SYNTHESIS AND CHARACTERIZATION OF CONDUCTING POLYANILINE/IRON OXIDE (PAni/Fe$_3$O$_4$) NANORODS FOR IMMUNOSENSING APPLICATION

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DEDICATION

This work is dedicated to my beloved parents; Alhaji Ado Mahmoud and Hajiya Zainab Musa, my darling wife, Fatima and my children, whose love, understanding and encouragement made the completion of this work possible.

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ABSTRACT

The development of the current electrochemical biosensors is geared towards the simplification and improvement of the transduction pathway. Nevertheless, recently the modification of transducers with nanomaterials has been extensively studied and conveys many advantages. In this work, electrochemical immunosensors were obtained after the modification of FTO coated glass with the synthesize conducting PAni-Fe_3O_4 nanocomposites forming a modified (PAni-Fe_3O_4/FTO) electrode on which anti-β-Amyloid was immobilized on the new electrode for detection of Alzheimer's diseases. The new improve physical and electrical properties of the nanocomposites such as enhanced conductivity and area/volume ratio facilitates transduction process and biomolecules adsorption. The PAni-Fe_3O_4 have a nanorod structure and are electrically conductive. The presence of small red shift and a slight blue shift in UV-Vis spectra is an indication of a successful electrode modification. FT-IR spectrum of immunosensor exhibits additional absorption bands at 1932 cm^{-1} (carbonyl stretch), 3213 cm^{-1} and 2113 cm^{-1} (N–H stretching), indicating the immobilization of anti-β-Amyloid on PAni/Fe_3O_4/FTO surface. The AFM studies showed the value of the roughness of the modified electrode to be 50.13 nm which is higher than that of the bare FTO (27.49 nm) thus, indicating increased available surface and porous morphology that allows more adsorption of antibodies on the surface. The immunosensor exhibited a specific response after incubation with β-Amyloid at the range of 0.01 down to 0.00001 µg mL^{-1}. The linear regression equation was found to be $y = 0.04431x + 0.00142$ and correlation coefficient of 0.947. The limit of detection was calculated to be 0.0023 µgmL^{-1} at 3σ. The modified electrode showed a better sensitizing effect and stability as immobilization support/matrices. The results of these studies have implications for the application of this interesting matrix (PAni-Fe_3O_4) toward the development of other biosensors.

ABSTRAK

Evolusi daripada biosensor elektrokimia terkini mencerminkan pemudahan dan peningkatan dalam laluan transduksi. Walau bagaimanapun, akhir-akhir ini, pengubahsuaian transduser dengan bahan nano telah banyak dikaji dan menunjukkan banyak kelebihan. Dalam kajian ini, immunosensor elektrokimia telah diperolehi selepas pengubahsuaian kaca bersalut FTO dengan pengalir komposit nano PAni-Fe_3O_4 membentuk elektrod yang diubah suai (PAni-Fe_3O_4/FTO) dimana anti-β-Amyloid dipegunkan pada elektrod baru untuk pengesanan penyakit Alzheimer. Peningkatan sifat-sifat baru fizikal dan elektrik daripada komposit nano ini membawa kepada peningkatan kekonduksian dan nisbah luas/isipadu memudahkan proses transduksi dan penjerapan biomolekul. PAni-Fe_3O_4 mempunyai struktur nanotiub dan konduktif secara elektrik. Adanya sedikit anjakan merah dan biru dalam spektra UV-Vis menunjukkan pengubahsuaian elektrod telah berjaya. Spektra FT-IR immunosensor memperlihatkan jalur penyerapan tambahan pada 1932 cm^{-1} (regangan karbonil), 3213 cm^{-1} dan 2113 cm^{-1} (regangan N-H), menunjukkan kepegunan anti-β-Amyloid pada permukaan PAni-Fe_3O_4/FTO. Kajian AFM menunjukkan nilai kekasaran elektrod yang diubahsuai menjadi 50.13 nm yang mana adalah lebih tinggi daripada yang FTO yang tidak disalut (27.49 nm), jadi ia menunjukkan peningkatan permukaan morfologi berliang yang membolehkan lebih banyak penjerapan antibodi di permukaan. Immunosensor itu menunjukkan respon tertentu selepas pengeraman dengan β-Amyloid pada julat 0.00001-0.01μg mL^{-1}. Persamaan regresi linear didapati y = 0.04431 + 0.00142 dan pekali korelasi 0.947. Had pengesanan telah dikira iaitu 0.0023 μgmL^{-1} pada 3σ. Elektrod yang diubahsuai tersebut menunjukkan kesan pemekaan yang lebih baik dan stabil sebagai penyokong pemegunan/matriks. Keputusan kajian ini mempunyai implikasi kepada penggunaan matriks yang berkesan ini (PAni-Fe_3O_4) ke arah pembangunan biosensor yang lain.

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LIST OF SYMBOLS AND ABBREVIATIONS

Ab	Antibody
AD	Alzheimer's disease
AFP	Alpha fetoprotein
Ag	Antigen
Ag/AgCl	Silver/silver chloride
APS	Ammonium peroxydisulfate
$A\beta$	Beta amyloid
BVDV	Bovine viral diarrhea virus
cm	Centimeter
Conc	Concentration
CV	Cyclic voltammetry
DBSA	Dodecylbenzenesulfonic acid
DC	Direct current
DDS	Drug delivery system
e^-	Electron(s)
EDC	N-ethyl-N′-(3-(dimethylamino)propyl) carbodiimide
ELISA	Enzyme linked immunosorbent assay
Emu/g	Magnetic moment
FESEM	Field emission scanning electron microscopy
FITC	Fluorescein isothiocyanate
FTO	Fluorine tin oxide
g	Gram(s)
HRP	Horseradish peroxidase
ICPs	Intrinsically conducting polymers
ITO	Indium tin oxide
KDa	Kilodalton
MALDI-TOF	Matrix assisted laser desorption ionization-time of flight

MDa	Megadalton
MINT-SRC	Microelectronics and nanotechnology-Shamsuddin research center
MS	Mass spectroscopy
NaDS	Dodecylbenzene sulfonic acid sodium salt
NHS	N-hydroxysuccinimide
NPs	Nanoparticles
NSA	Naphthalene sulfuric acid
$^{\circ}$C	Degree Celsius
Oe	Oersted
PAni	Polyaniline
PBS	Phosphate buffer solution
pI	isoelectric point
PNCs	Polymer nanocomposites
PoC	Point of care
PPC	Parallel plate capacitor
PPY	Polypyrrole
QCM	Quartz crystal microbalance
R_{ct}	Charge-transfer resistance
R_f	Final resistance
R_i	Initial resistance
R_s	Resistance of the solution
s	Second(s)
S/cm	Siemens per centimeter
t	Time
T3	Triiodothyronine
TEM	Transmission electron microscopy
V	Volts
XRD	X-ray diffraction
Z'	Real impedance component
Z''	Imaginary impedance component
Z_w	Warburg impedance
β	Beta

CHAPTER 1

INTRODUCTION

1.1   Background of the study

Nanostructures formed by conducting polymers showed properties differing from their bulk structures, such as increased conductivity of nanotubes with respect to the conventional conducting polymer (Martin-Yerga, Gonzalez-Garcia, & Costa-Garcia, 2014; Xia *et al.*, 2010), and increased charge transport rates compared to conventional conducting polymers (Dhand *et al.*, 2011; Van Dyke & Martin, 1990). The good conductivity and increased surface area of conducting polymers couple with its highly conjugated length (Xia *et al.*, 2010) makes them suitable candidates for electrochemical application including electronics, photonics, mechanics and sensing (Azak *et al.*, 2016; Buber *et al.*, 2017).

Conducting polymers (CPs), have become increasingly popular due to its unique electrical and optical properties. Material characteristics of CPs are similar to those of some metals and inorganic semiconductors, while retaining polymer properties such as flexibility, ease of processing and synthesis, generally associated with conventional polymers (Nambiar & Yeow, 2011).

CPs are often categorised into different types based on the type of electric charges (e.g., delocalized π electrons, ions, or conductive nanomaterials) responsible for conduction. Several CPs are known to interact with biological samples while maintaining good biocompatibility and hence, they qualify as interesting candidates for use in a numerous biological and medical applications (Nambiar *et al.*, 2011).

Synergies in nanotechnology and bioelectronics have open new possibilities to miniaturize and optimize present microscale devices at the nanoscale. It is now possible to accurately measure definite electrical properties in combination with several electrochemical transducers. The large surface-to-volume ratio exhibited by nano-objects makes their electrical properties increases to external influences, especially as the structures continue to shrink toward the atomic limit. Since the nanometer dimensions of these objects are similar to the size of the target biomolecules, it may result in enhanced sensitivity measurement (Patolsky, Zheng, & Lieber, 2006), and increase of sensitivity due to higher capture efficiency (Nair & Alam, 2007).

In another approach, the changes in conductance of an electrode as a result of the immobilization of *e.g.* enzymes, complementary antibody-antigen pairs, etc. onto the electrode surface can be directly monitored. The construction of multi-analyte conductance biosensors and conductive polymer-based devices has been made possible by the rapid development of semiconductor technology and sensor integration with microelectronic devices, such as field-effect transistor devices (Grieshaber *et al.*, 2008). Today there is growing interest in conductometric immunosensors with nanostructures like nanowires, for biosensing (Städler *et al.*, 2007). Although conductometric sensing has not been as widely implemented as it could be (Eggins, 2008), successful development of these devices for practical application, such as detection of drug in human urine and environmental pollutant detection are already established (Grieshaber *et al.*, 2008).

Conducting polymers have recently been considered as suitable matrices for immobilization of biomolecules (Dhand *et al.*, 2007). This has been attributed to a number of factors such as flexibility in chemical structure required for binding with biomolecules (Basniwal *et al.*, 2013), efficient signal transduction and unique electron transfer capabilities (Haldorai *et al.*, 2011). they are also capable of penetrating the insulating shell of biomolecule and provides a means for direct electrical communication between the redox center and the electrode surface (Dhand *et al.*, 2011). This is achieved by delocalizing redox charges over a series of conducting polymer groups, thereby acting as self-contained electron transfer mediators. Dhand *et al.*(2010) have described a new concept which is based on the fact that the conductivity of this class of polymers is very sensitive to the chemical potential of the microenvironment within a polymer matrix (Dhand *et al.*, 2010a).

Polyaniline (PAni) is interesting material for sensor and biosensor interfaces because it can act as an effective electron transfer mediator in redox or enzymatic reactions (Dhand *et al.*, 2011). PAni is ideally suited for covalent binding of biomolecules (DNA and enzymes) due to the available active functional groups. Furthermore, properties of PAni such as its shape and dimensions can be fine tune and control during the synthesis (Ding, Wan, & Wei, 2007), by varying synthesis parameters or processing conditions which normally results in a desired physical and electrochemical properties for biosensing applications.

Electrochemical methods have drawn more attention than other transduction methods (Martín-Yerga, González-García, & Costa-García, 2012)and have a wide range of uses, due to their simple pre-treatment procedure (Lucarelli *et al.*, 2008), fast analytical time, precise and sensitive current measurement, inexpensive and miniaturizable instrumentation (Dungchai, Chailapakul, & Henry, 2011; Zang *et al.*, 2012). In electrochemical immunosensors, the level of analyte is determine by detecting the changes of conductance, current, potential or impedance caused by the immunoreaction (Ronkainen, Halsall, & Heineman, 2010). In these methods, the amperometric immunosensor is especially promising because of its , simplicity (Wang & Tan, 2007), high sensitivity and relatively low detection limit (Chang & Park, 2010; Lin *et al.*, 2009).

1.2   Biosensors

Disease control is vital and it improves the quality of our lives, this is greatly dependent through diagnosing and monitoring various biochemical compounds in the body fluid (Rahman *et al.*, 2008). The diagnosis of a disease through measurement of constituents in the body fluids started long ago. For example, diabetes mellitus was first diagnosed by testing the sweetness of urine. The monitoring of the biochemical compounds in the body fluid involve typical analytical techniques for biochemical test, specialists to run the tests, and longer time for performing clinical tests. Since the levels of various compounds in a body system are directly related to some diseases, it is possible to monitor the development of diseases by monitoring the concentration of these compounds (Buszewski *et al.*, 2007). Therefore, constant, fast, and sensitive monitoring is required to efficiently measure the concentration of the body's fluid.

The methods of diagnostics must be simple, sensitive and able to detect multiple biomarkers which normally exist at low concentrations in biological fluids or test samples. In recent years, there is an increasing growth of demand in the field of health care for a user friendly, simple and disposable devices that also demonstrate fast response times, cost-efficient, and suitable for mass production (Yager, Domingo, & Gerdes, 2008). Biosensor technologies offer the potential to fulfil these criteria through an interdisciplinary combination of approaches from chemistry, nanotechnology and medical science. However, there is need to further developed and improved the biosensor devices so as to face the new challenges and to allow, for example, multiplex analysis of several biomarkers in which arrays of sensors need to be developed on the same chip. In this context, electrochemical biosensor becomes the promising analytical method for a sensitive and selective detection of biomolecules (Guo & Dong, 2009).

The general definition of a biosensor is an analytical device which is capable of converting a biological response into a processable and quantifiable signal (Grieshaber *et al.*, 2008). Figure 1.1shows the general outline of a typical biosensor: bioreceptors binds specifically to the analyte on a support or interface where the biological activity takes place, the resultant signal is picked up by the transducer element and is converted to an electronic signal and then amplified by a detector circuit and process by computer software to be converted to a meaningful physical parameter describing the process being investigated. Biosensors can be applied for the detection of many varieties of samples such as cell cultures, body fluids,food samples (Grieshaber *et al.*, 2008)
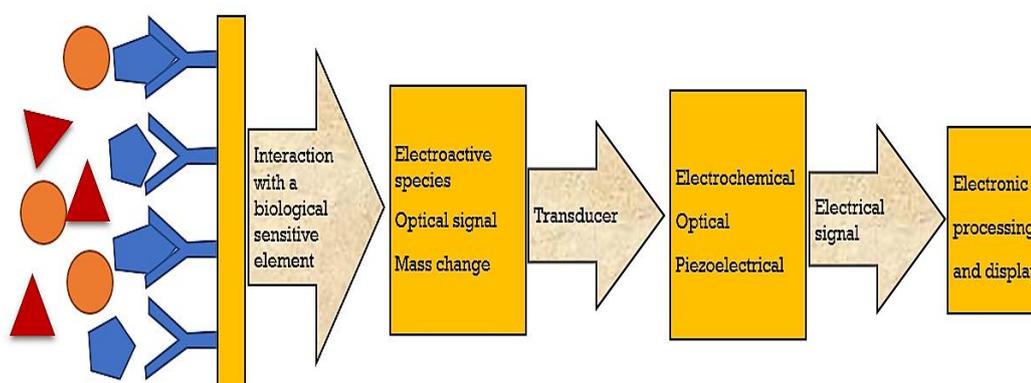
Figure 1.1: General outline of a typical biosensor

1.2.1   Immobilization techniques and bioreceptors

Biosensors may be classified according to the biological specificity or to the mode of signal transduction or, alternatively, a combination of the two. Figure 1.2 illustrates a typical biosensor transduction methods and some bio-receptors in a hierarchical format. Biological elements include antibodies, enzymes, micro-organisms, biological tissue, and organelles. The transduction technique depends on the type of physicochemical change resulting from the sensing event (Krejcova *et al.*, 2012). The most commonly used sensing elements and transducers are described in Figure 1.2 below.
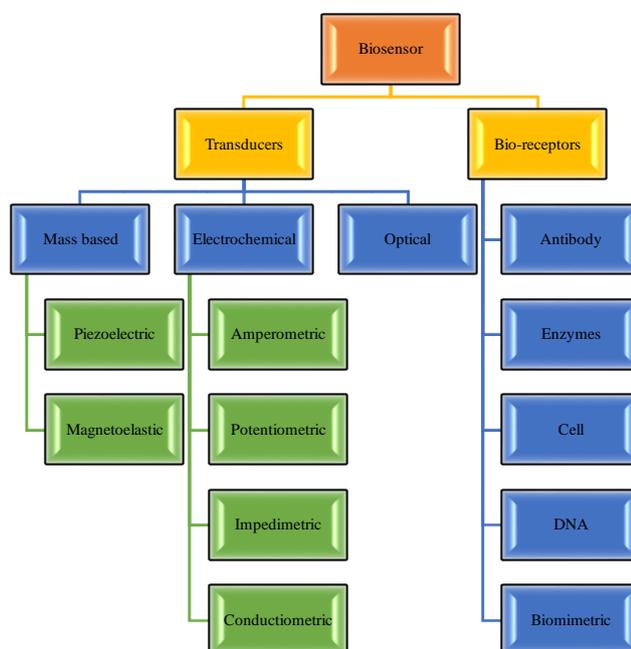
Figure 1.2: Classification of biosensors and transduction techniques

Biosensors can be classified as point-of-care devices (PoC) when it can be use for analysing clinical samples at home or at the surgical room. In order to develop appropriate biosensor technologies, specific markers need to be identified to ensure specificity of the devices. Biosensors provide advanced platforms for biomarker analysis with additional advantages of being inexpensive, rapid and robust as well as offering multianalyte testing capability (Tothill, 2009).

Until recently, most fabricated biosensors or bioelectronics devices are based on one or more biomolecules used in conjunction with an electrode. The redox reaction could be detected electrochemically by measurement of either loss or formation of a product or substrate, by the use of small mediator species that shuttle between the biorecognition element and the electrode, or sometime though not common by direct electron transfer (ET) between the biomolecule redox site and the electrode (Murphy, 2006).

Direct ET can be difficult to achieve, since the redox site of a biomolecule is most of the time hidden deep inside the biomolecule. Developments in achieving direct electron transfer have been made through the modification of the electrode and biomolecules surface by the use of conducting materials as mediators and the design of functional bio-interfaces (Hartmann, 2005). The next generation biosensors that will give highly reliable, stable and robust field-based diagnostic devices is considered to be a higher conductive organic base transducers (Dhand *et al.*, 2010a).

1.2.2 Electrochemical detection techniques

The measurement of electrical properties for extracting information from biological systems in bio-sensing is usually electrochemical in nature, in which a bio-electrochemical component serves as the main transduction element. Although bio-sensing devices employ different kind of recognition elements, electrochemical detection methods use predominantly enzymes. This is mostly as a results of their specific binding abilities and bio-catalytic activity (Grieshaber *et al.*, 2008). Other bio-recognition elements are nucleic acids, antibodies, micro-organisms and cells (Chambers *et al.*, 2008).

Ordinarily, in bio-electrochemistry, the reaction under examination would either generate a measurable potential or charge accumulation (potentiometric),a measurable current (amperometric), or the conductive measurable properties of a medium (conductometric) between the electrodes (Henríquez, Horstkotte, & Cerdà, 2014). Another types of electrochemical detection techniques are impedimetric, this technique measures the impedance (both reactance and resistance) (Wang *et al.*, 2016).

1.2.3   Materials for surface modifications

Currently, various materials are used in the preparation of support/surfaces for biosensing applications (Arya *et al.*, 2012). However, depending on the measurement method and technique, special requirements are required or need to be fulfilled, such as transparency for optical devices and electrical conductivity for electrochemical techniques. Glass and other oxide surfaces are the most commonly used due to their favorable optical characteristics (Harper *et al.*, 2012). Widely used are also gold, microporous gold, (Song, Ni, & Kokot, 2013b) graphite, glass carbon (Gao *et al.*, 2014)and Indium Tin Oxide (ITO) (Guo *et al.*, 2013).

After studying sensing layer ability of polyaniline for the immobilization of antibodies (Huan *et al.*, 2011; Wang *et al.*, 2009). Fe_3O_4 nanoparticle was disperse onto the conductive polyaniline nanorod to form a nanocomposite which was deposited on FTO coated glass to examine the properties of the new sensing layer and find out if a targeted antibody can be immobilized on it and wether the new composites will be used as transducing matrix. For a desired result, it is essential to get a uniform layer of the nanocomposite onto the surface of the electrode which was achieved via electrochemical deposition.

In the first part of this study, $PAni/Fe_3O_4$ nanocomposites was synthesized by means of ultrasonic irradiation of aqueous solutions of aniline, phosphoric acid, ammonium peroxydisulfate (APS) and some amount of aniline dimer-COOH attached to Fe_3O_4 nanoparticles. During the synthesis, the aggregates of Fe_3O_4 nanoparticles were broken down by the ultrasonic mixing and the particles were re-dispersed in the aqueous solution. APS was used as an oxidizing agent to initiate the polymerisation while Phosphoric acid (H_3PO_4) was use as doping agent and helps in the protonation of PAni which improves the electrical conductivity.

The fabrication of the modified bio-electrode for detection of "β-Amyloid" was done by electrochemically depositing $PAni/Fe_3O_4$ nanorods composite film on Fluorine tin oxide (FTO)-coated glass, with anti-β-Amyloid covalently immobilized on the composite surface. The synergy between PAni and Fe_3O_4 yields a conductive nanocomposite layer that exhibits better sensitizing effect and stability as transducing matrix and a platform for β-Amyloid detection.

To the best of our knowledge, the use of this synthesize nanocomposite (PAni/Fe$_3$O$_4$) as a sensing conductive layer for the immobilization of anti-β-Amyloid for β-Amyloid detection is new. The synthesized nanocomposites are conductive and paramagnetic, a combination of properties not exhibited by other known polymers nanocomposites. The Fabricated modified conductive PAni/Fe$_3$O$_4$/FTO electrode (transducer) can be used for immobilization of other disease biomarkers. The immunosensor was also found to detect β-Amyloid, a biomarker for Alzheimer's disease. The new properties of the nanocomposites, modified electrode, as well as the sensitivity of the immunosensor was checked and reported.

1.3   Problem statement

The early diagnosis of Alzheimer's disease is crucial for successful prognosis of the disease and for this reason sensitive and specific methods are required (Hampel *et al.*, 2010). It is now the sixth killer disease in the United States. Those with Alzheimer's live an average of eight years after their symptoms become noticeable to others, but survival can range from four to 20 years, depending on age and other health conditions. Alzheimer's has no current cure and although its current treatments cannot stop Alzheimer's from progressing, they can temporarily slow the worsening of dementia symptoms (Perrin, Fagan, & Holtzman, 2009).

Meanwhile, the early detection of Alzheimer's will help delay its onset, improve the quality and help prolong patient's life and their caregivers. Therefore, research tasks toward the fabrication technology of biosensor which is capable of early and accurate diagnosis of Alzheimer's disease are exceptionally necessary and crucial in this current period. Biosensor not only helps last the patient's life but also has correct prognosis in the successful treatment of the disease (Eccles *et al.*, 2013).

Electrochemical immunosensors (EI) has attracted numerous interests due to its inherent benefits over the other transduction schemes, such as a high sensitivity, ease of use, a possible automation and integration in compact analytical devices, mostly cheap and relatively simple technology of its production. Thus, EIs have great potential in point of care (POC) diagnostics for early detection of diseases. During last decades, numerous efforts have been put into EIs development. Firstly, different fabrication methods and amplification strategies have been employed to achieve high

sensitivity and now the use of nanostructured technology has been involved in the fabrication and signal amplification of EIs, which present great superiority.

One more reason why nanobiosensing becomes very important in nanobiotechnology is the urgent need for it. Early and low-cost point-of-care detection of disease markers is crucial when it comes to the diagnosing and managing health issues worldwide, especially in the third world countries.Due to their specificity, speed, portability, and low cost, biosensors offer exciting opportunities for numerous decentralized clinical applications, ranging from 'alternative-site' testing (e.g., physician's office), emergency-room screening, bedside monitoring, or home self testing.The outdated barrier between bioreceptors and transducers is now being replaced by the integrative approach as nano-transducers are taking part now in the recognition event and as the receptors start to become active transducing elements of the sensors.

Antibody molecules have enormous potential in the development of biosensors and therefore the need to develop a suitable transducing matrix support that will provide an optimum bioactivity is crucial and a challenge to many research scientist. In biosensing the use of conventional immobilization methods during immunoassays generally yield some unstable with mostly incorrect orientation of the antibodies that results in a reduced sensitivity during detection for the already lower analytes concentration.

Accurate detection as well as quantification of biochemical or biological processes are very crucial for medical health care, biotechnological and biological applications. However, the conversion of the biological information into a simple and processed electronic signal remains another big challenge because of the complexity involve during the fabrication process like the direct connection of the electronic device to the biological environment. Electrochemical biosensors provide an attractive means of analyzing the content of a biological sample due to its ability to directly converts the biological event into an electronic signal (Grieshaber *et al.*, 2008).

In recent years,(Gu *et al.*, 2012) conductive polymer nanocomposites are required for certain applications such as fire retardants (Kashiwagi *et al.*, 2005), biosensors (Feng *et al.*, 2011; Virji *et al.*, 2009) and electrodes for electrodeposition because of their homogeneity, relatively easy processability, and tunable physicochemical properties (Zhu *et al.*, 2010). However, fabrication of conducting polymer ($PAni/Fe_3O_4$) nanocomposite based immunosensor having high sensitivity,

selectivity and fast response time, is still required. Magnetite (Fe_3O_4) nanoparticles have novel electrical, mechanical and structural properties, it has attracted much interest not only in the field of magnetic recording media, magnetic fluids and data storage, but also in the areas of medical care such as drug delivery systems (DDS), medical diagnostics, cancer therapy and sensors, (El Ghandoor *et al.*, 2012). The synergy between PAni and Fe_3O_4 yields a conductive nanocomposite that exhibits better sensitizing effect and stability as transducing matrix and a platform for a successful immobilization of anti-β-Amyloid for β-Amyloid detection.

There is also a continued and sustained effort towards developing new immunoassays for other biological disease markers and lowering the limits of detection on the presently existing immunosensors.

1.3.1 Objectives of the study

(i) To synthesize polyaniline iron oxide ($PAni/Fe_3O_4$) nanocomposites and investigate the physical and electrical properties of the nanocomposites.

(ii) To fabricate $PAni/Fe_3O_4$/FTO modified electrode via electrochemical deposition of $PAni/Fe_3O_4$ nanocomposite to form a thin film conducting layer on the FTO coated glass and investigate the physical and electrical properties of the modified electrode ($PAni/Fe_3O_4$/FTO).

(iii) To develop the modified electrode into an immunosensor by immobilizing anti-β-Amyloid on the modified electrode.

(iv) To investigate the detection of β-Amyloid via electrochemical transduction.

(v) To evaluate the performance of the new immunosensor.

1.4 Scope of study

PAni nanorod containing Fe_3O_4 nanoparticle were synthesize by ultrasonic irradiation of the aqueous solutions of aniline, phosphoric acid, ammonium peroxydisulfate and quantitative amount of Fe_3O_4. Morphology studies were carried out with Field emission scanning electron microscopy (FESEM), Energy Dispersive Spectroscopy (EDS) and X-ray diffraction. Molecular structure was characterized by Fourier transform infrared spectroscopy.

Cyclic voltammetry method was used to deposit the PAni/Fe$_3$O$_4$ nanocomposites on FTO coated glass. The conductivity test of both the nanocomposites and the modified electrode were done using the standard Van der pauw (four-probe method) DC it was used to measure the electron transport behavior of the nanocomposites (PAni/Fe$_3$O$_4$) as well as the modified electrode. The dried PAni/Fe$_3$O$_4$ nanocomposites powders were pelletized by hydraulic press (10MPa for 5 min). DC resistance of pellets was measured and from the measured resistance and the sample dimensions, the conductivity value was calculated.

The new modified PAni/Fe$_3$O$_4$/FTO electrode was functionalized into an immunosensor by immobilizing anti-β-Amyloid (antibody) onto the surface of PAni/Fe$_3$O$_4$/FTO, this is achieved by immersing the immunosensor in a 1ml solution mixture containing the anti-β-amyloid, 1% glutaraldehyde and PBS in a specific volume ratio for 30 minutes at room temperature.

Electrochemical impedance spectroscopy (EIS) methods was employed to investigate the 'labelless' detection of analytes via impedimetric transduction. EIS was also used to study electrical characteristics of electrode surface for every layer formation and every interaction between molecules along with the obtained signal variations.Cyclic voltammetry was also use for the modified electrode interogations.The immunosensor was incubated under optimized conditions with different concentrations of β-Amyloid solution for 30 min before the cyclic voltammetry interrogations were performed at a scan rate of 0.1Vs^{-1}. EIS was use to probe the features of surface-modified electrodes. The optimized pH, incubation time as well as the selectivity of the new immunosensor was determined and subsequently used.

CHAPTER 2

LITERATURE REVIEW

2.1   Intrinsically conducting polymers (ICPs)

Intrinsically conducting polymers are inherently conducting in nature due to the presence of a conjugated π electron system in their structure. ICPs have a low energy optical transition, low ionization potential and a high electron affinity (Bhadra *et al.*, 2009; Unsworth *et al.*, 1992). A high level of conductivity (near metallic) can be achieved in ICPs through oxidation–reduction as well as doping with a suitable dopant (Li, Lu, & Wong, 2010). The first ICP to be discovered was polyacetylene, synthesized by (Shirakawa *et al.*, 1977).

Shirakawa *et al.* (1977) found that the conductivity of polyacetylene could be increased by several orders of magnitude through chemical doping and in reality, it can be converted from an insulator to a metal like conductor. Following the study on polyacetylene, other polymers such as polypyrrole (PPY), polythiophene, Polyaniline, poly(p-phenylenevinylene), and poly(p-phenylene), as well as their derivatives, have been synthesized and reported as a new group of polymers known as ICPs. Their conductivity, stability and processability are presented in Table 2.1. (Holze & Wu, 2014) .

Table 2.1: The conductivity, stability and processability of a number of doped intrinsically conducting polymers

Polymer	Conductivity (S/cm)	Stability	Processability
Polyacetylene	10^3–10^5	Poor	Limited
Polyphenylene	1000	Poor	Limited
Poly(phenylene vinylene)	1000	Poor	Limited
Poly(phenylene sulphide)	100	Poor	Excellent
Polypyrroles	100	Good	Good
Polythiophenes	100	Good	Excellent
Polyaniline	10	Good	Good

The conductivity of doped polyacetylene is comparable with that of metallic copper but its stability and processability are very poor compared to normal polymer. The conductivity of polyphenylene is quite high but from the environmental stability point of view it is poor. Conversely, the conductivity of polypyrrole, polythiophene and Polyaniline is comparatively less but these polymers have better stability (Holze *et al.*, 2014).

2.1.1 Electrical properties of ICP based nanocomposites

The organic conjugated polymers are insulators in their undoped forms e.g. room temperature electrical conductivity (σ_{dc}) of emeraldine base (EB) is ~10^{-9} S/cm (Gupta & Choudhary, 2011). However, controlled doping leads to enhancement of conductivity due to formation of charge carriers i.e. polarons/bipolarons (Saini, Jalan, & Dhawan, 2008) that can move under the influence of external potential and in the Columbic field of counter-ions distributed along the chain.

Therefore, increasing dopant concentration leads to increase in concentration and mobility of proto-generated charge carriers resulting in enhancement of conductivity. Furthermore, such a conductivity enhancement in conductivity is strongly dependent on nature and concentration of dopant and in some case conductivity well exceeds the required limit (Saini *et al.*, 2011) for exhibiting good shielding effectiveness.

2.2 Conducting polymer nanostructures and their application in biosensors

Studies on conducting polymers (CP) has continue to be the subject of high investigations globally by many research scholars,(Umare, Shambharkar, & Ningthoujam, 2010). Conducting polymers constitute those class of functional polymers that contain alternating single and double carbon–carbon bonds along the polymeric chains. The highly conjugated polymer chain can be assigned reversible chemical, electrochemical and physical properties controlled by a doping/de-doping process, which makes these polymers very attractive as transducer materials in various sensing devices (Xia *et al.*, 2010).

Conducting polymers emerges as potential candidates for electrochemical sensors because of their straightforward preparation procedures, unique properties like its stability in air, conducting polymers have been used in chemical and bio sensors, electrochemical devices, energy storage, memory devices, and also in electro-catalysts (Bhadra *et al.*, 2009).

Conducting Polymers are poly-conjugated polymers with electronic properties similar to those of metals, while still retaining the properties of conventional organic polymers. Since after the observation of this remarkably high electrical conductivity of poly-acetylene, a lot of other conjugated polymers have been converted from an insulating state into a highly conductive state (Bhadra *et al.*, 2009). Polyaniline, Poly-acetylene, poly-thiophene and poly-pyrrole are the most common and most widely investigated conducting polymers and are depicted in the Figure 2.1).

Polythiophene

Polypyrrole

Polyaniline

Polyacetylene
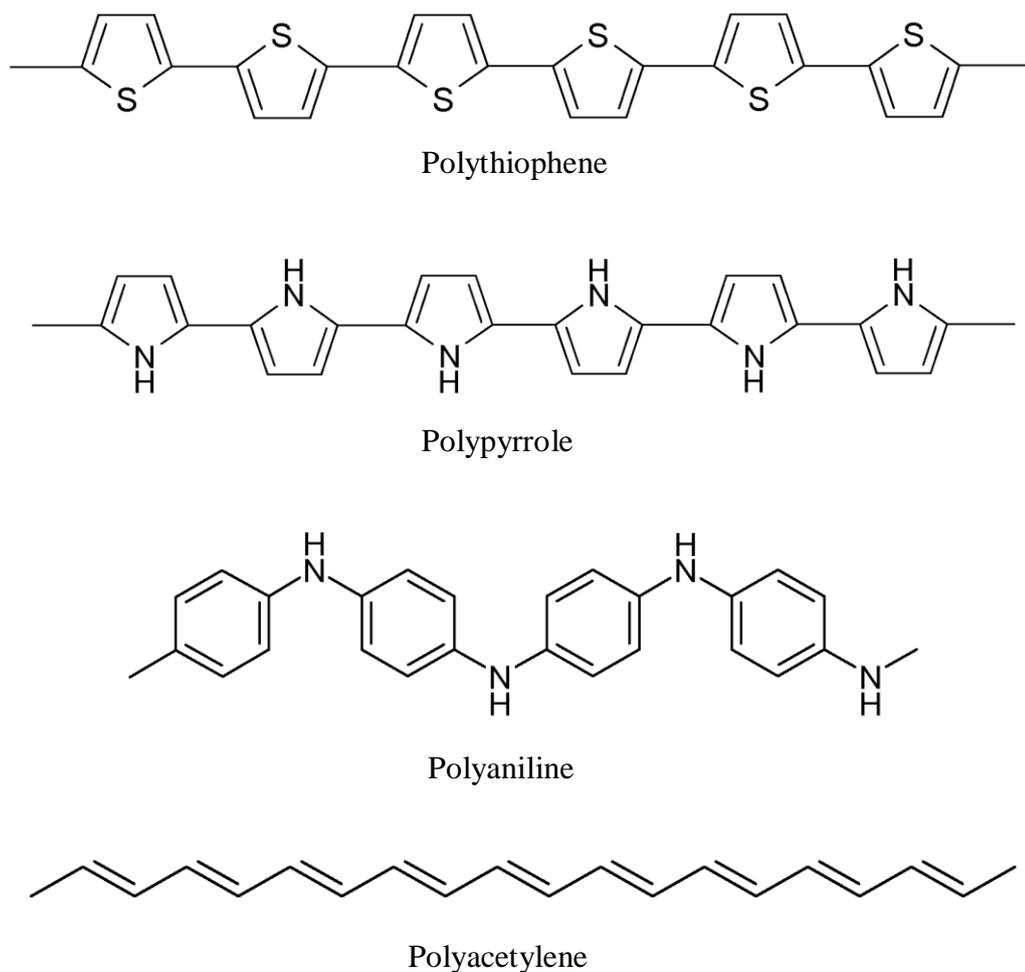
Figure 2.1: The structures of typical conducting polymers use for biosensing applications (Xia, Wei, & Wan, 2010)

As a special subclass of conducting polymers, these conducting polymer nanostructures did not only retain their unique properties, but also possess the characteristics of nanomaterials like large surface area, nanosize, and quantum effect, this new property further give the conducting polymers additional merit in designing and making novel sensors.

Conducting polymer sensors are based on the variation of either their capacity, optical properties or their redox properties. The sensitivity of conducting polymer-based sensors is primarily determined by the difference in a specific property before and after exposure to a test target molecule. Therefore, the specific surface area of the conducting polymers plays a key role in determining the sensitivity of a sensor. The variation of the conductivity caused by doping/de-doping processes is widely used in the conducting polymer-based sensors. The test target molecules can increase or

decrease the number and mobility of charge carriers, which lead to the overall change in conductivity (Xia *et al.*, 2010).

Conducting polymers are also known for their ability to be compatible with biological molecules in neutral aqueous solutions. Moreover, the polymer itself can be modified to bind biomolecules to a biosensor. Another advantage of conducting polymers is that the electrochemical synthesis allows direct deposition of a polymer film on the electrode substrate followed by biomolecules immobilization. It is thus possible to control the spatial distribution of the immobilized enzymes, the film thickness, and modulation of enzyme activity.

The response time is relatively long when bulk polymer is used to construct a sensor, this is due to slow penetration of the target molecules into the conducting polymer, and most of the time there is an accompanying hysteretic effect (Xia *et al.*, 2010). However, sensors that are constructed with conducting polymer nanostructures, the response time is expected to be significantly faster due to porous structure of the nanotubes or nanofibers. For instance, a nanofiber thin film of PAni synthesized by an interfacial polymerization responds much more rapidly than does a conventional film for both acid doping and de-doping. Especially, nanofiber films show no particular thickness dependence on their performance in the range of 0.2–2.5 µm (Xia *et al.*, 2010). Conducting polymer nanostructures with larges specific surface area and porous structure are therefore predicted to be excellent sensing materials for biosensors (Xia *et al.*, 2010).

2.2.1   Biosensors of conducting polymer nanostructures

Biosensors have recently attracted much interest because of their utilization in rapid detection and monitoring in food and clinical diagnostics, bioprocess monitoring and environmental defense monitoring. A biosensor is couples with an immobilized bio specific recognition or catalytic component like protein (antibody, enzymes, and antigens), DNA, RNA, polypeptides, cells, etc., to the transducer's surface, which will then converts the biological recognition event into a quantifiable or measurable electrical signal, indicating the presence of the target analyte (Cosnier, 2007). Moreover, the large contact area between the analyte and nanostructures result in enhanced and amplified sensitivity for bio-sensing.

Figure 2.2 is a schematic of the application of different conducting polymer nanostructures in biosensing. Conducting polymer nanostructures, such as nanowires, nanorods and nanoparticles, offer new opportunities as sensing matrix or platforms for biological and environmental applications (Ahuja & Kumar, 2009; Xia *et al.*, 2010).
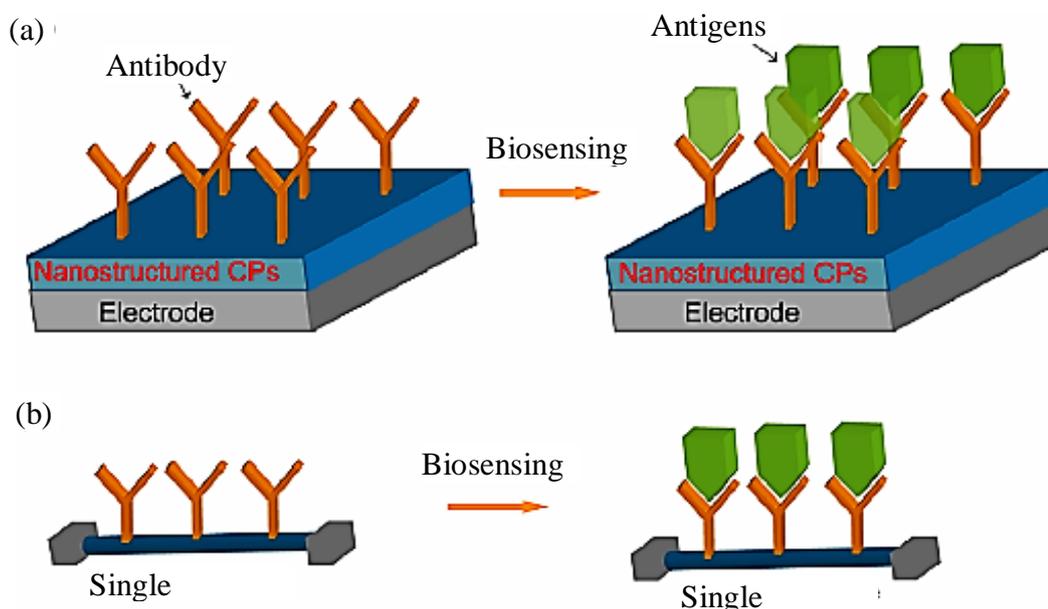
Figure 2.2: Schematic of the application of (a) conducting polymer nanostructures and (b) single conducting polymer nanowire in biosensing (Xia, Wei, & Wan, 2010)

Conducting polymers are adaptable materials that are used for sensing application because apart from possessing unique properties, they can also be used as immobilization matrices (Ahuja, Mir, & Kumar, 2007), receptors as well as redox for electric charge transport system (transducers) in a biosensor (Li, Huang, & Kaner, 2008a; Nambiar *et al.*, 2011) .

In conductometric sensors, the electrical conductivity of the conducting polymers changes after interaction with the analytes is measured (Lange, Roznyatovskaya, & Mirsky, 2008). The changes in conductivity can be generated from doping/de-doping the process, conformational change and the redox process. In potentiometric sensors, the concerned changes in the system's chemical potential are mainly resulted from shifts in the anion equilibrium within the polymer film that caused by doping/de-doping or redox processes (Parajuli, 2011; Seeber, Terzi, & Zanardi, 2014).

Essentially, a desirable biosensor may take advantage of numerous enhance element (nanostructure, composites, specific molecular design, etc.). On one hand, the nanostructures can be an enhance element for a nanocomposite based sensor and vice versa. Moreover, the processability of conducting polymer materials is often important for the formation of specific nanocomposites or nanostructures (Lu *et al.*, 2011).

2.2.2   Conducting polymer nanoparticles based biosensors

Nanoparticles can be used in several ways, such as for modification of electrode surfaces (Zhang *et al.*, 2012), or for modification of biological receptor molecules such as enzymes, antibodies, or oligonucleotides. Zero-dimensional nanostructure possesses unique physical and chemical properties that derive from their nanosize (Willner, Willner, & Katz, 2007). Nanoparticles provide an ideal structure for the application of biosensors due to the following reasons (Xia *et al.*, 2010).

(1)   Minimum diffusion limitations; nanoparticles are readily dispersed in aqueous media and over the electrode surface during the modification process.

(2)   Maximum surface-to-volume ratio; this provides a larger active area for the design of a biosensor.

(3)   High efficiency of bio-reagent loading; the immobilization of biomolecules can be realized by either physical entrapment or surface modification and

(4)   Enhanced process ability; they can be well-dispersed in aqueous solution.

In general, conducting polymer nanoparticles are dispersed on the electrode surface so as to increase the area/volume ratio, this action will favor the adsorption of biomolecules onto the electrode surface (Turkmen *et al.*, 2014). The self-encapsulation of a redox enzyme–glucose oxidase (GOx) within the conducting Polypyrol (PPy) has been achieved by a chemical method (Ramanavicius & Ramanaviciene, 2009). The possibility of applying PPy/GOx nanoparticles in the design of amperometric biosensors was illustrated in their studies.

Furthermore, an amperometric enzyme biosensor fabricated from PAni nanoparticles was reported (Morrin *et al.*, 2005). Electrodeposited polymer nanoparticles resulted in a highly ordered conductive nanostructured electrode surface, which enabled uniform electrostatic adsorption of protein, thereby exhibiting higher signal-to-background ratios (61±3) and shorter response times (0.62s) than electrochemically prepared PAni/polyvinylsulfonate films. Taking advantage of conducting polymer nanoparticles, sufficient amounts of enzyme were firmly immobilized during the fabrication of a phosphate biosensor (Rahman *et al.*, 2006).

The response time of the biosensors was about 6 s. A linear response was observed between 1.0 µM and 100 µM and the detection limit was determined to be about 0.3 µM (Xia *et al.*, 2010) . Besides, an ascorbic acid sensor has been fabricated via the drop-casting of PAni nanoparticles onto a screen-printed carbon-paste electrode (Xia *et al.*, 2010). The PAni nanoparticles not only enhanced the catalytic reaction, but also allowed the detection of ascorbate at the reduced applied potential of 0 V and operation at neutral pH, avoiding the problem of sample interference.

Significant research has also been devoted toward the design of conducting polymer-metal nanoparticle composite based biosensors (Li *et al.*, 2009), these hybrid nanomaterials are expected to display synergistic properties between the polymer and the metal nanoparticles. For instance, an acidic condition is generally required for the formation of the most highly conductive form of PAni, but most bio-electrochemical reactions require a near neutral pH. Silver chloride coated with PAni core shell nanocomposites (AgCl/PAni) showed an excellent electrochemical behavior at a neutral pH. Citrate-stabilized gold nanoparticles (AuNPs) could also be assembled on the surface of AgCl/PAni through coordination bonds. Since gold nanoparticles could provide a higher number of negative charges for anionic doping, the electroactivity of AuNPs–AgCl/PAni was better than that of AgCl/PAni (Li *et al.*, 2009).

2.3   Polyaniline (PAni)

Polyaniline (PAni) is among the oldest known conducting polymers (Reddinger & Reynolds, 1999) and has been extensively reviewed (Bhadra *et al.*, 2009; Genies *et al.*, 1990; MacDiarmid & Epstein, 1989; Syed & Dinesan, 1991), due to its several unique properties (Li, Jiang, & Xu, 2007; Umare *et al.*, 2010). It's easy synthesis, light weight, lower cost, (Xiao *et al.*, 2013), better electronic, optical properties (Haldorai *et al.*, 2011; Kim & Park, 2011) better stability in air and soluble in various solvents and good processibility (Li *et al.*, 2008b).

In electrochemical biosensing, among the most promising applications of polyaniline is that it can act as a suitable matrix for the immobilization of biomolecules. Polyaniline is compatible with biological molecules in neutral aqueous solutions. Furthermore, based on its excellent conductivity and electroactivity, polyaniline can act as an effective mediator for electron transfer in redox or enzymatic reactions, where polyaniline undergoes redox cycling and can shuttle electrons from the enzyme active centre direct to the electrode surface. Another advantage for the application of polyaniline in electrochemical biosensing is that polyaniline can be synthesized electrochemically directly on the electrode from aniline monomer, using potentiostatic, galvanostatic or potentiodynamic means.

The electropolymerisation method of preparation of polyaniline modified electrodes offers the potential to incorporate a wide range of dopants into the formed polyaniline film and this gives good control over the film thickness (Luo *et al.*, 2007). PAni has captured attention of scientific community recently due to the discovery of its low priced and high conductivity. Consequently, researchers are continuously exploring its potential applications that includes those in biosensors because of its number of useful features such as;

(1)   Direct and easy deposition on the sensor electrode.

(2)   Control of thickness.

(3)   Redox conductivity and polyelectrolyte characteristics.

(4)   High surface area.

(5)   Chemical specificities.

(6)   Long term environmental stability.

(7)   Tunable properties (Dhand *et al.*, 2011).

Furthermore, PAni is ideally suited for covalent binding of biomolecules (DNA and enzymes) due to the presence of active functional groups. Apart from this, control over the dimensions and shape of PAni via varying synthesis methods or processing conditions usually result in a desired physical and electrochemical properties especially for biosensing applications (Dhand *et al.*, 2010a).

The ability of PAni to combined with different nanoparticles to form the nanocomposite has great potential applications such as radar-absorber (Makeiff & Huber, 2006), indicators (Drelinkiewicz *et al.*, 2007), electrochemical and capacitors (Sun & Deng, 2008), Bioelectronic components (Willner *et al.*, 2007), catalysts (Amaya *et al.*, 2008) and gas separation membrane (Weng *et al.*, 2011). Suitable matrix for antibody immobilization can be fabricated from PAni by entrapping the biological sensing elements in the polymer matrix (Lu *et al.*, 2004; Muhammad-Tahir, 2007). This entrapment feature is extensively used for direct measurement of immunoreaction (antibody-antigen binding) and is further investigated in this research.

The advantages of PAni in the area of biosensor are indicated as enzyme amplifier that provide spectacular signal amplification and eliminates electrode fouling. Apart from this, PAni exhibits two redox couples in the convenient potential range to accelerate an efficient enzyme–polymer charge transfer (Dhand *et al.*, 2011). Economically, aniline monomer is inexpensive and therefore more economical than other monomers that are used for the synthesis of conducting polymers (Dhand *et al.*, 2011).

For catalytic and electroanalytical applications, PAni must be supported on the electrode such as gold electrode (Wan *et al.*, 2010), glassy carbon electrode (Dong *et al.*, 2012), and carbon nanotubes. Recently, the application of indium/tin oxide (ITO) film coated glass has attracted increasing interest due to its prominent characteristics and low cost. This is very important in sensor design and manufacture for reasons of cost (Wang, 2005).

2.3.1 Polyaniline nanostructure

Polyaniline (PAni) is one of the most investigated conducting polymers for the development and fabrication of biosensors due to its unique physical, optical and electrical properties (Deep *et al.*, 2012). It provides extraordinary stability, simplicity of synthesis and excellent electrochemical properties. PAni based sensors have been reported to function primarily through amperometric measurements (Arslan, Ustabaş, & Arslan, 2011) and most of the reported PAni based amperometric biosensors have been developed on noble metal electrodes such as Au, Pt (Ahmad *et al.*, 2009), and other inorganic metals. It may be of particular significance to develop and explore the biosensing properties of PAni/Fe_3O_4 substrates which may find further applications as alternative platforms for immobilization of antigen for immunosensing.

Polyaniline nanostructures have also been prepared by blending the PAni with an electrical,inorganic,optical and magnetic nanoparticles to produce the nanocomposites (Gu *et al.*, 2014; Shendkar *et al.*, 2016). Among the inorganic nanoparticles, Fe_3O_4 nanoparticles have gain a lot of attention due to their interesting magnetic properties as well as their extensive potential applications in magnetic recording media, color imaging, ferrofluids and soft magnetic materials (Kong *et al.*, 2009; Yang *et al.*, 2009). Significant research is also devoted toward the development of conducting polymer/ metal nanoparticle composite based biosensors (Braik *et al.*, 2016; Shrivastava, Jadon, & Jain, 2016). These nanomaterials hybrid are expected to show synergistic properties between the metal nanoparticles and the polymer.

2.3.2 Electrochemical properties and chemical structure of polyaniline

The doping level and redox state are the two most important factors that determines the chemical structure of polyaniline. Polyaniline primarily has three distinguishable oxidation states, which are; the fully reduced leucoemeraldine state, the half oxidized emeraldine state and the fully oxidized pernigraniline state, with nearly an infinite number of possible oxidation states existing in between. Hence, in principle, polyaniline do exist in a continuum of oxidation states ranging from a completely reduced to a completely oxidized form.

The chemical structure of the repeating units of polyaniline is shown in Figure 2.3 where the polymer chain consists two types of repeating units; the reduced and the oxidized unit and are illustrated (Song *et al.*, 2013a). The degree of oxidation is described by a variable x whose value ranges between 0 and 1, and it represents the fraction of the two repeating units.

(a)

(b)

(c)
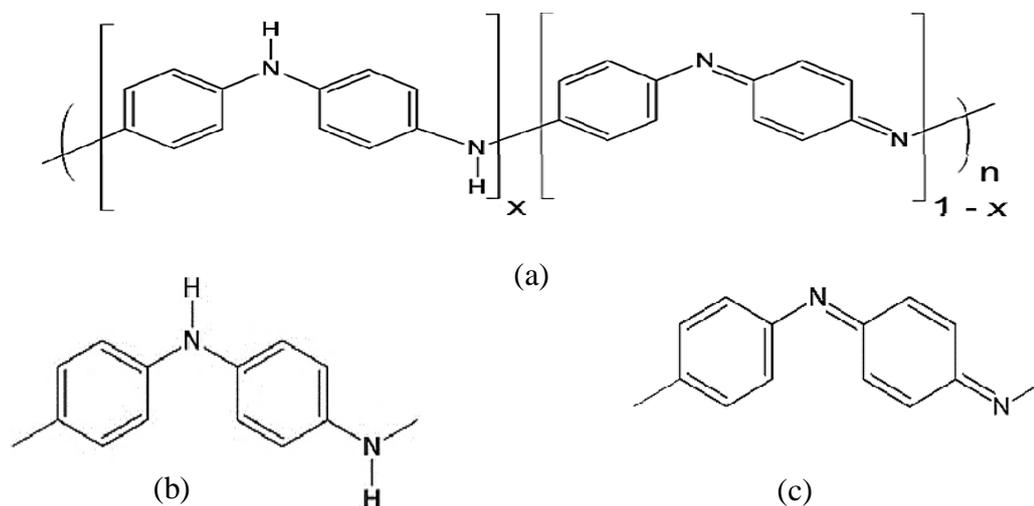
Figure 2.3: Polyaniline structure (a) General chemical structure, (b) Reduced repeating unit, and (c) Oxidized repeating unit (Song & Choi, 2013a)

The oxidation state can be defined by the value of (1-X). X can be varied continuously in principle from one (the completely reduced material) to zero (completely oxidized polymer).The completely reduced material (X=1) is called leucoemeraldine and contains only the amine nitrogen atom whereas the completely oxidized form (X= 0) is termed as pernigraniline and is compose of imine nitrogen atoms only (Keivani *et al.*, 2010; Song *et al.*, 2013b).The true emeraldine oxidation state X= 0.5 of polyaniline in which 50% of the nitrogen atoms are protonated (consists of equal numbers of reduced and oxidized repeat units) is the most highly conducting (metallic) form of the polymer (Keivani *et al.*, 2010). Table 2.2 shows the different redox forms of Polyaniline.

Table 2.2:The different oxidation states of polyaniline with their colours (Keivani *et al.*, 2010)

Form of polyaniline	Redox state	Oxidized groups (m value)	Reduced groups (n value)	Color
Leucoemeraldine	Fully reduced	0	1	Clear/White
Emeraldine	Partially oxidized	0.5	0.5	Blue/Green
Pernigraniline	Fully oxidized	1	0	Violet/Blue

Chemical polymerization of aniline in aqueous acidic media can be easily performed using an oxidizing agent such as $(NH_4)_2S_2O_8$ as shown in Figure 2.4. Ammonium persulfate is the most popular and frequently used chemical oxidant for polymerization of aniline in acidic aqueous solutions (Keivani *et al.*, 2010).
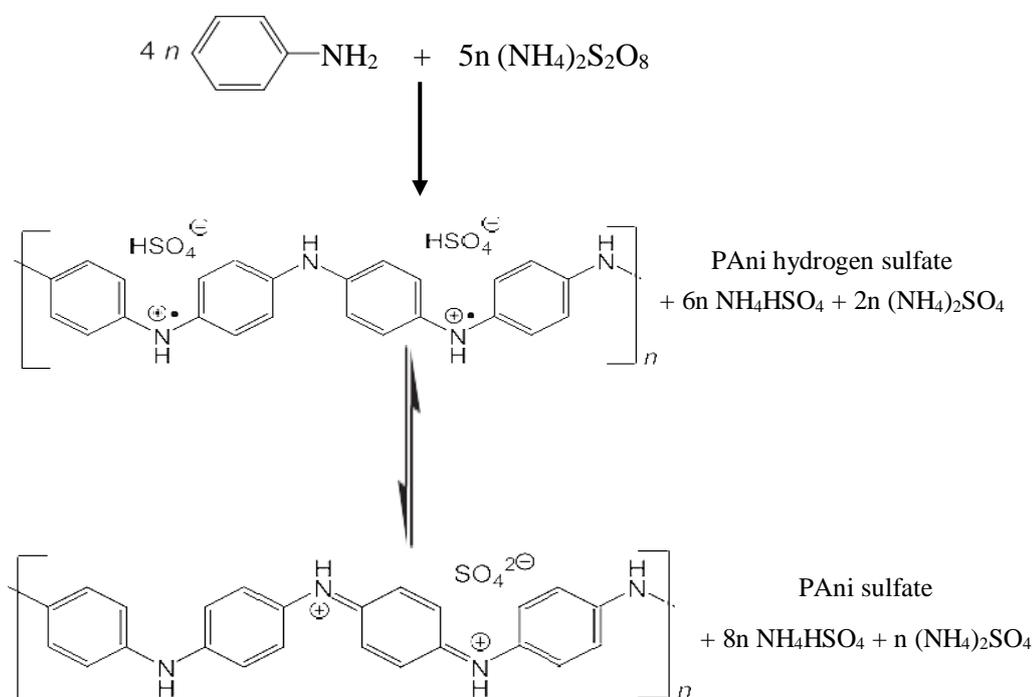
Figure 2.4: Overall polymerization reaction of polyaniline using ammonium persulfate (Sapurina & Shishov, 2012)

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