detectable signals. Biosensor depending on the transducer used can be recognized as electrochemical, optical, piezoelectric or calorimetric biosensor. Other biosensor types offered by different receptors are enzymatic, immuno, nucleic acid, cell and biomimetic biosensor (Pejcic et al., 2006).

Conventional methods such as physico-chemical techniques, nucleic acid tests, immunological techniques and others used for recognition and detection suffer from several pitfalls such as non-specificity, lower sensitivity, lengthy analysis, slower response, artefacts, need of high throughput instruments, technicians and non-availability of pure sample. Biosensors overcome all these drawbacks and offer faster, accurate, cheaper and sustainable analysis and monitoring (Thakkar et al., 2019). Biosensors find their applications in various arenas such as pharma and medical sector, food processing industry, environmental field, defense and security sectors. Medical diagnostic sector shares a very huge market of US$ 13 billion in biosensor field (Pejcic et al., 2006). Glucose biosensor for diabetic patients is a major achievement in this field (Thakkar et al., 2019)

The need for research to develop and explore biosensor field comes from the shortcomings suffered by the conventional techniques. The major goal of developing new prototypes of biosensors can be expressed as enhancing the detection capacity, sensitivity and reducing the detection time and cost. Amalgamation of nanomaterials with various assays (i.e., enzyme assay, nucleic acid assay, immune assay etc.) is considered to be very important and crucial step to accomplish these objectives. Nanomaterials offer high surface area, mechanical strength, thermal and electrical conductivity making them attractive for sensor application. Our laboratory has explored

functionalization and use of carbon nanotubes in electrochemical-enzyme biosensors. A suspension of carbon nanotubes in polyvinyl alcohol solution was used to immobilize glucose oxidase as a model enzyme. The resulting electrode showed higher glucose oxidase loading and hence better glucose detection limit on the fabricated electrode (Gupta et al., 2018, 2016).

However, sensors used nowadays also have some kind of limitations in term of specificity, selectivity, detection limit, range of detection, artefacts, high cost and invasive nature. Idea of the study is to develop prototypes of sensors which can overcome the drawbacks offered by both conventional methods and available sensors. The study focuses on advancement, fabrication, characterization and standardization of biosensors.

Pesticides being the common environmental contaminants enter into the food chain through water bodies, food and soil. Once exposed, pesticides may cause many diseases to humans by obstructing various metabolic pathways and processes. Agriculture, food and chemical warfare production sectors are the foremost source of these poisonous compounds (Arduini et al., 2013). Therefore, sensitive, rapid and real time detection of these impurities is necessary. The toxicity of pesticides depends on the type and structure of the pesticides. Among numerous pesticides used globally, 30% are organophosphorus pesticides. Organophosphorus compounds irreversibly interact with the enzyme AChE and inhibit its activity. This results into accumulation of the neurotransmitter acetylcholine in nerve synapses. This elevated level of acetylcholine in nerve synapses causes toxicity to respiratory and nervous systems or may lead to death in cases of severe exposure (Turan et al., 2016). Available methods for detecting pesticide residues such as TLC, GC, HPLC, LC/GC-MS and spectroscopic techniques are lengthy, expensive, need technicians and do not offer real-time testing. Electrochemical method offers cost effective approach in addition to rapid and onsite detection and thus rising above the shortcomings of conventional assays (Arduini et al., 2013). In the present study, organophosphorus is selected as a model pesticide for developing a prototype of enzymatic biosensor for electrochemical sensing of the pesticide. Carbon nanotubes in combination with AChE enzyme were employed to fabricate electrochemical sensor using glassy carbon electrode. The fabrication method avoids use of any membrane and crosslinker which may interfere in the electron transfer process. Cyclic voltammetry technique was used to characterize and standardize the biosensor thoroughly.

Triglycerides (TGAs), the natural fat transporters and energy source in the body are formed through the esterification of three fatty acid molecules with a glycerol molecule (Di Tocco et al., 2018). TGAs are considered to be indicators in serum samples for lipid associated metabolic disorders such as hypertension, atherosclerosis and coronary heart disease. Besides, increased levels of TGAs are also associated with liver obstruction, nephrosis, diabetes, endocrine disorders (Solanki et al., 2009). Normal TGA range in serum is up to 150 mg/dL whereas 150-199 mg/dL is considered to be indicator of borderline disease. High risk level is from 200-499 mg/dL, whereas above 500 mg/dL is considered to be at very high risk condition for TGAs (Di Tocco et al., 2018). Because of improved health awareness and food regulatory laws, TGA level has become a significant factor for food quality assurance (Wu et al., 2014). Standard available detection methods include use of high throughput instruments such as spectroscopy, chromatography, fluorimetry, NMR etc. Other colorimetric methods such as titration and lipid profile test also can be used for triglyceride estimation. All these techniques are complex, lengthy, slow, expensive, and need technicians. Non-portability or lack of onsite detection also limits their uses. On other hand, electrochemical sensor offers easy, selective, rapid and cheap method for detection with miniaturization possibility (Di Tocco et al., 2018). Lipase has a tendency to hydrolyze TGA ester into Fatty acid and glycerol. The chemistry was used to develop nanomaterial based electrochemical biosensor for the estimation of triglycerides in the samples. New fabrication chemistry which offers use of combination of nanoparticles such as reduced graphene oxide nanoparticles, carbon nanotubes, titanium oxide nanoparticles with polyethyleneimine have been explored. The fabrication chemistry allowed more enzyme loading and hence increased detection limit. Cyclic voltammetry technique was used to explore characterization and standardization of fabricated electrode.

Staphylococcus aureus is gram positive facultative anaerobe. It can cause numerous illnesses such as septicemia, endocarditis, pneumonia, meningitis, abscesses, skin diseases etc. The bacteria are very common in hospital acquired infections. Nearly 5,00,000 cases are detected every year with *S. aureus* infection only in America and approx. 94,000 cases are found to be with life-threating infection of *S. aureus.* Standard methods such as bacterial culture, PCR and other metabolic tests have some limitations such as lengthy analysis and processing time, need of technician and high

throughput facilities which can be combated by biosensor development (Chang et al., 2013). Molecular imprinting technique was used and explored to artificially develop recognizing receptor onto acrylamide polymer for *S. aureus* detection. Ease of preparation, low cost and specific recognition are the features offered by imprinting methodology.

Current study explored the use of different methodologies in combination with nanotechnology, electrochemistry and biochemistry to develop, characterize, standardize and advance prototypes of biosensors. Amongst the developed fabrication chemistries, pesticide sensor eliminated the use of crosslinker or membrane while triglyceride sensor offered novel fabrication chemistry and high conductivity due to the combination of nanoparticles and the polymer interface used. Molecular imprinting as a new detection methodology has been explored to detect *Staphylococcus aureus*. Fabrication of the biosensors, their characterization and standardization were done by various techniques such as cyclic voltammetry, spectroscopy, FT-IR, FE-SEM and AFM.

SPECIFIC OBJECTIVES:

Major objectives of the present study are:

1. Development, fabrication and characterization of biosensor for the detection of pesticide.
2. Development, fabrication and characterization of biosensor for the detection of triglyceride.
3. Development, fabrication and characterization of biosensor for the detection of *staphylococcus aureus*.
4. Development, fabrication and characterization of biosensor for the detection of a cancer biomarker.
5. Development, fabrication and characterization of biosensor for the detection of Covid-19.

Objective 1: Development, fabrication and characterization of biosensor for the detection of pesticide.

Present study is focused on coating, fabrication and characterization of the electrodes using nanomaterials and appropriate enzyme. Pesticide biosensor was fabricated by doping carbon nanotube (MWCNTs) with the acetylcholine esterase enzyme (AChE). Here, the electrode was fabricated without the use of any membrane, polymer or crosslinker. Glassy Carbon Electrode was

modified by layering MWCNTs having carboxylic groups on their outer surface. Following that, AChE was directly drop casted on to the MWCNTs surface. Carboxyl groups of MWCNTs intermingled with amine group of enzyme and resulted into amide bond formation. The biosensor performance is totally grounded on presentation and activity of AChE enzyme immobillized on the electrode. The enzyme acetylcholinesterase (AChE) attached on the electrode works on the basis of inhibition chemistry. When enzyme is exposed to acetyl thiocholine iodide substrate, it forms thiocholine. This response can be measured by the fabricated electrode in the form of current produced due to the oxidation of thiocholine.

Equation. 1

$$\text{Acetylthiocholine} + H_2O \rightarrow \text{Thiocholine(red.)} + \text{Acetic acid}$$

$$\text{(Anode) 2 Thiocholine(red.)} \rightarrow \text{Dithio-bis-choline(ox.)(Dimeric)} + 2H^+ + 2e^-$$

When the sensor is exposed to organophosphorus pesticide, it will block the activity of the enzyme AChE. The decreased magnitude of current can be calculated as inhibition percentage (Equation. 2) which can be seen as proportional to the added concentration of the pesticide.

Equation. 2

$$\% \text{ Inhibition} = (I_0 - I_1)/I_0 * 100$$

where, I_0 = peak current magnitude for ATChI substrate before pesticide exposure.
I_1 = peak current magnitude of ATChI substrate after pesticide exposure

Thus, the sensor was exposed to various concentrations of organophosphorus pesticide and current was measured before and after its exposure to the pesticide as a result of decreased enzymatic reaction.

a) Fabrication and optimization of AChE/MWCNTs/GCE coating:

Before starting fabrication procedure, glassy carbon electrode was taken for polishing with alumina slurry followed by rinsing in water and drying at room temperature. MWCNTs bearing carboxylic group were cast-off to GCE for coating the surface. Various concentrations of MWCNTs in DMF ranging from 3 mg/ml to 8 mg/ml were ultrasonicated. Out of it 5 μL of the suspension from each stock was drop-casted onto the electrode separately and the electrode was allowed to dry at room temperature for 1 hr to form nanofilm. The nanofilms had exposed carboxylic group on the surface. Following it, different aliquots from stock solutions of enzyme

in PBS (pH 7.2) were drop casted onto the MWCNTs layer. This resulted into amide bond formation between MWCNTs and AChE. Thus, the resulting electrode was washed with PBS and stored at 4°C in air. Standardization of coating and fabrication of the electrode was confirmed initially by cyclic voltammetry. It was further followed by other standard techniques such as FT-IR, FE-SEM, AFM. Potential used for cycling the voltage was +0.7 to -0.2 with the scan rate and sample interval of 100 mV/S and 0.001 V respectively. The electrolyte composition used throughout the experiments was as follows: 10 mM KCl + 0.1 mM $K_3[Fe(CN)_6]$ + 10 mM Phosphate buffer (pH 7.2).

b) Standardization and characterization of AChE/MWCNTs/GCE:

The coating parameters of the electrode such as concentrations of MWCNTs and AChE were characterized and standardized by running cyclic voltammetry and recording the peak current magnitude in electrolytic solution. The optimal concentration of MWCNTs and AChE to be coated on the electrode were found to be 0.03 mg/dL and 0.1 U respectively. Other parameters such as concentrations of acetylthiocholine iodide (ATChI) as a substrate and organophosphorus pesticide (OPP) an inhibitor were also optimized using the same parameters mentioned above. 12 mM ATChI was found to be optimal to carry out detection of organophosphorus pesticide (OPP). Inhibition time was standardized and was found to be 10 min. Detection limit and linear range of the sensor was also studied and was found to be 0.1-500 nM and 10-50 nM respectively. The sensor can be reused by treating it with 1mM pralidoxime iodide for 15 min. producing 90.68% of its actual activity. The sensor when stored at 4˚C in air dry condition, showed 54.28% of its initial activity after 32 days. The sensor can be refabricated with SD of 0.071.

Objective 2: Development, fabrication and characterization of biosensor for the detection of triglycerides.

Triglyceride biosensor has been fabricated using enzyme assay method in combination with electrochemistry. The Glassy carbon electrode was coated with GO followed by MWCNTS. Following it, nanopolymer mixture PEI-TiO2 was layered. Glutaraldehyde was added to cross link both the polymer and upcoming lipase. The electrode was dipped in lipase solution to immobilize it onto nano-polymer surface. The work proposes novel fabrication chemistry for lipase immobilization. Enzyme lipase hydrolyses the substrate tributyrin into glycerol and fatty acids and

releases protons. The proton release causes changes in pH of electrolyte solution which then results in the increase of current. Increase in the current magnitude was found to be proportional to triglyceride concentration.

$$\text{Tributyrin} \xrightarrow{\text{Lipase}} \text{Glycerol} + \text{Fatty acid} + 3H^{+}$$

The sensor was exposed to various concentrations of triglyceride to measure its performance by cyclic voltammetry.

a) Fabrication of Lipase/Glutaraldehyde/PEI-TiO_2/MWCNTs/GO/GCE electrode:

Glass carbon electrode (GCE) was polished with alumina slurry and was followed by rinsing in water. The electrode was dried at room temperature. GO was drop casted on to GCE and was allowed to dry at room temperature. It was then followed by MWCNTs cast-off to the surface. TiO_2 nanosuspension prepared in polyethylene imine polymer by ultrasonication was layered onto GO-MWCNT modified electrode surface. It was allowed to dry at room temperature. Glutaraldehyde as a cross linker was layered onto the nanopolymer surface. Lipase enzyme was then layered to bind it covalently. Glutaraldehyde cross links both polyethyleneimine polymer and lipase enzyme. The resulting electrode was washed with PBS and stored at 4°C in air. Fabrication of the electrode was confirmed initially by cyclic voltammetry. Other techniques such as FT-IR and FE-SEM were also used for confirming the fabrication chemistry. Potential used for cycling the voltage was -0.8 to +0.8 with the sample interval of 0.001 V. The electrolyte composition used during the experiments was as follows: 9% NaCl + 50 mM $K_3[Fe(CN)_6]$ + 50 mM Phosphate buffer (pH 8.0).

b) Standardization and characterization of Lipase/Glutaraldehyde/PEI-TiO_2/MWCNTs/ GO/GCE:

The electrode was characterized and standardized by running cyclic voltammetry and recording the peak current magnitude in electrolytic solution. The sensor was tested against different pH values in the electrolytic solution having 9% NaCl + 50 mM $K_3[Fe(CN)_6]$ + 50 mM Phosphate buffer. The sensor showed pH sensitivity and pH 8 was chosen to be optimum pH to carry out triglyceride detection. Interference of the sensor was tested with several interfering species such as cholesterol, urea, glucose and uric acid. The sensor showed negligible interference and good performance even in the presence of the interfering species. Various concentrations of tributyrin

were exposed to the fabricated sensor in the electrolytic solution. Change in the magnitude of the current was observed by running cyclic voltammetry from –0.8 to + 0.8 V. Detection limit and linear range of the sensor was also studied and was found to be 25 mg/dL and 25-600 mg/dL respectively. The sensor can be refabricated with SD of 0.346.

Objective 3: Development, fabrication and characterization of biosensor for the detection of *Staphylococcus aureus*.

In present study, Molecular imprinting technique has been explored to develop biomimetic receptor cavities on polyacrylamide polymer gel slab. In molecular imprinting, functional monomers are copolymerized with a desirable template whose receptors are supposed to get imprinted onto the polymer surface. It arranges functional groups of the polymer according to size, shape and molecular characteristics of the template material. The template is then allowed to get removed from it. Thus, monomers get arranged in such a manner that would generate cavities/receptors complementary to template. Stability, specificity and ease of preparation attracted us to imprint acrylamide gel slab with *staphylococcus aureus*.

Staphylococcus aureus bacteria were grown overnight at 37˚C and were inoculated for 4 hr. at 37˚C in Luria broth to get a growth of O.D.= 0.1 at 600 nm. The culture was then centrifuged at 10000 rpm for 15 min. at 4 ˚C. The pelleted cells were washed and suspended in phosphate buffer (PB) pH=8.0. The cells were then allowed to get entrapped in 2-10% of acrylamide polymer gel slab of 2 mm width. Further, gel slabs were Gram stained to check entrapment of the bacteria. Amongst 2, 4, 6, 8 and 10% only 4, 6 and 8% acrylamide gel showed entrapment. Mixture of NaOH and SDS was used as a lysis solution to remove entrapped bacteria. Again Gram's staining was used to ensure removal of the bacteria. 9 hr. of ultrasonication of the polymers into lysis solution resulted into complete removal of the bacteria. Thus, resulting whole bacterial cell imprinted polymers were allowed to re-adsorb bacteria into imprinted cavities in bacterial cell suspension. Only 4% of the acrylamide gel showed successful reabsorption over a period of time from 15-180 minutes. Incubation of the 4% acrylamide into *S. aureus* suspension at 75 minutes showed highest readsorption of the bacteria. Cross-reactivity or cross-readsorption with other bacteria by the imprinted polymer will be explored as a further study.

Conclusion:

Pesticide and triglyceride biosensors were successfully developed using the electrochemical technique cyclic voltammetry. Electrochemical biosensor developed for detection of OPP can be used for analytical applications directly. Response time and Detection limit are 10 min. and 0.1 nM respectively. Detection limit of the OPP sensor is 10-50 nM. Reusability and stability of the sensor were found to be 90.68% and 54.28% respectively. The sensor was used against real samples for detection and it showed 103.56% and 97.28% of sample recovery with SD of 4.44. Electrochemical biosensor developed for detection of triglyceride with novel fabrication chemistry can be subjected for miniaturization and analytical application directly. Detection limits of the triglyceride sensor are 25 mg/dL and 700 mg/dL with linear range of 25-600 mg/dL. Interference was found to be negligible. The sensor was used against real samples for detection and it showed reliable results for on field application. Different concentrations from 2 to 10% acrylamide gels were used to entrap and imprint *Staphylococcus aureus.* The bacteria were found to get entrapped in acrylamide polymer slab with 4, 6 and 8 % of the concentrations. Removal of the bacteria was successfully observed by treating the gel in lysis solution. Removal of the bacteria was confirmed by Gram's staining. Thus, molecularly imprinted acrylamide gel has been developed to make optical biosensor for the detection of *Staphylococcus aureus*. The bacteria were allowed to re-adsorb onto the gel, only 4% acrylamide gel showed adsorption. As a part of further study, cross readsoprtion with other bacteria onto imprinted cavities will be tested. Efforts for biosensor development for detecting a cancer biomarker and Covid-19 detection will be explored as a future plan of work.

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Turan, J., Kesik, M., Soylemez, S., Goker, S., Coskun, S., Unalan, H.E., Toppare, L., 2016. An effective surface design based on a conjugated polymer and silver nanowires for the detection of paraoxon in tap water and milk. Sensors and Actuators, B: Chemical 228, 278–286. https://doi.org/10.1016/j.snb.2016.01.034

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Peer Reviewed Publications:

Thakkar, J.B., Gupta, S., Prabha, C.R., 2019. Acetylcholine esterase enzyme doped multiwalled carbon nanotubes for the detection of organophosphorus pesticide using cyclic voltammetry. International Journal of Biological Macromolecules 137. https://doi.org/10.1016/j.ijbiomac.2019.06.162 **(Impact factor 5.2)**

International/National Poster Presentations during Ph.D. Duration:

1. ACS Publications symposium: Innovation in material science and technology, Singapore. November 2019.
2. Conference on advances in catalysis for energy and environment (CACEE 2018), TIFR, Mumbai, India. January 2018.

National Conferences attended during Ph.D. Duration:

1. "Omics…to structural basis of Diseases", Department of Biochemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara-Gujarat. October 2016.
2. "Advances in molecular biology", Department of Microbiology and Biotechnology, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara-Gujarat. January 2017.
3. "Recent advances in cell biology", Department of Microbiology and Biotechnology, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara-Gujarat. January 2018.
4. "Trends in Biochemistry and Inauguration of Prof. L. J. Parekh Memorial Lecture Series", Department of Biochemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara-Gujarat. September 2016.

Workshops Attended during Ph.D. Duration:

1. “Digital Microfluidics: Design and Applications”, Global Initiative of Academic Networks, MNIT Jaipur, India. December 2019.
2. “Workshop on Biological Applications of Magnetic Nanoparticles”, MagGenome Technologies Pvt. Ltd., The Maharaja Sayajirao University of Baroda, Vadodara-Gujarat. March 2019.
3. “Science Communication Workshop”, The Wellcome Trust/DBT India Alliance, The Maharaja Sayajirao University of Baroda, Vadodara-Gujarat. March 2016.

Awards received during Ph.D. Duration:

1. UGC Travel Award by The M. S. University of Baroda to attend TEEP international student research internship invited by Kaohsiung Medical University, Kaohsiung, Taiwan. March 2020.
2. Best Presentation at the “Science Conclave 2020” Organized by Faculty of Science, The M. S. University of Baroda, Vadodara. February 2020.
3. UGC Travel Award by The M. S. University of Baroda to present the paper at International Conference CACEE 2018 organized by TIFR, Mumbai, India. January 2018.
4. M.S. Patel Travel Award by The Department of Biochemistry, Faculty of Science, The M. S. University of Baroda to present the paper at International Conference CACEE 2018 organized by TIFR, Mumbai, India. January 2018.

E-Symposiums attended during Ph.D. Duration:

1. “Electrochemistry: Fundamentals and Applications in Engineering (EFAE20)”, Department of Chemical Engineering, National Institute of Technology, Rourkela, Odisha. October, 2020.
2. EMBL Conference: Microfluidics: Designing the Next Wave of Biological Inquiry, Heidelberg, Germany. July, 2020.
3. “Physics meets Biology for COVID19 Management”, Department of Physics, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat. June 2020.
4. “Electrochemical Biosensor Webinar-Covid19 detection”, Sinsil International Pvt. Ltd. June, 2020.

5. “Mitochondrial Copper in Human Disease”, Department of Biochemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Gujarat. May 2020.
6. “Loss of GSK-3Beta Kinase Mediated Phosphorylation in an HtrA2 Variant Contributes to Parkinsonian Phenotype”, The Maharaja Sayajirao University of Baroda, Gujarat. May 2020.

Date: **Signature of the candidate**

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