"Dynamic a.c. Impedance Study on Biofunctionalised Gold Nanoparticle-Polymer Nanocomposite Electrode"

A

Project Report

submitted in partial fulfillment of the requirement for the award of the degree of

MASTER OF TECHNOLOGY

in

NANOSCIENCE AND TECHNOLOGY

by

Sudhir Singh Pal

Roll. No. 13/NST/2K10

under the guidance of

Dr. N. K. Puri	**Dr. Rajesh**
Assistant Professor	**Senior Scientist**
DTU, New Delhi	**N.P.L., Delhi**

DEPARTMENT OF APPLIED PHYSICS
DELHI TECHNOLOGICAL UNIVERSITY
NEW DELHI-110042

JULY-2012

CERTIFICATE

This is to certify that the project entitled ***"Dynamic a.c. Impedance Study on Biofunctionalised Gold Nanoparticle-Polymer Nanocomposite Electrode"*** completed by Mr. Sudhir Singh Pal, student of M.Tech., Nanoscience and Technology in Applied Physics Department at Delhi Technological University, New Delhi embodies the original work carried out by him under my supervision and guidance. His work has been found very well for the partial fulfillment of the requirement of the degree of M.Tech.

It is further certified that, the student has developed the project during the period starting from 2nd January, 2012 to 30th June, 2012.

This report has not been submitted in part or full in any other University for award of any other degree or diploma.

Mr. Sudhir Singh Pal is student of good moral character. We wish him success in future.

(Dr. Rajesh)

Senior Scientist
Liquid Crystals & SAM Section
Engineering Material Division
National Physical Laboratory
Dr. K.S .Krishnan Road
New Delhi-110012

(Dr. Rajeev Chopra)

Head, HRD Group
National Physical Laboratory
Dr. K.S. Krishnan Road
New Delhi-110012

Department of Applied Physics
Delhi Technological University
Delhi

CERTIFICATE

This is to certify that **Mr. Sudhir Singh**, a student of final semester M.Tech (Nanoscience and Technology), Applied Physics Department, during the session 2010-2012 has successfully completed the project work on ***"Dynamic a.c. Impedance Study on Biofunctionalised Gold Nanoparticle-Polymer Nanocomposite Electrode"*** at National Physical Laboratory, Delhi and has submitted a satisfactory report in partial fulfilment for the award of the degree of Master of Technology.

The assistance and help received during the course of investigation have been fully acknowledged. He is a good student and we wish him good luck in future.

Dr. N. K. Puri	**Prof. R. K. Sinha**
Assistant Professor,	HOD
Applied Physics Department	Applied Physics Department
Delhi Technological University	Delhi Technological University
Delhi -110042	Delhi -110042

Candidate Declaration

I hereby declare that the work which is being presented in this thesis entitled ***"Dynamic a.c. Impedance Study on Biofunctionalised Gold Nanoparticle-Polymer Nanocomposite Electrode"*** my own work carried out under the guidance of Dr. N. K. Puri, Assistant Professor, Applied Physics Department, Delhi Technological University, Delhi and Dr. Rajesh, Senior Scientist National Physical Laboratory, New Delhi.

I further declare that the matter embodied in this thesis has not been submitted for the award of any other degree or diploma.

Date: ***Sudhir Singh Pal***

Place: New Delhi Roll. No. 13/NST/2K10

Dedicated to
My Parents

// ACKNOWLEDGEMENT

I sincerely thank my guide **Dr. Rajesh**, Senior Scientist, Liquid Crystal and SAM Section, National Physical Laboratory, New Delhi for his guidance, constant support and encouragement throughout my project.

It is my great respect and profound gratefulness that I record my indebt and deep felt devotion to my supervisors. **Dr. Rajesh**, Senior Scientist, Crystal and SAM Section, National Physical Laboratory, New Delhi and **Dr. N. K. Puri** , Assistant Professor, Department of Physics, DTU for their precious and encouraging supervision, valuable discussions and inspiring guidance throughout the work. Despite having a busy work schedule, they helped me a lot throughout this project with great interest. Things that I have learnt from them will continue to guide me further in my academic career.

I would like to extend my thanks to **Dr. R .K. Sinha**, Prof. & Head, Department of Physics, DTU for his kind guidance and support during the course for allowing me to carry out my project work in this esteemed research laboratory, NPL, New Delhi.

I would like to thank to **Dr. A. M. Biradar**, for his constructive suggestions, constant inspiration and timely help. Also I would like to thank to **Mr. Vikash Sharma**, **Mr. V. K. Tanwar, Mr. Mangeram** for their guidance and help at each and every step of my project. Besides my advisors, I would like to thank the rest of my thesis committee: **Sujeet Kumar Mishra**, who rescued me from various red tape crises and reviewed my work on a very short notice and **Nidhi Puri** for assistance and encouragement. I am grateful for the whole Liquid Crystal and SAM section for providing me all the facilities to complete my work.

I am extremely thankful to Prof**. Ramesh Chandra Budhani**, Director, National Physical Laboratory, New Delhi, for his kind permission to carry out the project work at NPL I express my sincere thanks to **Dr. Rajeev Chopra**, HRD Group and **Mr. Dharamvir Singh Saini**, HRD Group, NPL, for allowing me to do my M. Tech. research project at NPL.

I heartedly thank to my classmates for their support during my studies.

With deep sense of gratitude, I recorded here my happiness in thanking to my **Parents** with continuous presence and inspirations which guided me all along in developing this project work.

Sudhir Singh Pal

Roll. No. 13/NST/2K10

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ABSTRACT

A polypyyrole and gold nanoparticle composite film has been prepared for the quantitative estimation of any enzyme or antibody in aqueous solution. The gold nanoparticles were synthesized using sodium citrate as the reducing agent. The nanoparticles were capped with 3-mercaptopropionic acid. The protein BSA, was covalently linked to the carboxyl group (COOH) of 3-mercaptopropionic acid (MPA) capped gold nanoparticles, embedded in the surface of the polypyrrole film electrochemically deposited onto an ITO coated glass plate. The cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) techniques were used for the characterization of different surfaces formed over the ITO coated glass electrode.

GNPs provide a large surface area for immobilization of protein BSA and at the same time it provides high electrocatalytic activity to transfer electrons between BSA and electrode.The incorporation of gold nanoparticles into the polypyrrole film makes it more stable and conducting than simple polypyrrole film. This BSA/GNP(MPA)-PPy/ITO bioelectrode so prepared can be further use for biosensor application by immobilizing different antibody or enzyme.

CHAPTER-1

INTRODUCTION

1.1. BIOSENSORS

A biosensor is an analytical device which converts a biological response into an electrical signal. The term 'biosensor' is often used to cover sensor devices used in order to determine the concentration of substances and other parameters of biological interest even where they do not utilise a biological system directly.

Usually biological molecules are utilised as the active recognition entity within a sensor. These display unsurpassed selectivities; for example glucose oxidase will interact with glucose and no other sugar, and in this way will act as a highly selective receptor. In the case of glucose oxidase, the electrochemically inactive substrate glucose is oxidised to form gluconolactone along with the concurrent generation of the electro active species hydrogen peroxide. Enzymes also generally display rapid turnover rates and this is often essential to (a) avoid saturation and (b) to allow sufficient generation of the active species in order to be detectable.

A biosensor is a device that consists of a biological recognition system, often called a bioreceptor, and a transducer. The interaction of the analyte with the bioreceptor is designed to produce an effect measured by the transducer, which converts the information into a measurable effect, such as an electrical signal. [1]

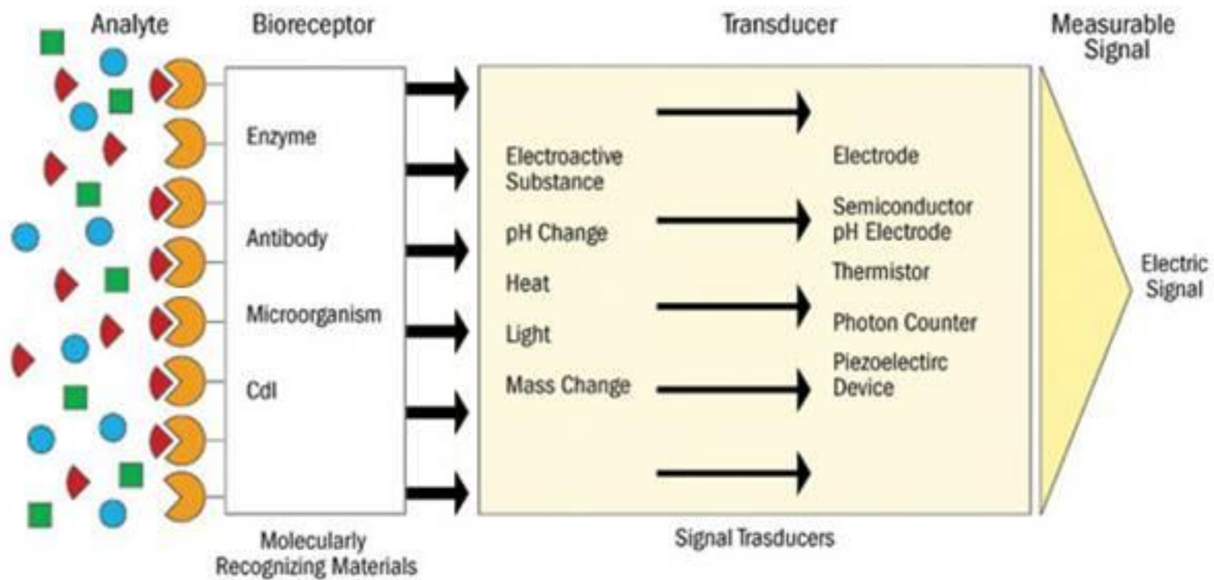


Fig.1: ***Schematic representation of a biosensor***

Biosensors can be classified either by their bioreceptor or their transducer type A bioreceptor is a biological molecular species for example an antibody, an enzyme, a protein, or a nucleic acid or a living biological system as cells, tissue, or whole organisms that utilizes a biochemical mechanism for recognition. The sampling component of a biosensor contains a bio-sensitive layer. The layer can either contain bioreceptors or be made of bioreceptors covalently attached to the transducer. The most common forms of bioreceptors used in biosensing are based on

1) antibody/antigen interactions

2) nucleic acid interactions

3) enzymatic interactions

4) cellular interactions(i.e. microorganisms, proteins)

5) interactions using biomimetic materials (i.e., synthetic bioreceptors).

For transducer classification, conventional techniques include:

1) optical measurements (i.e. luminescence, absorption, surface plasmon resonance, etc.)

2) mass-sensitive measurements (i.e. surface acoustic wave, microbalance,etc.).

3) electrochemical

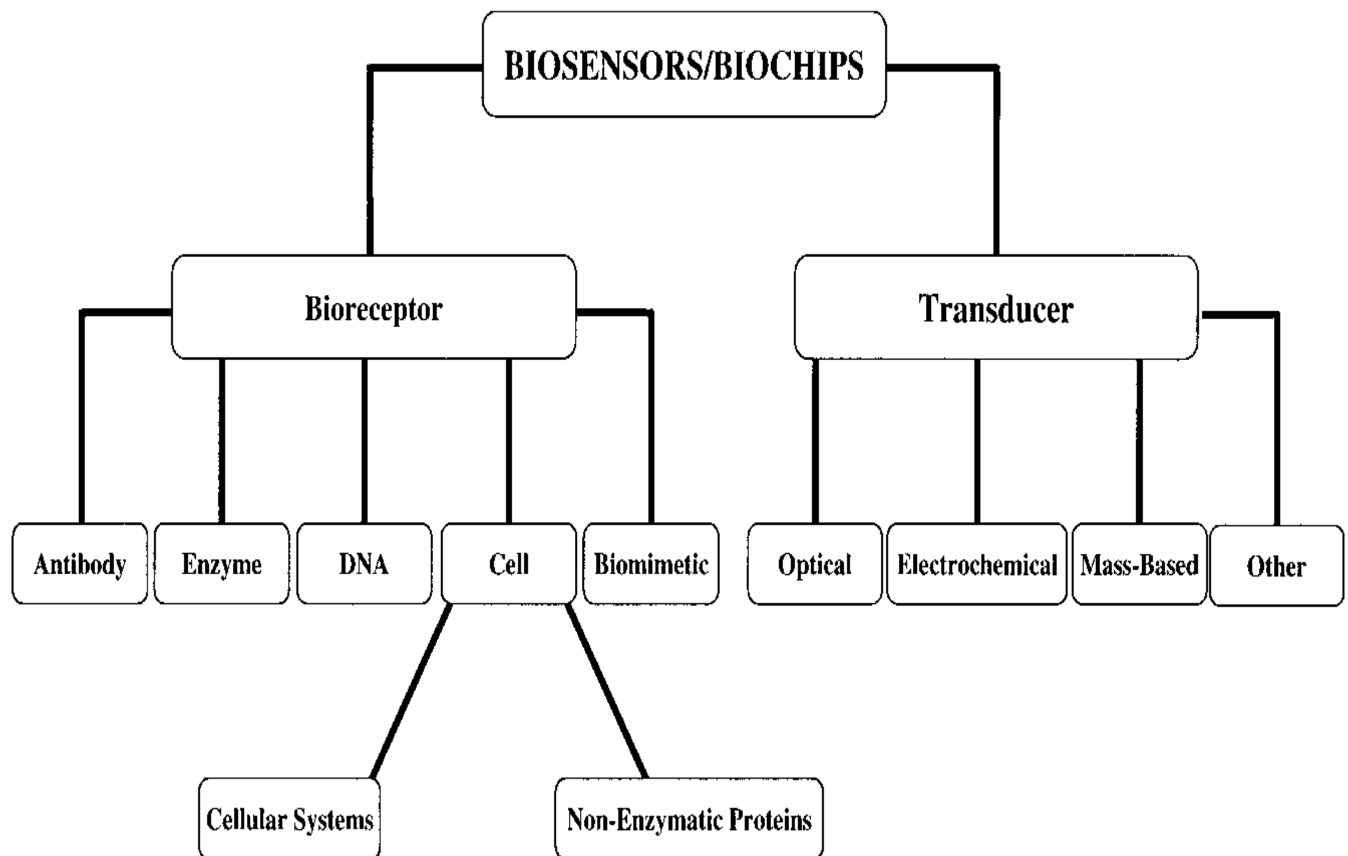


Fig.2: ***Classification of biosensors***

1.2. HISTORY OF BIOSENSORS:

Since the first biosensors were reported in the early 1960s[1] , there has been an explosive growth of research activities in this area . Biosensors have seen a wide variety of applications primarily in two major areas, biological monitoring and environmental sensing. This review covers the recent and significant advances in biosensor and biochip technologies for the analysis of samples of biological and biomedical interest. Since over 1500 articles have been published in the area of biosensors and biochip technology from January 1998 to August 1999,in this paper is not meant to be a comprehensive review, but rather a critical review, presenting a selection of the significant advances in the field of biosensors and biochips.

A computer search of the Science Citation Index provided most of the references for this review, and was in general limited to journal articles and generally did not include patents, conference proceedings, reports or dissertations.

The idea of a glucose enzyme electrode was proposed in 1962by Clark and Lyons from the Children Hospital in Cincinnati Their first device relied on a thin layer of GOx entrapped over an oxygen electrode (via a semi permeable dialysis membrane), and monitoring the oxygen consumed by the enzyme-catalyzed reaction:

Glucose + oxygen → glycolic acid +hydrogen peroxide

Guilbault and Lubrano described in 1973 an enzyme electrode for the determination of blood glucose based on amperometric (anodic)monitoring of the liberated hydrogen peroxide:

Good precision and accuracy were obtained in connection to 100 mL blood samples. A wide range of amperometric enzyme electrodes, differing in the electrode design or material, membrane composition, or immobilization approach have since been described.

1.2.1. CHRONICLE DEVELOPMENT OF BIOSENSORS

- **1916** First report on the immobilization of proteins: adsorption of invertase on activated charcoal.
- **1956** Invention of the first oxygen electrode [Leland Clark]
- **1962** First description of a biosensor: an amperometric enzyme electrode for glucose. [Leland Clark, New York Academy of Sciences Symposium]
- **1969** First potentiometric biosensor: urease immobilized on an ammonia electrode to detect urea. [Guilbault and Montalvo]
- **1970** Invention of the Ion-Selective Field-Effect Transistor (ISFET).
- **1972/5** First commercial biosensor: Yellow Springs Instruments glucose biosensor.
- **1976** First bedside artificial pancreas [Clemens et al.]
- **1980** First fiber optic pH sensor for in vivo blood gases.
- **1982** First fiber optic-based biosensor for glucose
- **1983** First surface Plasmon resonance (SPR) immunosensor.
- **1987** Launch of the blood glucose biosensor[MediSense]

1.1.2 GENERATIONS OF BIOSENSORS

Since the 1st biosensor developed by Clark, there are three 'generations' of biosensors:

a) First generation biosensors where the normal product of the reaction diffuses to the transducer and causes the electrical response.

b) Second generation biosensors which involve specific 'mediators' between the reaction and the transducer in order to generate improved response. The second generation biosensors involve two steps: first, there is a redox reaction between enzyme and substrate that is reoxidized by the mediator, and in second step eventually the mediator is oxidized by the electrode.

c) Third generation biosensors arise from the self-contained nature of the sensor where the reaction itself causes the response and no product or mediator diffusion is involved. Third generation sensors are accompanied by co-immobilization of enzyme and mediator at an electrode surface making the biorecognition component an integral part of the electrode transducer i.e. direct electrical contact of enzyme to electrode. Since neither mediator nor enzyme must be added, this design facilitates repeated measurements.[2]

1.3. BIORECEPTORS

Bioreceptors are the key to specificity for biosensor technologies. They are responsible for binding the analyte of interest to the sensor for the measurement. These bioreceptors can take many forms and the different bioreceptors that have been used are as numerous as the different analytes that have been monitored using biosensors. However, bioreceptors can generally be classified into five different major categories. These categories include:

1) antibody/ antigen

2) enzymes

3) nucleic acids/DNA

4) cellular structures/cells

5) biomimetic.

1.3.1. ANTIBODY - ANTIGEN

Antibodies are biological molecules that exhibit very specific binding capabilities for specific structures. This is very important due to the complex nature of most biological systems. An antibody is a complex biomolecule , made up of hundreds of individual amino acids arranged in a highly ordered sequence. For an immune response to be produced against a particular molecule, a certain molecularsize and complexity are necessary: proteins with molecular weights greater than 5000 Da are generally immunogenic. The way in which an antigen and its antigen specific antibody interact may be understood as analogous to a lock and key fit, by which specific geometrical configurations of a unique key enables it to open a lock. In the same way, an antigen-specific antibody "fits" its unique antigen in a highly specific manner. This unique property of antibodies is the key to their usefulness in immunosensors where only the specific analyte of interest, the antigen, fits into the antibody binding site.

Radioimmunoassay (RIA) utilizing radioactive labels has been the most widely used immunoassay method. Radioimmunoassay have been applied to a number of fields including pharmacology, clinical chemistry, forensic science, environmental monitoring, molecular epidemiology and agricultural science. The usefulness of RIA, however, is limited by several shortcomings, including the cost of instrumentation, the limited shelf life of radioisotopes, and the potential deleterious biological effects inherent to radioactive materials. For these reasons, there are extensive research efforts aimed at developing simpler, more practical immunochemical

techniques and instrumentation, which offer comparable sensitivity and selectivity to RIA. In the 1980s, advances in spectrochemical instrumentation, laser miniaturization, biotechnology and fiber optic research have provided opportunities for novel approaches to the development of sensors for the detection of chemicals and biological materials of environmental and biomedical interest.

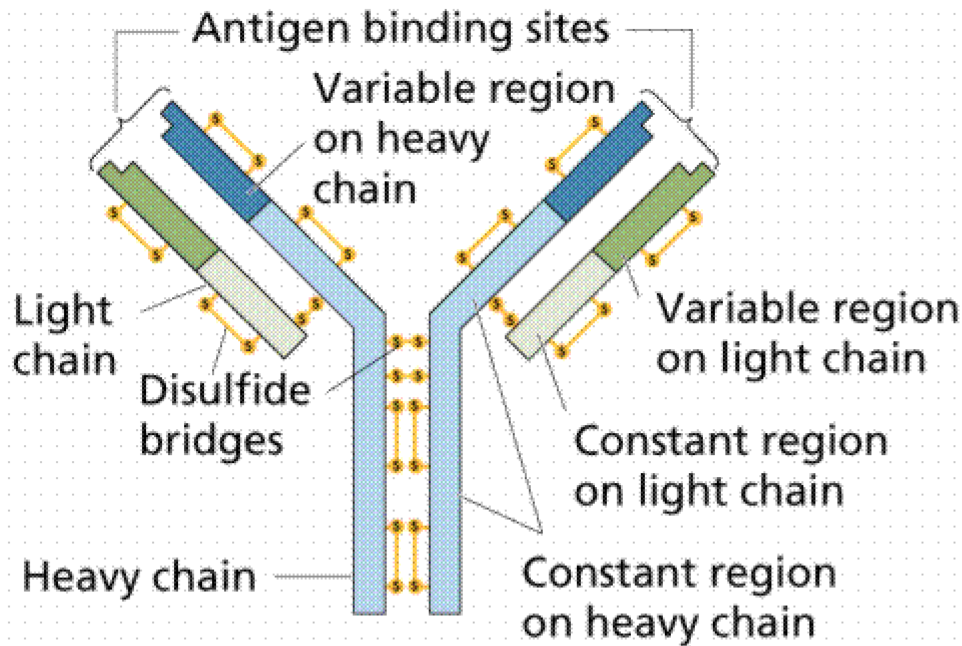


Fig.3: ***Structure of Antibody***

Since the first development of a remote fiberoptic immunosensor for *in situ* detection of the chemical carcinogen benzo[a]pyrene[3] , antibodies have become common bioreceptors used in biosensors today. Biomolecular interactions can be classified in two categories, according to the test format performed (i.e., direct and indirect). In a direct format the immobilized target molecule interacts with a ligand molecule or the immobilized ligand interacts with a target

molecule directly. For immunosensors, the simplest situation involves *in situ* incubation followed by direct measurement of a naturally fluorescent analyte [3]. For non fluorescent analyte systems, *in situ* incubation is followed by development of a fluorophor-labeled second antibody. The resulting antibody sandwich produces a fluorescence signal that is directly proportional to the amount of bound antigen. The sensitivity obtained when using these techniques increases with increasing amounts of immobilized receptor. The indirect format involves competition between fluorophor-labeled and unlabeled antigens[4]. In this case, the unlabeled analyte competes with the labeled analyte for a limited number of receptor binding sites. Assay sensitivity therefore increases with decreasing amounts of immobilized reagent. Due to the fiber-to-fiber differences in fiber optic biosensors, there is often a great difficulty in normalizing the signal from one fiber to the signal from another fiber. Ligler and coworkers reported on a method for calibrating antibody-based biosensors using two different fluorescent dyes[5]. To accomplish this, they labeled the capture antibodies, bound to the fiber, with one fluorescence dye and the antigen with a different dye. Both dyes were excited at the same wavelength and their fluorescence monitored. The resultant emission spectrum of the fluorescence signal from the capture antibodies was used to normalize the signal from the tagged antigen.

1.3.2. ENZYME

Enzymes are often chosen as bioreceptors based on their specific binding capabilities as well as their catalytic activity. In biocatalytic recognition mechanisms, the detection is amplified by a reaction catalyzed by macromolecules called biocatalysts. With the exception of a small group of catalytic ribonucleic acid molecules, all enzymes are proteins. Some enzymes require no

chemical groups other than their amino acid residues for activity. Others require an additional chemical component called a cofactor, which may be either one or more inorganic ions, such as Fe^{2+}, Mg^{2+}, Mn^{2+}, or Z^{2+} , or a more complex organic or metalloorganic molecule called a coenzyme. The catalytic activity provided by enzymes allows for much lower limits of detection than would be obtained with common binding techniques. The catalytic activity of enzymes depends upon the integrity of their native protein conformation. If an enzyme is denatured, dissociated into its subunits, or broken down into its component amino acids, its catalytic activity is destroyed. Enzyme-coupled receptors can also be used to modify the recognition mechanisms. For instance, the activity of an enzyme can be modulated when a ligand binds at the receptor. This enzymatic activity is often greatly enhanced by an enzyme cascade, which leads to complex reactions in the cell.[6]

1.3.3. NUCLIC ACID

Another bio recognition mechanism involves hybridization of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), which are the building blocks of genetics. In the last decade, nucleic acids have received increasing interest as bioreceptors for biosensor[7-12]. The complementarity of adenine : thymine (A :T) and cytosine : guanine (C :G) pairing in DNA forms the basis for the specificity of biorecognition in DNA biosensors, often referred to as genosensors. If the sequence of bases composing a certain part of the DNA molecule is known, then the complementary sequence, often called a probe, can be synthesized and labeled with an optically detectable compound (e.g., a fluorescent label). By unwinding the double-stranded DNA into single strands, adding the probe, and then annealing the strands, the labeled probe will hybridize to its complementary sequence on the target molecule.

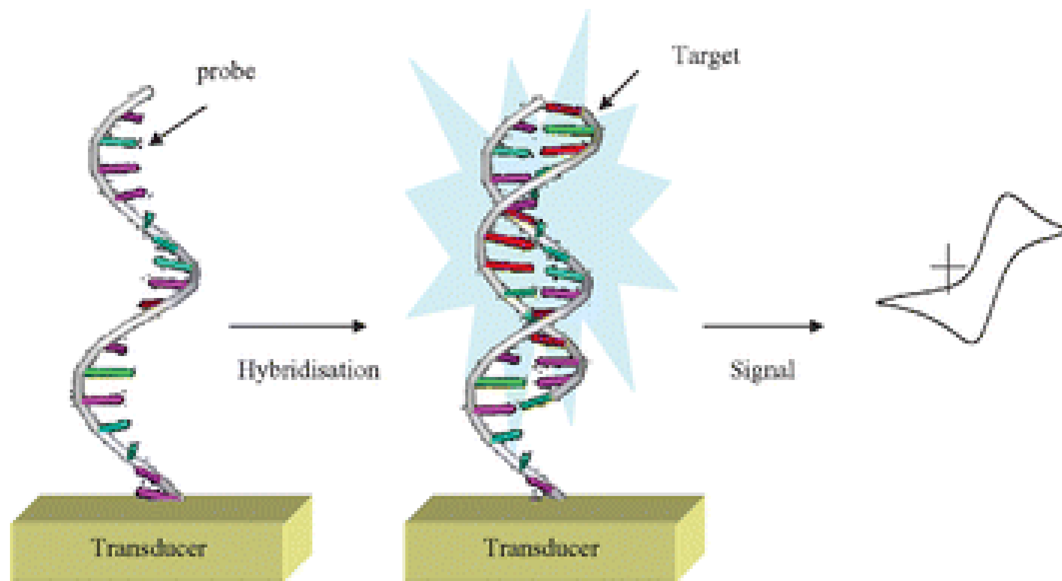


Fig.4: ***Interaction in DNA biosensors***

1.3.4. CELLULAR STRUCTURE AND CELL

Cellular structures and cells comprise a broad category of bioreceptors that have been used in the development of biosensors and biochips[13-20]. These bioreceptors are either based on bio-recognition by an entire cell/microorganism or a specific cellular component that is capable of specific binding to certain species. There are presently three major subclasses of this category:

1) cellular systems,

2) enzymes

3) non-enzymatic proteins.

Due to the importance and large number of biosensors based on enzymes, these have been given their own classification and were previously discussed. One of the major benefits associated with using this class of bioreceptors is that often the detection limits can be very low because of signal

amplification. Many biosensors developed with these types of bioreceptors rely on their catalytic or pseudo catalytic properties.

Cellular systems Microorganisms offer a form of bioreceptor that often allows a whole class of compounds to be monitored. Generally, these microorganism biosensors rely on the uptake of certain chemicals into the microorganism for digestion. Often, a class of chemicals are ingested by a microorganism, therefore allowing a class-specific biosensor to be created. Microorganisms such as bacteria and fungi have been used as indicators of toxicity or for the measurement of specific substances. For example, cell metabolism (e.g. growth inhibition, cell viability, substrate uptake), cell respiration and bacterial bioluminescence have been used to evaluate the effects of toxic heavy metals. Many cell organelles can be isolated and used as bioreceptors.

Since cell organelles are essentially closed systems, they can be used over long periods of time. Whole mammalian tissue slices or *in vitro* cultured mammalian cells are used as biosensing elements in bioreceptors. Plant tissues are also used ijn plant-based biosensors because they are effective catalysts as a result of the enzymatic pathways they possess.[6]

Non-enzymatic proteins. Many proteins that are found within cells often serve the purpose of bio-reception for intracellular reactions that will take place later or in another part of the cell. These proteins could simply be used for transport of a chemical from one place to another, such as a carrier protein or channel protein on a cellular surface.

In any case, these proteins provide a means of molecular recognition through one or another type of mechanism (i.e. active site or potential sensitive site). By attaching these proteins to various

types of transducers, many researchers have constructed biosensors based on non-enzymatic protein bio-recognition.

1.3.5. BIOMIMETIC RECEPTORS

A receptor that is fabricated and designed to mimic a bioreceptor is often termed a biomimetic receptor[21-31]. Several different methods have been developed over the years for the construction of biomimetic receptors. These methods include: genetically engineered molecules, artificial membrane fabrication and molecular imprinting. The molecular imprinting technique, which has recently received great interest, consists of mixing analyte molecules with monomers and a large amount of cross linkers. Following polymerization, the hard polymer is ground into a powder and the analyte molecules are extracted with organic solvents to remove them from the polymer network. As a result, the polymer has molecular holes or binding sites that are complementary to the selected analyte. Recombinant techniques, which allow for the synthesis or modification of a wide variety of binding sites using chemical means, have also provided powerful tools for designing synthetic bioreceptors with desired properties.

1.4. TRANSDUCER

Biosensors can also be classified based upon the transduction methods they employ. Transduction can be accomplished via a great variety of methods. Most forms of transduction can be categorized in one of three main classes. These classes are:

1) optical detection methods

2) mass detection methods.

3) Electrochemical techniques

However, new types of transducers are constantly being developed for use in biosensors. Each of these three main classes contain many different subclasses, creating a nearly infinite number of possible transduction methods or combination of methods.

1.4.1. OPTICAL TECHNIQUE

Optical transduction offers the largest number of possible subcategories of all three of the transducer classes[3,4,6] . This is due to the fact that optical biosensors can be used for many different types of spectroscopy (e.g., absorption, fluorescence, phosphorescence, Raman, SERS, refraction, dispersion spectrometry, etc.) with different spectrochemical properties recorded. These properties include: amplitude, energy, polarization, decay time and/or phase. Amplitude is the most commonly measured parameter of the electromagnetic spectrum, as it can generally be correlated with the concentration of the analyte of interest. The energy of the electromagnetic radiation measured can often provide information about changes in the local environment surrounding the analyte, its intramolecular atomic vibrations (i.e. Raman or infrared absorption spectroscopies) or the formation of new energy levels. Measurement of the interaction of a free molecule with a fixed surface can often be investigated based on polarization measurements. Polarization of emitted light is often random when emitted from a free molecule in solution, however, when a molecule becomes bound to a fixed surface, the emitted light often remains polarized. The decay time of a specific emission signal (i.e. fluorescence or phosphorescence) can also be used to gain information about molecular interactions since these decay times are very dependent upon the excited state of the molecules and their local molecular environment.

1.4.2 MASS-SENSITIVE TECHNIQUES

Another form of transduction that has been used for biosensors is the measurement of small changes in mass[32-40]. This is the newest of the three classes of measurements, however, it has already been shown to capable of very sensitive measurements. The principle means of mass analysis relies on the use of piezoelectric crystals. These crystals can be made to vibrate at a specific frequency with the application of an electrical signal of a specific frequency. The frequency of oscillation is therefore dependent on the electrical frequency applied to the crystal as well as the crystal's mass. Therefore, when the mass increases due to binding of chemicals, the oscillation frequency of the crystal changes and the resulting change can be measured electrically and be used to determine the additional mass of the crystal.

1.4.3. ELECTROCHEMICAL TECHNIQES

Electrochemical detection is another possible means of transduction that has been used in biosensors. This technique is very complementary to optical detection methods such as fluorescence, the most sensitive of the optical techniques. Since many analytes of interest are not strongly fluorescent and tagging a molecule with a fluorescent label is often labor intensive, electrochemical transduction can be very useful. By combining the sensitivity of electrochemical measurements with the selectivity provided by bioreception, detection limits comparable to fluorescence biosensors are often achievable.

(A) AMPEROMETRIC BIOSENSOR

In amperometric biosensor, the potential between the two electrodes is set and the current produced by the oxidation or reduction of electro active species is measured and correlated to the

concentration of the analyte of interest. The application of pH-sensitive amperometric biosensors is widespread nowadays because of their utilization in turbid media, instrumental sensitivity and amenability to miniaturization. The amperometric biosensors are known to be reliable, cheaper and highly sensitive for the clinical, environmental and industrial purposes. They generally have response times, dynamic ranges and sensitivities similar to the potentiometric biosensors.

Clark oxygen electrodes perhaps represent the basis for the simplest forms of amperometric biosensors, where a current is produced in proportion to the oxygen concentration. This is measured by the reduction of oxygen at a platinum working electrode in reference to an Ag/AgCl reference electrode at a given potential. Typically, the current is measured at a constant potential and this is referred to as amperometry. If a current is measured during controlled variations of the potential, this is referred to as voltammetry.

(B) CONDUCTOMETRIC BIOSENSOR

These devices measure the ability of an analyte (*e.g.* electrolyte solutions) or a medium (*e.g.* nano wires) to conduct an electrical current between electrodes or reference nodes. Conductometric devices can be considered as a subset of impedimetric devices. In most cases conductometric devices have been strongly associated with enzymes, where the ionic strength, and thus the conductivity, of a solution between two electrodes changes as a result of an enzymatic reaction. Thus, conductometric devices can be used to study enzymatic reactions that produce changes in the concentration of charged species in a solution. The variable ionic background of clinical samples and the requirement to measure small conductivity changes in media of high ionic strength limit the applicability of such enzyme-based conductometric devices for biosensing. Another approach is to directly monitor the changes in conductance of an

electrode as a result of the immobilization of e.g. enzymes, complementary antibody-antigen pairs, etc. onto the electrode surface.

(C) POTENTIOMETRIC BIOSENSOR

The most important type of electrochemical biosensors is the potentiometric biosensor. In this type of biosensor, the voltage that is produced during the oxidation or reduction of a product or reactant, usually at a constant current is measured. This type of measurement technique is very attractive for practical applications as it allows the use of small size, portable and low cost instruments. These devices operate under equilibrium conditions and measure the accumulation of charge density at the electrode surface brought about by some selective process.

Potentiometric devices measure the accumulation of a charge potential at the working electrode compared to the reference electrode in an electrochemical cell when zero or no significant current flows between them. In other words, potentiometry provides information about the ion activity in an electrochemical reaction.

1.5. BIOMOLECULE IMMMOBILIZATION METHODS

Immobilization of biomolecules such as protein, enzyme on the transducer surface is a fundamental step in the development of biosensors. The methods of immobilization is a critical factor in the construction of biosensor and can greatly affect its performance. Immobilization of biomolecules can be carried out using many different procedures, like physical adsorption, covalent binding, gel entrapment and electrochemical immobilization, while retaining the biological recognition properties of the biomolecules. The procedure of biomolecule

immobilization on conductive surfaces remains a key step for the performance of the resulting electrochemical devices.

For efficient deposition of a biomolecule, it must satisfy a few pre-requisites, that

- There must be an efficient and stable immobilization of the biological macromolecules on transducer surfaces,
- It must retain its biological properties completely
- It should be compatible and chemically inert towards host structure
- It should be accessible when immobilized.

1.5.1. PHYSICAL ADSORPTION

Physical adsorption, a commonly used immobilization technique, involves binding forces that include hydrogen bonds, electrostatic interaction, multiple salt bridges and vander Waals forces, making the binding susceptible to pH. Moreover adsorption of the biomolecule onto the matrix results in a weak binding that may lead to desorption and leaching of the biomolecule to sample solution during measurements. This decreases the lifetime stability of electrode. This method has been used for the preparation of many biosensors. Immobilization by physical adsorption also involves cross-linking. Cross-linking gives sensors with short response times but poor stability because enzyme is directly exposed to the bulk solution and partly denaturized by the cross-linking.

1.5.2. COVALENT IMMOBILIZATION

Amongst all the immobilization methods covalent immobilization is the most advanced one. In this method biomolecule is incorporated in the conducting polymer matrix through

complementary functional group chemistry. This way of immobilization confers strong interaction between polymer and biomolecule that can be achieved by amide or ester linkage in the presence of some coupling agent. It comes with an advantage of low diffusional resistance and such a sensor shows good stability under adverse conditions.

Covalent linking of a biomolecule to polymer matrix is accomplished in a two step process i.e. synthesis of functionalized polymer followed by covalent immobilization. Since, immobilization takes place only on the outer surface of the polymer, it permits selection of optimum reaction conditions for each step. Covalent immobilization not achieved in polymer only can be participate with other moieties (having functional group) present with conducting polymer after modification viz plasticizer or fillers. Covalent immobilization not only inhibits leaching of the enzyme but also provides thin coating of the enzyme which facilitates fast ionic movement thereby increasing sensitivity and long life stability of the biosensor. Generally, covalent immobilization is often preferred to physical adsorption to avoid leaching of the bioreceptor. Covalent binding through an amine group has a number of advantages that include high binding strength, binding of distinct functional groups, specific orientation of molecules and improved stability.

1.5.3 ELECTROCHEMICAL IMMOBILIZATION

The conventional procedures that are used for biomolecule immobilization like physical adsorption, covalent binding, entrapment and crosss-linking suffer from a low reproducibily and have a poor spatially controlled deposition. In recent years, the focus of immobilization has been shifted towards the entrapment of biomolecules in the layers of electrochemically synthesized polymers.[2]

The major advantages of electrochemical immobilization technique are : it is one step, fastest than all immobilization procedures; the distribution of the immobilized enzyme is spatially controlled irrespective of geometry, shape and dimension of the electrode, the film thickness can be precisely controlled through e.g. the charge involved in the deposition step and can be used to build up multilayer / multienzyme structures, it allows reproducible and precise formation of a polymer coating over surfaces whatever their size and geometry.[2]

1.6. IMMUNOSENSORS

An Immunosensor is a device comprising an immobilized antigen or antibody species coupled to a signal transducer, which detects the binding of complementary species. For immobilization of the immunoagents many methods have been developed on various substrates. The binding event is transformed into a measurable signal by the transducer. Transduction has been performed using optical (e.g., surface plasmon resonance), piezoelectrical (e.g., quartz crystal microbalance), surface scanning (e.g., atomic force microscopy) scanning electrochemical microscopy, and other electrochemical techniques. Electrochemical detection of immune interaction can be performed both with and without labeling. A frequently used format in electrochemical immunosensing is an amperometric immunosensor, where proteins are labeled with enzymes producing an electro active product from an added substrate. An indirect immunosensor uses a separate labeled speciesthat is detected after binding by e.g. fluorescence or luminescence. A direct device detects the binding by a change in the potential difference, current, mass, resistance, heat or optical properties. Direct detection without labeling can be performed by cyclic voltammetry, chronoamperometry, impedimentary, and by measuring the current during potential pulses (pulsed amperometric detection). These methods are able to

detect a change in capacitance and/or resistance of the electrode induced by binding of protein. These immunosensors have been developed using various substrates. Those built on silicon, silanized metal, or polypyrrole are often regenerable, while those based on self-assembled monolayers (SAMs), that is, monolayers formed spontaneously from sulfur-containing compounds on silver or gold, are not [2].

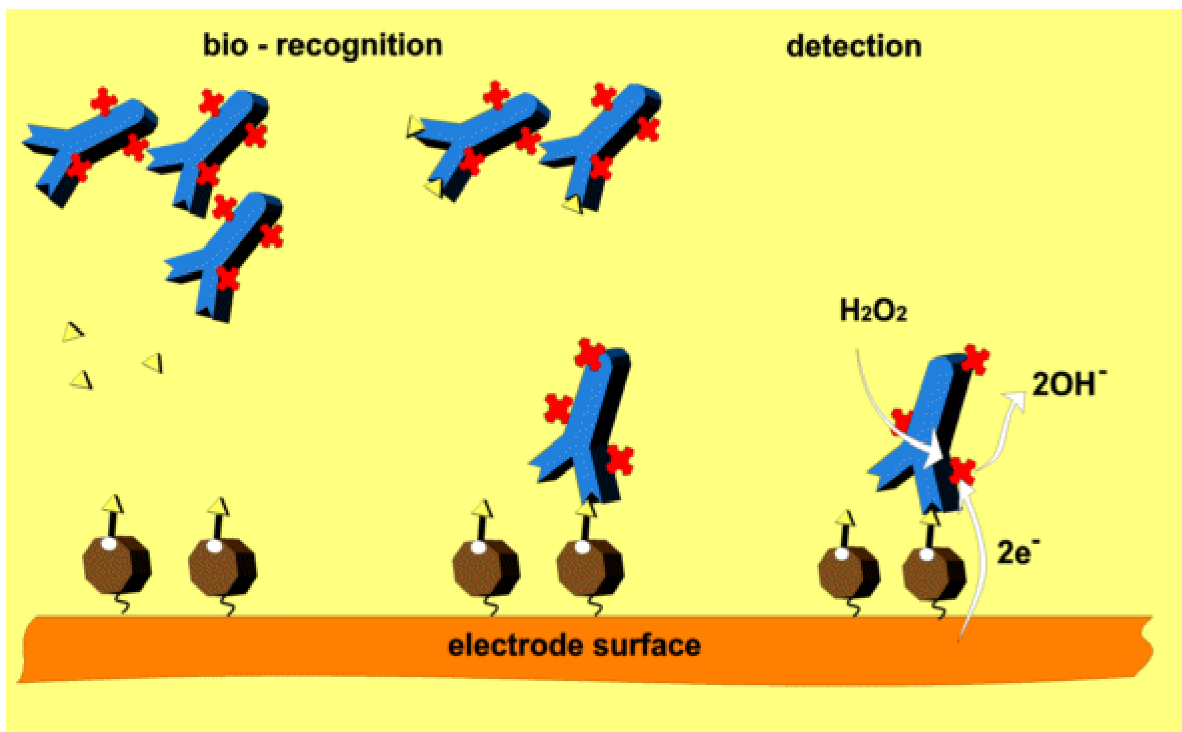


Fig.5: ***Principle of operation of an Immunosensor***

1.7. IMPORTANCE OF CONDUCTING POLYMER IN BIOSENSOR

Polymers, by virtue of their light weight and greater ease of fabrication, have replaced and are continuing to replace metals in several areas of applications; as often remarked – 'from buckets to rockets'. Polymers have traditionally been considered good electrical insulators and a variety of their applications have relied on this insulating property however a large number of organic compounds, which effectively transport charge are roughly divided into three groups i.e. charge transfer complexes/ ion radical salts, organometallic species and conjugated organic polymers. A new class of polymers known as intrinsically conducting polymers or electroactive conjugated polymers has recently emerged. Such materials exhibit interesting electrical and optical properties previously found only in inorganic systems. Electronically conducting polymers differ from all the familiar inorganic crystalline semiconductors e.g. silicon in two important features that polymers are molecular in nature and lack long range order . A key requirement for a polymer to become intrinsically electrically conducting is that there should be an overlap of molecular orbitals to allow the formation of delocalized molecular wave function. Besides this, molecular orbitals must be partially filled so that there is a free movement of electrons throughout the lattice.[41]

Conducting polymers contain π-electron backbone responsible for their unusual electronic properties such as electrical conductivity, low energy optical transitions, low ionization potential and high electron affinity. This extended (π-conjugated system of the conducting polymers have single and double bonds alternating along the polymer chain. The higher values of the electrical conductivity obtained in such organic polymers have led to the name 'synthetic metals'. Many applications of conducting polymers including analytical chemistry and biosensing devices have been reviewed by various researchers and . They have widened the possibility of modification of

surface of conventional electrodes providing new and interesting properties. They were applied in electrocatalysis, membrane separations and chromatography. They also create new technological possibilities in design of chemical and biochemical sensors.[41]

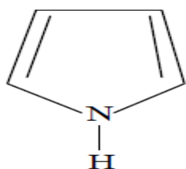
1.7.1 CONDUCTION MECHANISM

The mechanism of conduction in such polymers is very complex since such a material exhibits conductivity across a range of about fifteen orders of magnitude and many involve different mechanisms within different regimes. Conducting polymers show enhanced electrical conductivity by several orders of magnitude of doping. The concept of solitons, polarons and bipolarons has been used to explain the electronic phenomena in these systems. Conductivity in conducting polymers is influenced by a variety of factors including polaron length, the conjugation length, the overall chain length and by the charge transfer to adjacent molecules . These are explained by large number of models based on intersoliton hopping, hopping between localized states assisted by lattice vibrations, intra-chain hopping of bipolarons, variable range hopping in 3-dimensions and charging energy limited tunneling between conducting domains.

Common conducting polymers are polyacetylene, polypyrrole, polythiophene, polyterthiophene, polyaniline, poly-fluorine, poly-3-alkylthiophene, polycarbazole, poly tetrathiafulvalene, polynapthalene, polyphenylene sulfide, polyparaphenylene sulfide, and poly-diaminonaphthalene. Among the various conducting polymers poly-aniline, polythiophene, and polypyrrole are biocompatible and hence, cause minimal and reversible disturbance to the working environment and protect electrodes from fouling.

1.7.2 POLYPYROLE

Polypyrole is one of the widely used conducting polymers with some special electrical properties. These properties originate from the fact that polypyrole is an intrinsically conducting polymer and can be synthesized to have conductivities up to 1000 S/cm which approaches the conductivity of metals.[42]



Electrochemical synthesis of conducting polymer from their monomer is known as electrochemical polymerization. Electrochemical polymerization is normally carried out in a single or dual compartment cell by adopting a standard three electrode configuration in a typical electrochemical bath consisting of monomer and a supporting electrolyte both dissolved in an appropriate solvent. Generally, electrochemical polymerization can be carried out either potentiostatically (to obtain thin films) or galvanostatically (to obtain thick film). In electrochemical polymerization doping and processing takes place simultaneously.

Electrochemically polymerized conducting polymers has been studied extensively for the construction of biosensors, because of the (1) direct and easy deposition on the electrode surface (2) control of thickness (3) redox conductivity of and polyelectrolyte characteristics of polymer useful for sensor application. Polypyrrole fulfills the above requirements together with having the characteristics of easy oxidation, high chemical stability, low cost of monomer. Many studies have been done to construct polypyrrole based enzyme biosensors, in which entrapment of enzyme was used an immobilizations techniques. The entrapment of biomolecules a simple one step method during the electrochemical polymerization of pyrrole, but it suffers greatly from the poor accessibility of the target molecules due to its hydrophobicity. Another method is the

covalent immobilization; this method involves the attachment of enzyme molecules through chemical binding between enzyme and the carboxyl or amine groups on the surface. This functional group of the polymer surface introduced either by the post-functionalization of the PPy film or the initial polymerization of the functionalized pyrrole.

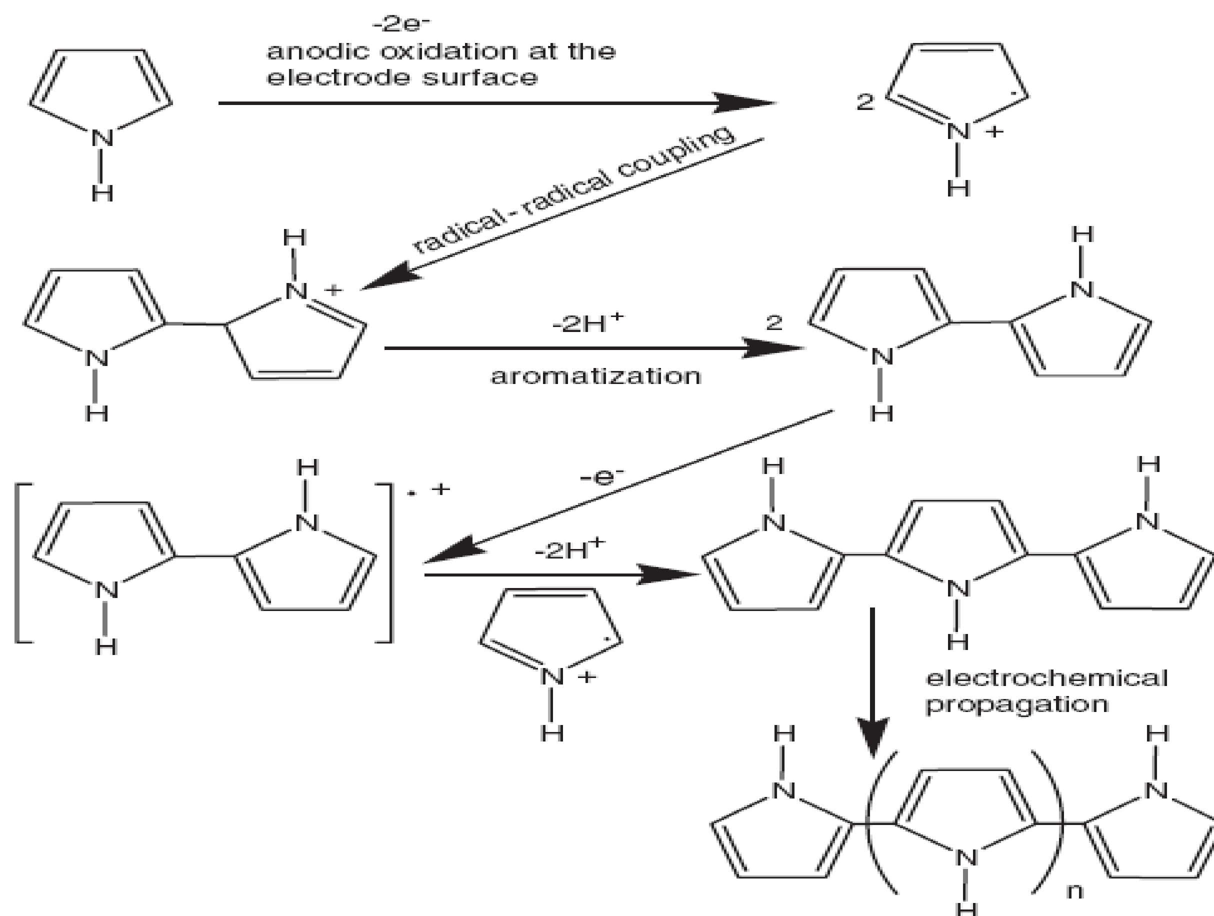


Fig.6: ***Mechanism of electrochemical polymerization of polypyrrole from pyrrole monomer.***

This approach allows better orientation for higher activity and long time stability of enzymes, making it a preferable method for construction of the enzyme electrode.

1.8. IMPORTANCE OF GOLD NANOPARTICLE IN BIOSENSORS

The unique properties of gold nanoparticles to provide a suitable microenvironment for biomolecules immobilization retaining their biological activity, and to facilitate electron transfer between the immobilized proteins and electrode surfaces, have led to an intensive use of this nanomaterial for the construction of electrochemical biosensors with enhanced analytical performance with respect to other biosensor designs. The advantageous operational characteristics of the biosensing devices designed making use of gold nanoparticles are highlighted with respect to non-nanostructured biosensors .

Electrochemical enzyme biosensors including those using hybrid materials with carbon nanotubes and polymers, sol–gel matrices, and layer-by-layer architectures are considered. Moreover, electrochemical immunosensors in which gold nanoparticles play a crucial role in the electrode transduction enhancement of the affinity reaction as well as in the efficiency of immunoreagents immobilization in a stable mode are reviewed. Similarly, recent advances in the development of DNA biosensors using gold nanoparticles to improve DNA immobilization on electrode surfaces and as suitable labels to improve detection of hybridization events are considered. Finally, other biosensors designed with gold nanoparticles oriented to electrically contact redox enzymes to electrodes by a reconstitution process and to the study of direct electron transfer between redox proteins and electrode surfaces have also been treated.[43]

1.9. BOVINE SERUM ALBUMIN

Bovine serum albumin (BSA) is a large globular protein with a good essential amino acid profile. It has been well characterized and the physical properties of this protein are well known

BSA binds free fatty acids, other lipids and flavor compounds, which can alter the heat denaturation of the protein. Isolated BSA has been reported to be a very functional protein . It is reported to partially unfold between 40 and 50^0C, exposing non-polar residues on the surface and facilitating reversible protein-protein interactions. Phospholipid -protein-calcium complexes are formed at pH levels below the isoelectric point of the BSA .Whether these interactions have any biological function is not known.

1.9.1. BIOLOGICAL FUNCTION

Bovine serum albumin has been given little attention in respect to its role in the functional properties of whey protein concentrates, and makes up only about 5% of the protein in whey protein concentrates .Its primary biological function has been associated with its lipid binding properties , but the mechanism of this role has not been clearly elucidated. It may play a role in mediating lipid oxidation, since BSA has been shown in-vitro to protect lipids against phenolic induced oxidation .

Denatured BSA might "reduce the probability of a person acquiring certain diseases, such as insulin dependent diabetes or auto-immune disease.

Bosselears, etal. (1994) compared the anti-mutagenic effect of BSA, soy protein, total whey protein, b-lacto globulin and pepsin-hydrolysed casein. Of these proteins, only the enzyme-hydrolysed casein and BSA were effective against genotoxic compounds.

Bovine serum albumin has been used as a component of cell media to regenerate plants from cultured guard cells and to provide for enhancement of production of plasminogen activator.

CHAPTER-2

MATERIAL AND METHODS

2.1. APPARATUS

Contact angles were recorded on Drop Shape Analysis System, model DSA10MK2 from Kru"ss GmbH, Germany. Atomic force microscopy (AFM) images were obtained on a Nanoscope 5, VEECO Instrument Ltd., USA Transmission electron microscopy (TEM) images were taken on high resolution TM model Technai G2 F30 S Twin, The Netherlands. Electrochemical polymerization and potentiometric measurements were done on a PGSTST302N, AUTOLAB instrument from Eco Chemie, Netherland. Potentiometric measurements were carried out in a conventional three electrode cell configuration where Ag/AgCl as reference electrode, platinum wire as counter electrode and a working electrode.

2.2 MATERIALS

Tetrachloroauric (III) acid ($HAuCl_4$) was obtained from Himedia Pvt. Ltd., India for preparation of GNPs, Sodium citrate dihydrate, ($Na_3C_6H_5O_7.2H_2O$) are available from Sigma–Aldrich, St. Louis, MO (800/325–3010) Bovine serum Albumin (**BSA**) was ensured from GENEI marked, pyrrole monomer from Aldrich. Ag/AgCl is purchased from CH Instrument Inc, N-hydroxy succinimide 98% (NHS) having chemical formula $C_4H_5NO_3$ and M.W = 115.09 and N-(3-Dimethylaminopropyl)-N'-ethyl carbodimide hydrochloride(EDC) having chemical formula $C_8H_{17}N_3HCl$ and M.W = 191.71 were obtained from Sigma-Aldrich. Tris, 3-Mercaptopropionic acid (MPA). Other chemicals were of analytical grade and used without further purification.

2.3. SYNTHESIS OF 3-MERCAPTO PROPIONIC ACID (MPA) CAPPED GOLD(Au) NANOPARTICLES

The synthesis of nanoparticles involves surfactant molecules that bind to their surface, which stabilize the nuclei and larger nanoparticles against aggregation by a repulsive force, and which generally control the growth of the nanoparticles in terms of rate, final size or geometric shape.

The molecule bound to the nanoparticle surface called ligand not only control the growth of the particle during synthesis, but also aggregation of the nanoparticle. The repulsive force between particles can be due to electrostatic repulsion or a hydration of the layer of the surface. The ligand molecule have to be bound to the particle surface by some attractive interaction, either by chemisorption, electrostatic attraction or hydrophobic interaction, most commonly provided by a head group of the ligand molecule. Generally it is found that strongly binding ligand molecules forming a dense layer stabilize particles better than weakly binding ones. In aqueous solutions, strongly charged ligand molecules, containing e.g. carboxylic or sulphonic acid group are found to stabilize the particles for longer time and also at more elevated salt concentration. In this way the exchange of ligand molecule on the surface by others improve the stability of the given nanoparticles and provide new properties or functionality to the particles.

Here gold nanoparticles are synthesized in an aqueous solution at room temperature by citrate reduction. Citrate not only acts as reducing agent but also as stabilizing agent. The resulting nanoparticles have negatively charged citrate ions adsorbed on their surface and are thus stabilized by electrostatic repulsion. The citrate layer is replaced by ligands binding stronger to the particle surface i.e. by 3-mercaptopropanoic acid (MPA) which is known as capping.

The stock solution (A) of 1 mM $HAuCl_4$ by dissolving 0.1 g of solid $HAuCl_4$ in 500 ml of distilled water and stock solution (B) of 38.8 mM $Na_3C_6H_5O_7.2HO$ (Sodium citrate) by dissolving 0.5 g of sodium citrate in 50 ml of distilled water were prepared. 20ml of 1 mM $HAuCl_4$ from stock solution was taken in round-bottom flask equipped with a condenser. The solution in the flask is heated until it boils. A magnetic stirrer is kept in flask to stir the solution when needed.

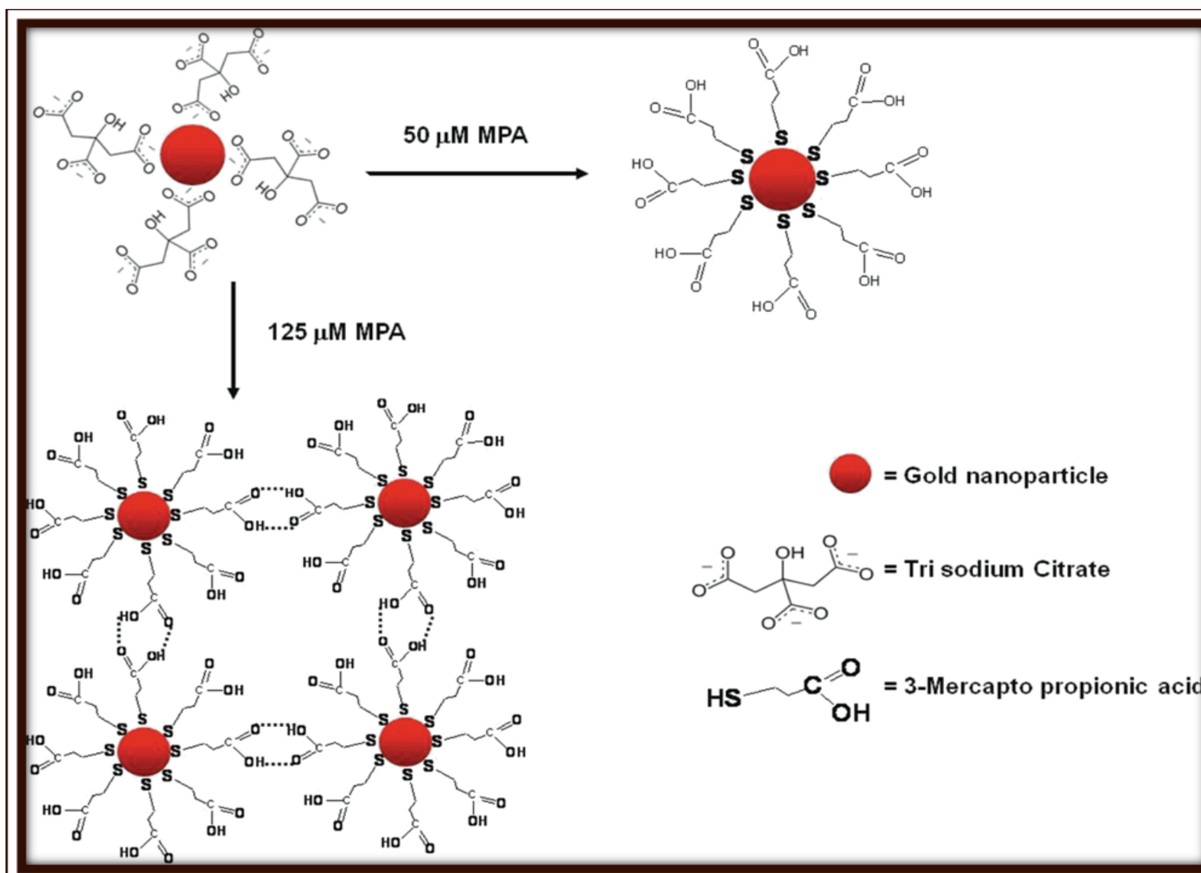


Fig.7: ***Schematic representation of capping of gold nanoparticles***

After the solution begins to boil, 2 ml 38.8 mM $Na_3C_6\ H_5O_7.2H_2O$ from stock solution(B) and continue to boil the solution and stir it until it becomes red in colour (approx. 10-15 mins). Now the above solution is taken in a conical flask and kept for 10 mins in room temperature.[44] Now the solution is stirred and 12.5 μM MPA solution is added and stirred for 10-15 mins. The free carboxylic groups of MPA on the surface of the gold nanoparticles provided more number of binding sites for covalent immobilization of the biomolecule. The size of Au nanoparticles are 10-15 nm characterized by transmission electron microscopy(TEM) as shown in figure. The gold nanoparticles manifest themselves as small dark spots in the TEM image.

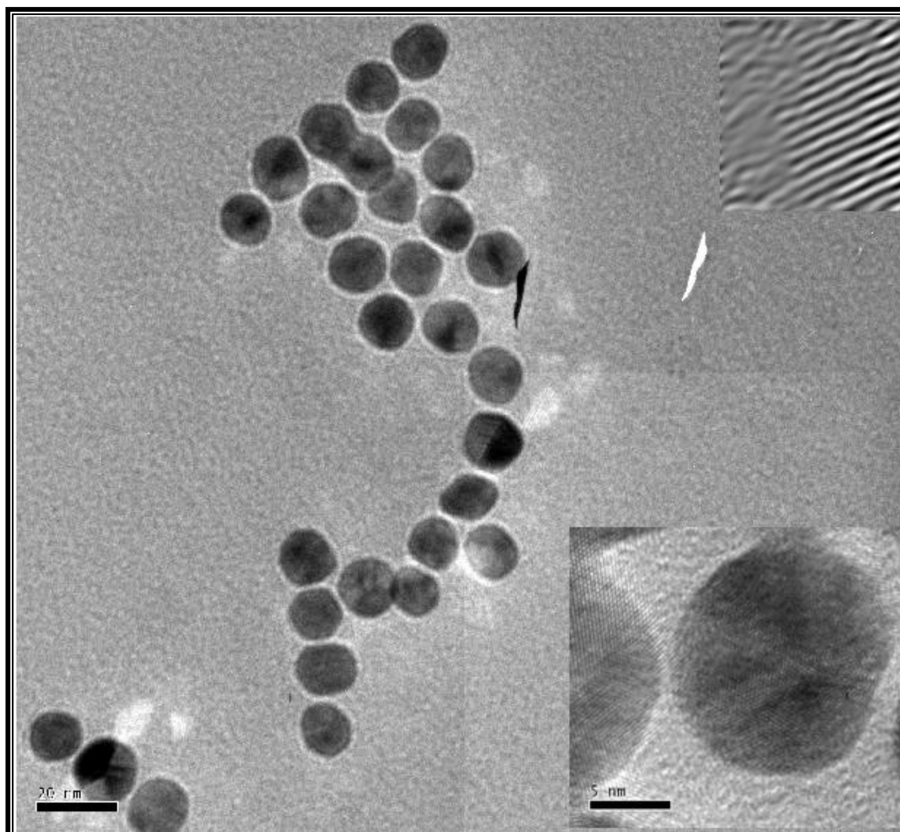


Fig.8. ***TEM micrograph of GNPs with average size of 10-15 nm***.

2.4. SYNTHESIS OF PBS BUFFER SOLUTION:

The step involved for preparation of PBS buffer are:

Prepare 1.0M buffer solution by making A and B as follows-

A: 0.2 M solution of monobasic potassium phosphate (6. 96gm in 200 ml water)

B: 0.2 M solution of dibasic potassium dihydrogen phosphate (2.72gm in 200 ml water)

81 ml of A + 21 ml of B + 1480 mg of KCl, diluted to a total of 200 ml.

by this we can prepare the stock solution of PBS buffer of pH-7.4.

2.5. FABRICATION OF BSA/GNP(MPA)-PPY/ITO/GLASS ELECTRODE

For preparing the this electrode, we took ITO (indium-tin-oxide) coated glass plates of (0.5 X 0.5) cm^2 area for the electrode preparation. These ITO plates were cleaned by sequential cleaning in soapy water (extran), acetone, ethanol, 2-isopropanol and distilled water for 10 minutes each and then dried under vacuum. Then the GNP(MPA)-PPy polymer nanocomposite film was formed on the required area by applying constant current of 1 mA cm^{-2} having charged densities of 250 mC cm^{-2}, using three electrode system on a PGSTAT302N, AUTOLAB instrument from Eco Chemie, The Netherlands.[45,46]

The GNP(MPA)-PPy/ITO glass plates were then immersed in aqueous solution of 30 mM NHS and 150 mM EDC for 1 hr followed by washing with distilled water and then dried under N_2 gas to have NHS-EDC/GNP(MPA)-PPy/ITO coated glass plates. These plates were then immersed in BSA solution prepared in phosphate buffered solution (PBS) of pH 7.4 for 2 hr to immobilize the BSA protein on the electrode followed by washing in PBS to remove the unbounded protein and finally dried under N_2. These BSA/GNP(MPA)-PPy/ITO coated glass plates were used as working electrode. These protein electrodes were stored at 4^0 C in a refrigerator when not in use.

2.6 INSTRUMENTATION

2.6.1. CONTACT ANGLE

The contact angle is a measure of the ability of a liquid to spread on a surface. The method consists to measure the angle between the outline tangent of a drop deposited on a solid and the surface of this solid. The contact angle is sensitive enough to show different results even

Fig.9: ***Photograph of Drop Shape Analysis System, model DSA10MK2***

with a small amount of contamination such as monolayer orders. That's why contact angle is used for evaluating cleanness of solid surface. Factors like temperature, humidity, solid surface roughness, and static electricity, etc. all affect results, so measuring in a controlled environment is important.

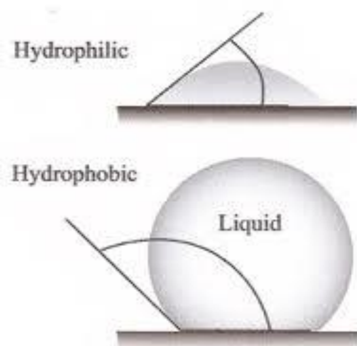


Fig.10: ***Contact angle measurement for hydrophobic and hydrophilic surfaces***

Contact angle is larger for hydrophobic surface while it is smaller for hydrophilic surface. Hydrophobic and hydrophilic forces are interactions that serve to keep chemical groups positioned close to one another. From the contact angle, physical properties of interaction between solid and liquid like wettability, affinity, adhesiveness and repellency can be studied.

2.6.2 ATOMIC FORCE MICROSCOPY(AFM)

The atomic force microscope (AFM), invented in 1986, is a new instrument which provides three-dimensional surface images of samples by scanning a sharp probing tip over the sample surface. Unlike electron microscopes (EM), the AFM has the advantage to obtain high-resolution pictures not only in a vacuous but also in a non-vacuous (i.e., air or liquid) environment. AFM is useful for observing biological structures such as DNA, collagen molecules, collagen fibrils and chromosomes. AFM images of living cultured cells in liquid can be used for investigating the movement of cellular processes in relation to sub cellular cytoskeletal elements. Recently, numerous AFM-related microscopes, or scanning probe microscopes (SPM), have been invented in parallel with the development of the AFM itself. These microscopes allow the simultaneous collection of topographical and other (e.g., viscoelastic, near-field optical) images of samples in

the same portions. Thus, the combination of AFM and the other SPM has great potential for providing valuable new findings on structure and function of cells and tissues.

2.6.3 CYCLIC VOLTAMMETRY (CV)

Cyclic Voltammetry (CV) is perhaps the most effective and versatile electroanalytical technique available for the mechanistic study of redox systems. It enables the electrode potential to be rapidly scanned in search of redox couples. Once located, a couple can then be characterized from the potentials of peaks on the cyclic voltammogram and from changes caused by variation of the scan rate.

Fig.11: ***Photograph of autolab instrument model no. PGSTST302N***

A typical electrode reaction involves the transfer of charge between an electrode and a species in solution.

The electrode reaction usually referred to as electrolysis, typically involves a series of steps:

1. Reactant (O) moves to the interface: this is termed mass transport

2. Electron transfer can then occur via quantum mechanical tunnelling between the electrode and reactant close to the electrode (typical tunnelling distances are less than 2 nm)

3. The product (R) moves away from the electrode to allow fresh reactant to the surface

The 'simplest' example of an electrode reaction is a single electron transfer reaction, e.g.[10]

$$Fe^{3+} + e^{-} \longrightarrow Fe^{2+}.$$

Cyclic voltammetry is generally used to study the electrochemical properties of an analyte in solution. The utility of cyclic voltammetry is highly dependent on the analyte being studied. The analyte has to be redox active within the experimental potential window. It is also highly desirable for the analyte to display a reversible wave. A reversible wave is when an analyte is reduced or oxidized on a forward scan and is then reoxidized or rereduced in a predictable way on the return scan as shown in the figure 8. The current at the working electrode is plotted versus the applied voltage to give the cyclic voltammogram trace. A cyclic voltammogram is obtained by measuring the current at the working electrode during the potential scans.

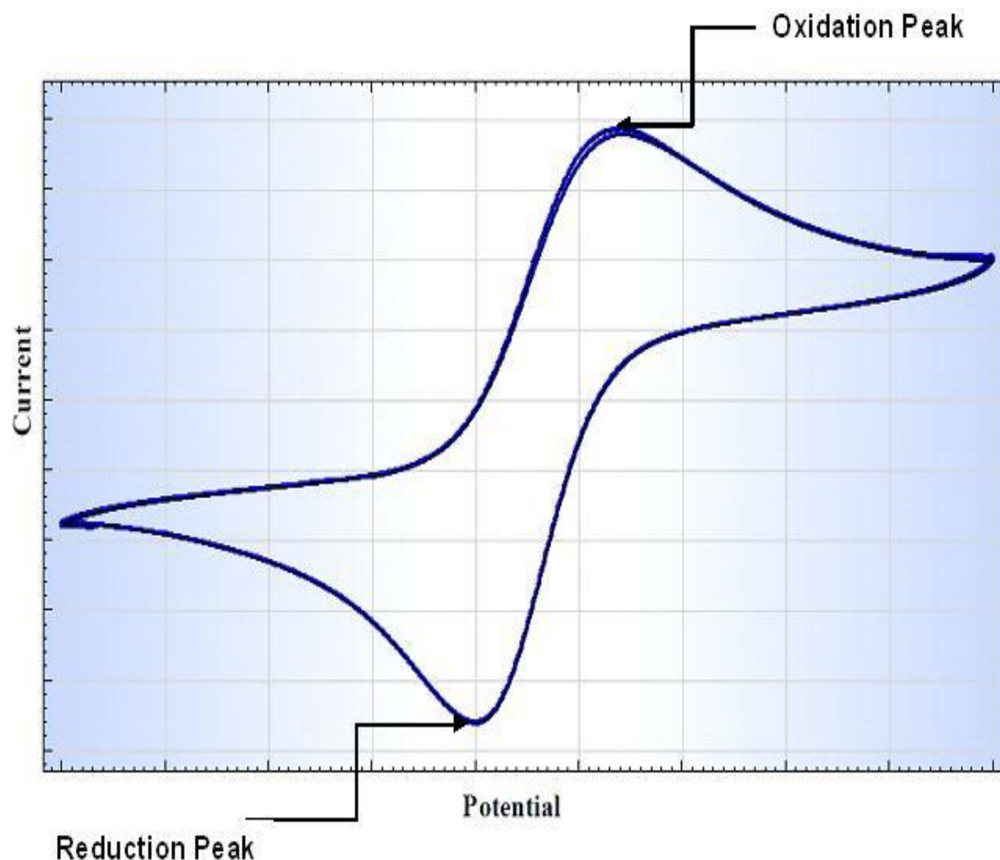


Fig.12: ***Cyclic voltammogram showing oxidation and reduction peaks***

The potential is applied between the reference electrode and the working electrode and the current is measured between the working electrode and the counter electrode. This data is then plotted as current (I) vs. potential (E). As the waveform shows, the forward scan produces a current peak for any analytes that can be reduced (or oxidized depending on the initial scan direction) through the range of the potential scanned. The current will increase as the potential reaches the reduction potential of the analyte, but then falls off as the concentration of the analyte is depleted close to the electrode surface. If the redox couple is reversible then when the applied potential is reversed, it will reach the potential that will reoxidize the product formed in the first reduction reaction, and produce a current of reverse polarity from the forward scan. This

oxidation peak will usually have a similar shape to the reduction peak. As a result, information about the redox potential and electrochemical reaction rates of the compounds are obtained. The CV experiment only samples a small portion of the solution, the material within the diffusion layer.

2.6.4. ELECTROCHEMICAL IMPEDANCE SPECTROSCOPY (EIS)

Electrochemical impedance spectroscopy (EIS) applied to the determination of the double-layer capacitance, to the characterization of electrode processes and complex interfaces, biomolecular interaction, and microstructural characterization. EIS studies the system response to the application of a periodic small amplitude ac signal. These measurements are carried out at different ac frequencies and, thus, the name impedance spectroscopy. Since an ac potential is applied to the cell, there will probably be a phase shift between the applied ac potential and the ac current response. Therefore, the impedance can be represented using a vector diagram displaying the in phase (Z') and out of phase (Z") impedances, the total impedance and the phase angle. Analysis of the system response contains information about the interface, its structure and reactions taking place there. Data obtained by EIS is expressed graphically in a Bode plot or a Nyquist plot. Often, EIS reveals information about the reaction mechanism of an electrochemical process: different reaction steps will dominate at certain frequencies, and the frequency response shown by EIS can help identify the rate limiting step.

The impedance spectrum, which includes a semicircle portion at higher frequencies, corresponds to the electron transfer limiting process and a linear part at the low frequencies resulting from diffusion limiting step of the electrochemical process. Diffusion can create impedance known as Warburg impedance. The ohmic resistance of the electrolyte solution (Rs)

and the Warburg impedance (Zw) represents the bulk properties of the electrolyte solution and diffusion features of the redox probe in solution, respectively. The diameter of the semicircle in the Nyquist plots represents the electron-transfer resistance of the layer, which can be used to describe the interface properties of the modified electrode.

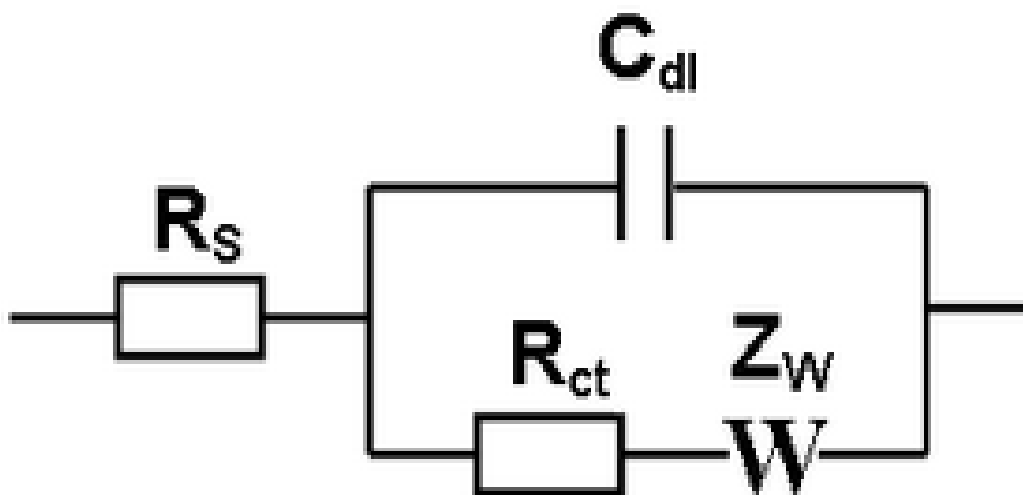


Fig.13: ***Schematic diagram of equivalent circuit for impedance spectroscopy in presence of redox couple: Rs, resistance of the electrolyte solution; Ret, electron-transfer resistance; Zw, Warburg impedance; Cd, double layer capacitance.***

All the impedance measurements were performed in the presence of a redox probe $[Fe(CN)_6]^{3-/4-}$ at the scanning frequencies from 0.1 to 100,000 Hz. The FRA analysis curve is shown in figure. From above Nyquist plot, one can determine the electron charge transfer resistance at the electrode surface. The X-axis shows the real part of impedance (electron charge transfer resistance) while the Y-axis shows the imaginary part of impedance (electron charge transfer resistance).

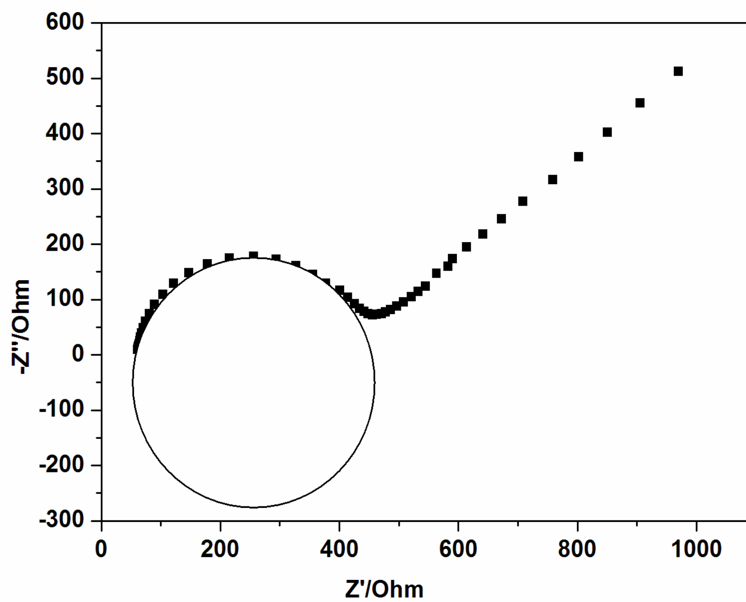


Fig.14: ***Frequency response analysis (FRA) curve of ITO electrode.***

(A) BODE PLOT

In impedance spectroscopy, a small sinusoidal voltage is placed on the sample over a wide frequency range, from 10^5 to 10^{-3} Hz. It is therefore and alternating current (AC) technique. The controlling computer system measures the magnitude of the current induced by the potential and in addition the phase angle between the potential and current maxima. A modified Ohms law is applied:

For DC conditions:-

$$V = i\ R$$

For AC conditions:-

$$V = i\ Z$$

where Z is the IMPEDANCE of the system.

From this modified Ohms Law for AC conditions the impedance can be calculated by setting the input potential and measuring the induced current. When the phase angle θ, between the voltage applied and the current induced is zero, then a pure resistance is present. When a phase angle of 90^0 is measured between the voltage and current at the same frequency, a pure capacitance is present. Angles between these values can mean a combination of a capacitor and resistor are present. It is therefore important to plot the impedance, usually the log of the impedance magnitude, as a function of frequency and in addition the phase angle as a function of frequency. These are known as Bode plots.

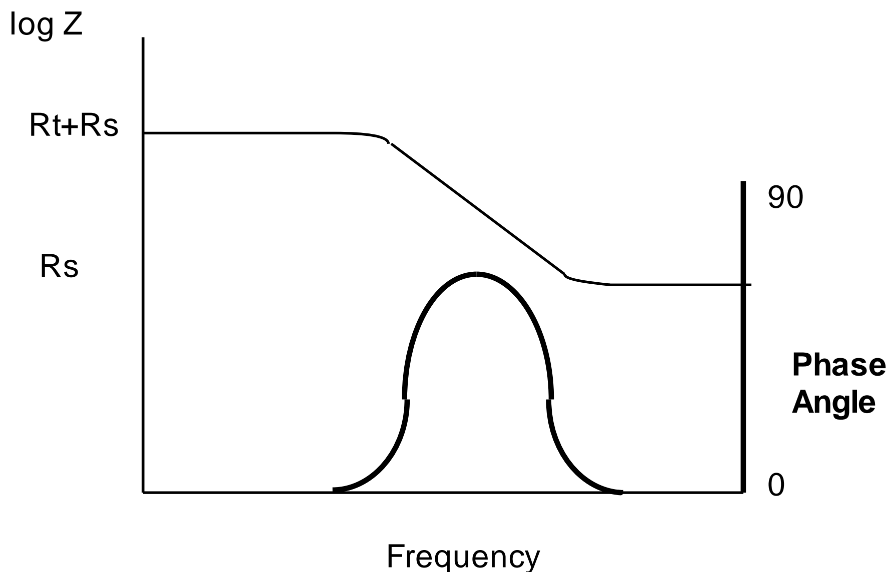


Fig.15: ***bode plot for a simple electrical circuit called a Randles circuit***

An example of a Bode plot is shown above for a simple electrical circuit called a Randles circuit. It consists of a resistor and a capacitor in parallel. This circuit simulates the type of data found for a simple corroding interface, such as steel in seawater. When the circuit has a maxima in the phase angle as shown in this case, it is caused by a combination of a resistance and a capacitor in parallel and is called an RC circuit. It also leads to models of electrochemical interfaces. One simple model is called the double layer model. In this the interface for a metal in electrolyte is envisioned as a layer of ions adjacent to the electrode surface with a further layer of solvated ions further away from the surface. The two layers of ions leads to a capacitor as they store charge, called the double layer capacitance, Cdl. It also leads to a resistor as charge leaks across the capacitor, called the charge transfer resistance, R_t. Therefore the models of electrochemical interfaces are represented as a capacitor and resistor in parallel. In series with this is a resistor representing the solution resistance, Rs. A typical interface is shown below along with its electrical analogs.

By using the electrical circuit analog approach, the data can be modeled to provide quantitative values for Rs, Cdl and most importantly Rt. Rt is proportional to corrosion resistance of the electrode. The higher this value the more resistant to corrosion.

CHAPTER-3

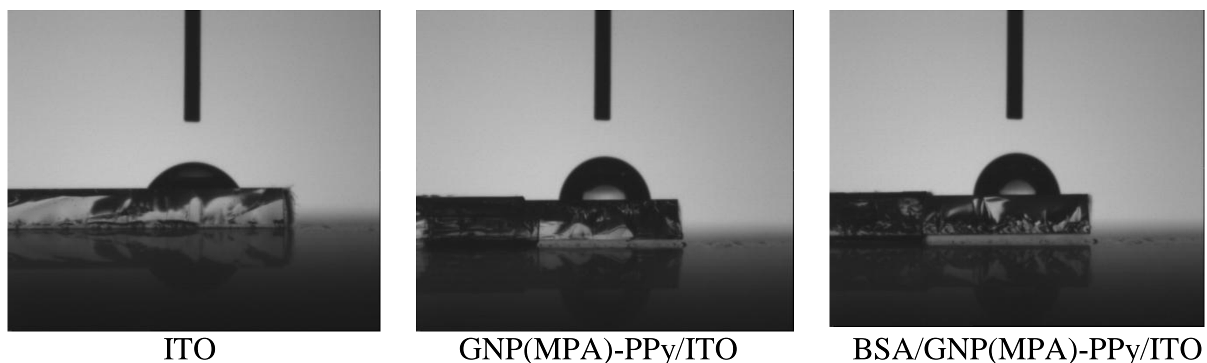
RESULT AND DISCUSSION

3.1. SURFACE CHARACTERIZATION OF THE BSA/ GNP(MPA)-PPy /ITO GLASS ELECTRODE

The surface morphology of the each modified step involved in the fabrication of GNPs modified electrode was characterized by using atomic force microscopy (AFM) images taken in a non contact mode and contact angle measurement.

3.1.1. CONTACT ANGLE

The contact angle measurement by sessile drop analysis method of all the deposited films and bare ITO glass electrode were taken as shown in figure .



ITO GNP(MPA)-PPy/ITO BSA/GNP(MPA)-PPy/ITO

Fig.16: ***Contact angle measurements before and after deposition of various films on ITO coated glass electrode***.

The drop image was stored and an image analysis system calculated the contact angle θ from the shape of the drop. Measurements were repeated on four drops at different regions as shown in table1. The hydrophilicity of the surface changes significantly with each step of surface modification.[47]

The initial low contact angle value of 40.38^0. obtained for bare ITO coated glass plate. This hydrophilic character of the surface is due to the presence of surface hydroxyl group**.**

However, after the formation of GNP(MPA)-PPy nanocomposite on the ITO glass plate, the contact angle was found to increase to 81.26^0 . This increase in water drop contact angle with respect to freshly cleaned ITO glass substrate is due to the hydrophobic nature of PPy. This on further subsequent treatment with BSA protein results in a significant increment in water contact angle to 91.08° due to the hydrophobic nature BSA protein.

Type of surface	Contact angle (Degree)
ITO	40.38^0
GNP-PPY/ITO	81.26^0
BSA/ GNP-PPY/ITO	91.08^0

TABLE 1: ***Contact angle values at various modification steps***

3.1.2. ATOMIC FORCE SPECTOSCOPY

The surface morphology of the each modified step involved in the fabrication of GNP(MPA) modified electrode was characterized by using atomic force microscopy (AFM) images taken in a non contact mode. The AFM images of GNP(MPA) modified GNP(MPA)-PPy/ITO surface indicate the well coverage of the surface by the GNP(MPA). However, the AFM images of BSA modified GNP(MPA)-PPy/ITO-glass exhibits an asymmetrical globular shaped surface with an increased roughness. This is indication of the immobilization of the BSA protein molecules well over the surface of GNP(MPA)PPy/ITO-glass.[47]

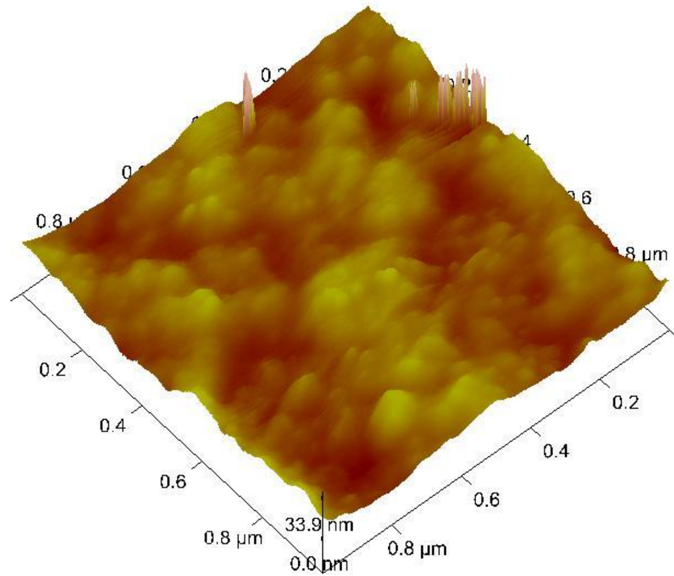


Fig17: *3D AFM images of GNP(MPA)-PPy/ITO glass electrode*

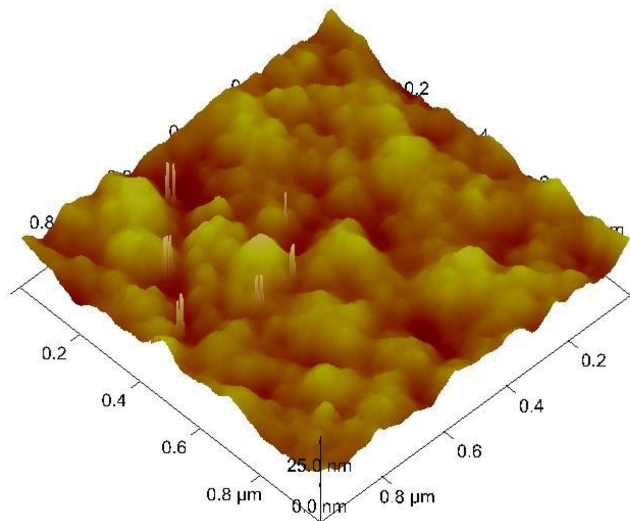


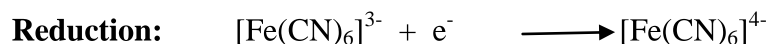
Fig18: *3D AFM images of BSA/GNP(MPA)-PPy/ITO glass electrode*

3.2 ELECTROCHEMICAL CHARACTERIZATION OF THE BSA/ GNP(MPA)-PPy /ITO GLASS ELECTRODE

The BSA/GNP(MPA)-PPy/ITO glass electrode was electrochemically characterized by cyclic voltammetric and frequency response analysis

3.2.1 CYCLIC VOLTAMMETRY

All the electrochemical measurements were performed in PBS of pH 7.4 containing 0.1 M KCl and 2 mM $[Fe(CN)_6]^{3-/4-}$. The $[Fe(CN)_6]^{3-/4-}$ redox probe was used as a marker to investigate the changes in electrode behavior after each surface modification step. Each step of surface modification of ITO-glass plate and protein immobilization were monitored by cyclic voltammetry.[47]



Reduction: $[Fe(CN)_6]^{3-} + e^- \longrightarrow [Fe(CN)_6]^{4-}$

Oxidation: $[Fe(CN)_6]^{4-} \longrightarrow [Fe(CN)_6]^{3-} + e^-$

The cyclic voltammograms (CV) of the modified electrode before and after the immobilization of the protein , BSA is shown in figure 19 . In all the CV experiments, 3rd cycle was considered as stable one, since no significant changes were observed in the subsequent cycles. PPy/ITO film shows a peak-to-peak potential (ΔE_p) of 139 mV and a peak-to-peak oxidation and reduction current (ΔI_p) of 0.185 mA. formation of GNP(MPA)-PPy nanocomposite film the peak-to-peak oxidation and reduction current (ΔI_p) is 0.246 mA and a potential difference (ΔE_p) is 113 mV can be found between the cathodic and anodic waves of the redox probe. This may be due to the

fact that the large surface area of the conducting GNPs of GNP(MPA)-PPy composite provides a fast exchange of electrons and thus indicating the formation of the GNP(MPA)-PPy surface

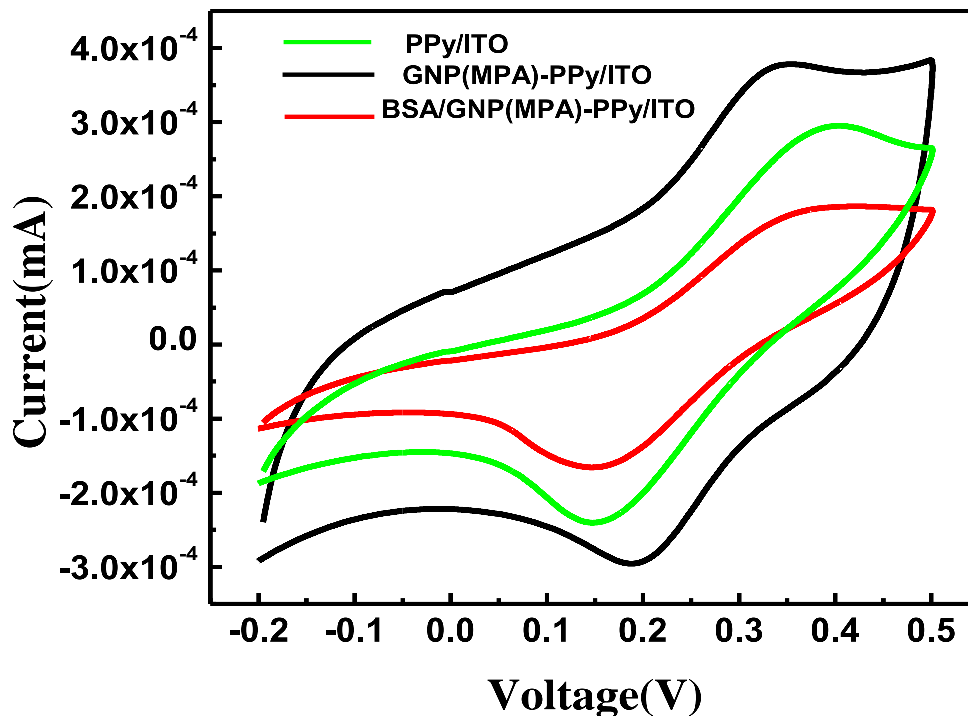


Fig.19: ***Cyclic voltammograms (CV) of (a) PPy/ITO, (b) GNP(MPA)-PPy/ITO electrode, and (c) BSA/GNP(MPA)-PPy/ITO electrode in 0.1 M KCl solution containing 2 mM $[Fe(CN)_6]^{3-}$; scan rate 25 mV/s; 3rd cycle voltammogram.***

The CV curve shows a further decline in the redox peak current (ΔI_p) of 0.026 mA and a large potential difference (ΔE_p) of 191 mV between the redox waves after the immobilization of protein molecules at the surface of the modified electrode.

This may be attributed to the formation of insulating layer of protein molecule, at the electrode surface, which perturbs the interfacial electron transfer, indicating an efficient covalent bonding of BSA.

3.2.2 ELECTROCHEMICAL IMPEDANCE SPECTROSCOPY

The step by step modification of the surface of bare ITO glass electrode to BSA/GNP(MPA)-PPy/ITO glass electrode was also characterized by electrochemical impedance spectroscopy. The semicircle diameter of the portion of the impedance spectrum gives the value of the charge transfer resistance of the layer which is used to describe the interface properties of the modified electrode.[48]

The electrochemical impedance spectra of the bare and the GNP (MPA)-PPy nanocomposite modified ITO glass electrode before and after the immobilization of the protein, BSA were obtained on a PGSTST302N, AUTOLAB instrument from Eco Chemie, Netherland. The charge transfer resistance (R_{et}) value of simple PPy/ITO film is 369 Ohm. After the formation of GNP(MPA)-PPy nanocomposite film on the ITO glass electrode the R_{et} value is 234 Ohm, which indicates an easy and fast exchange of electrons at the electrode surface interface. But immobilization of BSA on the GNP(MPA)-PPy/ITO glass electrode results in increase in R_{et} value to 679 Ohm. These results are in accordance with the pattern obtained in cyclic voltammetry measurements, clearly confirms the formation of the BSA/GNP(MPA)-PPy/ITO glass electrode.

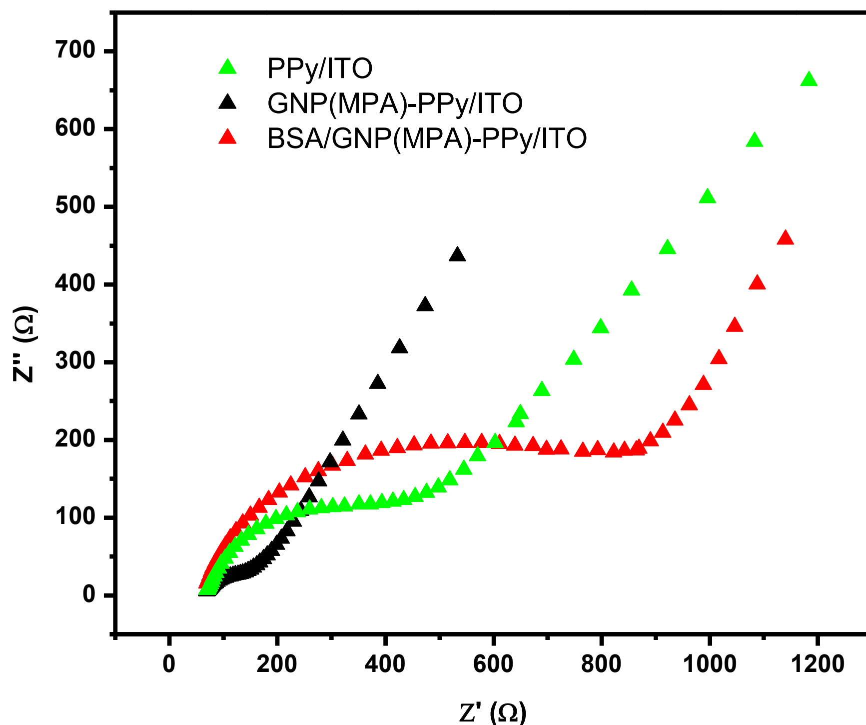


Fig.20:*Electrochemical impedance spectra of (a) PPy/ITO electrode, (b) GNP(MPA)-PPy/ITO electrode, and (c) BSA/GNP(MPA)-PPy/ITO electrode.*

The oxidation and reduction potential, peak to peak potential, peak to peak current and the corresponding electron-transfer resistance values are listed in table2.

Type of electrode	ΔE_P (mV)	ΔI_P (mA)	Charge Transfer resistance R_{et} (Ω)	Charge Transfer resistance R_{et} (KΩ-cm^2)
PPy/ITO	139	0.185	369	92.25
GNP-PPy/ITO	113	0.246	234	58.5
BSA/ GNP(MPA)-PPy/ITO	191	0.026	679	169.75

TABLE 2: ***CV peak potential difference (ΔEp) and charge transfer resistance before and after each step of ITO glass surface modifications and enzyme immobilization***

* Surface Area of ITO electrode = 0.25 cm^2

* ΔEp is peak to peak oxidation and reduction potential.

* ΔIp is peak to peak oxidation and reduction current.

(A)BODE PLOT:

The bode impedance plot of PPy/ITO exhibits a solution resistance (R_s) above 10 kH_z . The capacitive behavior is exhibited in the region from 50 H_z to 10 $kH_{z.}$ whereas the charge transfer resistance (Rc_t) behavior is dominant in the frequency range of 10 H_z to 50 H_z. The bode impedance plot of GNP(MPA)PPy/ITO exhibits a solution resistance (R_s) above 58 kH_z . The capacitive behavior is exhibited in the region from 28 H_z to 58 $kH_{z.}$ Below 100H_z the process occurring at electrode is mainly diffusion controlled. In the bode impedance plot of BSA/GNP(MPA)PPy/ITO, shows a solution resistance above 70 kH_z . A frequency region of 20 H_z to 70 $kH_{z.}$ belongs to capacitive behavior and 10 H_z to 20 H_z exhibited a charge transfer resistance behavior.

In the bode phase plot, phase angles of 5^0 and 10^0 was obtained for PPY/ITO and BSA/GNP(MPA)-PPy/ITO electrodes, respectively in low frequency region depicting a charge transfer behavior. While for GNP(MPA)-PPy/ITO, appearance of a phase shift of approximately 20^0 in the low frequency region is an indication of a diffusion control process.

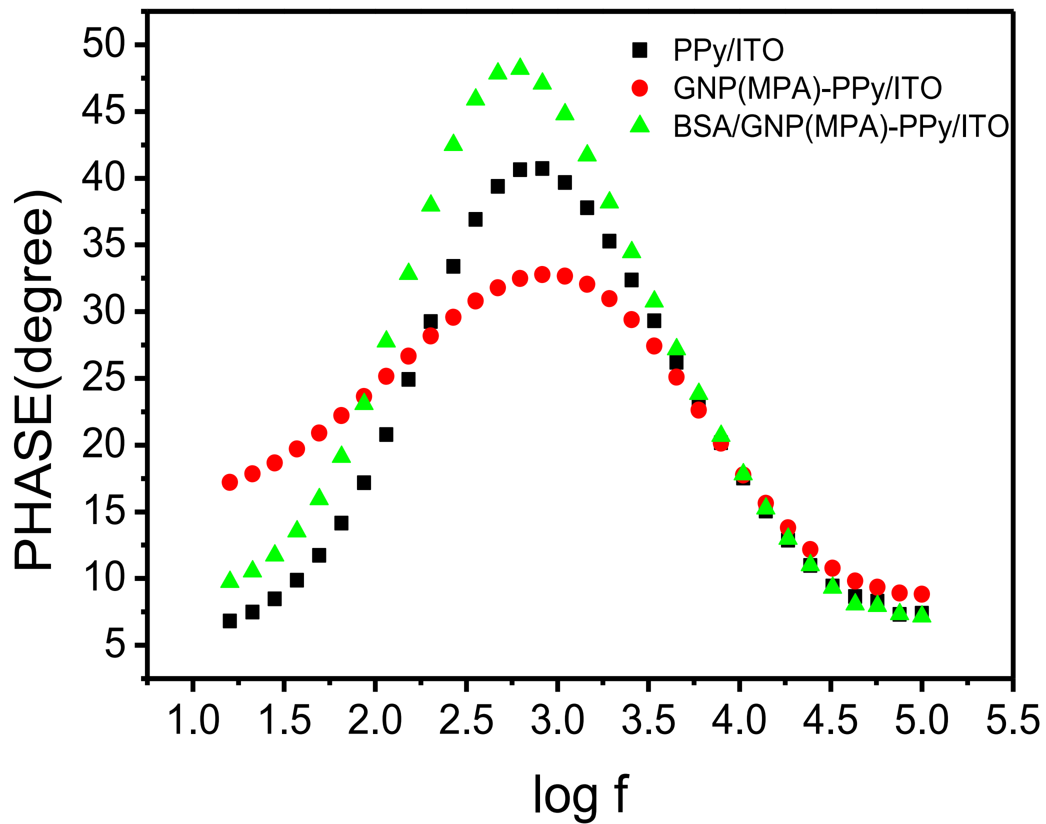


Fig.21. ***Bode plot of (a) PPy/ITO (b) GN(MPA)P-PPy/ITO (c) /BSA/GNP(MPA)-PPy/ITO electrode. This is between phase and log of frequency.***

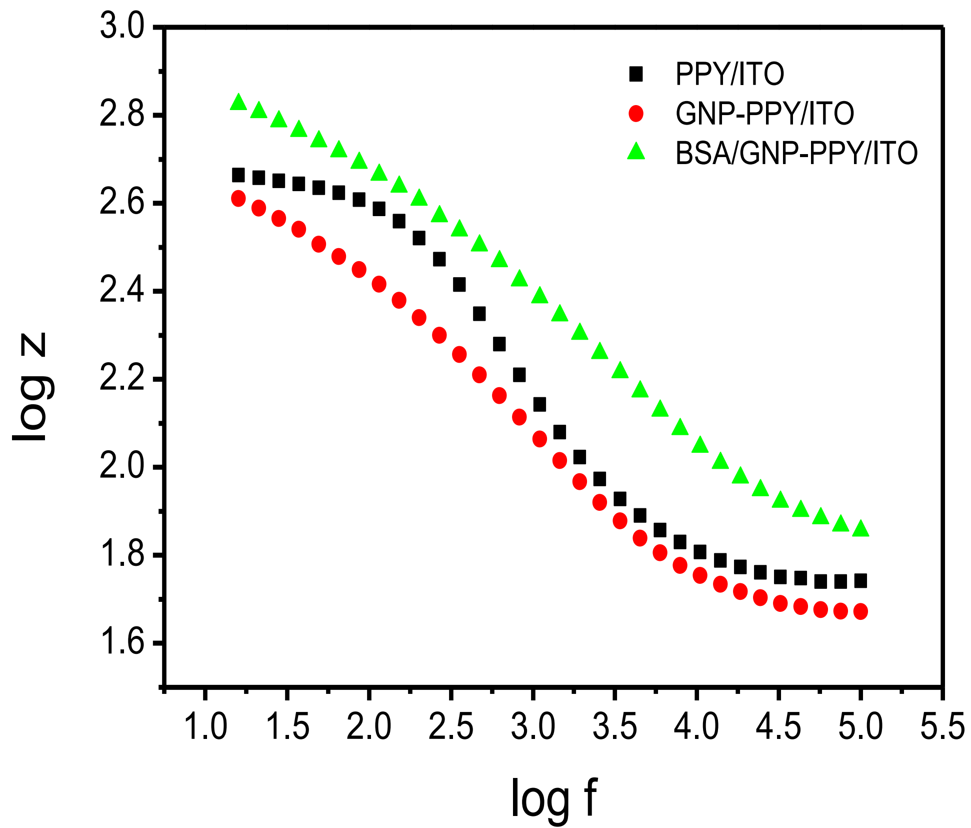


Fig.22: ***Bode plot of (a) PPy/ITO (b) GNP(MPA)-PPy/ITO (c) /BSA/GNP(MPA)-PPy/ITO electrode. This is between log of impedance and log of frequency.***

CHAPTER- 4

CONCLUSION

4.1. CONCLUSION

In this work, GNP(MPA)-PPy nanocomposite films were synthesized using a simple electrochemical polymerization technique. The GNP(MPA)-PPy nanocomposite prepared electrochemically shows an efficient covalent immobilization of BSA. GNPs provide a large surface area for immobilization of protein BSA and at the same time it provides high electrocatalytic activity to transfer electrons between BSA and electrode. The incorporation of gold nanoparticles into the polypyrrole film makes it more stable and conducting than simple polypyrrole film. The most important aspect of this electrode is its high protein loading over the polymer nanocomposite film.

Bioelectrode so prepared can be further use for biosensor application by immobilizing different antibody or enzymes for a particular detection of target molecule.

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