Synthesis of conducting biopolymer based biosensor for pollutants detection in environmental monitoring

to be submitted as Major Project in partial fulfilment of the requirement for the degree of

Master of Technology

In

Biomedical Engineering

Submitted by

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CERTIFICATE

This is to certify that the dissertation entitled **"Synthesis of conducting biopolymer based biosensor for pollution detection in environmental monitoring"** submitted by **SHREYA DUTTA (2K15/BME/10)** in the partial fulfilment of the requirements for the reward of the degree of Master of Technology, Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work carried out by her under my guidance. The information and data enclosed in this thesis is original and has not been submitted elsewhere for honouring of any other degree.

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I declare that my major project entitled **"Synthesis of conducting biopolymer based biosensor for pollution detection in environmental monitoring"** submitted to Department of Biotechnology, Delhi Technological University as a result of the work carried out by me at Biosensor Laboratory‖, Department of Biotechnology, as major project.

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ACKNOWLEDGEMENT

First and fore-most I bow down to the divine almighty for providing me inspiration, support and constant strength to achieve this milestone which can add meaning to my life.

I take this opportunity to express a deep sense of gratitude towards my guide Dr. Prakash Chandra, Department of Biotechnology, Delhi Technological University for providing excellent guidance, encouragement and inspiration throughout this investigation right from the imitation of work to the ship shaping of manuscript.

I heartily thank Prof. D. Kumar, Head, Department of Biotechnology, Delhi Technological University, for his kind support and help in providing all facilities in a timely manner.

I express my kind regards and gratitude to Prof. B. D. Malhotra, Department of Biotechnology, Delhi Technological University for his incessant help and allowing me to work in his laboratory.

I am highly indebted to Ms. Shine Augustine and Mr. Saurabh Kumar (Research Scholars) whose guidance and constant supervisison helped me to decide on my project work and complete it in time. I would also like to thank Mr. Chhail Bihari, Mr. Jitendra Singh and Mr. Mukesh Kumar for providing necessary chemicals and maintaining laboratory in good condition. I would also like to thank all my classmates for their valuable suggestions and helpful discussions

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ABBREBIATIONS

1. KH- Katira hydrogel
2. KG- Katira Gum
3. PANI- Polyaniline
4. APS- Ammonium persulphate
5. AAc- Acrylic Acid
6. ACN- Acetonitrile
7. DMSO- Dimethyl sulphoxide
8. ITO- Indium tin oxide
9. FTIR- Fourier transform infrared spectroscopy
10. SEM- Scanning electron microscope
11. TGA- Thermo gravimetric analysis
12. PBS- Phosphate Buffer Saline
13. NN-MBA- NN-methyl bisacrylamide
14. UV- Ultraviolet
15. CEP- Conducting electroactive polymers
16. IPN- Interpenetrating polymer network

Synthesis of conducting biopolymer based biosensor for pollutants detection in environmental monitoring

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1. Abstract

The increasing concentration of heavy metals in the water systems has been a major concern these days. A number of diseases have been reported, which is due to the bioaccumulation of these toxic elements in the human body. This thesis proposes a biocompatible, non-toxic method for the identification of these metal ions from the polluted aquatic samples. A conductometric urease biosensor, having Katira-cl-AAc/PANI hydrogel composite as the bio-sensing platform has been fabricated on the ITO electrode. The utilization of the natural polysaccharide, katira gum, has resulted in an enhanced conductivity along with the incorporation of the conducting polymer – polyaniline. Hydrogel provides greater surface area for the enzyme to react with the substrate, which has been used for the construction of the immobilization matrix. The hydrolysis of urea into carbon dioxide and ammonia are catalysed by the enzyme urease. The metallic inhibitors of urease are quantified in this work, using different electrochemical techniques. A comparative study of different concentrations of three heavy metal ions, cadmium, cobalt and mercury, has been done. The fabricated enzyme electrode, Urease/Katira-cl-AAc/PANI/ITO, exhibits greater stability, enhanced sensitivity and a wide range of detection limit (200 to 270 ng L^{-1}) with a shelf life of 28 days for both substrate and inhibitors.

Chapter 1

INTRODUCTION

2. Introduction

Heavy metals can be found in every ecosystem. The cause is due to different anthropogenic activities like agriculture and industrial wastes, leaching of soil etc. Toxicity due to the presence of these heavy metals has posed a negative effect on the aquatic flora and fauna. These enter the food chain through bio-magnification causing adverse effects on human health. Some metals are essential trace elements like copper, but in excess cause toxicity. Other metals like cadmium and mercury are fatal even in very low concentrations. Humans, when exposed to these metals for long duration can get affected by severe physical, neurological and muscular degeneration. Heavy metals can cause Alzheimer's disease, Parkinson's disease, muscular dystrophy, and multiple sclerosis. In recent years, many methods have been developed for the determination of the heavy metals in the waste water sources and in drinking water. Determination of heavy metals from the polluted water samples has been performed by many techniques like fluorescence spectrometry, voltammetry, mass spectrometry, potentiometry, etc., of which biosensors are gaining popularity due to its reusability, specificity, stability, in-situ analysis, fast response and cheap screening methods.

Various enzymes have been used for the detection of heavy metals. The interaction of the heavy metal ions with the enzyme or the enzyme-substrate complex plays a crucial role for the detection and estimation of the ions. The interaction can be competitive, non-competitive or uncompetitive. The utilization of urease enzyme for analyzing heavy metal toxicity has made it a sensitive and uncomplicated method for quantification. The strong inhibitory effect of the metal ions on the urease activity has been employed for the assessment of these pollutants. The hydrolytic breakdown of urea into ammonia and carbon dioxide by urease as a catalysis has been used as a base for the detection. The immobilization of urease has been done on a variety of matrices like metal nanoparticles, conducting polymers, metal oxides, polymeric gels, hydrogels, conducting papers, etc. The non-toxicity of the immobilization matrix is a very crucial factor when experimenting with different materials is taken into consideration. Considering this, a natural biopolymer based hydrogel has been used as a matrix for immobilization of urease enzyme in the fabrication of the biosensor. Katira gum, a biopolymer extracted from the plant *Cochlospermum religiosum* from the grooves of the bark. This is very commonly used in the in pharmaceuticals and food industries as an emulsifier, thickening agent, stabilizer and laxative. Katira gum is a hetero-polysaccharide having a water-swellable part, which in turn makes it a

desirable material for the formulation of hydrogel. Hydrogels have gained a large importance as an immobilization matrix in biosensors because of its different porosity and large swelling index, which is very crucial for the adsorption of the bio-recognition elements onto the surface. The three-dimensional (3D) structure of the hydrogel has a lot of similarity to the natural tissues and thus can mimic as an extracellular matrix for tissue growth and other biomedical applications like drug delivery, wound dressing, contact lenses, bone cement, catheters, tourniquets and biosensors. This application has led to their parallel development as biorecognition layer of amperometric, potentiometric, conductometric and fibre-optic based biosensors. The swelling of the hydrogels leads to increase in the surface area, hence providing a larger area for interaction. The conductivity of the hydrogel has been enhanced by the incorporation of emeraldine salt (conducting form of polyaniline). Inter-penetrating polymer network (IPN) of polyaniline and hydrogel has been formed by swelling the katira gum hydrogel into a solution containing aniline, an initiator and a cross-linker. A wide variety of natural polymers has been used for the synthesis of IPN composite hydrogels like chitosan, alginate, starch, cellulose, guar gum, etc. The final result of the IPN composite is an electroconductive hydrogel which conducts electricity due to the presence of the conducting polyaniline (PANI) doped with HCl.

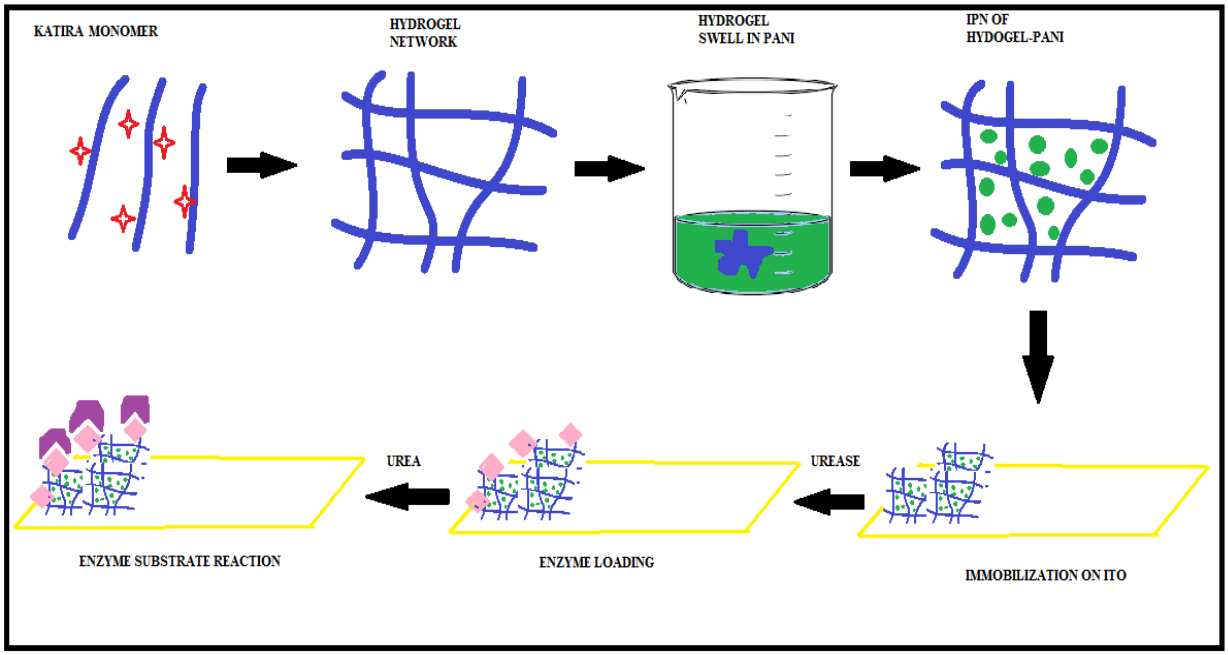

Figure 2.1 Fabrication step of Urease/KH/PANI-HCl/ITO electrode

In this study, indium-tin-oxide (ITO) electrodes have been used for the deposition of the Katira gum hydrogel (KH)/PANI-HCl by drop casting method. Urease has been

further immobilized onto the hydrogel composite ITO electrode. The electrode response studies in the presence of urea as an analyte has been achieved with the help of Electrochemical Analyzer. The magnitude of peak current obtained for the different electrodes PANI-HCl/ITO, KH/PANI-HCl/ITO and Urease/KH/PANI-HCl/ITO has been compared. The response of the biosensor was measured within 1 mM to 10 mM of urea concentration. The heavy metal ions irreversibly inhibit the activity of the urease, thus decreasing the magnitude of the current for the urease immobilized electrode. Different heavy metal ions in different concentrations have been used for the comparative study of the inhibitory effect on the enzyme. The salts of the heavy metals have been used for studying the catalytic effects of urease. The metal ions were evaluated to inhibit the activity of the urease in the following order: $Hg^{2+} > Cd^{2+} > Co^{2+}$.

Chapter 2

LITERATURE REVIEW

3. Literature review

3.1 Katira gum

Katira gum is a natural polysaccharide that is derived from the tree *Cochlospermum religiosum* and is commercially available in the market as gond katira. The tree is found all over India, mainly in the western Himalayan tracts, Orissa, West Bengal, Bihar, Jharkhand and the Deccan peninsula. The gum is produced as a result of physical injury or fungal attack or stress from the groves of the bark of the plant. This gum has been used for many years in pharmaceutical, cosmetic and food industries as stabilizing agents, emulsifiers and thickening agents. It is also used in cigar paste and is exported from India [1]. The gum is used for treating diarrhoea, dysentery, syphilis, pharyngitis, cough, trachoma and gonorrhoea, which utilizes the antipyretic, analgesic and sedative property of the gum [2].

The composition of the polysaccharide present in the katira gum is D-galactose, D-galactouronic acid and L-rhamnose in the ratio 2:1:3 respectively. It also has some traces of ketohexose. Physical appearance of the gum is yellowish-white pale colour which on immersing in water, imbibes water and swells into a transparent structure. The gum consists of two portions, one which is water-soluble which occupies 50-75% of the material and a water-swellable portion which occupies 25-50% of the material [3]. The solutions of katira gum are acidic, which ranges between pH 5-6, and is stable over a wide range of pH [4]. The biocompatibility, non-toxic nature and the potency for wound healing of this gum makes it a preferable choice for biomedical applications.

3.2 Hydrogels and its applications

Hydrogels are a novel class of materials which have the property to absorb a large amount of water or water-based fluids, swell and retain the solution to form hydrated interpenetrating polymer network in an aqueous medium. It has a three-dimensional elastic network of hydrophilic polymeric material with water filling the interstitial space. The swelling capacity of the hydrogel depends on the polymer backbone, the extent of cross-linking and the monomer components present in the hydrogel. Hydrogels have been used in the biomedical field since the early 50s [5]. Their unique soft elastomeric morphology, reduces the mechanical and frictional irritation to the tissue bed, their low interfacial tension decreases protein adsorption and hence bio-fouling and cell adhesion, and their swelling capacity leads to high permeability for low molecular weight drug molecules and metabolites [6]. In order to

make a hydrogel conductive, it is doped with conducting materials like- metals, metal-oxides, conducting polymers, etc. The property of such electroconductive hydrogels depends both on the concentration and the structure of the doped material that has been incorporated into the hydrogel matrix.

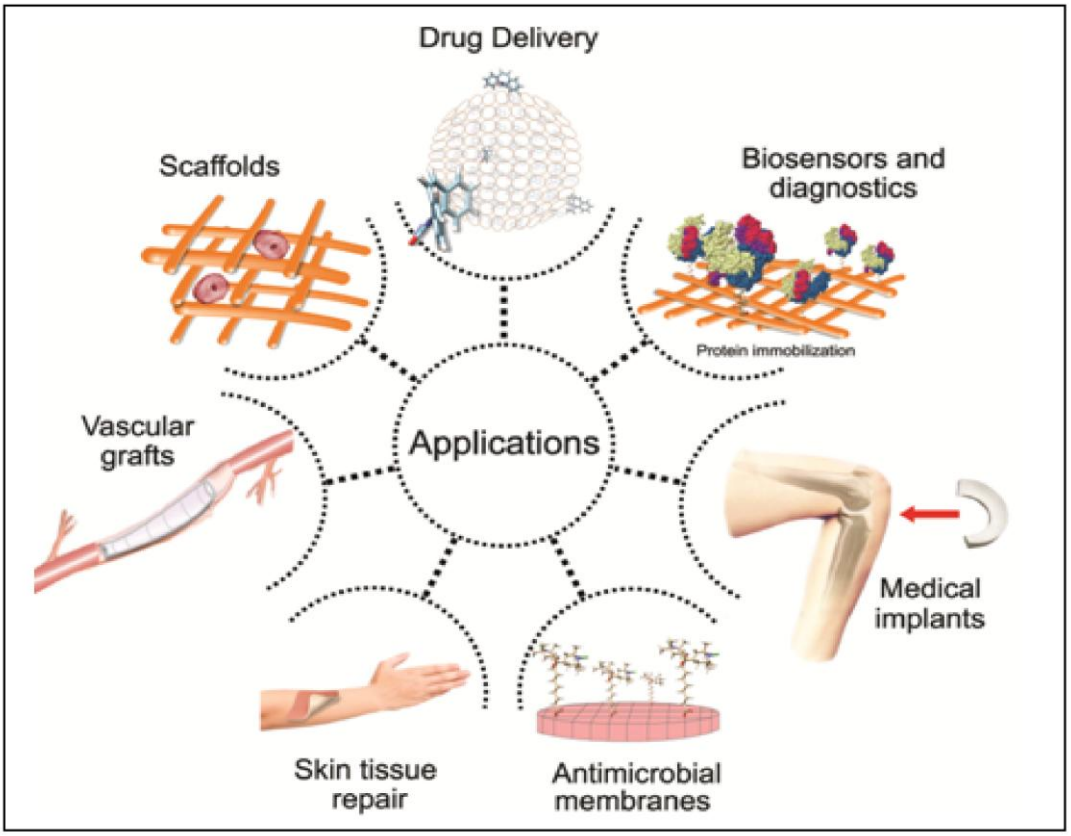

Figure 3.1 Different biomedical applications of hydrogels

Hydrogel finds its application in various fields. In biomedical applications, it is used as coatings for certain materials, as drug delivery devices, for wound dressing, as implantable devices, in tissue engineering (as scaffold for the growth of animal cells), as trans-dermal drug delivery, in contact lenses and in biosensors. In agriculture, it can be used as water storage granules, and for controlled release of pesticides. In industrial applications, it can be used in food industries, for electrophoresis, as corrosion inhibitor, in waste water treatment, in chromatography, and in cosmetic industries.

In this work, we have synthesized a hydrogel using katira gum which is a hetero-polysaccharide, made up of D-galactose, D-galacturonic acid, and L-rhamnose. Acrylic acid (AAc) has been added to the solution to increase the swelling property of the hydrogel. The hydrogel has been synthesized using ***N,N′***-

Methylenebis(acrylamide) (NN-MBA) as a cross-linker and ammonium persulfate (APS) as an initiator. The APS helps in generating free radicals for the polymerization process and produces sulphate anion free radicals (SO_4^{2-}), hence creates a reactive site on the polysaccharide. The sulphate anion in turn reacts with water to produce hydroxyl radicals (-OH^*). The hydroxyl radicals extract the hydrogen atom from the katira gum backbone, and hence produces radical sites for the attachment to the macromolecular backbone (KG^*). The hydrogen is abstracted from the carboxylic (–COOH) group of the galactouronic acid. During the chain propagation of the polymerization process, acrylic acid is homopolymerized to poly-acrylic acid and then is grafted to the katira gum backbone forming KG-*g*-polyAAc macro radicals. In the presence of cross-linker, these macro radical chains are linked with each other covalently to form hydrogel [7]. For initiation of the reaction, an external heat supply is required to increase the activation energy of the reaction. For this, the whole solution mixture is heated in water bath at 60°C. Further the swelling of the hydrogel in polyaniline solution results in the formation of interpenetrating polymer network. The lone pair present on the polyaniline helps in the conduction of the charge. The carboxylic group of the hydrogel plays a significant role in the binding of the enzyme onto the surface of the hydrogel composite.

Therefore, katira gum has a good film forming ability, large surface area, biocompatibility, non-toxic and fascinating electron transport ability due to the incorporation of polyaniline. Exploiting the properties of PANI, hybrid nanocomposite based electrode having thermal, electrical and tuneable mechanical properties can be prepared with a low production cost that can be further used for the construction of a biosensor.

3.3 Conducting electro-active polymers

Conducting electro-active polymers (CEPs) are a highly conjugated polymer family having p-bonding which is spatially extended that possesses distinctive electrical, electrochemical and optical properties [8]. Important applications of these properties are found in a variety of industrial process. Polymers like polypyrrole (PPy), polyaniline (PANI), polythiophene (PTh) and its derivatives, polyphenylene (PPP) and its copolymers and derivatives. Table 1 shows different CEPs listing their electrical conductivities and band gaps. They have wide applications in electrical conductors, nonlinear optical devices, light emitting diodes (LEDs), as photoresists, as

biosensors, as coatings for protection against corrosion, as antistatic coatings, as solar cells, as sensory elements, etc.

The properties of these CEPs vary with different conditions like temperature, pressure, pH, humidity, gas/ vapour concentration, medium ionic strength, etc. For many years CEPs have been incorporated into biosensors for measuring and detecting different analyte like enzyme substrates, DNA fragments, antibodies, whole cell microorganisms, drug metabolites, metal ions, etc. Biosensors having CEPs incorporated can be used as electrochemical, optical or gravimetric detecting devices for measuring different, very low concentration of samples and as continuous flow systems having quick response times, greater sensitivities, low detection ranges.

Since some of the conducting polymers are non flexible, so it limits its use as a device that can be used in vivo. To overcome this drawback, we have incorporated the conducting polymer (PANI) into hydrogel that is flexible. Though in our study we have used ITO electrodes which do not show any flexibility and elasticity, but if constructed in a flexible support like paper, the hydrogel composite with CEP can be easily coated and be used as a non-invasive biosensor. Apart from the highly beneficial properties of CEPs in biomedical field, there are some limitations. Amongst them are slow switching speeds in bio-electronic applications, the potential for unintended over oxidation leading to formation of reactive species, time-temperature drift of materials properties and questionable biocompatibility. These issues can be resolved to some extent by the incorporation of CEPs in hydrogels [9].

In our work, PANI has been incorporated into the katira gum hydrogel using oxidative polymerization of aniline monomers in acidic environment. Interfacial polymerization of PANI on hydrogel has been accomplished generating IPN of polyaniline incorporated hydrogel composite. There exists 3 forms of polyaniline namely- leucoemeraldine (yellow or colourless), emeraldine (green colour for emeraldine salt, and blue colour for emeraldine base) and per-nigraniline (blue or violet colour) [10]. Of the three forms, only the emeraldine salt is conductive and which is used in biosensors. Conducting polyaniline is formed under oxidative conditions in aqueous media, preferably, in the presence of acids.

S.No.	CONDUCTING POLYMER	d-d* GAP (eV)	CONDUCTIVITY (S/cm)
1.	Polyacetylene	1.5	10^3- $1.7x10^5$
2.	Polypyrrole	3.1	10^2 – $7.5x10^3$
3.	Polythiophene	2.06	10 - 10^3
4.	Polyphenylene	3.0	10^2 - 10^3
5.	Poly (p-phenylene vinylene)	2.5	$3x10^3$ – $5x10^3$
6.	Polyaniline	3.2	30-200
7.	Polyfuran	-	10^2
8.	Polyisothionaphthene	1.0	-
9.	Poly-4H-cyclo-pentadithiophene	1.2	-
10.	Polysquaraines	0.5	-
11.	Poly(thieno[3,4-b] thiophene	0.85	-
12.	Poly(2-dicyl thieno (3,4-thiophene-4,6-dicyl)	0.92	-
13.	Poly(1-alkyl-2,5-pyrrylene vinylene)	1.67	-
14.	Poly(2,2'-bithiophene)	1.32	10^{-1}
15.	Indole	1.26	$2x10^{-2}$
16.	Carbazole	1.30	10^{-3}
17.	Azulene	0.96	10^{-2}- 1
18.	Fluorence	1.82	10^{-4}
19.	Fluoranthene	1.68	10^{-5}-10^{-3}
20.	Triphenylene	1.83	10^{-4}
21.	Pyrene	1.33	10^{-1}- 1

Table 1- List of conducting polymers with their band gap and conductivity

3.4 Biosensors

Biosensors are devices that are used to detect biological elements with the help of a transducer that converts the physical signal into an electrical signal which can be conveyed to the detector for further processing. First biosensor was made in 1962, by scientist Leland C. Clark as an enzyme electrode. Since then, the work on biosensors has propagated across the globe very rapidly. Researches from different communities like physics, chemistry, material science, biology have shown their keen interest in developing different kinds of biosensors exploring the properties of a variety of materials. These sensing devices have a wide range of application in the field of medicine, biotechnology, agricultural, environmental as well as military and bioterrorism detection and prevention. Biosensor is a device that combines two elements, a biological element and a sensory element. Different types of biological elements are used which are specific for different analyte. The interaction between the biological element and the specific analyte produces a signal which is then converted into a measurable electrical signal with the help of a transducer. The bioelement is very analyte specific which makes the biosensor very sensitive towards a single analyte. It does not recognize any other analyte.

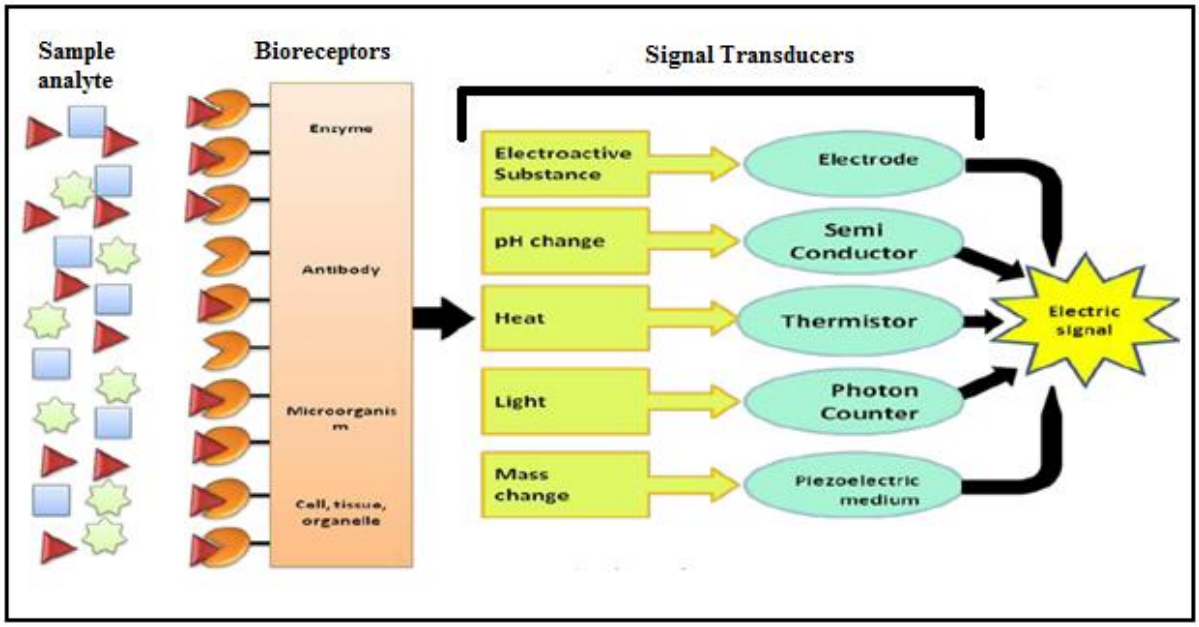

Figure 3.2 The basic structure of a biosensor

3.4.1. Components of biosensor

Biosensors are generally divided into three main components- (a) a biological recognition element such as enzyme, whole cells, antibody, nucleic acid etc for the identification of analyte known as bio-receptor, (b) an immobilization matrix like paper, polymer composites, nanomaterials, sol-gel films, hydrogels, self assembled monolayer, and LB films for the immobilization of a biomolecules and (c) a transducer to convert a signal from a biochemical reaction into a measurable and readable signal. The biosensor membrane comprises of the bioreceptor along with the transducer.

3.4.1.1 Bio-recognition element

The bio-recognition element or the bioreceptor is a biological molecule that has the capability of recognising substrates specific for that receptor. Many types of bioreceptors are used for the fabrication of a biosensor, the most common being enzymes, antibodies, whole cells, cellular fragments and nucleic acids (single stranded DNAs and RNAs). Suitable environment is required by these molecule for maintain their structural integrity and biorecognition activity. The reaction of the biomolecules with the analyte produces biochemical signals, which the transducer senses and converts it into a interpretable signal. Most widely used biomolecules for a biosensor are enzymes. But recently, antibodies and protein receptor molecules are being used progressively. These biorecognition elements should have the ability to recognize

biomarkers that are secreted in the body fluids such as saliva, urine, sweat, blood etc. The specificity greatly depends on the biomolecules that is specific for the receptor like the enzyme are specific for a particular substrate, and antibodies are specific for a particular type of antigen [11]. Receptor proteins, which are mostly membrane bound molecules, have specific affinity for enzymes, hormones, antibodies, micro-organisms and other biologically active compounds, are also used as a bioreceptor molecule.

3.4.1.2 Immobilization matrix

Immobilization matrix is a solid support onto which the biomolecules can get adsorbed or cross-linked. Immobilization is a process by which molecules are confined in any matrix, or membrane. The method of immobilization mainly depends on the mechanical and the chemical properties of the matrix. Many materials like glass, polymer films (conducting or non-conducting), screen printed electrodes, self assembled monolayers, nanoparticles, hydrogels, paper etc are used for immobilization of biomolecules. The characteristics of a favourable immobilizing matrix should be resistant to a wide range of temperature, ionic strength, pH and chemical composition [12].

Immobilization restricts the movement of the biomolecules to a definite space and makes it stable and reusable. There are many methods used for immobilization such as physical adsorption, physical entrapment, membrane confinement, covalent bonding and cross linking using different reagents. The method used for immobilization should be chosen in such a manner that there is very minimum loss of the biomolecules or there is no change in the active site or the reactive groups of that molecule which would hamper its binding affinity. The stability of the biocomponent in the matrices like gels, polymers, or pastes can be improved by entrapping the biomolecules and hence increasing the performance of it [13]. In case of enzyme immobilization, the enzyme should retain its catalytic activity as much as possible. In this study, hydrogel has been used as an immobilization matrix due to increasing in surface area after its swelling, which will allow the attachment of a huge amount of enzyme to the surface.

3.4.1.3 Transducer

Transducer is a component of the biosensor that converts one form of energy to another form. In context of the biosensors, it converts a biological signal received from a biochemical reaction into an electronic signal that is detected by the detector. There are various types of transducer based on the signals that it receives like optical, electrochemical, thermal or piezoelectric. Among the electrochemical transducer

aroused interest due to its simplicity, high signal-to-noise ratio, high sensitivity and fast response time. It is better than other transducer in a variety of way some of which including wide linear detection range, low detection limit, high sensitivity, low sampling amount, cheap, fast response, and good stability [12].

3.5 Electrochemical biosensors

According to the IUPAC definition, an electrochemical biosensor is a device, that can provide specific quantitative analytical information using a bioreceptor that can be preserved with the transducer [14]. Electrochemical biosensors are based on catalytic redox reaction that generates or expends electrons. The sensor substrate usually contains three electrodes; a reference electrode (RE), a working electrode (WE) and a counter electrode (CE). The reaction happening at the working electrode involves the target analyte, which is responsible for producing a current or a voltage. The current can be measured at a static potential or the potential can be measured at zero current. The electrochemical biosensors are further classified into conductometric, potentiometric and amperometric sensors.

3.5.1 Conductometric biosensor

These biosensors are based on change in conductivity of the medium when any substrate or analyte binds to the biorecognition element. The ionic species concentration changes during the reaction between the biomolecule and the analyte. This changes the conductivity, which is further decoded by the transducer.

3.5.2 Potentiometric biosensor

As the name suggests, a potentiometric biosensors measure the potential of an electrode in the electrolyte. When an equilibrium condition of zero current is achieved, the potential at the electrode is measured. Changes in pH and ionic concentration can be measured with the help of a potentiometric biosensor.

3.5.3 Amperometric biosensor

Amperometric biosensor depends on the transfer of electrons produced by an oxido-reductase enzyme at the working electrode. A specific potential is maintained at the working electrode with respect to the reference electrode. The electrochemical reaction proceeds at the electrode surface, generating a current which is related to the bulk concentration of the substrate.

3.6 Application of biosensors in environmental monitoring

The potential for environmental applications lies in the ability of biosensors to measure interaction of pollutants with biological systems through biomolecular

recognition capability. In the environmental area, pioneering work on an antibody-based biosensor for benzo[a]pyrene was done in the 1980s at the U.S. Department of Energy's Oak Ridge National Laboratory. Recently, biosensors for 2, 4, 6-trinitrotoluene (TNT) and RDX (hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine) have been used at the U.S. Naval Research Laboratory. Many biosensors are on the brink of commercialization, such as the continuous-flow immunosensor, which is expected to be licensed later this year. The continuous-flow immunosensor uses a clear plastic disposable immunoassay cartridge that is inserted into an analytical system; measurements are converted into database format with associated signal acquisition and analysis software. Biosensors are currently available for monitoring biochemical oxygen demand (BOD) and are in use at water treatment facilities in Europe and Japan. Other promising applications for environmental biosensors include groundwater monitoring, drinking water analysis, and the rapid analysis of extracts of soils and sediments at hazardous waste sites.

An assay optimized for flow cytometry with detection of genotoxic compounds in mind has been developed. They have applied the SOS– green fluorescent protein (GFP) whole-cell biosensor directly to soil microcosms. They have spiked the soil microcosms with a dilution-series of crude broth extract from the mitomycin C-producing streptomycete *Streptomyces caespitosus*. They reported hat thebiosensors extracted from these microcosms after 1 dayof incubation at 30 ?C were easily distinguished from extracts of non-contaminated soil particles using flow cytometry, and induction of the biosensor by mitomycin C was detectable at concentrations as low as 2.5 ng/g of soil [22].

Among the many possible combinations of biological recognition elements and transducers targeted to specific environmental compounds, certain combinations appear particularly promising in finding niche applications within the broad array of currently available field analytical methods. The measurement of phenolic and peroxide contaminants by using enzyme electrodes and the detection of small molecules by evanescent wave fiber-optic technology is two such applications. According to the Agency for Toxic Substances and Disease Registry, phenols and phenoxy acids are priority pollutants, based on the frequency of their occurrence at National Priority List sites, available toxicity data, and potential for human exposure . Phenolics are semi-volatile aromatic hydrocarbons frequently seen in waste streams and wastewater from coal, gas, and petroleum industries. EPA's Las Vegas National Exposure Research Laboratory is making research efforts to determine the

effectiveness of an enzyme biosensor to detect phenolic compounds in spiked and groundwater samples, using an enzyme based biosensor to monitor phenolic compounds in chromatographic effluents [23]. Simple and potentially portable liquid chromatography systems are used to separate phenols, followed by an enzyme electrode detector configuration. The biosensor is able to measure relative percentages of each phenolic compound present.

A microbial biosensor was developed for monitoring microbiologically influenced corrosion (MIC) of metallic materials in industrial systems [21]. They have immobilized the *Pseudomonas* sp. isolated from corroded metal surface on acetyl cellulose membrane. Respiratory activity of *Pseudomonas* sp. was estimated by measuring oxygen consumption. The biosensor was used for the measurement of sulphuric acid in a batch culture medium contaminated by microorganisms. They have reported the response time of 5 minute.

The Naval Research Laboratory has developed a fibre-optic biosensor and a continuous-flow immunosensor that can be used to measure explosives in discrete samples or monitor process streams. The fibre-optic system is based on a competitive immunoassay performed on the fibre core of a long optical fibre. The flow system is a displacement immunoassay with response measured by changes in the fluorescent signal in several minutes. Immunosensors such as these combine the advantages of conventional immunoassay methods with the option of obtaining real-time monitoring measurements with data integration capabilities. Laboratory confirmation is done with high performance liquid chromatography. The Oak Ridge National Laboratory has an ongoing biosensor research and development program within its centers for Manufacturing Technology. Among the biosensors being investigated are calorimetric micro-biosensors for DNA and acetylcholinesterase, a DNA biosensor microchip suitable for clinical and environmental use, and an antibody-based biosensor (immunosensor) for monitoring benzo[a] pyrene) and its DNA adducts.

3.7 Heavy metals

There are many definitions of heavy metals that have been proposed based on atomic weight, density, chemical properties or toxicity [15]. This category includes transition elements, lanthanides, actinides and some mettaloids. Heavy metals are found naturally in ecosystem in different concentrations.

There are 35 heavy elements that have toxic effects on the nature. Some of these are- bismuth, cerium, chromium, copper, cobalt, cadmium, arsenic, cesium,

mercury, manganese, nickel, lead, thallium, zinc etc. Some of these like copper, nickel,iron are essential for plants in very low amounts. Others like arsenic, cesium, cadmium are not essential as they do not play any physiochemical role in plants. When heavy metals are not metabolised in the body and starts accumulating in the tissues, they become toxic. Heavy metal toxicity can result in mental damage, impairment of the central nervous system and damage to the vital organs. Either natural means or human activities can cause the introduction of heavy metal in the ecosystem.

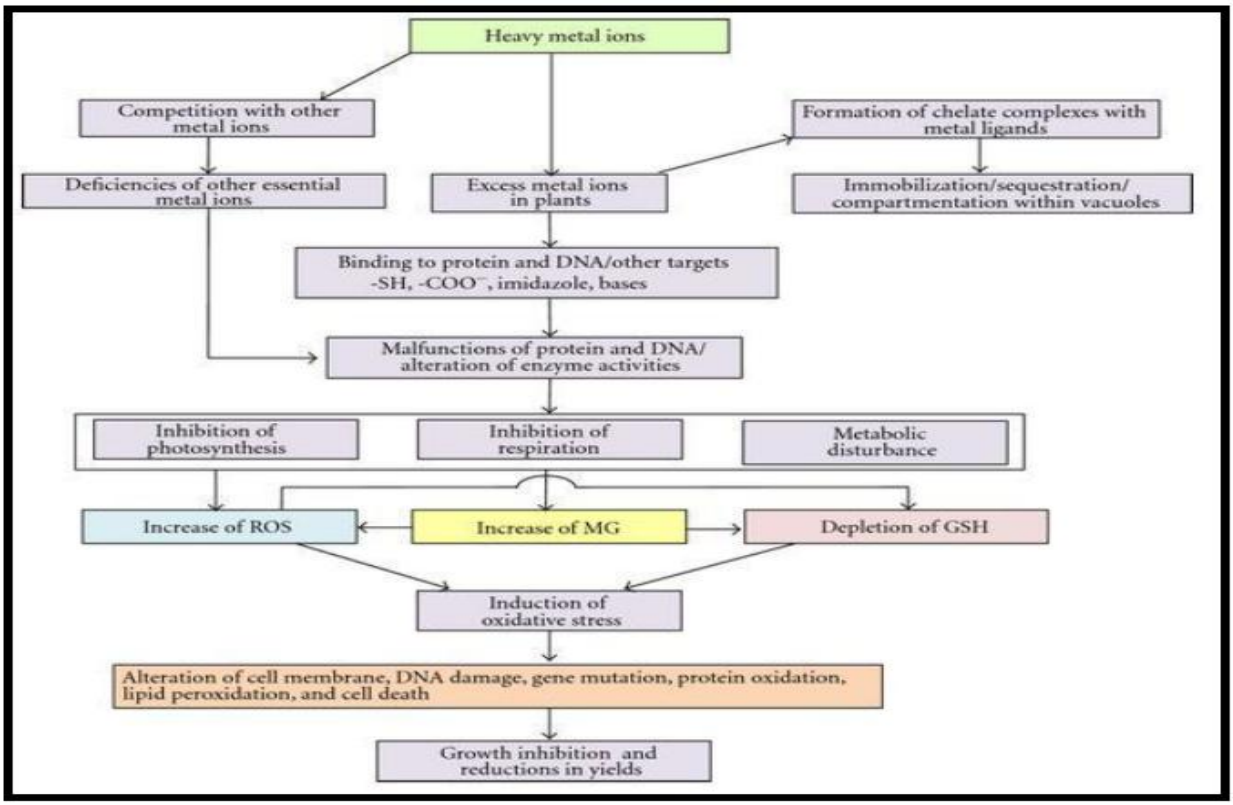

Figure 3.3 Mechanisms for toxicity of heavy metals

3.8 Conventional methods for detection of heavy metals

In recent years, a number of techniques have been developed for the detection of heavy metals from different sources of water. Because of the increasing demand for the detection of toxicity caused due to heavy metal poisoning, methods are developed which can detect them in very minimum amount of time i.e. having fast response. Listed below are some of the conventional methods which are used in sensing-

- **Atomic absorption spectroscopy –** This method has been used for detection of approximately 70 elements, quantitatively. The concentration of the element is measured by passing a specific wavelength of light through the sample. Hollow

cathode lamp is used as an energy source which is absorbed by the atoms. The concentration of the element is measured as the reduction in the intensity of light [16].

- **Flame atomic absorption spectrometer –** It determines the concentration of the elements in parts per million (ppm). It has air-acetylene or nitrous oxide flame atomizer. Aerosol samples are presented to the atomizer. This has a very quick response time of 10-15 seconds. This technique has been used in the determination of arsenic, zirconium, cadmium, lead, zinc and copper [17].
- **Inductively coupled plasma mass-spectrometry –** An argon plasma source is used for the dissociation of the sample into different components. The mass spectrometer receives the ions released from the plasma. The ions travel in different paths according to their mass-to-charge ratio which is detected by a magnetic analyser or a quadrupole. This technique has been used for the elemental analysis of sea water samples having high saline matrix. This normally detects in part per trillion (ppt) ranges [18].
- **X-Ray fluorescence –** As the name suggests, this involves the interaction of the metals with x-rays. The orbital electrons are dislodged when the high energy x-rays strikes the atom. The unoccupied spaces of the unstable lower orbital are filled by the electrons from the outer shells. In this process fluorescence (non-radiant energy) is emitted which is detected by the detector. This process has been used to detect different heavy metals from industrial complex samples [19].
- **Neutron activation analysis –** In this radioactive isotopes are generated when the sample is exposed to neutrons. These isotopes decay and emit gamma rays with characteristic energy. The concentrations of the elements are measured quantitatively by determining the intensities of the gamma rays emitted. It was used to determine heavy metals in green tiger shrimp and blue crab from the Persian Gulf.
- **Anodic stripping voltametry –** This is a technique that leads to the accumulation of reduced analyte on the surface of the electrode when reducing potential is applied. Then an equal amount of current is generated proportional to the amount of analyte deposited onto the electrode, when an oxidizing potential sweep is applied to the electrode. Mercury is generally used as an electrode for the detection of elements. These are very sensitive, low manufacturing price, and are consistent [20].

Chapter 3

MATERIALS AND METHODS

4.1. Materials

Reagents

1. Katira gum
2. Acrylic acid (AAc)
3. ***N,N′***-Methylenebis(acrylamide) (NN-MBA)
4. Ammonium persulfate (APS)
5. Aniline
6. 1,2-dichloromethane
7. Concentrated Hydrochloric acid (HCl)
8. Glycerol
9. Sodium hydroxide (NaOH)
10. Dimethyl sulfoxide (DMSO)
11. Acetonitrile (ACN)
12. Potassium ferrocyanide ($K_4Fe(CN)_6$)
13. Potassium ferricyanide ($K_3Fe(CN)_6$)
14. Phosphate Buffer Saline (PBS, pH 7.0)
15. Monobasic sodium phosphate
16. Dibasic sodium phosphate
17. Sodium chloride
18. Ethanol
19. Deionised water (H_2O)
20. Urease
21. Urea (NH_2CONH_2)
22. Cobalt (II) chloride ($CoCl_2.6H_2O$)
23. Mercuric chloride ($HgCl_2$)
24. Cadmium chloride ($CdCl_2.H_2O$)
25. Ammonia (NH_3)
26. Hydrogen peroxide (H_2O_2)
27. Potassium bromide (KBr)
28. Methanol (CH_3OH)
29. Acetone (CH_3COCH_3)

Apparatus

1. Beakers (50ml)
2. Conical flasks (50ml)
3. Glass rod
4. Petri plates
5. Measuring cylinders (10ml,50ml)
6. Eppendorf tube
7. Glass pipettes
8. Pestle and mortar
9. Pipettes
10. Spatula
11. Aluminium foil
12. Tissue roll
13. Whatman filter paper 1

Instruments

1. Water bath
2. Magnetic stirrer
3. Hot air oven
4. Weighing balance
5. Sonicator bath
6. Electrochemical analyzer
7. 4-probe system
8. 3 electrode system
9. Electrophoretic depositor
10. Spectrophotometer
11. Heating plate
12. Autoclave
13. Hydraulic press

4.2. Methodologies

4.2.1. Synthesis of hydrogel

1. 6% w/v of katira gum had been dissolved in Millipore water.
2. The mixture had been stirred with the help of a magnetic stirrer at 350rpm at room temperature till a homogenous solution was obtained.
3. Acrylic acid (0.874 M) had been added to the solution with continuous stirring.
4. NN-MBA (in varying concentration, of which 0.0389 M was chosen) had been further added to the solution with continuous stirring.
5. A freshly made solution of APS (in varying concentration, of which 0.0263 M was chosen) in Millipore water had been added to the above solution drop-wise with stirring until homogenous mixture was obtained.
6. After dissolving the mixture completely, it was kept in water bath at 65°C for 2hrs.
7. The cross-linked hydrogel had been stirred in distilled water to remove soluble fractions.
8. The hydrogel had been kept in hot air oven at 60°C and was dried until a constant weight was obtained.
9. Half of the dried hydrogel was then grinded into powder using mortar pestle and used for characterization. The other half was used for checking the swelling pattern of the hydrogel.

4.2.2. Incorporation of polyaniline in the hydrogel

1. Homogenous solution A of aniline in 20ml of distilled water had been made in a beaker. The concentration of aniline was varied from 0.1ml to 5ml.
2. The katira hydrogel was immersed in the aniline solution and was stirred for 15 to 20 minutes.
3. Another solution B had been made by adding 1.8 ml of hydrochloric acid and 0.45gms of APS (initiator) in 20ml of distilled water in a beaker.
4. The hydrogel was withdrawn from the aniline solution A and was immersed in the second beaker.
5. The hydrogel was kept undisturbed in solution B for 15- 20 minutes.

6. Change of colour of the hydrogel was observed. A colour change from brown to dark green showed the incorporation of the polyaniline in the hydrogel and the formation of emeraldine salt.
7. The hydrogel was then washed with methanol to remove the unreacted monomers.
8. After washing, the hydrogel was dried such that constant weight was obtained.
9. The dried hydrogel was grinded using mortar pestle and stored in an air tight container to avoid the incorporation of moisture.

4.2.3. Conducting swelling studies on hydrogel

1. The weight of the hydrogel was measured using a weighing balance and was noted down.
2. Hydrogel was immersed in 20ml dH_2O in a beaker and was left for 24 hrs. This step ensured that any unreacted impurities present were dissolved.
3. After 24 hrs the hydrogel was again dried until a constant weight was obtained. The weight was noted down as dry weight.
4. In a beaker, 20ml of PBS (pH 7.0) was taken and the hydrogel was immersed in it.
5. After every 10 minutes the hydrogel was taken out, patted dry on a blotting paper and weighed. This process was carried out for 1 hr.
6. After 1 hr, the same procedure of measuring was carried out by taking 20 mins, 45 mins and 1 hr difference. And the consecutive weight was noted down.
7. This process was carried out until a constant weight was obtained.
8. The swelling index of the hydrogel was calculated.
9. The hydrogel was dried and stored for further use.

4.2.4. Hydrolysation of ITO

1. A solution of NH_3:H_2O_2:H_2O had been taken in a ratio of 1:1:5.
2. The ITO slides were placed in this solution with their conductive sides facing upwards.
3. The slides were kept in this solution for an hour.
4. The slides were then washed with distilled water for 2-3 times.

5. Then it was followed by ethanol washing, dried and kept until further used.

4.2.5. Fabrication of biosensing platform

1. The powdered conducting hydrogel was dispersed in DMSO solution in 1mg/ml concentration.
2. This solution was then sonicated for 25-30 minutes in the sonicator bath to achieve proper dispersion.
3. 20µl of the solution was pipette out and was drop cast onto the hydrolysed ITO and was allowed to dry completely.
4. This electrode was stored in 4°C until further used.

4.2.6. Immobilization of enzyme

1. Different concentration of urease (0.5mg/ml, 1mg/ml and 2mg/ml) were made in PBS pH 7.
2. The electrodes were first dipped in 2.5% gluteraldehyde solution for 90 minutes.
3. The electrodes were then dried at room temperature.
4. 10µl of urease solution was drop cast over the functionalised electrode and was dried and stored in 4°C for 24 hrs.

4.2.7. Detection of heavy metals present

1. Voltammetry method was used for the detection of heavy metals like cobalt, cadmium and mercury in the solution.
2. The electrodes (1mg/ml) were first incubated in urea solution. 5mM concentration of urea was standardised for the further studies.
3. The oxidation peak current was noted in the cyclic voltammetry in the presence of the enzyme substrate reaction.
4. Varying concentration of heavy metals from 0.1mM to 5mM was used to see the inhibition effect of these metal ions on the enzyme electrode.

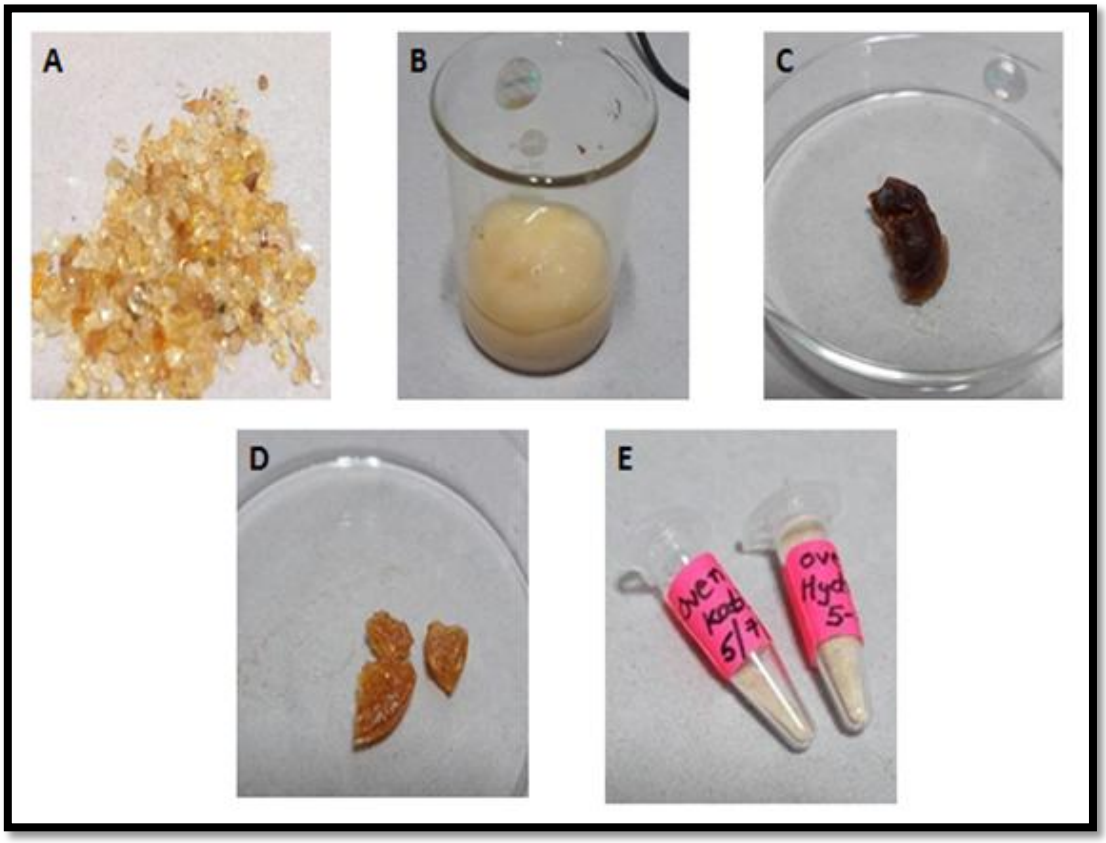

Figure 4.1. (A) Raw Katira gum, (B) Katira hydrogel, (C) Oven dried hydrogel, (D) Air dried hydrogel and (E) Powdered hydrogel

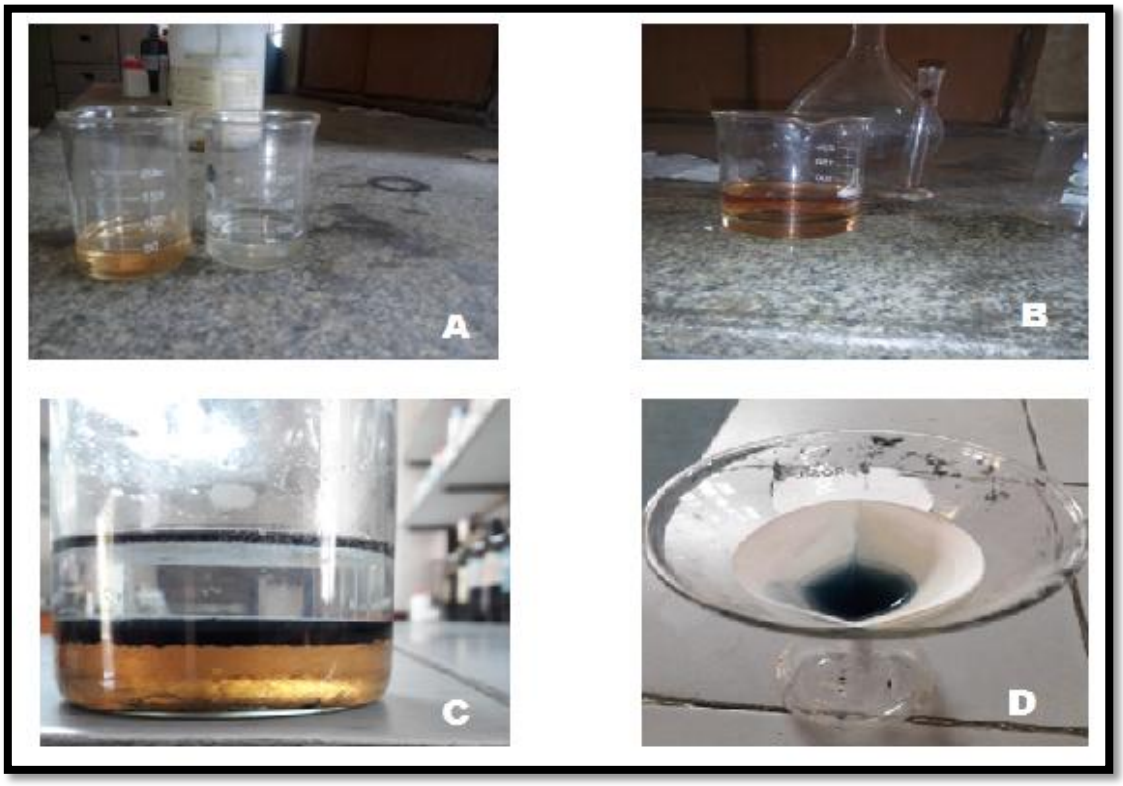

Figure 4.2. (A) Aniline and 1,2-dichloromethane solution; oxidizing solution, (B) Mixture of both the solutions, (C) Formation of emeraldine salt in the interface, (D) Filteration of polyaniline using whatman filter paper

Figure 4.3- Electrophoretic deposition of PANI films using two electrode system on ITO

4.3. Characterization

The morphology and the structure of the synthesized product was obtained by using scanning electron microscopy (SEM) [EVO 18 Special Edision]. The functional groups and bonds present in PANI, PANI/Katira gum hydrogel and hydrogel were investigated through Fourier transform infrared spectroscopy (FTIR) [Thermo Scientific, Nicolet 380]. The Autolab Potentiostat (Netherlands) was used for the electrochemical response studies using a three-electrode system. The fabricated electrode was used as working electrode, Platinum as reference electrode and Ag/AgCl as the counter electrode. The decay of the material with respect to the temperature was carried out using Thermo Gravimetric analysis. The conductivity of the synthesised PANI pellet was measured using the four-probe system. The compound quantitative analysis was done with UV-Vis Spectrophotometer [Perkin Elmer].

4.3.1. Scanning electron microscopy (SEM)

These are instruments which are used for the examination of objects on a very fine scale with the help of highly energetic electron beams. The surface morphology (shape and size of the particles making up the object), topography (surface density of the particles), the crystallographic composition (the arrangement and orientation of the particles or atoms) and the composition (the elements and the compounds that constitute the object and their relative ratios) can be examined using this technique. A two dimensional image is generated displaying all the spatial variations of these properties. The conventional scanning microscope can take images of areas ranging from 1 to 5 microns width with magnifications ranging from 20X to 30KX, and spatial resolution of 50 to 100 nm. It can analyze the sample at selected point

locations, which can be used for the quantitative or semi-quantitative analysis of chemical compositions.

The accelerated electron beams having kinetic energy are incident on the sample, which is mounted on the platform after the gold coating from the sputter coater. These produce signals which include secondary, diffracted, backscattered electrons, photons, visible light and heat which are used for imaging samples. The secondary electrons are generally used for showing the topography and the morphology of the samples. The compositions in multiphase samples are analyzed using the backscattered electrons. X-rays is produced by the collision of the electrons in the orbitals to the incident electrons which have discrete pattern for each element in the compound. This x-ray beam is focused onto the sample using a magnetic lens. The specimen is scanned by the electron beam in a series of lines and frames called raster. There are detectors that collect the scattered electrons producing a final image. These generated x-rays are non-destructive, so can be used for analysis. The figure 4.1 shows the components of the scanning electron microscope. The display is a histogram of the x-ray energy that is received by the detector, having individual peaks, the intensity of which is equivalent to the amount of a particular element present in the analyzed specimen.

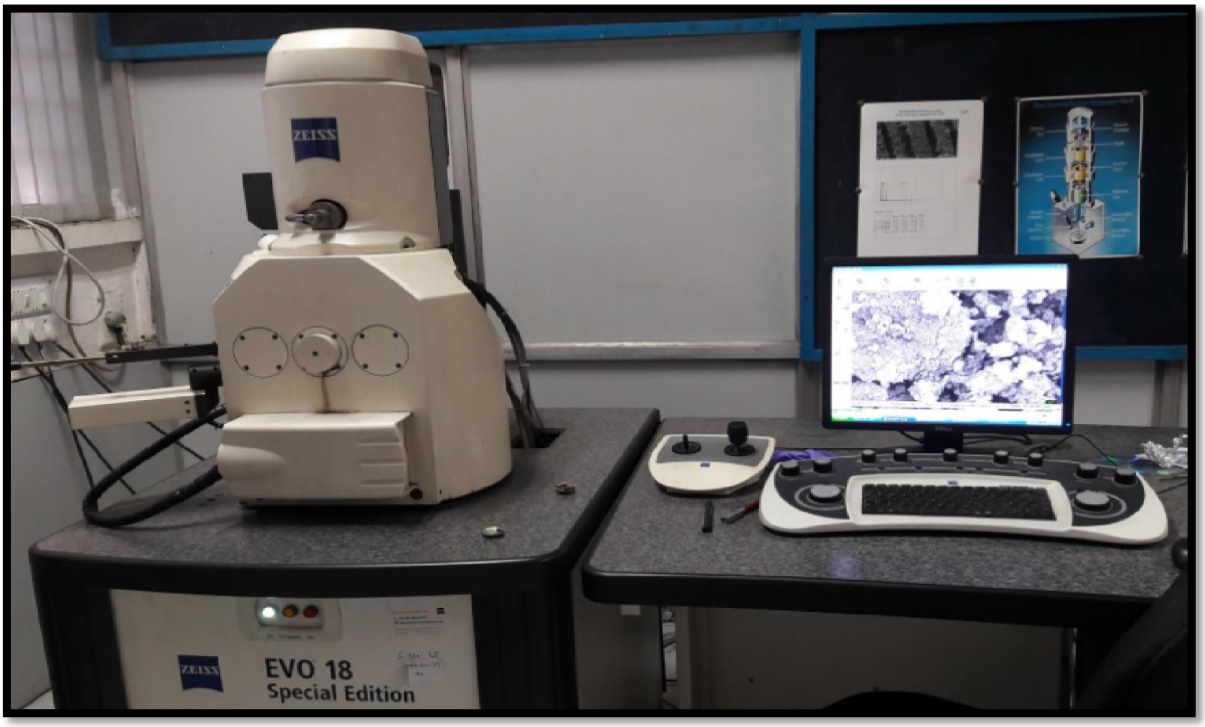

Figure 4.4- Scanning electron microscope

4.3.2. Fourier transform infrared spectroscopy (FTIR)

The Fourier transform infrared spectroscopy is an analytical technique which is used for obtaining an infrared spectrum of absorption for any material (solid or liquid). The infrared bands correspond to the functional groups of the material. In this technique the powdered samples are mixed with KBr (an IR-transparent material) to form a pellet, which is then mounted on the platform of the instrument. This material is irradiated with infrared radiation. The radiation gets absorbed in the material which then excites the molecules into a higher energy state. Each molecular structure absorbs characteristic wavelength which corresponds to the functional groups present in that structure.

The spectrometer uses an interferometer which modulated the wavelength from a broad IR-source. The intensity of the reflected light is measured by the detector as a function of its wavelength. The signal is obtained as an interferogram from the detector, which is analyzed using Fourier transforms in a computer system. A single beam infrared spectrum of the signal is obtained after analysis. The graph is plotted between intensity vs. frequency (in cm^{-1}, reciprocal of wavelength) to obtain the FTIR spectra of the material.

Figure 4.5- Fourier transform infrared spectroscopy (FTIR) instrument

4.3.3. Electrochemical Techniques

These are changes in the electrical signals due to the electrochemical reactions on the surface of the electrode, usually as a result of an imposed potential or current. The electrochemical analyzer has a three-electrode cell setup. The current flows between the counter electrode (CE) and the working electrode (WE). There is reference electrode made up of platinum electrode, which is kept at close proximity with the working electrode. The WE and CE potentials are not measured.

Differential pulse voltametry (DPV) is widely as a technique for the electrochemical studies. A potential pulse in series of fixed amplitude (10-100Mv) is superimposed on a base potential which slowly changes. 40-50 ms is the time interval for each potential step in this series. There are two time points when the current is measured in the pulse- before the starting and the ending of the pulse. The graph is then plotted as the difference of these two currents (δi) vs. the base potential. The differential pulse voltammogram is the plot δi vs. V, which consist of the current peaks which correspond to the analyte concentration.

Cyclic voltammetry (CV) is also an electrochemical technique that is used for the measurement of current that develops across the electrodes, where the voltage is predicted by the Nernst equation. The reference electrode is maintained at a constant potential and the potential of the working electrode is measured. An excitation signal is produced due to the applied potential. The slope of the excitation signal gives the scan rate.

Figure 4.6- Autolab Potentiostat/Galvanostat, EcoChemie, Netherlands

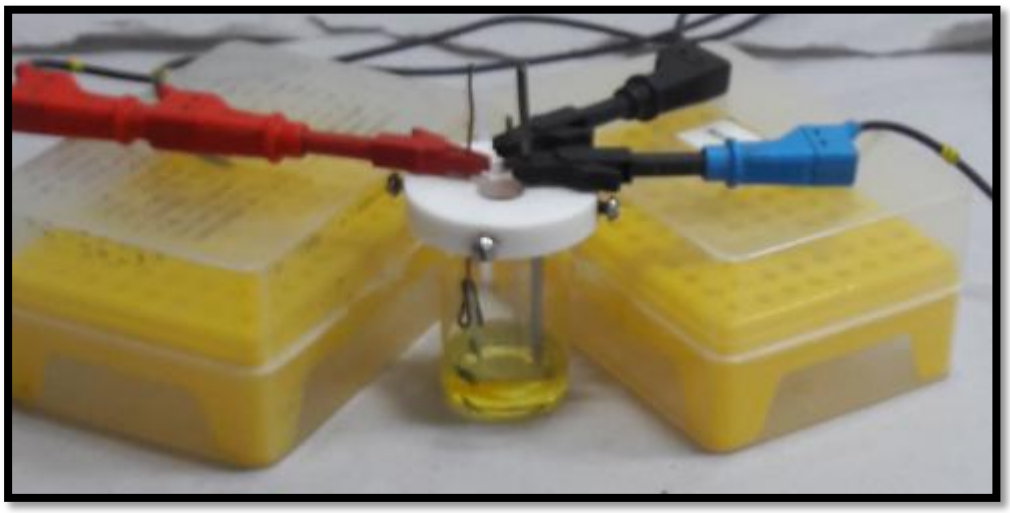

Figure 4.7- Three electrode assembly of Autolab Potentiostat, Electrochemical Analysis of Urease/katira-cl-AAc/PANI/ITO electrode

4.3.4. UV-Vis Spectrophotometer

This is the measurement of the attenuated light beam passing through the sample. The absorption can measured over a single wavelength or can be measured over a spectral range. This technique is very useful for the detection of functional groups, impurities, qualitative and quantitative analysis, any single compound without chromophore, drugs with chromophoretic reagents etc.

The UV absorption spectra develop by the transition of electron from the lower orbital to the higher energy orbital. This transition energy is calculated by the equation-

$$E_1 - E_0 = h\eta$$

When a sample is exposed to the light energy that matches the difference between a possible electronic transition within the molecule, a fraction of energy is absorbed by the molecules and the transition from the lower to higher energy state occurs. This absorption spectrometer records this degree of absorption at different wavelengths. A plot of absorbance (A) vs. wavelength (λ) is obtained from this analysis. There are basically four types of uv transisions.

$$\sigma \longrightarrow \sigma^*$$
$$n \longrightarrow \sigma^*$$
$$n \longrightarrow \pi^*$$
$$\pi \longrightarrow \pi^*$$

All the transitions require energy. The σ to σ^* transition requires large energy whereas the n to σ^* transition requires less energy. The absorbance spectroscopy follows the

Lambert-Beer's law. The spectrometer consists of a radiant source, wavelength selector, the solvent wherein the sample is dissolved, a photo-detector and readout.

Figure 4.7- UV-Vis Spectrometer [Perkin-Elmer]

4.3.5. Thermo-gravimetric analysis (TGA)

This is a technique that the decrease or decomposition of the material with a function of increasing temperature, in an atmosphere of nitrogen. Different type of materials like inorganic, organic, plastics, polymers, composites, ceramics etc can be analyzed with this technique. The range of the temperature is generally between 25°C to 900°C. A very small amount of sample 1-5 mg is placed in the crucible which is then exposed to a range of temperature and then purged with nitrogen to completely oxidise the material. A graph of temperature vs. weight loss is obtained from this analysis.

Figure 4.8- Thermo gravimetric Analyser [Perkin Elmer, TGA 4000]

Chapter 4

RESULTS AND DISCUSSIONS

5. Results and Discussion

5.1. Scanning electron microscopy studies

The SEM had been used for analyzing the morphology of the katira gum hydrogel, PANI and the hydrogel-PANI composite. SEM images clearly show the surface topology of the katira gum hydrogel as solid sheet like structures. The image shows a smooth structure. In the composite image, a clear visual of incorporation of the polyaniline particles into the hydrogel network can be seen. The rough clusters over the smooth sheets indicate the embodiment of PANI nanoparticles into the hydrogel network. For the surface topology to be analysed through SEM, the synthesized hydrogel limits the effectiveness of this analytical method as SEM can only be used with sample having very low or no moisture content. For this the sample must be dried which can alter the morphology. Figure 5.1 (a) and (b) shows the hydrogel and the PANI respectively. The PANI nanoparticles show a rough structure. This can be seen in the figure 5.1 (c) as deposited over the smooth sheets of the hydrogel.

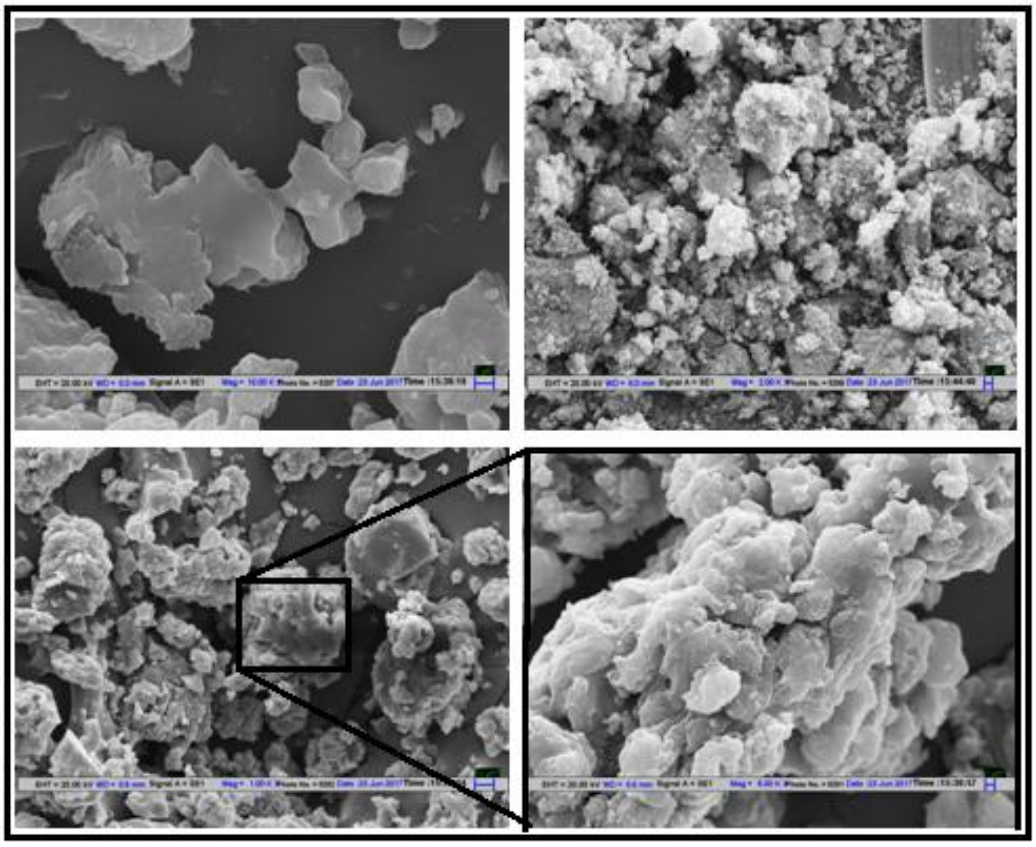

Figure 5.1. SEM images of (a)Katira gum hydrogel, (b)PANI, (c)Hydrogel-PANI composite, and (d)magnified image of composite.

5.2. Swelling studies

The results of the swelling studies conducted on the hydrogel were plotted as a graph of percentage swelling vs. Time. The swelling studies were conducted in distilled water and PBS (pH 7.4). The phosphate buffer saline showed greater swelling percentage than water. The optimized sample of hydrogel was further used in the biosensing membrane. Figure 5.2 shows the swelling percentage of the hydrogel in PBS is greater than the swelling percentage in water. PBS solvent showed a swelling percent of 534% and the water solvent showed a swelling of 218%. The difference in the swelling ratio is due to the changes in the pH of the swelling medium. The swelling ratio changes with increase in the pH. The swelling ratio is calculated by the formula

$$\text{Swelling ratio} = \{(W_s - W_d)/ W_d\} \text{ X } 100$$

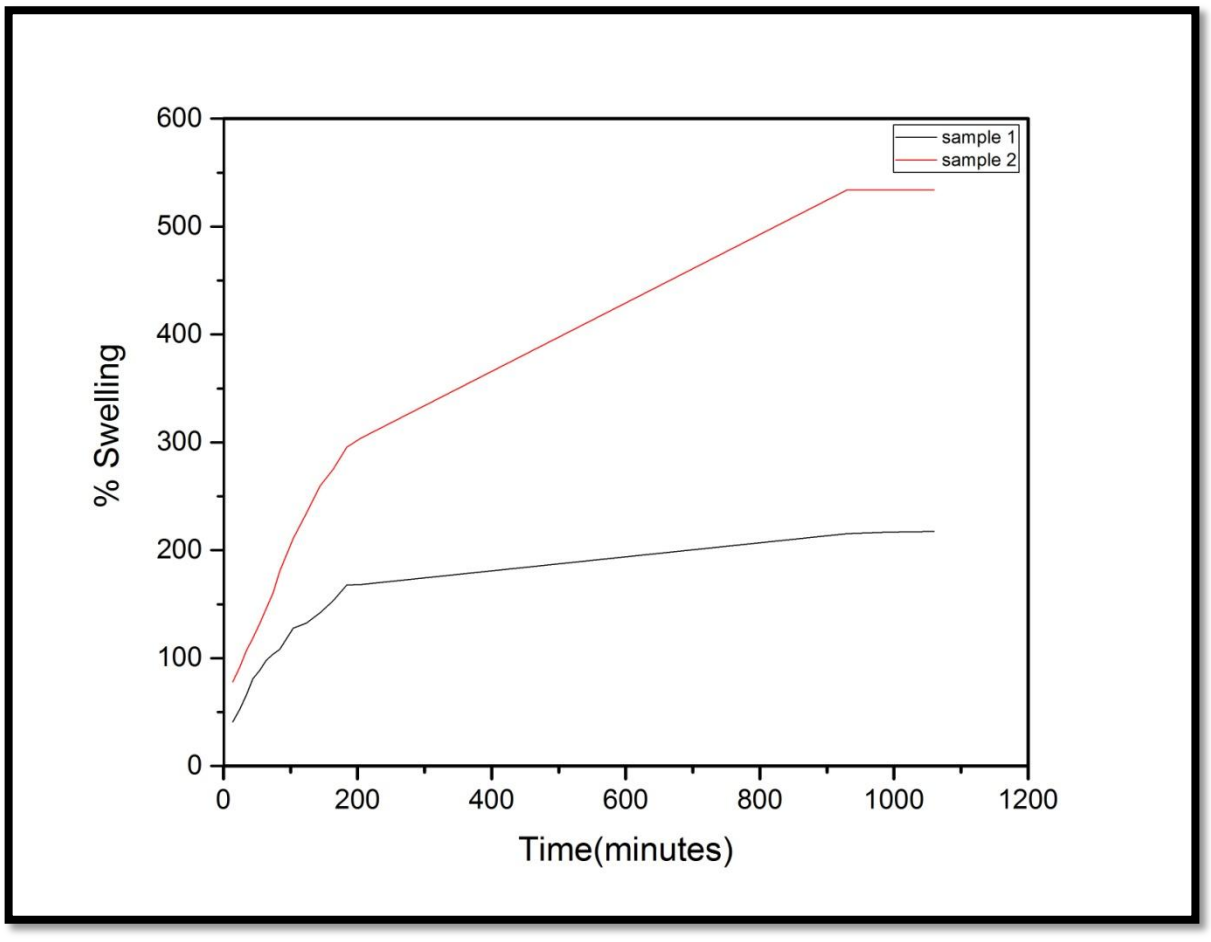

Figure 5.2 Slopes showing the swelling pattern of the hydrogel in (a) sample 1- PBS (pH 7.4) and (b) sample 2- water

5.3. FTIR studies

The FTIR spectra of Katira and AAc-Cl-Katira confirmed the copolymerization, as shown in figure 5.3. The cross-linking of acrylic acid on katira was evident by analyzing the characteristic peaks of the functional groups present in the hydrogel. Figure 5.3 also analyses the spectra of PANI and hydrogel incorporated PANI composite.

FTIR spectra of Katira gum and Katira-cl-poly(AAc) hydrogels, PANI and katira-cl-poly(AAc)/PANI are presented in Figure. In case of katira gum, a broad band at 3421.0 cm^{-1} which is due to O-H stretching vibrations has been observed. The peak in the region 1072.6 cm^{-1} has been assigned to C-O and C-C stretching vibrations of pyranose rings of polysaccharide. The shoulder at 1252.5 cm^{-1} is due to the asymmetric C-O-C vibrations in glycosidic groups of polysaccharides present in the gum. The absorption bands in the region 1620.9 cm^{-1} (C=O) is due to carboxylic acid of D-galactouronic acid present in katira. The peak at 1423.0 cm^{-1} is assigned, to C-OH bend.

FTIR spectrum of Katira-cl-poly(AAc) polymer shows absorption bands at 3446 cm^{-1} which is due to OH stretching vibrations, the band at 2927.0 cm^{-1} corresponds to the stretching vibrations of C-H. The peak at 2362.5 cm^{-1} shows the overtones and combinations of O-H bending and C-O stretching vibrations. A band at 1716.7 cm^{-1} corresponding to C-O stretching has been observed. A peak at 1396.4 cm^{-1} has been assigned to CH_2 bending. The peak at 1166.1 cm^{-1} is due to C-O stretching of –COOH bond. These are the bands that are exclusively present in the hydrogel beside the bonds in the raw katira gum sample. The presence of more predominant band at 1716.7 cm^{-1} as compared to gum and absorption band at 1635.8 cm^{-1} which is due to COO^- asymmetric stretching, indicate the incorporation of poly(AAc) on to the polysaccharide backbone.

The FTIR spectra of polyaniline, shows a peak at the 3437.1 cm^{-1} is due to the N-H bond stretching. The peak obtained at 2360.1 cm^{-1} is assigned to the NH unsaturated amine bond. The bend at 1568.9 cm^{-1} has appeared due to the C=N bond in the quinoidal units of the polymeric benzene ring. The 1480.5 cm^{-1} bend is assigned to the benzoid stretching vibrations. The band at the 1301.2 cm^{-1} region has appeared due to the C-N stretching vibrations in the alternate units of the quinoid-benzoid-quinoid rings. The bend at the 1126.6 cm^{-1} is due to the C-H plane bending.

The FTIR spectrum of the composite AAc-cl-katira/PANI shows peaks of both PANI and hydrogel confirming the incorporation of the emeraldine salt in the networks of

the hydrogel. The common peaks that are present in both PANI and the hydrogel include 3435.6 cm^{-1} which is due to the clubbing of the –OH and the N-H functional groups. That is why a broad band has been seen. The 2359.4 cm^{-1} peak is due to the N-H unsaturated amine. The peaks at 1630.9 cm^{-1} is a broad band which is due to the clubbing of the $–COO^-$ bond stretching, the –C=N stretching of the quinoid rings and the C=C stretches of the quinoid ring. And the last common band is observed at 1499.0 cm^{-1} which is attributed to the C-N stretching of the benzoid rings. Other peaks have been shown at 2926.2 cm^{-1} which is due to the C-H alkane stretching. The peak at 1720.9 cm^{-1} is assigned to the stretching of –C=O bond. Another peak has been observed at 1161.3 cm^{-1} which is due to the C-O-C stretch.

The results of the FTIR suggests that the composite has both hydrogel and polyaniline particles.

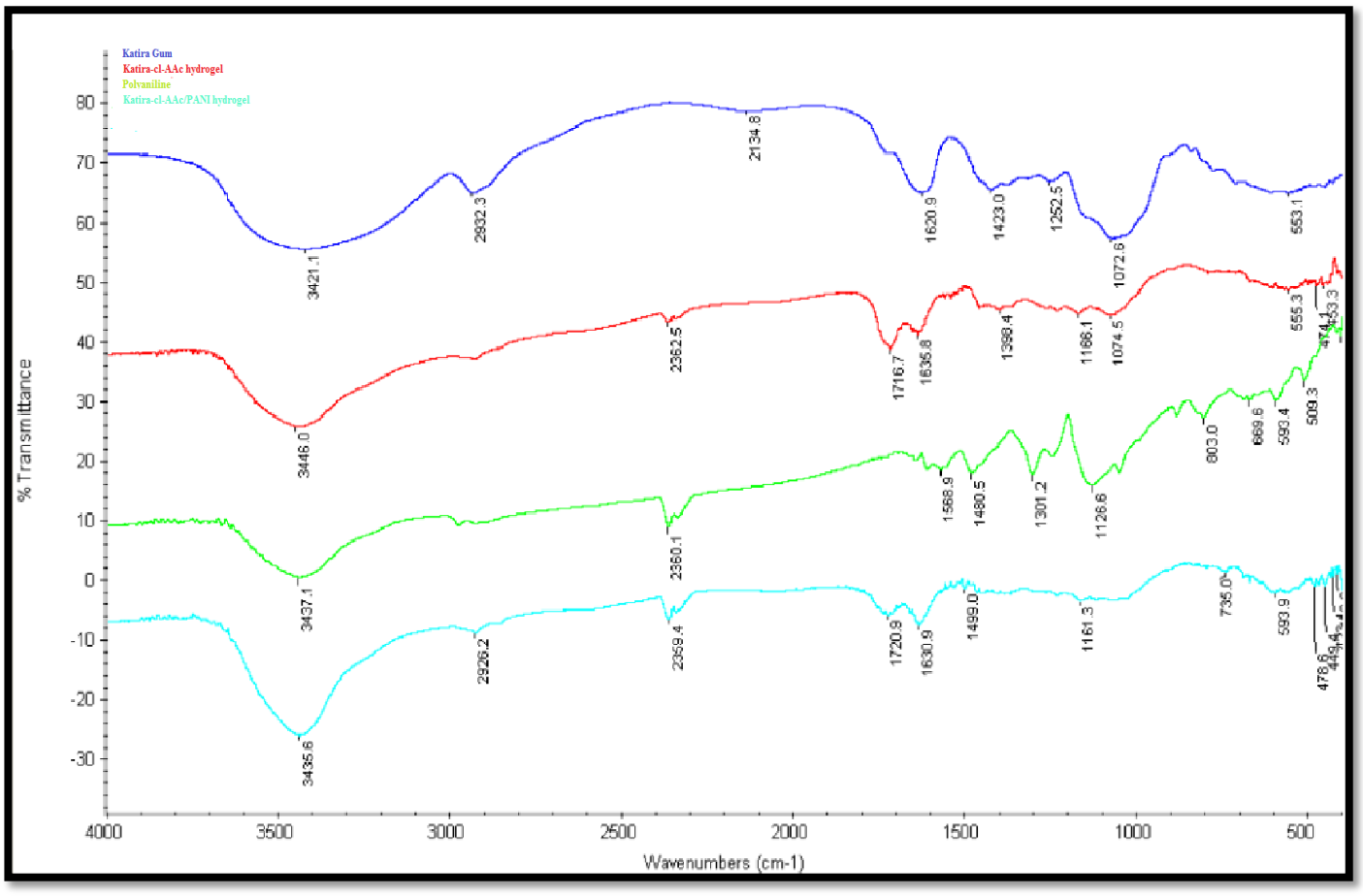

Figure 5.3. The FTIR spectra of katira gum, hydrogel, PANI, and PANI-Hydrogel

5.4. UV-Vis spectrometry results

The figure 5.4 shows the UV-visible spectra of PANI and Katira-cl-AAc/PANI hydrogel. PANI showed two absorption peaks at 360 and 680 nm. The band at 360 nm

is assigned to the benzonoid π-π^* transition. And the band at 680 nm is in the quinonoid rings due to the exciton like transition. The peak between 300-400 nm has been observed due to localized polarons. This is an essential characteristic of protonated PANI. The absorption band of the katira-polyaniline hydrogel shifts due to the interaction between the quinoids and the benzoid and the katira particles. The blue shift of the absorption from 360 to 300 nm and the blue shift of the 680 to 650 nm in the absorbance may be due to the interaction of the polyaniline particles with the katira gum hydrogel.

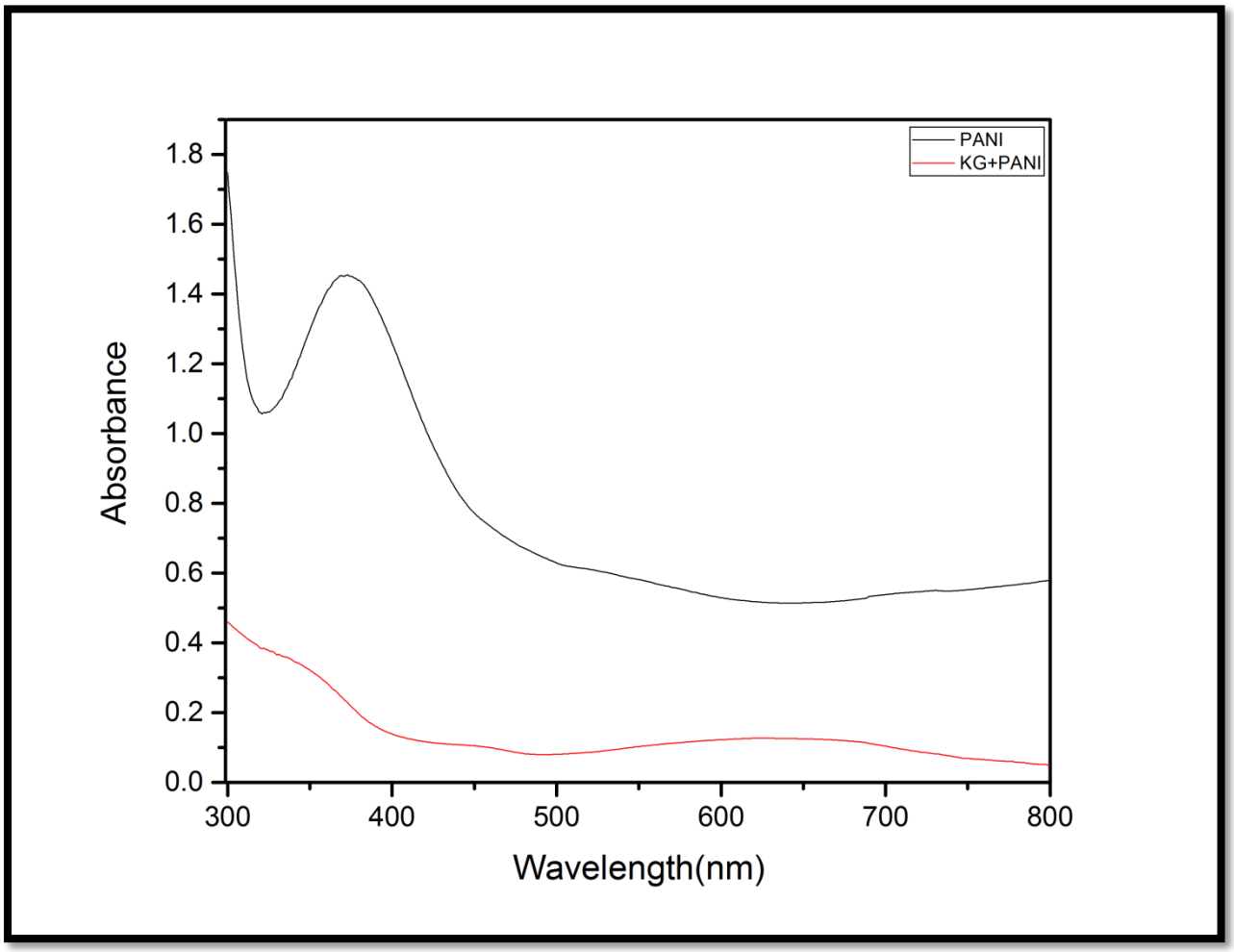

Figure 5.4. The UV-absorption spectrum of PANI and Katira-cl-AAc/PANI

5.5. Thermo gravimetric analysis

TGA is basically used for the finding the range of stability and for finding the decomposition temperature. In the figure 5.4, this shows the decomposition of the

Katira-cl-AAc hydrogel in an inert atmosphere of nitrogen. The first drop in the weight have been observered from 50°C to 175 °C, which is attributed to the loss of moisture content present in the sample. An abrupt bend in the slope has been observed from 175°C to 380°C, which signifies the constant decomposition of the compound. The decomposition, first starts with the volatile compounds present in the sample. The decomposition continues till 435°C. The range of stability is attained in the temperature range of 450-650 °C, before complete decomposition. At last nitrogen, is purged in the sample to ensure its complete decomposition.

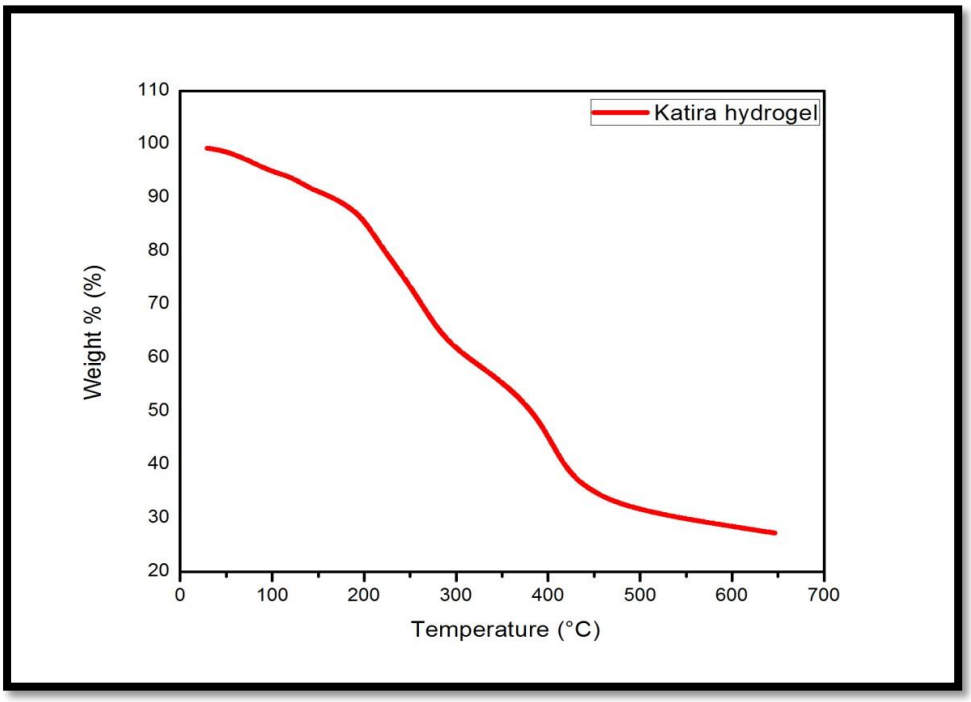

Figure 5.5 TGA of Katira-cl-AAc hydrogel

The figure 5.6 shows the thermal decomposition of polyaniline (emeraldine salt). In this the first dip in the slope has been observed due to the evaporation of the moisture. The abrupt change in the slope of the graph in the temperature range of 315 °C to 730 °C is due to the material loss other than the water loss. There is a sharp decrease in the sample weight in the temperature range of 730 to 750 °C, attributed to the attainment of stability before complete decomposition of the compound.

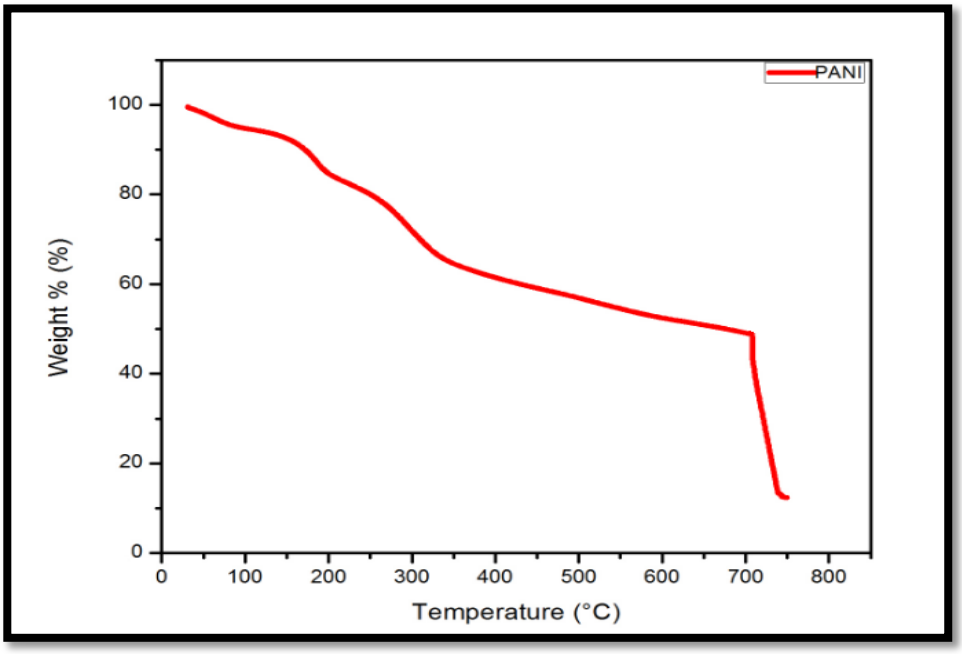

Figure 5.6. TGA of polyaniline

The figure 5.7, is the thermal degradation curve of Katira-cl-AAc/PANI composite. This is comparatively more stable than the other two materials. This composite has shown a gradual decrease in the weight upto 270 °C, which is due to the moisture content. Then there is a slight kink in the slope, attributing to the decomposition in material weight. A stability in the weight has been seen after 450 °C, before the complete decomposition of the compound.

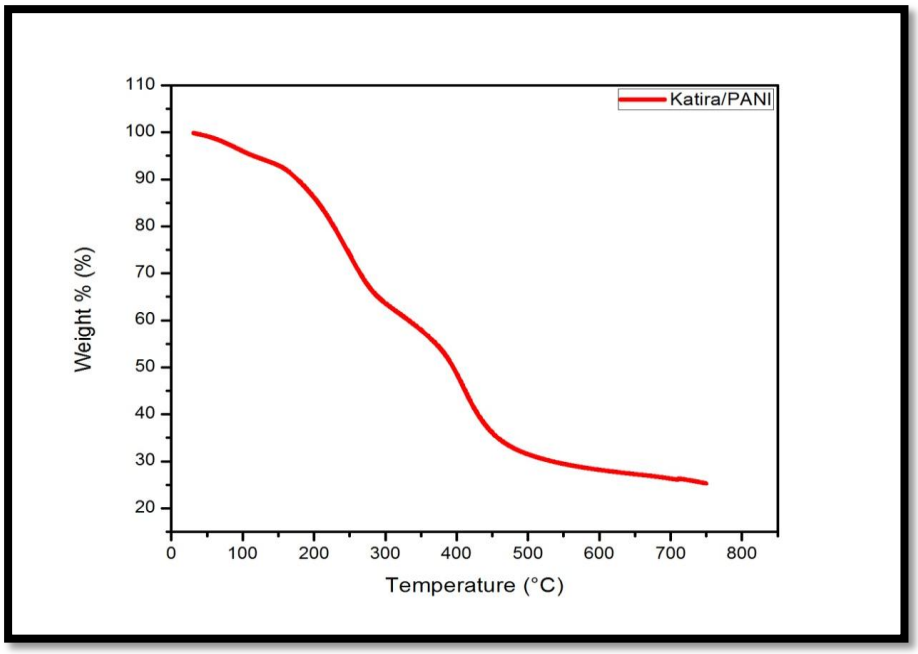

Figure 5.7. TGA of Katira-cl-AAc/PANI composite

5.6. Four-probe conduvtivity

The four probe is a technique which is used for the determination of conductivity and resistivity of the material. These are the fundamental properties of a semiconductor material. The graph of current vs. Voltage has been plotted to give the resistance of the PANI disc. The thickness of the disc was measured to be 0.38 mm. The R_{eq} calculated from the plot is 0.847 Ω. On the calculation of the conductivity of the PANI disc, it has been found to be 13.86 S/cm. The resistivity of the disc is 0.07215 Ω-cm. The conductivity of the PANI discs have been found to me moderately high due to the doping of the PANI with HCl. The emeraldine salt doped with HCl shows higher conductivity than the earlier reported PANI-CSA dopant. This change in conductivity is due to the change in the protonating agent. HCl is a strong protonator than CSA. Thus, makes the chain more electronegative and increases the conductivity. Using this property, this HCl doped PANI has been used for the biosensor platform fabrication in combination with the katira gum hydrogel, to immobilize urease onto the membrane.

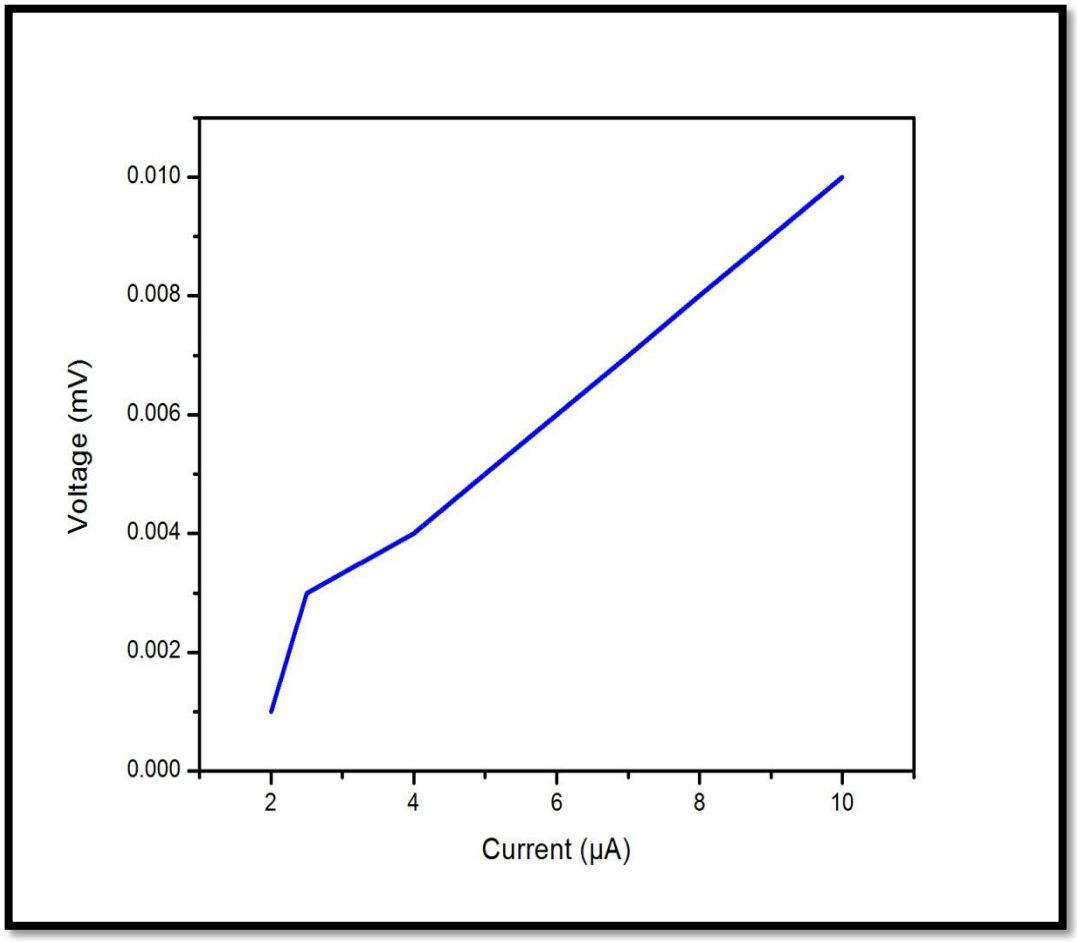

Figure 5.8 Graph of voltage vs. Current of PANI discs

5.7. Electrochemical Studies

5.7.1. Electrode studies

The differential pulse voltammerty of the electrodes PANI/ITO, Katira-cl-AAc/ITO, katira-cl-AAc/PANI/ITO, and Urease/Katira-cl-AAc/PANI/ITO had been conducted using the three-electrode system in the electrochemical analyser. The electrodes have been emmersed in PBS pH 7, 0.1M, having 5mM of $[Fe(CN)_6]^{3-/4-}$ with a scan rate of 50 mV/s. The potential has been changed from -0.3 to 0.5 V. It was observed that the magnitude of the peak current for PANI/ITO electrode was highest, 0.34 mA. The magnitude of the peak current decreased in the katira-cl-AAc/PANI/ITO electrode to 0.22 Ma, due to the decrease in the bulk concentration of polyaniline. This peak current magnitude is higher than the katira-cl-AAc/ITO electrode, which is attributed to the conducting property of the composite. The immobilization of urease onto the electrode further increases the peak oxidation current value to 0.24 mA. The highest current is seen in the case of PANI/ITO electrode, because of pure conducting polymer nanoparticles. In all the other electrodes there is a little hindrance due to the presence of the bulkier groups. Moreover, the conductivity of katira gum is less as compared to PANI, since it is a natural polysaccharide.

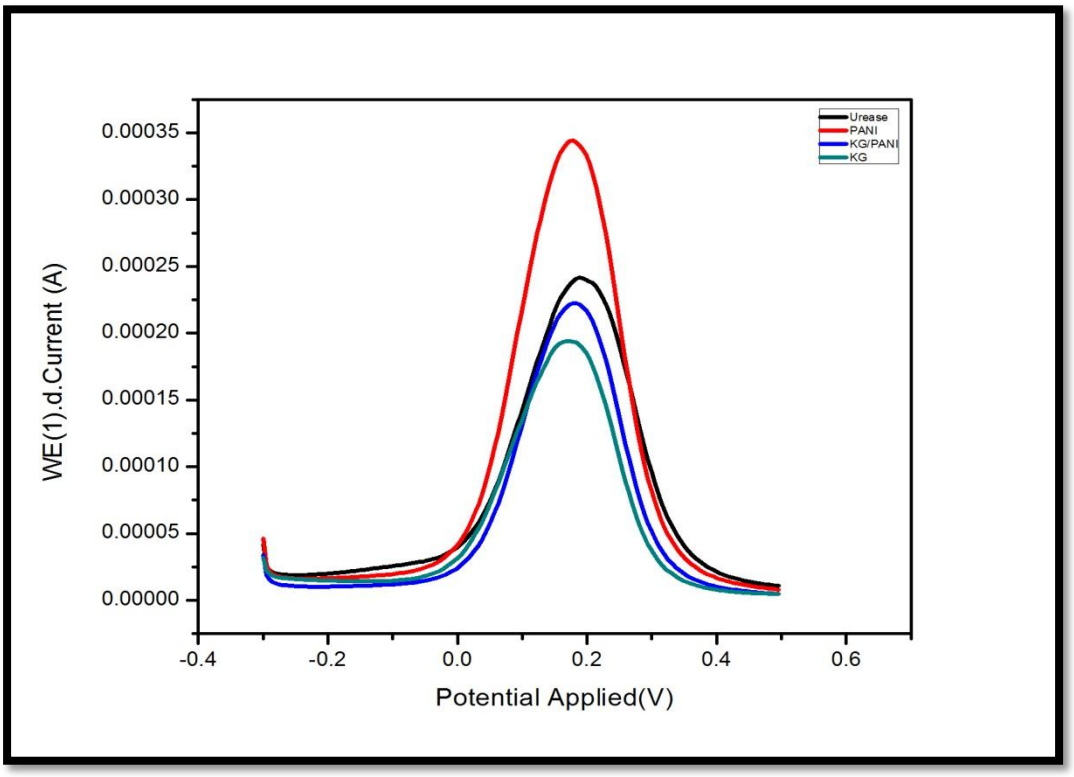

Figure 5.9. DPV of PANI/ITO, Katira-cl-AAc/ITO, Katira-cl-AAc/PANI/ITO and Urease/Katira-cl-AAc/PANI/ITO

Further, for the optimization of the concentration of urease, the cyclic voltammetry has been conducted on the Urease/Katira-cl-AAc/PANI/ITO electrode. The potential has been changed from -0.4V to 0.5V, with a scan rate of 50mV/s. Different concentration of urease, varying from 0.5mg/ml to 2mg/ml has been prepared in PBS buffer, (pH 7, 0.1M). The peak oxidation current of each electrode has been measured and compared. The urease concentration with 1mg/ml showing the highest oxidation current has been further used for conducting the experiment. Higher the concentration of the enzyme, slower becomes the mobility of the ions due to the increase in the bulk concentration. Thus 1mg/ml has been chosen as the ideal concentration of the enzyme for the substrate detection.

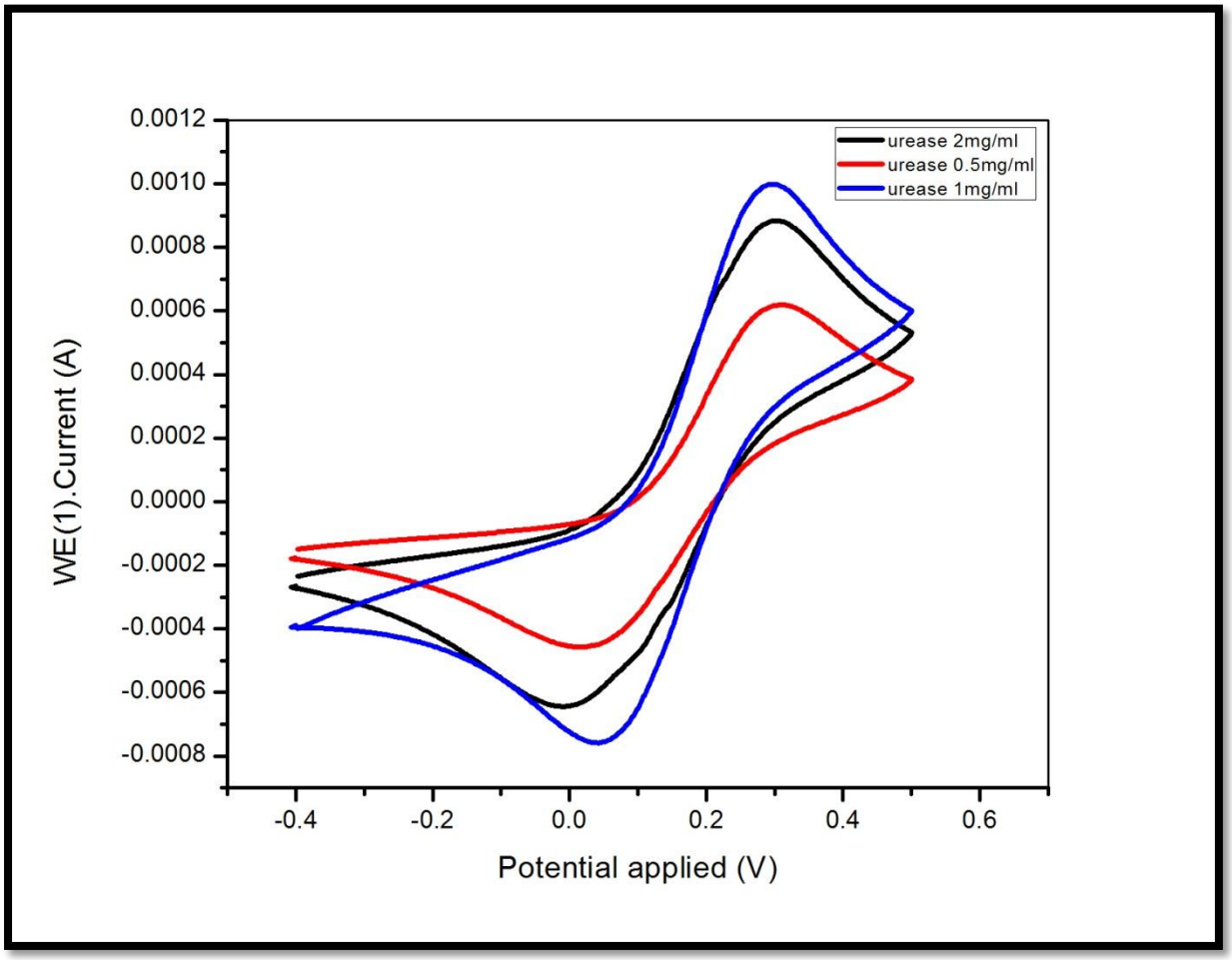

Figure 5.10. The cyclic voltammetry studies of different concentration of urease.

Increasing concentration of urea as a substrate, from 1mM to 10mM has been used for the biosensing. It has been seen that, at first, with increase in concentration of the substrate, the peak current increases. This is due to the presence of excess enzyme to substrate ratio. As the substrate, i.e., urea is increased, the enzyme catalyses the conversion of urea to ammonia and carbon-dioxide. A highest oxidation current has

been observed in 5mM concentration. Beyond this concentration, as the substrate is increased, there is a decrease in the peak current. This can be attributed to the cause that, all the active site on the enzyme has been occupied by the substrate molecules. Thus decreasing the charge transfer rate, and hence reducing the oxidation current.

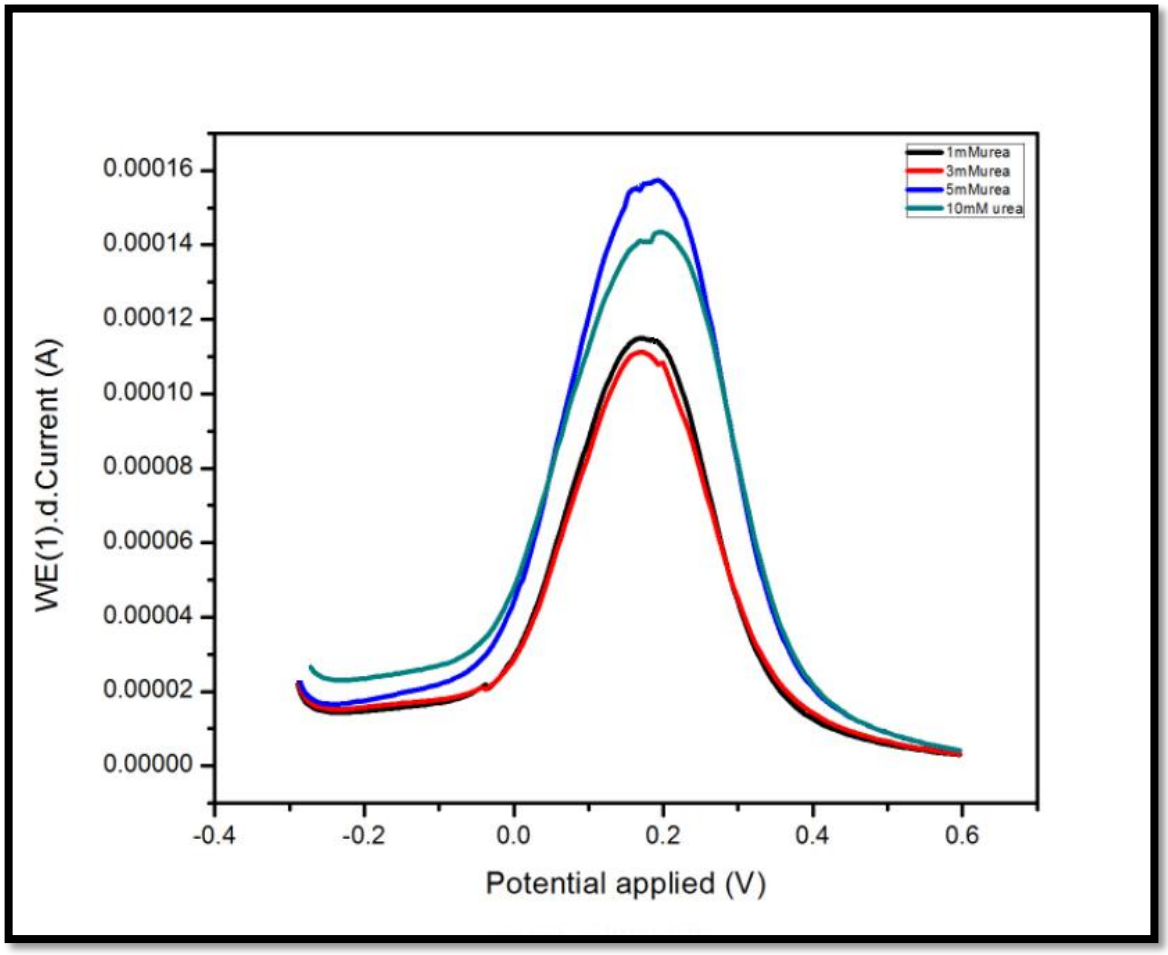

Figure 5.11. DPV of different concentrations of the substrate, urea for 1mg/ml urease electrode.

5.7.2. Incubation time studies

The figure 5.12 shows the response of the electrode Urease/Katira-cl-AAc/PANI/ITO in 5mM urea concentration with respect to different incubation times. It has been observed that there is an increase in the current with the increase in the incubation time from 5 minutes to 45 minutes. However, after 35 mins of incubation, the peak current of the electrode becomes constant. The electrode becomes stable at a particular oxidation current. Hence, the incubation time of 40

minutes has been selected for the further enzyme substrate response studies. After 40 minutes of incubation the enzyme substrate reaction reaches a steady state.

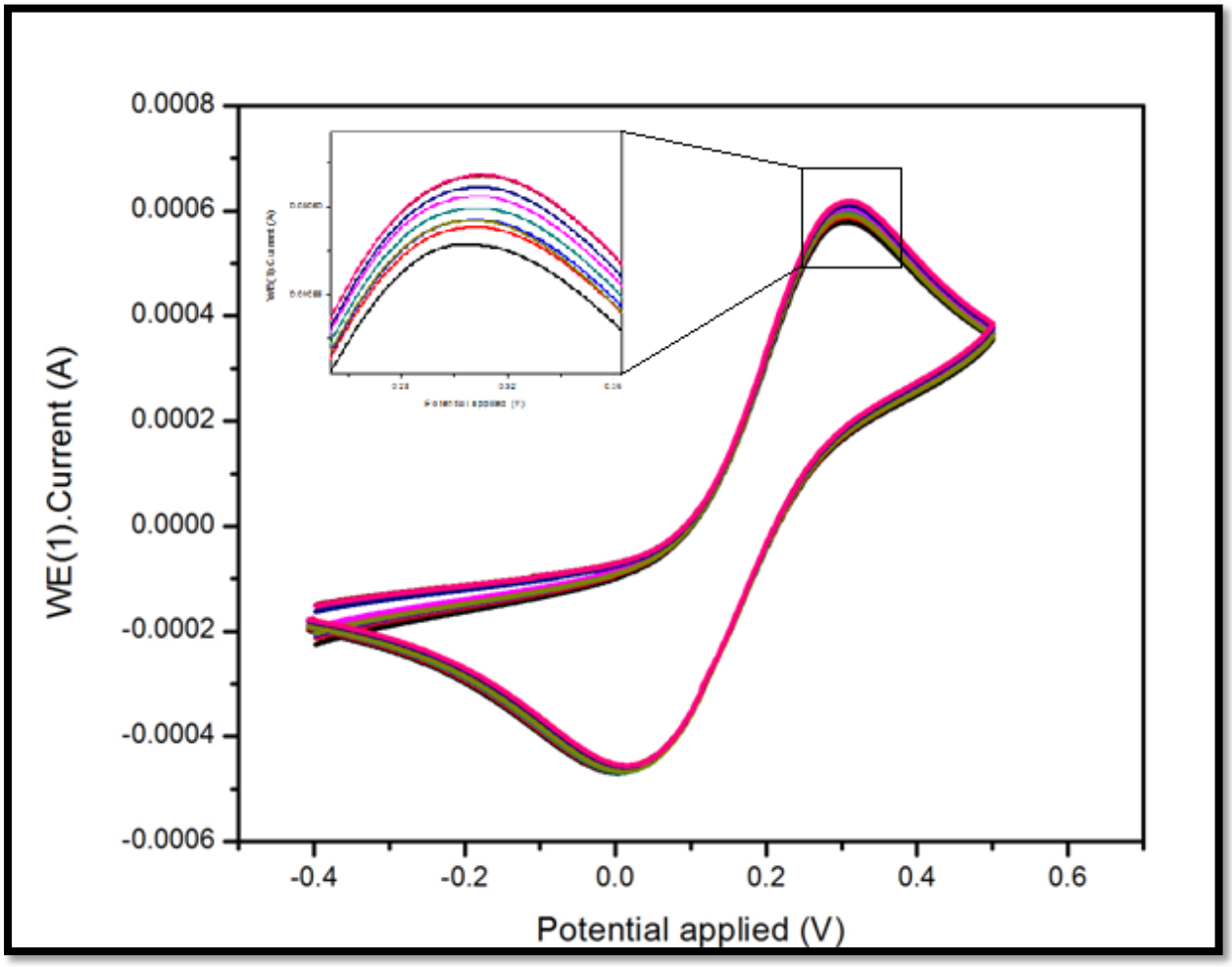

Figure 5.12. Incubation time study of the enzyme electrode using cyclic voltammetry.

Figure 5.13 shows a graph of current vs. Incubation time. The graph clearly shows that the current becomes constant for the Urease/katira-cl-AAc/PANI/ITO electrode at 0.619 mA after the incubation of 40 minutes. An incubation time of 40 minutes, with urea has been selected before every heavy metal testing to obtain the results further.

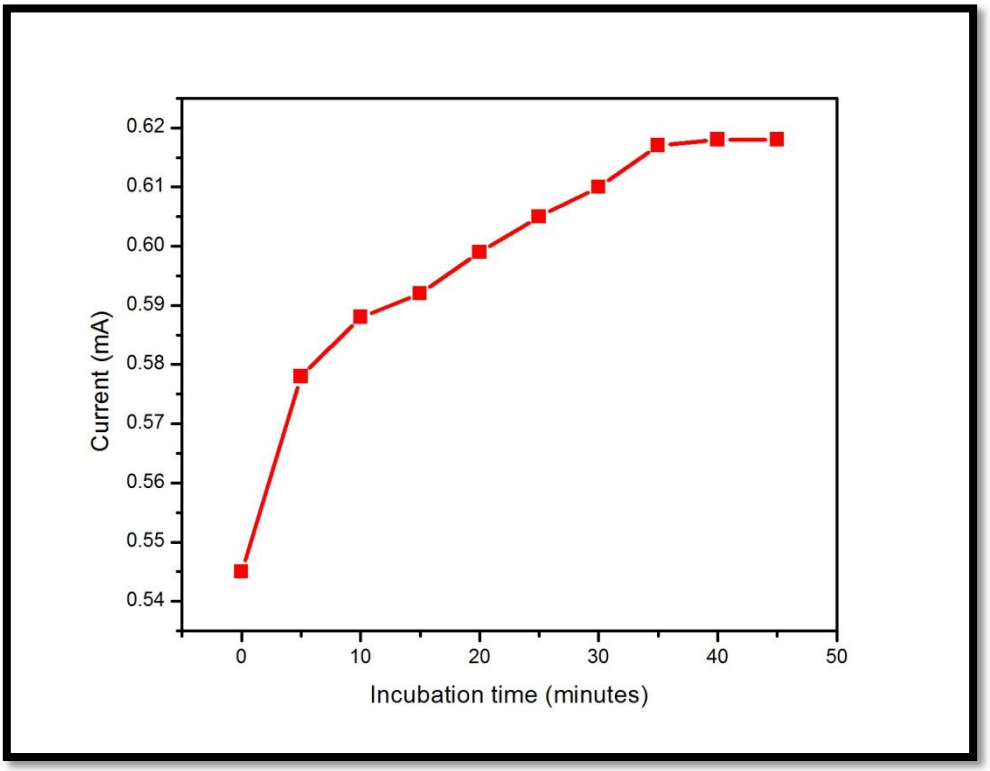

Figure 5.13. A plot of Incubation time vs. Current

5.7.3. Heavy metal salt inhibition of immobilized urease

The enzyme electrode were first incubated with different concentrations of heavy metal salts for 5 to 10 minutes with the subatrate urea. Cyclic voltammetry studies were done to analyze the divergence in the peak current from the enzyme electrode in 0.1M PBS (pH 7) containing 5 mM of $[Fe(CN)_6]^{3-/4-}$. The scan rate used was 10mV/s. Three different heavy metal salts were used for the comparative study of the percent inhibition of the enzyme urease activity. The measurement condition were kept identical for all the three metal ions. It was observed that, there is a retarding action of the immobilized urease due to reduction in the peak oxidtion current with increase in the concentration of the heavy metal salts. For each heavy metal the concentration was varied from 0.1 mM to 5 mM. Three salts of cobalt, mercury and cadmium were used to analyze the change in the enzyme activity. Percent inhibition was calculated using the formula,

$$\% \text{ Inhibition} = [(A_0\text{-}A)/A_0 \text{ X } 100]$$

Where A_0 is the initial peak current of the Urease/Katira-cl-AAc/PANI/ITO electrode and A is the inhibition peak current at differnt concentrations of metal salts.

A graph of Concentration of the heavy metal ions vs. % Inhibition was plotted for each metal ion. The caused was attributed to the non-competitive inhibition, as cited in previous papers, in the case of Cadmium ion, cobalt and mercuric ion. Non-competitive inhibition is due to the binding of the metal ions to the sites other than the allesteric sites of the enzyme. The inhibitor reduces the catalytic activity of the enzymes by binding to the enzyme, despite of the fact that the substrate has already bound to the enzyme or not. The slope of the graphs showed the following order of inhibition

$$Hg^{2+} > Cd^{2+} > Co^{2+}$$

Mercuric ion showed a sharp increased inhibition in the concentration range of 1.8 mM to 5 mM. As compared to the mercuric ion, cadmium ion showed greater inhibition when the concentration range of 0.1 mM to 2 mM. The cobalt ion showed a constant increase in the inhibition of the enzyme activity from 0.1 mM to 5 mM. The graphs 5.14 (a), (b) and (c) shows the % Inhibition of the urease activity with respect to the increasing concentration of the metal ions. In case of the mercuric ion, an increase in the peak oxidation current has been observed durin the incresing concentration of upto 1.8 mM, beyond this concentration, there is a sharp decrease in the value of the oxidation current. This is due to some discripany in the addition of the mercury salt which has increase the rate of charge transfer in the fabricated electrode system. There may be other cause like the Hg ion operates via other means including direct binding to the active site. A bar graph in the figure 5.14 (d) shows the comparative study of the percentage inhibition of the catalytic activity of the enzyme urease with respect to the differnt concentrations of the three heavy metal ions, Hg^{2+}, Co^{2+} and Cd^{2+}. The bars have been divided in two range of concentration, first being 0.1 mM to 1.2 mM in which cobalt ion has shown minimum inhibition rate as compared to the other two metal ions. And the second is the range between 1.8 mM to 5.0 mM where the inhibition rates of mercury and cadmium is almost same.

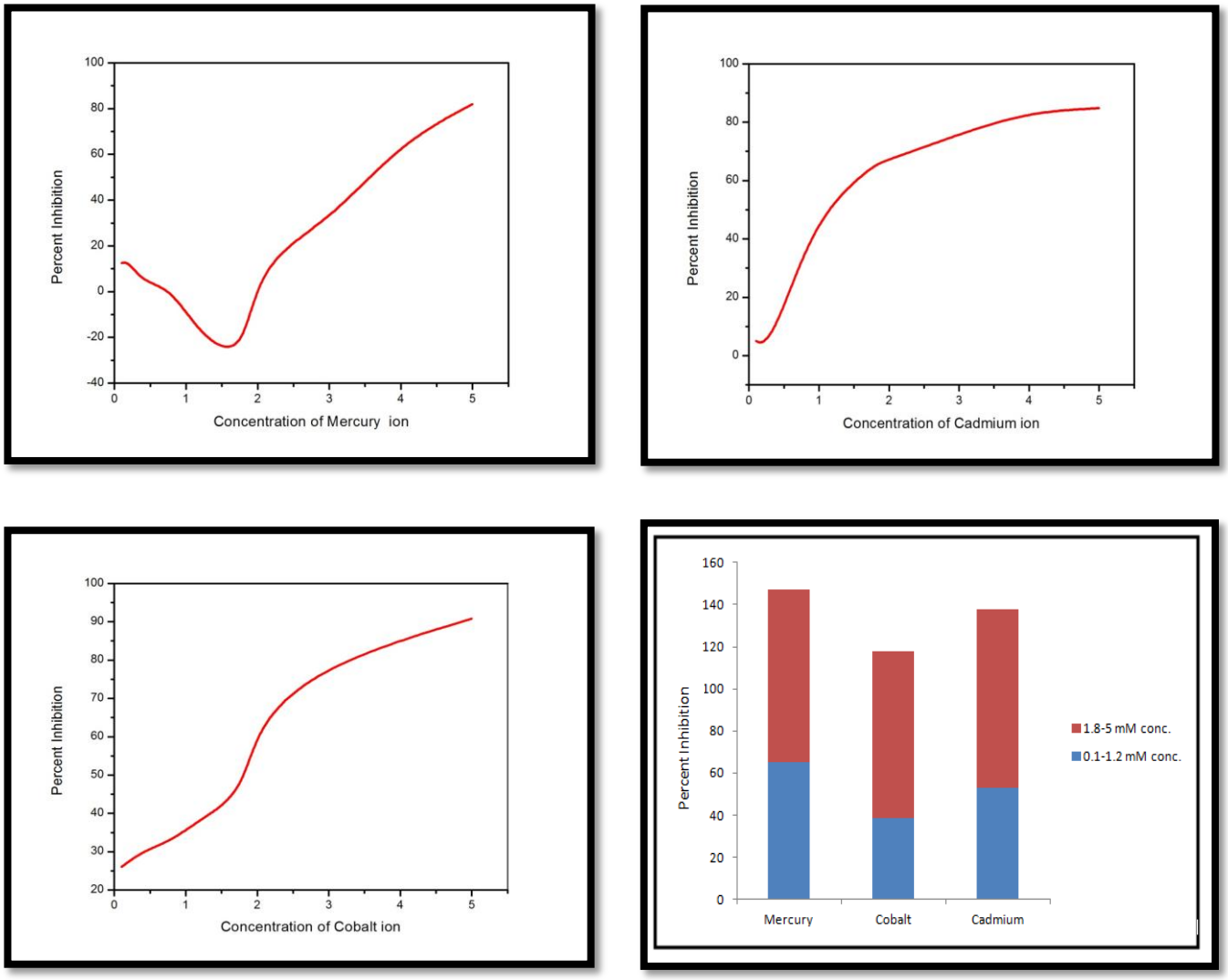

Figure 5.14. % Inhibitio of urease with respect to (a) Hg^{2+}, (b) Cd^{2+}, (c) Co^{2+} and (d) Bar graph showing the comparative inhibition rates of heavy metals

Detection technique	Sample	Platform	Detection limit	Reference
Conductometric	$Hg(NO_3)_2$	Urease/Au/Al_2O_3	0-40 mM	[24]
Potentiometric	Heavy metals (Cu,Cd, Hg)	Urease/Carbon/Rhodium/Carboxymethyl cellulose	0-20 ppb	[25]
Conductometric	Heavy metals	Sol-gel screen printed electrode	0.1-10 mM	[26]
Conductometric	Heavy metals	Urease/TMOS sol-gel/IDA	0.1 mM	[27]
Electrochemical	Hg	Urease/Gold electrode	0-50 µM	[28]
CV	Hg, Cd, Co	Urease/Katira-cl-AAc/PANI/ITO	0.1-5 mM	Current work

Table 2- Characteristics of various detection techniques for heavy metals using urease

The comparative data in the table 2 shows the previous works on heavy metal sensing using urease enzyme. Our work, has shown a more efficient method for the estimation of heavy metals like Cadmium, cobalt and mercury. The detection of metal concentration based on pure samples could be as low as 200 to 270 ng L^{-1}. Moreover, the biosensor is highly sensitive, disposable, low cost due to the use of single enzyme and natural polymers and has minimum loss of activity of the enzyme. This system is easy to use and can be employed for real time analysis of the samples.

Chapter 5

CONCLUSION

6. Conclusion

In this study, we have proposed a biocompatible, non-toxic hydrogel based biosensor for the detection of the heavy metal ions present in any water sample. PANI doped with HCl, a conducting polymer has been incorporated in the hydrogel which has helped in enhancing the conducting properties of the electrode. The ratio of katira gum: aniline monomer has been polymerized in the proportion of 2:1. Firstly the katira gum hydrogel has been synthesized, and then this hydrogel has been swelled in the solution of aniline to get an interpenetrating network of conducting hydrogel composite, Katira-cl-AAc/PANI. This composite has been dried at 60°C to remove any moisture content present. The dried product has been dispersed in an organic solvent, DMSO to form a solution, which was then drop cast over the hydrolysed ITO electrode. The area for the drop cast was kept one-third of the total surface of the electrode. Immobilization of urease enzyme was also carried out with the help of the drop casting method, wherein 20µl of the enzyme was taken. Prior to the enzyme immobilization, the electrode was functionalized with 2.5% glutaraldehyde solution for the activation of the groups in the hydrogel composite which would ease the attachment of urease onto the electrode surface. The fabricated Urease/Katira-cl-AAc/PANI/ITO electrode is simple and has been used for the quantification of urea as well as the heavy metal ions. The composite Katira-cl-AAc/PANI has shown good conducting properties, which can be used as a bioreceptor membrane for any other electrode fabrication. The hydrogel has increased the efficiency of the electrode as its swelling property has provided a large surface area for the attachment of the enzyme. This in turn has provided a greater surface area for the interaction of the substrate urea with the enzyme. The application of the immobilized enzyme has several benefits than the free enzyme, first being the reusability of the enzyme electrode, the second being the low consumption of the enzyme.

The heavy metal ions that has been used for the inhibition studies are found in the aquatic system in high concentrations and has effected a wide population causing many types of diseases and increasing the level of toxicity in humans. The non-competitive inhibition of the enzyme urease by the metal salts has been used for the quantification of the metal ions.

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