Synopsis of the thesis on

Development of Prototypes of Biosensors for the Detection of Pathogens, Cancer Biomarkers and Environmental Pollutants

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 Date:
The Registrar (Academic Section),
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Subject: Submission of synopsis of the Ph.D. work entitled **"Development of Prototypes of Biosensors for the Detection of Pathogens, Cancer Biomarkers and Environmental Pollutants"**

Respected Sir/Madam,

Kindly accept the synopsis of my Ph. D. work entitled **"Development of Prototypes of Biosensors for the Detection of Pathogens, Cancer Biomarkers and Environmental Pollutants"**. My date of registration is 25/01/2021 and registration no. is FOS/2250.

Thanking you,

Yours sincerely,

Dimpal Jayantilal Aghera

Prof. C. Ratna Prabha
Guide

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INTRODUCTION:

A biosensor can be defined as an analytical device used to identify and measure specific analytes by incorporating a biological element with a physicochemical sensor. These devices belong to the category of biophysical devices capable of quantifying and detecting the concentrations of particular analytes within different environmental contexts.

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 and utility aspect such as reusability, disposability, simplicity, portability, sensitivity and selectivity. Biosensors consist of three fundamental elements: Analyte, Receptor, and Transducer. They are categorized based on these fundamental components. The Receptor plays an essential role in identifying and binding with the target analyte, facilitating the transmission of recognition signals to the Transducer. The Transducer, in turn, converts these signals into detectable forms. Depending on the specific type of Transducer employed, biosensors can be categorized as electrochemical, optical, piezoelectric and calorimetric biosensors. Additionally, various receptors give rise to various biosensor types, such as enzyme, antigen or antibody, nucleic acid, cell, and biomimetic biosensors (Perumal et. al., 2014).

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. The global biosensor market will reach a range of USD 31.5 billion by 2028 (Cagnani et. al., 2022). Glucose biosensor for diabetic patients is a major achievement in this field (Gupta et al., 2018)

The incentive of research and exploration within the biosensor domain rises from the inherent Shortcomings experienced by conventional approaches. 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 applications. Our laboratory has carried out an investigation into the functionalization and utilization of carbon nanotubes (MWCNTs) in the development of electrochemical-enzyme biosensors. Specifically, a suspension containing multiwalled carbon nanotubes was employed in polyvinyl alcohol to immobilize the glucose oxidase enzyme, serving as a model for the construction of enzyme based biosensor. The resultant electrode exhibited an enhanced glucose oxidase loading, thereby leading to a better glucose detection limit on the fabricated electrode (Gupta et al., 2018, 2016).

However, modern sensor technologies are afflicted by various limitations, including issues related to specificity, selectivity, limit of detection (LOD), vulnerability to manufactured articles, cost factors, and invasiveness. The fundamental aim of this study is to develop sensor prototypes aimed to tackle the inherent deficiencies in both conventional methods and existing sensor designs and applications. The study places its primary focus on advancing sensor technology, optimizing fabrication processes, introducing methods for thorough characterization, and establishing standardized protocols for biosensors.

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, phenol sensor eliminated the use of membrane while lactose sensor offers high sensitivity, triglyceride sensor offered novel fabrication chemistry and high conductivity due to the combination of nanoparticles and the polymer interface used and also detects real samples. Aptamer assay as a new detection methodology has been explored to detect SARS-Cov-2 for real sample analysis. Fabrication of the biosensors, their characterization and standardization were done by various techniques such as cyclic voltammetry, FT-IR and FE-SEM.

SPECIFIC OBJECTIVES OF THE THESIS:

Major objectives of the present study are:

1. Development, fabrication and characterization of biosensor for the detection of **Environmental pollutants**.
2. Development, fabrication and characterization of biosensor for the detection of **Lactose**.

3. Testing of **Covid-19** real samples of Nsp3 gene of SARS-CoV-2 using DNA chip.
4. Testing of **triglyceride** real samples using conductive Nano-PEI-lipase based biosensor.
5. Development, fabrication and characterization of biosensor for the detection of **pathogens.**
6. Development, fabrication and characterization of biosensor for the detection of **cancer biomarker.**

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

Phenol is a natural organic compound which is commonly utilized in many organic preparations or in separation methods using phenolic resins. Phenols are released to the environment through degradation of natural organic substances or by burning wood. Phenol has brought in significant attention as a priority pollutant under the Jurisdiction of the US Environmental Protection Agency (EPA), as indicated by the Emergency Planning and Community Right-to-Know Act of 2014. The EPA has established severe guidelines for the allowable concentration of phenol in surface water, stipulating that it must not exceed 0.001 mg/L, while concentrations in the range of 9-25 mg/L are considered toxic to the environment and aquatic life, as reported earlier (Kulkarni et al. in 2013).

Hydroquinone (1,4-benzenediol is a common phenolic compound that finds extensive utility in diverse industries such as medicine, cosmetics, and pesticide production. Hydroquinone, which is known to be produced during the initial stages of oxidation of phenol, significantly enhances the toxicity of phenol-contaminated wastewater. This raises concerns about the elevated toxicity and reduced degradability of hydroquinone compared to the original phenol contaminants. It is important that even low concentrations of hydroquinone can be a severe threat to animals, plants, and the environment. High levels of hydroquinone exposure can lead to a range of health problems, including headaches, fatigue, tachycardia, kidney damage, and even cancer.

Traditional methods for detecting phenolic compounds, such as gas chromatography, liquid chromatography, spectroscopy, and flame ionization detection, have been widely utilized, with some success (Clement et al. in 1995). These techniques definitely offer precision and accuracy. Nevertheless, they come with significant drawbacks, including complexity, labor-intensive procedures, the necessity for well-trained technicians, and the requirement for high-throughput instrumentation. Additionally, conventional methods frequently encounter interference from other substances in the samples and involve intricate sample purifications. These limitations often necessitate the centralization of testing in specialized laboratories, leading to time-consuming processes, from sample collection to report preparation and delivery.

Phenolic biosensor involves the use of laccase (Lac), a polyphenol oxidase enzyme that contains multiple copper ions and efficiently utilizes molecular oxygen to oxidize various phenolic compounds through a four-electron reduction of oxygen to water (Claus in 2004 and Barton et al. in 2001). This remarkable enzymatic capability enables laccase-based biosensors to directly catalyze the oxidation of hydroquinone to p-quinone without the need for solvents, thereby enabling the quantification of hydroquinone. Laccase-based biosensors are employed in a variety of contexts, including food analysis and environmental monitoring.

In the present study, hydroquinone is selected as a model phenol for developing a prototype of anenzymatic biosensor for electrochemical sensing. To fabricate electrochemical sensor MWCNTs having carboxylic groups on their outer surface were layered on a screen-printed carbon electrode. Carboxyl groups of MWCNTs reacted with amine groups of laccase enzyme and resulted into amide bond formation. The biosensor's performance is based on the activity of Laccase enzyme immobilized on the electrode. When enzyme is exposed to hydroquinone substrate, it forms p-benzoquinone + $2H_2O$ + $2H^+$ + $2e^-$. Current produced due to this reaction was measured by the fabricated electrode. Thus, the sensor was exposed to various concentrations of hydroquinone pollutant and current was measured before and after its exposure. The fabrication method avoids the use of any membrane, which mayinterfere with the electron transfer process. Cyclic voltammetry technique was used to characterize and standardize the biosensor.

Fabrication and optimization of Lac/MWCNTs/SPCE coating:

MWCNTs bearing carboxylic group were cast-off on screen printed carbon electrode (SPCE) for coating the surface 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, aliquot from stock solution of enzyme in phosphate buffer (pH 7.0) was drop casted onto the MWCNTs layer. This resulted into amide bond formation between MWCNTs and laccase. Thus, the resulting electrode was washed with phosphate buffered saline (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 FE-SEM. Potential used for cycling the voltage was +0.5 to -0.3 with the scan rate and sample interval of 100 mV/S and 0.001 V respectively.

Standardization and characterization of Lac/MWCNTs/SPCE:

Other parameters such as concentrations of hydroquinone as a substrate also optimized using the same parameters mentioned above. Detection limit and linear range of the sensor was also studied and was found to be 5 µM and 800-1200 µM respectively. Sensitivity/interference analysis done using various substrates like Phenolic compounds (2-Nitro phenol, 4-Nitrophenol, Hydroquinone), protein (BSA), small molecules (glucose, cysteine, L- lysine and ascorbic acid, Folic acid), $CuSO_4$, EDTA, $FeCl_3$, KCl, $MgSO_4$, $MnSO_4$, NaCl, DNSA, uric acid using CV. All these compounds do not interfere when present in low amounts. The sensor when stored at 4˚C in air dry condition, showed 75% of its initial activity after 30 days. The sensor can be refabricated with SD of 0.56%.

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

Lactose is a disaccharide found in milk and dairy products. Lactase, an enzyme, breaks down lactose into galactose and glucose monomers. While children typically have sufficient lactase, most adults retain only 10% of their infant-level lactase activity, leading to lactose intolerance. This condition causes symptoms like abdominal pain, diarrhoea, nausea, and flatulence after consuming lactose-containing foods. Therefore, dairy industries are making efforts to produce lactose-reduced or lactose-free products.

Various methods can estimate lactose content, including spectrophotometry, polarimetry, infrared spectroscopy, titration, and chromatography. However, these methods have limitations, such as involvement of expensive equipment, trained professionals, and extensive sample preparation. Electrochemical methods offer an attractive alternative, as they provide precise, selective, and onsite results.

Researchers have explored biosensors for lactose detection. Marrakchi et al. developed a biosensor using β-galactosidase and glucose oxidase immobilized in films with glutaraldehyde for quantitative lactose detection in milk (2008). Tasca et al. created a third-generation biosensor using a single-walled carbon nanotube electrode modified with cellobiose dehydrogenase and aryl diazonium to determine lactose content (2013). In summary, lactose intolerance is a common issue, and electrochemical biosensors offer a promising approach for efficiently detecting lactose content in dairy products.

In the present study, Carbon nanotubes in combination with CuO-TiO_2-PEI and β-galactosidase-galactose oxidase enzymes were employed to fabricate electrochemical sensor using screen-printed carbon electrode.

SPCE was modified by layering MWCNTs with carboxylic groups on their surface. Following that, CuO-TiO_2-PEI mixture was directly drop casted on to the surface of the MWCNTs followed by β-galactosidase (β-gal) and galactose oxidase (Gox). Carboxyl groups of MWCNTs reacted with amine groups of the enzymes forming amide bonds. When β-gal and Gox are exposed to lactose substrate, O_2, $2H^+$ and $2e^-$ are formed in the successive reactions as given below:

$$\text{Lactose} + H_2O \longrightarrow \text{Glucose} + \text{Galactose}$$

$$\text{Galactose} + O_2 \longrightarrow \text{d- Galacto- hexodialdose} + H_2O_2$$

$$H_2O_2 \longrightarrow O_2 + 2H^+ + 2e^-$$

The release causes changes in pH of electrolyte solution which then results in the increase of current. This response can be measured by the electrode in the form of current produced due to this reaction. The sensor was exposed to various concentrations of lactose to measure its performance by cyclic voltammetry.

Fabrication and optimization of β-gal-Gox/CuO-TiO_2-PEI/MWCNTs/SPCE coating:

MWCNTs bearing carboxylic group were cast-off to SPCE for coating the surface 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, CuO-TiO2-PEI were drop casted onto the MWCNTs layer. Following it, aliquot from stock solutions of enzymes (β-gal & Gox) in PB (pH 7.0) were drop casted onto the MWCNTs layer. This resulted into amide bond formation between MWCNTs and β-gal & Gox. Thus, the resulting electrode was washed with PB 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 FE-SEM. The electrolyte composition used throughout the experiments was as follows: 0.1 mM KCl + 50 mM $K_3[Fe(CN)_6]$ + 0.1 M Phosphate buffer (pH 7.0) with the scan rate and sample interval of 100 mV/S and 0.001 V respectively.

Standardization and characterization of β-gal-Gox/CuO-TiO2-PEI/MWCNTs/SPCE:

Other parameters such as concentrations of lactose as a substrate was optimized using the same parameters mentioned above. Detection limit and linear range of the sensor was studied and was found to be 0 mM and 0-5 mM respectively. Importantly, the thesis included the stability, regeneration, reproducibility, analysis of real samples, showcasing the practical applicability of the developed biosensor for Lactose estimation.

Objective 3: Testing of Covid-19 real samples of Nsp3 gene of SARS-CoV-2 using DNA chip.

The COVID-19 pandemic, caused by the novel coronavirus SARS-CoV-2, has been declared a global health crisis by the World Health Organization (WHO) since January 9, 2020 (Mojsoska et al., 2021). To date, it has affected over 77.1 million people worldwide, resulting in approximately 6.9 million reported deaths. Early diagnosis of COVID-19 is crucial to isolate and treat infected individuals and slow the virus's spread.

Various testing methods, including CT scans, RT-PCR, and ELISA, are available to detect COVID-19. CT scans offer high sensitivity but low specificity. RT-PCR is the gold standard for definitive diagnosis, but it may yield false results. ELISA is cost-effective and rapid but its accuracy is questioned (Mojsoska et al., 2021). All these methods require skilled technicians and specialized facilities, causing delays in analysis.

Developing onsite point-of-care (POC) tests could alleviate these challenges and expedite testing. Over 140 commercial tests for SARS-CoV-2 detection have been introduced, most of which are RT-PCR-based. Some companies offer one-step RT-PCR kits, but their accuracy is not optimal due to low amplification rates.

One way to reduce testing delays is by using electrochemical DNA-chips. Samples can be subjected to reverse transcriptase PCR to produce cDNA, which can then be quantified directly using electrochemical biosensors, eliminating the need for cDNA amplification through qPCR and providing results within minutes. This approach can significantly accelerate the testing process. Even though the electrochemical biosensor based on this principle was constructed earlier in our laboratory, its validation using real samples was pending.

The DNA-chip was designed for the electrochemical detection of a nonstructural protein Nsp3 gene of SARS-CoV-2. To create this chip, a single-stranded DNA oligonucleotide probe representing the sequence from the gene of the nonstructural protein was covalently attached to functionalized multi-walled carbon nanotubes (MWCNTs). The resulting ssDNA-

MWCNT complex was mixed with polyvinyl alcohol (PVA) polymer and applied as a coating on a screen-printed carbon electrode. This film was crosslinked using glutaraldehyde (Jinal Thakkar, Ph.D. thesis 2022). However, Validation of performance of the electrode with real samples remained to be established. **This study focused on real sample analysis by using previously made DNA-chip.**

Real Sample analysis:

To investigate the application of the biosensor in the detection of SARS-CoV-2 positive samples was investigated. Serum samples of Covid-19 were purchased and isolation of RNA was done. Reverse transcriptase PCR was carried out with RNA from SARS-CoV-2 as the template. The validation of the DNA chip sensor was carried out with 2 cDNA samples of 1 fM & 10 fM concentrations and CV peak current (Ip) was monitored with respect to the oligonucleotide probe. The change in Ip with respect to probe, negative control (aptamer free coated electrode) confirms that hybridization occurred. The change in Ip was observed in both the positive samples.

Objective 4: Testing of triglyceride real samples using conductive Nano-PEI-lipase based biosensor.

Triglycerides (TGAs), the natural transporters of dietary fat 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 and 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 and is considered to be indicator of borderline disease. High risk level is from 200-499 mg/dL, and > 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 expensive 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 the 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 research focused on the applied analysis of real samples using a previously developed TGA biosensor. This biosensor was designed for the electrochemical detection of triglycerides (TGA). To enhance its performance, the study utilized a combination of reduced graphene oxide nanoparticles, carbon nanotubes, titanium oxide nanoparticles, and polyethyleneimine. The fabrication process was optimized to increase enzyme loading on the sensor, consequently improving its detection limit. The electrode after fabrication was characterized and standardized using cyclic voltammetry. However, real sample analysis was not completed and needed to be accomplished (Jinal Thakkar's thesis, 2022). In the present study real sample analysis was done using sunflower oil, coconut oil and groundnut oil (Thakkar et al., 2023).

Real Sample analysis:

To check the performance of the bioelectrode GCE/GO/MWCNTs/ PEI-TiO_2/GA/Lipase for its practical use, the electrode was tested against commercially available double-filtered ground nut oil, coconut oil and sunflower oil. The oils were separately mixed in 50 mM phosphate buffer (K3[Fe(CN)6], 9 % NaCl, pH 8). The samples were scanned at an 80 mV/s scan rate. Stock solutions of the ground nut oil, coconut oil and sunflower oil were made in ethanol and were dissolved at three different concentrations into the reaction mix, just before adding Triton X-100 (0.2 % W/V). The results showed relatable current change at the anodic sweep segment of cyclic voltammetry. The average recovery of the real sample was found to be 101.05 %, 98.03 % and 95.47 % with coconut oil, groundnut oil and sunflower oil respectively (Thakkar et. al., 2023).

Conclusion:

Phenol and Lactose biosensors were successfully developed using the electrochemical technique cyclic voltammetry. Electrochemical biosensor developed for detection of Hydroquinone can be used for analytical applications directly. Response time and Detection limit are 10 sec. and 5 µM respectively. Stability of the sensor were found 75%. The sensor was used against real samples for detection and it showed 98.9% and 101.5% of sample recovery. Electrochemical biosensor developed for detection of lactose with novel fabrication chemistry can be subjected for miniaturization and analytical application directly. Detection limits of the lactose sensor are 0 mM with linear range of 0-5 mM. Surface characterization, interference, stability and real sample analysis is ongoing. The biosensor for Covid-19 was tested for its performance with cDNA from prepared from Covid-19 positive serum. Real samples coconut oil, groundnut oil and sunflower oil were used to validate the performance of TGA biosensor. The efforts to develop biosensors for the detection of cancer biomarker for breast cancer Her2 and for the identification of *Mycobacterium sp.* are underway.

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Jinal B. Thakkar [a], Dimpal J. Aghera [a], Bhavana Trivedi [b], C. Ratna Prabha [a],*. Design and characterization of a biosensor with lipase immobilized nanoparticles in polymer film for the detection of triglycerides. International Journal of Biological Macromolecules 229 (2023) 136–145.

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Peer Reviewed Publications:

Jinal B. Thakkar [a]**, Dimpal J. Aghera** [a]**,** Bhavana Trivedi [b], C. Ratna Prabha [a,*]. Design and characterization of a biosensor with lipase immobilized nanoparticles in polymer film for the detection of triglycerides. International Journal of Biological Macromolecules 229 (2023) 136–145.

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

A one-day summit on ‘Low-cost diagnostics for affordable healthcare’ was organized by Wadhwani Research Centre for Bioengineering (WRCB), IIT Bombay, on 3rd June 2022.

The International Conference on "Celebrating Proteins on the Birth Centenary of Dr. G. N. Ramachandran " The Maharaja Sayajirao University of Baroda, Vadodara, on 4th March 2023.

Workshops Attended during Ph.D. Duration:

Hands on workshop on “Electrochemical biosensor technology”, Svatah bioinnovations LLP, GTU Ahmedabad, India. October 2023.

Date: **Signature of the candidate**

Dimpal Jayantilal Aghera

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Guide

Head

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