CHRONOAMPEROMETRIC SYNTHESIS AND CHARACTERIZATION OF POLYANILINE FILMS FOR GLUCOSE BIOSENSING.

A DISSERTATION THESIS SUBMITTED FOR THE PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF DEGREE

Of

MASTER OF SCIENCE

IN

BIOTECHNOLOGY

SUBMITTED BY

MAHIMA YADAV

2K20/MSCBIO/12

UNDER THE SUPERVISION OF

Prof. JAIGOPAL SHARMA
DEPARTMENT OF BIOTECHNOLOGY
DELHI TECHNOLOGICAL UNIVERSITY.

Dr. GAJJALA SUMANA
SENIOR PRINCIPAL SCIENTIST
CSIR- NPL

SUBMITTED TO

DELHI TECHNOLOGICAL UNIVERSITY

(FORMERLY DELHI COLLEGE OF ENGINEERING)

BAWANA ROAD, DELHI-110042

(2020-2022)

DECLARATION BY THE CANDIDATE

I hereby declare that the work presented in this dissertation entitled **"Chronoamperometric Synthesis And Characterization Of Polyaniline Films For Glucose Biosensing"** has been carried out by me under the guidance of internal supervisor **Prof. Jaigopal Sharma**, Professor, Department of Biotechnology, Delhi Technological University, Bawana Road, Delhi and external supervisors **Dr. Sumana Gajala**, Senior Principal Scientist, National Physical Laboratory, Delhi. This work is as per scientific ethics. No scientific dishonesty has been incorporated. Due recognition has been given to the authors whose work has been summarized here by means of correct references and citations. Any part of this work will not be published in print or electronic media without prior permission of the supervisor and the Institute (NPL, CSIR). This work has not been submitted elsewhere for the award of any other degree or diploma of the university or another institute of higher learning.

Mahima

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Mahima Yadav

Master of Science (Biotechnology)

2K20/MSCBIO/12

Date: 06-05-2022

Place: New Delhi.

CSIR-National Physical Laboratory
Dr KS Krishnan Marg, Delhi-110012

CERTIFICATE -II

This is to certify that the Project entitled "**Chronoamperometric Synthesis And Characterization Of Polyaniline Films For Glucose Biosensing**" submitted by **Ms. Mahima Yadav**, Roll no. **2K20/MSCBIO/12** in partial fulfilment of the requirements for the award of degree of Masters in Biotechnology from Delhi Technological University, Delhi, has done her dissertation work from Biomedical Instrumentation Section, Material Physics and Engineering National Physical Laboratory (NPL)-CSIR and it is an authentic work carried out by her under my supervision and guidance.

To the best of my knowledge, the matter embodied in this project report submitted by her is authenticated and has not been submitted to any other University/Institute for the award of any degree. Duration 2/3/22 to 5/5/2022

.....

Dr. Gajjala Sumana
Senior Principal Scientist
(External Supervisor)
National Physical Laboratory, CSIR.

डॉ. रीना शर्मा / Dr. RINA SHARMA
प्रमुख, मानव संसाधन विकास समूह
Head, Human Resource Development Group
सी.एस.आई.आर. -राष्ट्रीय भौतिक प्रयोगशाला
CSIR - National Physical Laboratory
डॉ. के. एस. कृष्णन मार्ग, नई दिल्ली-110012
Dr. K. S. Krishnan Marg, New Delhi-110012

NATIONAL PHYSICAL LABORATORY
(HRD GROUP)
Endorsement No. 361a/5a/21

ACKNOWLEDGEMENT

It is my privilege to thank my Internal Supervisor **Prof. Jaigopal Sharma**, Department of Biotechnology, Delhi Technological University. His guidance, knowledge and discussions were invaluable. His support and encouragement has been great source in completion of this project work. I wish to express my sincere thanks to **Ms. Neha Tiwari**, the senior research fellow, for her timely and scholarly advice. Her scientific approach helped me to a very great extent. Her friendly advice proved to be very enthusiastic.

I would like to express my deepest gratitude to my External Supervisor**, Dr. Gajjala Sumana**, for her excellent guidance, patience, and providing me with a very supporting atmosphere like DST-BECPRL, NPL for doing research. She guided me in each and every step of this project and motivated me to do this work. The help rendered by **Mrs. Rajrani, Ms. Priyanka, Mrs. Chandani, Ms. Samridhi** at every point of this project, by healthy discussions and encouragement is greatly acknowledged. Words cannot express my gratefulness to all of them for providing their valuable help and time whenever required. Special thanks to my friend and my fellow trainee **Varsha Yadav** who made this period memorable with her affection and care. Most importantly none of this would have been possible without the love and patience of my Parents. They have been a constant source of love, concern and support.

Mahima

MAHIMA YADAV
M.Sc. Biotechnology
2K20/MSCBI0/12

ABSTRACT

In medical diagnostics, medicines, food, and fermentation sectors, glucose level control is critical. The creation of an enzyme-based amperometric sensor employing an electrochemically deposited polyaniline layer on ITO electrode is described in this thesis. The integration of polymer films plays vital role in improving the performance of electrochemical devices. These polymer films provide active site for immobilization of metal nanoparticles that contribute towards the better performance of electrochemical devices. In the electrochemical devices PANI plays an very important role because it serves as a conductive material for transfer of electron, create a sensitive layer for detection, and for the immobilization of enzyme it act as a biocompatible matrix. Various conducting polymer-metal nanoparticles composites have been researched that enhances the electrocatalytic behavior of the device. These have found application in development of glucose sensors. The demand for detection of glucose has led to the advancement in the advancement of ideal enzymatic glucose sensor. In the present work polyaniline based conducting polymer have been investigated towards development of enzymatic glucose sensors. Thin films of Polyaniline has been deposited on ITO glass slides in the presence of Hydrochloric acid that act as an electrolyte. These electrochemically synthesized PANI films have been characterized with the help of Cyclic Voltammetry. Glucose Oxidase enzyme is immobilized on the PANI/ITO electrode by the process of physical adsorption. The redox characterization of GOx/PANI/ITO and PANI/ITO films has been performed through Cyclic Voltammetry (CV) method. A linear graph is obtained between the current and the concentration of glucose. Therefore the synthesized GOx/PANI/ITO electrode could be a promising candidate for the biosensing of glucose.

Keywords: *Amperometric, Biosensors, Cyclic Voltammetry, Electrochemical Devices, Glucose Oxidase, ITO Glucose Biosensing.*

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LIST OF ABBREVIATIONS

CPs - Conducting Polymers.

PTH – Polythiphene.

PA – polyacetylene.

PF – Polyfuran.

PPy – Polypyrrole.

PPP - Poly para phenylene.

PPV - Poly (phenylenevinylene).

PANI - Polyaniline.

ITO – Indium Tin Oxide.

Ab – Antibody.

DNA – Deoxyribonucleic Acid.

ES - Emeraldine Salt.

PS – Pernigraniline Salt.

LS – Leucomeraldine Salt.

PBS – Phosphate Buffer Saline.

CV – Cyclic Voltammetry.

CE – Counter Electrode.

HCl – Hydrochloric Acid.

CA – Chronoamperometry.

GOx- Glucose Oxidase.

RNA- Ribonucleic Acid.

CHAPTER 1
INTRODUCTION

1.1 Conducting Polymers:

Prior to the creation of conducting polymers (CPs), polymers were thought to be electrical insulators, however these organic polymers show remarkable optical and electric properties that are equivalent to those of semiconductors. The highly delocalized, polarised, and electron-dense bonds are responsible for the electrical and optical behaviour of a conjugated carbon chain, which is made up of alternating single and double bonds (K & Rout, 2021).

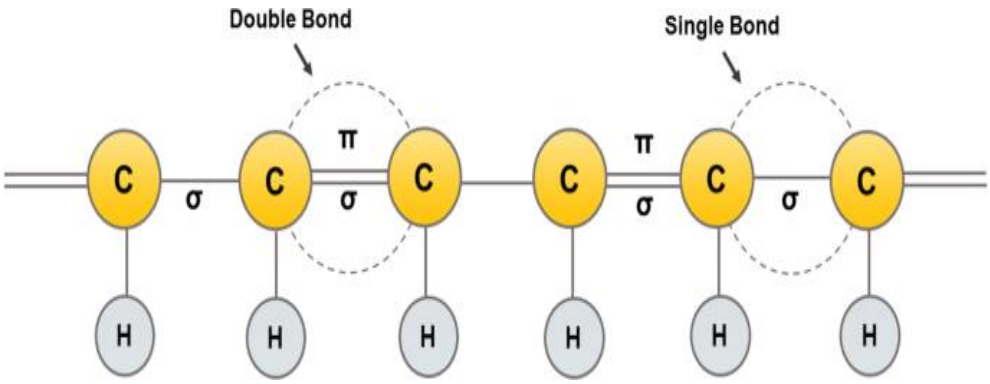


Figure 1: Schematic diagram of a conjagte backbone having alternating single and double bonds.

Typical conducting polymers include poly-thiphene (PTH), poly-acetylene (PA), poly-furan (PF), poly-pyrrole (PPy), poly-paraphenylene (PPP), poly-phenylenevinylene (PPV), and polyaniline (PANI). Alan and J. Heeger are the scientist discovered $(SN)_x$ sulfur nitride metal, an inorganic substances, which showed higher electrical conductivity when it is doped with bromine, and this finding led to the investigation of conducting polyacetylene. Polyacetylene doped with bromide has a conductivity a thousand times higher than that of pristine polyacetylene and this investigation was rewarded by a Nobel Prize in 2000 (Zhao et al., 2016). CPs can exist in both forms localized and de-localized and the difference between them is that the delocalized form helps in the formation of charged carriers including bipolarons, polarons, solitons and the delocalization of π bonds majorly depends on the disorder (Rahman et al., 2015).

Conjugate polymers' conductivity serves as an insulator to a semiconductor in their pure form, and the conductivity grows as the dopant concentration rises (Lu et al., 2018).

1.1.1 Conduction Mechanism:

Because the carbon atoms on the polymer network are sp2 hybridised, the alternating single- and double-bond structure can be electrically conductive. In the z direction, the p orbitals of carbon atoms are parallel to each other, forming a continuous p bond that may be used to transport charge carriers down the polymer chain. External charge carriers should thus be added to the polymers to make them conductive (Lanzalaco & Molina, 2020).

Electron acceptors can partially oxidise CPs, whereas electron donors can partially decrease them.Whenever a conducting material is doped with a dopant, the conduction band or valence band are either partially filled or polarons are formed. The band theory may be supported using Molecular Orbital Theory (Guo & Ma, 2018). 2 additional molecular orbitals can be formed by combining one p orbital from one carbon atom with another p orbital from some other carbon atom. The energy of one of the newly generated orbitals is lower than the energy of the original p orbital, while the other has a greater energy than the original p orbital. The bonding molecular orbital is the lower-energy orbital. The anti-bonding molecular orbital has a greater energy than the other orbitals (Inoue et al., 2020).

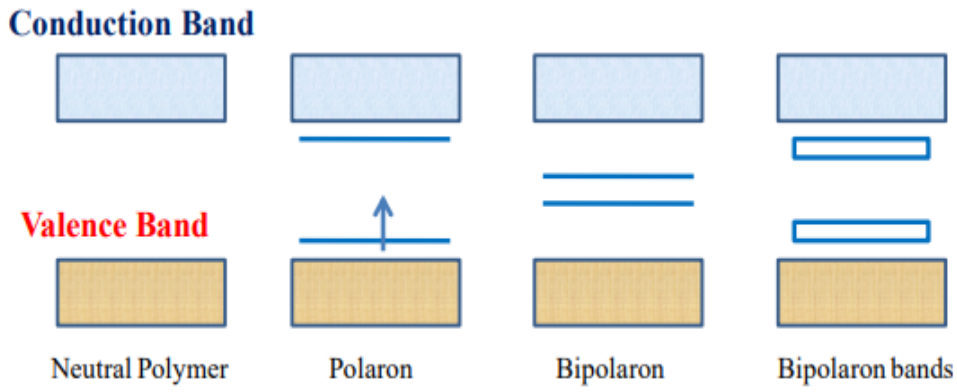


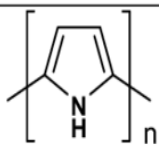
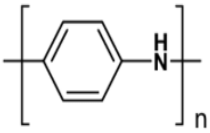
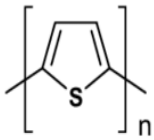
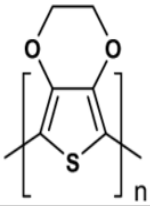
Figure 2: Band theory of conducting polymers.

1.2 Conducting Polymers in Fabrication of Sensors:

Conducting polymers (CPs) are a point of interest because of their exceptional electrical, optical, and electrochemical properties. The pioneer research in the area of conducting

polymers was based on the achievement of high electrical property of polyacetylene with oxidizing agents by Heeger ((Yuk et al., 2020). Sensors have been considered to be one of the most important practical applications of the CPs. Some of the CPs utilized in fabrication of sensors has been summarized in Table below. Each of these CPs have their own features to be used in sensor application. For example, polypyrrole (PPy) has excellent biocompatibility and low oxidation potential while PEDOT has characteristics of optical transparency and solubility in water (Diaz & Hall 1983). On the other hand, the monomer of PANI is inexpensive. In sensor application, the sensing mechanism of CPs may involve redox behaviour, weight and volume change, ion adsorption and desorption, charge transfer, and chain conformational changes. CPs have diverse chemical structure which imparts the property of attaining high selectivity and sensitivity as compared to their inorganic counterparts. Also, CPs have high flexibility, property of synthesis at low temperature, and are cost effective (Maity & Dawn, 2020).

TABLE 1: CPs utilized in fabrication of sensors.

Name	Structure
Polypyrrole (PPy)	
Polyaniline (PANI)	
Polythiophene (PTh)	
Poly(3,4-ethylenedioxythiophene) (PEDOT)	

1.3 Biosensors:

Biosensors are small integrated devices that employ biological elements like antibodies, enzymes, receptor proteins, nucleic acids, cells or tissue section because the sensor that's including the transducer for reception.

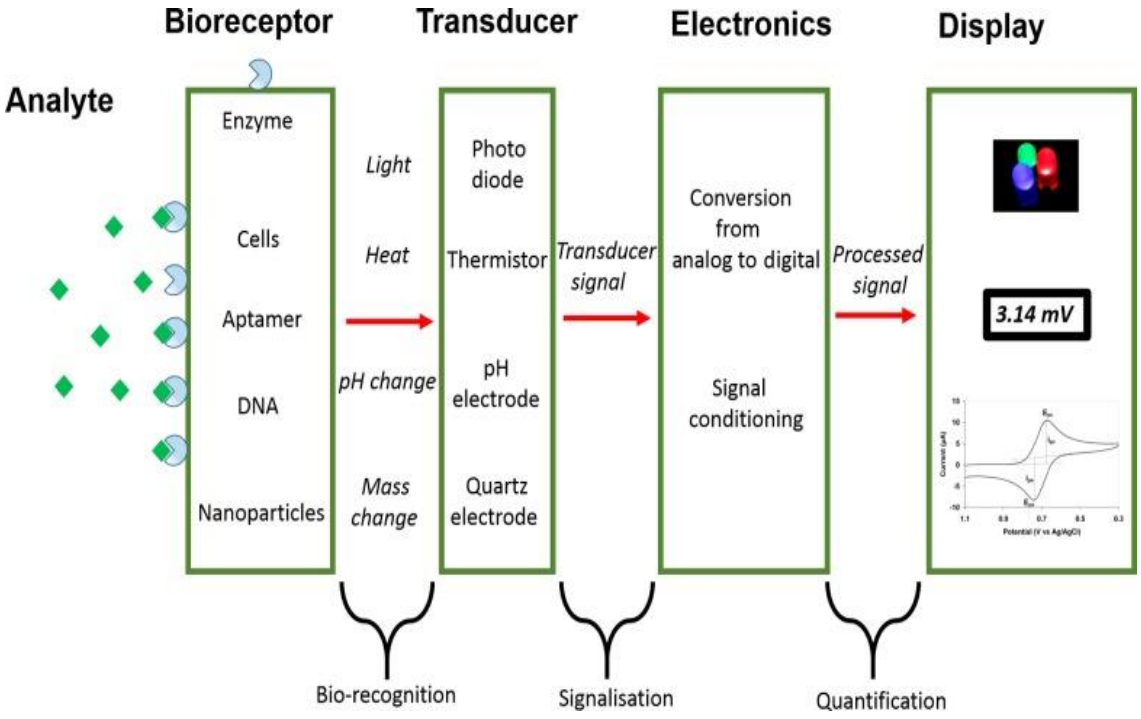


Figure 3: Schematic diagram of Biosensor.

1.4 Classification of Biosensors:

Based on the bioreceptor and the transducer the biosensors are mainly classified into two types. The biorecoginition elements contain enzyme, cell, nucleic acids, nanoparticles, aptamers, antibodies etc. and on the basis of transducer it conatins pezioelectric, amperometric, optical, and thermal biosensors (Naresh & Lee, 2021). The amperometric and the potentiometric biosensors are generally considered under electrochemical sensors. The amperometric and the poteniometric electrochemical biosensors are most extensively used biosensors as they offer easy handling, low cost, and low limit of detection etc.

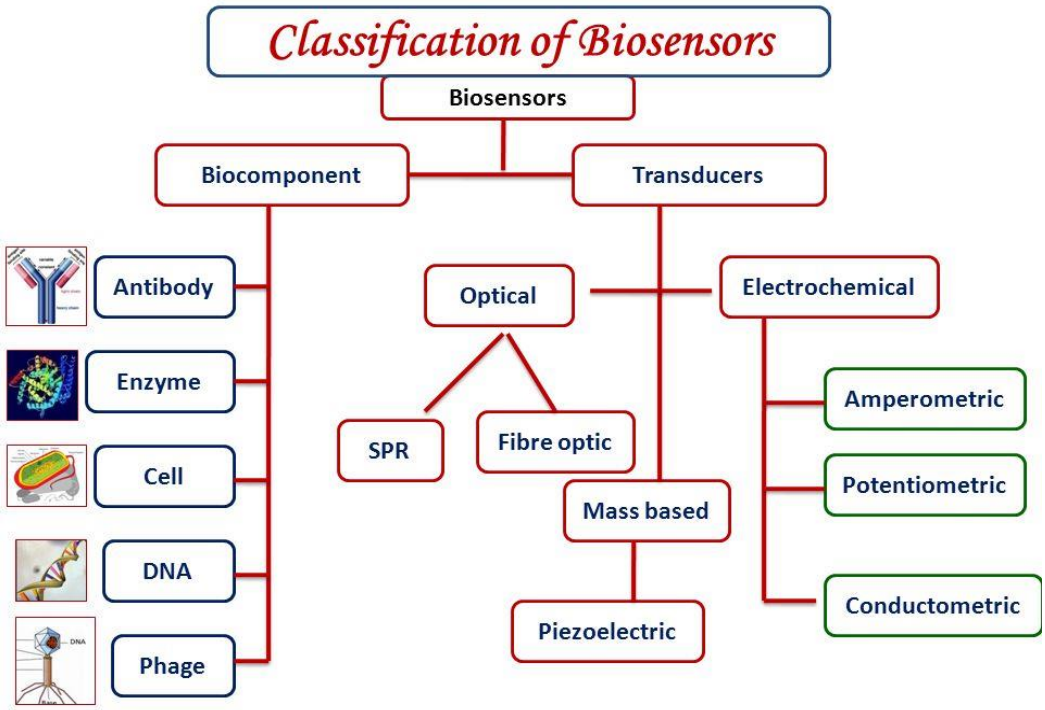


Figure 4: General classification of Biosensors.

1.4.1 Bioreceptor:

Bioreceptors are molecular assemblies that have the ability to recognise the target analyte and are thus regarded to be the most important component in the construction of specialised biosensors. Bioreceptors are divided into five primary groups, which include: Antibodies, enzymes, cells, tissues, and nucleic acids are the five categories (Bhalla et al., 2016; Kamel et al., 2019).

Bioreceptors for Antibodies: An antibody is a complex biomolecule with unique functions. It binds because it is made up of a sequence of amino acids organised in a highly structured structure. Antigen is a term used to describe a substance that is particular to its target . This one-of-a-kind feature is that it is the secret to their success applicability in the manufacture of immunosensors when the analyte of interest is detected.AB–antigen interactions that are particular However, AB binding is reliant on it capacity and irreversible interaction of test conditions (e.g., pH and temperature).

Enzyme Bioreceptors: Enzymes have catalytic activity in addition to specialised binding capabilities, making them ideal bioreceptors for sensing tests. The enzymes are all proteins, with the exception of a few catalytic RNA molecules. Antibodies' catalytic activity is dependent on the integrity of their natural protein structure, allowing for far lower detection limits than other

bioreceptor classes. Apart from their capacity to catalyse biological activities, enzymes as receptor molecules have a number of advantages, including the ability to detect a variety of analytes and compatibility with a variety of transduction mechanisms. The biosensor may be used again because enzymes are not consumed in reactions, but its lifetime is limited by their stability.

Cellular Bioreceptors: Cellular structures contain another type of bioreceptor that recognises a cell/microorganism or a specific cellular component capable of binding to a specific species. These have a tendency to attach to the surface and are therefore readily immobilised. They are also sensitive to their surroundings and can respond to a wide range of stimulation. However, compared to pure enzymatic biosensors, cell-based biosensors have limitations in terms of delayed reaction, resulting in poor specificity. This might be due to unfavourable side reactions catalysed by other enzymes in the same cell. The sensor duration is further limited by the cellular receptors' poor stability.

Tissue Bioreceptors: Tissues are employed as bioreceptors since they contain a large number of enzymes. Their benefits over biological bioreceptors include quicker substrate immobilisation, improved ability to maintain enzyme activity in natural environments, easier availability, and a lower price. However, key drawbacks include a lack of selectivity due to interference from other enzymes and a longer interval due to the transport barrier.

Table **2:** Bioreceptors based biosensors.

Receptor	Type
Enzyme	Bioaffinity/Biocatalysis
Antibody/Antigen	Bioaffinity (Immunosensor)
Nucleic Acids/DNA	Biocatalysis
Biomimetic Materials	Bioaffinity
Cellular Structures/Cells	Biocatalysis
Ionophore	Bioaffinity

1.4.1 Transducer:

The purpose of the transducer is to monitor the physiochemical changes caused by particular interactions between the target analyte and the bioreceptor. It transforms a biological signal into an electrical signal, which is then transformed into an analogue or digital signal. The transducer can perform both qualitative and quantitative measurements since the concentration of an analyte is related to the number of signals produced.

Table 3: Types of Biosensors based on the transduction mechanism.

Transduction Mechanism	Method
Mechanical	Stress sensing Mass sensing
Optical	Fluorescence Chemiluminescence Bioluminescence Surface Plasmon Scattering Evanescent Waves Interferometry
Electrical	Conductometric Capacitive
Piezoelectric	Quartz Crystal Microbalance (QCM) Surface Acoustic Wave (SAW)
Electrochemical	Potentiometric Amperometric Ion Sensitive FET[1] (ISFET) Chemical FET (ChemFET)
Thermal	Calorimetric

In a label–free procedure, detection is very simple and inexpensive since biomolecules do not appear to be altered, and so the target is recognised in its normal state, although sensing parameters are inadequate (Kamel et al., 2019). Electrochemical biosensors have gotten a lot of interest in biosensor development because of their simplicity, cost–effectiveness, accuracy, and high sensitivity for target analyte detection. Electrochemical biosensors commonly use amperometric and potentiometric transducers. Electrochemical biosensing devices are marketed in clinical, environmental, industrial, and agricultural domains due to inherent downsizing possibilities (Y. Li et al., 2020).

1.5 Characteristics of a sensor:

The performance parameters of a sensor determine its efficiency for specific application. These parameters are listed below:

i. **Sensitivity:** It is defined as the change in response of the glucose sensor to change in concentration of analyte. The sensitivity of sensor can be calculated from the slope of the calibration curve. For a sensor, sensitivity should be large and also constant in the linear range of concentration (Kalantar-zadeh & Fry, 2008).

ii. **Selectivity:** It is known as ability of the sensor to distinguish targeted molecule in presence of other interfering species of similar structures. The chemical and physical nature of analyte affects the selectivity of the sensor (Kristoffersson et al., 2021).

iii. **Detection limit:** It is termed as the lowest concentration of analyte which is measured by the sensor. For an ideal sensor the limit of detection can be in ppb values.

iv. **Response time:** It is defined as the time taken by the sensor to respond from zero concentration to a change in concentration of the analyte.

v. **Linear range:** It is the range from the lowest to the upper concentration values that can be accurately measured by the sensor. Generally, sensors with wide linear range of analyte concentration are preferred for practical purpose.

vi. **Stability:** It is the change in characteristics of the sensor over the period of time. Usually, it is termed as ability of the sensor to sustain its performance parameters over a period.

vii. **Reproducibility:** It is defined as the similarity among responses from identical sensors fabricated with same procedure. Generally, an ideal electrochemical sensor should show high selectivity and sensitivity, less response time, wide linear glucose concentration range, and low limits of detection. In addition, sensor should be portable, cost effective, stable, and accurate.

1.6 Glucose sensor:

In recent years, intensive research has been involved in designing of novel glucose sensor as well as improving their performance parameters. This involves the use of different material such as conducting polymers, metal nanoparticles, composites, biomaterials, cellulose and surfactants. Among these, metal nanoparticles conducting polymers and their composites have been extensively researched in fabrication of sensors (Kang et al., 2022). The blend of sensor technology and nanotechnology acts as a market propeller in the development of glucose sensors. However, still much of the attention is needed towards development of glucose sensors

that are thermally stable and have high sensitivity, stability, and wide linear range (Sahoo et al., 2019) (Cano et al., 2022) (Rafiqi & Moosvi, n.d.).

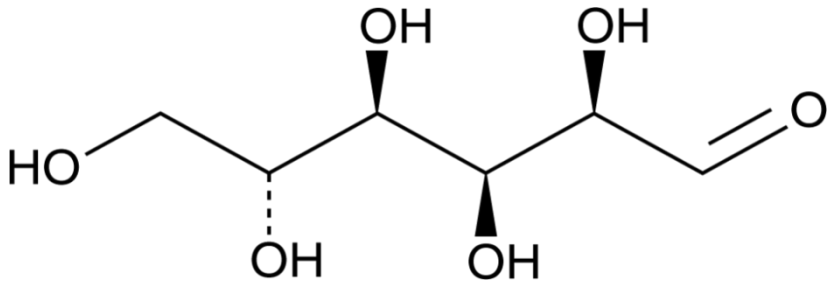
1.7 Need for Glucose Sensors:

The constant estimation of glucose is not only important in clinical diagnosis but also vital in industries like fermentation, beverage, and food (Batool et al., 2019). Glucose is significant carbohydrate subjected to need for continuous monitoring and accurate detection. It is the main source of energy in living organisms and in metabolic activities. Of the two forms of glucose, L-glucose and D-glucose, only latter form is biologically active and can be metabolized by cells (Neethirajan et al., 2018). Glucose is essential for the metabolic activities in the living organisms. The normal glucose level in blood is 80-120 mg/dL. The change in the level of concentration of glucose has adverse affect on the health (Pullano et al., 2022). Diabetes mellitus is due to the insulin deficiency that further result in hyperglycemia. Thus, it is required to maintain the adequate levels of glucose which can be attained with its continuous and accurate detection (Y.-C. E. Li & Lee, 2020). The selective, precise, and simple way for detection of glucose is a great challenge to be accomplished by the researchers.

Table 4: Different Glucose Levels in Body.

Blood Glucose Levels (mg/dL)	Blood Glucose Levels (mmol/L)	Interpretation
< 53	< 3	Severe hypoglycemia
< 70	< 3.9	Hypoglycemia
< 125	< 7	Normal
< 200	< 10	High (Take action)
>200 - 500+	>10 - 27.7+	Metabolic Consequences (Take action)

Glucose plays a vital role in fermentation industry as a growth limiting substrate. Also, it is the main source of energy and carbon for the growth of microbes. The variation in level of concentrations of glucose from desired value affects the quality and yield of the product. Hence, continuous monitoring of glucose is essential (Ridhuan et al., 2018).



Mostly, the electrochemical sensors for detection of glucose are based on the enzyme, glucose oxidase (GOx) that oxidizes glucose to gluconolactone which is further hydrolyzed to hydrogen peroxide and gluconic acid, (Duerkop et al., 2006).

Glucose + 0_2 → H_20_2 + Gluconic Acid

H_20_2 →0_2 + 2H+ + 2e.

1.8 Objective of Study:

The need for glucose sensors is increasing day by day. In this perspective, an ideal glucose is desirable that is highly reliable, accurate, stable, sensitive, and selective. This research work would be focused towards developing enzymatic glucose sensors based on conducting polymer (PANI). The main objectives of the present studies are as follows:

- **i.** To develop enzymatic sensors of high sensitivity, stability, wide linear range of glucose concentration, very less response time, sensitivity.
- **ii.** To study the characterization of the bioelectrode using cyclic voltammetry (CV) technique.
- **iii.** To evaluate the various analytical parameters using chronoamperometry (CA) technique.

CHAPTER 2

REVIEW OF LITERATURE:

2.1 The polyaniline:

Of the numerous conducting polymers that are being used in non-enzymatic glucose sensors, PANI has contributed most due to long range of conductivity that has been reported over the range of 11 orders and is stable to environmental and thermal conditions (Bera et al., 2017). In an acid solution, PANI exists in three different oxidation forms. Of these, emeraldine salt (ES) is conductive form that can either be reduced or oxidized, termed as pernigraniline salt (PS) and leucomeraldine salt (LS), respectively (Nicolas-Debarnot & Poncin, 2003). However, of the three forms, only ES-PANI is conducting, therefore is the only oxidative form of PANI that has been used in fabrication of sensors. All these three forms can be mutually converted with ease by chemical and electrochemical reaction (Stejskal et al., 2015).

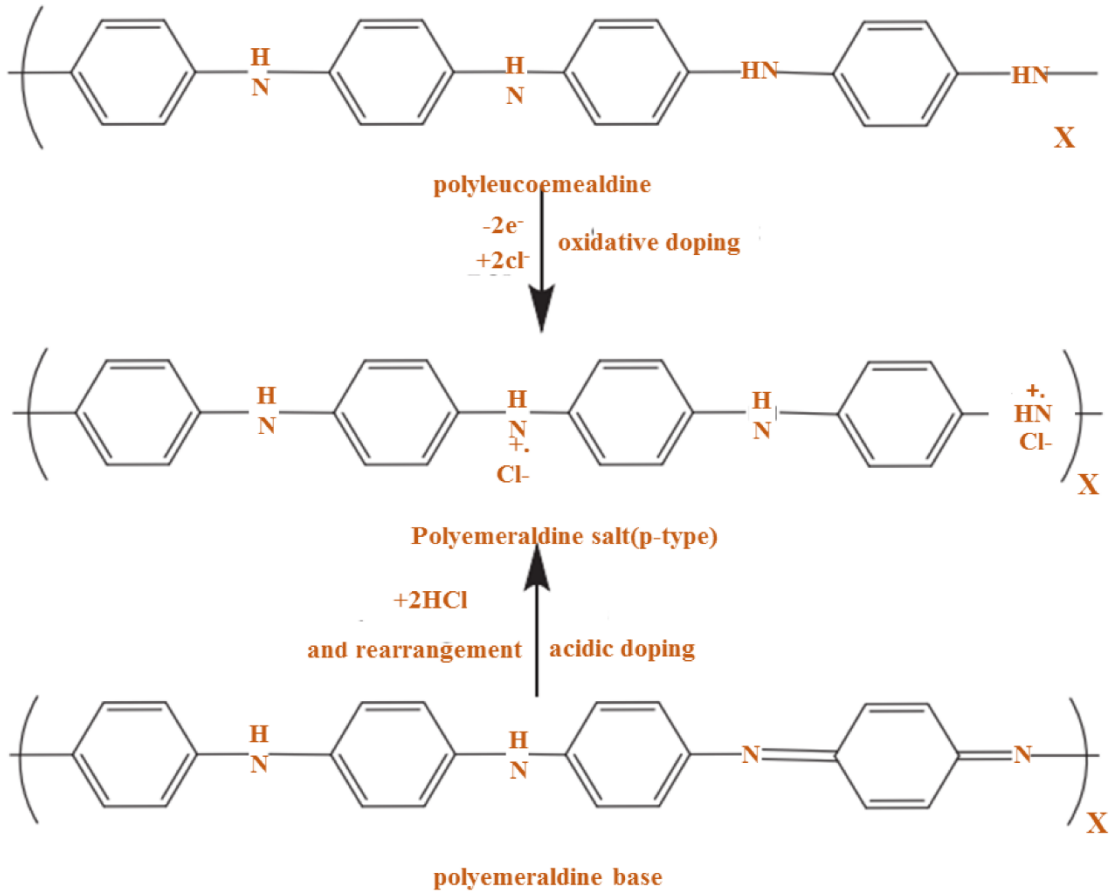


Figure 5: Oxidative forms of PANI in acidic solution.

2.2 Mechanism of Polymerization of aniline:

The mechanism behind the electrochemical polymerization process of aniline has been investigated extensively (Tang et al., 2011). The polymerization process of aniline is performed in acid electrolyte as the high pH values results in the formation of short conjugated oligomer

with altered nature . According to Zotti and coworkers, the rate determining step in the polymerization of aniline through electrochemical process involves the formation of aniline cation radicals on the surface of electrode (Moorthy et al., 2018). The radical formation was further ascertained by Mu et al in the experiment where they introduced molecules such as hydroquinone and resorcinol which were capable of retarding the reaction (Briseno et al., 2004). The following step involves the coupling of the anilinium dical and elimination of two protons resulting in oligomer. The latter is oxidized at anode a 10de along with aniline. Next, the chain propagation step involves the coupling of aniline radical cation with oligomer radical cation. The polymer is doped with the counter anion present in the electrolyte (Wang et al., 1987). The detailed mechanism is shown in the figure below.

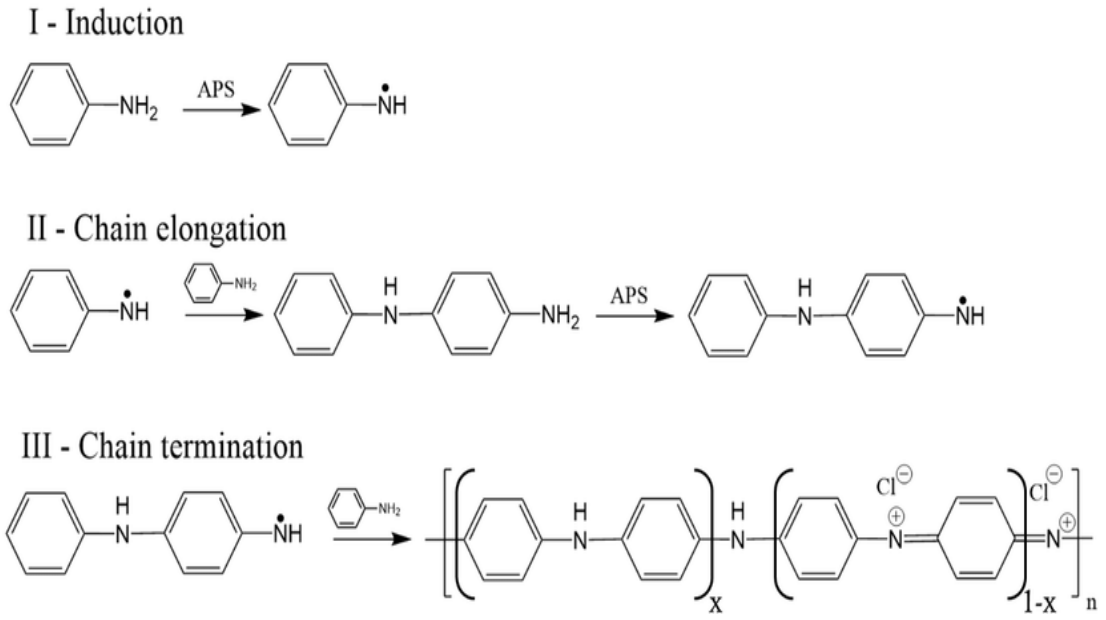


Figure 6: Mechanism of electrochemical polymerization of aniline.

2.3 Immobilization of Enzyme:

The mechanism of immobilization of the electrocatalysts (enzymes) into the polymeric films has been based on various assumptions (Fernandes et al., 2003). The studies show that the electrostatic interaction is the key phenomenon in the incorporation of enzymes. This speculation has been effectively shown by experimental facts (Li et al. 2005). However, this

assumption could not hold true for the non-ionic polymer where incorporation of enzyme depends upon the pre-adsorption of enzyme on electrode surface (Green et al. 2008; Murugan et al. 2005). As discussed, numerous polymers have been studied in fabrication of glucose sensors. Of these, this study focuses on the PANI polymer (Crespilho et al., 2009).

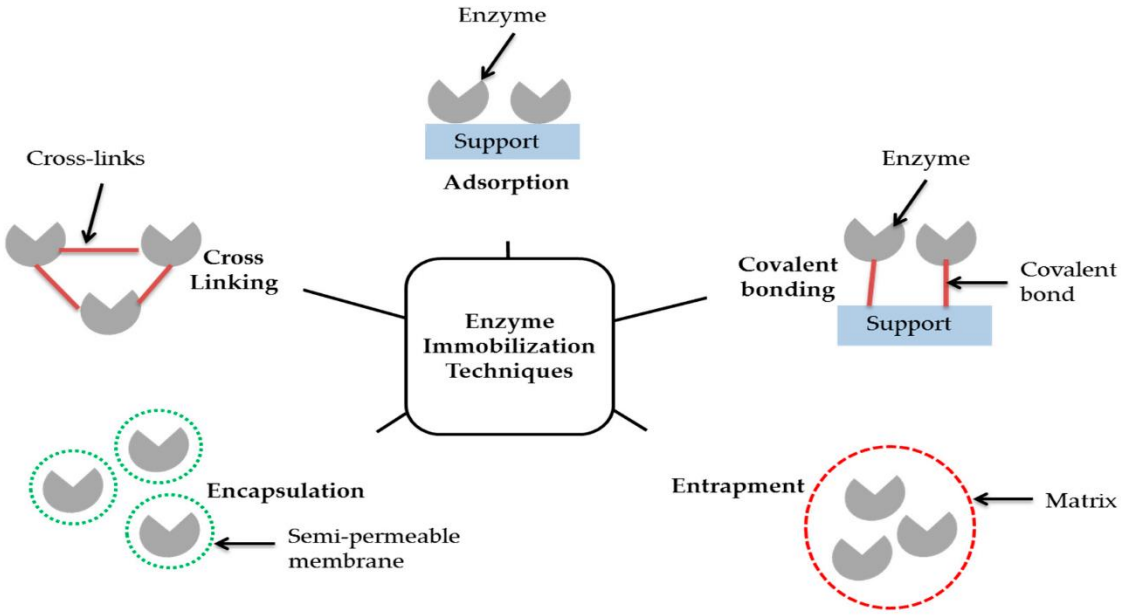


Figure 7: Different methods of enzyme immobilization on the substrate.

2.4 Properties of Polyaniline:

PANI have many unique features including ease of synthesis, inexpensive, easyily be doped with protonic acids, stability in any environmental condition, and many applications (Beygisangchin et al., 2021). Firstly PANI was came according to its oxidation level in different forms and it was known as black aniline. PANI is among most widely used CP and synthesis can be done by chemical and electrochemical methods while electrochemical methods are generally used to create highly pure coatings. It has significant electrical properties, remarkable environmental stability, high surface to volume ratio pH change properties. It can exist in different nanostructures like nanofibers, nanotubes, nanospheres depending upon their synthesis method (Ziadan & Saadon, 2012)

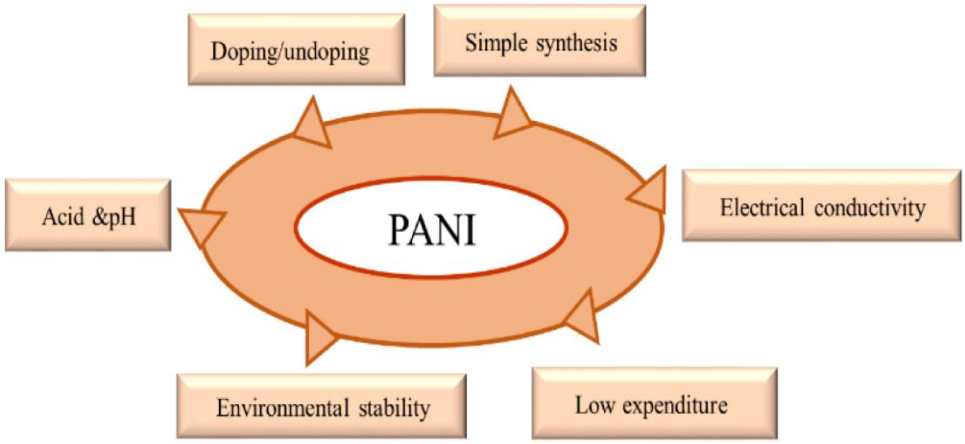


Figure 8: Unique properties of PANI.

2.5 Applications of PANI:

Because of the unique electrical properties, PANI posses diverse application in many fields. PANI can be used in different fields for biosensing which includes (Malhotra et al., 2015):

1. Glucose Biosensing.
2. PANI based Immunosensors.
3. Cholesterol Biosensing.
4. Choline Biosesning
5. Phenol Biosensing.
6. Viral and Bacterial Biosensing.
7. Nucleic acid Biosensing.
8. Cancer Biosensing.

CHAPTER-3

MATERIALS AND METHODS

3.1 Materials:

The details of chemicals and biochemical used in various experiments are as follows:

Sodium phosphate monobasic (NaH2PO4)(Acros organics),Sodium phosphate dibasic (Na2HPO4) (Merck), Sodium Chloride (NaCl) (Sisco research laboratories), ,Hydrochloric acid (HCl) (Merck), Distilled water, Glucose D+ ($C_6H_{12}O_6$), Aniline ($C_6H_5NH_2$), Ammonium hydroxide (NH4OH) (Merck), Ammonia (NH_3), Glucose Oxidase, Horse Radish Peroxidase (HRP), (Hydrogen Peroxide (Sigma –Aldrich) ,Potassium ferrocyanide (K4[Fe(CN)6]) ,Potassium ferricyanide (K3[Fe(CN)6),ITO coated glass substrate, Acetone etc.

3.2 Equipments:

Petri dish, reagent bottles, measuring cylinder, beaker, pipette, oven, funnel, pipette, vials, cylindrical flask, round bottom flask, burner, condenser, dropper, cyclic voltammetry (CV), originPro8.5 software, weighing balance, multimeter, electrochemical cell, pH meter, magnetic stirrer etc.

3.3 Synthesis and Characterization Techniques:

3.3.1 Electrochemical Cell:

The performance of electrochemical sensors is dependent on the material of the electrode, its surface and dimensions that is used to construct electrochemical cell. Basically three types of electrode can be employed for the construction of the electrochemical sensors. There are

1. **Working electrode:**

At the working electrode reaction of the electrochemical system occurs. The working electrode can be cathode or an anode subject to the reduction or the oxidation reaction. Different kinds of working electrodes are employed in the electrochemical sensors such as glassy carbon electrode, Pt electrode, Au electrode, carbon paste. Multiwalled carbon nanotube, and Ag electrode.

2. **Reference electrode:**

The electrode has constantly maintained potential which is used by other electrodes of the system for measurement. Reference electrode used in the electrochemical cell include hydrogen electrode, calomel electrode, and Ag/AgCl electrode.

3. **Auxillary electrode:**

Also known as counter electrode. If the working electrode is cathode, then auxillary electrode acts as anode and vice-versa. The surface area of the auxillary electrode is much larger than the

working electrode. Inert material are used to make auxillary electrode such as graphite, Au, and Pt.

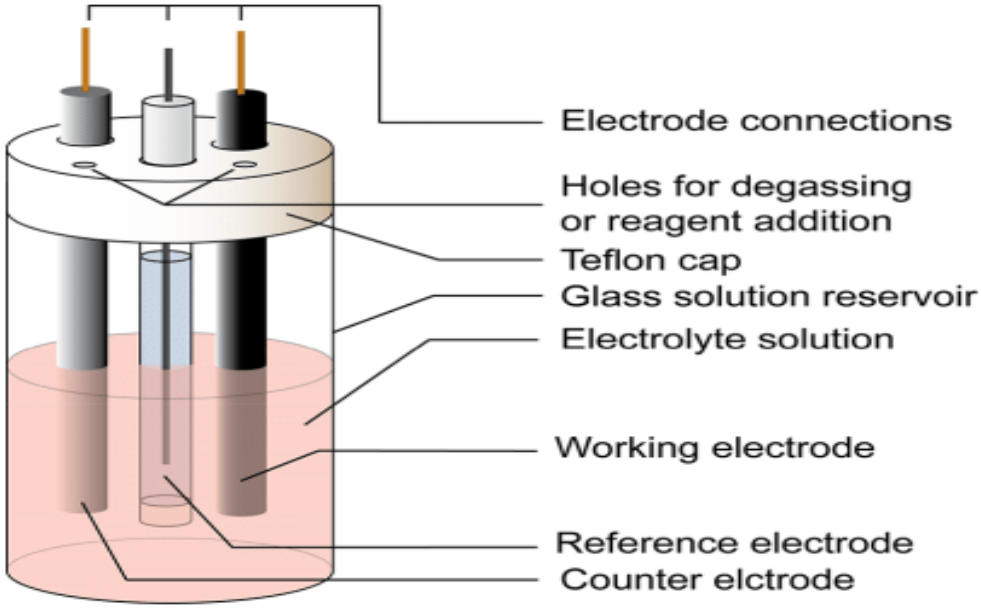


Figure 9: Electrochemical Cell and the different electrodes.

3.3.2 Cyclic Voltammetry:

Cyclic voltammetry is based on the potential sweep of the working electrode at a fix scan rate (in volts/second) from Initial E to either High E or Low E compute the current vs. time curve. At specific potential the sweep is back hence named cyclic voltammetry. As the scan rate is constant and initial high E values are known and current is produced as function of potential. The experimental parameters have to be specified for carrying out the CV experiment. These include initial and low/final E values, scan rate, and number of segments.

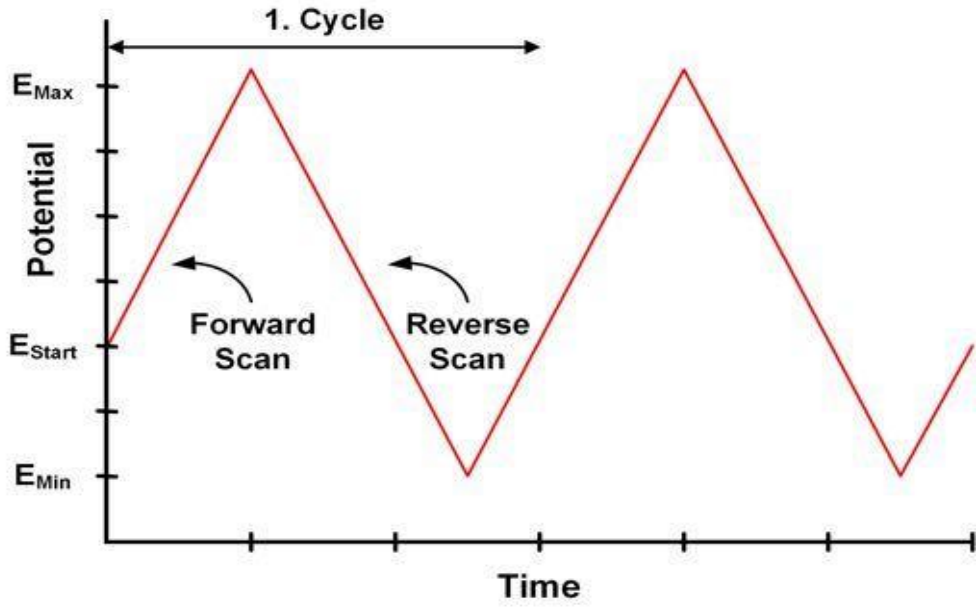


Figure 10: Potential-time Excitation signal in a cyclic Voltammetry.

The potential E in the voltammogram is given by Nernst Eq.(1)

$$E = E^0 + 2.303\ RT/nF \log [ox]/[red]$$

Where E^0 is standard potential, [ox] and [red] are the concentration of oxidising and reducing species respectively, R is universal gas constant, F is Faraday's constant, T is temperature and n is the no. of electrons.

For the process involving fast electron transfer, the reaction is reversible and the peak separation is given by Eq.2:

$$\Delta Ep = Epa - EPc = 2.303RT/nF$$

The slow electron transfer process are associated with irreversibility for which

$$\Delta Ep > 0.0592/nV$$

For the reversible couple reduction potential (E^0) is given by Eq. 3

$$E^0 = (Epa+Epc)/2$$

Where EPa is anodic potential, Ipa is anodic peak current, EPc is cathodic peak current. The expected response of current potential peak is a typical reversible redox couple is illustrated in the figure below.

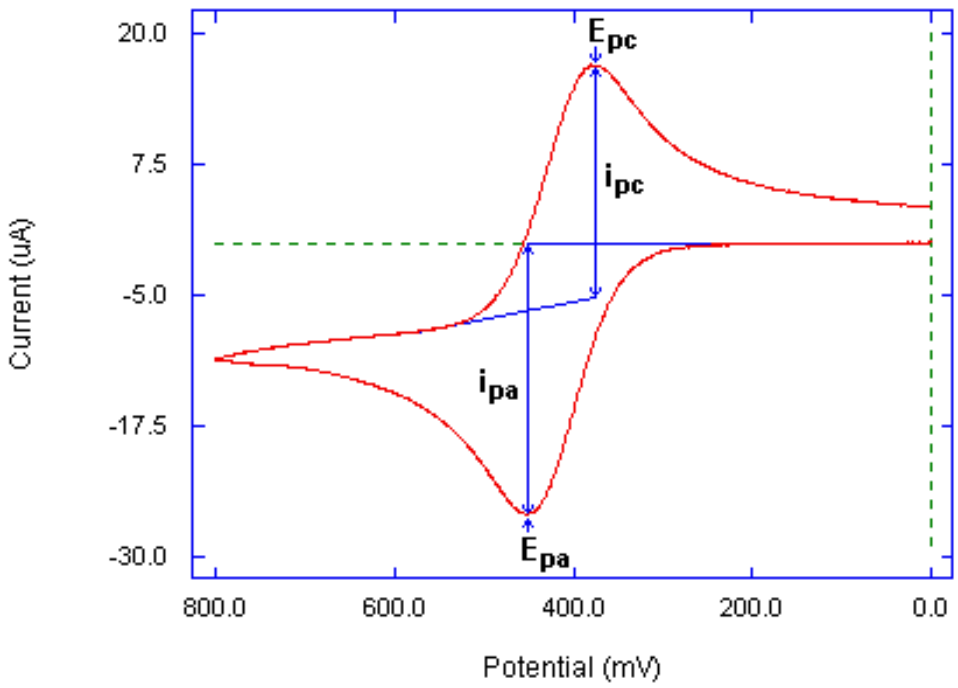


Figure 11: Current potential peak in reversible redox couple cyclic voltammetry.

While setting the parameters for cyclic voltammetry experiment, scan rate controls the speed at which the applied potential is scanned. More the value for scan rate implies decrease in diffusion layer and hence higher current observed in voltammograms. The glucose sensors developed in the work were used for the oxidation of glucose at different scan rate in CV. To understand the surface phenomenon for the oxidation of glucose, a graph is plotted for Ip against scan rate.

For the reversible process, the variation in V with Ip is used to calculate the active surface area of the electrode using the Randles-Sevcik Equation (Eq.4)

$$Ip = 268600n^{(3/2)} AD^{(1/2)} Cv^{(1/2)}$$

Where Ip is peak current (A), n is no. of electrons transfer, D is the dilution coefficient (cm^2 /sec), C is bulk concentration (mol/cm^3), A is surface area of the electrode (cm^2), and v is the scan rate (V/s).

3.3.3 Chronoamperometry:

Chronopotentiometry (CA) is the simplest volatmmetric technique used for the electrochemical measurements. In the CA techniques the potential is stepped from the initial E to higher E where electron transfer occur resulting in a current that is recorded as a function of time. Figure shows that the iput given to the system at an initial voltage ($E_{initial}$) to to the final voltage (E_{final}). As depicted voltage impulsively step up from $E_{initial}$ to E_{final} at t=0. Simultaneously, the output is observed in the current density vs time voltammograms.

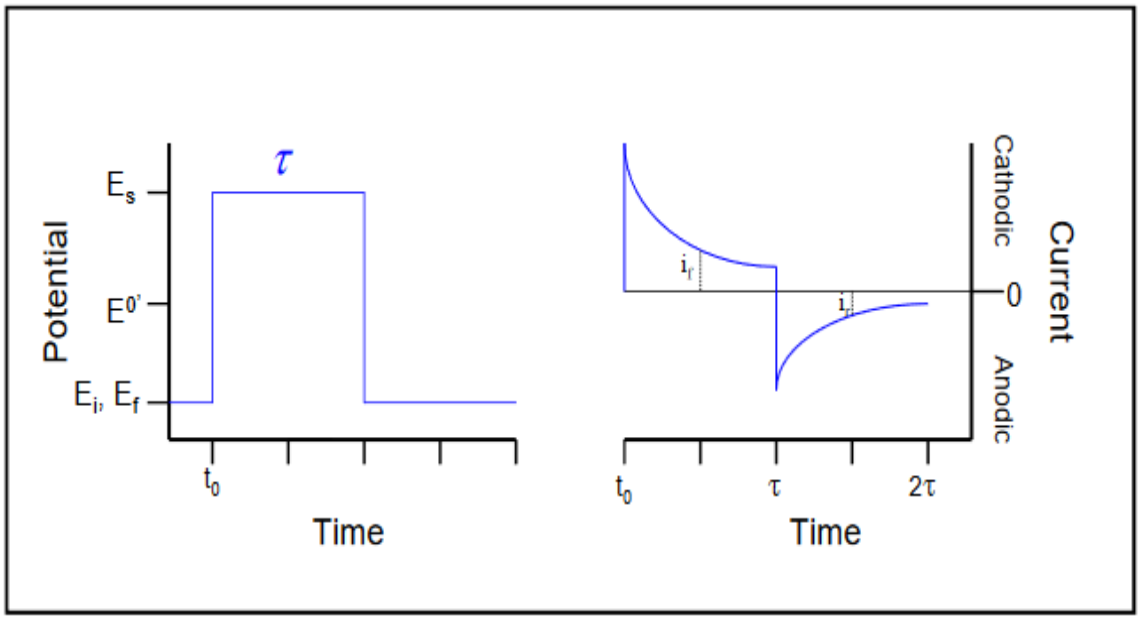


Figure 12: Voltage and current density using chronoamperometric techniques.

The behaviour of current flow with the application of potential step is defined by Cottrell Eq.(5).

$$I = nFACD^{1/2} / \pi^{1/2} t^{1/2}$$

Where, n is the number of electrons, A is the surface area, D is the diffusion coefficient, F is the Faraday constant, C is the concentration, and t is time.

The simplicity of the chronoampermetric experiment can be considered from the following experiment.

Consider an electrochemical cell in which analyte is present in its oxidized form. At E, no electron transfer occurs and thus no current flows through the cell. As potential applied is stepped to more negative value, E_l at time, t_o, the reduction of takes place. If more of the oxidized form of the analyte is present at the electrode surface it is also immediately reduced to maintain zero concentration of oxidized form of analyte at the electrode. After the specified

period, t_s, potential is stepped back and the reduced form of analyte is again oxidized and the experiment is done. The time after t_o should be sufficiently long that the current is not decayed and at this duration negligible electrode capacitance is charged. This current helps to determine the concentration of the analyte.

3.3.3 Potentiostatic Method:

The principle behind potentiometric sensors is the electron exchange between sensing element and sample in solution. An ion-selective electrode is used for measurement for selectively measuring the activity of compound. These electrodes have characteristics of measuring concentration over a wide linear range and fast response time.

The potential of E of the system is given by Nernst Equation. Eq.1

$$E=Eo-2.303RTn_eFlog10\ (\alpha A+\beta AB\alpha B)$$

Where E is the potential of electrode, Eo is the standard potential dependent on electrode material, T is the absolute temperature, n_e is the no. of electrons participating in the reaction. R is the universal gas constant, F is Faraday's constant, αA is the activity of species A, αB is the activity of species B, βAB is the selectivity coefficient of A species sensitive electrode to species B. Activity of species is function of concentration.

3.3.4 Galvanostatic Method:

A steady current is sustained in an electrolyte between the working and counter electrodes, and the resulting signal is recorded by a galvanostat across both electrodes. Along with the chronoamperometric and chronopotentiometric systems, this approach is commonly employed to assess corrosion rate in an electrochemical process. In this diagram, the workings of a galvanostat are shown schematically.

This method is used to keep the current constant in a electrolytic cell. This majorly consist a high voltage source that generates a high voltage. In the measurement of polymerization the current of the counter electrode and the working electrode is kept in control and to maintain constant current it can be easily adjusted. And along with the high voltage there is a resistor

present. The resistors are generally present in a series so that the constant current flow is maintained. It is simply based on Ohm's law

R=U/I

And the voltage and the current are directly proportional to each other.

Uc = Rv * Io

Where Uc is controlled voltage.

Rv is variation is resistance.

Io is current which is constant.

The application of this technique is to generate thin films deposition.

CHAPTER- 4

EXPERIMENTAL WORK

4.1 Hydrolysis of Indium Tin Oxide (ITO):

Hydrolysis Procedure:

Cleaning is a very basic but very important step as it decides the quality of a film as if cleaning is not done properly then impurity on ITO substrate will not allow formation of a uniform film.

The main steps are discussed below in detail:

• Dip ITO films in ethanol

• Then Dip these ITO films in DI water.

• Then Dip in 1:1:5 mixture of NH3 (ammonia) or NH_3OH (ammonia hydroxide), H_2O_2 and DI water respectively.

• Cover the petridish and heat at 80°C for 30 min in oven.

• Take out ITO films from the solution and dip in DI water.

• Take out and soak the water from the films with the help of a tissue paper.

• Leave for drying overnight or dry by keeping in oven for again half an hour at 80°C.

• Hydrolysis of ITO is done so that the ITO can become active with the OH- group and can become more hydrophilic for the films to be deposited on it.

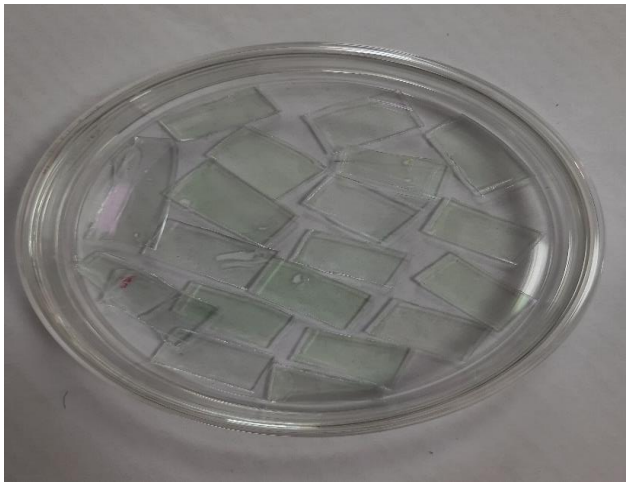


Figure 13: ITO films after hydrolysis and drying in oven.

29.2 Steam Distillation for purification of Aniline:

Steam distillation is an organic compound separation and purification technique. Basically, this operation entails volatilizing a substance by injecting steam into a combination of the compound and water.

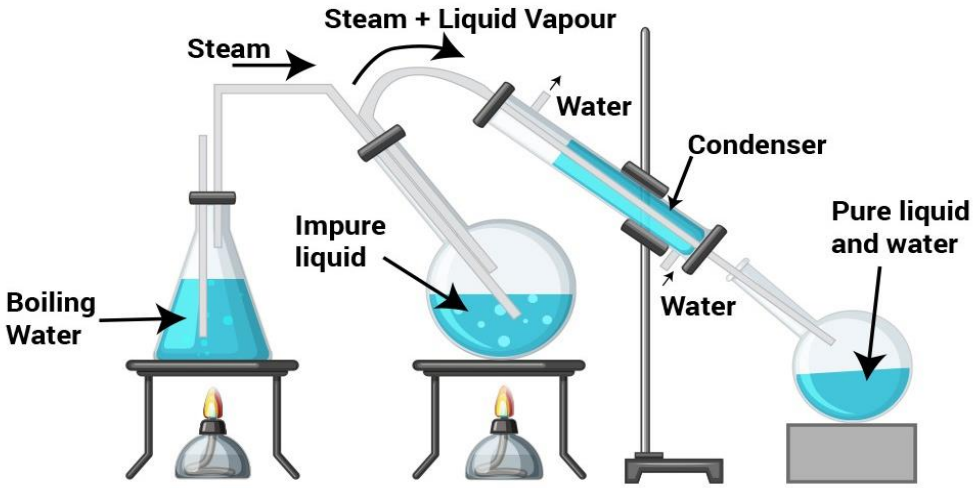


Figure 14: Steam distillation apparatus.

Water and aniline are used in this separation method. The volatile vapour is carried by steam from boiling water, which can be drained and returned to a liquid or solid condenser. In the water bath, the non-volatile chemical persists. Because aniline is steam volatile and insoluble in water, it is refined by the steam distillation process.

Procedure:

1. Take 5-6 ml aniline and 20ml of water in a round bottom distillation flask and set up the apparatus.
2. Pass the steam at a certain pressure.
3. Note down the temperature of distillation.
4. Pass the steam till about 80-90 % of the liquid distills.
5. Stop passing the steam and allow the distillate and residue to get cooled.
6. By using a separating funnel, separate aniline and water in residue and distillate.
7. Add sodium sulphate in the distilled aniline to remove excess water.
8. Cover the bottle containing aniline with foil paper.
9. Store the aniline at 4 °C

Figure 15: Steam distillation of aniline.

Figure 16: Distilled Aniline

4.3 Synthesis of PANI thin films:

Conductive surface of ITO was used for the deposition of Polyaniline film. It takes 4-5 minutes for the deposition of uniform thin film of ITO.

4.3.1 Synthesis of Aniline Solutions:

Aniline was distilled by boiling at its boiling point (161°C) and then cooling it by at 4 °C and we collected it. This distilled is stored in dark place to avoid its photo degradation.

The solution of aniline for deposition is prepared in acidic medium and here it is prepared in Hydrochloric Acid (HCl). 1M 15ml HCl was mixed with 400µl of aniline.

4.3.2 Electrodeposition of Polyaniline on ITO:

Deposition of PANI was done using *chronoamperometry method. The deposition was carried out using and Electrochemical Analyzer (*Cyclic voltammetry). For deposition a three electrode was used: ITO electrode was used as a working electrode, Platinum as a Counter electrode and (Ag/AgCl) as Reference electrode. Polarity-platinum electrode was attached to the positive terminal The working electrode-ITO (in this case) was attached to the negative terminal for the deposition to occur. The depositions were seen at varying conditions (time) for the best conditions to be detected.

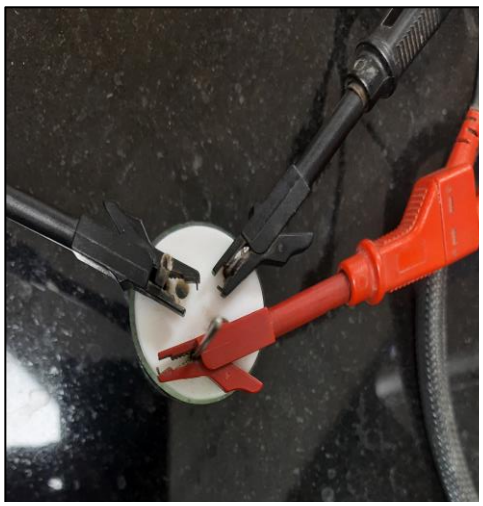
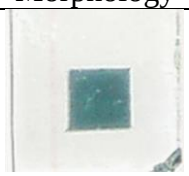
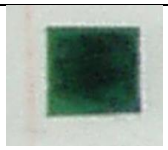
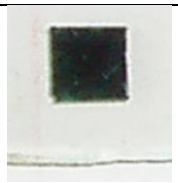
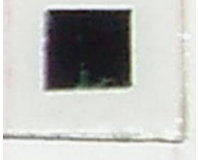


Figure 17: Different electrodes of electrochemical cell (Working- ITO, Reference –Ag/AgCl and Counter- Platinum).

During the exercise it was observed that the polyaniline thin film were successfully deposited on, ITO coated glass plate further study on their sensing properties has been done.

Table 5: Chronoamperometric deposition of PANI films on ITO at different time.

Sample	Voltage (V)	Time (Sec)	Morphology
1	0.85 V	60	
2	0.85V	120	
3	0.85V	180	
4	0.85V	300	

Deposition was carried out in an acidic medium of Hydrochloric acid and distilled polyaniline with the help of software and a suitable method (chronoamperometry) is first selected and then particular parameters are entered. After entering the particular values the cycle is run. Deposition of PANI was carried out at 0.85V for 60sec, 120secs, 180secs and 300secs. After each cycle ITO substrate was taken out and then another ITO slides was put as working electrode for next cycle. An Emerald greenish thin film was observed on ITO substrate.

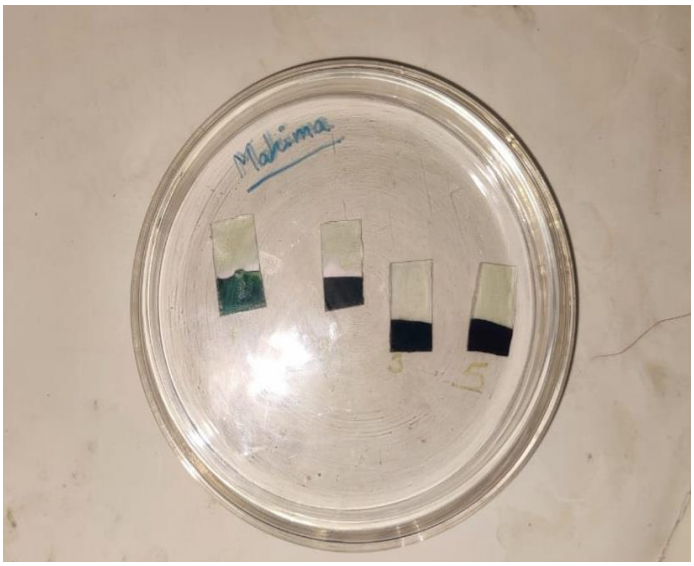
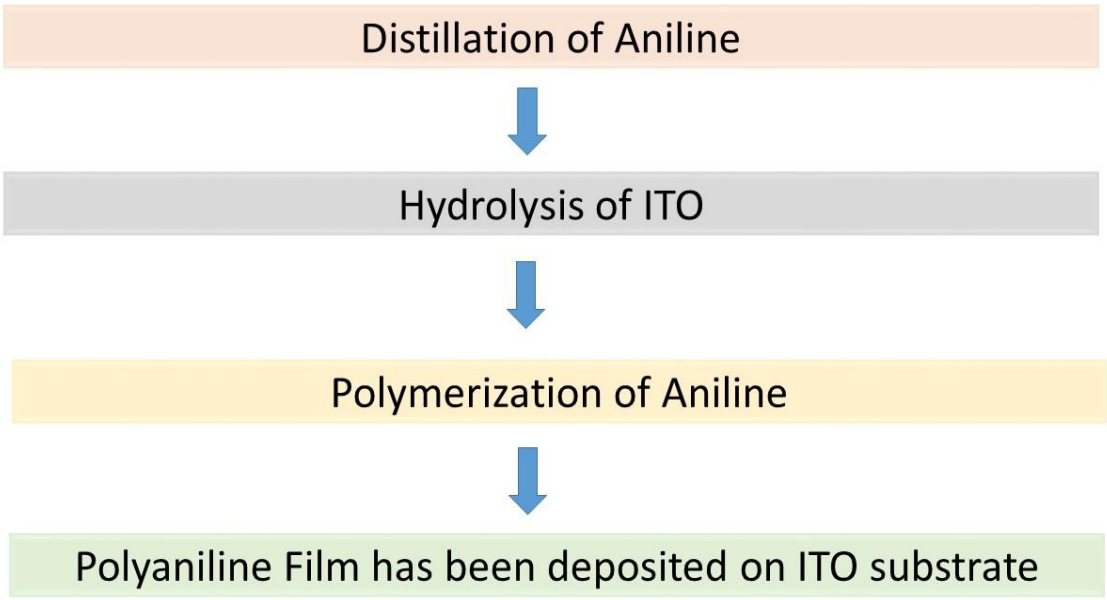


Figure 18: Electrochemical deposition of PANI on ITO substrate.



4.4 Characterization Techniques:

4.4.1 Cyclic Voltammetry: Cyclic Voltammetry (CV) studies were carried out in a cell comprising of modified working electrode (ITO/PANI) of diameter 2mM, counter Pt wire electrode of diameter 0.5mM and Ag/AgCl electrode as reference electrode by using electrochemical workstation. PANI is electrochemically synthesized on the ITO glass, and for the electrochemical sensing it is employed as a working electrode. PANI/ITO electrode CV was recorded in Phosphate buffer saline (PBS) solution of 0.1M, pH = 7 at a particular scan rate of 50Mv/sec with potential range of -7mV to +7mV. The ferri-ferrocyanide redox couple is used in the PBS buffer. Cyclic voltammetry (CV) and other studies have been used for characterization of the ITO modified electrode. CV of different modified electrodes in 5mM ferro-ferri buffer was taken where peak current increased due to the good electrocatalytic activity and high electroactive surface area of the PANI/ITO.

Figure 19: Potentiostat-Galvanostat.

4.5 Immobilization of Enzyme:

4.5.1 Preparation of Solutions:

The solution of Glucose Oxidase and Horse Radish Peroxidase (HRP) were freshly prepared in Phosphate Buffer of concentration 50mM, pH- 7.4, NaCl prior to being used.

4.52.Preparation of Phosphate Buffer:

(i) 1M solution of K2HPO4 (dibasic monohydrogen phosphate/dibasic potassium phosphate) and KH2PO4 (monobasic dihydrogen phosphate/monobasic potassium phosphate) is prepared.

1M KH2PO4 = 1.2g KH2PO4 in 100 ml DI water, 1M K2HPO4 = 1.41g K2HPO4 in 100 ml DI water.

(ii) For 0.1M(100 millimolar) PBS at 25°C having pH=7.4 Volume of 1M KH2PO4= 9.5ml.

Volume of 1M K2HPO4 = 40.5 ml.

(iii)To this 100 ml solution of A and B, 100 ml DI water was added to get total of 100 ml phosphate buffer solution.

(iv) Take 100ml of phosphate buffer solution and add 1.5g of NaCl to get phosphate buffer saline (For 100ml PBS add 1.g NaCl).

Preparation of ferro-ferri buffer:

Using the molecular weight of K4 [Fe (CN) 6].3H2O (MW – 422.39g/mol) and K3 [Fe (CN) 6] (MW –329.24g/mol), Make a ferro-ferri solution of desired concentration.

For 5mM of 100ml ferro-ferri solution.

Weight of K4[Fe(CN)6]=184mg, Weight of K3 [Fe (CN) 6] =164.5mg.,K4 [Fe (CN) 6] =Potassium Ferro cyanide, K3 [Fe (CN) 6] =Potassium ferricyanide.

Figure 20: Bottle containing Ferro-ferri solution in PBS buffer.

Figure 21: pH meter

Stock Solution of Glucose:

Stock solution of 1g glucose was prepared in 1ml PBS buffer and was stored at 4°C. Afterwards glucose stock solution was left for 24 hours so that it get muta-rotated before we further use it. The glucose stock solution was further diluted in the concentration of 50µL, 100µL, 150 µL,200µL, 300µL.

Immobilization of Glucose Oxidase onto PANI/ITO electrode:

Freshly prepared solution of Glucose Oxidase (5mg into 1ml of phosphate buffer, pH 7.4) was used to get 1unit of enzyme per µl. 5µl of this solution is mixed with 10 µl of HRP and this mixture was immobilized onto electrochemical synthesized PANI/ITO electrode with the help of physical adsorption method. The immobilization of enzyme requires 4-5hrs at room temperature. This reaction takes place in humid chamber which was form with the help of funnel and petri plate containing water. The process of immobilization is important to enhance the activity of enzymes and to improve the stability of biocatalyst. The function of GOx is to catalyst the oxidation of β-D-glucose to produce gluconic acid along with hydrogen peroxide that utilizes molecular oxygen as an acceptor of electron.

Figure 22: Immobilization of GOx onto PANI/ITO electrode in Humid Chamber.

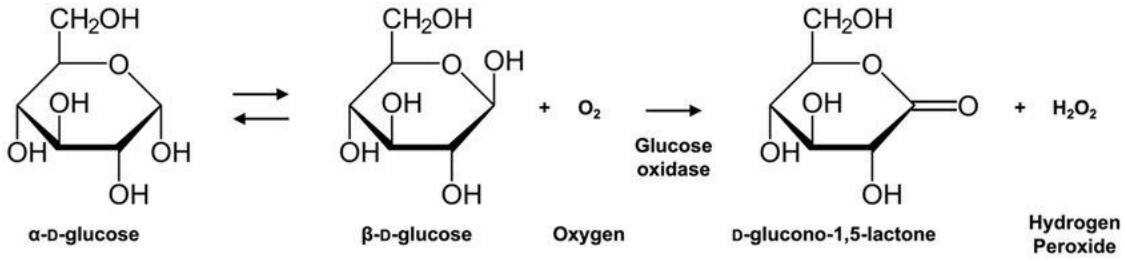


Figure 23: Conversion of glucose into gluconolactone and hydrogen peroxide by the help of glucose oxidase enzyme.

4.6 Electrochemical analysis of GOx/PANI/ITO electrode:

The immobilized GOx/PANI/ITO electrode was characterized with the help of CV in the PBS buffer (50Mm, pH-7.4, NaCl). The GOx/PANI/ITO was used as working electrode and platinum as auxiliary electrode and Ag/AgCl was taken as reference electrode. CV analysis of ITO, PANI/ITO, and GOx/PANI/ITO were performed and after that a comparison has been made between their conductivity.

4.7 Sensing of Glucose:

After immobilization of GOx on PANI/ITO, the electroactive nature of GOx/PANI/ITO electrode was measured with the help of CV in the presence of Phosphate buffer and glucose. The response of the oxidation of glucose by enzyme was measured by reduction of current. The electrochemical studies was carried out on GOx/PANI/ITO bioelectrode for different glucose concentration (50µl, 100µl, 150µl, 200µl) in PBS containing Potassium Ferro Cyanide and Potassium Ferri Cyanide (50mM, pH-7.4, NaCl). In this case the working electrode is GOx/PANI/ITO and reference electrode is Ag/AgCl and the counter electrode is platinum.

$$\text{Glucose} + \text{GOx} - \text{FAD}^{+} \rightarrow \text{Glucolactone} + \text{GOx} - \text{FADH}_2$$

$$\text{GOx} - \text{FADH}_2 + \mathbf{O}_2 \rightarrow \text{GOx} - \text{FAD} + \mathbf{H}_2\,\mathbf{O}_2$$

$$H_2O_2 \rightarrow 2H^{+} + O_2 + 2e$$

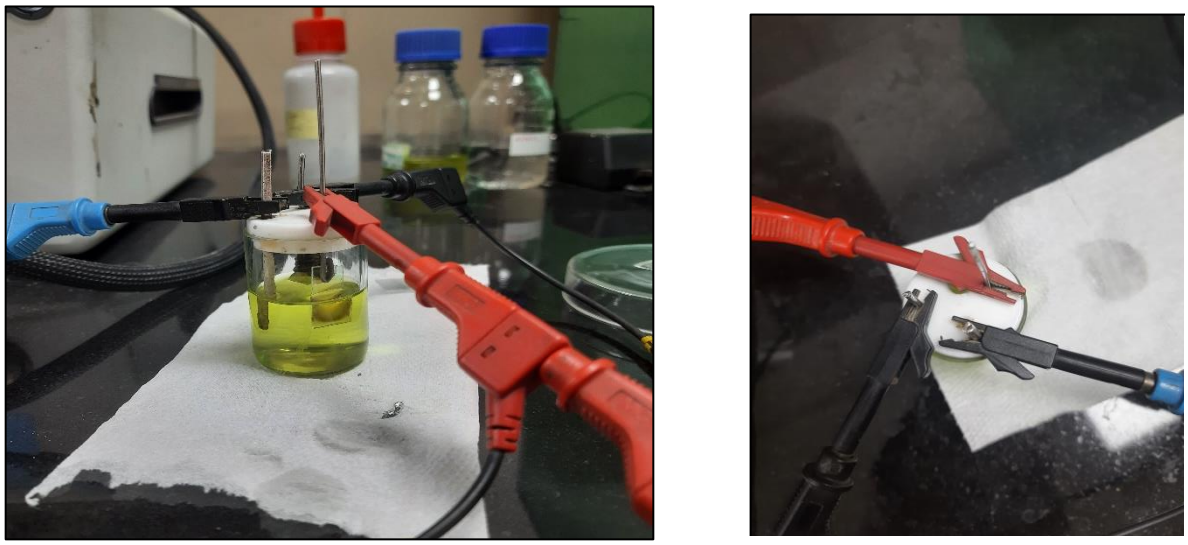


Figure 24: Electrochemical cell containing GOx/PANI/ITO as working electrode, Platinum as counter electrode and Ag/AgCl as reference electrode, and Top view of electrochemical cell electrodes.

CHAPTER- 5

RESULTS AND DISCUSSIONS

5.1 Morphology of PANI:

The picture of PANI films amperometrically deposited at 0.85V on the ITO glass slide is shown below in the Figure. In this figure it can be easily seen that thin films of PANI has been electrochemically deposited on the glass slide of ITO. With the increase in duration of deposition, the layer of thickness was increased significantly and the PANI films colour became darker and darker (Emeraldine Green).

The fabrication of PANI on ITO glass slides requires many pre and post treatment of different electrode and the solution of electrolyte. After evaluation with different treatment, immediate rinsing of electrode is required after the deposition, this is very crucial for getting electrochemically active film of PANI on ITO glass slide. This is because there are many unreacted monomers and oligomers that can hamper the overall performance of PANI.

5.2 Cyclic Voltammetric studies:

The electrochemical analysis of ITO and PANI/ITO and GOx/PANI/ITO has been carried out using cyclic voltammetry.

CV characterization of different PANI films on ITO:

The PANI films are deposited on ITO at different time period i.e. 60secs, 120secs, 180secs, 300secs. These are the Cyclic voltammograms of all the films which are combined in one graph. This is done to check which out of all the varying conditions had the most stable film. The sample that shows the highest current peaks i.e., both reduction and oxidation peaks, is chosen to be the best sample because of its highest conduction.

In this graph, we can see the olive green peak is the highest, which is of the sample 1 of PANI/ITO. The highest oxidation peak is coming out to be at 1000 µA and the lowest reduction peak is at - 1000 µA. It shows change in the redox process due to addition of carbon. On the basis of this characterization, Sample 1 was chosen to be the most stable sample and best for the performance of electrochemical sensor

Further characterization of this film was performed.

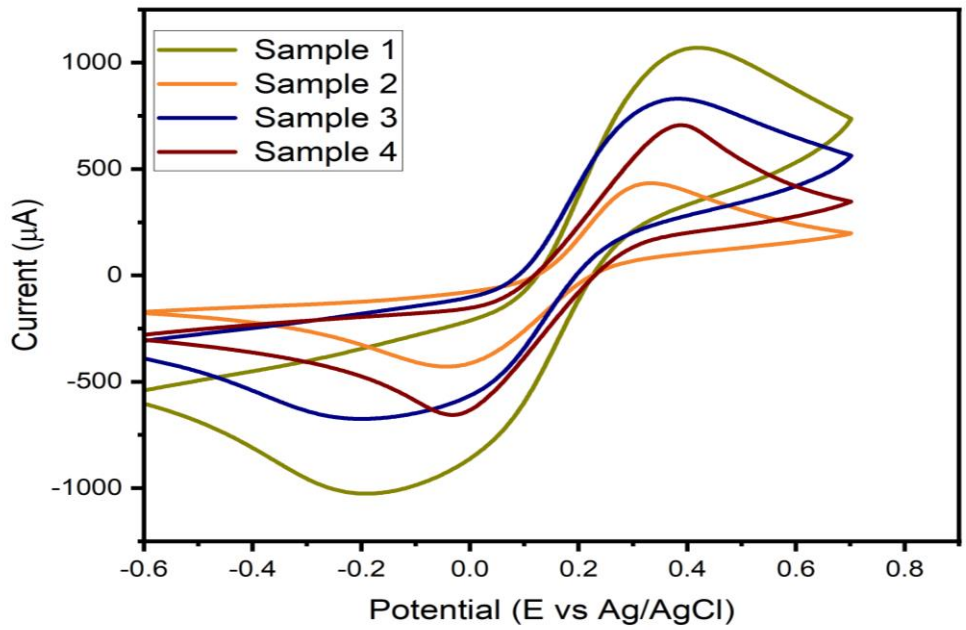


Figure 25: Cyclic Voltammograms of different samples of PANI/ITO deposited at different time period.

Comparison of conductance of plane ITO and PANI/ITO:

The comparison has been drawn between the sample 1st sample of PANI film with 50mV/cycle scan rate and the plane ITO. From the above graph, we can clearly see that the conductance of PANI deposited film on ITO is more than the plane ITO.

Figure shows cyclic voltammograms of PANI/ITO and ITO. The scan rate 50mV/cycle and it is in the range of -7mV to +7mV in the presence of PBS buffer. The PANI films were deposited on ITO with chronoamperometric process.These are the Cyclic voltammograms of the films and ITO which are combined in one graph. The highest oxidation peak of PANI/ITO is coming out to be at 1000 µA and the lowest reduction peak is at – 1000 µA. It shows change in the redox process due to addition of polyaniline. On the basis of this characterization, this sample was chosen to be the most stable sample and best for the performance of electrochemical sensor. In the case of ITO the oxidation peak is around 400µA and reduction peak is at 400µA. These peaks in the figures shows that oxidation of aniline oligomers takes place. This shows that there is an increase in conductivity of ITO after polymerization of PANI onto it. This reveals that there is increase in active surface area which results in the transfer of electron between the medium and the electrode.

By all these parameters that we have performed on the PANI films, specifically on the PANI 1ST sample, we can say that this PANI/ITO based sensor is very well suited for the detection of glucose. The 1st PANI sample was deposited by chronoamperometry at 0.85V for 300 seconds.

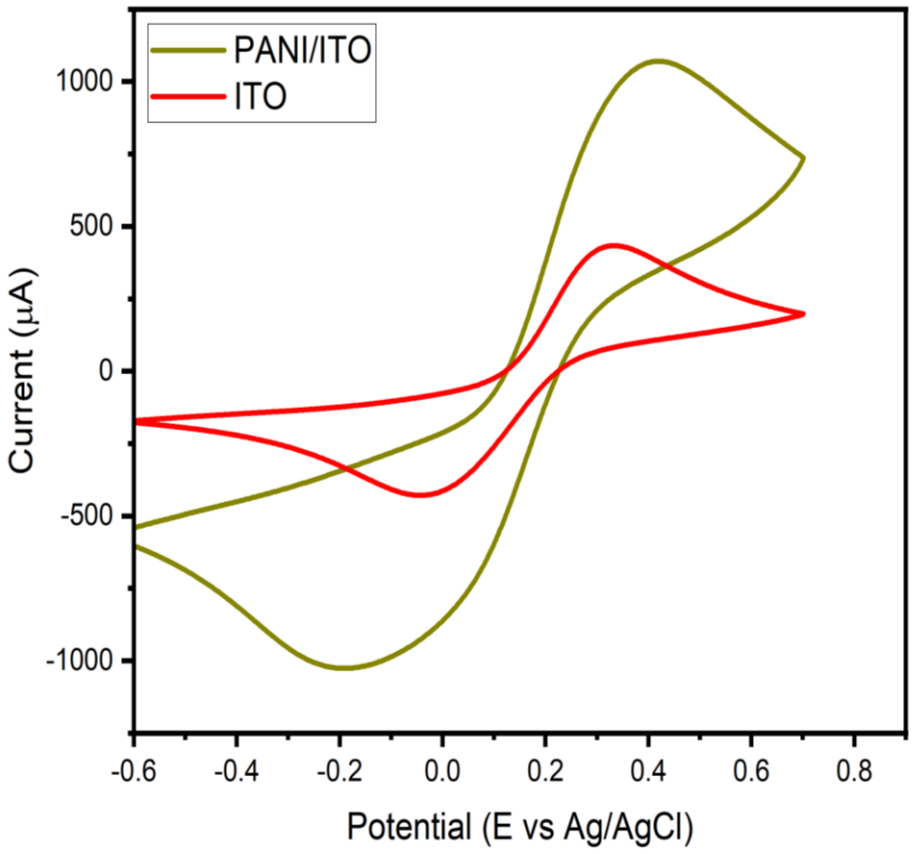


Figure 26: Comparison of Cyclic voltammograms of PANI/ITO AND ITO.

CV- comparison of conductance of ITO, material and bioelctrode:

Figure shows cyclic voltammograms of ITO, material (PANI/ITO) and the bioelectrode (GOx/PANI/ITO). ITO shows oxidation peak at 400µA and in the case of material (ITO/PANI) oxidation peak is at 100 µA but in the case of bioelctrode (GOx/PANI/ITO) the peak of oxidation is at 600 µA. The bioelctrode shows less current then the material that is may be due to insulating property of glucose oxidase enzyme. The immobilization of glucose oxidase onto

the PANI/ITO matrix blocks the charge carriers that leads to the slow redox process in GOx/PAN/ITO bioelectrode during the electro-biochemical reaction.

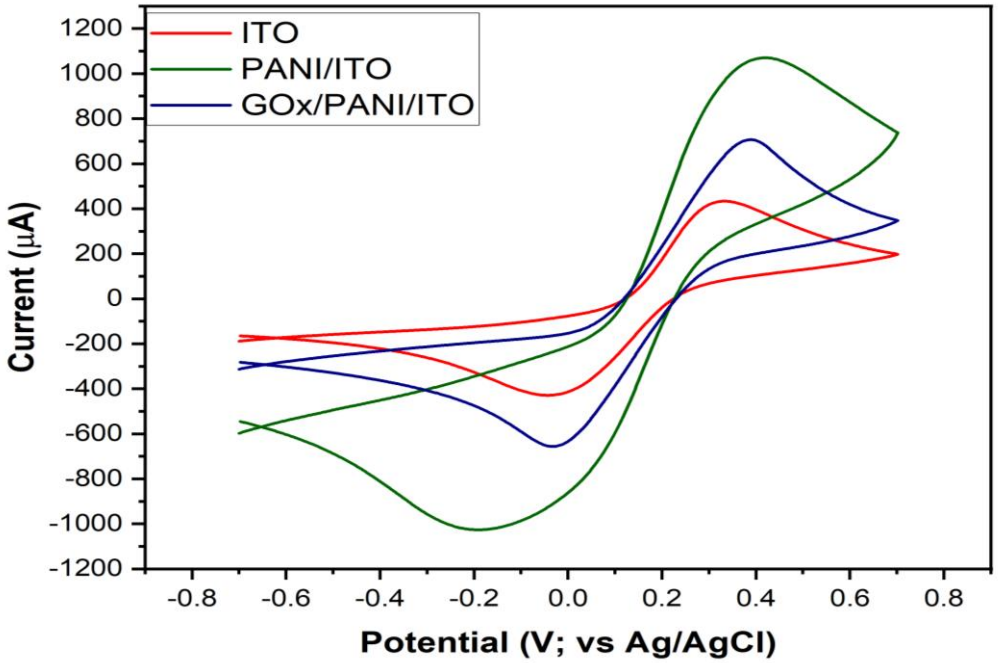


Figure 27: Cyclic Voltammograms of ITO, PANI/ITO and GOx/PANI/ITO.

Sensing characteristics of GOx/PANI/ITO bioelectrodes

The electrochemical studies of bioelectrode has been carried out for different glucose concentration (50µl, 100µl, 150µl, and 200µ) in phosphate buffer (50Mm, ph-7.4, NaCl) containing [Fe (CN)6] 3−/4−]. When ferro/ferri is added onto electrode, it should be reduced by the enzyme and the oxidation current because of reoxidation at the electrode should increase.

With different concentrations of glucose, increase in current can be observed. Glucose behaves as free charge carrier in the acid medium and results in re-polymerization and thus increase in current can be observed which can be used as a sensing property of glucose. The electronic characteristics of induces which results in higher charge transfer within the bioelectrode which leads towards better electrolytic performance for the biosensing of glucose. The characteristic peak denotes that there is a reversible electron transfer of glucose oxidase during the oxidation of glucose. The conductance is increasing with increase in concentration of glucose.

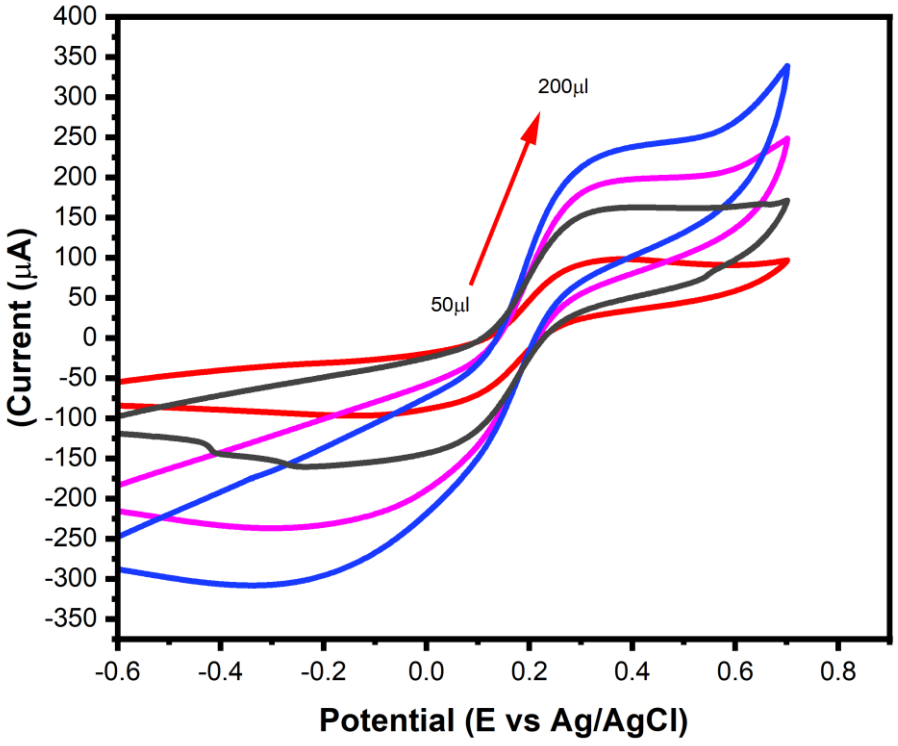


Figure 28: Glucose sensing using different concentration by the GOx/PANI/ITO bioelectrode.

With different concentrations of glucose, increase in current can be observed. Glucose behaves as free charge carrier in the acid medium and results in re-polymerization and thus increase in current can be observed which can be used as a sensing property of glucose. The electronic characteristics of induces which results in higher charge transfer within the bioelectrode which leads towards better electrolytic performance for the biosensing of glucose. The characteristic peak denotes that there is a reversible electron transfer of glucose oxidase during the oxidation of glucose. The conductance is increasing with increase in concentration of glucose.

Current Response:

By plotting a calibration curve between the concentration of glucose and current, the current response is found linear. It can be seen from the linear curve of GOx/PANI/ITO bioelectrode that this bioelectrode can be used to estimate glucose from 50mM to 200Mm. The amperometric response of the bioelectrode has been carefully recorded towards the increasing concentration of glucose. A general trend of increase concentration with increase in current clearly denotes the high sensitivity of constructed bioelectrode (GOx/PANI/ITO). The current

of most of the glucose biosensors increases very rapidly with increase in concentration of glucose .

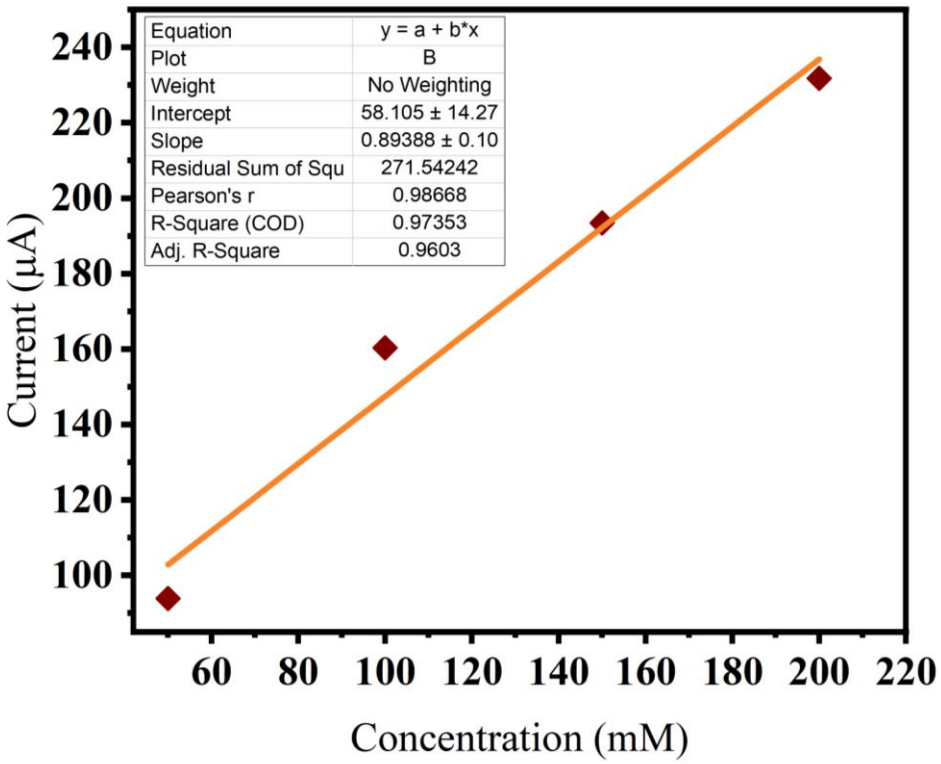


Figure 29: Current response curve (current magnitude vs concentration of glucose).

Because PANI had a large specific surface area, good electrical characteristics, and certain electrocatalyticones, the current of glucose reduction increased and the peak potential positively moved. The peak current of glucose dramatically increased for PANI/ITO and the peak potential moved even more positively. This behaviour indicated that PANI on ITO electrode has an excellent electocatalytic activity for glucose sensing.

In addition, the effect of glucose concentration on the cathodic peak current was examined by changing the concentration of glucose from 50 to 200 microliter in 5 mM PBS (pH 7.4) at the scan rate of 50mV/sec. The figure shows the cyclic voltammetric response of bioelctrodes at the different concentrations of glucose. The peak current values show a strong linear correlation (range 50-200 microliter) with glucose concentrations.

CHAPTER-6

CONCLUSION AND FUTURE SCOPE

Glucose biosensors have improved in terms of reliability, speed, and accuracy, as well as being more compact and simple to use. Advanced technology research, such as electrodes, membranes, immobilisation techniques, and nanomaterials, is still ongoing. Despite significant advancements in glucose biosensor technology, there are still a number of obstacles to overcome in order to obtain effective glucose monitoring. The ADA requires that a blood glucose POC test be accurate to within 5% of the detected value.

.It has been shown that PANI can be prepared by electrochemical technique using 1M Hydrochloric acid as electrolyte. These polymeric films were characterized spectroscopically using cyclic voltammetry which shows that the conductivity of ITO is improved because of deposition of PANI. It has also been demonstrated that electrochemically prepared PANI films can be utilized for physical adsorption of GOx. These bioelctrodes (GOx/PANI/ITO) have been shown to monitor the glucose concentration in the range of 50-200mM. The electrochemical approach was used for the polymerization of PANI on ITO slide. Further, the PANI coated ITO glass electrode (ITO/PANI) was employed for electrochemical sensing of glucose. This ITO/PANI also showed descent detection limit, wide linear range (50-200µM), repeatability and stability using cyclic voltammetry

Cyclic voltammetry (CV) and other studies have been used for characterization of the ITO modified electrode. CV of different modified electrodes in 5mM ferro-ferri buffer was taken where peak current increased due to the good electrocatalytic activity and high electroactive surface area of the PANI/ITO. The peak current increasing linearly with concentration confirmed the successful adsorption of glucose. So,in the future we can perform our experiments by using the bioelectrode . To summarise, we used two unique ways for direct electrical deposition for synthesising PANI nanofilms on surfaces of ITO substrates in this study. These PANI nanofilms modified ITO coated glass electrodes fabricated and used in the electro chemical bio sensors. These methods can be employed industrially on a larger and wider scale for mass production and fabrication of large quantities of sensors at low monetary value.

To guarantee trustworthy and precise testing, a more thorough examination of the sensitivity and selectivity of glucose biosensors is required. Good linearity, accuracy, and correlation when compared to a medical laboratory reference technique, as well as tolerance to common interferences, are all analytical criteria for appropriate hospital or home POC devices. The calibration and quality check of the devices should be done on a regular basis, as directed by

the manufacturer. Data integrity and, by implication, treatment results might be influenced by user-dependent variables. The most often mentioned issues include inappropriate test strip use, a lack of quality control method, filthy fingers, and dirty instruments. Several research has found that learning and ongoing training can help to decrease measurement mistakes caused by the aforementioned variables and enhance monitoring efficiency.

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