POLYANILINE MODIFIED FLEXIBLE CONDUCTING PAPER FOR CANCER DETECTION

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

M. Tech (Industrial Biotechnology)

Submitted by

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DECLARATION

Certified that the major thesis entitled **" Polyaniline Modified Flexible Conducting Paper for Cancer Detection ",** submitted by me is in fulfilment of the requirement for the award of the degree of Master of Technology in Industrial Biotechnology, Delhi Technological University. It is a record of original research work carried out by me under the supervision of Prof. B.D. Malhotra, Department of Biotechnology, Delhi Technological University, Delhi 110042. The matter embodied in this thesis is original and has not been submitted for the award of any other degree/diploma.

Anindita Sen

2K14/IBT/17

CERTIFICATE

This is to certify that the M.Tech thesis entitled "**Polyaniline Modified Flexible Conducting Paper For Cancer Detection"**, submitted by **Anindita Sen (2K14/IBT/17)** in fulfilment of the requirement for the major thesis during M.Tech, Delhi Technological University (Formerly Delhi College of Engineering), is an authentic record of the candidate's own work which was carried out by her under my guidance. The information and data enclosed in this dissertation is original.

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CONTENTS

ABBREVIATIONS AND SYMBOLS

Ab	Antibody
Au	Gold
BSA	Bovine serum albumin
CEA	Carcinoembryonic Antigen
PANI	Polyaniline
EIS	Electrochemical impedance spectroscopy
EDX	Energy dispersive x-ray
FTIR	Fourier transform infrared
PBS	Phosphate buffer saline
SEM	Scanning electron microscopy
XRD	X-ray diffraction
$[Fe(CN)_6]^{4-/3-}$	Ferro-ferricyanide

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Chapter 1

Abstract

Polyaniline modified flexible conducting paper for cancer detection

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1. ABSTRACT:

This dissertation contains results of studies relating to the fabrication of a flexible, disposable, and label free biosensing platform for detection of the cancer biomarker (carcinoembryonic antigen, CEA). Polyaniline (PANI) has been electrochemically deposited over gold sputtered paper (Au@paper) for covalent immobilization of monoclonal carcinoembryonic antibodies (anti-CEA). The bovine serum albumin (BSA) has been used for blocking nonspecific binding sites at the anti-CEA conjugated PANI/Au@Paper. The PANI/Au@Paper, anti CEA/PANI/Au@Paper and BSA/anti-CEA/PANI/Au@Paper platforms have been characterized using scanning electron microscopy, X-ray diffraction, Fourier transmission infrared spectroscopy, chronoamperometry and electrochemical impedance techniques. The results of the electrochemical response studies indicate that this BSA/anti-CEA/PANI/Au@paper electrode has sensitivity of 13.9 μA ng^{-1} ml cm^2, shelf life of 22 days, and can be used to estimate CEA in the range of 2–20 ng ml^{-1}. This conducting paper sensor has been validated by detection of CEA in serum samples of cancer patients via immunoassay technique.

Chapter 2

Introduction

2. INTRODUCTION:

Cancer is currently the leading cause of morbidity and mortality worldwide. An estimated 14.1 million new cases of cancer occurred in 2012 and it is expected to reach 22 millions in the next two decades (WHO factsheet). The diagnosis of cancer via a biomarker has recently aroused much interest. More than 160 different types of cancer biomarkers have been identified till date (Kumar *et al,* 2013). Among these, carcinoembryonic antigen (CEA) is one of the most widely used cancer biomarker that has been associated with colon, ovarian, breast and lung cancer (Wanebo *et al,* 1978; Sorenson *et al,* 2011; Myers *et al,* 1978; Kulpa *et al,* 2002). Its abnormal pre and post operative serum levels have been correlated with the depth of tumor invasion and these are being used routinely for the prognosis of cancer. Many methods such as immunohistopathology (Goldenberg *et al,* 1978), radioimmuno assays (Thompson *et al,* 1969), enzyme linked immune sorbent assay (ELISA) (Zhou *et al,* 2012), reverse transcriptase polymerase chain reaction (RT-PCR) (Yonemura *et al,* 2001) and positron emission tomography for CEA detection have been developed (Flanagan *et al,* 1998). These techniques are time consuming, expensive, require expert handling and involve exposure to harmful radiations. In this context, biosensors offer a number of advantages such as simplicity, high sensitivity, cost effectiveness, flexibility and require low sample volume (Malhotra *et al,* 2003; Bandotkar *et al,* 2014). Glassy carbon, ITO and gold coated glass electrodes are currently being used as a substrate for fabrication of biosensing platform. However, the rigidity, brittleness and cost, limit their applications towards the development of a wearable, flexible, cost effective and disposable point of care device. Recently, paper based electrochemical biosensors have been attracting considerable attention because of their light weight, flexibility, portability, high sensitivity, fast response time and disposability (Desmet *et al,* 2016; Kumar *et al,* 2015; Chen *et al,* 2015).

For the fabrication of an efficient biosensing platform, immobilizing matrix plays a crucial role. In this context, polyanilinie (PANI) may serve as a promising immobilization matrix due to its solution processability, electrical conductivity, flexiblity and large surface area. Moreover, the presence of amine groups provide an additional benefit for covalent immobilization of a biomolecule with enhanced stability and biosensing characteristics (Crean *et al,* 2011; Dhand *et al,* 2011; Kumar *et al,* 2013). Singh et al used nanostructured polyaniline and Soni et al used polyaniline-gold nanocomposite, for detection of sexually transmitted disease (gonorrhoeae) (Singh *et al,* 2009; Soni *et al*, 2015). Further, Tiwari et al demonstrated chitosan-co-polyaniline (CS-co-PANI) copolymer for breast cancer biomarker (BRCA1) detection (Tiwari *et al,* 2009). These authors used rigid and brittle glassy substrates as biosensing platforms.

This report contains results of studies relating to the development of a flexible, label free and disposable electrochemical biosensor for CEA detection. The fabricated polyaniline modified paper electrode (BSA/anti-CEA/PANI/Au@paper) exhibits wide linear detection range with good sensitivity. The results obtained using this electrochemical biosensor have been validated using CEA concentration of serum samples from cancer patients obtained via immunoassay technique.

Chapter 3

Literature Review

3.1 Conducting Polymer

A conducting polymer is a polymeric material that has the electrical, electronic, optical and magnetic properties of a metal along with the processability and mechanical properties associated with a conventional organic polymer (Inzet *et al*, 2012). Alternatively, any organic polymer that has conjugated π electrons or holes by doping (chemically or electrochemically) can posses metallic conductivity. Electronically conducting polymer possess spatially delocalized band like structures and are extensively conjugated molecules. The band structure possessed by them is similar to that of a solid state semi-conductor (Okamoto *et al*, 1964). The band gap is defined by the energy difference between the conduction and the valence bands as shown in figure 3.1.

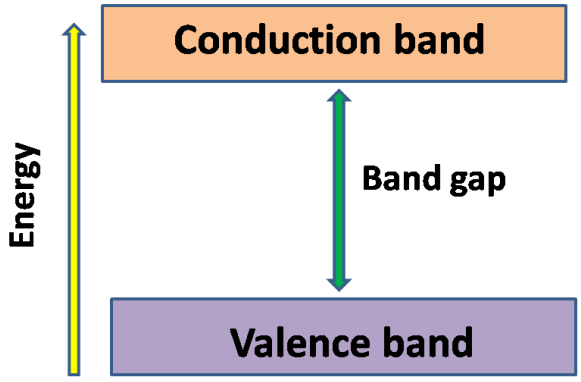


Figure 3.1: Band structure in an electrically conducting polymer

These properties have been observed in a number of conjugated systems such as polyaniline, polypyrrole, polythiophene and poly(p-ethylene). Moreover, apart from their role as electrical conductors, conjugated polymers have also gained application in areas such as optics, nonlinear optics, microelectronics, microelectromechanical systems, electromagnetic devices and membranes (Ferraris *et al*, 1973).

3.1.1. Polyaniline

Polyaniline (PANI) is one of the oldest and the most widely studied organic polymer ever synthesized (in 1862). In earlier works published in 1910s, it was described as existing in four different oxidation states (as an octomer). In 1967, it was reported by Josefowios et al. that the conductivity of polyaniline increased several folds when doped in protonic acids with decreasing pH values. They also proposed that polyaniline could be a potential electrode material for rechargeable batteries. However, these findings were fraught with problems relating to uncertain composition and were lost in literature. Finally in 1980 Dias and Logan rejuvenated research on polyanilines followed by several other research groups in the proceeding decades. MacDiarmid demonstrated the conductive states of polyaniline which arose upon protonic doping of the emeraldine form of polyaniline (MacDiarmid *et al,* 2001; Chiang *et al,* 1986).

Polyaniline is polymerized from the monomer aniline and is found in one of the three oxidation states namely:

- Leucomeraldine- white/clear and colourless $(C_6H_4NH)_n$
- Emaraldine- green due to the emaraldine salt, blue due to emaraldine base $([C_6H_4NH]_2[C_6H_4N]_2)_n$.
- Pernigraniline- blue/violet $(C_6H_4N)_n$

Leucomeraldine is the fully reduced state. Pernigraniline is the fully oxidized state with imine links instead of amine links. The emaraldine form of polyaniline, often referred to as emaraldine base is neutral unless doped which results in emaraldine salt with the imine nitrogens protonated by an acid. Protonation helps to delocalize the otherwise trapped electrons in diiminoquinone-diaminobenzene state. The emaraldine state is of utmost importance due to its high stability and ability to form the highly electrically conducting emaraldine salt on doping (Chiang *et al,* 1986).

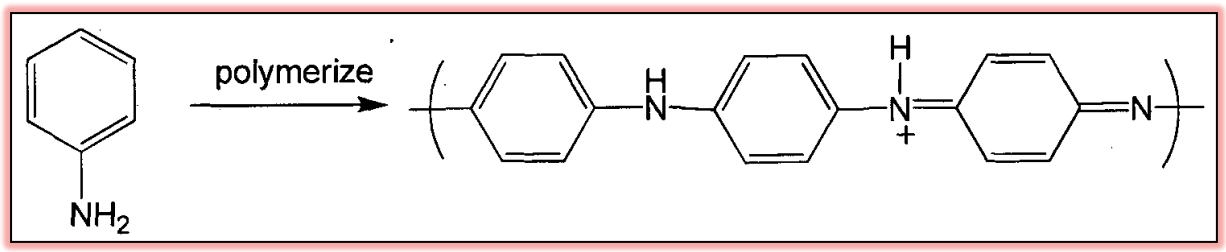


Figure 3.2: Polymerization of aniline to polyaniline.

There are two main techniques for the deposition of polyaniline namely chemical and electrochemical. The most widely used technique for the synthesis of PANI is electrochemical anodic oxidation. The electrochemical technique has various advantages such as absence of catalyst, direct grafting of doped conducting polymer onto the electrode surface and easy control of film thickness. Cyclic voltammetry (CV) is an excellent technique for studying the best operative conditions. Also, chronoamperometric technique has been explored by various research groups for the deposition of polyaniline. Mohd et al deposited polyaniline on ITO-coated glass at different potentials and times (MacDiarmid *et al*, 2001; Chiang *et al*, 1986).

Polyaniline has potential applications in electronics, energy storage devices and biomedics due to their light weight, conductivity, solution processability, mechanical flexibility and low cost. Recently, biosensors are gaining a lot of interest due to their unique characteristics and PANI has proved to be an attractive candidate as a transducer or immobilization matrix in biosensors due to the colour change associated with it and the availability of free NH2 groups which facilitates immobilization of biomolecules (Inzelt *et al*, 2012; Ferraris *et al*, 1973).

3.2 Biosensor

A biosensor is an analytical device, which converts a biological response into a measureable signal. Biosensor can be used to estimate the concentration of desired substances and other parameters of biological interest without using the biological system directly. Enzymes, DNA and antibodies are the most commonly used bio-sensing materials in the development of the biosensors because of their specific nature. But these are not stable in the solutions, so it is necessary to immobilize them onto a matrix that can provide a bio-compatible environmental to the bio-molecule.However, as per the IUPAC recommendations "**A biosensor is a self-contained integrated electronic device capable of providing specific quantitative or semi- quantitative analytical information using a biological recognition element which is retained in direct spatial contact with a transduction element**" (Gerard *et al*, 2002; Daniel *et al*, 2001).

3.2.1. Components of a biosensor

Biological- Enzymes, antibodies/antigens, tissues, bacteria, yeast, organelles, nucleic acids, liposomes etc. Biological components perform following functions.

a. Specifically recognize the analyte of interest.

b. Interact with analyte to produce some physical or chemical changes that can be detected through a transducer.

Physical- Transducer, amplifier and microprocessor.

(a) Transducer

A transducer is a device which converts any type of signal to an electrical signal. The signal produced as a result of interaction of bio-molecules with the analyte may be in the form of electrochemical (change in potential or current), optical (color change), calorimetric (heat

measurement), piezoelectric (mass change) response and will be converted to electric signal with the choice of suitable transducer.

(b) Amplifier

The electronic signal produced by transducer is very small and is amplified by an amplifier.

(c) Microprocessor

The amplified signal is fed into the microprocessor. The signal is then processed and interpreted and is displayed in suitable units.

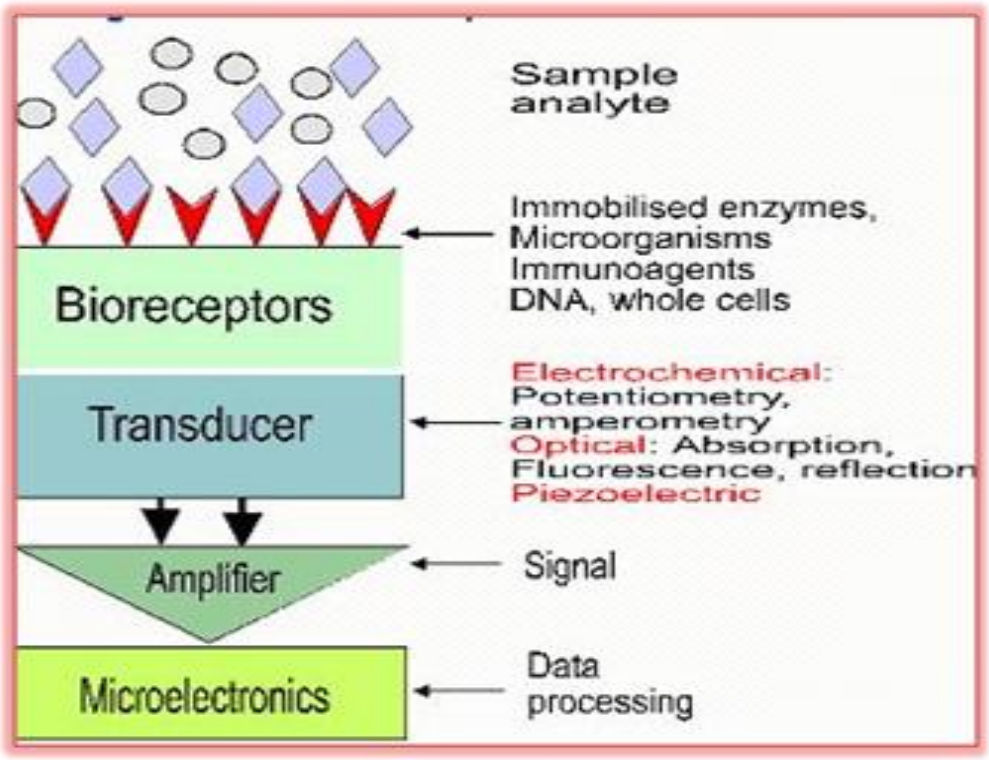


Figure 3.3: Schematic of Biosensor

3.2.2. Characteristics of a biosensor

An ideal biosensor should have features mentioned below:

a. The biocatalyst must be highly **specific** for the purpose of the analyses, be **stable** under normal storage condition.

b. The reaction should be as **independent** of such physical parameters as stirring, pH and temperature as is manageable.

c. The **response** should be accurate, precise, reproducible and linear over the useful analytical range, without dilution or concentration. It should also be free from electrical noise.

d. If the biosensor is to be used for invasive monitoring in clinical situations, the probe must be **tiny and biocompatible**, having no toxic or antigenic effects.

e. The complete biosensor should be **cheap, small, portable** and capable of being used by semi-skilled operators.

3.2.3. Conducting polymer based biosensor:

Conducting polymers due to their conducting nature and biocompatibility have become the materials of choice. Malhotra et al. have reviewed the prospects of conducting polymers in biosensors and have shown that conducting polymers can be utilized for the fabrication of a wide variety of biosensors (Malhotra *et al,* 2003). In cholesterol biosensor fabrication conducting polymers are the most studied materials for the matrix preparation. Among the conducting polymers, polyaniline is the oldest and the most widely studied polymer. Chetna et al. used electrophoretically deposited conducting polymer film derived from nanostructured polyaniline colloidal suspension (on indium-tin-oxide (ITO) glass plate) derived from nano-structured polyaniline (PANI) colloidal suspension using *N*-ethyl-*N'*-(3-dimethylaminopropyl) corbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) chemistry for covalentaly immobilization of cholestol oxidase (Dhand *et al,* 2007). Renu et al used nanostructured polyaniline and Soni et al used polyaniline-gold nanocomposite, for detection of sexually transmitted disease (gonorrhoeae). Further, Tiwari et al demonstrated chitosan-co-polyaniline (CS-co-PANI) copolymer for breast cancer biomarker (BRCA1) detection. These authors used rigid and brittle glassy substrates as biosensing platforms. In this context, paper

seems to be a promising alternative that is being currently explored by researchers all over the world.

3.3 Cancer:

Cancer is a group of diseases that are delineated by irregular cell growth with the potential to penetrate or spread to other parts of the body. Not all tumours are cancerous. The benign tumours do not spread to other parts of the body unlike malignant tumours. Tobacco use is the cause of 22% of cancer deaths. Another 10% is due to obesity, poor diet, lack of physical activity and drinking alcohol. Other factors include infections, exposure to ionic radiations and environmental pollutants (Anand *et al*, 2008).

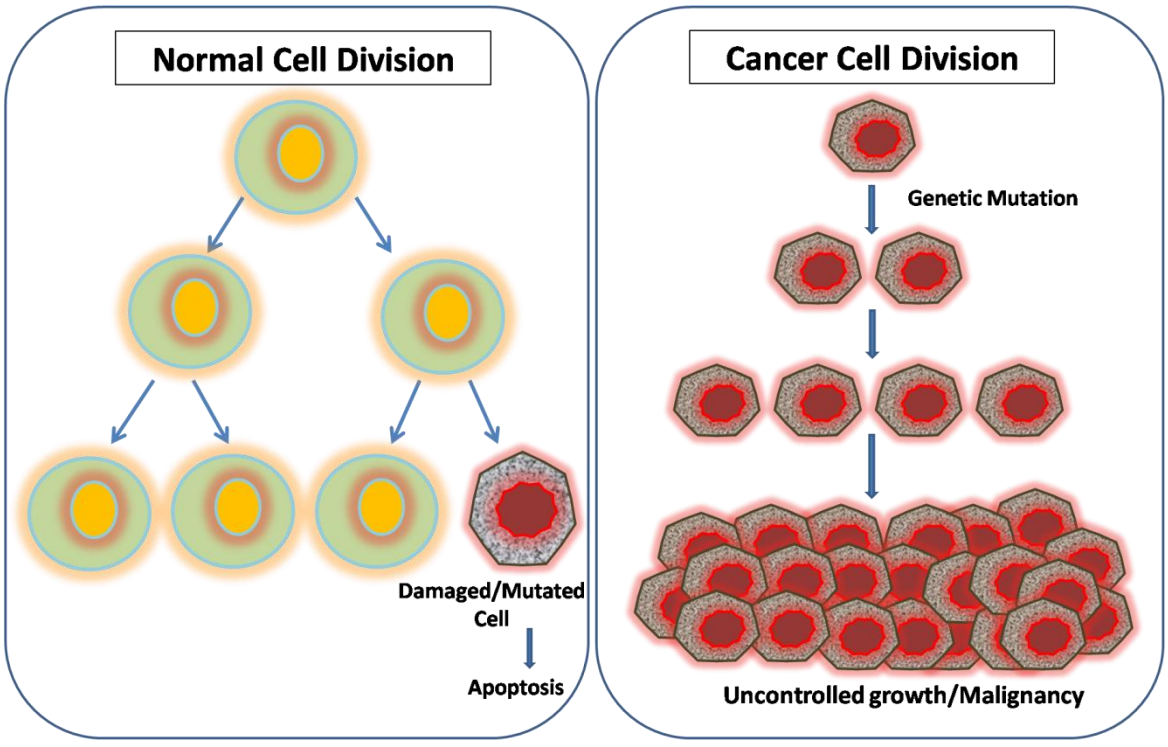


Figure 3.4: Normal cell division versus Cancer cell division

3.3.1. Biomarkers:

Each cell type has a unique molecular indication, referred to as biomarkers, which are identifiable aspects such as levels or activities of a myriad of proteins, genes or other molecular hallmarks. Biomarkers are therefore, an accurate standard or evaluation of natural biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention1. This involves all diagnostic tests, imaging technologies, and any other objective measures of a person's health status. Biomarkers are subject to powerful modulation, and are demanded to improve our knowledge of drug metabolism, drug action, potency and safety. These can also promote molecular representation of diseases, provide information about the course of disease and predict response to therapies.

3.3.2. Carcinoembryonic antigen (CEA):

CEA stands for carcinoembryonic antigen. CEA is a type of glycoprotein molecule that is produced by cells of the gastrointestinal tract during embryonic development. It is produced in very small amounts after birth. The level of CEA in the bloodstream is thus relatively low unless certain diseases - including certain forms of cancer- are present. CEA is most frequently tested in blood. It can also be tested in body fluids and in biopsy tissue. The normal range for CEA in an adult non-smoker is <2.5 ng/ml and for a smoker <5.0 ng/ml.

Conventional techniques such as immunohistopathology (Goldenberg *et al,* 1978), radioimmuno assays (Thomson *et al*, 1969), enzyme linked immune sorbent assay (ELISA) (Zhou *et al,* 2012), reverse transcriptase polymerase chain reaction (RT-PCR) (Yonemura *et al,* 2001) and positron emission tomography for CEA detection have been developed (Flanagan *et al,* 1998). These techniques are time consuming, expensive, require expert handling and involve exposure to harmful radiations.

The urgent need for a rapid, reliable, specific and sensitive method for diagnosis of cancer markers at an early stage of disease has focused on point of care (POC) diagnostics. Biosensors have a number of potential advantages over other methods of cancer diagnosis, especially reduced assay time, portability, high sensitivity,selectivity, simplicity, miniaturized and flexibility. Biosensor-based diagnostics can assist cancer screening and improve the rates of earlier diagnosis and attendant improved forecast. This technology can be particularly useful for enhanced health care delivery in the public setting and to underserved diasporas. Biosensors have potential for multi-target analyses, automation, and cost effective testing**.**

3.4 Paper

Paper is a popular material for writing, printing, drawing and packaging. The potential utility of paper beyond these traditional means arises from its physical properties. It is a highly versatile material since it can be made thin, lightweight and flexible depending on its pulp processing. The chief constituent of paper is cellulose fibre, and this can be highly desirable for various applications as it allows the liquid to flow within its hydrophilic fibre matrix (via capillary action) without the necessity of an active pump or external source. Moreover, cellulose fibres can be suitably functionalised, thus changing properties such as hydrophilicity as well as its permeability and reactivity. Paper has induced much interest as a potential material for sensors and devices in analytical and clinical chemistry given a highly versatile, highly abundant and low cost substrate. These analytical devices can be combined in a fashion that is flexible, portable, disposable and easy to operate. Following the contraption of paper chromatography in the early 20th century, diagnostic devices based on paper began to rise. A paper device for the semi-quantitative detection of glucose in urine was demonstrated in 1956, that further evolved into immunochromatographic paper test strips (lateral flow or dipstick tests), with the pregnancy test kit being a well-known example. Paper

offers many advantages in view of its cheap production, renewable raw materials and a porous structure useful for electrical purposes. Paper can also be a versatile electronic substrate.

3.4.1. Conducting paper:

Conducting papers were reported several decades ago and can be produced by the addition of conducting materials onto it. The conducting materials can be polymers or metalized fibers etc. In this work ,however, paper was made conducting via ion-sputtering technique. Sputtering results in the deposition of metals on the surface of paper and renders it conducting. Alternatively, it is also possible to make paper conducting by deposition of a conducting material simply by dip coating, printing or layer-by-layer coating. Conducting paper electronics has prospects in wide range of applications such as electric field emitters, antistatic coatings, electromagnetic shields and cost effective diagnostics. The advantage of using paper as a substrate for sensors is in the high porosity and large interfacial surface area it has to offer. A fully inkjet-printed passive ultrahigh frequency wireless gas sensor was fabricated by Yang et al. on a paper substrate. Hu *et al.* used conducting paper integrated with 1D nanomaterial (CNT modified silver nanowire films) as a replacement of its metallic counterparts in lithium ion batteries. Trnovec *et al*. utilized gravure and offset printing with poly (3,4-ethylenedioxythiophene)/poly (styrene sulfonic acid) (PEDOT/PSS) to get a paper of surface resistivity $2.6\ \mathrm{k\Omega\ sq^{-1}}$. Approach towards the deposition of nanomaterials on paper has also been made. PEDOT:PSS coated conducting paper with a resistivity of 5.5Ω sq-1 was achieved by Zhou *et al*; Mäkelä *et al*. reported a roll-to-roll printing method for producing polyaniline–dodecylbenzenesulfonic acid (PANI–DBSA) patterns on the paper substrate. Gomes et al developed characterized polyaniline films via thermal inkjet printing

on bond and photographic paper. Kumar et al synthesized a polyaniline based conducting paper employing screen printing for the selective detection of troponin.

Chapter 4

Materials and Methods

4.1. Chemicals and reagents

Whatman filter paper (procured from GE healthcare, UK) was cut into strips (1×2.5cm) to make electrodes, aniline was purchased from Merck India Ltd and was distilled before use. Carcino embryonic antibody monoclonal (anti-CEA), carcino embryonic antigen (CEA), bovine serum albumin (BSA), 1-ethyl-(dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma Aldrich India. Deionized water obtained from Millipore water (18.2 MΩ-cm) purification system has been used in all buffer and solution preparation.Hydrochloric acid (HCl), sodium Chloride (NaCl) and other analytical regaents purchased from Merck India.

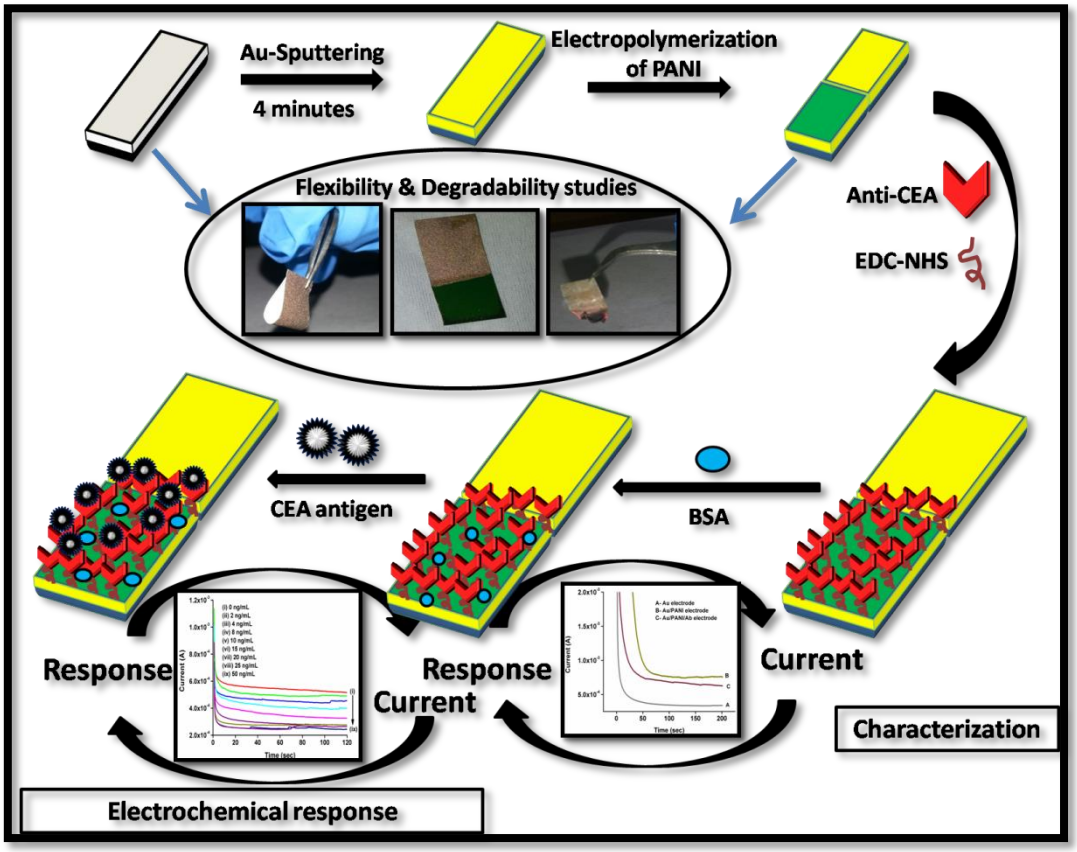
4.2. Experimental:

4.2.1. Fabrication of conducting electrode (PANI/Au@paper electrode):

Whatman filter paper 1 was used as a substrate for the fabrication of paper based biosensing platform. Initially, filter paper was sputtered with gold for 4 minutes using ion sputter (used in coating of SEM sample). Further, electropolymerization of polyaniline was accomplished by adopting cyclic voltametry using a conventional three-electrode cell wherein gold coated paper (Au@paper) a cts as a working, platinum as auxiliary and Ag/AgCl as reference electrodes into a solution containing 1.4 M aniline in 1M HCl in water and is exposed to a potential cycle (n=10) between -0.2V to 0.9V with a scan rate of 20 mV/s. Thus obtained uniform green color stable film of PANI/Au@paper electrode was rinsed with milli Q water to remove any unbound polyaniline molecules.

4.2.2. Fabrication of bioelectrode (BSA/anti-CEA/PANI/Au@paper)

For the fabrication of bioelectrode, monoclonal CEA (1 mg/mL) anti-bodies were immobilized onto PANI/Au@paper via covalent binding using the EDC-NHS chemistry. Where EDC-NHS is used to activate and couple the carboxylic (-COOH) group of the antibody with amine group of polyaniline. Further, BSA (0.1% in PBS) was used for blocking of non-specific active sites of anti-CEA/PANI/Au@paper electode. The fabricated BSA/anti-CEA/PANI/Au@paper bioelectode was washed several times to remove any unbound antibodies and BSA, respectively. These bioelectrodes were stored at 4°C till further use. Scheme 1 shows the step wise fabrication of polyaniline modified paper sensor.



Scheme 1: Schematic of fabricated anti-CEA/PANI/Au@paper electrode.

4.3 Instrumentation:

4.3.1. Sputter equipment:

Sputtering is a technique that can be used to deposit thin films of a material onto a surface (substrate). By first creating a gaseous plasma and then accelerating the ions from this plasma into some source material (target), the source material is eroded by the arriving ions via energy transfer and is ejected in the form of neutral particles - either individual atoms, clusters of atoms or molecules. As these neutral particles are ejected they will travel in a straight line unless they come into contact with something - other particles or a nearby surface. If a substrate such as paper is placed in the path of these ejected particles it will be coated by a thin film of the source material. In this work filter paper was coated with gold using Hitachi Ion Sputter E1010.

Figure 4.1: Ion sputter instrument

4.3.2. Fourier Transform – Infra Red Spectroscopy:

FT-IR is an analytical technique used for the structural characterization of organic materials. This technique measures the absorption of various infrared light wavelengths by the material of interest. These infrared absorption bands identify specific molecular components and structures. In order for a vibrational mode in a molecule to be IR active, it can be associated with changes in the permanent dipole. An infrared spectrum represents a fingerprint of a sample having absorption peak that correspond to the frequencies of vibration between the bonds of the atoms making up the material. Because each different material is a unique combination of atoms, no two compounds produce the exact infrared spectrum. FTIR spectrophotometer has a spectrum in the range of 400-4000 cm-1. Fourier transform infrared (FTIR) spectra of modified paper electrodes were recorded using Bruker Optics, Vertex 70V in transmission mode.

Figure 4.2: FTIR instrument

4.3.3. X-ray diffraction:

X-ray diffraction has been in use in two main areas, for the fingerprint characterization of crystalline materials and the determination of their structure. Each crystalline solid has its unique characteristic X-ray powder pattern which may be used as a "fingerprint" for its identification. Once the material has been identified, X-ray crystallography may be used to determine its structure, i.e. how the atoms pack together in the crystalline state and what the interatomic distance and angle are etc. The crystallinity of the modified paper electrode was investigated via X-ray diffraction (XRD) studies (Bruker D-8 Advance). A monochromatic X-ray beam with Cu Kα radiation ($\lambda = 1.5406$ Å) was used to record the spectrum.

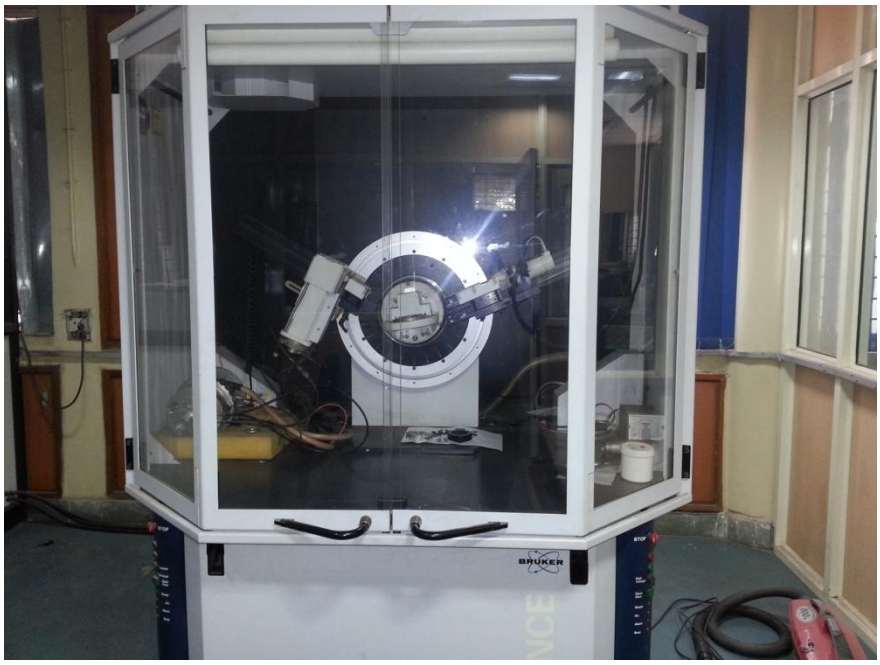


Figure 4.3: XRD instrument

4.3.4. Scanning Electron Microscopy & Energy Dispersive X-ray Spectroscopy:

The scanning electron microscopy is a type of electron microscope that produces images of a sample by scanning it with a focused beam of electrons. The types of signals produced by SEM include secondary electrons (SE), reflected or back-scattered electrons (BSE), photons of characteristic X-rays and light absorbed current and transmitted electrons. The most common SEM mode is detection of secondary electrons emitted by atoms excited by the electron beam. By scanning the sample and collecting the secondary electrons that are emitted using a special detector, an image is formed displaying the topography of the surface.

EDX is an analytical technique that can be used for the elemental analysis or chemical characterization of samples. The surface morphology was investigated by the scanning electron microscopy (Hitachi S-3700N) and energy dispersive X-ray (EDX) analysis was performed with same instruments.

Figure 4.4: SEM instrument

4.3.6. Electrochemical Impedance Spectroscopy:

The electrochemical impedance spectroscopic technique is used to study the changes occurring at the interface of the electrode and solution as it is sensitive and is capable of providing information about the modification carried out on the electrode surface. It is a powerful technique that reveals the changes occurring in the charge transfer processes at the electrode/solution interface. The resistance is given by Ohm's law: R = E/I, when a dc signal is applied to an interface where, E and I are the applied voltage and resulting current, respectively. When an AC signal is applied to an interface, Ohm's law is again applicable, but the measured quantity is called the impedance Z and is given by Z = Ep / Ip, where Ep and Ip are the applied peak voltage and the measured peak current respectively. The AC impedance technique is commonly applied for the investigations of electrode kinetics and the reaction rates are related to the charge transfer resistances. Electrochemical impedance is usually measured by applying an AC potential to an electrochemical cell and measuring the current through a cell.

4.3.7. Chronoamperometry:

Chronoamperometry is a technique which a step potential is applied at working electrode and steady state current is measured in a quiet unstirred cell as a function of time. This technique can be utilized to determine diffusion constant and can be helpful in investigating kinetics and mechanisms. During this process a diffusion layer is formed between the surface of electrode and solution, which controls the transfer of analyte from the solution of higher concentration towards electrode surface and thus there is a concentration gradient present between solution to the electrode surface. In most electrochemical cells this decay is much slower than the charging decay, cells with no supporting electrolyte are notable exceptions. This technique is used to with a three electrode system. Since the current

is integrated over relatively longer time intervals, chronoamperometry technique gives a better signal to noise ratio in comparison to other amperometric techniques. The electrochemical studies were carried out by an Autolab Potentiostat/Galvanostat (Metrohm,Netherlands) using a conventional three-electrode cell with the polyaniline modified paper (PANI/Au@paper) as working electrode, platinum as auxiliary electrode and Ag/AgCl as the reference electrode in phosphate buffer saline (PBS,50mM,pH 7.4) containing 5mM $[Fe(CN)_6]^{3-/4-}$.

Figure 4.5: Electrochemical analyzer

Chapter 5

Results and Discussion

5.1 Electrochemical deposition of PANI

Figure 5.1 shows results of the cyclic voltammtery studies relating to the formation of polyaniline (PANI) on the surface of gold coated paper (Au@paper) electrode. Oxidation peak seen near 0.2V corresponds to the formation of emaraldine, a partially oxidized form of aniline and peak near 0.8V relates to the formation of pernigraniline, a fully oxidized form of aniline (Akundy *et al*, 2002). With increase in the thickness of the film there is a gradual shift in both anodic and cathodic peak potential (Attasi *et al*, 2013). During electrochemical deposition, polyaniline is switched from its reduced form (leucoemaraldine, transparent), to its oxidized form (emaraldine/ perningraniline, green). The reduced form of polyaniline depicts its insulating nature whereas the oxidized form indicates its conducting behavior. Inset of figure 9 shows the fabricated PANI/Au@paper electrode that exhibits a green colour uniform deposition of polyaniline.

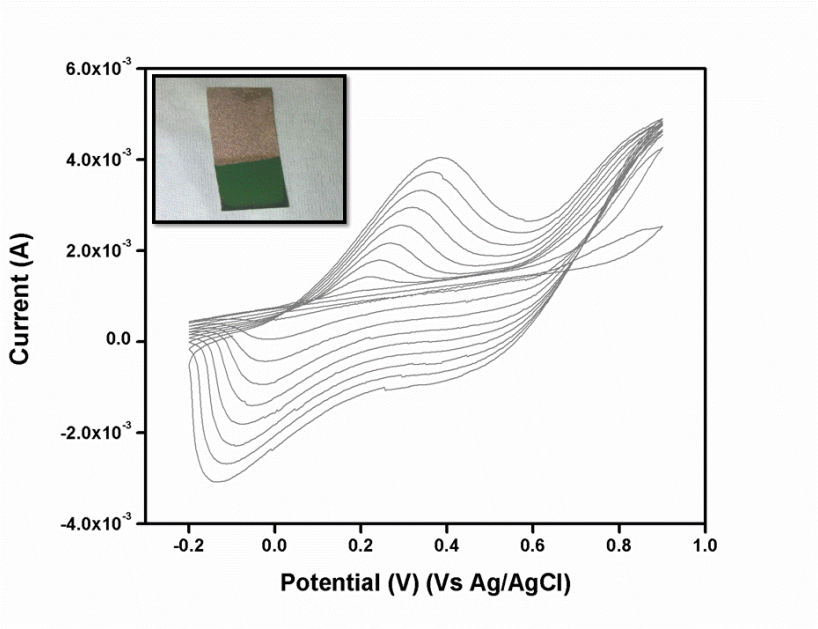


Figure 5.1: Electrochemical polymerization of aniline via cyclic voltametery on gold sputtered paper electrode (Au@paper); Inset showing the fabricated PANI/Au@paper electrode.

5.2 Structural and morphological studies

5.2.1. X-ray diffraction studies:

The results of X-ray diffraction (XRD) studies of the fabricated PANI/Au@paper electrode conducted in between 2θ angle 30˚ to 70˚ are shown in figure 5.2. The diffraction peaks observed at 34.2° and 46.3° correspond to (221) and (115) planes, indicating the formation of PANI over Au@paper electrode (JCPDS No: 53-1891) (Subramanian *et al*, 2012).

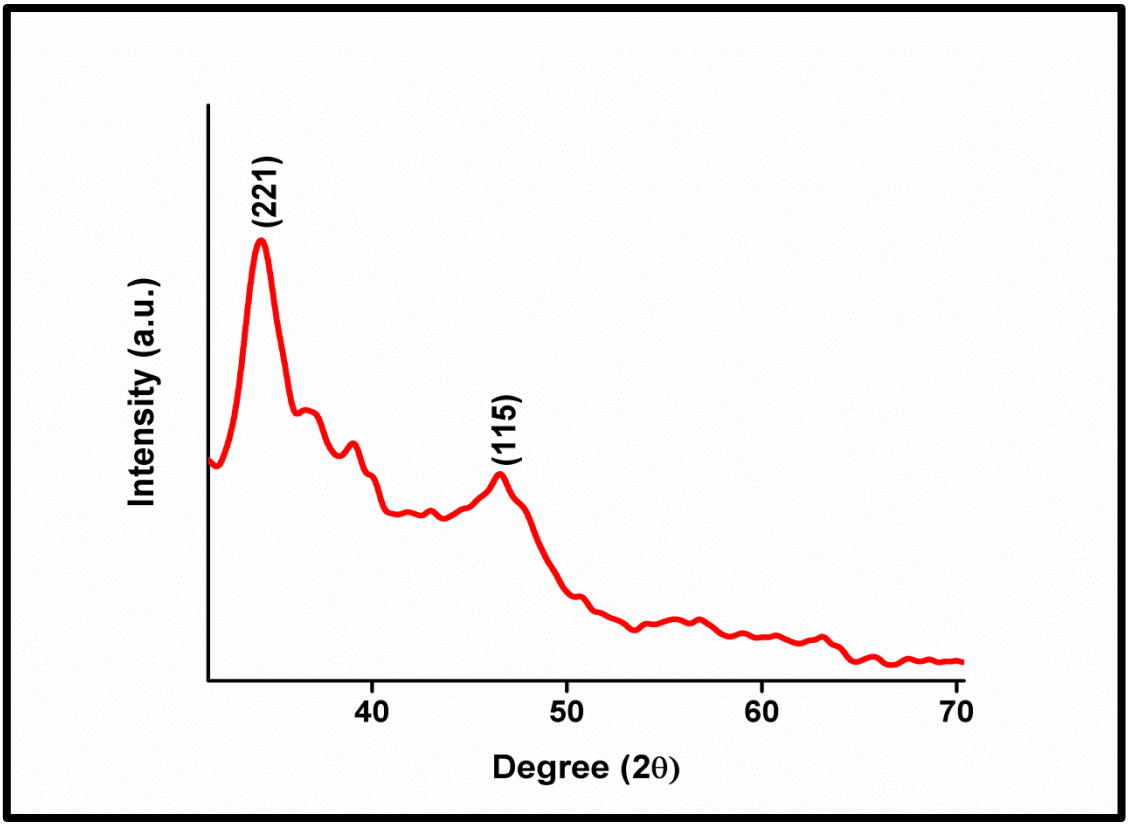


Figure 5.2: XRD pattern of PANI/Au@paper electrode

5.2.2. Scanning electron microscopy studies:

The surface morphology of Au@paper, PANI/Au@paper and anti-CEA/PANI/Au@paper electrodes was investigated via scanning electron microscopy (SEM) as shown in figure 5.3A-C. The SEM micrograph of Au@paper electrode (Figure 5.3A) shows smooth and homogenous surface whereas in figure 5.3B the thread like porous structure is found to cover the Au@paper surface confirming the uniform deposition of polyaniline. However, after anti-CEA immoblization (Figure 5.3C) surface morphology of the PANI/Au@paper electrode shows the filled pores in the fibrous structure of PANI with shiny appearance.

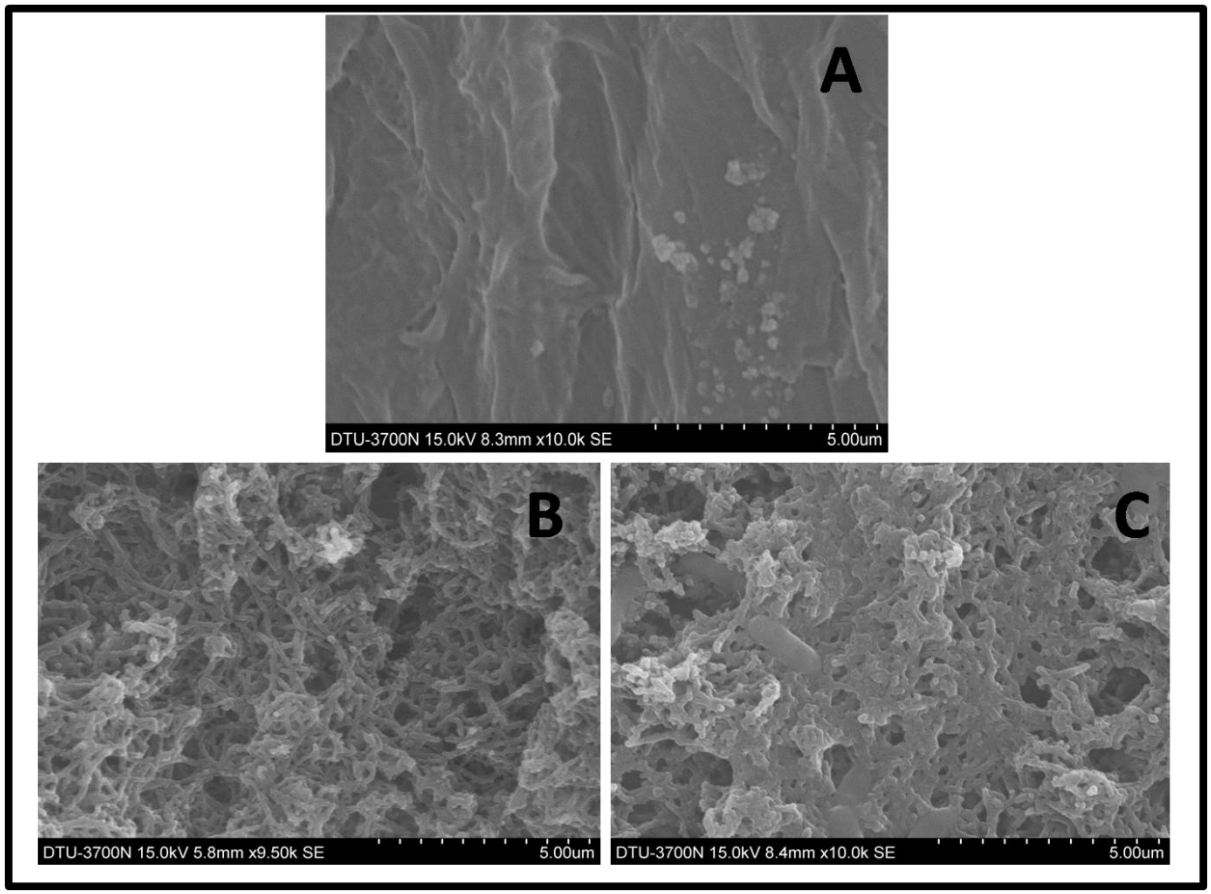


Figure 5.3: SEM images A: SEM image of Au@paper electrode; **B:** SEM image of PANI deposited Au@paper electrode (PANI/Au@paper); **C:** SEM image of anti-CEA functionalized PANI/Au@paper electrode (anti-CEA/ PANI/Au@paper).

5.2.3. Fourier transformed infrared spectroscopy (FT-IR) studies

The FT-IR spectra recorded for PANI/Au@paper and anti-CEA/PANI/Au@paper are shown in figure 5.4A and B, repectively. The FTIR spectrum of PANI/Au@paper exhibits characterstic absorption band near 1466 and 1618 cm^{-1} representing benzenoid and quinoid ring of PANI (Jagadeesan *et al*, 2012). The other high intensity peak found at 1108 cm^{-1} corresponds to the C-H in plane bending motion of the quinoid ring. On the other hand the peak present at 1298 cm^{-1} indicates C-N stretching of aromatic amine whereas the broad band at 3440 cm^{-1} is due to N-H stretching of $-NH_2$ groups present in PANI that helps in covalent immobilization of antibodies (Xu *et al*, 2010). After the anti-CEA immobilization, the peaks observed at 1531 cm^{-1} correspond to the C–N bond formed between $-NH_2$ group of PANI and –COOH groups of F_c region of antibodies, indiating covalent immobilization of anti-CEA (Kumar *et al*, 2013). The additional peak found at 1700 cm^{-1} and 3300 cm^{-1} may be attributed to C=O stretching for amide I and –NH stretch in amide II present in protein (anti-CEA), respectively (figure 5.4B).

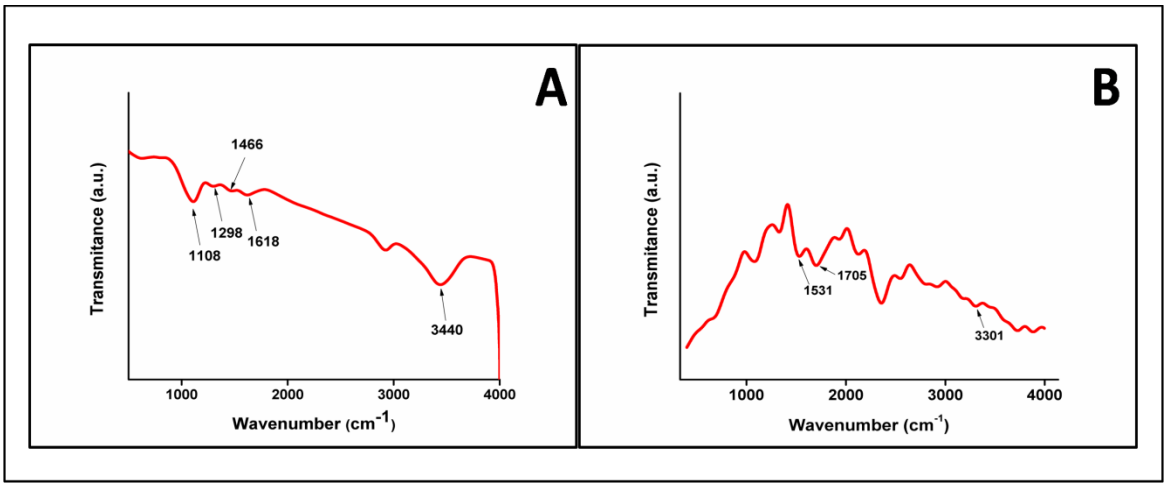


Figure 5.4: FTIR spectra of A: PANI/Au@paper and B: anti-CEA/PANI/Au@Paper.

5.3 Flexibility studies:

The results of flexibility studies of the PANI/Au@paper electrode are shown in figure 5.5A-D. The optical image in figure 5.5A exhibits green colour coating of PANI over Au@paper with high degree of flexibility. The SEM image in figure 5.5B shows the dark and light area of PANI and Au, respectively at PANI/Au@paper electrode which confirms the successful coverage of PANI over the modified paper fibers (Au@paper). The after effect of bending (+180° and – 180°) of Au@paper electrode results in the formation of crack along the line of bending as shown in the SEM micrograph (figure 5.5C). This may perhaps be attributed to the deformation mismatch between the metal (Au) and the paper substrate when subject to stress (Siegel *et al,* 2010). It appears that the PANI modified Au@paper overcomes this problem by conferring improved flexibility to the Au@paper electrode as shown in figure 5.5D. Further, it was observed that no cracks developed during bending of PANI/Au@paper electrode. This clearly proves that polyaniline provides a strong hold over the sputtered gold layer to prevent the delamination of Au from paper surface.

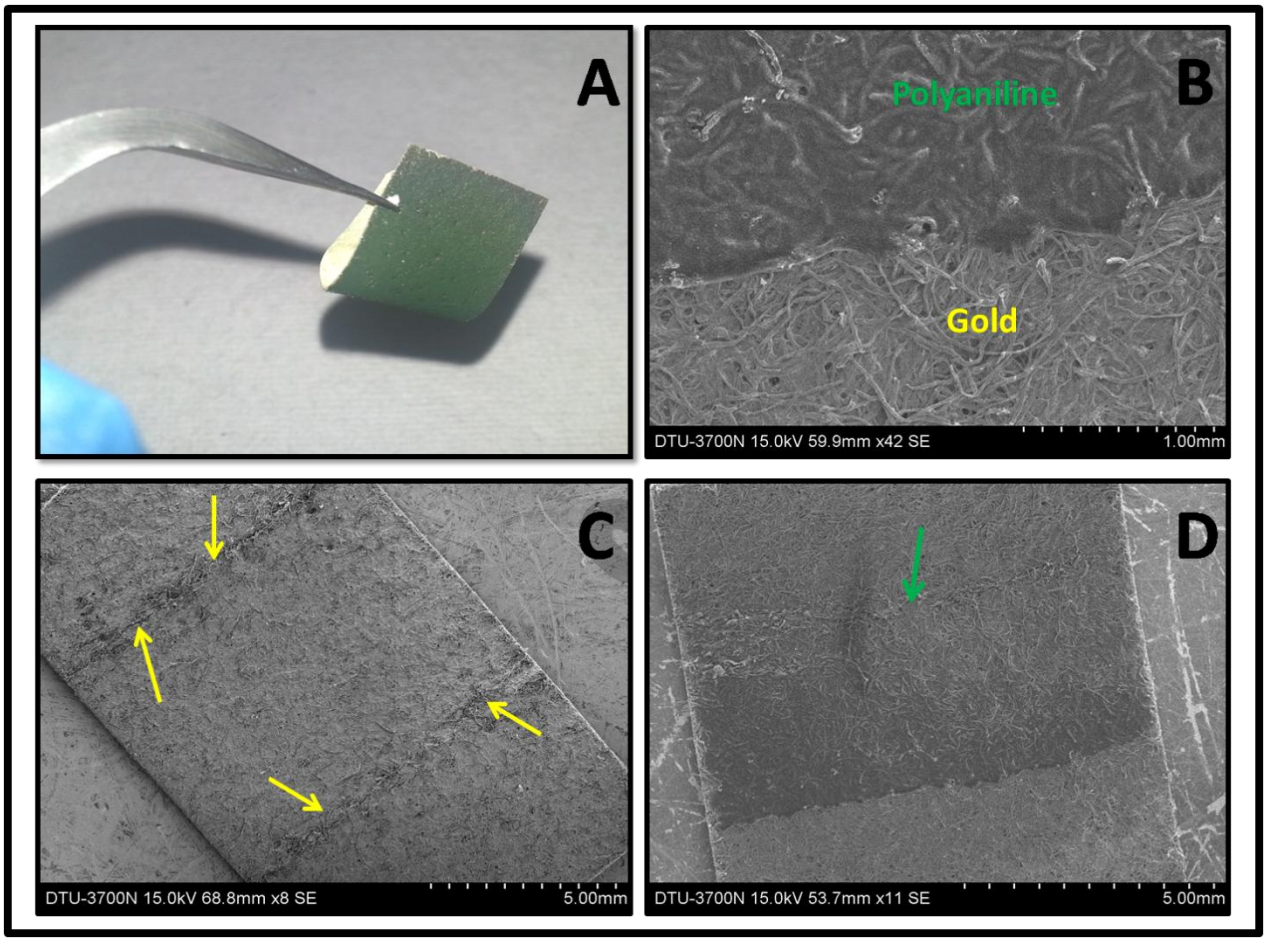


Figure 5.5: (A)**:** Optical image of PANI/Au@paper, showing flexible nature. The SEM images of (B) Au@paper and PANI/Au@Paper, confirm uniform deposition of PANI that tightly bound the modified paper fibers. (C) Au@paper electrode after folding (+180° and –180°), developed cracks along the line of folding indicated by the yellow arrows and (D) PANI/Au@paper electrode, no visible cracks have developed along the line of folding after deposition of PANI

5.4 Electrochemical studies:

Electrochemical impedance spectroscopy (EIS) is an effective tool for studying the interfacial properties of surface modified electrodes. In order to investigate the charge transfer phenomena occurring at the paper/solution interface EIS studies were carried out. The Nyquist plot obtained for gold coated paper (Au@paper), PANI deposited on gold coated electrode (PANI/Au@paper) and anti-CEA immobilized on PANI coated gold electrode (anti-CEA/PANI/Au@paper) at 0.03 V biasing potential conducted in the frequency range,

100 kHz to 0.1 Hz is shown in figure 5.6A. The diameter of the semicircle in the Nyquist plot gives magnitude of the charge transfer resistance (R_{ct}) of electrode that depends on the dielectric characteristics of the electrode/electrolyte interface. The curve fitting has been done assuming Randles circuit [$R_s(R_{ct}C_{dl})$] of the electrochemical cell. The R_{ct} value obtained for PANI/Au@paper electrode (~20.6 **Ω**, curve ii) is found to be lower than that of Au@paper electrode (~268**Ω**, curve i). This suggests that the deposition of PANI onto the Au@paper surface enhances the electron transfer kinetics between electrolyte and Au@paper electrode resulting in the enhancement of the electrochemical properties of PANI/Au@paper electrode. However, the observed R_{ct} value (24.6 **Ω**, curve iii) for the anti-CEA/ PANI/Au@paper electrode is higher than that of the PANI/Au@paper electrode (~20.6 **Ω**,curve ii). The significant increase in the R_{ct} value is attributed to the immobilization of anti-CEA that cause the hindrance due to macromolecular structure of antibodies that perhaps obstruct electron transfer owing to their insulating nature. The heterogeneous electron transfer rate constant (K_{ct}) of Au@paper, PANI/Au@paper electrode and anti-CEA/PANI/Au@paper electrode can be calculated using Eq.1 (Bardea *et al*, 2000):

$$K_{ct} = RT/n^2F^2AR_{ct}[S] \qquad \text{Eq. 1}$$

Where R is gas constant, T is absolute temperature (T=298 K), n is the number of electron transferred per molecule of redox probe, [S] is the concentration of redox probe (mol/cm3), A is the electrode area (cm^2) and F is faraday constant. The heterogenous electron transfer rate constant (Kct) value of the Au@paper, PANI/Au@paper and anti-CEA/PANI/Au@paper electrode has been estimated to be $1.98x\ 10^{-4}$ cms^{-1}, $2.5x10^{-3}$ cms^{-1} and $2.1x10^{-3}$ cms^{-1}, respectively. This indicates that the PANI/Au@paper electrode exhibits faster electron transfer kinetics as compared to the anti-CEA/PANI/Au@paper and Au@paper electrode (Table 5.1). Interestingly, Kct of the PANI/Au@paper electrode is found to be nearly 2 orders

of magnitude higher compared to previous reported works of similar kind (Kumar *et al*, 2015a;Kumar *et al*, 2015b).

S.No	Material coated on paper	Charge Transfer Resistance (R_{ct})	Heterogeneous electron transfer rate constant (K_{ct})
1	Gold (via sputtering)	268.1 Ω	$1.98x\ 10^{-4}$ cms^{-1}
2	PANI/Au electrode	20.59 Ω	$2.5x10^{-3}$ cms^{-1}
3	Anti-CEA/PANI/Au electrode	24.60 Ω	$2.1x10^{-3}$ cms^{-1}

Table 5.1: Electrochemical properties of modified paper electrode

Figure 5.6B shows results of the chronoamperometric studies (current versus time) obtained for (i) Au@paper (ii) PANI/Au@paper (iii) anti-CEA/PANI/Au@paper, at 0.5 V on every 0.1s. The increase in the value of the electrochemical current of the PANI/Au@paper electrode (~0.76 mA) than that of the Au@paper electrode (~0.34 mA) confirms polymerization of PANI on the Au@paper surface which enhances permeability of the redox couple $[Fe(CN)_6]^{3-/4-}$. Next, a decrease in the electrochemical response current of the anti-CEA/PANI/Au@paper electrode (~0.68 mA) than that of PANI/Au@paper electrode (~0.76 mA) is due to the immobilization of anti-CEA which perhaps hinders the electron transport owing to its insulating and macromolecular nature.

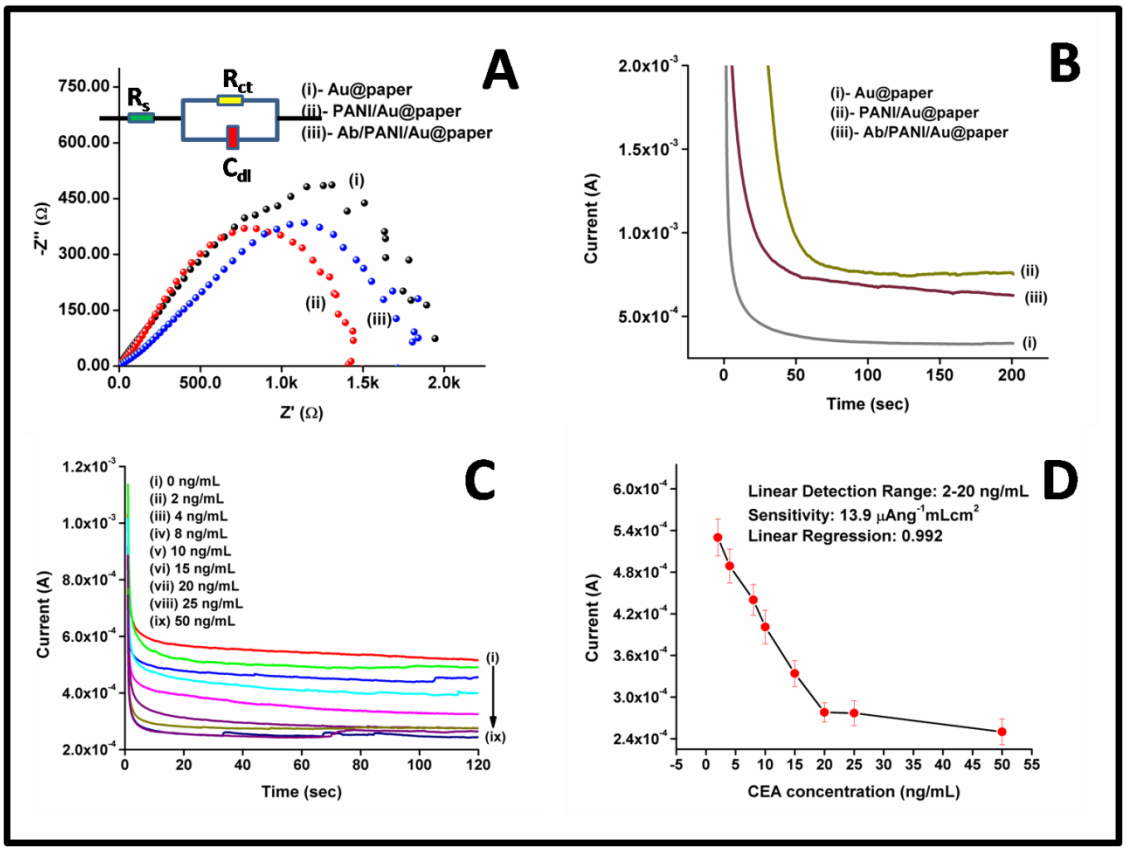


Figure 5.6: Electrochemical studies conducted on modified paper electrode. A: Electrochemical impedance spectra and B: chronoamperometry plot obtained for (i) Au@paper (ii) PANI/ Au@paper (iii) anti-CEA/ PANI/ Au@paper. C: Electrochemical response studies of anti-CEA immobilized conducting electrode (BSA/ anti-CEA/ PANI/ Au@paper) at different concentrations of CEA. D:calibration plot between magnitudes of current recorded and CEA concentration.

The electrochemical response of the anti-CEA/PANI/Au@paper electrode (Figure 5.6C) was conducted as a function of carcinoembryonic antigen (CEA) concentration (2-50 ng/mL) in PBS (50mM, pH 7.4, 0.9% NaCl) containing 5mM $[Fe(CN)_6]^{3-/4-}$ using chronoamperometry with an incubation time of 10 min. For this purpose, bovine serum albumin (0.1% in PBS) was added to the anti-CEA/PANI/Au@paper electrode to block the non-specific sites. After rinsing with PBS, the electrode was used for the sensing studies. Thereafter, magnitude of the current measured on addition of every CEA concentration. It can be seen that current decreases on addition of CEA due to formation of electrically insulating antigen-antibody complexes due to specific interaction of the CEA and anti-CEA that perhaps diminishes

charge transfer via $[Fe(CN)_6]^{3-/4}$ leading to reduction in amperometric current at the paper electrode surface. Figure 5.6D shows the calibration curve obtained between the response current and CEA concentration in the range of 2-50 ng/mL. However, a linear relationship obtained between 2-20 ng/ml with a sensitivity of 7.7 $\mu A ng^{-1} mL cm^{-2}$ and follows Eq.(2)

$$I\ (A) = -13.9\ \mu A\ mL\ ng\text{-}1 \times [\text{CEA concentration}] + 549\ \mu A;\ R^2 = 0.99 \qquad \text{Eq. 2}$$

Furthermore, a control experiment was performed to check cross reactivity of the PANI/Au@paper with CEA antigen (in the absence of antibodies). However, no significant change in current response was observed for the PANI/Au@paper in the absence of antibodies as a function of CEA concentration (Figure 5.7A), which clearly indicates the absence of cross reactivity.

5.5 Reproducibility, Selectivity and Stability studies

Figure 5.7B shows the electrochemical response of five different paper electrodes fabricated via the same set of procedure with constant surface area as evident by the low value of relative standard deviation (RSD) of 2.73% (mean value 604 μA). This suggests appreciable repeatability of BSA/PANI/Au@paper electrode. The selectivity of BSA/anti-CEA/PANI/Au@paper electrode was investigated in the presence of cardiac troponin 1 (CTn-1), endotheline-1 (ET-1) and cytokeratin-19 fragment (CYFRA-21-1) (2 ng/ml). Figure 5.7C shows decrease in the electrochemical current response on the addition of carcino embryonic antigen (CEA) (2 ng/ml). Thereafter, we did not observe any significant current change in electrochemical current response on addition of the other interferants indicating high selectivity of BSA/anti-CEA/PANI/Au@paper electrode. Figure 5.7D shows the results of storage stability studies conducted on BSA/anti-CEA/PANI/Au@paper electrode at regular interval of 2 days. It has been found that the electrode remains fairly stable uptil 22 days. The

fabricated immunosensor anti-CEA/PANI/Au@paper electrode retained about 86% activity even after 19 days when stored at 4°C after which the response decreased to about 73% in 22 days.

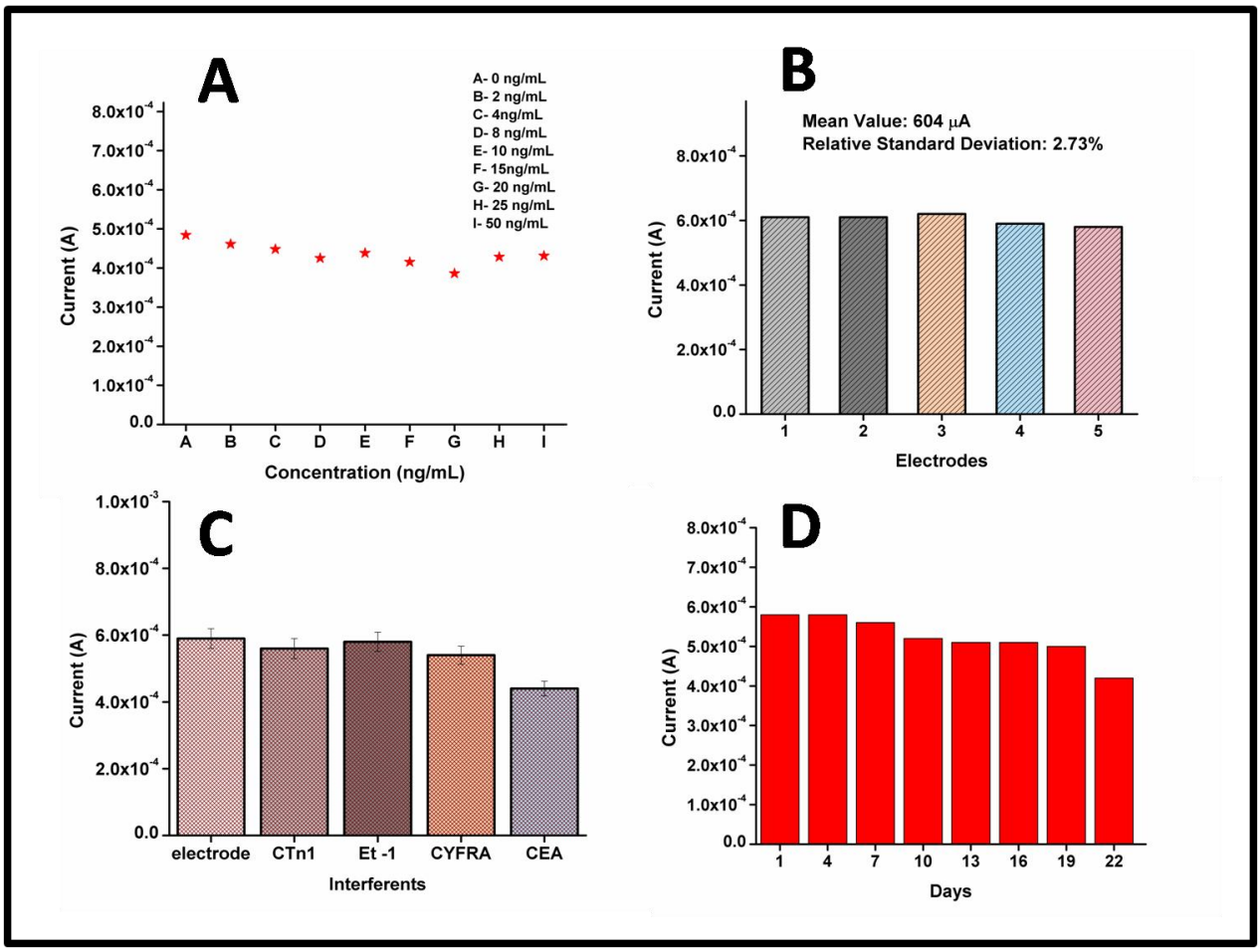


Figure 5.7: A: Control experiment of PANI/Au@paper electrode as a of CEA concentration (0-50 ng/mL). B: Electrochemical current response of five different BSA/anti-CEA/PANI/Au paper electrode fabricated via the same set of procedure. C: Electrochemical current response of obtained as a function of other interferants D: electrochemical current response of BSA/anti-CEA/PANI/Au paper electrode measured as a function of time (days).

5.6 Real sample analysis

Further, BSA/anti-CEA/PANI/Au@paper electrode was validated using CEA concentration obtained by serum samples of cancer patients via immunoassay technique. The serum samples were obtained from blood samples of cancer patients and processed in Rajiv Gandhi

Cancer Institute and Research Centre (RGCI & RC) in Rohini, Delhi, India. Concentration of CEA in the serums of cancer patients was measured at RGCI & RC using the VITROS CEA reagent Pack and the VITROS CEA Calibrators on the VITROS ECi/ECiQ Immunodiagnostic Systems, VITROS 3600 Immunodiagnostic System and VITROS 5600 Integrated System using Intellichecks Technology. The sensing results obtained (Table 5.2) with the help of the fabricated paper electrode were in accordance with the results of those obtained using immunoassay. The results are indicative of a high positive correlation between the serum samples and the standard samples with acceptable RSD values.

S.No	CEA Concentration (ng/mL) Determined using ELISA	Amperometric Current (mA) obtained with standard CEA sample	Amperometric Current (mA) obtained with serum sample	%RSD
1	2	0.52	0.53	1.35
2	4	0.48	0.49	1.46
3	8	0.44	0.47	4.66
4	10	0.40	0.44	6.73
5	15	0.33	0.38	9.96

Table 5.2: Determination of carcinoembryonic antigen concentration in serum samples using BSA/anti-CEA/PANI/Au paper electrode.

The sensing characteristics of BSA/anti-CEA/PANI/Au paper electrode are summarized in Table 5.3 along with those reported in literature. It can be clearly seen that the fabricated electrode is more efficient than those reported in conventional electrodes and is much higher in comparision to the paper electrode. This electrode can be easily decomposed by simple incineration method (Figure 5.8). The resulting ash was examined by energy dispersive X-ray (EDX) technique. The results of EDX analysis confirm the absence of any toxic or pernicious elements in this conducting paper ash (Figure 5.9).

Table 5.3 shows response characteristics of BSA/anti-CEA/PANI/Au@paper electrode along with those reported in literature

S. No	Substrate	Material	Fabrication Method	Detection Method	Linear range and Sensitivity	Biodegradability	Stability (days)	References
1	ITO	Au-Chitosan	Drop cast	Cyclic Voltammetry	2-20 ng/mL	No		(Lin *et al.* 2007)
2	ITO	Thiol derivated nanogold	Drop cast	Electrochemical Impedance Spectroscopy	5-80ng/mL	No	20	(Zeng *et a*l. 2015)
3	Glassy carbon electrode	AuNP,MWCNT ,Chitosan	Drop cast	Differential Pulse Voltametry	0.3-2.5 & 2.5-20 ng/mL and ------	No	30	(Huang *et al.* 2010)
4	Whatman paper 1	PEDOT:PSS/RGO, Ethylene Glycol	Dip Coating	Amperometry	2-8 ng/mL and 25.8 µA(ng/mL)-1cm2	Yes	21	(Kumar *et al.* 2015)
5	Whatman paper 1	Au/Polyaniline	Sputtering of Au followed by electrochemical deposition of PANI	Amperometry	2-20 ng/mL and 13.9 µA mL ng-1	Yes	22	Present work

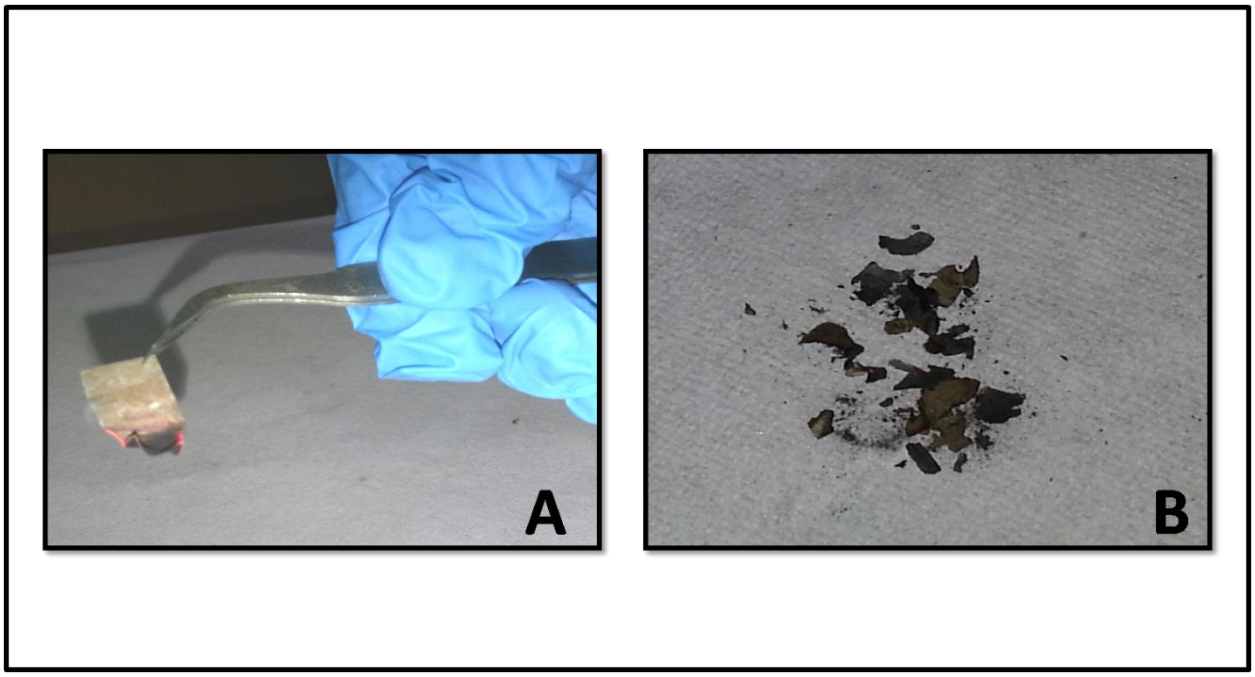


Figure 5.8: A and B showing easy to dispose off nature of the electrode

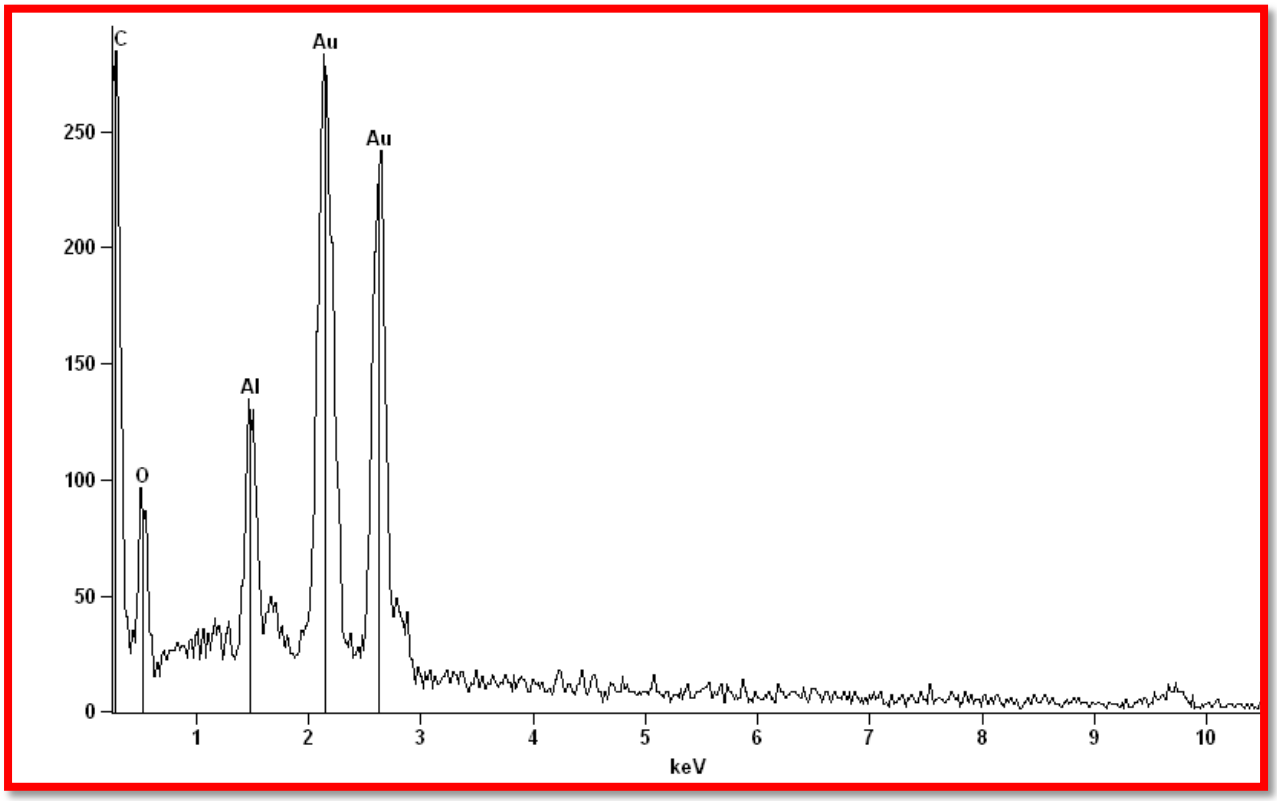


Figure 5.9: Energy dispersive X-ray (EDX) analysis

Chapter 6

Conclusions

It has been demonstrated that a flexible, lightweight and disposable paper sensor based on polyaniline modified paper, can be fabricated . It has been found that polyaniline modified paper exhibits improved flexibility, high heterogenous electron transfer rate constant, large surface area and provides a suitable matrix for covalent immobilization of biomolecules. The fabricated PANI/Au@paper electrode has been used for cancer biomarker (CEA) detection, and is found to be highly sensitive (13.9 μAng^{-1} $mLcm^{-2}$) with good linearity in the physiological range 2-20 $ngmL^{-1}$ and a lower detection limit of 1.36 $ngmL^{-1}$. This simple, flexible, lightweight platform has immense potential for smart point-of-care devices, gas sensors, strain sensors and flexible electronics etc.

Chapter 7

Future Perspective

Moving the society's dependence away from the conventional biochemical assays to low cost clinical diagnostic devices may be regarded as a quintessential progression in the POC and effective disease management. The fabricated electrode serves as a potential platform for applications in point-of-care diagnostics. Future efforts should be directed towards further improving the sensitivity and stability of the fabricated electrode. A possible strategy involves the integration of metal oxide doped conducting polymers for better sensitivity and lower detection limits. Moreover, the fabricated electrode should be explored for applications in multianalyte systems.

The electrochemical biosensors integrated with miniaturized electronics hold huge prospects for fabrication and commercialization of point-of-care devices. Conventional potentiostats which allow several electrochemical techniques like chronoamperometry, cyclic voltammetry, electrochemical impedance spectroscopy are pretty costly which is why alternative cost effective potentiostats can be developed which would allow the characterization and detection of analytes via chronoamperometry or impedance spectroscopy. Moreover, the miniaturized potentiostat can be interfaced with a microfluidic system integrated immunosensor for the detection of cancer biomarkers. The fabricated immunosensor could be incorporated into a microfluidic system that can be integrated with a miniaturized potentiostat for the electrochemical detection of cancer biomarkers.

Chapter 8

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