FUNCTIONALIZATION AND FABRICATION OF POLYMER BASED DEVICE PLATFORM ARCHITECTURES FOR SENSOR APPLICATIONS

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

FUNCTIONALIZATION AND FABRICATION OF POLYMER BASED DEVICE PLATFORM ARCHITECTURES FOR SENSOR APPLICATIONS

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In this thesis, two types of sensors: Conjugated polymer based amperometric biosensors and a chemiresistive sensor (polymer/SWCNT) for clinical and food applications were designed, fabricated and characterized. Three chapters in this thesis focused on ethanol, glucose and cholesterol biosensors based on functional polymer/MWCNT composition, different peptide sequence containing polymer, and also copolymer of two different functional monomers, respectively. After successful electrochemical deposition of the polymers on the graphite electrode surfaces, immobilization of alcohol oxidase (AOx), glucose oxidase (GOx) and cholesterol oxidase (ChOx) were carried out. Since conjugated polymer based biosensors are recognized to be a next generation building architecture for highly sensitive and fast biosensing systems, in this thesis, it is aimed to create new biosensor systems for the quantitative detection of ethanol, glucose and cholesterol which are thought to play an important role to prevent several diseases.

To construct a chemiresistive sensor, nanowires (NWs), especially carbon nanotubes (CNTs), as the basis for chemiresistor research were used. In this work, single walled

carbon nanotubes (SWCNTs) due to their excellent conductivity, exceptional aspect ratios, and numerous methods available for functionalization were favored. The first step generates a surface wherein P4VP-SWCNT dispersion was spray-coated and covalently linked to the surface. The P4VP dispersant not only displays a favorable interaction with SWCNTs to stabilize debundled dispersion, but it also allows further modifications on its nucleophilic pyridyl nitrogens with alkyl halides. In the second step, a post-functionalization of P4VP-SWCNT surface via reacting with 2-bromoethanol was implemented. Finally, GOx was immobilized on the modified surface to detect glucose in beverages.

As a conclusion, all devices constructed in this thesis present four new examples in biosensing applications that overcome challenges associated with analysis time, selectivity and also requirement of pretreatment of the samples. Sensor performances were also evaluated for each sensor. Additionally, surface features of the sensors and molecules were fully characterized using NMR, SEM, XPS, CV, FT-IR and Raman techniques depending on the type of sensor. To further test the sensing performance, the sensors were tested towards their specific analytes and showed promising feasibility for the quantitative analysis in beverages and human blood samples.

Keywords: Surface modifications for sensor applications, detection of glucose and ethanol in beverages, cholesterol detection in human blood samples, conjugated polymer based electrochemical biosensors, chemiresistive glucose sensor

ÖZ

SENSÖR UYGULAMALARI İÇİN POLİMER TABANLI CİHAZ YÜZEYLERİNİN FONKSİYONLANDIRILMASI VE ÜRETİLMESİ

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Bu tez çalışmasında iki farklı sensör klinik ve gıda uygulamaları için dizayn edildikten sonra karakterize edilmiştir; iletken polimer bazlı amperometrik biyosensör ve kemirezistif sensör (polimer/SWCNT). Bu tezde üç bölümde sırasıyla fonksiyonlandırılmış polimer/MWCNT komposizyonu, farklı peptit dizilimi içeren polimer ve farklı fonksiyonel gruplara sahip monomerlerden oluşan kopolimer bazlı etanol, glikoz ve kolesterol biyosensörleri oluşturulması üzerine odaklanılmıştır. Grafit elektrot yüzeylere polimerlerin başarılı bir şekilde depozit edilmesinden sonra, alkol oksidaz (AOx), glikoz oksidaz (GOx) ve kolesterol oksidaz (ChOx) immobilizasyonu yapılmıştır. İletken polimer tabanlı biyosensörler son derece hassas ve hızlı olan biyoalgılayıcı sistemler için yeni nesil bir yapı mimarisi olarak tanımlandığı icin, bu tez çalışmasında, birçok hastalığın önlenmesinde önemli bir role sahip olduğu düşünülen etanol, glikoz ve kolesterolün kantitatif olarak tayin edilebilmesi için yeni biyosensor sistemlerinin geliştirilmesi amaçlanmıştır.

Kemirezistif sensörü oluşturmak için, nanoteller (NWs), özellikle karbon nanotüpler (CNTs) kullanılmıştır. Bu çalışmada, tek duvarlı karbon nanotüpler (SWCNTs) yüksek iletkenlikleri, olağanüstü en-boy oranı ve fonksiyonlandırılması için birçok metodun var olmasından dolayı kullanılmıştır. İlk stepte P4VP-SWCNT karışımının sprey yöntemi ile kaplandığı ve kovalent olarak tutunduğu bir yüzey oluşturulmuştur. P4VP sadece karışımı stabilize etmek için SWCNT ile iyi bir şekilde etkileşim yapmakla kalmaz aynı zamanda alkil halitlerle nükleofilik piridil nitrojenlerinde modifikasyon yapılmasına olanak sağlamaktadir. İkinci stepte, P4VP-SWCNT yüzeyinin ileri modifikasyonu icin 2-bromoetanol ile fonksiyonlandırılmıştır. Son olarak, içeceklerde glikoz tayini için modifiye edilen yüzeylere GOx immobilize edilmiştir.

Sonuç olarak, bu tez çalışmasında oluşturulan tüm cihazlar, numunelerin ön işlem gereksinimi, hassasiyet ve analiz süresi ile ilişkili olarak meydana gelen sıkıntıların üstesinden gelebilecek biyosensör uygulamalarında dört yeni örnek teşkil etmektedir. Sensör performansları herbir sensör için değerlendirilmiştir. Sensörlerin yüzeyleri ve moleküllerin tüm karakterizasyonları sensöre bağlı olarak NMR, SEM, XPS, CV, FT-IR ve Raman tekniklerinin kullanılmasıyla karakterize edilmiştir. Sensör performanslarının ileriki testleri için, sensörlerin onlara spesifik analitlere cevap verdiği ve insan kan örneklerinde ve içeceklerde kantitatif analizleri için umut verici olduğu belirlenmiştir.

Anahtar kelimeler: Sensör uygulamaları için yüzey modifikasyonları, iletken polimlerler, glikoz ve etanolün içeceklerde tayini, kolesterolün insan kan örneklerinde tayini, iletken polimer tabanlı elektrokimyasal biyosensörler, kemirezistif glikoz sensörü

To my precious husband, baby and family

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ABBREVIATIONS

ACN	Acetonitrile
AOx	Alcohol Oxidase
ChOx	Cholesterol Oxidase
CNT	Carbon Nanotube
CPs	Conjugated Polymers
CV	Cyclic Voltammetry
DCM	Dichloromethane
E_g	Bandgap
EDC	N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride
FAD	Flavin Adenine Dinucleotide
f-MWCNT	Carboxylic Acid Functionalized Multi-walled Carbon Nanotube
FTIR	Fourier Transform Infrared Spectrophotometry
GA	Glutaraldehyde
GOx	Glucose Oxidase
HOMO	Highest Occupied Molecular Orbital
HPLC	High Performance Liquid Chromatography
I_{max}	Maximum Current
K_M^{app}	Apparent Michaelis-Menten Constant
LOD	Limit of Detection
LUMO	Lowest Unoccupied Molecular Orbital
MWCNT	Multi-walled Carbon Nanotube
NHS	N-Hydroxysuccinimide
NMR	Nuclear Magnetic Resonance
PA	Polyacetylene
PANI	Polyaniline
PEDOT	Poly (3,4-ethylenedioxythiophene)
PBS	Phosphate Buffer Saline
PPy	Polypyrrole

PTh	Polythiophene
RSD	Relative Standard Deviation
SD	Standard Deviation
SEM	Scanning Electron Microscope
SWCNT	Single-walled Carbon Nanotube
VB	Valance Band
XPS	X-ray Photoelectron Spectroscope

CHAPTER 1

INTRODUCTION

1.1 Biosensors

The main concern for public health and environment has given rise to a significant interest in the analysis of some important analytes. The accurate determination of such important analytes is of great importance in environmental and human health analysis. Undesirable accumulation of such analytes is an indicator in the diagnosis and prevention of several diseases. Up to date, various methods have been proposed for their analysis including spectrophotometric, chromatographic and electrochemical methods. However, some methods have certain disadvantages such as lack of selectivity, high cost, long analysis time and requirement of pre-treatment of the samples. On the contrary, for developing fast, reliable and sensitive methods, a large number of studies have devoted to develop biosensors. Biosensors have been considered to be one of the most popular devices due to their fast response, miniature size, reliability and high sensitivity. In literature, different types of materials can be used for designing biosensors. This thesis (for the three chapters), focused on conjugated polymers (CPs) based biosensors and concentrated on their major importance in biosensor design by considering the recent studies. Biosensors based on CPs and their derivatives attract a great deal of attention. A number of CPs such as poly(aniline) (PANI), poly(thiophene) (PTh) and poly(pyrrole) (PPy) have been studied for the development of biosensors to be used in clinical and environmental diagnostics.

A biosensor can be defined as “a device that uses biological molecules, especially enzymes or antibodies, to transform chemical information into an analytical signal to detect the presence of chemicals”. Biosensors are promising analytical devices which lead to provide specific quantitative or semi-quantitative analytical information using a biological recognition element that is sustained in direct contact with a transducer.

They are diagnostic devices typically used for detection of biological analyses with a physico-chemical detector.

In recent years, biosensors have attracted great attention throughout the world. With the emerging technology specific, rapid and simple to operate sensors are needed [1]. Hence, at present, numerous biosensors have been developed for simultaneous determination of number of analytes in our daily life such as in diagnosis, food technology, biotechnology, genetic engineering and environmental monitoring etc. [2]. A biological detection element and a transducer are mainly the two parts of the biosensors. In the construction, catalytic or non-catalytic biological components can be used. Enzymes, tissues and microorganisms are examples for catalytic groups whereas the non-catalytic group stands for antibodies, nucleic acids and receptors. Transducers convert the biological signal into an understandable signal. There are several transducers for the fabrication of biosensor namely electrochemical, optical, colorimetric and acoustic ones [3]. As shown in Figure 1.1, biosensors can be categorized according to types of transducer and biological detection element. In the construction of electrochemical biosensors, the transducer transforms the biochemical information to an understandable signal, designating selectivity of the sensor. Especially, electrochemical transducers are preferred for construction of a biosensor, which can be classified as conducting, semiconducting, ionic conducting material. Electrochemical species are consumed or produced during the biological reaction, and the electrochemical signal is recorded by an electrochemical detector [4].

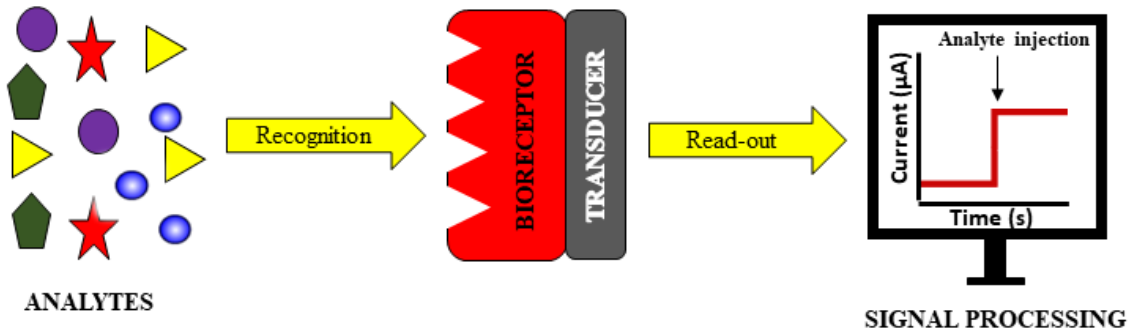

Figure 1.1 Schematic representation of a biosensor

In order to construct a successful biosensor number of conditions should be provided:

- ✓ The biocatalyst should be highly specific depending on the analysis. For example, if someone wants to construct a glucose sensor it should be highly specific for glucose.
- ✓ The sensor response should be accurate, precise, reproducible and linear over the concentration of interest. It should also be electrical or other transducer induced noise free.
- ✓ For rapid measurements of analytes from human samples like blood it is desired to obtain a biosensor which allows real-time analysis.
- ✓ The final constructed biosensor should be cheap, small, portable, easy to fabricate and stable under many of the measurements.

1.1.1 Electrochemical Biosensors

Electrochemical techniques are divided into subgroups depending on the type of transducer; conductometric, potentiometric and amperometric biosensors. Among the diversity of sensing methods applied, amperometric detection is the main focus in this thesis.

In the biosensor construction, considerable attention has been paid for developing the amperometric electrochemical biosensors. They detect substrate with the advantages of high sensitivity and reliability, fast response, good selectivity, and low detection limit [5]. This method employs the differences in the current produced by the reaction of an electroative species occurring in the bio-recognition layer upon applied constant potential. The reaction brings a change in the current and the change is associated with the amount of analytes in the reaction. The transducer surface can be modified with mediators and CPs for such purposes [1]. During amperometric measurements, a reference electrode keeps the applied potential stable which improves the repeatability of the electrochemical reaction. The electron transfer mechanism between the catalytic molecule, usually oxidase or dehydrogenase, and the working electrode surface

involving intervention or conducting polymer is the most important point affecting the functionality of the amperometric biosensor. Oxidase or dehydrogenase type enzymes can take part in oxidation-reduction reactions with the help of their active sites, including flavin adenine dinucleotide (FAD) or metal centers. As a result of this, they direct the electrons to the electrode.

Clark and Lyons developed the first amperometric type enzyme electrode in 1962 [6]. They designed the sensor for glucose analysis using the glucose oxidase enzyme with the Clark oxygen electrode via monitoring oxygen consumption. Their construction mechanism is based on recording either the production of H_2O_2 or the consumption of O_2 during enzymatic reaction. Platinum was used as the counter electrode and Ag/AgCl electrode was used as the reference electrode. Upon application of -0.68 V to the cathode, a current proportional to the oxygen concentration was obtained. Hence, the reduction of the diffused oxygen concentration is detected by following the current change. In addition to this approach, amperometric biosensor can be followed by the current change upon applied +0.68 V potential. During this method, the production of hydrogen peroxide was measured. Since the applied potential is much higher than the oxygen electrode, electroactive species in the reaction medium may cause a decrease in the biosensor performance. Preferring the lower working potential method can overcome this limitation [7]. In this thesis, during the enzymatic reactions, oxygen consumption is determined at -0.7 V (vs. Ag reference electrode) applied between the working and reference electrodes. When a potential of -0.7 V is applied, a current proportional to the oxygen content is generated. During the catalytic reactions of oxidase enzymes, oxygen is consumed. The rate of this reduction is derived from the rate of diffusion of the oxygen from the bulk solution which is based on the concentration of oxygen in the bulk. Therefore, when the catalytic reaction occurs, the depletion of the diffused oxygen concentration can be easily detected via the current change due to the establishment of a new equilibrium. Since the consumed oxygen is proportional with the consumed substrate amount, the concentration of the analyte is proportional to the current change upon oxygen consumption. Moreover, it is significant to apply low detection potentials in order to overcome the interference problem of the common species showing interference in biosensor systems, such as

ascorbic acid and uric acid. This brings the correct measurement with no interference in the measured signal. However, the oxidation of H_2O_2 usually requires a relatively high positive potential (usually over 0.6 V vs. SCE). Using -0.7 V in amperometric measurements is not an unusual choice, in contrast, it is the best widely used potential in the literature [8-17]. Moreover, application of high voltages to working electrode can bring interference problems due to the high possibility of oxidation of several materials in the samples. To follow peroxide is also bringing these consequences due to the application of oxidation potential of hydrogen peroxide (+0.7 V). In addition to these, to follow the production of peroxide in the system is quite difficult compare to following oxygen consumption in the biosensor system. Moreover, mediator use is important in some systems where the electron transfer is hard to achieve. Mediator enables faster electron transfer. Due to its redox, it is important to apply its redox potential, generally, which is quite high compare to -0.7 V. However, in our system, the conducting polymer does the electron transfer job perfectly with no need of a mediator. This also enhances the signals and reduces the interference effects.

Amperometric biosensors can be divided into three generations depending on the level of integration (Figure 1.2.). In “first-generation” biosensor construction, the creation of the product or consumption of reagent can be recorded to detect the analyte concentration. During the biosensor design, there are two ways to fix the enzyme molecule on the transducer surface: bounding or entrapment in a membrane. The “second-generation” biosensors involves “mediators” between the receptor and the transducer to improve the biosensor signals and facilitate the electron transfer. A redox reaction occurs during the enzymatic reaction that is re-oxidized by the mediator. Additionally, using mediator lowers the applied potential via eliminating the interference effect. The mediators can consist of the transition metal cations and their complexes as oxidizing agents and can be integrated into the enzymatic biosensing system [18, 19]. The choice of the appropriate meadiator is very essential in biosensor construction. A suitable mediator should have low cost, be experimental processibility and be non-toxic for biomolecules. By this way, ferrocene derivatives, organic dyes, ruthenium and osmium complexes can be used as mediators depending on their specific reactions. However, due to the weak attachment of the mediators, leaching of

the non-bonding mediators from the electrode surface occurs which cause different variations in the biosensor performance [20, 21]. "Third-generation" approach in biosensor defines the direct electron transfer between active sides of biomolecule and the transducer [1]. That means the redox enzyme is directly immobilized on the electrode surface and interacts with the electrode to give biosensor response. Such a design encourages the repeated measurements and makes better the biosensor performance [2].

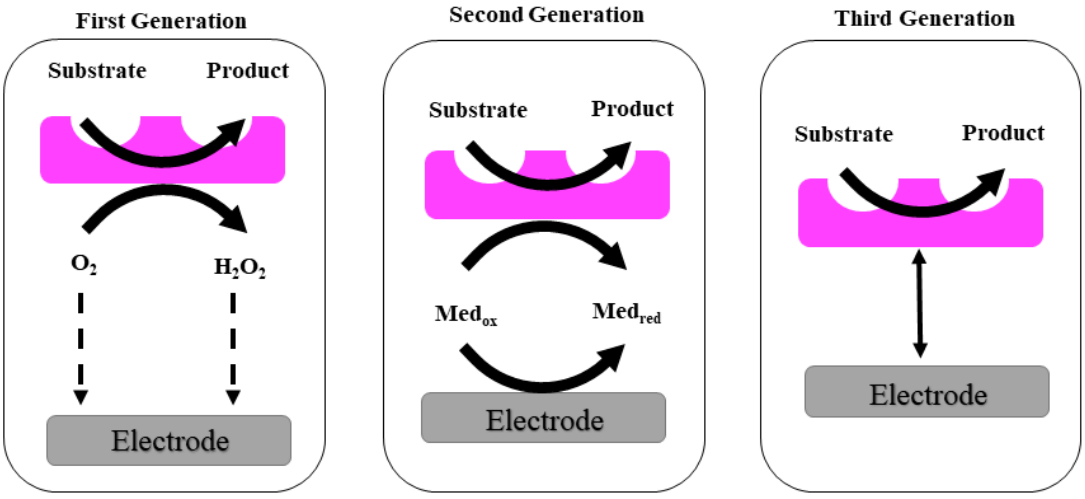

Figure 1.2 Schematic representation of three generations of amperometric biosensors

1.1.2 Immobilization Methods

Enzymes have several applications due to their unique properties such as high specificity, high catalytic efficiency and ability to lower the activation energy. There are several problems as regards to loss of enzyme on a support surface and maintenance of enzyme stability and shelf life of the biosensors. In order to overcome these problems, use of immobilized enzyme is the best solution [22]. The term "immobilized enzymes" means "*enzymes physically localized on the electrode surface*

with the retention of their bioactivity during construction of a biosensing system." The procedure of biomolecule immobilization on electrode surfaces remains as a fundamental step for the production of mechanically durable and stable biosensors. To improve the performance of the biosensor, it is preferable to find a manageable immobilization method and a stable material that can maintain the bio-catalytic activity of the biomolecules. Selected biorecognition molecule needs to be fastened on the electrode surface to provide rigidification of three-dimensional structure. Increase of the advantages of the enzyme catalysis which is possible using a suitable support with low cost and high binding capacity are the main objective of the enzyme immobilization [23]. To create an efficient and stable immobilization, the activity and accessibility of the enzyme should be considered after the immobilization. Thus, compare to free enzymes, it is expected that immobilized enzymes should be durable and resistive to environmental conditions. Therefore, for successful biosensor architecture, the immobilization should lead to promote good connection between the enzyme and the transducer surface. Otherwise, the performance, sensitivity, response time and lifetime of the biosensor are affected badly. All these are closely related with the choice of immobilization technique. In the case of enzyme denaturation or conformational change because of the immobilization, modification on the active site occurs and sensitivity decreases [24]. Hence, the choice of immobilization way is the key step in fabrication of the biosensors. The interaction between the support (transducer surface) and the enzyme can be weak or robust according to the purpose. These interactions can be broadly classified in terms of physical and chemical interaction between support and enzyme. Immobilization of enzymes can be performed using different methods: adsorption, covalent binding, entrapment and cross-linking which have been commonly used techniques to fabricate desired biosensors [25]. Each immobilization method presents some advantages and drawbacks.

1.1.2.1 Physical adsorption

Physical adsorption is the simplest immobilization method and the process is based on Van der Waals forces, ionic and hydrogen bonding as well as hydrophobic interactions,

which are very weak forces, however they can enable sufficient binding [26]. Such a method involves noncovalent immobilization and can be reversed by changing the conditions such as pH, ionic strength, temperature, and polarity of the solvent. Physical adsorption is favored by many researchers due to the simple adsorption of the enzyme molecules onto the number of CPs where it is fixed on the surface by hydrogen bonding and Van der Waals forces [27, 28]. By the help of these bonding forces, attachment of enzymes in CPs provides the localization of biologically active molecules on electrodes of any size or three-dimensional geometry to fabricate amperometric biosensors [1]. This method has been used for the preparation of many biosensors published in literature [29-31].

Immobilization by adsorption is a mild, easy process and usually protects the catalytic activity of the enzyme. The contribution of the process in other methods should be considered, such method is therefore economically attractive and has a short time processable method, however it may suffer from some problems such as enzyme leakage from matrix when the interactions are relatively weak. A using of a dialysis membrane may overcome such a problem via covering the biomaterials and preventing the leakage of the enzyme on the transducer. The representative scheme of the enzyme adsorption is shown in Figure 1.3 (A).

1.1.2.2 Entrapment

Enzyme entrapment is the most straightforward method among the immobilization methods. The use of physical entrapment of an enzyme was first achieved in 1963 [32]. Enzyme can be entrapped into the network on the electrode surface with creating three-dimensional network formation for the fixation of the enzyme. Polymer [33], dialysis membrane [34], sol-gel encapsulation [35], biological matrices [36] etc can be used as a network. Once biomolecule dissolved in a solution contains some chemicals, it is entrapped into the network. The entrapment of the biocomponents into the polymeric materials has been widely used in our groups, previously [37(a), 38]. During this one-step method, the transducer is soaked into an aqueous solution in the presence of the

monomer and enzyme. Once electropolymerization was started, the enzyme molecules entrapped in the polymeric matrix on the electrode surface during electropolymerization of the monomer. It is very simple and has a high reproducibility. However, stability of the enzyme under potential during electropolymerization and interference possibility of the supporting electrolyte in the reaction medium are main concerns for this technique.

The advantages of enzyme entrapment method are its simplicity and reproducibility of the one-step preparation procedure. Additionally, the method gives a simple possibility immobilizing of mediators or enzymes simultaneously by adding them to the polymerization solution. Such a method is different from other techniques since the biomolecule does not bind directly to the surface using specific functional groups. However, diffusion problems and long response time of sensor are some drawbacks of this technique because of restricted movement of enzymes in a porous gel. Since enzyme molecules entrapped within the network or a membrane the pore size of the network may not be sufficient to facilitate the diffusion of substrates and products [39, 40]. A representative scheme for entrapment immobilization method is shown in Figure 1.3 (B).

1.1.2.3 Covalent Binding

This method is based on the formation of covalent bonds among the biomolecule and the support matrix and the most profoundly method to construct stable biosensor (Figure 1.3 (C)). An advantage of these methods is that, because of the stable nature of the bonds formed between enzyme and matrix, the enzyme is not released into the solution upon use. There are many commercially available supports for immobilization; the best choice in each case requires the consideration of some relevant properties of the catalyst and the intended use. The binding reaction should be performed under conditions that do not cause loss of enzymatic activity, and the active site of the enzyme must be unaffected by the reagents used. Covalent association

of enzymes to supports occurs owing to their side chain amino acids like arginine, aspartic acid, histidine and degree of reactivity based on different functional groups like imidazole, indolyl, phenolic hydroxyl, etc [26].

To construct covalent attachment two sequential steps are necessary. Firstly, the supporting surface is modified via several techniques like coating the electrode with a functional polymer [41], incorporation of functional nanomaterials [42(a)], formation of self-assembled monolayers (SAM) [43] or addition of sol-gel composites [44]. Then, biomolecule is introduced to the prepared support material. At this stage, biomolecule forms a covalent binding on the electrode surface using linkers. A great number of activation methods can be used for attachment, for instance using glutaraldeyhe or carbodiimide. Glutaraldehye is used as the activating agent and allows the binding between amino groups of the support and amino groups of the enzyme [45]. Carbodiimide provides the binding between carboxylic acid of the support and amino groups of the enzyme or vice versa [12].

An enzyme structure contains free amino and carboxylic acid groups. These groups are free to attach to the functionalized electrode surface covalently with the help of activation agents used for the covalent attachment. As a conclusion, enzyme is strongly bounded to the surface providing the stability in many cases. During the covalent integration of the enzymes, the redox centers of the matrix closes to the active sides of enzyme resulted with the efficient charge transfer

1.1.2.4 Crosslinking

Schematic representation of intermolecular crosslinking immobilization method is summarized in Figure 1.3 (D). Attachment of a biomolecule is achieved with intermolecular crosslinking of protein molecule or covalent binding to the biological support. There are several proteins to crosslink the biomolecules such as bovine serum albumin, glutaraldehyde or carbodiimide. This method is used to stabilize the adsorbed protein on the surface via preventing the enzyme leakage. However, more compact

protein structure may cause leaching out of the enzyme on the electrode surface. Additionally, excess use of crosslinkers to prevent enzyme linkage may also lead to extra binding which brings activity loss. Such a method has several drawbacks, the biosensor constructed with this method presents a good operational and storage stability.

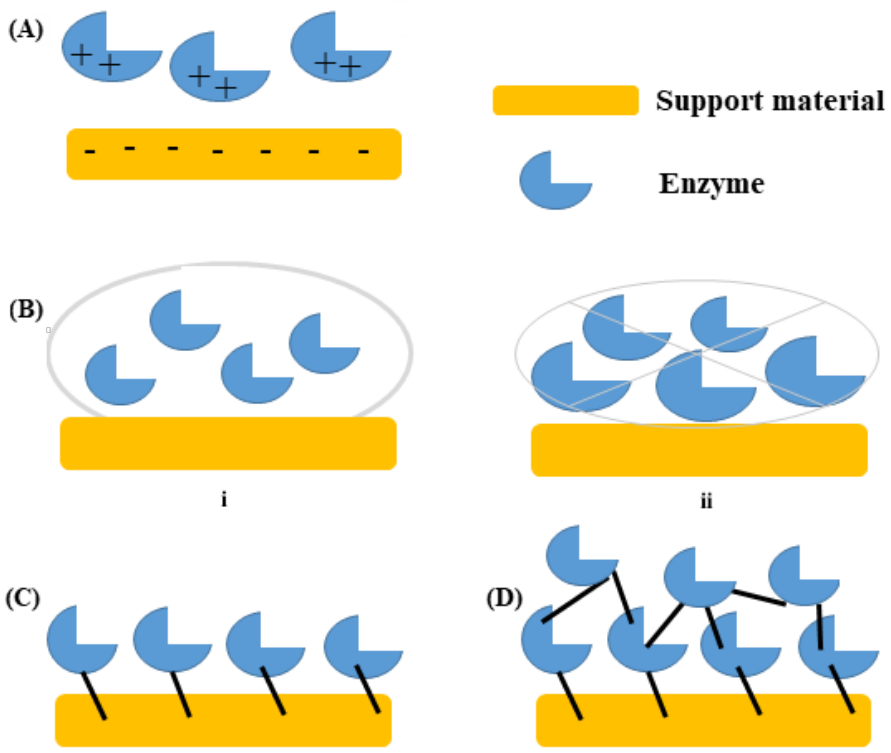

Figure 1.3 Schematic representation of (A) physical adsorption of enzymes, (B) entrapment of enzymes (i) in a dialysis membrane and (ii) in a polymeric matrix, (C) covalently attached enzyme molecules and (D) crosslinked enzyme molecules.

1.2 Conjugated Polymers in Biosensing Design

1.2.1 General Information About Conjugated Polymers

Conjugated polymers (CPs) are organic macromolecules possessing a backbone chain of alternating single and double bonds. The broader awareness of the conductivity of conjugated polymers increased since their overlapping p-orbitals create a system of delocalized π-electrons, which can result in improved electronic and optical properties [46, 47]. The increasing attention in π-conjugated polymers as a new class of electronic materials appeared with the discovery of the synthesis of polyaniline as a blue-black colored shiny powder via anodic oxidation of aniline in sulphuric acid solution by Letheby and coworkers in 1862 [48]. However, the poly(acetylene) (PA) did not attract much attention until 1977 since it was produced as an insoluble and infusible powder as well as its irrespective preparation method [49].

CPs have emerged as feasible semiconducting electronic materials for numerous applications since a new age began at the end of 1970s with the investigation of high electrical conductivity in doped PA [50]. During the study, Shirakawa and coworkers were synthesized the first highly conductive PA from Ziegler–Natta polymerization of acetylene using nearly thousand times of catalyst by mistake. Professor H. Shirakawa prepared a ‘many molar’ concentration of catalyst instead of ‘milimolar’, and obtained a bright silvery films instead of black powder. Then, because of this interesting discovery of him, Professor A. G. MacDiarmid invited Professor H. Shirakawa to the university of Pennsylvania to investigate in detail this new form of polyacetylene. In collaboration with Professor A. J. Heeger, they reported that the conductivity of semiconducting polyacetylene enhanced over 100 S cm-1 when they exposed the polyacetylene film to halogens [50]. As a result, it was concluded that PA could be transferred from insulator to a semiconductor upon doping process. This discovery brought the 2000 Nobel Prize in Chemistry award to Alan Heeger, Alan Mac Diarmid, and Hideki Shirakawa [51-53]. This trio advanced the field of “plastic electronics”.

A polymer plastic contains alternating single and double bonds along the polymer chain. Due to the localized position of the bonds, the electrons on the polymer backbone cannot carry the electric current. However, in case the material is doped with strong electron acceptors, electrons move freely and plastics turn into conductive materials. Since PA is air sensitive, new CPs were designed and synthesized over the past decades. To solve these problems, researchers from both academia and industrial laboratories focused on more stable and processable CPs such as; polythiophene, polyfluorene, polypyrrole, polyaniline, and polycarbazole. This newly synthesized CPs led to new uses as supporting materials in modern science. Moreover, since these polymers could be functionalized easily, they have a broad range of applications such as electrochromic devices [54], organic solar cells [55], organic light emitting diodes [56], organic field effect transistors [57] and biosensors [12].

1.2.1.1 Chemical and Electrochemical Synthesis of Conjugated Polymers

Chemical and electrochemical polymerization methods are most commonly used procedures to synthesize conjugated polymers. Additionally, there are also different techniques to synthesize them such as photochemical polymerization, metathesis polymerization, and solid-state polymerization [58].

One of the method to synthesize conjugated polymers via chemically is chemical oxidative polymerization. Chemical oxidative polymerization is widely used for polymerization of heterocyclic compounds in the presence of oxidizing agents. During polymerization anhydrous Lewis acid like $MoCl_5$, $FeCl_3$, $RuCl_3$ were used as catalysts. Generally, $FeCl_3$ is the mostly preferred oxidant through the chemical polymerization [59-61]. During this polymerization, the oxidants are able to oxidize the monomers in appropriate solution, leading to electroactive cation radicals and then they react with the monomer molecules to form the oligomers and polymer [62].

In another method (metal-catalyzed cross-coupling reactions), chemical polymerization can be carried out by reacting a monomer with Mg in THF, followed by self-coupling with nickel(bipyridine)dichloride ($Ni(bipy)Cl_2$) as the catalyst. Yamamoto's method is the first technique among the many types of metal catalyzed polymerization reaction in the literature. The suggested method contains the cross coupling between heteroaryl halide and Grignard reagent (Mg in THF) [63]. In addition to this methods, palladium-mediated cross-coupling reactions such as Suzuki–Miyaura, Sonogashira, Heck, and Stille reactions have been widely used in the synthesis of π-conjugated semiconducting materials. Among them Suzuki and Stille cross coupling reactions are mostly preferred to obtain donor-acceptor type polymers [64]. In Suzuki coupling reactions palladium-catalyzed cross coupling between organoboronic acid and halides is achieved with the formation of C-C bonds. Pd (0) catalyst is used for this reaction to start the cross coupling reaction. Similar to the Suzuki coupling, in Stille coupling reactions generally accepted process involves an oxidative addition of Pd from the aryl halide step [65].

Electrochemical synthesis of conductive polymers is very essential and efficient way for the bio-functionalization of electrodes because it consists of a one-step procedure that brings easy preparation of electrodes. Moreover, electrochemically generated polymeric films are highly robust during the measurements in aqueous medium and growth of conducting polymer can be controlled. Hence, electrogenarated polymers establish a powerful platform for the development of biosensors and they permit easy localization of biomolecules on transducer surface as well as enhance the charge transfer between active side of enzymes and electrode [66]. Hence, considering their compelling properties, electropolymerized films are promising materials for biomolecule immobilization in biosensing systems [67].

Although chemical polymerization provides low cost polymerization and allows to prepare large amount of conjugated polymers use of strong oxidizing agents may cause over oxidation and decomposition of the polymer as well as causing side reactions [58, 68].

Electrochemical polymerization technique overcomes these disadvantages and offers many advantages over chemical polymerization.

Main advantages of the electropolymerization methods are listed below:

- ✓ This technique allows to control the thickness of the conjugated polymer coated surface in terms of charge passing through the cell. Additionally, the film thickness could be calculated with the charge under the area of voltammogram.
- ✓ The method is simple, reproducible and obvious process for growth of the conjugated polymers on the electrode surface which allows well defined and finely controlled deposition.
- ✓ A small amount of monomer is enough to produce polymeric film on the electrode surface.
- ✓ During the electropolymerization via in-situ growing process whole electrochemical and spectroelectrochemical analyses can be completed rapidly which allows time saving.
- ✓ Electrochemically coated polymers on the electrode surface brings easy surface characterization of it for further analysis.

Yet, major drawback is that the synthesized polymer is insoluble; hence, it is difficult to characterize via traditional methods. The proposed electrochemical mechanism for the thiophene is illustrated in Figure 1.4. This mechanism is also valid for other conducting heterocycles such as pyrrole, furan and also their derivatives with the same steps. The conjugated polymer is electrochemically synthesized onto the electrode by cyclic voltammetry technique via repeated electrochemical cycling [66].

The electrochemical polymerization consists of electrochemical (E), chemical (C) and electrochemical (E) reactions resulting in E(CE)n mechanism. In this mechanism, first, radical cations of the monomers were created and later polymerization continues via dimerization and so on [69, 70].

In detail, electrochemical polymerization involves various steps as follows: Oxidation of the monomer is the first step to produce a radical cation (E); In the second step two radical cations can coupling to produce dimers followed by a proton loss to yield a neutral dimer (C), since oxidation potential of the resulting dimer is lower than the monomer oxidation potential the extended conjugation oxidation of the dimer occurs as a next step to produce its radical cation undergoes coupling to form oligomers.

Electrochemical polymerization is normally achieved in a single- or dual-compartment cell using a standard three-electrode configuration in the presence of a supporting electrolyte, both dissolved in an appropriate solvent. Electrochemical polymerization is carried out either potentiostatically or galvanostatically using a suitable power supply (potentiogalvanostat). Compare to this two techniques potentiostatic conditions are preferred to obtain thin films whereas galvanostatic conditions are used to produce thick films [58].

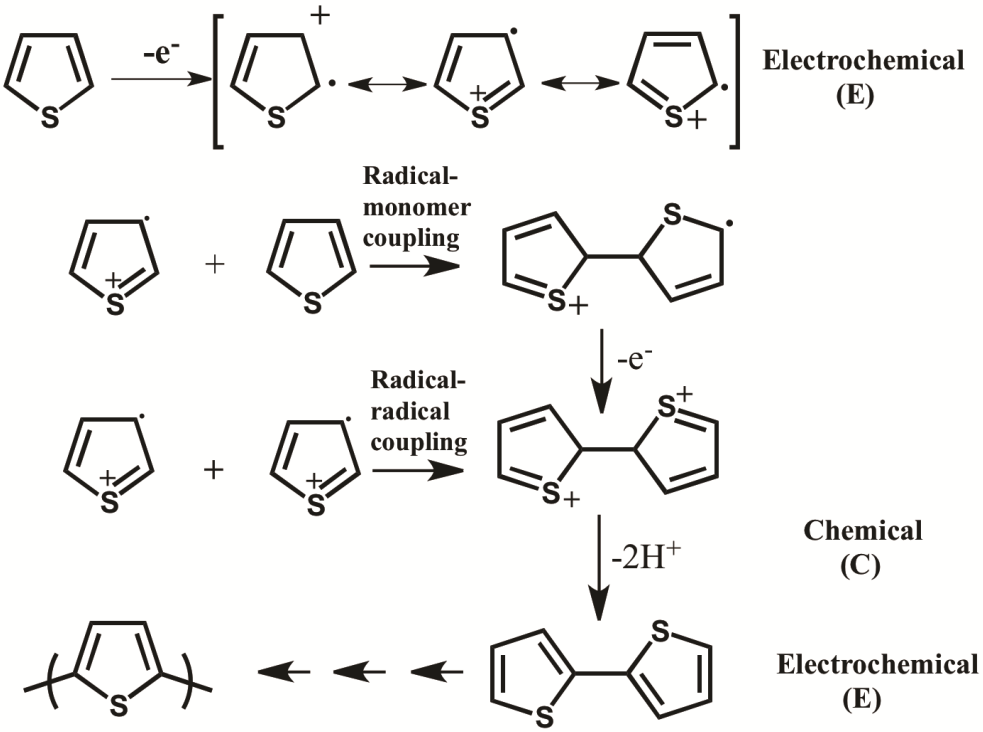

Figure 1.4 Electropolymerization mechanism of thiophene.

Cyclic voltametry (CV) is widely used potentiodynamic technique for electropolymerization of the monomers to investigate the electrochemical characterization of the resulting polymer films. During this experiment, using solvent and supporting electrolyte are very crucial steps. To say the solvent is proper for the experiment it should has high dielectric constant in order to satisfy ionic conductivity of the electrolytic medium and have high resistance against decomposition to prevent any electrochemical process in the working potential range. Most common solvents are acetonitrile, propylene carbonate, nitromethane which have high dielectric constants. Additionally, to ensure electrical conductivity in the solution and dope the polymer as the counter ion choice of a proper supporting electrolyte is very important. Using anions like ClO_4^-, $PF_6^-$and BF_4^- associated with lithium/sodium or tetraalkylamonium cations electropolymerization can be achieved. Moreover, Indium–tin oxide (ITO) coated glass, platinum, gold, glassy carbon and graphite electrodes are examples for widely used working electrodes (anodes) for electrochemical studies.

1.3 Importance of Conjugated Polymers in Biosensor Applications

Conjugated polymer interfaces offer a suitable platform for localizing biomolecules onto the electrode surfaces. They can be utilized to improve sensitivity, stability and selectivity of the biosensors fabricated for different purposes [71-73]. Use of CPs in biosensor construction enhances the electrocatalytic properties of biomolecules since the electrons can move freely on the conjugated π electron backbones [2]. Hence, this property improves rapid electron transfer and satisfies direct communication between the transducer and the biomolecule [74]. In addition, CP modified surfaces give extensive stability to enzymes on the electrode surface [75]. In enzyme immobilization strategy, one important factor is holding the biomolecule onto the substrate for long-term stability without losing enzyme activity. Thus, CPs are excellent candidates for their superior properties.

CPs can be deposited on the electrode surface using electropolymerization technique by arranging thickness of the film upon demand [76]. Besides, CPs can be electrochemically produced at room temperature and there is no need to construct

harsh conditions to produce polymers. During the electropolymerization of CPs, they can be deposited on various electrode surfaces such as Pt-, Au- or C-based electrodes or ITO coated glasses. Moreover, they can be deposited on such surfaces precisely to be independent from the size and shape of the solid substrate. This brings high reproducibility of the biological system and easy fabrication of the substrates for biosensor applications. They can be modified by incorporating specific functional moieties that can link to biomolecules of interest. This attachment leads retaining the enzyme activity for a long time [77, 78] via creating a suitable three dimensional matrix on the electrode surface. The polymer structure may include carboxyl, amino etc. groups which are open to covalent bond to achieve a robust biosensor. Moreover, upon demand, the chemical structure of conjugated polymers can be modified with different materials such as dendrimers or amino acids via modulating and synthesizing the new structures [79]. In the case of hydrophobicity, polymeric material can be modulated by introducing hydrophilic groups in order to get the highest interaction with biomolecules. Additionally, because of the biocompatible property of CPs with biological molecules they can be easily utilized for biochemical reactions and hence, biomolecules can retain their activity for long duration [80].

In medical diagnostics and detection of environmental pollutants, highly precise and rapid detection sensing methods are of great importance. In this regard, as a sensing platform, CPs are very attractive materials since their use gives variable binding interactions with biomolecules that may offer sensitive and long-term stability during measurements.

1.3.1 Conjugated Polymer Based Alcohol Biosensors

The quantitative detection of ethanol, in the quality control of alcoholic beverages during the fermentation process as well as for the clinical analysis, with biosensors is of great importance. Up to now, a number of matrices and a wide range of polymers have been employed for the development of different ethanol biosensors. However, most of the studies suffered from the average lifetime of the devices. This refers to the

chemical instability of the immobilization bonds. Furthermore, in general, the biosensors prepared with conventional techniques where the biological component is immobilized to the surface of a transducer showed poor manufacturing reproducibility. Hence, fast, economic and simple biosensing systems are necessary in order to control the quality of the products. With this motivation, the present thesis work describes the construction of an alcohol biosensor based on a newly designed matrices made of alcohol oxidase, conjugated polymer and carbon nanotube combination. The results revealed that the proposed sensor is very suitable to detect alcohol in beverages with its long-shelf life, stable and wide linear range properties which provides superiority amoung the other sensing devices.

Alcohol oxidase (AOx) is one of the most used enzymes due to its substrate specificity and availability and stability over a useful range of reaction conditions, which makes this enzyme a promising catalyst for biosensor applications. AOx (Alcohol: O_2 oxidoreductase, EC 1.1.3.13) is an oligomeric enzyme consisting of eight identical sub-units arranged in a quasi-cubic arrangement, each containing a strongly bound flavin adenine dinucleotide (FAD) molecule [81]. AOx oxidizes low molecular weight alcohols to the corresponding aldehyde in the presence of molecular oxygen (O_2) as the electron acceptor, according to the following reaction:

$$\mathrm{RC}H_2\mathrm{OH} + O_2 \xrightarrow{AOx} \mathrm{RCHO} + H_2O_2$$

Conjugated polymer based ethanol bio-sensing systems have attracted considerable attention than the others due to the unique properties of CPs. Conjugated polymeric materials led scientists to explore their distinctive and charming properties in various applications. Recently, there is a need of rapid and reliable detection of ethanol in many of areas due to its commercial importance. At this point, choice of appropriate matrix and stability of the immobilization are the most significant factors to create this fascinating analysis. An appropriate immobilization matrix should be biocompatible,

non-toxic to biomolecule, durable and long-life via conserving the bioactivity of the biological molecules. At this point, functional surfaces generated by conjugated polymers assess functionality on the electrode surfaces with the pendant groups on their backbones. Herein, most recent studies related to the conjugated polymer based amperometric biosensors prepared with alcohol oxidase are main topic for this part.

In our group, a few of conjugated polymer based ethanol biosensors with the different surface modifications have been developed. For example, a novel monomer; 9-methyl-9*H*-carbazole-3-carbohydrazine (MCCH) was designed, synthesized and electrochemically polymerized it on a graphite electrode to achieve an effective immobilization matrix for biomolecule deposition [41]. The presence of amino groups in the structure of poly(MCCH) allows covalent immobilization of alcohol oxidase onto the electrode surface. N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC)/N-hydroxysuccinimide (NHS) chemistry was used for linking carboxylic acid groups of the enzyme molecules to amino groups of the polymer. The using of CPs provided a considerably wide linear range, high sensitivity and very low limit of detection. Moreover, the poly(MCCH)/AOx biosensor was used for the determination of ethanol content in some commercial alcoholic beverages and the results were comparable with the labels provided by the manufacturers. Additionally, proper enzyme immobilization platform was achieved with the polymerization of conjugated polymers of 3,4-ethylenedioxythiophene and (EDOT) and 4,7-dithien-2-yl-2,1,3-benzothiadiazole (TBTD) [38]. In this study, entrapment method was used to immobilize AOx to achieve amperometric alcohol biosensor. Platinum electrode was coated with polymer and AOx was entrapped by polymerizing EDOT via a constant potential onto conjugated polymer coated surface. The novel designed biosensor was tested for kinetic parameters, optimum pH and optimum charge. Moreover, kinetic parameters were obtained for poly(EDOT/TBTD)/AOx and also for only poly(3,4-ethylenedioxythiophene) (PEDOT) matrix. The best results were obtained for poly(EDOT/TBTD)/AOx bio-sensing system.

Similarly, alcohol biosensors based on conducting PPy, PEDOT and poly(3,4-ethylenedioxypyrrole) were constructed [37(a)]. Moreover, the alcohol contents of the distilled beverages (vodka, dry cin, whisky, and rakı) were determined with the designed sensors and the authors reported that there is a good match with the chromatography and biosensor results. In another study, conducting polymer bearing polypeptide segments and ferrocene moieties containing surface was designed [82]. To construct such a surface, the electroactive polypeptide macromonomer and independently synthesized ferrocene imidazole derivative of dithiophene were electrochemically polymerized on the graphite electrode surface. It was reported that a novel biosensor was evaluated as an ethanol sensing system providing fast response time (9s), wide linear range (0.17 mM - 2.25 mM), low detection limit (0.28 mM) and high sensitivity value (12.52 μA mM^{-1} cm^{-2}). Moreover, obtained biosensor was tested by determining the alcohol content in several alcoholic beverages and results revealed that it is a reliable sensing strategy for alcohol determination in real samples.

Since the unique properties and potential technological applications of carbon nanomaterials have attracted increasing interest, Huangxian et al. designed an amperometric ethanol biosensor based on a stable poly(thionine)–carbon nanofiber/AOx biocomposite film. To construct such a surface, they performed a thin polymeric film on the glassy carbon electrode with an electropolymerization process. Finally, ethanol was detected with improved analytical capabilities [83]. Furthermore, the quantitative detection of ethanol is reported based on an alcohol oxidase/graphite/polymer biocomposite. Such a biocomposite is made of a graphite powder, an epoxy resin and alcohol oxidase. It was reported enzyme retained its stability and preserved its activity in the matrix [84].

1.3.2 Conjugated Polymer Based Glucose Biosensors

Glucose is an essential carbohydrate circulating in the physiological system. The beta cells of the pancreas produce the hormone insulin which converts the sugar into the skeletal muscle and adipose tissue. Improper function of pancreas results in failure to

abnormal blood glucose concentration. Therefore, the accurate determination of glucose level in any physiological fluid is crucial in diagnostics and in the long term treatment of diabetes.

Glucose detection is a crucial issue since diabetes mellitus is one of the leading causes of death and disability in the world [85]. Abnormality of the glucose level in human blood causes several disorders such as blindness, nerve degeneration and kidney failure [86]. The diagnosis and management of diabetic patients require exact monitoring and control of the glucose level in the body. Therefore, continuous testing of glucose level is crucial to prevent long-term complications.

Glucose oxidase (GOx) was often used as a model enzyme to analyze various redox centers. The redox centers lead electron transfer during the enzymatic reaction. GOx catalyzes the oxidation of β -glucose to D-glucono-δ-lactone in the presence of molecular oxygen which subsequently is non-enzymatically hydrolyzed into gluconic acid and hydrogen peroxide [87].

Ramanavicius et al reported amperometric glucose sensors to investigate effect of PANI layer on biosensor responses [88]. The GOx was self-encapsulated within PANI matrix and an increase in upper detection limit and sensor stability were detected compared to those of unmodified GOx electrode. The same fabrication process was followed by the same author and co-workers using PTh [89]. Polymerization of PTh was defined as "*green chemistry*" since no hazardous chemicals were involved during the reaction. Poly(1,10-phenanthroline-5,6-dione) (PPD) was also used as the immobilization matrix for GOx conducted [90].

One of the important reasons of preferring CPs as the support matrices in biosensor design is that CPs allow the structural and electronic modifications of various surfaces to be used as immobilization matrices [91]. The polymer backbone can be tuned by according to desired properties. For instance, presence of hydrophobic alkyl chain in the structure of poly((2-dodecyl-4,7-di(thiophen-2-yl)-2*H*-benzo[d][1,2,3]triazole

(PTBT) improved the interaction between the enzyme solution (GOx and isoleucine) and polymer coated support electrode [11]. In our previous study, two different CPs containing benzotriazole and benzoselenadiazole units were used for the fabrication of glucose biosensor [17]. Effects of selenium and sulfur heteroatom containing polymer backbones on biosensor performance were investigated. Due to better interaction ability of Se with NH, C=O, COO- and C-N groups as well as higher affinity of Se to protein, Se-containing polymer showed high biosensor responses and stability. Moreover, it provides enzyme mimic environment for the biosensor since Se has also the role of an antioxidant preventing the cell degeneration in tissues [92].

CPs can be functionalized with several groups like carboxyl, -COOH; amine, -NH_2; hydroxyl, -OH that are reactive towards biomolecules and used in covalent immobilization with the help of specific cross linking agents to achieve lifetime stability of the enzyme electrode [2]. It involves formation of covalent attachment using pendant functional groups on the immobilization matrix with the enzyme. Carboxyl group containing poly(2-(2,5-di(thiophen-2-yl)-1H-pyrol-1-yl)acetic acid was utilized to immobilize GOx successfully via EDC/NHS [12]. Electrodeposited poly([2,2';5',2"]-terthiophene-3'-carbaldehyde) film serves as an excellent host matrix for immobilization of biomolecule via covalent immobilization due to the free aldehyde groups of the polymeric structure [93]. Timur et al synthesized a conjugated polymer, poly(5-(4H-dithieno[3,2-b:29,39-d]pyrrol-4-yl)naphthalen-1amine) where GOx was deposited onto the free amino groups containing polymeric support matrix using glutaraldehyde as the crosslinking agent [94]. Poly(N-phenylglycine) (PPG) was also used to fabricate an enzyme electrode to immobilize GOx covalently on the PPG modified transducer by the condensation reaction of amino groups of GOx and the carboxyl groups present on the film [95]. N-substituted pyrrole polymers were used to investigate their matrix properties in biosensor applications [96]. Amperometric biosensors were fabricated through immobilizing GOx onto the synthesized polymers; N-(p-benzoicacid)polypyrrole (NpbPPy); N-(o-aminophenyl)polypyrrole (NoaPPy); N-(m-nitrophenyl) polypyrrole (NmnPPy).

Since the amount of immobilized GOx was enhanced with the help of condensation reaction of -COOH groups on NpbPPy matrix, the best biosensor performance was succeeded for NpbPPy matrix.

Copolymer films based on integration of different polymeric structure in a same scaffold have the properties of individual components. These copolymers are expected to embody the superiority of both parent polymers; thus, tailor-made design of such copolymers allows obtaining innumerable kinds of materials with desired functionalities [97]. Moreover, electrochemical copolymerization technique provides opportunities to deposit the copolymer at the electrode surface by controlling the several parameters as well as to obtain the desired polymer with no need of purification. Totir et al generated a copolymer film of poly (azulene-co-3-thiophene acetic acid) which was tested as the host matrix for the construction of a glucose biosensor [98]. The pendant carboxyl groups in the copolymer chain are open to form covalent attachments with the enzyme molecules. Such strong attachments provided to determine wide substrate concentrations ranging from 40 to 200 μM with a sensitivity of 0.7 $nAcm^{-1}\mu M^{-1}$ at the –0.07 V vs. Ag/AgCl. Co-electrodeposition of poly(N-acetylene)-Prussian blue (PNAANI-PB) film exhibited remarkable synergistic effects compared to prussian blue films [99]. The sensitivity was improved and working range of the glucose sensor was expanded. Moreover, 1,3-bis(2-pyridylimino)isoindoline derivative bearing 3,4-ethylenedioxythiophene (EDOT-BPI) and its palladium complex (EDOT-PdBPI) were synthesized and polymerization of EDOT-PdBPI and copolymerization with 4-amino-N-(2,5-di(thiophene-2-yl)-1*H*-pyrrol-1-yl)benzamide (HKCN) were carried out by an electrochemical method. P(EDOT-PdBPI-co-HKCN) modified graphite rod electrode was used for glucose detection in beverages [100].

Another approach to merge various polymers onto the electrode surface is the mixing of polymers or their solutions onto the electrode surface without any treatment. Yilmaz et al synthesized a copolymer of glycidyl methacrylate with 3-thienyl methacrylate (poly(GMA-co-MTM); then the polymer solution was drop-casted onto the electrode

surface to construct glucose biosensor [101]. Brett and co-workers developed a PEDOT/poly (methylene blue) (PMB) modified glassy carbon electrode (GCE) for a GOx-based biosensor [102]. PEDOT films generated on top of PMB modified bare electrode was used to enhance stability of PMB modified electrode. The proposed sensing architecture (GOx/PEDOT/PMB/GCE) showed superior biosensor performance compared to GOx/GCE and GOx/PEDOT/GCE biosensors. A conducting film composed of PANI and poly (acrylic acid) (PAA) were prepared by electropolymerization of aniline in the presence of various concentrations of PAA [103]. The biosensor response to a given concentration increased as PAA content in PANI/PAA film increased. This behaviour was related to conductivity of the PANI/PAA film since carboxyl groups within PAA chain caused protonation of nitrogen atoms on PANI.

Immobilization of mediators in the sensing matrices generates enhanced electron transfer efficiency of redox enzymes since active sites of the biomolecules are deeply embedded inside the protein [40]. Among other mediators, ferrocene and its derivatives are one of the most popular choices in biosensor design due their ability to generate stable redox species [104]. Electrodeposited co-polymer of pyrrole and ferrocene carboxylate modified pyrrole p(Py-FcPy) [105], co-polymer of pristine pyrrole, carboxylate-modified pyrrole and ferrocene-modified pyrrole [106], co-deposition of 4-(2,5-di(thiophen-2-yl)-1*H*-pyrrol-1-yl) aniline (SNS-NH_2) and 4-(2,5-di(thiophen-2-yl)-1*H*-pyrrol-1-yl) amidoferrocenyldithiophosphonate (SNS-NH_2-Fc) [107] and co-polymer of O-4-(1H-pyrrol-1-yl)-ferrocenyldithiophosphonate (TPFc) with 4-(2,5-di(thiophen-2-yl)-1H-pyrrol-1-yl)butane-1-amine (TPA) [108] were employed as the electron-mediating support materials for developing GOx-immobilized electrodes.

In biosensor design, the wrapping of carbon nanotubes (CNT) with CPs leads producing new composite materials with combined properties of each component. The synergistic effect gave rise to a remarkable improvement in the biosensor efficiency [109]. High-performance glucose biosensors were constructed by immobilization of

GOx onto a poly(2,6-diaminopyridine)/MWCNT transducer [110] or 4-(2,5-di(thiophen-2-yl)-1*H*-pyrrol-1-yl) benzenamine (SNS-NH_2) modified with CNT surface [111]. The sensors were envisaged for the determination of glucose level in various real samples which could make them a promising bio-probe for precise detection of glucose. Adronov group employed a water soluble conjugated polymer, poly [3-(3-N, N-diethylaminopropoxy) thiophene] (PDAOT) in supramolecular functionalization of single-walled carbon nanotubes (SWCNT) [112]. The PDAOT-SWCNT combination offers a universal platform for enzyme deposition within coated films revealing good film quality, improved electron-transfer kinetics and high electronic conductivity. A nafion overcoat results in improved selectivity for this sensor design. This interface effectively suppressed the response to possible interferents like ascorbic acid, uric acid etc. while maintaining much of the sensor sensitivity to target substrate; glucose. Furthermore, our group incorporated nylon 6,6 nanofibers and multi-walled carbon nanotube (MWCNT) with an aldehyde functionalized conducting polymer (PBIBA) to investigate sensor properties for detection of glucose for the first time [113]. Nylon nanofibers provide excellent reproducibility and high enzyme loading with their highly porous structure [114]. Remarkable compatibility between MWCNTs and nylon which improves the dispersion and the interfacial adhesion is a reason to select nylon nanofibers as the host matrix for the MWCNTs. Furthermore, the two dimensional nanocarbons; graphenes have attracted growing interests in the field of biosensor technology. They are assumed to have superior properties compared to CNT counterparts owing to their larger surface area, easy production with no hazardous byproducts and higher intensity of active edge planes per unit mass [115]. Highly sensitive GOx biosensors based on graphene-PEDOT: PSS modified electrode [116] and graphene-PANI-chitosan modified electrodes [117] were developed for electrochemical detection of glucose.

As an immobilization platform, the use of supramolecular compounds for bio-sensing systems creates one of the most remarkable topics. Calixarenes are the host molecules which have a cavity composed of several cyclic phenolic units [118]. They can be modified according to desired properties.

Also, their cavity properties enable easy deposition of the guest molecules on any surface [119]. Thus, these macrocyclic molecules are promising materials for enzyme immobilization. Various types of sensors combined a conducting polymer and calixarene have been designed and tested for biological components in any test solution. Safarnavadeh et al reported capability of calixarene containing parasulfonate as the anion dopant for loading GOx on PPy film in biosensor construction [120]. They emphasized that leaching of enzyme was prevented by calixarene dopant anions. This fact enhanced the stability of the suggested biosensor. In another work, the sensor matrix included AuNPs apart from a calixarene derivative and selenium containing conjugated polymer (poly(SBTz)) [121]. The proposed calixarene compound contained free thiol and carboxyl groups. By taking advantage of high affinity of AuNPs to thiols and covalent binding between the calixarene and GOx using carbodiimide chemistry, the design resulted in improved sensor stability together with a superior analytical performance.

Poly(amidoamine) (PAMAM) dendrimers with different generations are also strong candidates for the design of immobilization matrix in biosensor construction. Şenel and Nergiz synthesized different generations of amidoamine-pyrrole dendrimers with branched amine periphery and focal pyrrole functionality [122]. GOx was covalently immobilized onto the co-polymer of amidoamine-pyrrole dendrimers. Different biosensors were constructed using PAMAM G1, PAMAM G2, PAMAM G3 and without any PAMAM dendritic wedges. Also, the biosensor fabricated with pyrrole linked to any PAMAM dendron showed better biosensor signal than the one with pyrrole alone concerning the steady state current response curves of the electrodes. The reason is that PAMAM dendrons enable covalent linkage with biomolecules, serving higher enzyme loading capacity. This conclusion is also confirmed by our group [42(a)]. A carboxyl group containing conjugated polymer film was functionalized with PAMAM dendrimers; G2 and G4 and their matrix properties were investigated for glucose biosensor applications. The PAMAM G2 and G4 modified biosensors illustrates wider linear range with higher current response.

Also, it was emphasized that the crowded microenvironment of G4 around enzyme molecules had a higher K_M^{app} value than that of the G2 modified one while achieving higher I_{max}.

1.3.3 Conjugated Polymer Based Cholesterol Biosensors

The estimation of cholesterol in human blood is one of the central issues in clinical diagnosis since cholesterol is an essential lipid with several biological functions in organisms [123]. An imbalance of cholesterol level in blood is considered to be one of the most severe threats to human health and is related with several serious illnesses. High levels of cholesterol is associated with cardiovascular diseases, such as atherosclerosis, hypertension, diabetes mellitus and brain vascular diseases whereas a low level of cholesterol is associated with anemia and wasting syndrome etc. [124, 125]. Hence, several researchers have focused on the development of analytical devices for the detection, quantification, and monitoring of blood cholesterol level. Among the various analytical methods developed so far, amperometric enzyme based biosensors have been mostly preferred to detect cholesterol. In recent years, electrochemical biosensors have shown to be very efficient tools for the analysis of biologically important molecules. They are fast, inexpensive, portable, and have high selectivity and sensitivity. Moreover, this method offers a sensitive, selective and quick detection of the cholesterol in blood samples.

Cholesterol oxidase (ChOx) is a (FAD)-containing enzyme that catalyzes the oxidation of cholesterol by molecular oxygen generating cholest-4-en-3-one and H_2O_2 [126, 127]. ChOx a key enzyme in the cholesterol metabolism due to its high selectivity to cholesterol. A number of bio-sensing matrices have been developed previously, including conjugated polymers, nanomaterials or their combinations. Among the diversity of sensing materials, conjugated polymer containing surfaces are our main focus in this thesis.

In our group, different functional moiety containing conjugated polymers for construction of a cholesterol biosensor have been designed and synthesized. A monomer, (Z)-4-(4-(9*H*-carbazol-9-yl) benzylidene)-2-(4-nitrophenyl) oxazol-5(4*H*)-

one (CBNP) was synthesized, and electrochemically polymerized on an electrode where ChOx was deposited onto the polymeric support matrix using glutaraldehyde as the crosslinking agent [66]. Due to the free nitro group of the polymer, hydrogen bonding with the enzyme molecules was achieved. In addition to this, possible π–π stacking between the aromatic residues of enzyme and polymer enables strong attachment for the enzyme on the polymer surface. Since enzyme molecule is properly attached on the polymeric surface, the proposed biosensor showed a wide linear range for the substrate compared to those of previously reported studies [127, 128]. The similar fabrication process was followed in our group using a fluorine containing polymer [129]. In this study, use of a functional fluorine moiety in the structure provides a sensitive and reliable biosensor where no membrane or covalent bond was used for that matter.

Furthermore, through the successful adsorption of the biomolecules on the polymer surface, three dimensional feature of the protein molecules was protected due to the efficient H-bonding and static interactions in the polymer/enzyme interface. In our another study, the combined use of poly(10,13-bis(2,3-dihydrothieno[3,4-b] [1,4] dioxin-5-yl) dibenzo [a, c] phenazine) (PPHED) and sepiolite enhanced the stability of tertiary structure of proteins. Although PPHED do not have functional pendant groups to link the enzyme molecules with the support, efficient binding was achieved using sepiolite through adsorption. Moreover, after the enzyme immobilization the morphology of the surface changed drastically. Rather than using a pristine polymer, creating a modified one for a target application and tuning the material properties for a certain purpose was a worthy step in scientific progress. Cyclodextrins (CDs) are cyclic oligosaccharides that are composed of both hydrophobic and hydrophilic moieties in their molecular structure. This enables them to form new supramolecular complexes with a variety of molecules. An alternative approach for the easy preparation of biosensing surface based on a poly(2-(2-octyldodecyl)-4,7-di(selenophen-2-yl)-2H-benzo-[d][1,2,3]triazole)) (PSBTz)-bearing β -CD was described in our group, as well [67]. The best interaction and satisfactory immobilization of the enzyme molecules were achieved with an optimum amount of β-CD that provides an ideal conformation to create host-guest system. The successful

coating of electrode surface allows reliable and accurate detection of cholesterol in real samples.

Brett and co-workers [130] developed a PEDOT/poly(methylene blue)(PMB) modified glassy carbon electrode (PEDOT/PMB/GCE) as a platform for a cholesterol biosensor. It was reported that the poor stability of PMB modified glassy carbon electrodes was improved using a hydrophobic PEDOT electropolymerized film. The proposed bio-sensing system showed a superior sensitivity value (79.0 mA cm^{-2} mM^{-1}). Moreover, the developed ChOx/PEDOT/PMB/GCE biosensor was tested for cholesterol detection in whole cow milk and egg yolk. In another study, Shin and coworkers [131] were designed a PANI coated polyester film to obtain an amperometric cholesterol biosensor. In this study, PANI and polystyrene were used to improve sensor properties. When PANI was used alone, proper film was not generated on the electrode surface. Therefore, the combination of the PANI and polystyrene served as a proper immobilization matrix by making use of an electrostatic layer-by-layer adsorption technique. Electrospun PANI nanofibers were used to design a cholesterol biosensor by Shin and Kameoka [132]. After they constructed PANI nanofibers, biomolecule was immobilized on the PANI nanofibers using an electrostatic layer-by-layer adsorption method. A highly branched polymer (PEI) was used for reduction and simultaneous derivation of graphene oxide (GO) to form a biocompatible polymeric matrix on reduced graphene oxide (RGO) nanosheet. Ferrocene redox moieties were then wired onto RGO nanosheets through the polymer matrix. The proposed sensing material was used to accommodate enzymes stably and the sensor showed high stability, excellent selectivity, good reproducibility and fast sensing response [133].

To fabricate graphene (G), polyvinyl pyrrolidone (PVP) and PANI nanocomposites based biosensor (G/PVP/PANI), Ruecha et al [134] designed a cholesterol sensor via electrospraying technique. Under optimum conditions, linear range and LOD values were determined. The amperometric measurements were achieved during 2 weeks to test the reproducibility of the sensor. It was reported there was only 10.9 % decrease

in its initial response indicating that the constructed paper-based sensor had good stability for bio-applications.

In the light of all these studies (alcohol, glucose and cholesterol biosensors), it is concluded that the designed sensors have an important impact on easy detection of analytes in real samples. All the polymers and surface designs used in the construction of amperometric biosensor were used for the first time in this thesis. Moreover, the proposed sensors were easily applied for detection of important analytes in real samples without using any membrane or mediator which the leakage of the mediator from the surface is the main problem of reagentless devices. Hence, the designed sensors showed great stability, high sensitivity and fast response to analyte when compared to the most of the published studies in the literature.

1.4 Chemiresistive Sensors

Chemiresistive sensors can be readily integrated into portable and low-cost devices that have been widely investigated for various applications to detect different types of the analytes [135-137] Sensor arrays of chemiresistive materials are naturally produced in this way. Such sensors are based on arrays of chemiresistive materials wherein their resistance can be altered by environmental effects. Chemiresistor sensor arrays are a promising technology to replace current laboratory-based analysis instrumentation, with the advantage of its low cost, manufactured easily and facile integration into portable devices for in-field use.

The assertion that nanowires (NWs) offer advantages in sensors is often made without justification since they have high surface area to volume ratios, which allows more interactions with analytes. Nanowires in the form of carbon nanotubes (CNTs) are well suited for use in sensor applications due to their mechanical and electrical properties [138-141]. Amongst all the NWs that have been explored for this application, in this work single walled carbon nanotubes (SWCNTs) were favored because of their excellent conductivity, exceptional aspect ratios, and numerous methods available for functionalization. Moreover, SWCNTs have been employed as a key component for

the construction of biocompatible platforms in biotechnological applications [142-144]. The typical SWCNTs based chemiresistive sensor is illustrated in Figure 1.5. A resistor (chemiresistor) is the simplest device with two same electrodes (adhesive (Cr) and metal (Au) layer) on an insulating glass support connected by NWs. Chemiresistors are fabricated by depositing the pristine semiconducting CNTs (conductive channel) between two electrodes where a small potential is supplied and the current is monitored. Upon analyte exposure, the CNT film exhibits changes in conductance.

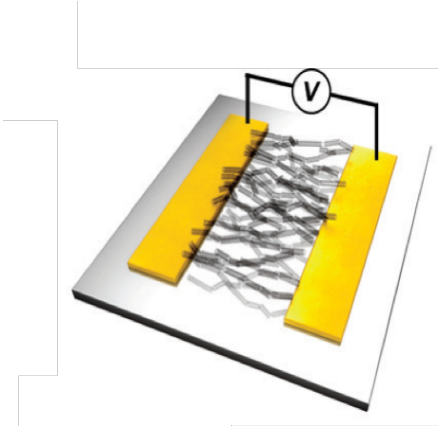

Figure 1.5 A chemiresistor based on a randomly oriented network of SWCNTs spanning two metallic electrodes [145].

CNTs can be functionalized either covalent or non-covalent. Since covalent modification disturbs the p-electron system of nanotubes and adds defects, non-covalent functionalization is mostly preferred by researchers. Noncovalent modification is less invasive than covalent functionalization as it mainly based on π interactions and Van der Waals interactions between molecules and CNTs. A common strategy is to use polymers. To reduce the dispersion challenge of SWCNT in solution, composites of CNTs with polymers were found to be useful to improve the solubility of CNTs as well as to prevent nanotube aggregation. Since the poor solubility of SWCNTs is a major disadvantage that limits their applicability and precise assembly in sensor applications, Swager group recently reported dispersions of SWCNTs stabilized by wrapping with poly(4-vinylpyridine) (P4VP) to create a diversity of transition metal compositions [146].

The P4VP dispersant not only displays a favorable interaction with SWCNTs to stabilize debundled dispersion, but it also allows further modifications on its nucleophilic pyridyl nitrogens with alkyl halides or metal ions. Moreover, P4VP has a potential to provide a suitable immobilization matrix for enzymes, antibodies, and nucleic acids. Based on this consideration, herein, focused on the P4VP-SWCNT nanocomposite as a sensor platform to create glucose sensor.

1.4.1 Carbon Nanotube Based Chemiresistive Sensors

Carbon nanotube (CNT) resistance-based chemical sensors have gained tremendous research interest and have been widely investigated in recent years [142, 143, 147, 148]. A typical CNT-based chemiresistor consists of one or several pairs of electrodes that make electrical contact with a thin film or network of CNTs. A change in the electrical resistance of the CNT film upon exposure to an analyte is measured as the output signal. The advantages of chemiresistors include minimal electronic components, low power consumption, and simple operation [136]. These features enable miniaturization of the sensing unit with no sample preparation and unobtrusive integration into a wearable system.

SWCNTs have found promising use in sensor applications since their electrical conductivity can be modified through interaction with chemical [149] or biological [150] species. Thus, in this thesis a SWCNT based chemiresistive sensor was designed and examples of literature discussions based on it were given. Different types of SWCNT devices, such as SWCNT field-effect-transistors (FETs), SWCNT chemiresistors and chemicapacitors have been developed for analyte detection. Among them, the chemiresistors which are based on the simple changes in resistance in response to the binding of analytes are very attractive because of their simple structure and the ease of high precise measurement [136,151]. Complexity associated with device fabrication has largely been limited with the use of CNT-FETs transistors for sensor applications. So, the objective of this part of the thesis was to design a simple

and cheap device via creating a surface wherein allows highly selective and sensitive analyte detection using a SWCNTs-based chemiresistor.

Since chemiresistor research has predominantly focused on vapor sensing [137, 152-154] the potential of utilizing chemiresistors directly in aqueous environments is rare in literature. SWCNTs-based chemiresistor/FET transducers have been modified with antibodies, enzymes, and aptamers, for highly sensitive and selective detection of biomolecules. Mulchandani et al [155] designed ZnS nanocrystals decorated single-walled carbon nanotube (SWNT) based chemiresistive sensor for DNA. They reported that free carboxyl groups surrounding the ZnS nanocrystals provided large loading of single strand DNA (ssDNA) probe. This allows an easy hybridization of ssDNA with target complementary c-ssDNA resulting in large electron transfer to SWNT. Thus the proposed ZnS/SWCNT/FET chemiresistor system provided a significant improvement in sensitivity toward c-ssDNA.

In another work, a chemiresistive sensor based on 1-pyrenemethylamine (PMA) functionalized SWCNT platforms was designed for detection of 2,4,6- trinitrotoluene in water [156]. They concluded that interaction of TNT with PMA on the surface of SWCNTs caused significant change in the conductance of the SWCNT sensors, that causes easy detection of TNT, without the need of pre-concentration of the analytes.

A label-free chemiresistive biosensor was designed for the detection of cardiac specific biomarker troponin-I (cTnI) [157]. The system based on mercaptopropionic acid capped gold nanoparticles (GNP) functionalized SWCNT hybrid. The functional surface allows covalently immobilization od cTnI antibody on gold nanoparticles through carbodiimide coupling reaction. The cTnI interaction to its corresponding antibody was followed with respect to changes in conductance in SWNTs channel.

To detect antigen myoglobin (Mb) in phosphate buffer saline, the device surface was modified with platinum nanoparticles (PtNP) and SWCNT hybrid. The device showed target protein antigen Mb-specific concentration-dependent response over a range of 0.1–1000 ng ml^{-1}.They reported the proposed sensing platform makes it a useful analytical tool in a large variety of applications [158].

Graphene, carbon nanotube, and gold nanoparticle chemiresistor sensors were also constructed for the detection of petroleum hydrocarbons in water [159]. In the work, aqueous solutions of petroleum hydrocarbons: cyclohexane, naphthalene, benzene, toluene, ethylbenzene, and the three isomers of xylene (BTEX analytes) were used as the analytes. Authors used different chemiresistors made from thin film assemblies of four different nanomaterials: gold nanoparticles (AuNP), single-wall carbon nanotubes (SWNT), multiwall carbon nanotubes (MWNT), and reduced graphene oxide nanosheets (RGON) to assess their sensitivity over the analytes.

Lee et al. designed a non-enzymatic chemiresistive sensor to detect glucose with combination of a synthetic receptor with aligned SWCNTs device [160]. Basically, they immobilized boronic acid (as a multivalent sugar receptor) on carbon nanotubes through amide bond formation. Using the interation between three common sugars (D-glucose, D-fructose and sucrose) and boronic acid modified SWCNT device, they detected such sugars. Such a system exhibited a high priority to bind fructose over other sugars.

In another work, displacement-based chemiresistive affinity (bio)sensor was designed [161]. The sensing system is based on glucose-concavalin A-dextran as a model system to demonstrate the displacement-based chemiresistive mode of sensing. They proposed that upon introduction of glucose to a ConA-dextran complex, glucose displaces dextran from ConA and the binding of ConA to carbohydrates results in changes to its conformation that causes a change in its isoelectric point. Finally, to evaluate the selectivity of the system over other sugars, they tested the ConA

displacement from the sensor surface and they concluded ConA showed higher affinity for glucose than sucrose and no affinity for galactose.

Choi et al designed enzyme based-chemiresistive glucose sensor using inkjet printing method. They used platinum nanoparticles and polyaniline layer. Using the conductivity properties of polyaniline they detected glucose [162].

As can be evidenced from the literature examples, SWCNT based chemiresistive systems showed good selectivity towards analytes. For this purpose, in this thesis, a SWCNT based chemiresistive sensor was designed and its sensor performances and surface characterizations were investigated in detail. Such a sensor design allowed a greatly simplified sensor fabrication process via selective detection of glucose in beverages.

CHAPTER 2

DEVELOPMENT OF AN EFFICIENT IMMOBILIZATION MATRIX BASED ON A CONDUCTING POLYMER AND FUNCTIONALIZED MULTIWALL CARBON NANOTUBES: SYNTHESIS AND ITS APPLICATION TO ETHANOL BIOSENSOR

2.1 Background and Motivation

Material design is important in surface modification depending on the purpose of the application. Rather than using a pristine material, creating a modified one for a target application and tuning the material properties to make it useful for a certain purpose is a worthy step in scientific progress. Functionalized conducting polymers are favorable materials towards surface modification and they can be used for detection and modulation of biomolecules [163]. Carbon nanotubes (CNTs) are important nanomaterials for their incorporation into electrochemical and biological sensing devices due to fast electron transfer, high surface area, electrochemical stability and biocompatibility [164]. Moreover, CNTs can act as immobilization matrices and as electrochemical transducers, resulting in the improvement of performance of the immobilized enzyme which enhances their use in biosensors [165, 166]. CNTs are classified as single wall carbon nanotubes (SWCNTs) and multiwall carbon nanotubes (MWCNTs) which have the advantages of good dispersion and low cost. Additionally, MWCNTs have also extensively used in biosensor applications since they have good compatibility with biomolecules in addition to their mechanical strength and stability properties. They enlarge the electroactive surface area of the electrodes, which results in an increased charge transfer and conductivity. As a result, they can catalyze biochemical reactions and promote charge-transfer between biomolecules and electrode surfaces [167-169]. So far, most studies have focused on the development

of an effective immobilization matrix using f-MWCNTs [170]. For the preparation of mechanically robust and stable biosensors, CNTs with the superior electron transfer properties are considered as appropriate electron transfer agents between the redox side of the enzyme molecules and the electrode.

In this study, with these motivations, a novel DAD type monomer, 2-(4-nitrophenyl)-4,7-di(thiophen-2-yl)-1*H*-benzo[d]-imidazole (BIPN), was used and its polymer was successfully synthesized electrochemically. Alcohol oxidase (AOx) was immobilized onto the conducting polymer coated electrode to construct the biosensor. To improve the immobilization and enhance the interaction between the polymer and enzyme molecules, carboxylic acid functionalized multiwall carbon nanotubes (f-MWCNTs) were introduced within the polymer coating. By this appraoch, not only covalent immobilization between the enzyme and carboxylic acid functionalized carbon nanotubes was achieved towards enhanced immobilization and prolonged stability, but also fast electron transfer on the electrode surface was achieved. Hence, the electrochemical properties of the polymer and the performance of the biosensor were improved. We developed a sensitive, simple and effective matrix for the preparation of an alcohol biosensor. The use of a conducting polymer of BIPN as the host matrix for immobilization of AOx enhanced the sensitivity, stability and film quality. Besides, the electrogenerated polymer film provides an easy control over the properties of the polymeric coating such as morphology and thickness.

CNTs and CPs are both interesting for their unique electrochemical properties. Without CNTs, the thin conducting polymer films provide a reasonable amperometric response time, but they generally suffer from low conductivities. On the other hand, when the thickness of the polymeric film increases, charge transport between the active side of the enzyme molecule and the transducer becomes slow. To overcome these problems efficient binding between enzyme molecules and immobilization matrix was achieved with the combination of CPs and functionalized CNTs. The presence of –COOH or –OH groups on the nanotube surface improves the attachment of organic or inorganic materials since a variety of chemical reactions can be conducted with this group.

Moreover, wrapping of functional CNTs in polymeric chains was found to be useful to improve their solubility as well as prevent nanotube aggregation, which helps to disperse and stabilize the CNTs in a polymer matrix. With the help of the combination of CNTs, ordered and homogeneous free defect films have been easily achieved [171]. This combination process is constructed through the Van der Waals interactions and π–π stacking between CNTs and polymer chains.

Conducting polymers are attached to CNT surfaces by in situ polymerization to improve the processability, and electrical, magnetic and optical properties of CNTs. AOx was immobilized onto the CNT modified conducting polymer coated graphite electrode with the help of EDC/NHS crosslinking agents in order to construct an amperometric alcohol biosensor. The presence of the free carboxylic acid groups on the nanotube backbone can be utilized for the covalent attachment of enzymes via formation of amide bonds. In this case, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were used to activate the free carboxylic acid groups of the conducting nanotube backbone. Through functional carboxylic acid groups of the CNT, amide bond formation between enzyme molecules and CNTs was generated. In addition to this covalent binding, a physical adsorption process occurs where the enzyme molecules were adsorbed in the polymer interface due to p–p stacking and the hydrogen bond between the functional groups of the enzyme molecule and NO_2 of the conducting polymer. Therefore, the research is increasingly focused on the preparation of a novel immobilization matrix with the combination of CNTs and conducting polymer. Moreover, the affinity of biological molecules to the benzimidazole unit is well-known. This idea was also used while synthesizing the monomer. By this way, it was aimed to have a polymeric immobilization matrix with high affinity to enzyme molecules in order to well immobilize them onto the electrode surface. This allows better contact between the biomolecule and the electroactive layer thereby improving the biocompatibility of enzyme molecules. The immobilization matrix was characterized by scanning electron microscopy (SEM), Fourier transform infrared (FTIR) spectroscopy and X-ray photoelectron microscopy (XPS) techniques. The practical application of this modified electrode was tested via determining alcohol in various beverages.

2.2 Experimental

2.2.1 Materials and Apparatus

Alcohol oxidase (E.C.1.1.3.13) from *Pichia pastoris* (28 Units/mg protein), methanol, $NaClO_4$ and $LiClO_4$ were purchased from Sigma-Aldrich and used with no further purification. Dichloromethane (DCM), acetonitrile (ACN) were purchased from Merck (Darmstadt, Germany). 2-Propanol and tert-butanol were obtained from (Merck). Ethanol (Carlo Erba) was used as received to prepare the substrate solution (1.7 M) at room temperature. For enzyme immobilization, a phosphate buffer solution (pH 7.0) consisting of 0.025 M Na_2HPO_4 (Fisher Scientific Company) and 0.025 M NaH_2PO_4 (Fisher Scientific Company) was used. N-hydroxysuccinimide (NHS) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) were purchased from Fluka (Buchs, Switzerland) and Sigma, respectively. All chemicals were of analytical reagent grade. Multi-wall carbon nanotube (O.D. × L 6-9 nm × 5 μm, >95% carbon) was purchased from Sigma-Aldrich. Alcoholic beverages were of commercial types.

Electrochemical measurements were performed with Ivium Compact Stat (The Netherlands) potentiostat in a cell equipped with three electrodes. Electropolymerization was performed with a Voltalab 50 potentiostat in a three-electrode cell consisting of graphite electrode (RingsdorffWerke GmbH, Bonn, Germany, type RW001, 3.05 mm diameter and 13 % porosity) as the working electrode. A platinum wire as the counter electrode, and a Ag wire as the pseudo reference electrode were employed. In order to perform the spectroelectrochemical studies of the polymer films Varian Cary 5000 UV-Vis spectrophotometer was used. For surface imaging of the cholesterol electrode, scanning electron microscope (SEM) (JEOL JSM-6400 model) and X-ray photoelectron spectroscopy (XPS) (PHI 5000 Versa Probe (F ULVACPHI, Inc., Japan/USA) with monochromatized Al Kα radiation (1486.6 eV) 10 as an X-ray anode at 24.9 W were used. Infrared (IR) spectra were recorded on a Varian 1000 FT-IR spectrophotometer in KBr pellets.

2.2.2 Synthetic procedure

2.2.2.1 Synthesis of 2-(4-nitrophenyl)-4,7-di(thiophen-2-yl)1*H*-benzo[d]imidazole (BIPN)

The corresponding monomer was prepared using the procedure described before [172].

2.2.3 MWCNT Functionalization

Pristine MWCNT (0.3 g) was dispersed in concentrated nitric acid (HNO_3, 70 mL, 65 %) and refluxed for 48 hours. Afterwards, the reaction was cooled to room temperature and filtered under vacuum. The residue was washed with distilled water until the pH of the solution became neutral. Then, thin film of f-MWCNT was dried at 80°C for overnight and a black powder was obtained [173].

2.2.4 Biosensor Preparation

For the construction of the bioactive layer on the graphite electrode, graphite rods were polished on emery paper and washed completely with distilled water. f-MWCNT modified poly(BIPN) based electrodes were prepared as follows: The functionalized MWCNT (0.2 mg) was mixed in 0.001 M BIPN monomer solution in 0.1 M $NaClO_4$/$LiClO_4$/DCM: ACN (5:95) by ultra sonication for 4 h. The f-MWCNT comprising conducting polymer layer was obtained through the electropolymerization by cycling the potential between 0 and 1.3 V at a scan rate of 0.1 V/s on a graphite electrode. The electrode was washed with distilled water to remove the organic impurities. After successful deposition of f-MWCNT/poly(BIPN) by cyclic voltammetry, 10 µL of AOx solution (50 mM pH 7.0 sodium phosphate buffer solution containing 3 U AOx, 0.4 M N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC) and 0.1 M N-hydroxysuccinimide (NHS)) was spread over the modified graphite electrode and left to dry for 3 h at room temperature. The enzyme electrode was stored at 4 °C for overnight. Finally, to get more uniform films, it was rinsed with distilled water.

Hence, we obtained an adherent, robust and higher response signals via efficient combination of enzyme molecules with modified electrode containing f-MWCNT/poly(BIPN)/AO_X successfully (Figure 2.1).

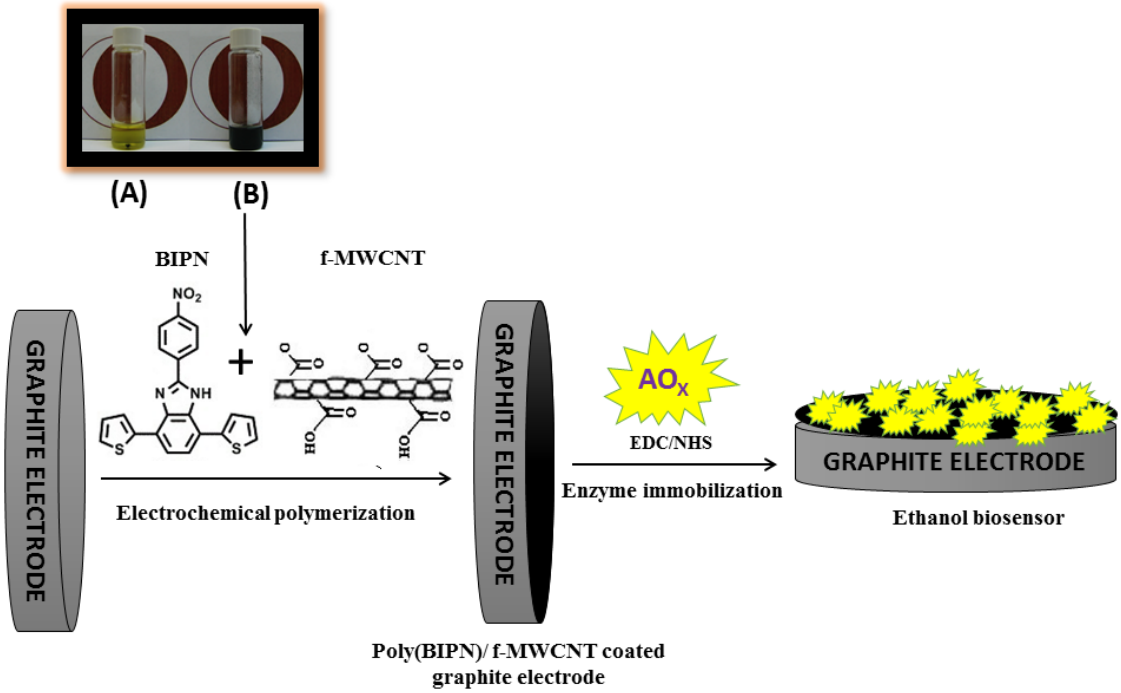

Figure 2.1 Typical preparation of the proposed biosensor ((A) before and (B) after dispersibility behavior of f-MWCNT in BIPN monomer solution given as inset).

2.2.5 Amperometric Biosensor Measurements

Amperometric studies were achieved in a reaction cell containing 10 mL phosphate buffer solution (50 mM, pH 7.0) under continuous stirring. After each measurement, working buffer solution was refreshed and electrodes were washed with distilled water and kept in phosphate buffer solution (50 mM, pH 7.0). Decrease in oxygen level as a result of enzymatic reaction was monitored at -0.7 V vs Ag in phosphate buffer. After background current reached a steady state, freshly prepared ethanol solution was injected into the medium and the current change was recorded.

All the experiments were carried out at ambient conditions. Measurement of amperometric analyses were calculated as average of three measurements and standard derivations were given as ±SD.

2.3 Results and Discussion

2.3.1 Electrochemical Polymerization of f-MWCNT/BIPN

The purified f-MWCNT (0.2 mg) was mixed with 0.001 M BIPN monomer solution in 0.1 M $NaClO_4$/$LiClO_4$/DCM: ACN (5:95) electrolyte/solvent system by ultra sonication for 4 h at room temperature. After this treatment a black suspension was obtained as depicted in Figure 2.1. During this step, functional groups of BIPN were adsorbed on f-MWCNT surface with the help of π-π conjugation of BIPN. The polymer film of f-MWCNT/poly(BIPN) was potentiodynamically coated on graphite electrode with 10 scans between 0 V and 1.30 V versus Ag wire pseudo-reference electrode via cyclic voltammetry with a scan rate of 100 mVs^{-1} (Fig. 2.2 (B)).

Charge involved in the film formation were calculated as 0.32 mC (thickness; 7.0 nm) for pristine poly(BIPN) and 0.50 mC (thickness; 11.0 nm) for f-MWCNT/ poly(BIPN). This difference can be related to the presence of f-MWCNT on the electrode surface. Moreover, oxidation potentials for monomer, polymer and f-MWCNT incorporating polymer can be calculated from the cyclic voltammograms. From Figures 2.2 (A) and (B), E_{ox}^{mon} were calculated using the first cycle of repeated potential scan polymerizations as 1.05 V and 0.99 V, respectively. Insertion of functionalized MWCNT on poly(BIPN) had significant affect both on electroactivity (current, charge) and oxidation potentials. After insertion f- MWCNT, ΔE^{ox} was calculated as 60 mV from the cyclic voltammetry results. Tremendous increase in the current density of f-MWCNT compared to pristine one also proves the previously discussed argument (Figures 2.2 (A) and (B)). Therefore, it can be concluded that insertion of f-MWCNT into the corresponding polymer solution increases the electroactivity [164].

CNTs have high specific surface area making them promising candidates for the construction of highly porous skeleton [174]. Especially, carboxylic acid group functionalized CNTs, have advantages in the immobilization of biomolecules through covalent bond formation and hence, highly specific biomolecule detection can be achieved. This combination offers a useful platform for immobilizing biomolecules to enhance sensor performances. It also provides a good interaction between the active site of the enzyme and CNTs inside the polymer layer. These results were evidenced by cyclic voltammetry where higher amperometric responses and charge involved in the film formation were obtained compare to the matrix without CNTs (Fig. 2.2 (C)). The formation of f-MWCNT/poly(BIPN) has been explored for a possible improvement in the electrical properties of polymers. Polymerization changes with the introduction of f-MWCNT into the monomer solution as seen in the second cycles of each electropolymerization (Fig. 2.2 (C)). The effect of carbon nanotube addition can be clearly seen and discussed above. However, when the amount of f-MWCNT increases in the monomer solution (0.4 and 0.6 mg), the charge deposition of the films decreases. This may be associated with the defect formation in the polymer film due to the excess presence of CNTs on the electrode surface. The polymer chains may form shorter or the stacking of the chains may be destroyed; hence, ordered structure of the polymeric film may be affected after a certain amount of f-MWCNT during the polymerization.

CPs can be used as immobilization matrices due to their good electrochemical and physical properties. Moreover, thanks to their easy decoration with functional groups during their synthesis, more powerful and robust polymeric films can be achieved for desirable enzyme immobilization. On the other hand, CNTs have received considerable attention as an electrode material due to their good electrocatalytic properties. As seen in Figure 2.2 (D), addition of optimum amount of modified CNTs (0.2 mg) improved biosensor performance. In Fig. 2.2 (D), the second cycles for the polymerizations of BIPN and f-MWCNT/poly(BIPN) were seen. The amounts and conditions in the electropolymerization were kept constant whereas electropolymerization shown with black line depicts only the polymerization of the monomer.

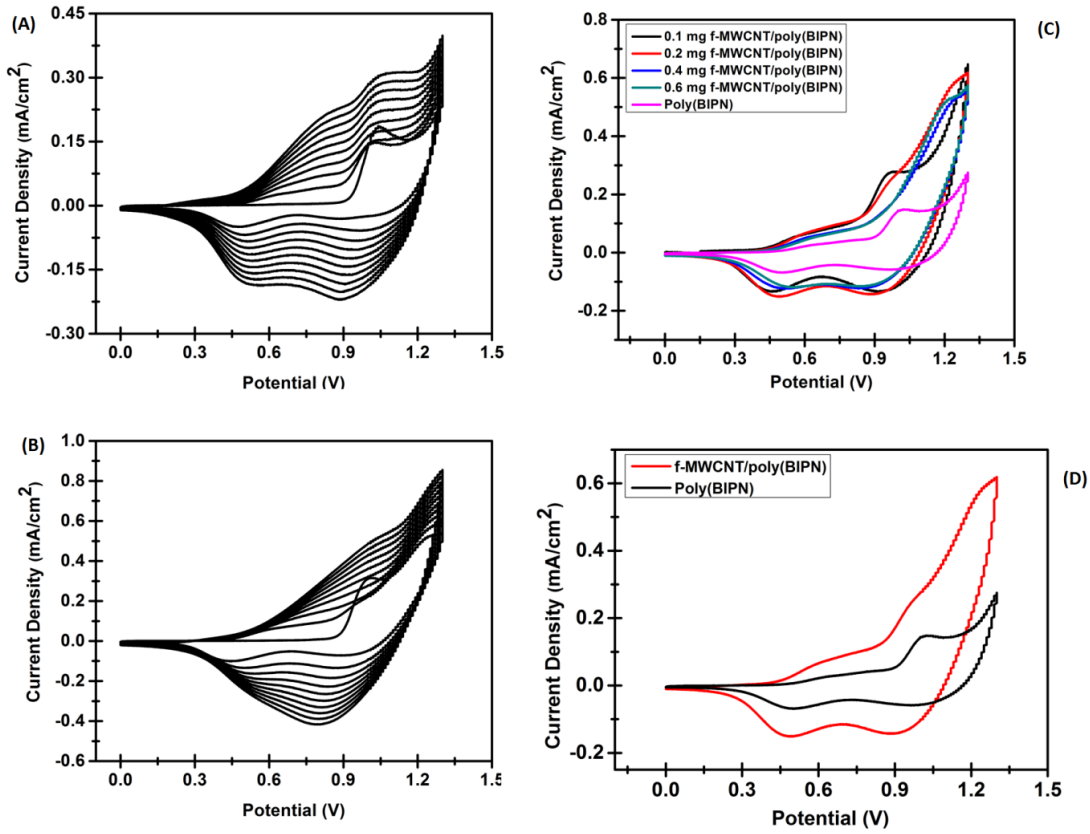

Figure 2.2 Repeated potential scan polymerization of (A) Poly(BIPN) and (B) f-MWCNT/poly(BIPN) in 0.1 M $LiClO_4/NaClO_4$, DCM/ACN (5:95, V: V) solution at 100 mV s^{-1} (C) Cyclic voltammograms of poly(BIPN) and f-MWCNT/poly(BIPN) at different f-MWCNT concentrations (D) Cyclic voltammograms of poly(BIPN) and optimum f-MWCNT/poly(BIPN) electrode in 0.1 M $LiClO_4/NaClO_4$, DCM/ACN (5:95, V: V) solution at 100 mV s^{-1}.

2.3.2 Optimization Studies

The thickness of the polymer/f-MWCNT layer can be adjusted with scan number during the electropolymerization. To investigate the effect of the film thickness on a biosensor performance, electrodes were prepared with different scan numbers and biosensor responses were determined (Fig. 2.3). Since the released electrons during the enzymatic reactions should be properly transferred to the transducer, thickness of the film is very important. To keep the enzymatic layer stabilized on the electrode

surface, a proper thickness is desired. On the other hand, enzyme molecules together with the modified layer due to the covalent and physical binding may leach from the surface. In a small thickness huge protein molecules could not be stabilized on the electrode surface. In order to investigate the effect of conducting polymer and functionalized carbon nanotube layer thickness, an optimum concentration (0.2 mg carbon nanotube in 2 mL monomer solution) were used in polymerization on the graphite electrode via using different scan numbers (20, 30, 40 and 50 scans). During these experiments, other than polymeric layer thickness, all other parameters were kept constant. Optimum value was found with 30 scan electropolymerization.

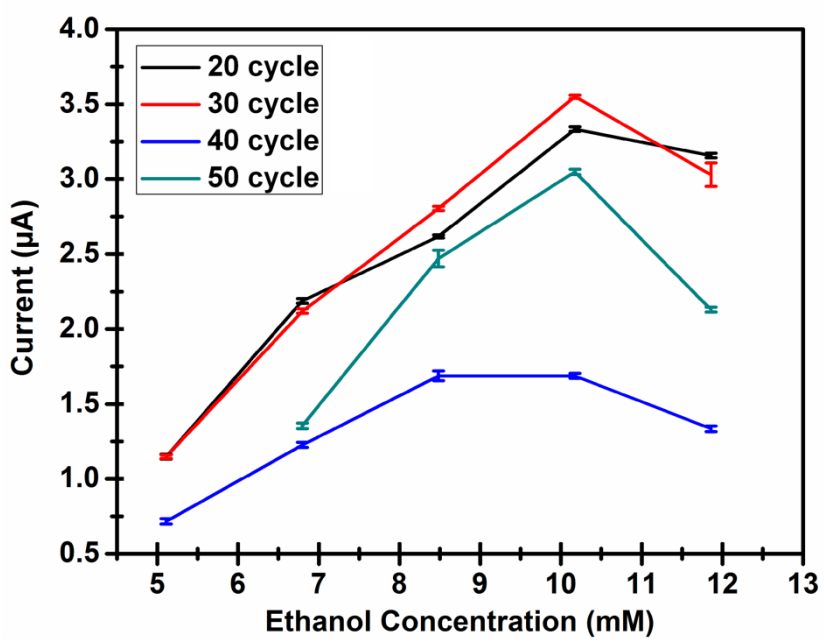

Figure 2.3 Effect of cycle number (in 50 mM phosphate buffer, pH 7.0, 25 °C, -0.7 V). Error bars show standard deviation (SD) of three measurements.

For the investigation of sensor coating composition, different compositions of f-MWCNT/poly(BIPN) suspension were tested. In these experiments, other than carbon nanotube amount, all other parameters were kept constant. For this reason, five different biosensors were prepared on graphite electrode in 0.1 M $NaClO_4$/$LiClO_4$/DCM: ACN (5:95) electrolyte/solvent system at room temperature via

scanning the potential between 0 V and 1.3 V (vs. Ag reference electrode) with a scan rate of 100 mVs^{-1} for 30 cycles. Fig. 2.4 shows that the response to ethanol does not continually increase or decrease with the amount of nanotubes incorporated. Higher carbon nanotube amounts caused decrease in the responses and this might be due to the diffusion problem for the substrate as well as limited orientation for the enzyme molecules. High amounts of CNTs in the films allow electrical communication but at some point, the response is limited by the electron transfer from the enzyme to the polymer's redox center. Moreover, in such excess amounts, the polymer coating on the electrode surface loses its quality due to the defects in the polymer chains in the presence of CNTs. This result is in accordance with previous studies. The large surface area due to the presence of CNTs on the transducer surface may cause an increase in the background current. In similar works [175, 176], after some point, the introduction of CNTs into the surface affects signal in an unfavorable manner resulting in a decrease in biosensor responses.

On the other hand, with low amounts of CNTs, enzyme molecules could not be fixed properly onto poly(BIPN) coated electrode which resulted in lower responses and leaching of enzyme from the electrode surface. Fig. 2.4 shows that the optimum f-MWCNT amount was found as 0.2 mg in 0.001 M monomer solution. Maximum interaction and satisfactory immobilization of the enzyme molecules were achieved with this amount. The effect of f-MWCNT amount is also in accordance with the results in cyclic voltammograms taken for different amounts of f-MWCNT containing polymerizations. The most electroactive coating also gives the best results in amperometric studies. This shows the reliability and effectiveness of the prepared matrix.

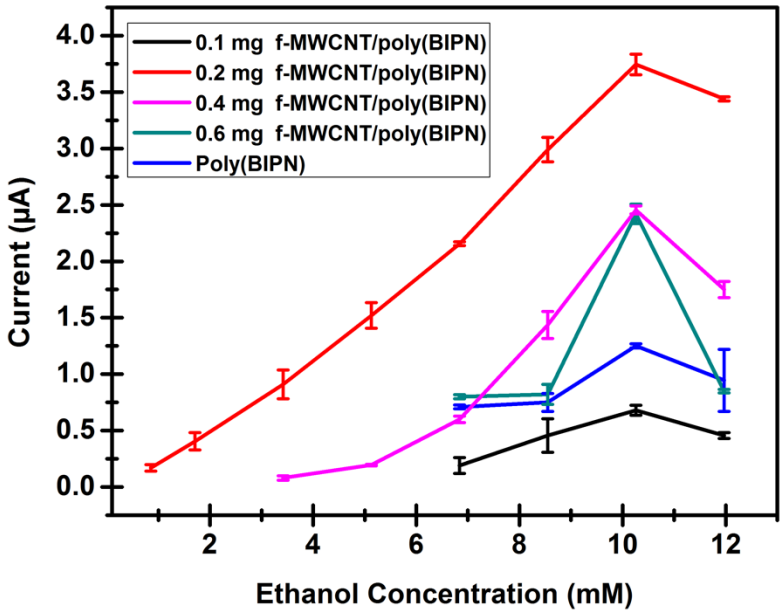

Figure 2.4 Effect of f-MWCNT amount on the biosensor response (in 50 mM phosphate buffer, pH 7.0, 25 °C, -0.7 V). Error bars show standard deviation (SD) of three measurements.

The effect of different amounts of AOx on the amperometric response was also investigated in the presence of 8.55 mM ethanol. Different amounts of the enzyme (2 U, 3 U, 4 U and 5.2 U) were immobilized on the modified electrode surface and the highest signals were recorded by the biosensor with 3 U AO_X in bioactive matrix. Higher enzyme amounts caused leaching from the surface. Hence, for the further experimental steps biocomposite matrix with optimum amount of AO_X was used.

Since enzyme stability is highly affected by certain environmental conditions, pH is the one of the main factors for biosensors. Effect of pH on the amperometric biosensor response was examined using 50 mM phosphate buffer solution in a range of pH 4.5-10 in the presence of ethanol as the substrate (8.55 mM). Maximum current responses were found at pH 7 (Fig. 2.5.). If the pH of the environment is extremely out of range, protein can be denatured [177].

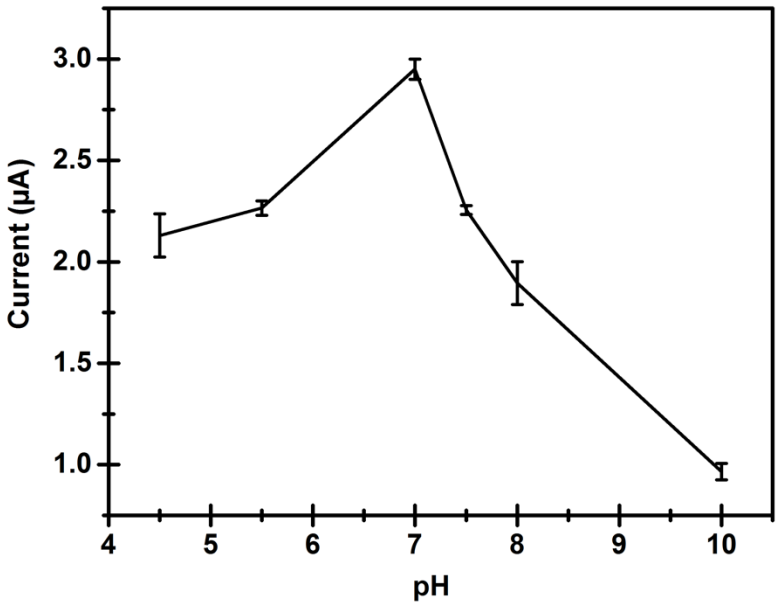

Figure 2.5 Effect of pH on the biosensor response. Error bars show standard deviation (SD) of three measurements.

2.3.3 Characterization

2.3.3.1 Scanning Electron Microscopy (SEM) Studies

The scanning electron microscopy (SEM) was used to characterize the surface morphologies of modified electrodes. Fig. 2.6 shows SEM images of the conducting polymer coated graphite electrode poly(BIPN), f-MWCNT, poly(BIPN)/f-MWCNT and poly(BIPN)/f-MWCNT/AOX modified electrode surfaces, respectively. The surface morphology of the only f-MWCNT film (Fig.2.6 (A)) shows typical fibrous shape. For the pure polymer, Fig. 2.6 B depicts the porous and cauliflower structure of a typical conducting polymer. In Figures 2.6 (A–C), it can be observed that, the poly(BIPN) and f-MWCNTs films reveal a morphology composed of uniform surface due to the good interaction between nanotubes and polymer's functional groups. This structural differences may explain different electrochemical behaviors for poly(BIPN) and f-MWCNTs/poly(BIPN).

As shown in Fig. 2.6 (C) the deposited poly(BIPN) is mostly wrapped around the f-MWCNTs. This fact leads to a higher conductivity and improved electrochemical properties for the films. The polymer and carbon nanotubes uniformly cover the electrode surface and provide porosity and a network. Such a surface design acts as a perfect layer for enzyme immobilization which is also supported by the amperometric results. Enzyme molecules are well-adhered on the coated electrode surface with the help of covalent binding due to the functional groups. In Fig. 2.6 (D), after immobilization of AO_X onto the modified electrode, the morphology of the surface changed drastically. This change can be related to the interaction between the functional groups of conducting polymer/nanotubes and protein molecules. In addition to this, comprehension of enzyme molecules through the structure of nanotubes may form electrostatic interactions between enzyme and poly(BIPN). This clearly shows that the enzyme was well-organized onto the modified surface.

2.3.3.2 X-ray Photoelectron Microscopy (XPS) Studies

Using X-ray photoelectron spectroscopy, carboxylic acid functionalization of MWCNTs and preparation of biolayer on the electrode surface were monitored (Figures 2.7 (A-E). All samples were degassed before each analysis. XPS data recorded for each sample was used to determine the specific binding energies via a fitting program. Figures 2.7 (A) and (B) depict the C1s spectrum of MWCNT and f-MWCNT which could be resolved into several characteristic peaks. In addition to peak at 284.8 eV representing unmodified carbon (C-C/C=C), the peaks at 286.4 and 288.7 eV are attributed to C-O/C=O and O=C-OH confirming the functionalization and introduction of COOH substituent [178]. The peaks at 285.3 and 286.4 eV for unmodified MWCNT (which was used as-received in XPS studies) can be related to sp^3-hybridized carbon atoms and structural defects on the nanotube structure [179] and atmospheric oxidation or remaining oxides arising from purification process of MWCNT. These results are also coherent with the FTIR results [178]. Moreover, the polymerization and immobilization were also confirmed via XPS studies using C1s spectrum (Fig. 2.7 (C)). In the C1s spectrum of conducting polymer, the peak at 284.3

eV can be attributed to aromatic carbons in the structure of polymer backbone and benzene unit and C-S in thiophene units [13]. The peaks at 285.6 and 287.8 eV belong to C-N in benzimidazole and C-N bond of nitrobenzene group, and C=N in benzimidazole ring, respectively [180, 181]. XPS of f-MWCNT containing conducting polymer coated surface was also performed. Due to the presence of f-MWCNT aromatic units in the polymer, the peak at 284.0 eV is attributed to C-C/C=C. 285.4 eV shows the C-N bond. 287.0 eV indicates the benzimidazole unit and nitrobenzene presence as well as C=O bond in the f-MWCNT structure. In addition, a peak at 288.1 eV belongs to O=C-OH substituent which confirms the presence of f-MWCNT within the polymeric structure (Fig. 2.7 (D)).

Successful immobilization of alcohol oxidase and covalent binding were confirmed via XPS results (Fig. 2.7 (E). In the C1s spectrum of enzyme immobilized f-MWCNT/conducting polymer coated electrode surface, the increase in intensity of the peak at 285.2 eV shows the presence of C-N bond, imidazole, nitrobenzene and MWCNT [178, 182]. The peak at 287.1 eV is attributed to O=C-N bond which confirms the covalent immobilization via amide bond formation between carboxylic acid groups of the f-MWCNT and amino groups of the enzyme molecules [178]. Besides, the increase in the signal at 288.2 eV confirms the presence carboxylic acids due to f-MWCNT and protein molecules.

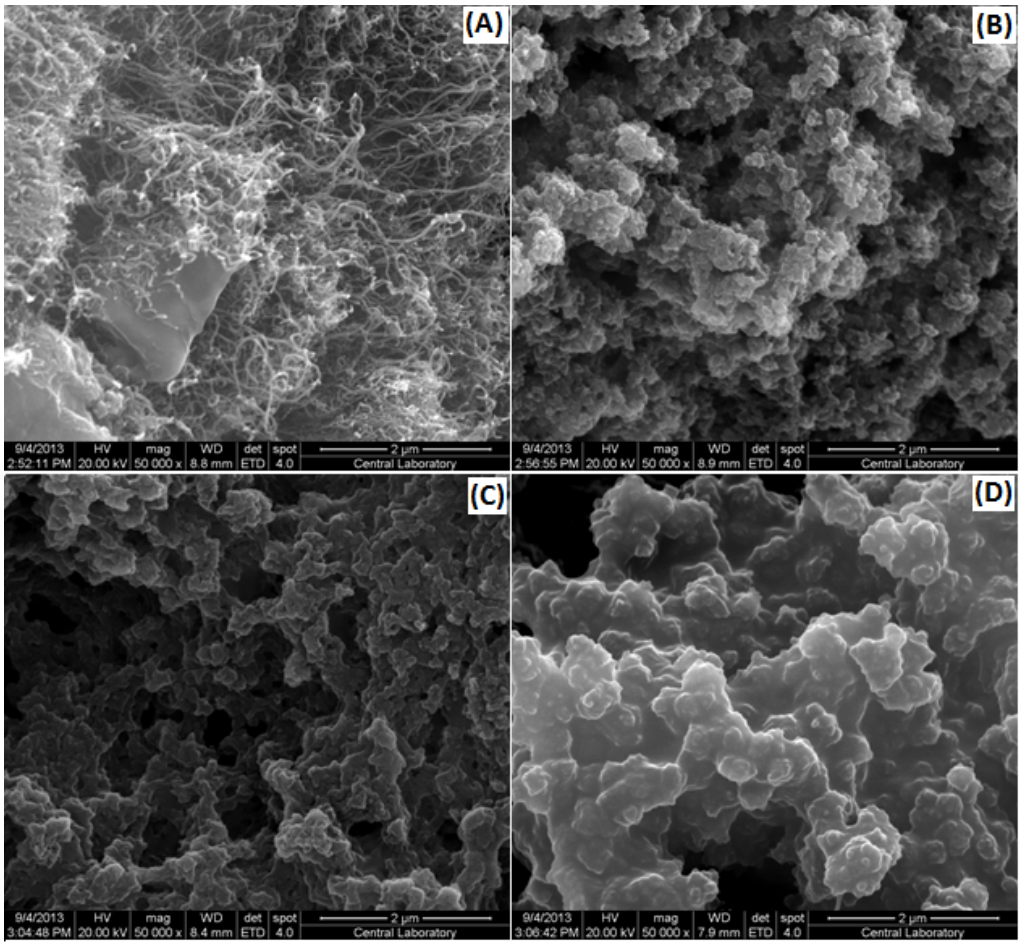

Figure 2.6 Surface characteristics of (A) Only f-MWCN (B) Only poly(BIPN) (C) f-MWCNT/poly(BIPN) and (D) f-MWCNT/ poly(BIPN)/AOx via SEM images.

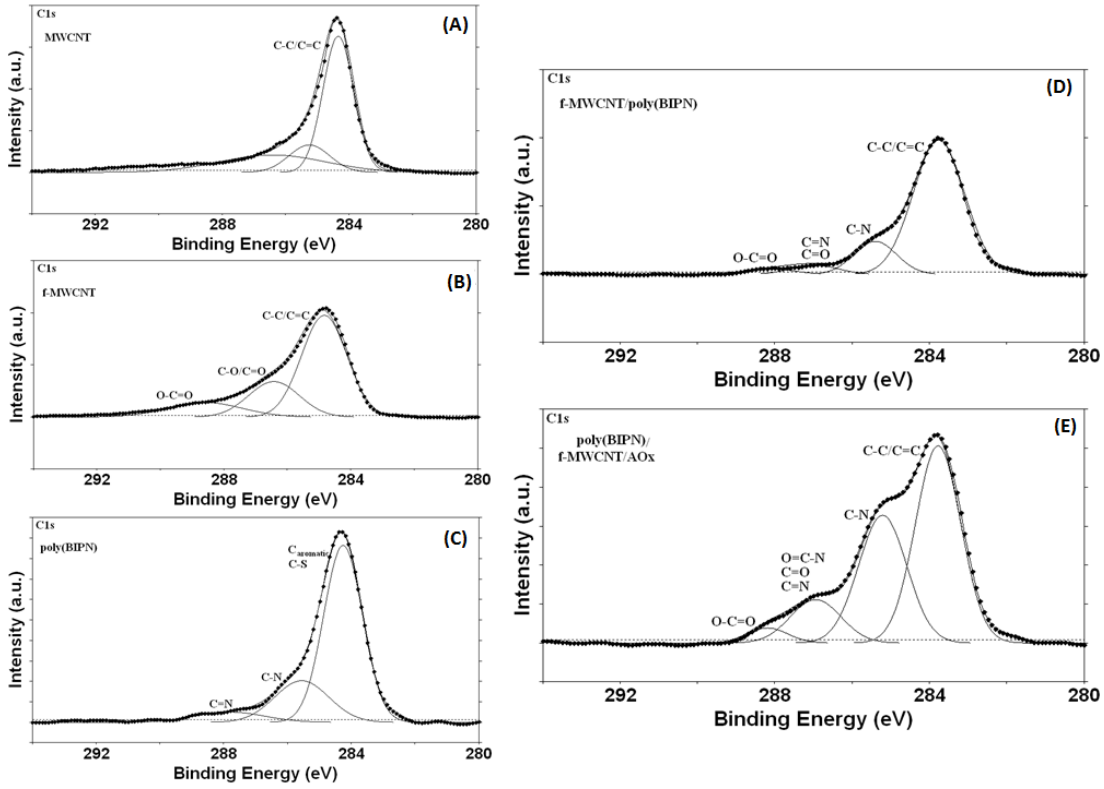

Figure 2.7 XPS C1s spectra for (A) Pristine MWCNT, (B) f-MWCNT, (C) Poly(BIPN), (D) f-MWCNT/poly(BIPN) and (E) AOx immobilized f-MWCNT/poly(BIPN).

2.3.3.3 Fourier Transform Infrared (FTIR) Studies

FTIR spectra of pristine MWCNT, functionalized MWCNT, poly(BIPN) and poly(BIPN) with f-MWCNT are represented in Fig. 2.8. In pristine MWCNT, characteristic peak of C=C vibration was observed at 1560 cm^{-1} (Fig. 2.8 (a)) [183]. After functionalization of MWCNT, three new peaks appeared at 3440 cm^{-1}, 1730 cm^{-1} and 1400 cm^{-1} referring to O-H stretching, C=O stretching and O-H bending vibrations, respectively (Fig. 2.8 (b)) [170]. This result clearly shows that the -COOH group was successfully obtained on the surface of MWCNTs. Fig. 2.8 (c) shows the spectrum of electropolymerized PBIPN. The peak at 3392 cm^{-1} indicates the characteristic N-H stretching of imidazole moiety. Another observation related to aromatic imidazole ring is the C-N stretching peak at 1402 cm^{-1} [184]. The

symmetrical stretching vibrations of nitro group are assigned to the peaks at 1379 cm^{-1} and 1344 cm^{-1} [185]. The C-H bending vibrations are summarized as 1143 cm^{-1}, 1111 cm^{-1} and 1086 cm-1 in-plane, 856 cm-1 and 798 cm^{-1} out of plane (Fig. 2.8 (c)) [185]. The structure of BIPN was also characterized by ^{1}H NMR spectroscopy. In Fig. 2.8 (d) O-H and N-H bending for f-MWCNT and poly(BIPN) overlapped and represented as a broad peak at 3414 cm^{-1}. The C=O stretching peak was shifted from 1730 cm^{-1} to 1697 cm^{-1} as a result of the hydrogen bonding formation between –COOH and $-NO_2$ groups due to the presence of poly(BIPN) (Fig. 2.8 (d)) [170].

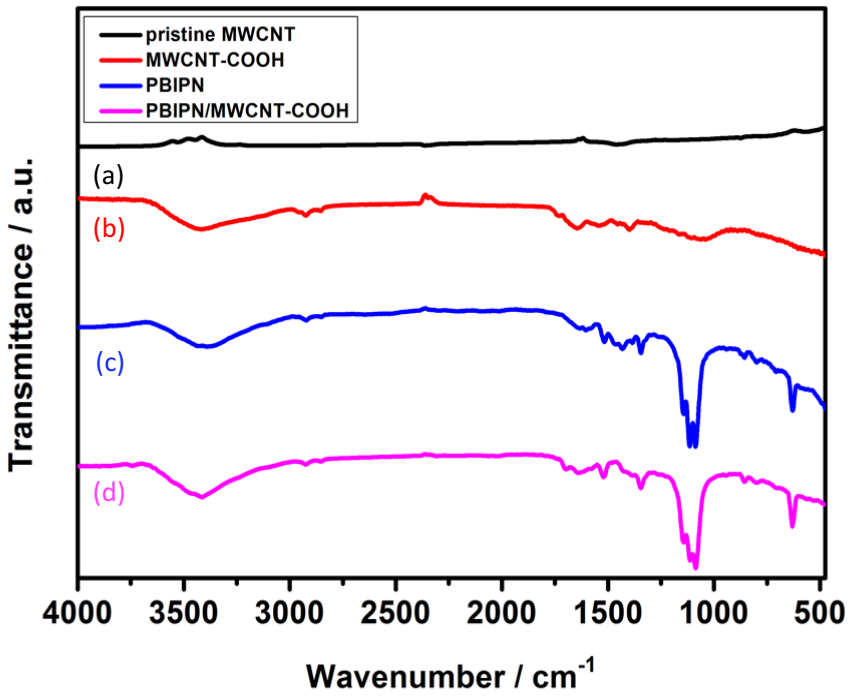

Figure 2.8 FTIR spectra of (a) Pristine MWCNT (b) f-MWCNT (c) Poly(BIPN) and (d) Poly(BIPN)/f-MWCNT.

2.3.4 Analytical Properties, Repeatability and Storage Stabilities of the Ethanol Biosensor

After all optimizations, biosensor responses for different amounts of ethanol were recorded to construct a calibration curve (Fig. 2.9). Limit of detection (LOD) was calculated as 0.17 µM based on S/N=3. Some characteristic parameters including kinetic parameters (K_M^{app}, I_{max}) and sensitivity were calculated as 16.949 mM, 3.31 µA and 476 µA/mM cm^2 respectively. A perfect linearity was obtained between 0.855 mM and 11.97 mM ethanol in 50 mM sodium phosphate pH 7.0 buffer as given in Fig. 2.9. SD and the RSD were calculated as 0.067 and 2.38 %, respectively. K_M^{app} and Imax values were calculated from Lineweaver-Burk plot.

A low K_M^{app} value is an indication of higher substrate affinity. Hence, with the help of the modified layer, immobilized enzyme molecules exhibit higher affinity toward ethanol. Moreover, K_M^{app} value for the poly(BIPN)/f-MWCNT/AO_X biosensor is extremely smaller than the ones reported in earlier studies (Table 2.1). This shows the effectiveness and success of the immobilization matrix, sensitivity of the biosensor and applicability in other operations. In recent years, several studies related to conducting polymer and carbon nanotubes based ethanol biosensors were investigated in literature and summarized in Table 2.1.

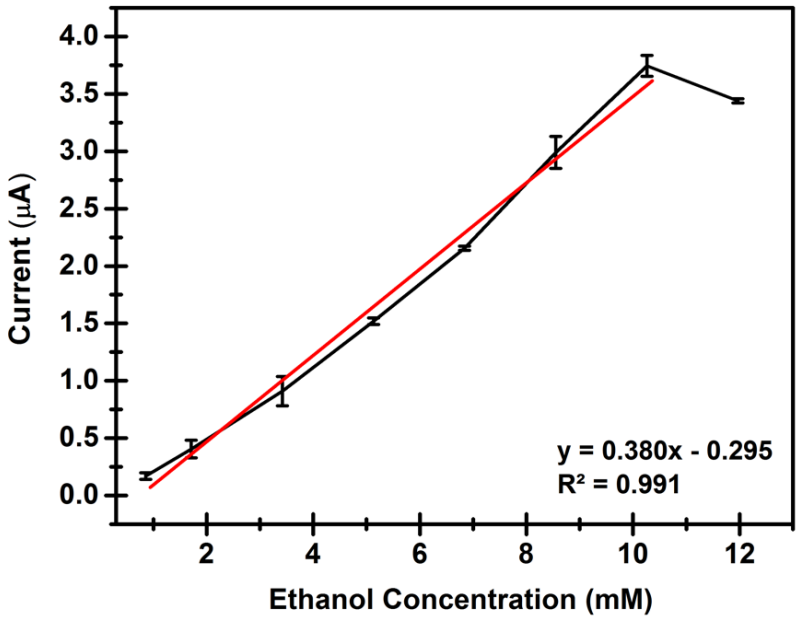

Figure 2.9 Calibration curve for ethanol (in 50 mM phosphate buffer, pH 7.0, 25 °C, -0.7 V). Error bars show standard deviation (SD) of three measurements.

Table 2.1 Comparison of biosensor, using the same technique, examples from the literature involving ethanol biosensors.

Immobilization matrix	Linear range	Sensitivity	References
Carbon paste matrix/AOx-HRP-osmium	250–2000 μM	NR	[193(a)]
MWCNT-Nafion/AOX/Au electrode	8.0 - 42 μM	3.0 μA/mM	[193(b)]
Hydrogel/AOX/platinum electrode	0.02 - 3.75 mM	10.6nA/mM	[193(c)]
Chitosan/AOx-eggshell membrane	60–800 μM	NR	[193(d)]
Poly(neutral red) (PNR)/carbon film electrode	0-1.0 mM	171.8 nA/mM^{-1}	[193(e)]
f-MWCNT/poly(BIPN)/ AOx	**0.855-11.97 mM**	**476μA/mM cm^2**	**This work**

NR: Not reported

Over the past few years, various approaches on carbon nanotubes, especially the functionalized ones, have been developed in order to improve the communication between the active site and electrode. Moreover, carbon nanotube based biosensors exhibit higher sensitivity, better stability and especially a broad linear response range. For example, polyaniline/carboxy functionalized MWCNT nanocomposite was developed as an interesting biosensor [170]. Functionalized carbon nanotubes were mixed with chemically polymerized polyaniline, stirred and filtered to get the composition. Then the composition was mixed with HRP enzyme and transferred to the Au electrode surface. It was proved that the linear range was expanded from 26-8000 μM to 886 μM to 10 mM with the help of both carbon naotubes and conducting polymer [170]. Liu et al., demonstrated that bio-composite film constructed via layer by layer containing MWCNT, chitosan and PAMAM nanocomposite could be used as dopamine and uric acid sensor with improved sensitivity and very extensive linear range [186]. An amperometric lactate biosensor based on conducting polymer and MWCNT composite on a gold electrode was reported by Rahman et al [187]. Results of this biosensor proved that sensitivity; stability as well as reproducibility had been improved significantly via using MWCNT. With this information, use of nanotube for the construction of biosensor has been very promising to obtain fast response and an efficient biosensor. Also in our case use of f-MWCNT improved the performance of biosensor as well as lowered the detection limit compared to the pristine poly(BIPN) biosensor.

There are multistep applications of similar methods for preparation of CNT containing surfaces in the literature [188, 189]. To the best of our knowledge, one step preparation of MWCNT and conducting polymer composite biolayer is rare in the literature [176, 190-192]. Hereby, a novel monomer for conducting polymer was synthesized and applied for the formation of a composite layer with MWCNT. The combination of a novel conducting polymer of BIPN with functionalized MWCNT is not explored for the detection of ethanol. The novel monomer is dissolved in organic solvents; nevertheless, the system works well. The one step procedure is easy to apply, simple, effective and homogeneous. Moreover, the preparation of alcohol biosensor with this

method brings novelty and improved results with the help of effective surface design and modification.

To determine substrate selectivity of the biosensor, 8.56 mM of various substrates such as ethanol, methanol, 2-propanol and tert-butanol were tested. The results indicated that the biosensor responded to primary aliphatic alcohols, and the maximum responses were obtained for ethanol (Table 2.2). Compared with the primary aliphatic alcohols, the biosensor response decreased with an increasing chain length. The decrease can be attributed to steric effects of branched alcohols which make it hard to reach the biosensor surface. Hence, proposed biosensor was very suitable for the ethanol determination [193].

Table 2.2 Substrate selectivity of the biosensor (amperometric response of methanol accepted as 100%).

Substrate	Activity (%)
Ethanol	97
Methanol	100
2-Propanol	32
Tert-butanol	2

The lifetime of the modified electrode was determined by measuring the amperometric response during 30 days. During the experiments, an activity loss of 2 % was observed on the 30th day. This result is the superior for the construction of long-life biosensor. The biosensor was kept at +4 °C when not in use.

In addition, investigation of effects of various materials as interferents was carried out with the proposed biosensor. Possible interferents like ascorbic acid, cholesterol, glucose and urea (between 1 mM and 10 mM) were studied as the substrate in a reaction cell containing 50 mM, pH 7.0 phosphate buffer solution. Amperometric measurements were done under optimized working conditions by applying -0.7 V where no interference effect was detected.

During the enzymatic reactions, oxygen consumption can be determined at -0.7 V. When a potential of -0.7 V is applied to cathode, a current proportional to the oxygen content is generated. During the catalytic reactions of oxidase enzymes, oxygen is consumed. Hence this proposed biosensor can be used in various applications even in the presence of such interferents in the analyte.

2.3.5 Sample Application

To investigate the performance and reliability of the constructed biosensor, it was tested for various beverages. Real samples with no dilution were analyzed with f-MWCNT/poly(BIPN)/AO_X biosensor (Table 2.3). The experiments were carried out at optimum conditions. Traditional conventional methods are often slower and with high cost which utilize undesirable routine analysis whereas this proposed biosensor has the advantages such as simple measurement procedure, easy fabrication and efficient sensitivity and selectivity. Thus, construction of this enzyme based amperometric biosensor has important for detection of ethanol samples. Results prove that there exists no significant difference between the two methods showing the reliability and accuracy of the biosensor. Hence, our system enables us to determine ethanol with an acceptable accuracy and rapid analysis.

Table 2.3 Ethanol detection in various samples.

Sample	Label value (%)	poly(BIPN)/f-MWCNT/AOx (%)	Relative Error (%)
Y Brand Raki	40.0	39.9	0.25
J Brand Whiskey	40.0	43.0	7.50
B Brand Wine	21.0	21.3	1.43
S Brand Liquor	20.0	22.1	10.5

2.4 Conclusion

In this study, novel conducting polymer was used for the construction of ethanol biosensor. The surface modifications of proposed biosensor provide perfect immobilization matrix for the alcohol oxidase enzyme. The fabricated biosensor exhibits excellent kinetic parameters such as K_M^{app}, I_{max}, low LOD and high stability. Combination of the CPs and nanotubes serves as proper immobilizing platform for biomolecules and preserve enzyme activity for a long period. For the proper enzyme immobilization, it is known that immobilization matrix has to preserve the stability and biological activities besides their orientation on the electrode surface. Moreover, it was successfully applied to the alcoholic samples with satisfactory results.

CHAPTER 3

POLYMERIZATION AND BIOSENSOR APPLICATION OF WATER SOLUBLE PEPTIDE-SNS TYPE MONOMER CONJUGATES

3.1 Background and Motivation

Peptides are important class of molecules which cover organic and biochemical materials and as a consequence of this, they have a large variety of applications in biomaterials [194, 195], medicinal chemistry [196], drug delivery systems [197], biosensors [198], organic electronics [199] and molecular recognition [200]. It is possible to attain different structures (alpha-helices, beta sheet etc.) and properties (hydrophilic, hydrophobic surfaces) just by proper selection of the amino acids. They also play a critical role in the generation of hybrid materials such as peptide-inorganic systems [201, 202] and peptide-polymer conjugates [203]. Peptides with polar side chains such as aspartic acid, glutamic acid, arginine are mostly soluble in water. Conjugation of the peptide having these amino acids to a poorly soluble hydrophobic molecule would enhance its solubility in water [204]. Conjugation of hydrophilic groups or even polymers such as PEG to a hydrophobic monomer to increase its water solubility is a common strategy especially in the preparation of water soluble conjugated polymers for biosensor applications [205]. Same strategy was also used to solubilize drug molecules which are very hydrophobic and poorly soluble in water [206]. Peptide-polymer conjugates are new classes of materials that are applicable to wide range of areas utilizing the features of both components to generate unique properties [207, 208].

Moreover, peptides are fascinating biomaterials mimicking natural proteins [209, 210] and they can show great enhancement in the solubility of the hydrophobic molecules in water and polar organic solvents. However, utilization of peptides to increase the solubility of the monomers has not been investigated throughly to the best of our

knowledge. Hence, one of the aims of this theses study was to design such a structure. Peptide conjugation to monomers [211, 212] can also exhibit a good platform for the immobilization of the enzymes when the monomer is polymerized. For a biosensor design, electrogenerated film matrices provide good stability and improvements for biomolecule immobilization [213]. Furthermore, peptides on the polymer surface and enzyme molecules would show versatile interactions since they are both made of amino acids. This phenomenon may allow to produce high performance biosensors and long term storage in immobilized surfaces.

Taking into consideration of the above-mentioned views, herein the water soluble SNS-type monomers bearing peptide side chains have been reported for sensor applications. In this work, firstly a SNS-type carboxylic group containing monomer was used and then, two different peptides (sequences of glutamic acid-arginine-arginine (ERR) and glutamic acid-arginine-arginine-arginine (ERRR)) anchored SNS-type carboxylic group containing monomer were used to increase their solubility in water. This modification allows well-organized molecular structure of the conjugated polymers on the substrates, and they constitute wonderful three dimensional matrices for the biomolecule. These biomolecules maintain their biological activity for a long term period. The design makes the biosensor an ideal candidate for developing cheap sensing devices. Moreover, the corresponding monomer design was really attractive since it enables covalent binding of the biomolecules through their available functional groups. Peptides are acting as linkers between polymer and glucose oxidase to generate a biocompatible medium between the enzyme and the electrode surface. To access the conjugated biopolymer architecture, the constructed monomers (SNS-COOH, SNS-ERR, and SNS-ERRR) were electrochemically polymerized on the graphite electrode via cyclic voltammetry. The electropolymerization of the monomers comprises an E(CE)n (electrochemical, chemical, electrochemical) mechanism consisting, as the first step, the formation of a radical cation [69, 70]. After electropolymerization of the monomers, GOx was immobilized on these polymeric surfaces and used for glucose detection. In a subsequent step, the matrix was fixed using glutaraldehyde (GA) as the crosslinking agent. In this study, it was demonstrated that conjugated coatings based

on peptide sequences in combination with glucose oxidase provide a simple route to manufacture surfaces that can act as a high performance glucose biosensor.

3.2 Experimental

3.2.1 Materials and Apparatus

Glucose oxidase (GOx, β-D-glucose: oxygen 1-oxidoreductase, EC 1.1.3.4, 17300 units/g solid) from A. niger and glucose were purchased from Sigma-Aldrich. Glutaraldehyde (GA) was obtained from Sigma–Aldrich Co., LCC. (St. Louis, USA). Chemicals needed for enzyme immobilization and buffer solution preparation were described in Chapter 2. As the substrate, glucose solution (0.1 M) was prepared by dissolving 0.18 g of glucose in 10 mL pH 7.0 PBS solution.

PalmSens potentiostat (PalmSens, Houten, The Netherlands) was used for the amperometric measurements. Three electrode system containing a graphite rod (Ringsdorff Werke GmbH, Bonn, Germany, type RW001, 3.05 mm diameter and 13% porosity), a platinum wire (Metrohm, Switzerland) and a Ag wire were used as the working, counter and reference electrodes, respectively. Prior to electrochemical polymerization reaction, spectroscopic grade graphite rods were polished on an emery paper and washed thoroughly with distilled water. For biosensor applications, electrochemical polymerizations were carried out on cleaned graphite electrodes via cyclic voltammetry using Gamry Instruments Reference 600 potentiostat/galvanostat (GAMRY Instruments Inc., Pennsylvania, USA). During electrochemical and spectroelectrochemical studies, indium tin oxide (ITO) doped glass slides were used. Spectroelectrochemical studies were performed via UV-Vis spectra (Agilent G1103A spectrophotometer). The potential for spectroelectrochemical studies was controlled using a Solatron 1285 potentiostat/galvanostat. All experiments were conducted under ambient conditions (25°C). In amperometric analyses, the data were given as the average of three measurements and standard derivations were recorded as ±SD. For surface imaging of the fabricated biosensor, scanning electron microscope (SEM) (JEOL JSM-6400 model) was used.

3.2.2 Synthetic procedure

3.2.2.1 Synthesis of Methyl 4-(2,5-di(thiophen-2-yl)-1*H*-pyrrol-1-yl) benzoic acid (SNS-COOH), SNS-Peptide Conjugate (SNS-ERR and SNS-ERRR)

The SNS-COOH monomer and SNS-peptide conjugates (SNS-ERR and SNS-ERRR) were synthesized using the procedure described in the literature [214].

3.2.3 Electrochemical Biosensor Fabrication and Amperometric Measurements

Initially graphite electrodes were coated with SNS-ERRR via electrochemical deposition according to the same procedure used for biosensor preparation. Electrochemical polymerization of the monomer (SNS-ERRR) was performed in a cell containing 10^{-3} $molL^{-1}$ of monomer, 0.1 $molL^{-1}$ solution of $NaClO_4/LiClO_4$ (1:1) as the supporting electrolyte in the potential range between -0.3–0.5 V. Afterwards, it was used as the functional platform containing peptide handles on the surface for biomolecule deposition. For the GOx immobilization, an enzyme solution (0.75 mg in 10.0 μL, 50 mM potassium phosphate buffer, pH 7.0) was spread over the polymer coated surface. Then, 1.0 % of 5.0 μL glutaraldehyde in potassium phosphate buffer (50 mM, pH 7.0) were cast on the electrode as the cross linker, and the electrode was allowed to dry at ambient conditions for 120 min. Then, loosely bound enzyme molecules were removed by rinsing the electrode surface with the distilled water. It was stored at 4 °C when not in use (Figure 3.1).

All amperometric measurements were carried out at room temperature in a reaction cell filled with 10 mL PBS, pH 7.0 under mild stirring and biosensor response signals were recorded as the current (μA) via following the oxygen consumption at −0.7 V due to the result of enzymatic activity in the bioactive surface. The calibration curves were obtained by plotting the current signal values of a series of standard glucose solution (μA) against different glucose concentrations. After the current was reached equilibrium, a certain amount of analyte solution was injected into the reaction medium, then equilibrium was established again.

The difference between the two constant current values gave the biosensor response.

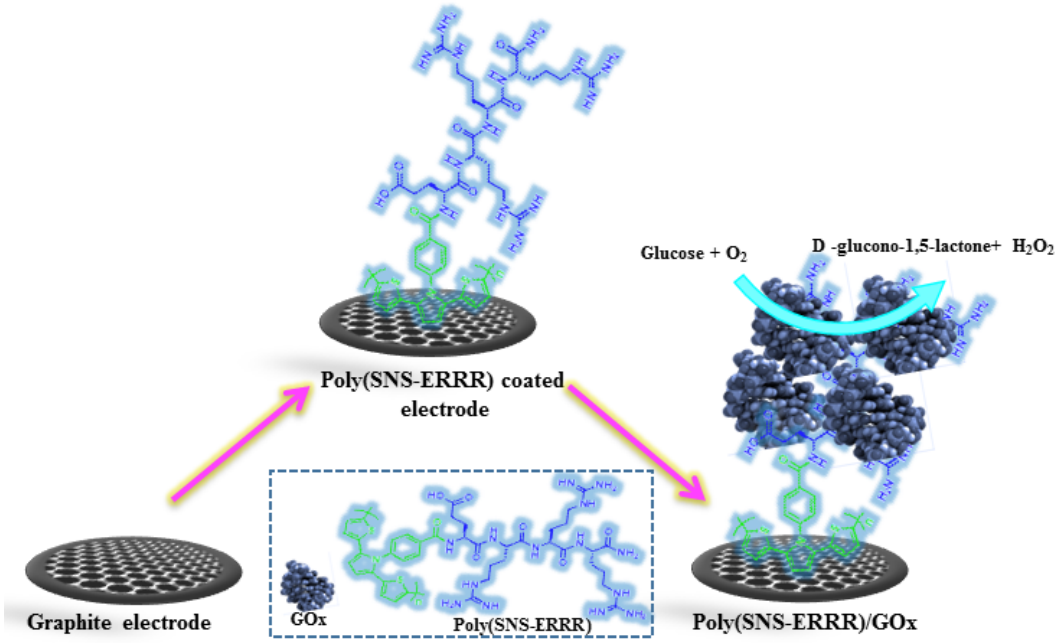

Figure 3.1 Schematic representation of proposed glucose biosensors.

3.3 Results and Discussion

3.3.1 Electrochemical and Spectroelectrochemical Properties of poly(SNS-ERR) and poly(SNS-ERRR)

3.3.1.1 Electrochemical Properties

Electroactivities of monomers were investigated using cyclic voltammetry technique. Cyclic voltammograms of pristine SNS-COOH monomer were obtained in ACN solution using $NaClO_4$-$LiClO_4$ (0.1 M) as the supporting electrolyte due to the poor solubility of the monomer in water. The electrochemical oxidations of SNS-ERR and SNS-ERRR were determined in a solution of $1x10^{-2}$ $molL^{-1}$ monomer and 0.1 $molL^{-1}$ $NaClO_4$-$LiClO_4$/H_2O electrolyte–solvent couple at a scan rate of 100 mV/s. Water solubility of the proposed monomer-peptide conjugates enable several advantages. One of the main advantages of such system is that SNS-ERR and SNS-ERRR can be easily electropolymerized in aqueous solutions that provides easy production of

polymers via green chemistry. Additionally, electrodeposition could be performed at relatively low positive potentials.

Electropolymerization of SNS-COOH was performed between 0.0 V and +1.0 V in non-aqueous solution. The electroactive film was developed on the ITO surface as accompanied with an increase in the current. An irreversible oxidation peak was observed at +0.72 V referring the monomer oxidation. Upon anodic scans, polymer oxidation and reduction peaks evolved at +0.58 V and +0.30 V (Figure 3.2 (A)). In the first cycle of voltammograms shown in Figure 3.2 (B), an irreversible monomer oxidation peak for SNS-ERR was centered at +0.30 V while reversible polymer redox waves for poly(SNS-ERR) were observed at +0.25 V and +0.14 V accompanied with an increase in the current density. Since polymerization has been carried out potentiodynamically ecah run exhibits a higher current density. This is due to the electrode area increasing at every run. In Randles Sevcik equation all the parameters are fixed whereas the electrode area is increasing during polymer coating on the surface. Since the polymer itself is electroactive, this may last until all monomer are consumed. Hence the first cycle refers to the least current whilst the last one stands for the highest current. The reversible redox couple proves that electrochemical polymerization is proceeding at the ITO electrode surface to form an electroactive polymer film. An irreversible anodic wave for SNS-ERRR at +0.32 V stands for monomer oxidation (Figure 3.2 (C)). Cyclic voltammetry studies indicated that SNS-ERR gets oxidized at lower potentials than that of SNS-ERRR. Slight differences in the oxidation potentials of monomers are probably due to long chain peptide moieties in the monomer structure. During anodic scan a doping/dedoping couple was detected for poly(SNS-ERRR) as +0.23 V and +0.12 V at a scan rate of 100 mV/s.

3.3.1.2 Spectroelectrochemistry

Spectroelectrochemical studies were carried out to determine the optical properties of poly(SNS-ERR) and poly(SNS-ERRR) upon applied potentials. For these measurements, polymer films were electrochemically deposited on ITO-coated glass plates from $1x10^{-2}$ $molL^{-1}$ monomer solution in 0.1 $molL^{-1}$ $NaClO_4$-$LiClO_4/H_2O$.

Changes in optical properties of poly(SNS-ERR) and poly(SNS-ERRR) were investigated in a monomer free solution via applying increasing potentials. The newly produced electronic bands were recorded as a function of applied potential (Figure 3.3). The neutral state absorption maxima of poly(SNS-ERR) and poly(SNS-ERRR) were observed as wide absorptions at 470 nm and 432 nm, respectively. The maximum absorptions in the visible region can be attributed to the high energy π- π^* transition of neutral state polymer.

Upon incremental stepping of the potential from 0.0 V to +0.5 V, the absorption of π-π^* transitions decreases, and two new optical transitions appear at lower energy, corresponding to the polaronic and bipolaronic charge carriers. The charge carriers led to different coloration for polymer films by generating new energy transitions. As the potential increases, the absorption peaks at about 537 and 915 nm for poly(SNS-ERR) and 667 nm and 1055 nm for poly(SNS-ERRR), which correspond to polaron and bipolaron species, respectively, also increase.

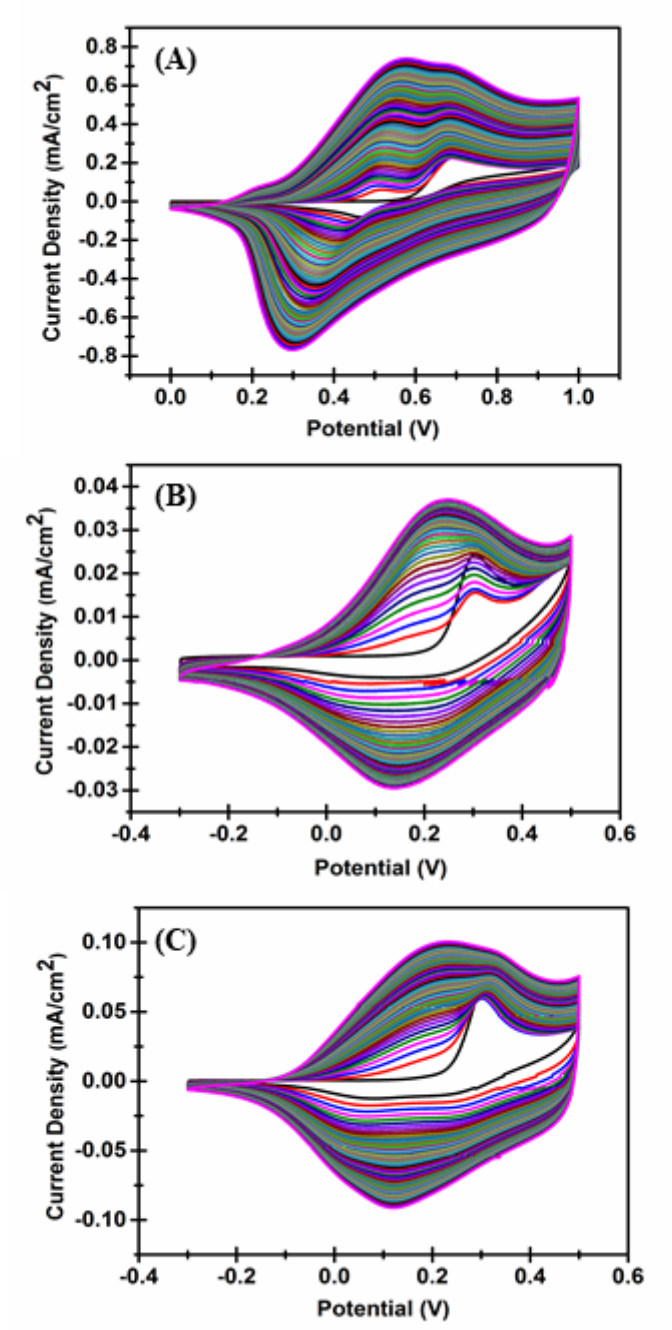

Figure 3.2 Repeated potential scan electropolymerization of (A) SNS-COOH in $NaClO_4$-$LiClO_4$/ACN electrolyte–solvent couple (B) SNS-ERR and (C) SNS-ERRR in 0.1 M $NaClO_4$-$LiClO_4$/H_2O electrolyte–solvent couple at a scan rate of 100 mV/s (up to 15 cycles).

3.3.1.3 Colorimetry Studies

Colorimetry studies were performed to evaluate the colors. Both polymers exhibited electrochromic properties. Poly(SNS-ERRR) reveals multi-colored electrochromic behavior with three distinct colors. The color analyses were done according to CIE 1931 Yxy color space. For the polymers color states for the pristine and oxidized forms are shown in Table 3.1.

Table 3.1 The colors and color coordinates of conducting polymers in accordance with CIE standards.

Poly(SNS-ERR)		**Poly(SNS-ERRR)**		
0.0 V	0.5 V	0.0 V	0.3 V	0.5 V
				
L: 92.734 a: -10.762 b: 21.783	L: 84.602 a: -3.040 b: -1.057	L: 88.019 a: -8.261 b: 45.918	L:87.953 a: -6.259 b:18.678	L:85.681 a: -1.757 b:2.716

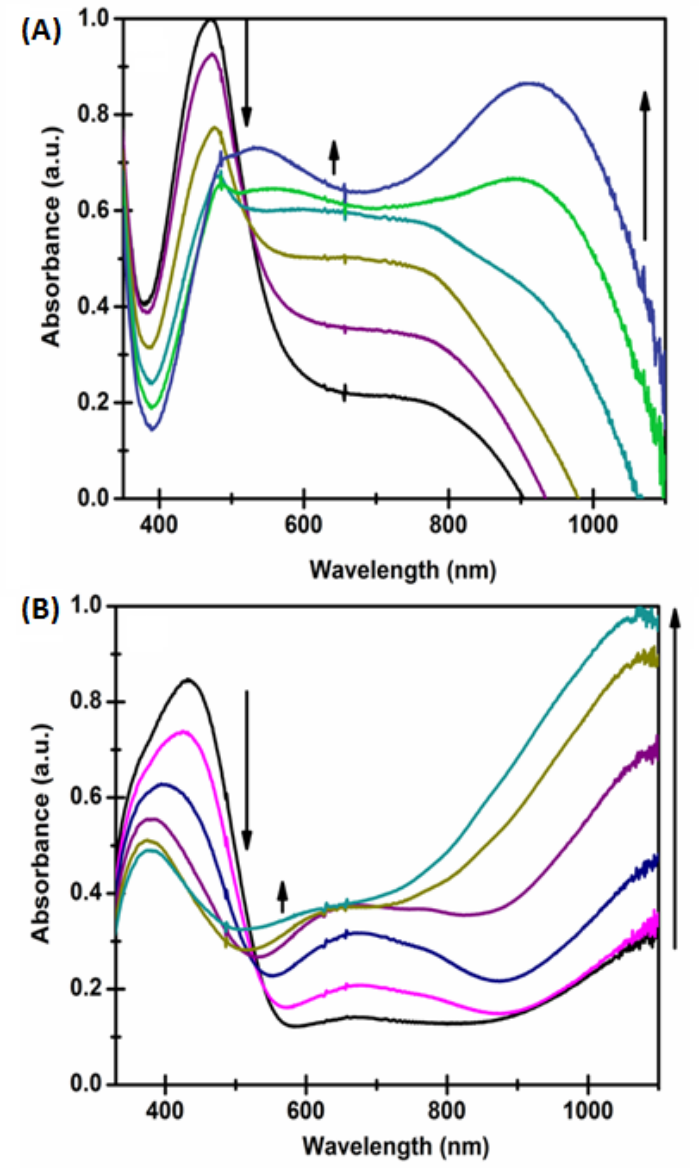

Figure 3.3 Spectroelectrochemistry of (A) Poly(SNS-ERR) and (B) Poly(SNS-ERRR) polymer films on ITO coated glass electrodes in monomer-free 0.1 M $NaClO_4$-$LiClO_4/H_2O$ at various applied potentials.

3.3.2 Optimizations and Surface Characterization of the Biosensors

In order to obtain a proper orientation and effective binding of the enzyme on the polymer surface, optimum polymer film thickness was investigated by adjusting the cycle number during electropolymerization. The cycle number in polymerization determines the deposited charge on the polymer film, which is very much related with the thickness of the polymer layer. Stability of the enzyme on polymer film is affected by the distance between the active site of the enzyme molecule and transducer layer. Too thick polymer films hinder the electron transfer between the enzyme and the electrode causing low charge transfer rate [129]. On the other hand, thin polymer films cannot protect the enzyme from the environmental effects which may cause deformation of the matrix. To find the optimum polymer film thickness, the monomer was polymerized on a graphite electrode with different scan numbers (30, 40, 50 and 60 scans) by keeping all the other parameters constant. The highest biosensor response was obtained with 50 scans which corresponds to 39 nm (charge involved in the film formation is 25.4 mC/cm^2) in thickness (Figure 3.4 (A)).

Then, the enzyme amount was optimized in order to obtain the highest biosensor response. If there is an excess loading of enzyme, the immobilized enzyme may not be fixed properly on the electrode surface since the matrix has an enzyme loading capacity. This may result in leaching out from the surface. On the other hand, if the enzyme amount is not sufficient, the desired sensitive responses cannot be recorded [172]. In order to optimize the enzyme amount, different electrodes having 0.5 mg (8.65 U), 0.75 mg (12.98 U), 1.00 mg (17.3 U) and 1.25 mg (21.63 U) GOx were prepared. When their responses were compared, the highest signal and sufficient stability were obtained with 0.75 mg GOx (Figure 3.4 (B)).

Finally, the optimum value for the pH value of the buffer solution was determined to provide an effective biosensor platform since enzyme molecules are affected by pH changes [215]. Amperometric measurements were performed using different buffer solutions in the range of pH 5.0–8.0 (sodium acetate buffer at pH 5.0, sodium phosphate buffer at pH 6.0-7.5, tris buffer at pH 8.0, 25 °C). The highest enzyme

activity; therefore, the highest stable biosensor response was obtained with pH 7.0 and used for further experiments.

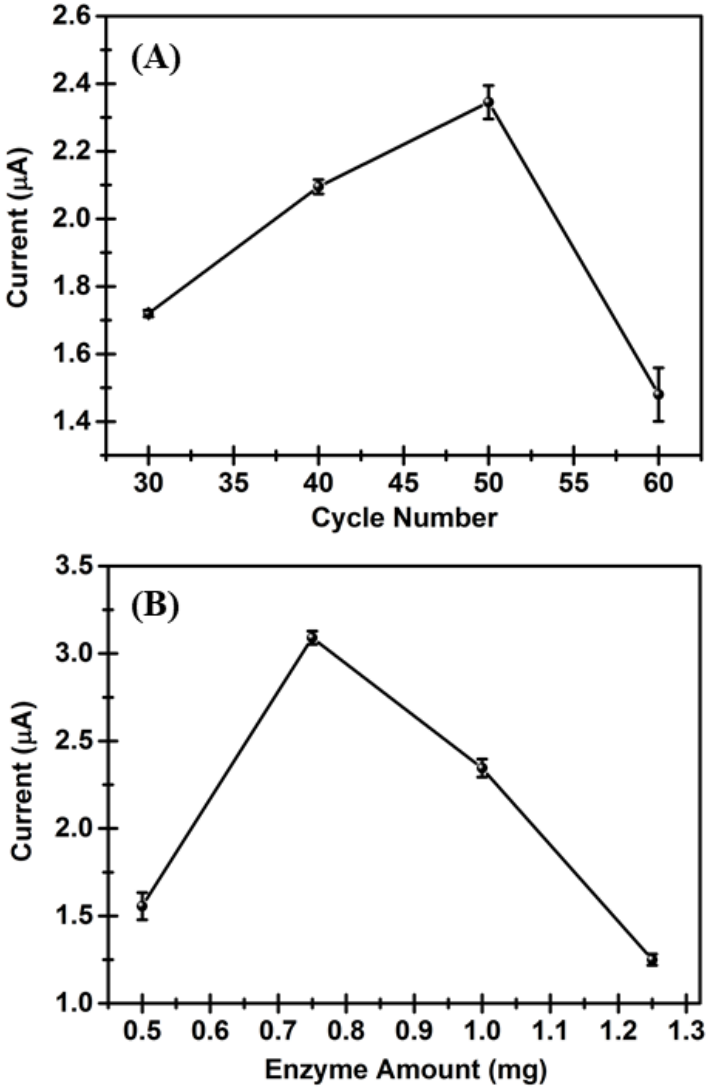

Figure 3.4 The effect of (A) Scan number and (B) Loaded enzyme amount (in 50.0 mM phosphate buffer, pH 7.0, 25 °C) on biosensor response. Error bars show the standard deviation (SD) of three measurements.

Cyclic voltammetry (CV) was used to investigate the charge transfer process on the biofilm surface. Effective electroactive surface areas for each surface modification were calculated using the Randless-Sevcik equation:

$$Ip = 2.69 \times 10^5 AD^{1/2}n^{3/2}v^{1/2}C$$

where n is the number of electrons involved in the redox reaction, A is the electrode area (cm^2), D is the diffusion coefficient of the molecule in solution ($cm^2\ s^{-1}$), C is the concentration of the probe molecule in the bulk solution (mol cm^{-3}), and v is the scan rate ($V\ s^{-1}$). According to the equation, increase in the peak currents can be attributed to an increase in effective surface area. For this purpose, cyclic voltammetry responses at bare electrode and modified electrodes were studied in a solution containing 5.0 mM $Fe(CN)_6^{3-/4-}$, 0.1 M KCl and 50.0 mM PBS pH 7.0 at the potential between 0 and 1.0 V with a scan rate of 100 mV s^{-1}. The voltammograms of different electrodes; bare graphite, poly(SNS-ERRR), and poly(SNS-ERRR)/GOx under optimum conditions were given in Figure 3.7. The electroactive surface areas for bare graphite, poly(SNS-ERRR) and poly(SNS-ERRR)/GOx modified electrodes were calculated as 0.072 cm^2, 0.11 cm^2 and 0.09 cm^2, respectively. In Figure 3.5, the poly(SNS-ERRR) modified graphite electrode had a higher peak current (121.4 μA) than the one on bare electrode (80.88 μA). This result revealed that the increase in peak current can be attributed to the increase in the effective surface area due to the generated conducting polymer film on the electrode surface. By this way, the released electrons as a result of the enzymatic reaction can be easily transferred to the transducer surface which is important for the biosensor response. After biomolecule immobilization, the decrease in the peak current confirmed the proper attachment of the biomolecule on the surface which diminished the electron transfer properties because of a possible diffusion layer. The results clearly confirm that the immobilization step for biosensor fabrication was achieved successfully.

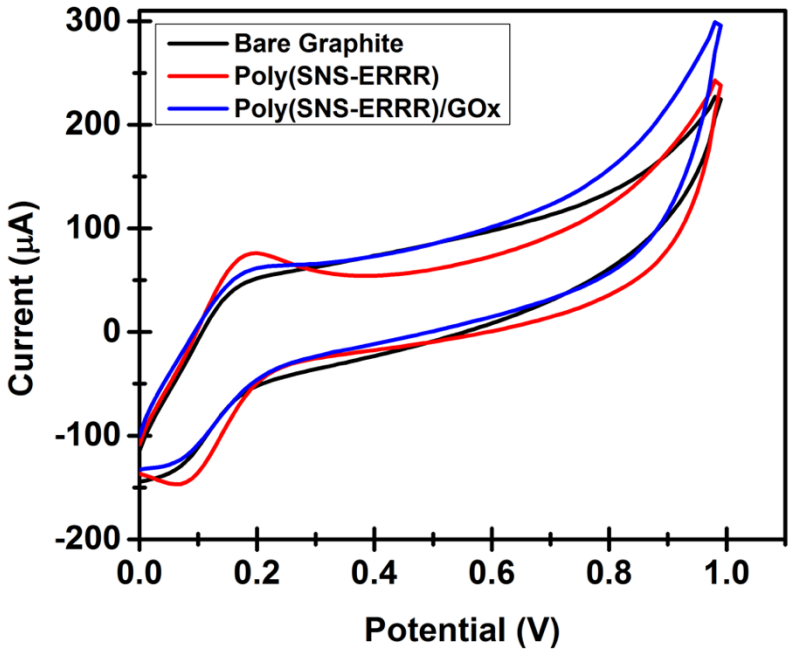

Figure 3.5 CVs of the bare graphite electrode, poly(SNS-COOH)-coated electrode, and poly(SNS-COOH)/GOx electrode (in 5.0 mM $Fe(CN)_6^{3-/4-}$, at a scan rate of 100 mV/s).

Scanning electron microscopy (SEM) technique was used to assess the surface morphology of the modified electrodes (Figures 3.6 (A- D)). In the case of the typical cauliflower like structure of conjugated polymer (poly(SNS-COOH)) (Figure 3.8 (A)), peptide integrated polymer surfaces (poly(SNS-ERR) and poly(SNS-ERRR)) showed completely different surface morphologies (Figures 3.6 (B-C)). A homogenous film observed on the graphite surface in the case of the combination of biolayer with the poly(SNS-ERRR) provided more ordered like structure (Figure 3.6 (D)). This ordered structure allows to get more reliable and stable biosensor responses that was proved by the analytical results.

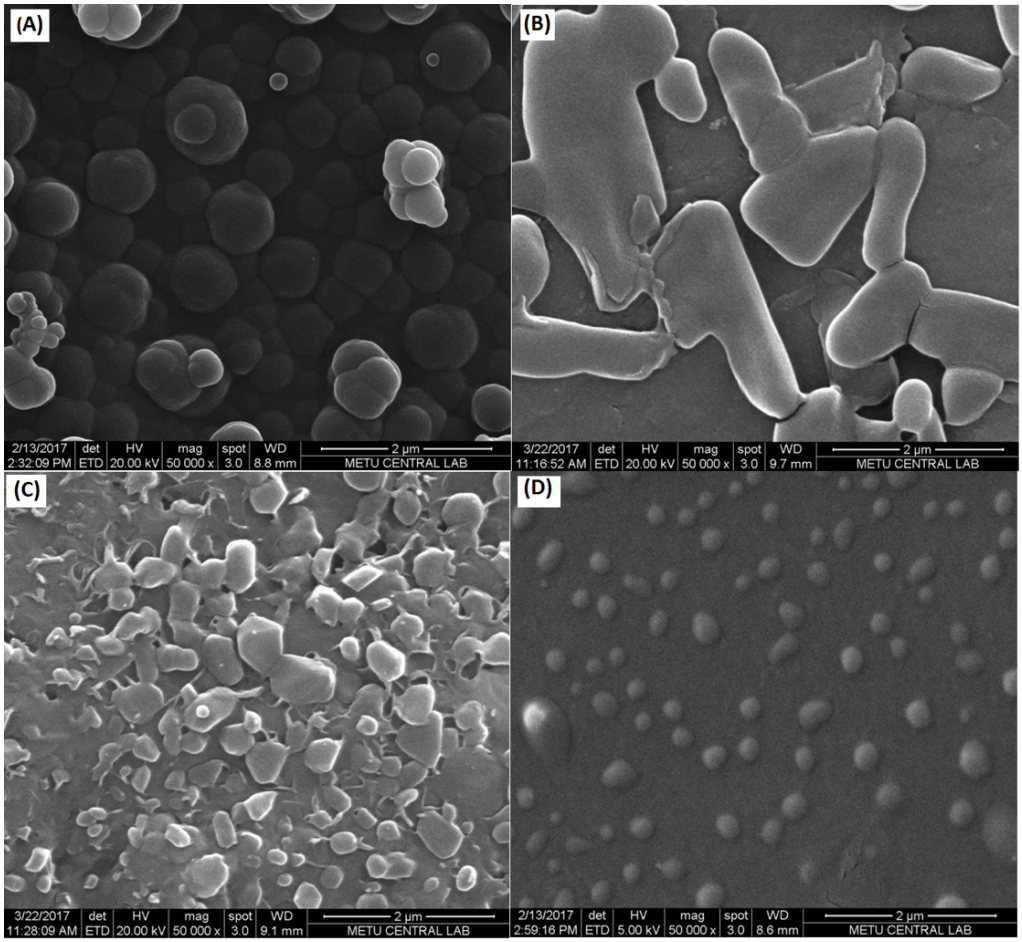

Figure 3.6 SEM images showing the surface characteristics of (A) Poly(SNS-COOH), (B) Poly(SNS-ERR), (C) Poly(SNS-ERRR), and (D) Poly(SNS-ERRR)/GOx via under optimized conditions.

3.3.3 Analytical Characterization of the Biosensors

Amperometric responses of the biosensor were recorded by adding different concentrations of glucose to buffer solution. The characteristic calibration curves for glucose were plotted and responses of biosensors were shown in Figure 3.7. Linear ranges for each biosensor were determined as 0.1–0.5 mM for the poly(SNS-COOH)/GOx and the poly(SNS-ERR)/GOx and 0.01-0.75 mM for poly(SNS-ERRR)/GOx modified electrodes with the correlation coefficients of 0.984, 0.997 and 0.998, respectively.

Sensitivity values of the biosensors were also calculated as 19.62 μAmM^{-1} cm^{-2} for the poly(SNS-COOH)/GOx, 42.0 $\mu AmM^{-1}cm^{-2}$ for the poly(SNS-ERR)/GOx and 91.37 $\mu AmM^{-1}cm^{-2}$ poly(SNS-ERRR)/GOx, respectively. Poly(SNS-ERRR) modified biosensor showed the best results among the three biosensors as given with the equation; y = 6.2875x + 0.0571 (R^2= 0.998). For optimum biosensor, the limit of detection (LOD) value was calculated by setting the intercept of the linear range of the calibration curve to zero using S/N (signal-to-noise ratio) = 3 criterions as 4.69 µM. When compared to other modifications, this biosensor showed also very low LOD value (92.7 µM for poly(SNS-COOH) and 72.14 µM for poly(SNS-ERR) modified electrode). The results revealed that the poly(SNS-ERRR) modified biosensor yields enhanced stability and affinity towards the substrate than to that of the other modifications. Furthermore, this modification displays that enzyme molecules were anchored more precisely and well-oriented, and hence, the analytical parameters were turned out to be better. Additionally, the biosensor has a rapid and sensitive response to glucose and reaches a steady-state equilibrium current in 2 s that allows easy detection of glucose in samples.

Kinetic parameters of the proposed biosensor were investigated using Lineweaver–Burk plot (1/I vs 1/[S]) at constant temperature and pH 7.0 and are listed in Table 3.2 comparatively with literature results. From the Lineweaver-Burk plot, K_M^{app} value was calculated as 0.208 mM. K_M shows the affinity of the enzyme molecules to their substrate. When compared to other studies in the literature, the present biosensor showed a considerably low LOD, high sensitivity and low K_M^{app} values. Liu et al proposed a glucose biosensor based on water-dispersible chitosan-functionalized graphene (CG) and further modified it with Fe_3O_4 [216]. The proposed biosensor has a sensitivity value of 5.658 $\mu AmM^{-1}cm^{-2}$ with a detection limit of 16 µM. In another study, a biosensor was developed for the detection of glucose with an electrode modification of PdNPs-electrochemically reduced graphene oxide [217]. The biosensor has the Michaelis constant of 5.44 mM.

Adronov et al designed a biosensor by entrapping glucose oxidase within the poly[3-(3-N,N-diethylaminopropoxy)thiophene] and single-walled carbon nanotubes films and they obtained the K_M^{app} value of 3.4 mM [112].

Moreover, a glucose biosensor utilizing polyaniline and chitosan-coupled carbon nanotubes was fabricated by Kang et al [218]. This biosensor construction showed K_M^{app} value of 5.35 mM and the sensitivity value of 21 $\mu AmM^{-1}cm^{-2}$. Another glucose biosensor based on the glucose oxidase/one-dimensional hierarchically structured TiO_2 modified electrode proposed the sensitivity of 9.9 $\mu A\ mM^{-1}cm^{-2}$ and the K_M^{app} value of 1.54 mM [219]. Poly(SNS-ERRR) matrices exhibited higher affinity toward the glucose substrate; hence, the designed biosensor served our purposes perfectly.

Furthermore, a glucose oxidase biosensor prepared with methyl viologen-mediated, as the redox mediator, and glutaraldehyde (GA) crosslinker in the presence of bovine serum albumin (BSA) as carrier protein gives LOD as 20 μM [220]. In an another example, based on glucose oxidase immobilized by glutaraldehyde co-crosslinking with bovine serum albumin and Nafion® cation-exchange polymer, on a cobalt(II) phthalocyanine–cobalt(II) tetra(5-phenoxy-10,15,20-triphenylporphyrin), (CoPc–$(CoTPP)_4$) pentamer film modified glassy carbon electrode (GCE), the LOD was found as 10 μM [221]. These results also confirm the proper surface design in the present study to achieve a very low LOD without any need for membrane, redox mediator or carrier protein. The designed peptide-modified conjugated polymer layer shows both mediator and carrier property for enzymatic reactions and immobilization, respectively. This is one of the main advantages of the sensing platform for real-time applications. Other advantages of the system are its easy preparation (not requiring a multi-step preparation) and a fast response time. When compared to other modifications summarized in Table 3.2, the response time of our biosensor was also shortened by the help of surface orientation of the enzyme molecules. This easy preparation procedure is easy to apply, simple and effective for detection of glucose. A detailed comparison of the properties of the biosensor is summarized in Table 3.2.

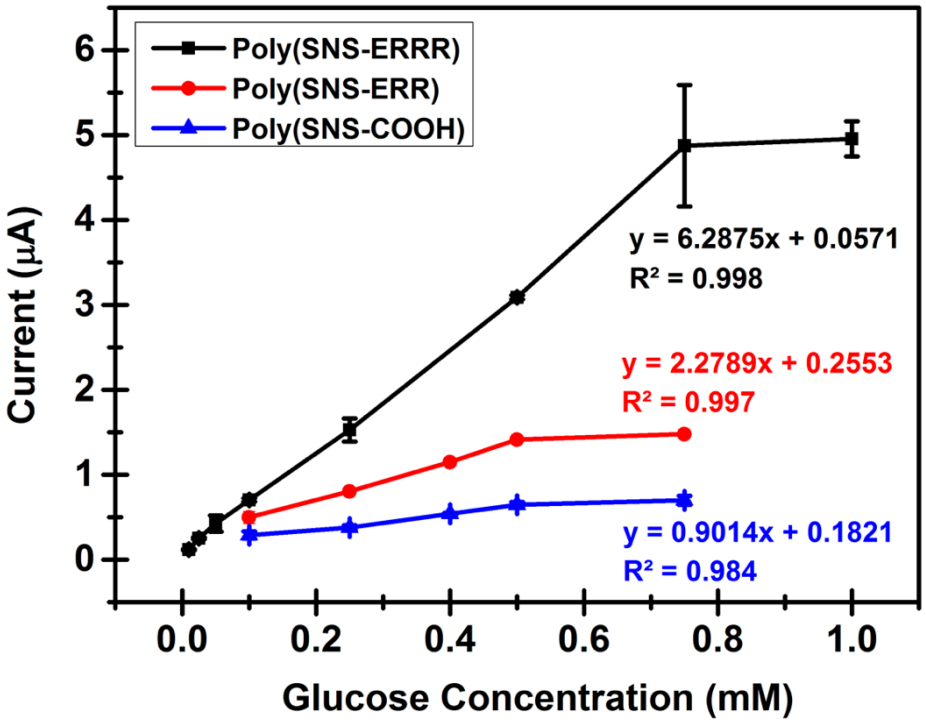

Figure 3.7 Calibration curve for glucose (in 50 mM phosphate buffer, pH 7.0, 25 °C). Error bars show standard deviation of three measurements.

Table 3.2 Comparison of the various glucose biosensors for the detection of glucose.

Matrices on electrodes	LOD (µM)	Sensitivity ($\mu AmM^{-1}cm^{-2}$)	K_M^{app} (mM)	References
Poly(SNS-anchored carboxylic acid) /PAMAM-G4	2.92	NR	1.59	[42(a)]
GOx/Au/MXene/Nafion/GCE	5.9	4.2	NR	[42(b)]
Graphene–AuNPs–GOD	35	NR	4.73	[42(c)]
Au cypress/PB grid	NR	74.3	1.48	[42(d)]
PA-g-PEG/GOx/graphite	20	47.72	0.97	[42(e)]
GOD/graphitic-nanocage modified GCE	8	13.3	NR	[42(f)]
BNNTs-PaniPt-GOD	6	19.02	3.4	[42(g)]
PET/VACNTs-Al-foil/PFLA/GOx	7.035	65.816	0.193	[42(h)]
$(PAH/CdTe)_{12}(PAH/PSS)_3(PAH/GOD)_3$	500	NR	8	[42(i)]
GOx/MWCNTs/CS/GCE	10	13	2.2	[42(j)]
GOD/Pt/OMC/Au	50	12	NR	[42(k)]
Poly(SNS-ERRR)/GOx	**4.69**	**91.37**	**0.208**	**This work**

NR: Not reported

Other analytical parameters such as repeatability and shelf-life of the biosensor were also evaluated. Shelf life of the biosensor was measured by recording the biosensor responses for 0.5 mM glucose during 31 days. In a period of 31 days, there was only 5.0% decrease in the biosensor response. When not in use, the biosensor was kept at 4◦C. The device was still active with 95% efficiency after a month (Figure 3.8 (A)). Additionally, the operational performance of poly(SNS-ERRR)/GOx was investigated for 20 successive measurements (with a standard deviation of 0.034% and relative standard deviation of 4.01%). The stability of the biosensor for repetitive uses in that period is quite good. Furthermore, the investigation of the biosensor selectivity toward possible interfering substances is a mandatory step in the development of biosensors. Hence, some potential interferents like ascorbic acid and urea (0.5 mM) were injected to the reaction cell instead of glucose as the substrate. The biosensor responses were followed and shown in Figure 3.8 (B). No responses were recorded for these interferents at -0.7 V potential under the optimized conditions that proves the biosensor can be comfortably used for the glucose determinations in various applications even within an environment with such interferents.

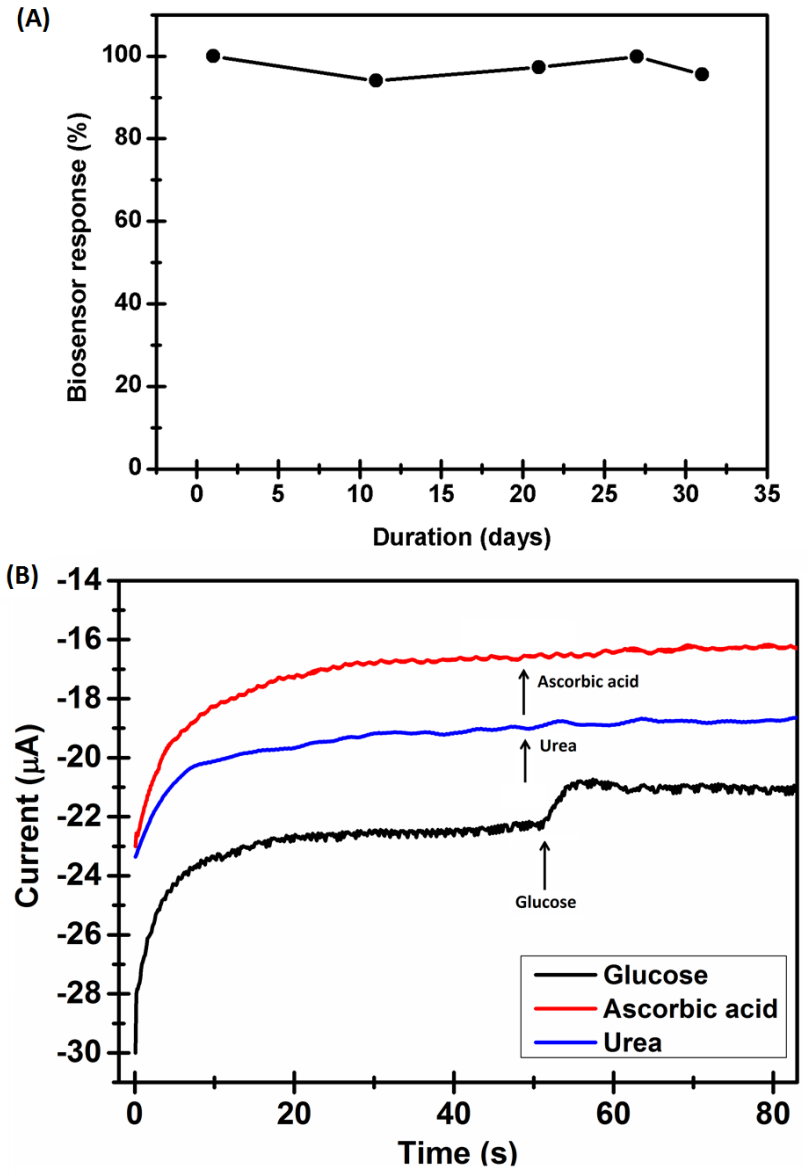

Figure 3.8 (A) Shelf-life analysis of the biosensor during 31 days and (B) Biosensor responses to glucose and interfering substances (in 50 mM phosphate buffer, pH 7.0, 25 °C).

3.3.4 Sample Application

To test the convenience of the biosensor glucose content in several beverages were determined. For this purpose, the samples were injected into the cell instead of the glucose substrate without any pretreatment. Each sample (around 10 μL) was injected into 10 mL of buffer containing cell and an automatic dilution occurs. By this way, the detected glucose amounts were in the range of our detected linear range. The responses of the biosensor were recorded for each sample and glucose contents were estimated from the calibration curve. All these experiments were achieved under the optimum conditions. Percent relative error was calculated and the biosensor results were compared with those given on the labels provided by the manufacturers. The measurements were carried out at ambient conditions (25 °C). The proposed biosensor design is suitable for use in real time analysis to investigate glucose content in beverages. The obtained results are summarized in Table 3.3.

Table 3.3 Results of glucose analyses in beverages.

	Glucose Content (mM)		
Sample	**Product Label**	**poly(SNS-ERRR)/GOx biosensor**	**Relative Error (%)**
S® ice tea	0.37	0.35	5.41
S® milk	0.25	0.23	8.0
C® coke	0.62	0.59	4.83

3.4 Conclusion

Two new water-soluble monomers were designed and successfully used for a glucose biosensor. Peptide-SNS-type monomer conjugation allowed green chemistry to achieve electropolymerization in aqueous medium. Moreover, the proposed monomers were electrochemically polymerized on graphite electrode via cyclic voltammetry and their biosensor results were evaluated using important parameters which are good indicators a for biosensor performance. Moreover, the surface characteristics of the electrodes were investigated using SEM and CV techniques. The optimum biosensor exhibited good analytical characteristics: linear range of 0.01−0.75 $mmolL^{-1}$ (R^2 = 0.998), sensitivity 91.37 $\mu AmM^{-1}cm^{-2}$, and K_M^{app} 0.208 mmol L^{-1}. In virtue of the good biocompatibility and remarkable interaction of the anchored peptides with enzymes, the biosensor showed high sensitivity and favorable selectivity, and was also efficient enough to detect glucose in beverages, indicating feasible potential for practical application. Furthermore, the proposed sensing system showed a good shelf-life stability of 31 days with a very stable and reproducible results.

CHAPTER 4

A NOVEL CONDUCTING COPOLYMER: INVESTIGATION OF ITS MATRIX PROPERTIES FOR CHOLESTEROL BIOSENSOR APPLICATIONS

4.1 Background and Motivation

Conducting polymers (CPs) have received considerable attention in recent years since they can be used for a variety of technological applications. They are suitable matrices for enzyme immobilization since conducting polymer based enzyme electrodes exhibit the high operational stability and fast response. Moreover, conducting copolymers have been proposed for biosensing applications due to a number of favorable characteristics such as: (1) easy and direct deposition on the electrode surface, (2) thickness control, (3) redox conductivity. Copolymerization is one of the most feasible methods that widely used to change various properties of the polymers. Copolymer based biosensors have been also widely studied previously [97, 222-224]. However, to the best of our knowledge a cholesterol biosensor prepared using BImTh and Fmoc-Gly-OH has not yet been reported. In this thesis, a novel conducting copolymer based biosensor was successfully achieved to construct a biosensor for the detection of cholesterol in human blood.

In this work, copolymer based graphite electrode was prepared by electropolymerizing 2-heptyl-4,7-di(thiophen-2-yl)-1*H*-benzo[d]imidazole) (BImTh) and 2-(((9H-fluoren-9-yl)methoxy)carbonylamino)acetic acid (Fmoc-Gly-OH). Electrochemical copolymerization was undertaken by cyclic voltammetry with a mixture of BImTh and Fmoc-Gly-OH monomers in acetonitrile (ACN)/dichloromethane (DCM) solution. BImTh was chosen as the comonomer since it has a hydrophobic alkyl chain as a pendant group and Fmoc-Gly-OH was chosen for its free carboxylic acid group which

is open for amide bonding. Enzyme molecules have hydrophobic parts and free amino groups. Hence, utilizing the copolymer, efficient binding between enzyme molecules and copolymer was achieved. Here, we anticipated that the electrochemical oxidation of a mixture of BImTh and Fmoc-Gly-OH will lead to the formation of the copolymers with improved characteristics compared to those of the BImTh and Fmoc-Gly-OH. The conducting copolymer was electrochemically synthesized onto the electrode by cyclic voltammetry technique via repeated electrochemical cycling. The electrochemical polymerization consists of electrochemical (E), chemical (C) and electrochemical (E) reactions resulting in E(CE)n mechanism.

In this mechanism, first, radical cations of the monomers were created and later polymerization continues via dimerization and so on. Cholesterol oxidase (ChOx) was immobilized on the copolymer coated graphite electrode in order to construct an amperometric cholesterol biosensor. Through functional carboxylic acid groups of the copolymer, amide bond formation between enzyme molecules and polymer was generated. In addition to this covalent binding, hydrophobic side chains of both copolymer and enzyme molecules interact to contribute the immobilization of the biomolecules. The presence of the free carboxylic acid groups on the copolymer backbone can be utilized for the covalent attachment of enzymes via formation of amide bonds. In this case, 1-ethyl-3-(3-dimethylamiopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were used to activate the free carboxylic groups of the conducting polymer backbone. The obtained copolymer was characterized by UV–vis absorption spectroscopy (UV–vis), scanning electron microscopy (SEM) and X-ray photoelectron microscopy (XPS) techniques. The practical application of copolymer modified electrode was tested via determining cholesterol in serum samples.

4.2 Experimental

4.2.1 Materials and Apparatus

Cholesterol oxidase (E.C.1.1.3.6) (26.4 U/mg protein) from *Pseudomonas fluorescens*, cholesterol, Triton-X 100, $NaClO_4$, $LiClO_4$ and Fmoc-Gly-OH were purchased from

Sigma-Aldrich and used with no further purification. 2-Propanol (Merck) was used as received to prepare the cholesterol stock solution (0.005 M) at room temperature via gently mixing to obtain a clear solution. Triton-X 100, the nonionic surfactant providing solubility of cholesterol in aqueous solutions was added to the analyte solutions in the ratio of 1% (v/v) just before the experiments. Chemicals needed for enzyme immobilization and buffer solution preparation were described in Chapters 2 and 3. All chemicals were of analytical reagent grade. Human serum samples were obtained from the Middle East Technical University (METU) Medical Center from patients volunteered for that matter. The data obtained from the hospital analyses were compared with the biosensor responses.

Electrochemical measurements were performed with Ivium CompactStat (The Netherlands) potentiostat in a cell equipped with three electrodes. Electropolymerization was performed with a Voltalab 50 potentiostat in a three-electrode cell consisting of graphite electrode (Ringsdorff Werke GmbH, Bonn, Germany, type RW001, 3.05 mm diameter and 13% porosity) as the working electrode. A platinum wire as the counter electrode, and a Ag wire as the pseudo reference electrode were employed. In order to perform the spectroelectrochemical studies of the polymer films Varian Cary 5000 UV-Vis spetrophotometer was used. For surface imaging of the cholesterol electrode, scanning electron microscope (SEM) (JEOL JSM-6400 model) and X-ray photoelectron spectroscopy (XPS) (PHI 5000 Versa Probe (F ULVACPHI, Inc., Japan/USA) with monochromatized Al Kα radiation (1486.6 eV) 10 as an X-ray anode at 24.9 W were used.

4.2.2 Synthetic procedure

4.2.2.1 Synthesis of 2-Heptyl-4,7-di(thiophen-2-yl)-1*H*-benzo[d]imidazole (BImTh)

BImTh was prepared using the procedure described in the literature [225].

4.2.3 Electrochemical Biosensor Fabrication and Amperometric Measurements

Prior to use graphite rods were polished on emery paper and washed completely with distilled water. The electrochemically prepared copolymer was constructed on the bare graphite electrode. Electrochemical copolymerization on the graphite electrode was carried out with cyclic voltammetry scanning the potential between 0.6 and 1.6 V at 100 mVs^{-1} for 30 cycles in a mixture of 0.017 M BImTh and Fmoc-Gly-OH (concentration ratio=1:1, M/M). After polymerization, polymer film was rinsed with distilled water to remove unreacted monomer. Cholesterol oxidase was immobilized onto the carboxylic acid functional conducting polymer using N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC)/N-hydroxysuccinimide (NHS). 10 µL of ChOx solution (50 mM pH 7.0 sodium phosphate buffer solution containing 21 U ChOx, 0.4 M EDC and 0.1 M NHS) was spread over copolymer coated graphite electrode and then, left to dry for 3 h at room temperature. Then, the enzyme electrode was stored at 4 °C for overnight. Before use, it was rinsed with distilled water to remove un-bound enzyme molecules and reagents. Hence, we obtained copolymer modified graphite electrode successfully (Figure 4.1).

Amperometric studies were achieved in a reaction cell containing 10 mL phosphate buffer solution (50 mM, pH 7.0) under carefully and mildly stirring. After each measurement, working buffer solution was refreshed and electrodes were washed with distilled water and kept in the phosphate buffer solution (50 mM, pH 7.0) for 3 min. Decrease in oxygen level as a result of enzymatic reaction was monitored at -0.7 V vs Ag in a phosphate buffer. After the background current reached a steady state, freshly prepared cholesterol solution was injected to the medium; the current change was recorded. All the experiments were carried out at ambient conditions. In order to see the effect of oxygen saturation, control experiments were done.

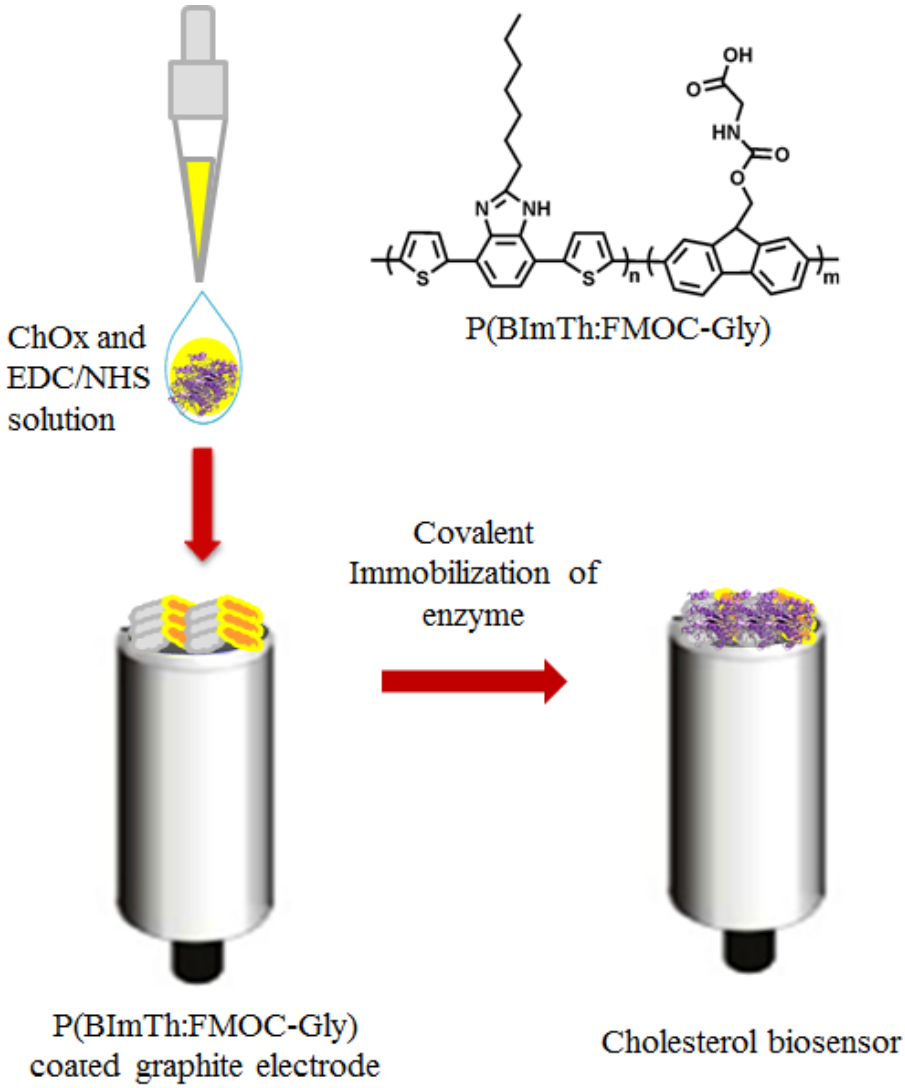

Figure 4.1 Typical preparation of the proposed biosensor.

4.3 Results and Discussion

4.3.1 The Preparation of P(BImTh), P(Fmoc-Gly-OH) and Copolymer Film by Electropolymerization

BImTh was polymerized potentiodynamically on indium tin oxide (ITO)-coated glass slides using equimolar 0.1 M sodium perchlorate ($NaClO_4$) and lithium perchlorate

($LiClO_4$) as the supporting electrolyte in a mixture of dichloromethane (DCM) and acetonitrile (ACN) (5:95, v/v) with repeated scan intervals between 0 and 1.30 V versus Ag wire pseudo-reference electrode [225]. Polymerization of Fmoc-Gly-OH was also performed potentiodynamically on ITO-coated glass slides using 0.1 M tetrabutylammonium hexafluorophosphate containing 5:95 dichloromethane (DCM): acetonitrile (ACN) solution with repeated scan intervals between 0.50 and 1.85 V. It was seen that P(BImTh) is forming an orange polymer film on the electrode surface. In case of P(Fmoc-Gly-OH), it is known that the carboxylic acid group would form amide bond with the enzyme, but electrochemical polymerization of Fmoc-Gly-OH did not result in a proper polymer film. Copolymer films using different concentrations of BImTh and Fmoc-Gly-OH solutions were performed in order to combine the film quality of P(BImTh) and enzyme immobilization property of P(Fmoc-Gly-OH).

Electrocopolymerization was achieved in different ratios of comonomers such as 1:1, 1:10 BImTh:Fmoc-Gly-OH. Electropolymerization of 1:1 (M/M) and 1:10 (M/M) BImTh:Fmoc-Gly-OH containing copolymer was performed in 0.017 M BImTh, 0.017 M Fmoc-Gly-OH and equimolar 0.1 M $NaClO_4$, $LiClO_4$ supporting electrolyte containing 2.0 mL 5:95 (v/v) DCM:ACN solution with repeated scan intervals between 0.60 and 1.60 V versus Ag wire pseudo-reference electrode (Fig. 4.2 (A)). This range enables the initiation of both monomers since their oxidation potentials are 1.0 V and 1.4 V, respectively [225]. Polymer redox potentials were found to be in between the polymer redox potentials of P(BImTh) and P(Fmoc-Gly-OH) (Table 4.1) which indicates the formation of a copolymer. The polymer redox potentials of 1:1 BImTh:Fmoc-Gly-OH containing copolymer indicate the excess presence of Fmoc-Gly-OH unit in the copolymer chain. The comparison of polymer redox potentials is given in Fig. 4.2 (B).

To further investigate the copolymer formation, absorption spectra of P(BImTh) and copolymers with 1:1 and 1:10 ratios were recorded (Fig. 4.2 (C)). As P(BImTh) is orange and P(Fmoc-Gly-OH) is colorless (absorbs in UV-region), increasing the

Fmoc-Gly-OH ratio has decreased the absorption intensity in VIS-region and shifted the absorption to lower wavelengths (Table 4.1).

Table 4.1 Redox potentials and absorption wavelengths of polymers.

Polymer	**E_{ox} / E_{red} (V) vs. Ag wire**	**λ_{max} (nm)**
P(BImTh)	0.62 / 0.51	450
1:1 BImTh:Fmoc-Gly-OH containing copolymer	0.96 / 0.84	438
1:10 BImTh:Fmoc-Gly-OH containing copolymer	1.01 / 0.90	402
P(Fmoc-Gly-OH)	1.41 / 1.29	UV-region

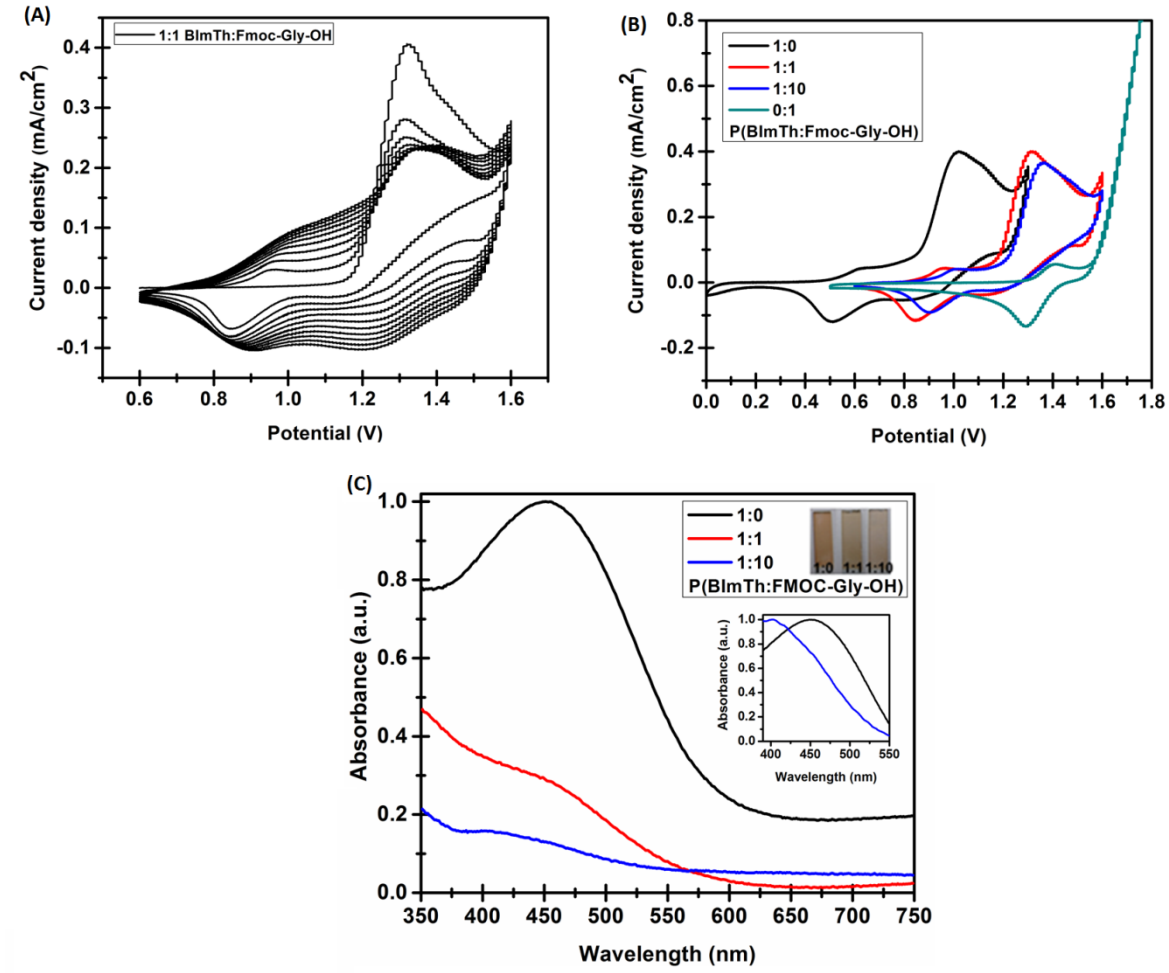

Figure 4.2 (A) Electropolymerization of 1:1 (M/M) BImTh:Fmoc-Gly-OH in 0.1 M $NaClO_4$, $LiClO_4$ supporting electrolyte containing 2.0 mL 5:95 (v/v) DCM:ACN solution, (B) Cyclic voltammograms of the polymers in 0.1 M $NaClO_4$, $LiClO_4$ supporting electrolyte containing 2.0 mL 5:95 (v/v) DCM:ACN solution, (C) Absorbance spectra of the polymers.

4.3.2 Effect of the Ratio of BImTh:Fmoc-Gly-OH in Copolymerization for the Formation of Cholesterol Biosensor

There are several main factors that affect the preparation of this copolymer based cholesterol biosensor. The preparation of this biosensor is simple but the copolymerization ratio, polymer layer thickness, amount of the enzyme and pH of the

biosensor environment are very important parameters to construct the biosensor. It was observed that when a biosensor with P(BImTh) was constructed, during the immobilization, due to the incompatibility of the enzyme with the hydrophobic polymer pendant alkyl chains, it was hard to fix the enzyme molecules on the polymer coat. It was noted that enzyme molecules leached from the electrode surface. Accordingly, copolymerization with a functional monomer; Fmoc-Gly-OH was realized. By this way, covalent amide linkages can be formed between protein and polymer via amine groups of enzyme molecules and carboxylic acid groups of copolymer. On the other hand, copolymerization brings the two functional pendant groups in the same polymeric structure. Since enzyme molecules bear hydrophobic and hydrophilic parts in its huge structure, alkyl chains in copolymer also involve in the interactions with the protein which brings the enhanced stability and well immobilization. The surface which is coated with the conducting polymer has the functional carboxylic acid groups as well as the alkyl chains providing both hydrophilic and hydrophobic nature to the matrix. In the immobilization procedure, amino groups of the enzyme molecules were used. Moreover, due to the hydrophobic side chain-containing amino acids such as valine, leucine, isoleucine, there are also hydrophobic sites in the structure of the protein molecules. With the help of hydrophobic alkyl chains in the polymer, these sites interact and the immobilization is enhanced. These interactions are also optimized via copolymerization ratio and thickness optimizations. Since the density of the alkyl chains change with the copolymerization ratio, different copolymers were prepared and related biosensors were prepared. The optimum one shows the best response. Hence, the presence and importance of the physical interactions were proved by changing the characteristic of the copolymer film.

First of all, the ratio of BImTh:Fmoc-Gly-OH in copolymerization was optimized. For this optimization study, amperometric responses of the biosensors with different P(BImTh:Fmoc-Gly-OH) copolymer ratios were investigated. In these experiments, amount of enzyme (21 U ChOx), crosslinker amount and scan number during polymerization (30-cycle) in biosensor construction were kept constant. Five different biosensors with P(Fmoc-Gly-OH) and copolymers with varying ratios such as 1:1, 1:3,

1:10, 2:1 (BImTh:Fmoc-Gly-OH) were prepared and calibration curves for cholesterol were recorded. When the ratio was changed, different surface characteristics prevail on the electrode surface. The hydrophobic and hydrophilic groups influence the immobilization so do the stability and quality of the biosensor. Throughout the copolymerization studies, according to the change of surface properties, three dimensional structures of enzyme molecules in the immobilization alter due to the differences in linkages. This brings the variations in the success of the immobilization and performance of the biosensors. Fig. 4.3 reveals that the optimum copolymer ratio was found as 1:1 (BImTh:Fmoc-Gly-OH 0.017 M/ 0.017 M) in respect to amperometric responses of these biosensors. Maximum interaction and satisfactory immobilization of the enzyme molecules were achieved with the biosensor containing copolymer with the ratio of 1:1 (M/M) (BImTh;Fmoc-Gly-OH). Hence, the best results were recorded with this copolymerization ratio and this was kept in following studies. During the experiments with different copolymer ratio, due to the insufficient properties of the conducting polymer films, same quality was not achieved for each biosensor. With better copolymer films, better sensitivity and higher responses were achieved. With lower quality of conducting copolymers, lower responses were acquired; hence, lower concentrations of analyte could not be detected with these biosensors. Due to these differences, different ranges were obtained in the calibration studies.

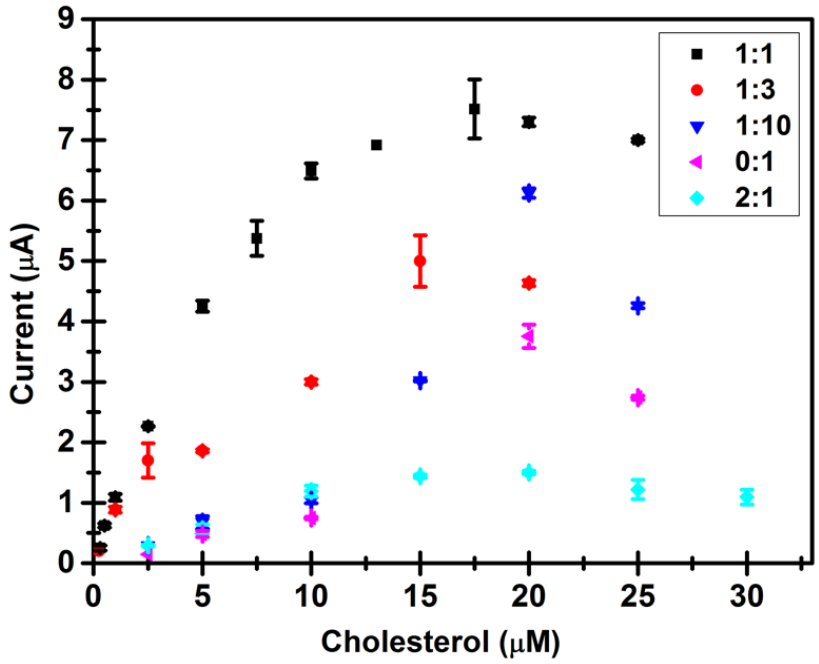

Figure 4.3 Effect of monomer ratio (BImTh:Fmoc-Gly-OH) on the biosensor response (in 50 mM phosphate buffer, pH 7.0, 25 °C, -0.7 V). Error bars show standard deviation (SD) of three measurements.

4.3.3 Effect of Scan Number in Electrocopolymerization, Enzyme Amount and pH

The copolymer thickness was controlled by adjusting scan number during electropolymerization. When the copolymer film is too thick, the distance between graphite electrode and active site of enzyme molecule may be unfavorably large. Thus, when the substrate associates with the recognition element, the released electrons during the enzymatic reactions may not be properly transferred to the transducer. On the contrary, very thin films may be unable to protect the enzyme molecule from the environmental effects. Moreover, due to the lack of enough functional groups, immobilization may not take place efficiently and precisely. In order to investigate the effect of copolymer layer thickness, comonomers in optimum ratio (1:1, M/M) were

polymerized on the graphite electrode with different scan numbers (20, 30, 50 and 70 scans) (Fig. 4.4 (A)). Optimum cycle was found by 30 scan electropolymerization.

The effect of different amounts of ChOx on the amperometric response was also investigated in the presence of 25 μM cholesterol. Different amounts of the enzyme such as 0.5 mg (13 U), 0.8 mg (21 U) and 1.2 mg (31 U) were immobilized on the modified electrode surface and optimum amount of enzyme was found as 0.8 mg. As illustrated in Fig. 4.4 (B), sufficient connection with enzyme molecules and polymeric layer were not observed under the optimum amount of enzyme. And also, the enzyme molecules leached from the surface due to excess loading in higher amounts.

Since ChOx is affected by the pH of the media, it is important to determine optimum pH value for the efficient measurements. For this reason, the effect of pH on the amperometric biosensor response was examined using 50 mM phosphate buffer solution in a range of pH 6.0-7.5 in the presence of cholesterol (25 μM). For acidic medium; 4.5, 5.0 and 5.5, sodium acetate buffer solutions and for basic one, 8.0, 10.0, sodium bicarbonate buffer solutions were used. Suitable phosphate buffer solution concentration was found to be 50 mM by Sing et al. [223]. For all the experiments, concentration of phosphate buffer concentration was used as 50 mM. During the experiments freshly prepared enzyme electrodes were used. As given in Fig. 4.4 (C), it was shown that at the conditions of high acidity or basicity, enzyme molecules lose their activity. The optimum enzyme activity was found at pH 7.0.

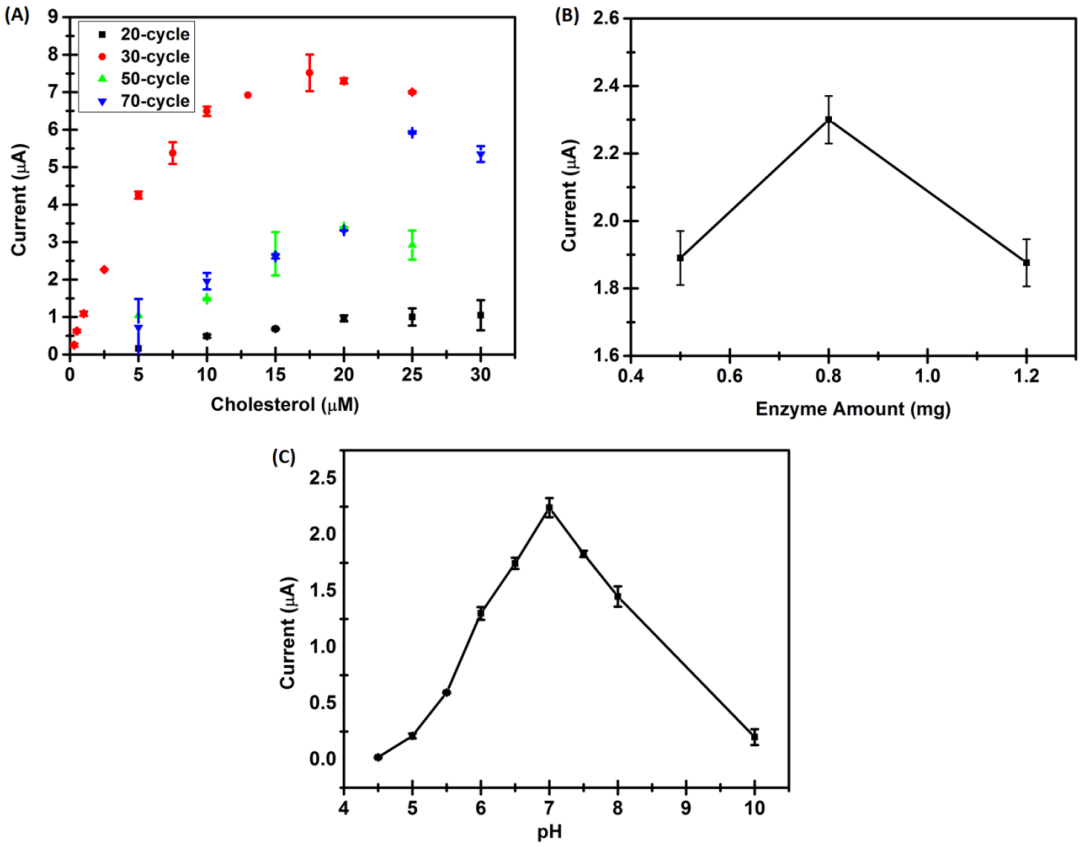

Figure 4.4 (A) The effect of conducting copolymer film thickness, (B) Loaded enzyme amount (in 50 mM phosphate buffer, pH 7.0, 25 °C, -0.7 V) and (C) pH on the biosensor response. Error bars show standard deviation (SD) of three measurements.

4.3.4 Characterization

4.3.4.1 Scanning Electron Microscopy (SEM) Studies

The surface morphology of copolymer coated graphite electrode before and after ChOx immobilization was investigated by SEM technique. In Fig. 4.5 (A), the image was taken after 30 cycles of electrocopolymerization on graphite. The surface morphology of the copolymer film depicts the porous and cauliflower structure of the

conducting polymer. In Fig. 4.5 (B), on immobilization of ChOx, the porous and cauliflower of the conducting polymer changes into the regular form due to the formation of covalent bonds between enzyme and polymer. This clearly shows that the enzyme was well-immobilized onto the conducting copolymer film.

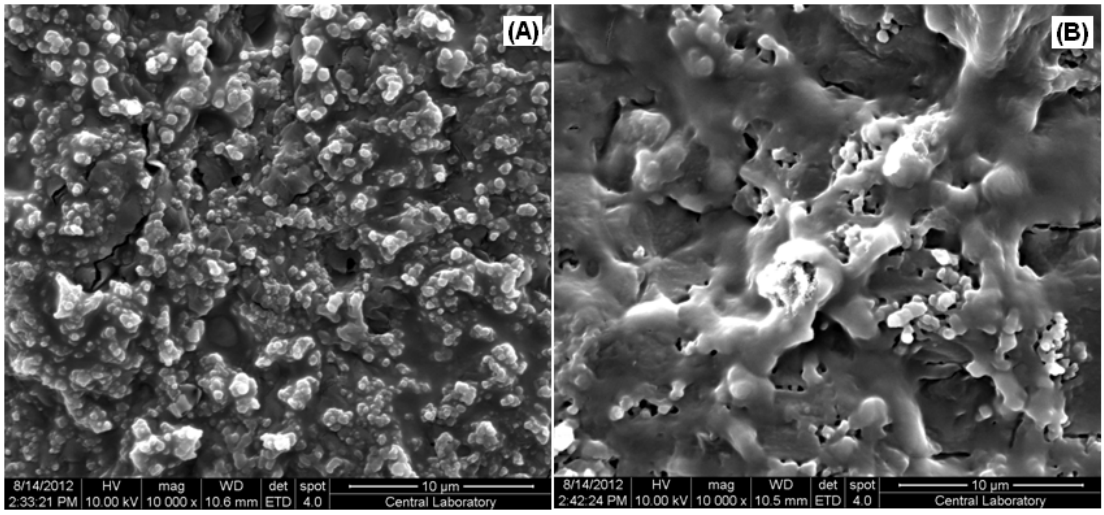

Figure 4.5 Surface characteristics of (A) Conducting copolymer (B) ChOx immobilized conducting copolymer via SEM images.

4.3.4.2 X-ray Photoelectron Microscopy (XPS) Studies

XPS spectra of P(BImTh-Fmoc-Gly-OH) and P(BImTh-Fmoc-Gly-OH)/ChOx were analyzed for their peak components hence they can be assigned to different characteristic chemical species. In the C1s spectra of P(BImTh-Fmoc-Gly-OH), the peaks at 283.6 and 284.5 eV can be attributed to aromatic, alkyl carbons and C-S in thiophene, respectively. The peaks at higher energies (288.7 eV) can be assigned to the carbons atoms of O=C-N and (O=C-N)-OH in the polymeric structures due to the presence of Fmoc-Gly-OH unit [226]. The increase in the peaks at 285.4 and 287.5 eV for C-N and O=C-N and decrease in the intensity of functional groups prove the amide bond formation and successful immobilization of the protein molecules (Fig. 4.6 (A) and (B)).

Moreover, Fig. 4.6 (C) and (D) show the N1s spectra for both surfaces. In Fig. 4.6 (C), the peaks at 401.1 and 398.8 eV appear due to the presence of imidazole ring in the structure of copolymer [13, 227, 228]. Also, after immobilization, the amide (399.7 eV) can be observed in Fig. 4.6 (D) supporting the covalent bonding.

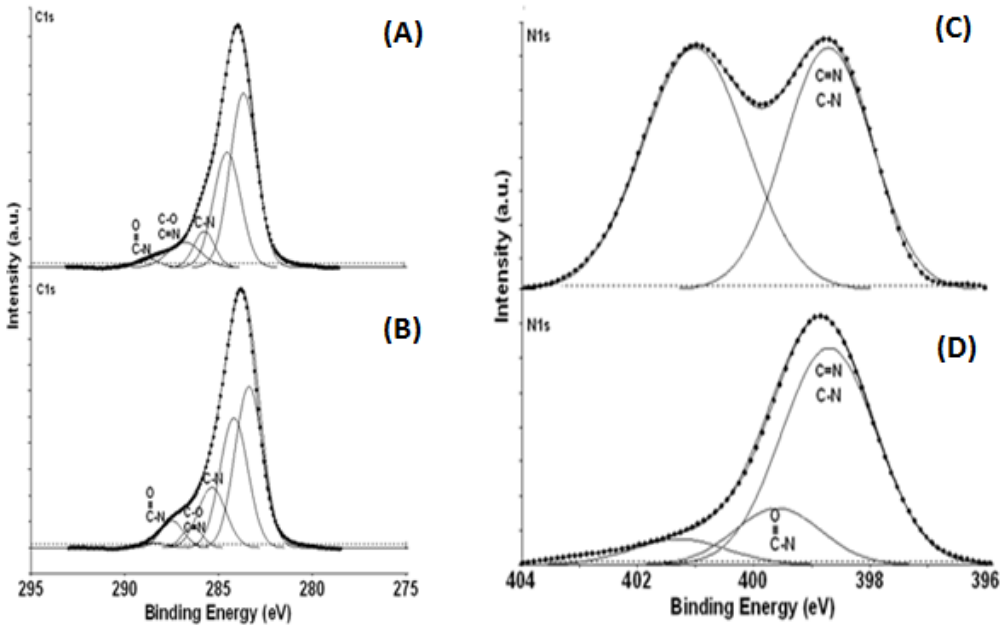

Figure 4.6 XPS C1s spectra for (A) Copolymer and (B) ChOx immobilized copolymer, N1s spectra for (C) Copolymer and (D) ChOx immobilized copolymer.

4.3.5 Analytical Properties, Repeatability and Storage Stabilities of the Cholesterol Biosensor

After all optimizations, biosensor responses for different amounts of cholesterol were recorded to construct the calibration curve (Fig. 4.7). Linear response range was illustrated as 0.3-10 µM (Fig.4.7 as inset). Moreover, limit of detection (LOD) was calculated as 0.17 µM for cholesterol based on S/N=3. Furthermore, K_M^{app}, I_{max} from the Lineweaver-Burk plot and sensitivity values were calculated as 6.25 µM, 9.69 µA and 2.47 mA/mM cm^2, respectively. Lower K_M value is the indication of higher substrate affinity. Moreover, K_M^{app} value for the P(BImTh:Fmoc-Gly-OH)/ChOx

biosensor is extremely smaller than the ones reported in earlier studies (Table 4.2). This shows the effectiveness and success of the immobilization matrix, sensitivity of the biosensor and applicability in other operations. On the other hand, with the help of functional matrix, covalent binding was achieved excellently affording high sensitivity and affinity to substrate as well as stability. In recent years, several studies related to conducting polymer based cholesterol biosensors were done in literature and summarized in Table 4.2.

For repeatability of the cholesterol biosensor response, ten successive measurements were obtained in the same day with 25 μM cholesterol solution. Standard deviation (SD) and the relative standard deviation (RSD) were calculated as ± 0.094 and 4.4%, respectively. The lifetime of the copolymer modified electrode was determined by measuring the amperometric response during 28 days. During the experiments, activity loss of 20 % was observed on the 28th day. The biosensor was kept at +4 °C when not in use.

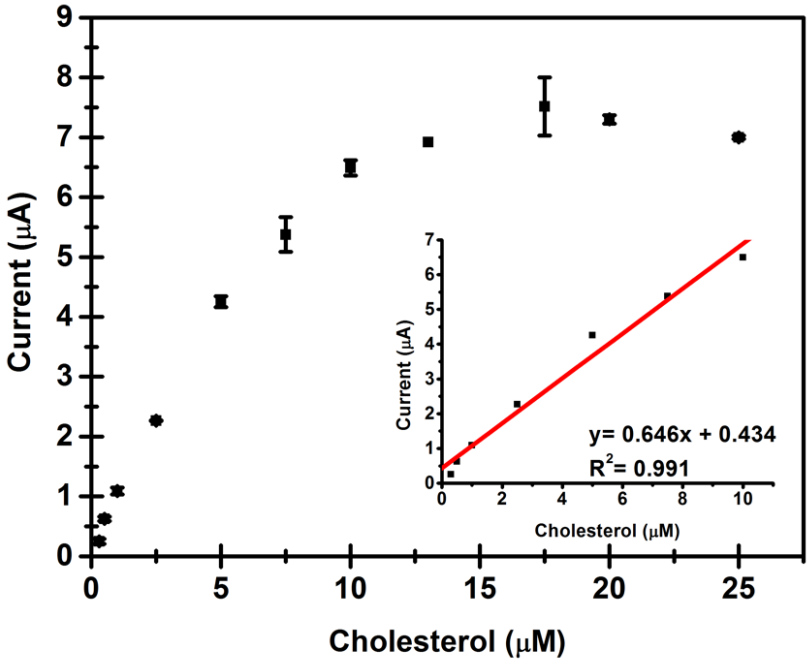

Figure 4.7 Calibration curve for cholesterol (in 50 mM phosphate buffer, pH 7.0, 25 °C, -0.7 V) (Lineer sensor responses were inserted as inset). Error bars show standard deviation (SD) of three measurements.

In order to be able to work on various samples comfortably, the biosensor must work interference free. Hence, investigations of effects of various materials as interferents were carried out. In particular, ascorbic acid, urea and glucose were used, since these compounds are easily oxidized at bare electrodes.

For this study, ascorbic acid, urea and glucose solutions were injected instead of substrate into the reaction cell containing 50 mM, pH 7.0 phosphate buffer solution. No interference effect was observed in all applications. As seen in Fig. 4.8, we concluded that copolymer based ChOx enzyme electrode can easily detect cholesterol with no interference. When cholesterol was injected into the reaction mixture, it was shown that the change of current was detected clearly. As a result, the biosensors can be used in various applications readily.

Table 4.2 Various studies from the literature based on conducting polymer containing cholesterol biosensors.

Conducting polymer	**Immob. tech.**	**K_m/V_{max} or I_{max}**	**Linear range**	**References**
Poly(3,4-ethylenedioxypyrrole)	Entrapment	3.4 mM/ 34 μAcm^{-2}	NR	[37(b)]
Poly(3,4-ethylenedioxythiophene)	Entrapment	1.3 mM/17.9 μAcm^{-2}	NR	[37(c)]
Polypyrrole(Ppy)	Entrapment	9.8 mM/NR	1-8 mM	[37(d)]
Poly(3-thiopheneacetic acid)	Covalent binding	NR	0-8 mM	[37(e)]
P(NMPY)-PTS	Entrapment	4.35 mM/NR	2-12 mM	[37(f)]
PANI	Covalent binding	0.62mM/NR	25-400 $mgdL^{-1}$	[37(g)]
P(BImTh:Fmoc-Gly-OH)	**Covalent binding**	**6.25 µM/9.69 µA**	**0.3-10 µM**	**This work**

NR: Not reported

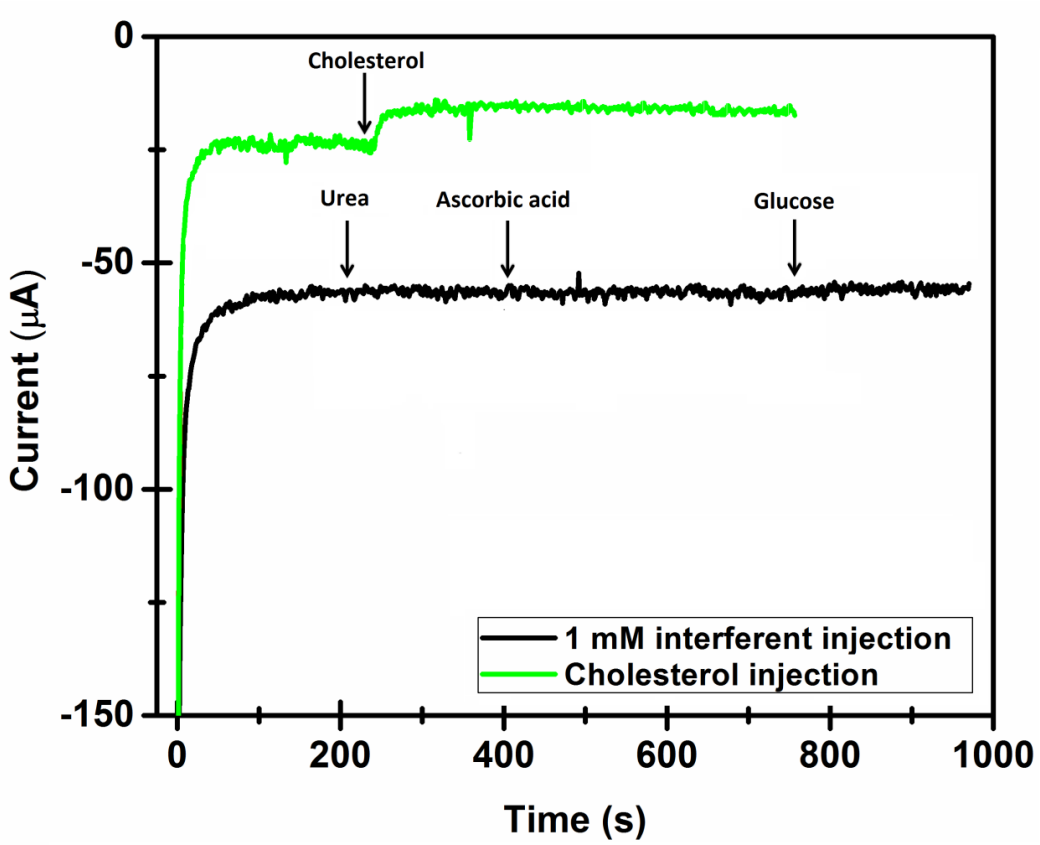

Figure 4.8 Amperometric responses of P(BImTh:Fmoc-Gly-OH)/ChOx biosensor to cholesterol and interference studies with ascorbic acid, urea and glucose solutions in 50 mM phosphate buffer, pH 7.0, 25 °C, -0.7 V.

4.3.6 Detection of Cholesterol in Serum Samples

The constructed copolymer based biosensor was tested for real human serum samples to determine the cholesterol level. Serum samples were obtained from Middle East Technical University (METU), Medical Center. This study was approved by the METU Medical Ethics Committee and a formal consent was obtained from all subjects who were not suffering from any disease and were not on any medication. Firstly, these human serum samples were analyzed for cholesterol at the METU Medical Center via enzymatic and colorimetric method (with Biochemistry analyzer (Beckman Coulter CX9, USA)) and the cholesterol levels were determined with a reference method.

After, these same serum samples were injected (act as substrate) into 50 mM phosphate buffer solution (pH 7.0) during amperometric measurement and current change was monitored. The experiments were achieved at the optimum conditions. It was shown that our results were very similar to the ones obtained in the hospital (Table 4.3).

Table 4.3 Determination of cholesterol in serum samples by constructed and reference procedures.

Sample	Hospital Data (mM)	P(BImTh:Fmoc-Gly-OH)/ChOx (mM)	Relative Error (%)
1	3.13	3.11	0.63
2	4.16	4.62	10.50
3	3.70	3.64	1.62
4	3.17	3.16	0.31
5	4.13	4.26	3.15

4.4 Conclusion

A novel copolymer film by electrocopolymerizing BImTh and Fmoc-Gly-OH was synthesized successfully for the construction of a cholesterol oxidase biosensor. The amperometric biosensor based on conducting copolymer was fabricated through covalent immobilization of the enzyme. The presence of the protein and amide bond between the surface and the enzyme molecules were confirmed by SEM and XPS studies. The fabricated biosensor exhibits excellent kinetic parameters such as K_M^{app}, I_{max}, low LOD and high stability. Moreover, it was successfully applied to the human blood serum samples with satisfactory results.

CHAPTER 5

QUATERNIZED POLYMER−SINGLE-WALLED CARBON NANOTUBE SCAFFOLDS FOR A CHEMIRESISTIVE GLUCOSE SENSOR

5.1 Background and Motivation

Accurate detection and quantification of glucose concentration is vital in clinical and biological samples and food processing since abnormal blood glucose concentration level in human body causes several health problems like diabetes mellitus. As a result, new methods for glucose detection and the monitoring of its accumulation level continues to be of interest.

In this study, to fabricate a chemiresistive glucose sensor, we began with our previous procedure wherein a P4VP-SWCNT dispersion was spray-coated and covalently linked to the surface [146]. We then implemented a post-functionalization of P4VP-SWCNT surface by reacting with 2-bromoethanol as illustrated in Figure 5.1. The modified quaternized surface was then treated with enzyme glucose oxidase (GOx) that is commonly used as a biological element of the glucose sensor for diabetics. The quaternized P4VP-SWCNT scaffold with hydroxyl end group functions as a bioactive layer that immobilizes GOx through electrostatic and hydrophilic interactions. The strong electrostatic interaction between the positively charged surface and the negatively charged enzyme results in a sensor with an efficient electron transfer in order to produce a higher response to glucose [229]. In a subsequent step, the P4VP-SWCNT-GOx matrix was fixed by glutaraldehyde (GA) as a cross-linking agent. Then, it was measured the resistance change of the sensor upon exposure to glucose as the analyte. P4VP-SWCNTGOx nanocomposites demonstrates high performance as

glucose sensors. This versatile chemiresistive sensor scaffold is expected to guide into a number of biosensor applications with different types of suitable enzymes.

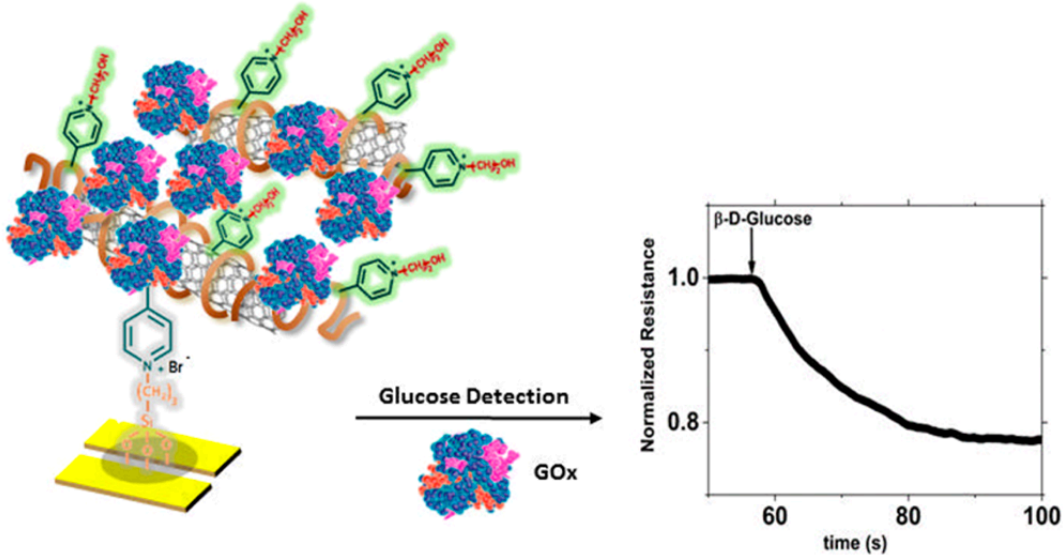

Figure 5.1 Schematic representation of the proposed sensor.

5.2 Experimental

5.2.1 Materials and Apparatus

All chemicals and reagents were purchased from Sigma-Aldrich and used without additional purification, unless otherwise noted. SWCNTs (purified ≥95% as SWCNT, batch #UPT 1188-58BL) were obtained from Nano-C, Inc. Poly(4-vinylpyridine) (Mv = 200,000 g/mol) and 3-bromopropyltrichlorosilane (BPTS) were purchased from Scientific Polymer Products, Inc. and Gelest, respectively. ß-D-Glucose and gluconolactone were purchased from TCI and EMD Millipore Corp., respectively. 2-Bromoethanol (97%), D-fructose (99%), and sucrose (99%) were purchased from Alfa Aesar. Glucose oxidase (from *Aspergillus Niger*, 17300 units per g solid), glutaraldehyde (GA) as a cross linking agent and glucose assay kit (GAGO-20) were obtained from Sigma-Aldrich and used as received.

ATR-FTIR spectra were recorded using a Thermo Scientific Nicolet 6700 FTIR with a Ge crystal for ATR. Raman spectra were collected with excitation at 633 nm laser using a Horiba LabRAM HR800 Raman spectrometer. Surface morphology of the device was investigated using a scanning electron microscope (SEM) (JEOL JSM-6700F FESEM), with an accelerating voltages of 3 and 10 kV. The static water contact angles on the surfaces before and after quaternization were measured using a Ramé-Hart goniometer (Model 590) by vertical deposition of 2 μL of water droplet. Glucose sensing experiments were performed using a Keithley 2400 source meter with a combination of Keithley KUSB-488B cable. Glucose sensing results were recorded using a custom Labview program. All measurements were performed at ambient conditions (25 ^{o}C).

5.2.2 Fabrication of the Chemiresistive Device

5.2.2.1 Preparation of a P4VP-SWCNT Dispersion

To prepare the P4VP-SWCNT dispersion, the SWCNTs (5 mg) were first added into the solution of P4VP (Mv = 200,000 g/mol, 50 mg) in N,N-dimethylformamide (DMF, 10 mL), then the resulting mixture was sonicated for 1 h in an ultrasonic bath (Branson, 3510) chilled with ice. Finally, the dispersion was allowed to reach room temperature, and the suspension was centrifuged for 30 min at 15,000 g and allowed to stand overnight undisturbed as reported in the literature [146]. The supernatant was carefully removed from the mixture and directly used for the device fabrication via spray coating.

5.2.2.2 Fabrication of a Quaternized P4VP-SWCNT Scaffold

Glass substrates were cleaned and prepared according to a literature procedure [230]. First, the glass substrates (VWR Microscope Slides) were cleaned using acetone in an ultrasonic bath for 5 min followed by UV-ozone treatment using a UVO cleaner (Jelight Company Inc., Model 42) for 20 min. The cleaned glass substrates were fitted

with a home-made stainless steel mask, and then layer of chromium (10 nm) and a subsequent layer of gold (120 nm) (99.99%, R.D. Mathis) were deposited onto the glass substrates using a thermal evaporator (Angstrom Engineering). Each device contains a gold pattern of electrode pair on the glass substrate. The gap between gold electrodes is 1 mm. Before the surface modification with 3-bromopropyltrichlorosilane (BPTS), the glass substrates were cleaned using acetone and isopropyl alcohol sequentially in an ultrasonic bath for 15 min to remove dust. Then, the glass substrates were treated with piranha solution (a mixture of sulfuric acid and 30% hydrogen peroxide (1:1, v/v)) for 1 h followed by cleaning and drying with distilled water and nitrogen after each step, respectively. For BPTS modification, these cleaned glass substrates were incubated in anhydrous toluene containing 10% of BPTS solution at room temperature under argon atmosphere overnight. This modification was used to react with the P4VP-SWCNT to provide a surface anchor. Excess BPTS was removed by washing the electrodes with pure toluene followed by drying with N_2. After drying of substrates, they were thermally annealed at 130 ^oC for 1 h. To produce a P4VP-SWCNT film a homemade transparency film (CG3700, 3M) mask was used to deposite the material selectively onto the gaps between electrode pairs, and the dispersion was manually spray coated with 400 μL of P4VP-SWCNT dispersion using an air brush (Revolution BR, Iwata). The rate of deposition was fixed as 40 μL/min at a distance of 10 cm with the substrate placed on a 130 ^oC hot plate under N_2 carrier gas of 2 bar pressure to obtain homogenous surface. After this process, following procedures were applied to obtain final substrate: The substrate was thermally annealed at 130 ^oC overnight to ensure the quaternization reaction between the surface bound alkyl bromides and pyridyl groups. Excess polymer and materials not covalently bound to the surface were removed by sonication in pure dichloromethane (DCM) for 1 min followed by drying under under N_2. For the quaternization reaction with 2-bromoethanol, 1 mL of 2-bromoethanol (97%) was added into the 4 mL of acetonitrile (ACN), and mixed well. Then, the modified device was placed into the ACN solution and then heated at 30 oC for 24 hours. Finally, the device was rinsed with pure ACN, and dried under a stream of N_2.

5.2.2.3 Fabrication of a P4VP-SWCNT-GOx Chemiresistive Sensor

Following the surface modification, the device was rinsed with distilled water and dried under N_2. Then 2 µL of glucose oxidase solution (10 µL of 2.5 mM pH 7.0 phosphate buffer solution containing 30.27 U GOx) was electrostatically immobilized onto the quaternized P4VP-SWCNT surface. GOx was chosen as the model enzyme. After 2 min, 2 µL of glutaraldehyde (GA) solution (1%) was added on the surface and allowed to dry in ambient air for 2 h to finalize the fabrication of the robust P4VP-SWCNT-GOx devices. The final resistance across the SWCNT network was between 350 kΩ -1 MΩ as measured by a multimeter. Devices were thoroughly washed with distilled water prior to use in order to remove unbound enzyme molecules. The use of GA not only provides intermolecular cross linking in proteins but also enhances anchoring of the enzyme molecules on the supporting material [231]. The fabricated sensor is depicted in Figure 5.2.

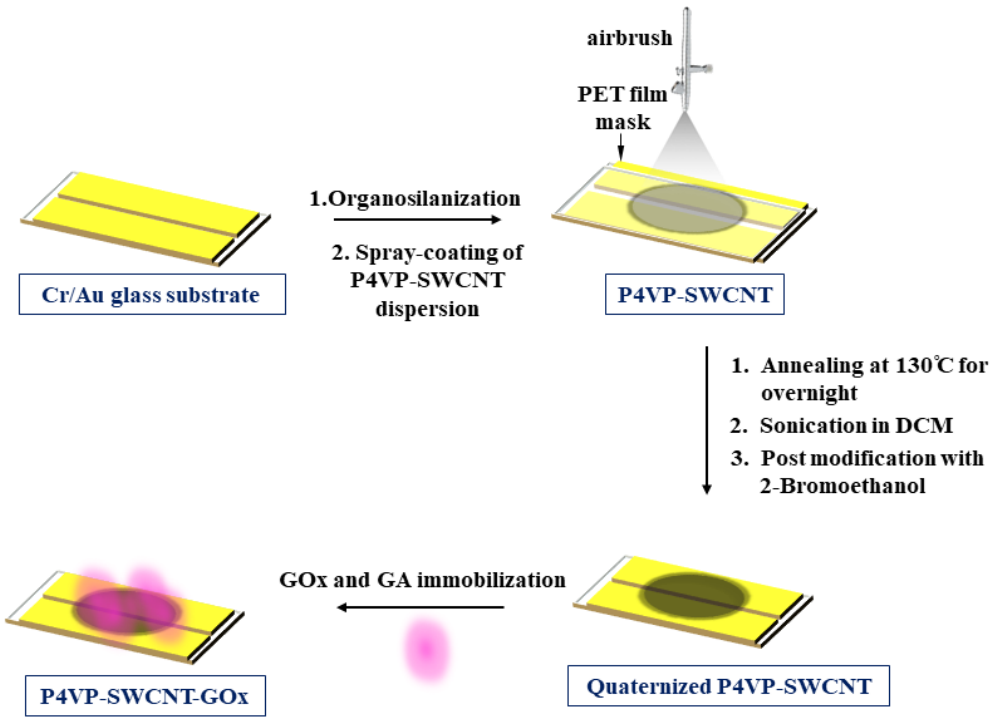

Figure 5.2 Preparation of the proposed chemiresistive glucose sensor.

5.2.3 Glucose Detection Measurement

Chemiresistive measurements with the devices were carried out at room temperature in the reaction cell containing 10 mL of NaOAc (2.5 mM, pH 5.5) by applying 0.1 V potential. Under the constant potential, the resistance change was monitored and recorded using a custom Labview program with the combination of Keithley 2400 Source Meter. The change in resistance resulted from exposure to glucose was calculated using the equation of ΔR (%) = $(R_0–R)/R_0$ x 100%, R_0 is the initial resistance of the P4VP-SWCNT-GOx, and R is the resistance after exposure to glucose. After the background reached at a steady state, a certain amount of glucose was injected in the reaction cell and the resistance change was recorded as the sensor response. All experiments were carried out at ambient conditions. For consecutive measurements on a single device, the working buffer was refreshed and the device was rinsed with distilled water after each measurement.

Glucose oxidase (GOx) is a well-known oxido-reductose enzyme that catalyses the conversion of glucose and oxygen to give gluconolactone and hydrogen peroxide as follows: [232, 233]

$$\beta\text{-D-Glucose} + O_2 \rightarrow \text{D-Glucono-1,5-lactone} + H_2O_2$$

The quantification of glucose can be monitored via the detection of enzymatically liberated hydrogen peroxide under constant potential. When the reaction occurs each glucose produces an equivalent of hydrogen peroxide and subsequently alters the resistance of the system. As desired, in the absence of GOx, no change in resistance was observed. We hypothesize that SWCNT based chemiresistive response to hydrogen peroxide produces a selective and sensitive glucose sensor. Moreover, this sensor showed different resistance changes upon exposure to different concentration of glucose. Following the optimization and characterization studies, the P4VP-

SWCNT-GOx device was tested for glucose in beverage samples that were used without any pretreatment.

5.3 Results and Discussion

The nature of the supporting and transducing materials is critical to create biosensing platforms. Carbon nanotubes are not natural materials for these applications and must be modified. To test our new platform, we have turned our attention to glucose oxidase (GOx) based sensors, which have been studied extensively. For an intergrated sensor GOx needs to be properly fixed onto the substrate. To create a stable and robust platform, herein, we demonstrate that a quaternized P4VP-SWCNT chemiresistor scaffold is capable of glucose monitoring via immobilization of enzyme molecules. This system makes use of a simple and noncovalent functionalization/surface-imobilization of SWCNTs with P4VP, and subsequent quaternization with 2-bromoethanol to create biomimetic and cost-effective devices. The quaternized surface, being hydrophilic in nature, is known to interact with the biomolecule via hydrogen bonding and π-stacking, thus providing a fixation for enzyme molecules. Additionally, the cationic character of the surface creates electrostatic binding. GOx has an isoelectric point of about 4.2 [234] and hence carriers a net negative charge in pH 7.0 phosphate buffer solution. Strong binding of negative biomolecules will occur as a result of the electrostatic attraction and the entropy released by counterions when two complementary polyelectrolytes come together. The close interaction of the enzymes with the SWCNTs results in a chemiresistive sensor with more efficient electron transfer, as is the case here, for high sensitivity [229]. Carbon nanotubes provide excellent performance in chemiresistive sensor designs, [145] and a strong electrical coupling to glucose oxidase was achieved by our designs [235].

To prepare hydrophilic and positively charged surface for the GOx immobilization, we functionalized P4VP-SWCNT with 2-bromoethanol. Figure 5.3A presents the ATR-FTIR spectra of P4VP-SWCNT composites before and after 2-bromoethanol treatment. The composite films were prepared by spraying 2.4 mL of the dispersion

for the FTIR spectroscopy study. The shift of the pyridyl C=N stretching band from 1601 to 1638 cm−1 is indicative of the quaternization reaction on the pyridiyl nitrogen of P4VP. The quaternized P4VP-SWCNT was also confirmed by a decrease in the water contact angle (Figure 5.3B). The P4VP-SWCNT composites are covalently anchored to the substrate, and consistent with the SEM images in Figure 5.3C(a) and (b) show that the composites remain on the substrate after the quaternization reaction. We then immobilized GOx to the cationic quaternized P4VP-SWCNT surface by electrostatic assembly and then treated with GA as a cross-linking agent. We note that SWCNTs were not observed by SEM in Figure 5.3C(c) as a result of the top layer being composed of crosslinked GOx. The electrical resistance of the P4VP-SWCNT-GOx films soaked in pH 5.5 acetate buffer solution ranges between 350 kΩ and 1 MΩ. Figure 5.4A summarizes the Raman spectra of P4VP-SWCNT, quaternized P4VP-SWCNT, and P4VPSWCNT-GOx composites using an excitation wavelength of 633 nm. We found that there was no significant change in the D/G band intensity, which indicates that the quaternization reaction and GOx immobilization do not add significant defects to the SWCNT sidewalls. This aspect is one of the advantages of our approach, wherein with noncovalently wrapped P4VP to SWCNT, the π electronic states in the SWCNT sidewalls remain intact with functionalization thereby maintaining the critical electrical transport properties of the nanotubes.

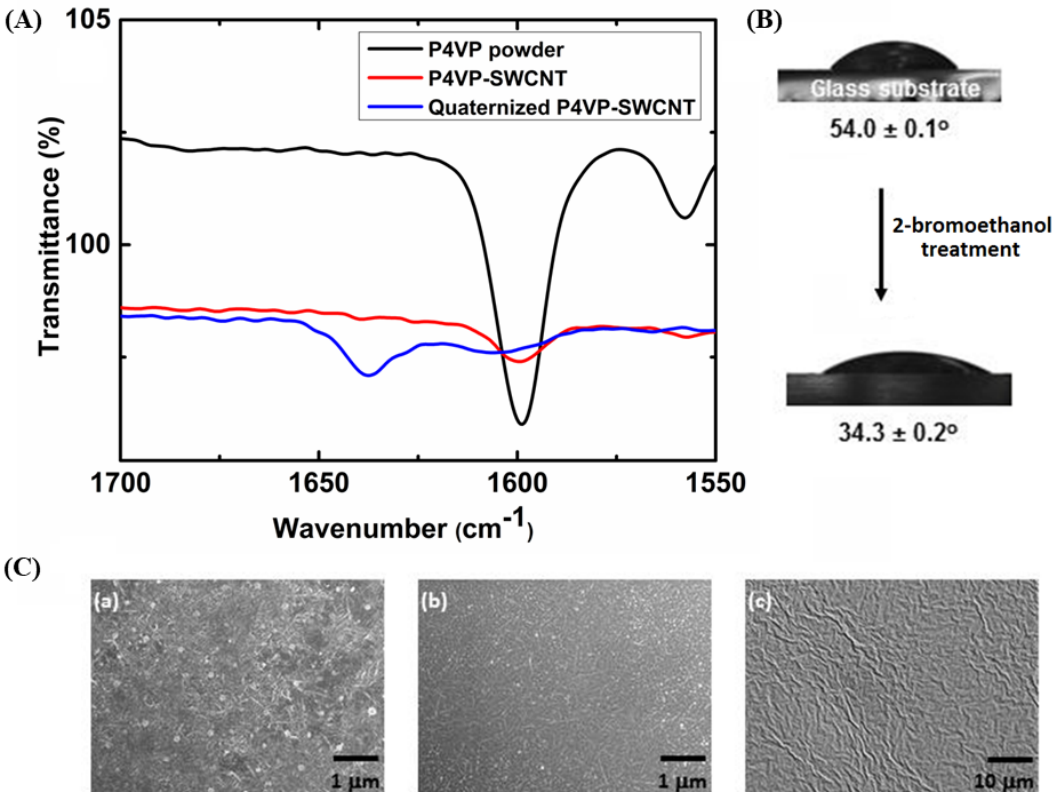

Figure 5.3 (A) ATR-FTIR spectra of P4VP powder, P4VP-SWCNT, and quaternized P4VP-SWCNT composites on glass substrates (B) Water contact angle measurements of P4VP-SWCNT and quaternized P4VP-SWCNT composites (C) SEM images of (a) P4VP-SWCNT, (b) Quaternized P4VP-SWCNT, and (c) P4VP-SWCNT-GOx films on glass substrates.

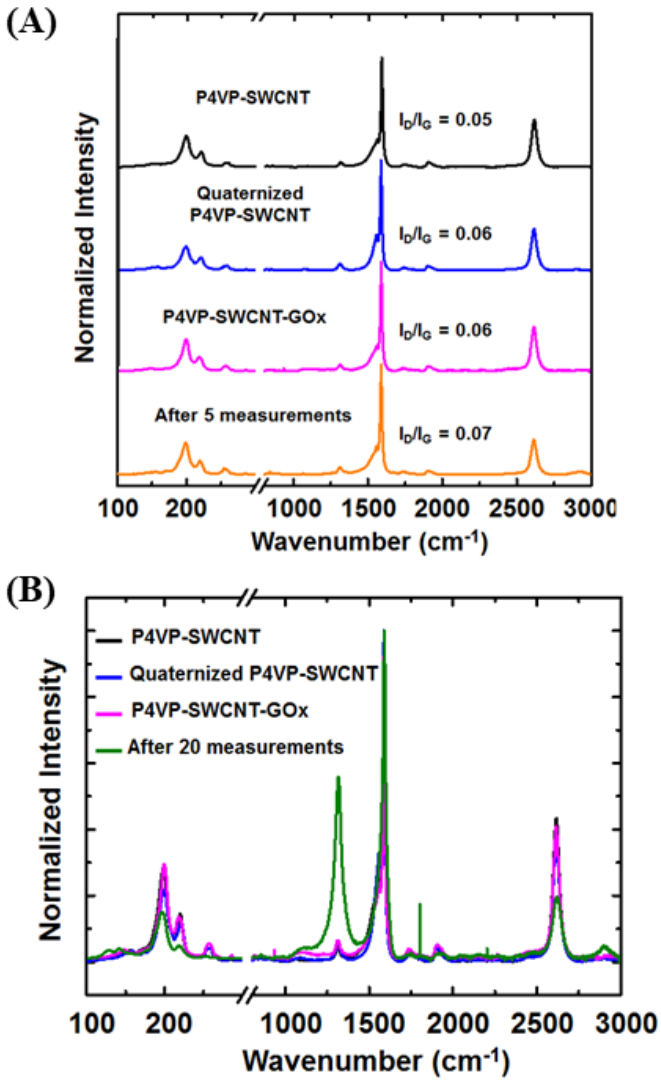

Figure 5.4 (A) Raman spectra of P4VP-SWCNT, quaternized P4VP-SWCNT, P4VP-SWCNT-GOx film, and P4VP-SWCNT-GOx film after five consecutive measurements with 2.0 mM of glucose. (B) Superimposed Raman spectra of P4VP-SWCNT, quaternized P4VP-SWCNT, P4VPSWCNT-GOx film, and P4VP-SWCNT-GOx film after 20 consecutive measurements with 2.0 mM of glucose (excitation at 633 nm).

Following the preparation of P4VP-SWCNT-GOx device with optimum conditions, we proceeded chemiresistive measurement of the devices at room temperature in a reaction cell containing 10 mL of NaOAc (2.5 mM, pH 5.5) with a 0.1 V applied potential between the electrodes. Under the constant potential, we monitor the current to determine the resistance change. The change in resistance is $\Delta R\ (\%) = ((R0 - R)/R0) \times 100\%$, where R0 is the initial resistance of the P4VP-SWCNTGOx and R is the resistance after exposure to glucose. The immobilized GOx catalyzes the conversion of glucose and oxygen to gluconolactone and hydrogen peroxide and we can detect glucose by monitoring a decrease in resistance of the sensor caused by the action of the enzymatically liberated hydrogen peroxide.

A calibration curve generated from the addition of different concentration of glucose solutions to the device is shown in Figure 5.5 A. Linearity is achieved between 0.08 and 2.2 mM glucose, as given by $y = 140.6x + 3.239$ and $R2 = 0.991$. Typical sensing traces of the sensor to different concentration of glucose are given in Figure 5.5 B. As shown in Figure 5.5 B, without the glucose addition, there is no signal change in the resistance. As expected when the concentration of glucose is increased, a greater response was observed in accord with a larger quantity of hydrogen peroxide production from the enzymatic reaction. However, at higher concentrations of glucose (above 2.2 mM glucose concentration), we observed a decrease in the response as a result of reaching the saturation point of the enzyme molecules (it is a general phenomenon for enzymatic based sensor systems). Additionally, considering that the transduction involves the injection of carriers (holes) in the SWCNTs, nonlinearity at high concentrations (doping levels) is expected. As a result, the reliable linear range of the sensor was obtained from 0.08 to 2.2 mM. In blank experiments, the sensor fabricated at the optimized conditions was tested that lacks glucose oxidase. Addition of the glucose solution to the reaction cell in this case did not change the resistance of the system. Additionally, when only the buffer was added to the medium instead of glucose, no change in resistance was observed. These controls confirm that the resistance change can only be observed as a result of enzymatic reaction with P4VPSWCNT-GOx surface and glucose.

Difficulties in accurate measurement of glucose concentration can arise from the interference by other compounds. To examine the robustness of sensor response, we tested the P4VP-SWCNT-GOx sensor for the selective determination of glucose over different sugars. Figure 5.5 C shows a comparison of the sensor response to the same concentrations of sucrose, fructose and glucose (2.0 mM). During these experiments, there was no response either to sucrose or fructose.

The reproducibility and stability are two important parameters for evaluating the performance of any sensor. The reproducibility of the system was examined using seven different electrodes in the buffer solution containing 2.0 mM glucose. All devices were fabricated under the same conditions. The responses for each device were recorded as illustrated in Figure 5.5 D. The averaged percent response with one sensor is ranged from 23.9% to 40.6%, and the relative standard deviation of seven sensors was found as 18.9%. Additionally, the sensor signals corresponding to the 2.0 mM glucose solution were measured 10 times consecutively in order to prove the repeatability of the sensor response. The SD and the relative standard deviation were calculated as ±0.365 and 11.3%, respectively.

Moreover, the long-term stability of the proposed sensor was investigated under the optimum conditions. We examined the stability of P4VP-SWCNT-GOx for a 45 days operational performance. The fabricated sensor device was first kept at 4.0 °C for 45 days and then exposed to 2.0 mM of glucose. It was found that the sensor retained 83.3% of its initial response, showing the prospects for long-term stability of the device. The stability of the P4VP-SWCNT-GOx electrode is related to the excellent biocompatibility of the alkylated P4VP-SWCNT, which preserves the activity of the biomolecules. Nevertheless, some decrease in the response after a long-term storage was observed, which is probably the result of the enzyme denaturation. Mitigating this issue is beyond the initial scope of this investigation. One advantage of our chemiresistive glucose sensor is its fast response (~3 s), which creates a real time sensor. All devices containing GOx revealed an instant decrease in resistance with each glucose addition.

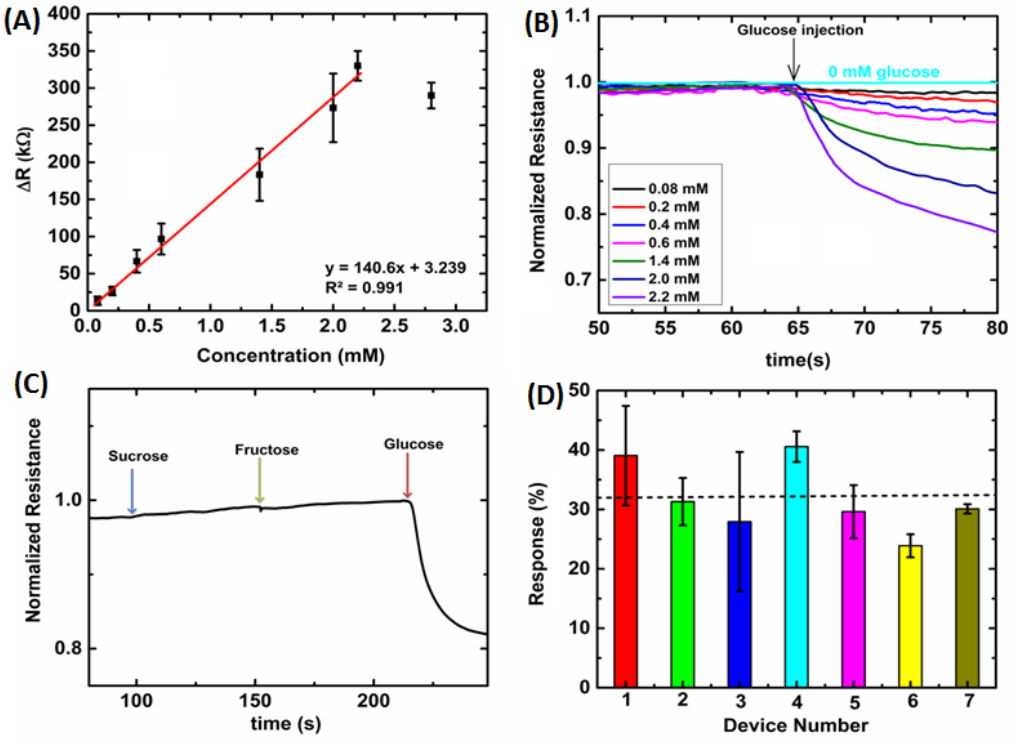

Figure 5.5 (A) Calibration curve for glucose (in 2.5 mM NaOAc buffer, pH 5.5) (error bars show three consecutive measurements with the same device), (B) The chemiresistive device response to various glucose concentrations between 0.08 and 2.2 mM, (C) Sensor selectivity for different sugars (2.0 mM in 2.5 mM NaOAc buffer, pH 5.5), and (D) Responses of glucose using different devices under the optimum conditions: The dotted line indicates the average value of the devices (error bars show three consecutive measurements with the same device).

It is noted that when the sensor is exposed to 2.0 mM glucose solution for 5 consecutive times, there was a modest increase in the D/G band in Raman spectra from 0.06 to 0.07 (Figure 5.4A, bottom). However, after 20 consecutive exposures to glucose, the D/G band intensity ratio of the P4VP-SWCNT-GOx surface increased to 0.56 indicating the reactions of the SWCNT sidewalls with hydrogen peroxide (Figure 5.4B).

Oxidation of CNTs by H_2O_2 was also a central topic when using CNTs as H_2O_2 sensors or sensitive glucose sensors based on oxidase [236, 237]. The oxidation of

CNTs with H_2O_2 causes defects on the nanotube structure. To explain the reason of the increase in the Raman disorder signal of SWCNT by H_2O_2, we prepared a device with P4VP-SWCNT not containing GOx and obtained its Raman spectrum after exposure to an appropriate concentration of H_2O_2 for 20 times. Figure 5.6 (A) shows the Raman spectra of the P4VP-SWCNT before and after H_2O_2-treatment (20 times exposure). The results revealed that after exposure to H_2O_2, there was a slight increase in the D/G band ratio from 0.05 to 0.07. This is an evidence of the defect by H_2O_2, which has been reported in other studies [238, 239]. The structure distribution of semiconducting SWCNTs has been strongly modified by the peroxide treatment. To test the effect of the gluconolactone, which is the product of the enzymatic reaction, Raman spectra were taken for the P4VP-SWCNT-GOx after exposure to an appropriate concentration of gluconolactone and H_2O_2 for 20 times each. In Figure 5.6 (B), the red line shows the Raman spectrum of the gluconolactone-treated P4VP-SWCNT-GOx with the D/G band ratio of 0.06 while the one exposed to H_2O_2 for 20 times showed a notable increase in the D/G band ratio of 0.15 (Figure 5.6 (B), blue line). Interestingly, we found that the enzymatic reaction caused a huge increase in the D/G band intensity (0.56) after exposure to glucose (Figure 5.4 B). We presume that there is a synergetic effect of the enzyme that affects the electronic nature of the SWCNT under hydrogen peroxide. GOx is flavoprotein, containing two tightly bounded flavine adenine dinucleotide redox centers. The redox centers lead an electron transfer during the enzymatic reaction. The GOx from *Aspergillus niger* catalyzes the oxidation of β-glucose to D-glucono-δ-lactone and hydrogen peroxide with molecular oxygen. Presumably, this reaction may affect the electronic structure of the nanotube. Moreover, the oxidation process of β-glucose to D-glucono-δ-lactone may disturb the π electronic states in the SWCNT sidewalls. Therefore, sensors based on this system is limited to five consecutive exposures with a single sensor chip, which would be envisioned as a consumable in a real-world applicaton.

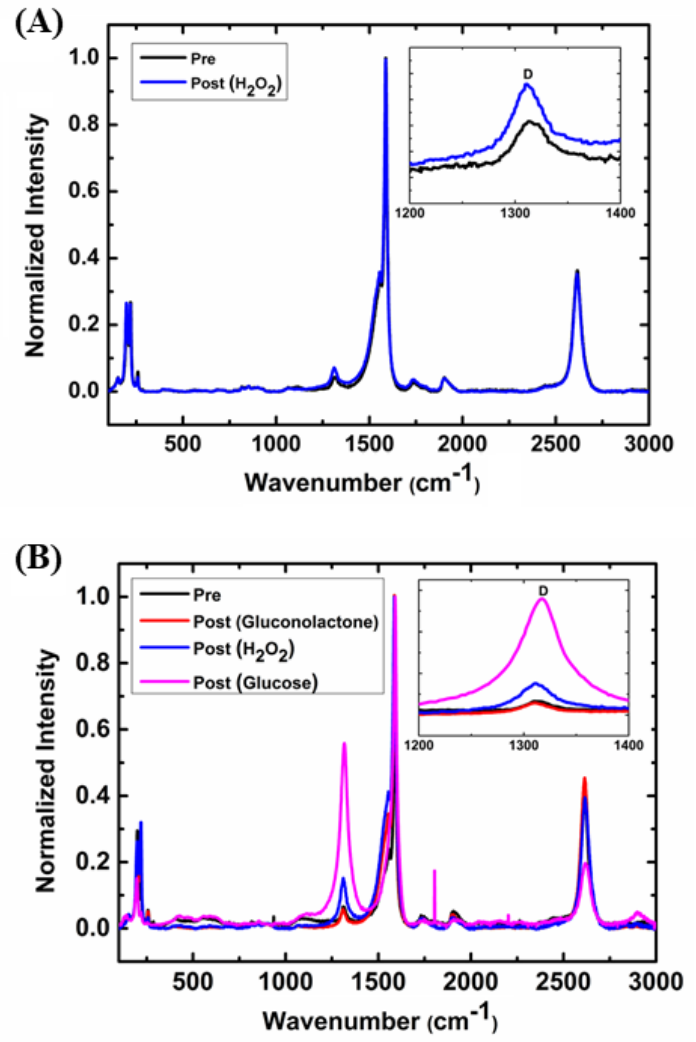

Figure 5.6 Raman spectra of (A) P4VP-SWCNT before and after exposure to H_2O_2 (20 times exposure), and (B) P4VP-SWCNT-GOx before and after exposure to gluconolactone, H_2O_2 and glucose (20 times exposure) in 2.5 mM NaOAc buffer solution.

To further test the sensing performance we have endeavored to measure the concentration of glucose in two different beverages (S fruit punch juice and L ice tea). Two real samples were analyzed using the designed sensor, and the results were compared with the values obtained using a commercial glucose kit with spectrophotometric detection. Table 5.1 shows the results obtained by both methods.

The untreated beverage samples were injected into the device cell (automatic dilution occurs in 10 mL reaction medium). The sensor responses for each sample were recorded, and glucose concentrations were estimated from the calibration curve. The difference between the values given by the commercial kit and those obtained with our sensor reveal that it is likely the result of interference from nonglucose components in the beverages. Difficulties in accurate measurement of glucose concentration can arise from the interference from other compounds [112]. Pretreatements of samples or making use of a sensor array could improve this analysis. Since the commercial methods have several drawbacks like lack of selectivity, long analysis time, high cost, and requirement of pretreatment of the samples, our sensors can be suitable for glucose determination in real-world samples after some improvements. Hence, the chemiresistive glucose sensor offers several advantages over traditional methods, and also improvements are the subject of ongoing research with specific attention to the design of new surface components.

Table 5.1 Determination of glucose in beverage samples.

Glucose (mol/L) **Sample**	**Spectrophotometric Assay**	**Chemiresistive Sensor**	**% Error**
S fruit punch juice	0.204	0.231	13.23
L ice tea	0.130	0.125	-3.84

5.4 Conclusion

In conclusion, we have fabricated a promising chemiresistive glucose sensor using a P4VP-SWCNT derived sensing scaffold to achieve effective attachment of biomolecules. Surface immobilized P4VP-SWCNT composites were functionalized with 2-bromoethanol to generate hydrophilic and positively charged surface to increase the compatibility to GOx. The quaternized P4VP-SWCNT scaffold not only provides a prolonged stability and an enhanced immobilization, but also allows an excellent communication between the biomolecule and the electroactive layer thereby improving the biocompatibility of enzyme molecules. The designed system is a rare example of an enzymatic-based chemiresistive sensor system and enables rapid responses for real-time glucose sensing for inline processes. The development of a low-cost, fast, and robust glucose sensor will likely find utility in selected applications.

CHAPTER 6

CONCLUSIONS AND OUTLOOK

6.1 Conclusions

Considering the explanations described in this thesis, conjugated polymer based amperometric biosensors and SWCNT based chemiresistive sensors have attractive merits in material science and chemistry and have main importance for detection of various analytes in any test solution. For development of an excellent sensor system, appropriate support material should be provided. Three chapters of this thesis are devoted to the use of conjugated polymers for the fabrication of electrochemical biosensors that allow the achievement of enhanced biosensor performance with respect to other designs. Moreover, incorporation of conjugated polymers with other structures like carbon nanotubes and peptides scaffolds exhibited superior properties for the fabrication of a biosensor. Using such a system common important analytes (ethanol, glucose and cholesterol) were detected in beverages and human blood samples. In addition to this, using chemiresistive sensor with novel architecture enables rapid responses for real-time glucose sensing for inline processes. The development of a low-cost, fast, and robust glucose sensor will likely find utility in selected applications.

In Chapter 1, introductory information, importance of conjugated polymers in sensor applications and current status of conjugated polymer based amperometric biosensors were mentioned. Additionally, chemiresistive sensors and the role of nanowires (NWs), specifically carbon nanotubes (CNTs), as the basis for chemiresistor research were also explained. Then, it was summarized different device platform architectures with selected examples from the literature about conjugated polymer based amperometric biosensors and SWCNT based chemiresistive sensors.

In the second chapter, it is aimed to design and characterize novel surfaces based on conjugated polymers in order to use in the design of biosensing devices. Firstly, a novel functional monomer, 2-(4-nitrophenyl)-4,7-di(thiophen-2-yl)-1*H*-benzo[d]imidazole (BIPN), was used for matrix generation through electrochemical polymerization. Its conducting polymer was successfully used for the biolayer construction in the biosensor preparation. The electrochemical and morphological properties were improved by the introduction of carboxylic acid functionalized multiwall carbon nanotubes (f-MWCNTs). Carboxylic acid functionalization of MWCNTs was carried out via acid treatment. The electrode surface was modified with the polymer and f-MWCNTs during electropolymerization to achieve a perfect immobilization matrix for alcohol oxidase. In order to prepare a new alcohol biosensor, alcohol oxidase (AOx) was immobilized onto the modified electrode. The modified electrode was characterized by scanning electron microscopy (SEM), X-ray photoelectron microscopy (XPS) and Fourier transform infrared (FTIR) spectroscopy techniques. Electrochemical responses of the enzyme electrodes were monitored at -0.7 V vs. Ag reference electrode by monitoring oxygen consumption in the presence of ethanol. Kinetic parameters, operational and storage stabilities were investigated. K_M^{app}, I_{max}, LOD and sensitivity were calculated as 16.946 mM, 3.31 μA, 0.806 mM and 476 μA mM^{-1} cm^{-2}, respectively. Finally, this biosensor was applied to estimate the alcohol content in various beverages successfully.

In the third chapter, a simple and efficient approach for the preparation of biosensing platform was developed based on newly designed peptide-SNS type monomer conjugates. The approach involves the electrochemical polymerization of the peptide-SNS type monomer on the electrode surface. Utilization of peptides to increase the solubility of the monomers was first investigated with this thesis. The obtained monomers, soluble in water, were fully characterized by spectral analyses and utilized as matrices for biomolecule attachment. Polymerization of monomers in water has the potential to provide an alternative process for the electrochemical preparation of the polymers in aqueous medium, without using any organic solvent.

Under the optimized conditions, the biosensor responded to the target analyte; glucose, with a strikingly selective and sensitive manner, and showed promising feasibility for the quantitative analysis of glucose in beverages. K_M^{app}, LOD and sensitivity were calculated as 0.208 mM, 4.69 μM and 91.37 μA/mM cm^2, respectively. Although glucose sensors have been widely reported in the literature, water soluble peptide-conjugated monomer, which showed better biosensor performance when compared to other reported studies, was design for the first time.

In the fourth chapter, 2-heptyl-4,7-di(thiophen-2-yl)-1*H*-benzo[d]imidazole) (BImTh) was used to detect cholesterol in human serum samples. For this reason, electrochemical copolymerization of this monomer with 2-(((9H-fluoren-9-yl)methoxy)carbonylamino)acetic acid (Fmoc-Gly-OH) was achieved on a graphite electrode and used as a matrix for amperometric cholesterol biosensing studies. In order to prepare a new cholesterol biosensor, cholesterol oxidase (ChOx) was covalently immobilized onto the copolymer coated graphite electrode. Cholesterol was used as the substrate and the decrease in oxygen level as a result of enzymatic reaction was monitored at −0.7 V vs Ag reference electrode in a phosphate buffer (50 mM, pH 7.0). Kinetic parameters, storage stabilities and surface characteristics were investigated. K_M^{app}, I_{max}, LOD and sensitivity were calculated as 6.25 μM, 9.69 μA, 0.17 μM and 2.47 mA/mM cm^2, respectively. This biosensor was applied to the determination of total cholesterol in serum samples.

In the fifth chapter, a promising chemiresistive glucose sensor using a P4VP-SWCNT derived sensing scaffold have been fabricated to achieve effective attachment of biomolecules. Surface immobilized P4VP-SWCNT composites are functionalized with 2-bromoethanol to generate hydrophilic and positively charged surface to increase the compatibility to GOx. The quaternized P4VP-SWCNT scaffold not only provides a prolonged stability and an enhanced immobilization, but also allows an excellent communication between the biomolecule and the electroactive layer thereby improving the biocompatibility of enzyme molecules. The modified surfaces were investigated using different techniques such scanning electron microscopy (SEM),

Fourier transform infrared (FTIR) and Raman Spectroscopy. In addition to this, the surface hydrophilicity was investigated using contact angle measurements before and after quaternization reaction. Finally, this sensor was applied to estimate the glucose content in beverages successfully.

6.2 Outlook

Nowadays, it is such an interesting subject that reliable, fast and high sensitive methods for the determination of cholesterol, glucose and ethanol have progressed for research to prevent several important diseases. Diabetes mellitus (DM) is a serious metabolic disease, which is related to the abnormal accumulation of glucose concentration in the blood. The worldwide prevalence of diabetes has continuously increased although there has been considerable progress in modern medical science and technology. The estimation of cholesterol is also an important for the diagnosis and prevention of several heart diseases and arteriosclerosis. Moreover, estimation of ethanol with high sensitivity, selectivity and accuracy plays an important role in the quality control of alcoholic beverages as well as clinical analysis. Thus, to reduce and prevent costly treatment of these diseases, the monitoring of glucose, cholesterol and ethanol level is required. As a result, new methods for detection of them and the monitoring of their accumulation level continues to be of interest.

Since the commercial methods have several drawbacks like lack of selectivity, long analysis time, high cost, and requirement of pretreatment of the samples, it was believed that created alternative matrices in this thesis to obtain better sensing devices may become a complementary method to conventional chromatographic sensing techniques for detection and quantification of target analytes in our daily life in the near future. The combination of carbon nanotubes and peptides with conjugates polymers or copolymerization method to improve the analytical characteristics of the biosensors has the potential for use in the construction of new biological macromolecular architectures. In this thesis, the polymers used in amperometric biosensor construction are designed for the first time and used in sensor experiments.

Especially, polymerization of monomers in water has the potential to provide an alternative process for the electrochemical preparation of the polymers in aqueous medium, without using any organic solvent. That not only provides easy production of polymers via green chemistry but also relatively lowers the electrodeposition potentials of the polymers. Additionally, short peptide sequence allowed easy synthesis in small quantities, making the scale up of the production of the device economically affordable. The design makes the biosensor an ideal candidate to develop a cheap device.

Moreover, designing a chemiresistive sensor is very important since it can be readily integrated into portable and low-cost devices that have been widely investigated for various applications to detect different types of the analytes. In addition, they have low power requirements. With the insight of this knowledge, the chemiresistive sensor proposed in this thesis was used to analyze glucose content in a real sample and was proposed as an alternative sensing system for glucose analysis in beverages. Since an enzymatic-based chemiresistive sensor system is a rare example in literature, the design and development of a rapid responses sensors for real-time glucose sensing for inline processes, a low-cost and robust glucose sensor are major theme in sensor development.

We believe that this thesis will put an important insight for detecting some important analytes with highly sensitive, selective and reliable sensor systems using newly desogned architectures.

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