DEVELOPMENT OF A NEW ETHANOL BIOSENSOR BASED ON POLYFLUORENE- g- POLY(ETHYLENEGLYCOL) AND MULTIWALLED CARBON NANOTUBES

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ABSTRACT

DEVELOPMENT OF A NEW ETHANOL BIOSENSOR BASED ON POLYFLUORENE- g- POLY(ETHYLENEGLYCOL) AND MULTIWALLED CARBON NANOTUBES

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In this thesis study, a novel amperometric biosensor containing a conjugated polymer and a multiwalled carbon nanotube was developed for ethanol detection. Conjugated polymers have gained interest in biosensing and biomedical applications recently. Their ability to increase the effective surface area and adjustable surface morphology make them convenient candidates for suitable enzyme immobilization. The conjugated polymer poly(ethylene glycol) is soluble in both water and organic solvents. Besides, it interacts with the enzymes readily due to its hydrophilic nature. In this thesis, poly(ethylene glycol) with fluorene functionality was synthesized via a one-step procedure. The construction of biosensor was achieved through the modification of graphite electrode with synthesized conjugated polymer in addition to carbon nanotubes. Carbon nanotubes were used as supporting materials due to their fast electron transfer and electrochemical stability. Surface characterization and electrochemical characterization of the biosensor was performed using scanning electron microscopy and cyclic voltammetry, respectively. Combination of

conjugated polymer and multiwalled carbon nanotubes improved the performance of biosensor and showed high sensing ability compare to similar control biosensor (without PF-g-PEG or MWCNT). Alcohol oxidase enzyme (AOx) was immobilized on the modified electrode surface and several parameters effecting the performance of modified biosensor were optimized. The optimized biosensor exhibited good analytical parameters such as low limit of detection and high sensitivity. Linear range of proposed biosensor was between 0.0085-5.95 mM with detection limit of 0.11 mM and sensitivity value of 7.99 $\mu AmM^{-1}cm^{-2}$. Finally, to show the applicability of proposed biosensor, the biosensor was tested for different alcoholic beverages for ethanol analysis.

Keywords: Amperometric ethanol biosensor, Conjugated polymers, Alcohol oxidase, Carbon nanotubes, Polyfluorene

ÖZ

POLİFLUORENE-g-POLİ(ETİLENGLİKOL) VE ÇOK DUVARLI KARBON NANOTÜP YAPISINA DAYALI YENİ ETANOL BİYOSENSENSÖR GELİŞTİRİLMESİ

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Bu tez çalışmasında etanol tespiti için konjuge polimer ve çok duvarlı karbon nanotüp içeren yeni amperometric biyosensör geliştirilmiştir. Konjuge polimerler, biyoalgılama ve biyomedikal mühendisliğinde ilgi görmektedirler. Etkin yüzey alanını arttırma yetenekleri ve ayarlanabilir yüzey yapıları onları enzim immobilizasyonu için uygun aday haline getirir. Konjuge polimer, poli(etilen glikol) hem suda hem de organik çözücülerde çözünür, Ayrıca hidrofilik yapısı sayesinde enzim molekülleri ile kolaylıkla etkileşime girer. Bu çalışmada, floren fonksiyonel gurubuna sahip poli(etilen glikol) tek aşamalı bir prosedürle sentezlenmiştir. Biyosensör tasarımı, grafit elektrotun karbon nanotüplere ek olarak konjuge polimer ile modifikasyonu sayesinde sağlandı. Hızlı elektron transferi ve elektrokimyasal kararlılık sağlama özelliklerinden dolayı karbon nanotüp modifikasyon materyali olarak kullanılmıştır. Buna ek olarak, yüzey karakterizasyonu taramalı elektron mikroskobu (SEM) kullanılarak, elektrokimyasal karakterizasyon ise döngüsel voltametre (CV) tekniği ile incelenmiştir. Bu çalışma sayesinde, konjuge polimer ve çok duvarlı karbon nanotüp kombinasyonun, benzer cihazlarla (PF-g-PEG veya MWCNT içermeyen) kıyaslandığında biyosensör performansını arıttırdığı kanıtlandı. Alkol oksidaz enzimi (AOx) modifiye edilmiş elektrot yüzeyine

immobilize edildi. Çalışmanın devamında, modifiye edilmiş biyosensörün performansını etkileyen değişkenler optimize edildi. Optimize edilen biyosensör düşük tespit sınırı ve yüksek hassasiyet gibi çok iyi analitik parametreler göstermiştir. Elde edilen biyosensör 0.0085- 5.95 mM arasında çalışma aralığına, 0.11 mM algılama sınırına ve 7.99 $\mu AmM^{-1}cm^{-2}$ duyarlılık değerine sahiptir**.** Son olarak, hazırlanan biyosensörün uygulanabilirliğini göstermek için alkollü içecekler etanol analizi için test edilmiştir.

Anahtar Kelimeler: Amperometrik etanol biyosensör, Konjuge polimerler, Alkol oksidaz, Karbon nanotüp

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ABBREVIATIONS

ACN	Acetonitrile
Ag	Silver
AOx	Alcohol Oxidase
CE	Counter Electrode
CP	Conjugated Polymer
CV	Cyclic Voltammetry
DCM	Dichloromethane
FAD	Flavin adenine dinucleotide
GA	Glutaraldehyde
LOD	Limit of Detection
MWCNT	Multi-walled Carbon Nanotubes
NTI	Narrow therapeutic index
PBS	Phosphate buffer solution
Pt	Platinum
RE	Reference Electrode
RSD	Relative Standard Deviation
SEM	Scanning Electron Microscopy
SD	Standard Deviation

CHAPTER 1

INTRODUCTION

1.1 Biosensors

There are several definitions for a biosensor from past to present in literature. Based on one common definition, a biosensor is defined as an analytical device that transforms a chemical reaction to useful analytical signal. [1] According to another definition, a biosensor combines a bioactive sensing layer with a suitable transducer to give appropriate signal. [2]

History of biosensor goes back to 1955 to oxygen electrode developed by L.C. Clark to detect glucose in blood. [3] L. C. Clark is considered as 'the father of the biosensors' due to his invention. Later, a similar system was developed by G. P Hicks and S. J. Updike using glucose oxidase enzyme in 1967. [4] Before that G. Guilbault was demonstrated the first analytical application of immobilized cholinesterase biosensor in 1962. [5] Since that day, biosensors continue to gain importance and continue to be used in many different areas.

A typical biosensor consists of two main components as shown in Figure 1.1: a bioreceptor or a biorecognition element and a transducer. Biorecognition element recognizes analyte specifically. Interactions between the analyte and the biorecognition element give rise to signal production. Transducer transforms chemical process that takes place between the bioelement and the analyte into a measurable signal form. Signalization process can be electrical or thermal depending on the transducer type. [6]

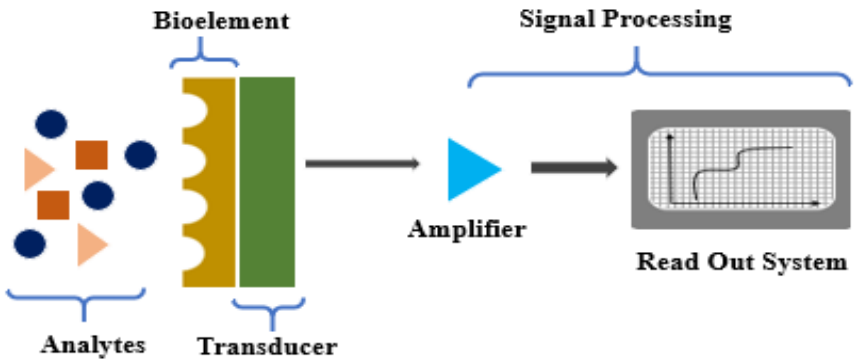

Figure 1.1. General representation of a biosensor

Since their development biosensors have found applications in various fields such as food quality and environmental monitoring, drug delivery, disease and toxin detection, medical field etc. They can be employed in food industry to maintain the safety and quality of products. They can be used in fermentation process to monitor and optimize the reactors. Apart from the food industries, biosensors can be applied in medical field; for instance glucose biosensors have an important place to control the glucose level in blood with high precision. They provide more accurate and cost-efficient results compared to other traditional techniques due to their fast response time, low cost, and selectivity. [7]

1.1.1 Biosensor Types

Biosensors can be classified based on their components: transducer and bioreceptor. The bioreceptor that recognizes analyte can be an enzyme, whole cell, antibody, nucleic acid, tissue, and microorganism.[8] Apart from the bioreceptor element, biosensors can also be classified based on the transducer, detecting element, as thermal, optical piezoelectric and electrochemical. [8] Figure 1.2 displays the types of biosensors based on the transducer and bioelement.

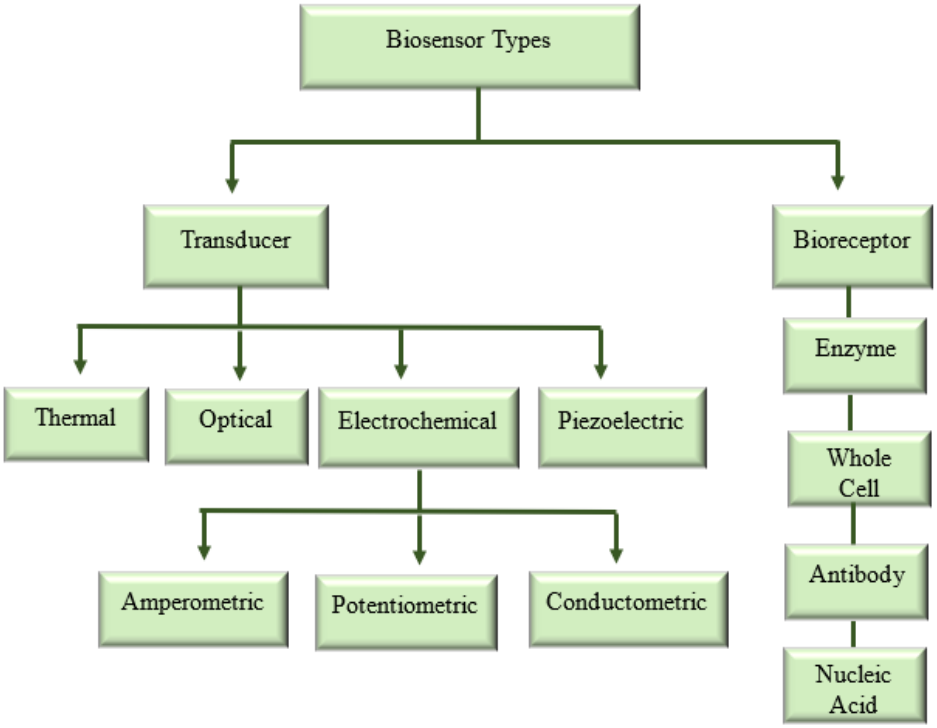

Figure 1.2. Types of biosensor based on the transducer and bioreceptor

Optical biosensors are devices that depend on changes in optical measurements such as fluorescence, chemiluminescence or absorbance. In this type of biosensors, the interaction between the optical field and biosensing element are measured. Generally, enzyme and antibodies are used as biorecognition elements in optical biosensors. Optical biosensors are examined in two categories. These are direct optical detection and labeled optical detection biosensors. An important advantage of these system is their high sensitivity. Also, another advantage of optical biosensor is that they do not need reference sensors since with similar light source, comparative signal can be generated. Optical biosensors are used to monitor risk of toxicity of . [9]

Thermal biosensors or calorimetric biosensors measure of the heat that is produced due to an enzymatic reaction. Main working principle of the thermal biosensors is to convert the heat produced from an enzymatic reaction into a readable electrical signal. This conversion process was performed by thermistors. Heat is measured with the help of insulated jacket in order to prevent the heat loss. In thermal biosensors, enzymes are used as biosensing elements and appropriate analytes are used depending on the enzyme. Although thermistors are cheap and have long term stability, the sensitivity of these devices is generally low. Another disadvantage of thermal biosensors is their bulky and the requirement of an insulated jacket. Thermal biosensors are generally used for the prediction of serum cholesterol. [10]

Piezoelectric biosensors are mass sensitive devices. Working principle of the piezoelectric biosensors is the measurement of the change in frequency during the combination of antigen and antibody. The frequency change is related to the change in mass. Piezoelectric biosensors have found application in wide range of industries such as medical, nuclear, and aerospace. For instance, they are used to detect hepatitis C virus detection DNA hybridization. [11]

1.1.1.1 Electrochemical Biosensors

In electrochemical biosensors, bioelement reacts with an analyte and an electrical signal is generated. Produced signal is proportional to the concentration of the target analyte. Electrochemical biosensors are preferred due to cost efficiency, portability, and accessibility. Besides the electrochemical techniques do not require sample pretreatment and achieve a very low limit of detection. Apart from these advantages, a small amount of analyte is enough for the analysis, to obtain stable and sensitive results.[12]

In electrochemical biosensors, two or three electrode systems are used. In two electrode system, working electrode and counter electrode are used. Current flow is generated between these two electrodes. To provide a reference potential, the three-

electrode system was developed. Reference electrode was used to control the potential in the system. Working electrode should be a conductive material that contains the biorecognition element. While platinum, gold and graphite can be used as working electrodes, Ag/AgCl is generally used as the reference electrode (RE).

Electrochemical biosensors are examined in two categories depending on the biological recognition process.

- Affinity sensors
- Biocatalytic devices

Affinity sensors are based on the interactions between the bioelement and analyte. Nucleic acids and antibodies are two of the bioelements used in the affinity sensors. Binding between the bioelement and analyte is highly specific and strong. Having higher affinity of for the analyte makes the sensor sensitive. [13]

Biocatalytic devices use whole cell, tissue, or enzyme due to their biocatalytic activity. Biocatalytic species can recognize the analyte and generate electroactive products. Especially, enzymes are preferred in this technique since they have high, specific biocatalytic activity towards to the analyte. Biocatalytic devices are inexpensive and, also easy to use. [13]

Reaction between the analyte and biorecognition element can be monitored via the change in current, potential, or conductivity. Depending on the monitored signal, biocatalytic devices are classified in three categories: Conductometric, potentiometric and amperometric.

Conductometric biosensors are used to detect alteration in electrical conductance or resistance in sample solution. Changing the field between two electrodes causes a change in conductivity. In conductometric biosensors, enzymes are used as bioelements since the charges on an enzyme can cause a change in conductivity. Although conductometric biosensors are portable and do not require reference electrode, they have relatively low sensitivity. Conductometric biosensors are generally used in pathogenic analysis and drug detection. [12]

Potentiometric biosensors rely on the measurement of the redox potential of the reaction cell. Main principle is the application of a ramp voltage to the reference electrode. Thus, current arises due to the electrochemical reaction and with the help of the generated current the change in potential is measured. Two-electrode system is used: reference electrode and indicator (working) electrode. Disadvantage of potentiometric biosensor is the need for frequent calibration. Potentiometric biosensors are used especially in ammonia and CO_2 detection. [14]

Amperometric biosensors are based on the measurement of change in current at constant potential during a certain period. Constant potential is applied between the counter and working electrode. This results in the occurrence of nonspontaneous oxidation reduction reactions. Generated current is directly related with the concentration of analyte in the sample. [15] Amperometric biosensors have high selectivity and sensitivity. Therefore, they gained great importance compared to other electrochemical biosensors. Having such simplicity makes them to be used in a wide area of applications. Amperometric biosensors can be used in both clinical and nonclinical analysis. Glucose, ethanol, and pathogen detection are some of clinical application fields of amperometric biosensors. Also, amperometric biosensors are used in fermentation quality control and lactate detection for environmental analysis.

1.1.2 Immobilization Methods

Nature of the matrix used in biosensor construction has great importance when deciding on the proper bioelement system. Biomolecule should be cost efficient, easily derivatized and environmentally stable. Biomolecule can have biocatalytic (enzyme, whole cell) or bioaffinity (antibody, nucleic acid) properties. However, enzymes are generally used as biorecognition elements for a biosensor system due to their biocompatibility. [16]

Enzyme immobilization as biorecognition element provides many advantages. One of the main advantages is to provide stability for the matrix. Also, enzymes decrease possibility of the formation of contamination and reaction time.

Enzyme immobilization methods can be classified in two main categories as shown in Figure 1.3:

- Reversible immobilization
- Irreversible immobilization

In reversible immobilization method, enzyme – matrix combination can be separated under mild condition. Reversible immobilization methods are highly advantageous due to their economic viability.[17] Enzyme can be reused without losing its activity. Physical adsorption is a reversible immobilization method.

In irreversible immobilization method, enzyme is bind to matrix and without destroying matrix, enzyme cannot be separated, and it cannot be reused. This is the basic difference between reversible and irreversible enzyme immobilization method. Covalent bonding, entrapment and crosslinking are the types of irreversible immobilization methods.

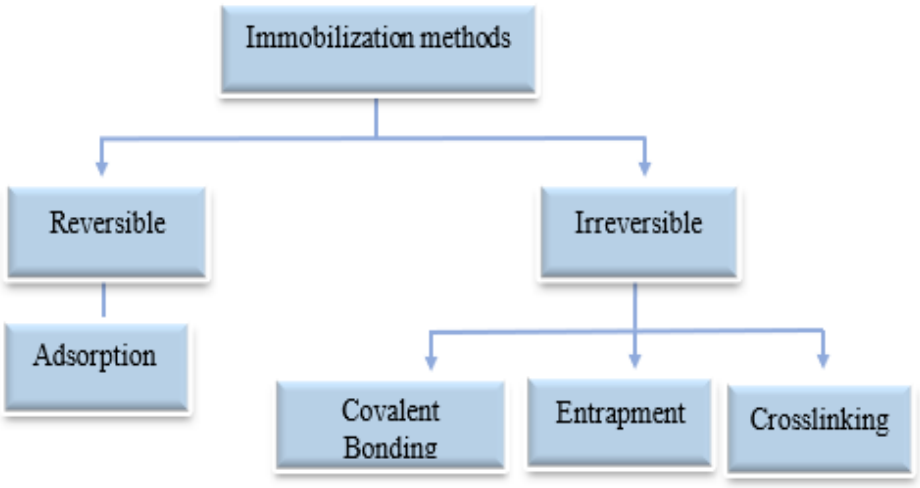

Figure 1.3. Types of enzyme immobilization methods

1.1.2.1 Physical Adsorption

Enzyme is adsorbed physically to the outer surface of the matrix in physical adsorption immobilization technique. (Figure 1.4) There is no formation of the covalent bond between enzyme and matrix. Bonding is provided through weaker interactions as hydrogen bonding and van der Waals interactions. Physical adsorption techniques allow the protection of the catalytic activity of the enzyme. Besides, in adsorption technique, conformational changes in enzyme structure do not occur. Apart from the advantages of adsorption technique, enzyme linkage is one of the problems that might be encountered physical adsorption technique. [18]

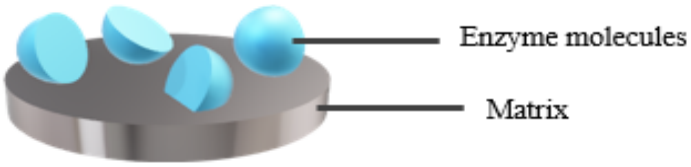

Figure 1.4. Representation of Physical Adsorption Method

1.1.2.2 Entrapment

In this type of immobilization, enzyme is trapped inside the matrix which has a porous structure. (Figure 1.5) An appropriate matrix can be formed from gels, conducting polymers, agar, or fiber. To prevent the leakage of the enzyme, pore size of the support can be adjusted by changing the concentration of the bioelement. The bonding between the enzyme and the matrix can be covalent or noncovalent. Although the entrapment method is widely applicable for enzymes, there is possibility of the denaturation of the enzyme. [18]

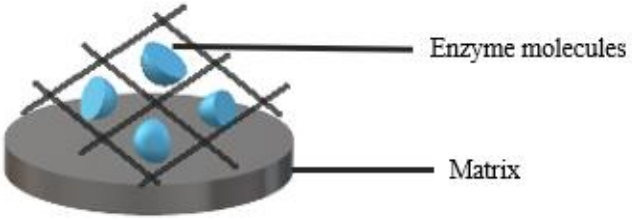

Figure 1.5. Representation of Entrapment Method

1.1.2.3 Crosslinking

Croslinking immobilization method creates intermolecular crosslinking between the enzymes with the help of polyfunctional reagents as represented in Figure 1.6. This polyfunctional reagents will crosslink the enzyme molecules via covalent bonding. The most important advantage of this method is the provided biocatalyst stabilization. Diazonium salts and glutaraldehyde are commonly used as crosslinking agents. [19] Although crosslinking provides strength, reaction is usually difficult to control.

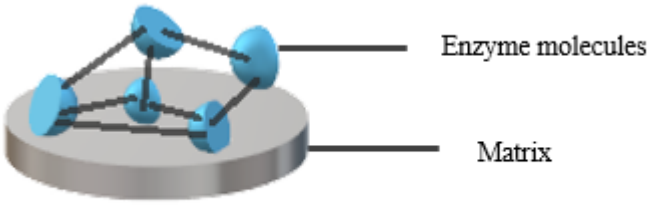

Figure 1.6. Representation of Crosslinking Method

1.1.2.4 Covalent Bonding

Chemical bonding immobilization occurs between the matrix and a functional group of the enzyme such as carboxyl, amino or sulfhydryl group etc. as represented in Figure 1.7. [18] Covalent bonding provides strong and stable enzyme matrix combination and enzyme leakage does not occur. However after some time, enzyme can lose its biological activity. Also, supporting matrix and the enzyme cannot be reused. [20]

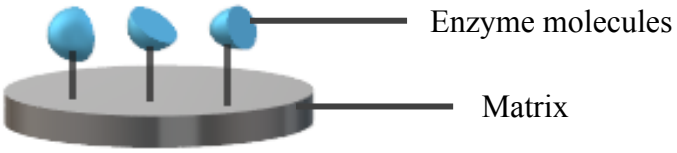

Figure 1.7. Representation of Covalent Bonding Method

1.1.3 Ethanol Biosensors

Ethanol detection plays an important role in food industries and clinical analysis. Illegal alcoholic beverage consumption can cause serious health problems. According to World Health Organization, three million people died because of alcohol abuse in 2006. Yet, the total unrecorded alcohol consumption is through to be around 26 % in the world. [21] Apart from this issue, alcohol detection in blood or urine in clinical laboratories is also important to prevent intoxication and overdose. [22] Hence accurate and sensitive alcohol detection is curial. To detect alcohol, there are several analytical techniques such as high-performance liquid chromatography, mass spectrometry, gas chromatography etc. However, these techniques require costly instruments and, they require longer analysis time. Therefore, these techniques are not useful for practical and cost-efficient ethanol detection. [23] Electrochemical biosensors are also used to detect ethanol in a sample. Electrochemical biosensors provide many advantages. These biosensors have high analytical performance and are cost efficient. Electrochemical biosensor for ethanol sensing can be constructed using enzyme-based sensors. Generally, two types of enzymes are used to detect ethanol: alcohol oxidase (AOx) and alcohol dehydrogenase (ADH). There are several research related with alcohol oxidase based electrochemical biosensors for ethanol detection using various biosensing architecture. Alhadeff et al. developed electrochemical biosensor creating silver nanoparticles/ polyaniline/ graphite/ epoxy composites. Alcohol oxidase (AOx) and horseradish peroxidase (HRP) were immobilized onto modified surface with adsorption. [24] In another work, Barsan et al. fabricated amperometric ethanol biosensor using poly(neutral) red (PNR) on carbon film electrode. Alcohol oxidase and glutaraldehyde as crosslinking were immobilized on coated surface. [25]

1.1.3.1 Alcohol Oxidase

Alcohol oxidase (AOx) has oligomeric structure with eight identical subunits. These subunits have quasicubic arrangements and each subunit have covalently bound cofactor, flavin adenine dinucleotide (FAD). Alcohol oxidases are obtained from methylotrophic yeasts which are known as peroxisomes. [32] Alcohol oxidases play significant role in alcohol detection because they are rapid and efficient catalysts for oxidation.

Main role of alcohol oxidase enzyme is to oxidize alcohol (low molecular weight) to the corresponding aldehyde. During the reaction, alcohol oxidases use molecular oxygen (O_2) as electron acceptor. Reaction takes place as following:

Since molecular oxygen is a strong oxidizing agent, alcohol oxidation is an irreversible enzymatic reaction. During the oxidation reaction the cofactor, FAD, is reduced to $FADH_2$. [27] Later on $FADH_2$ can be oxidized back to FAD. Molecular oxygen is converted to hydrogen peroxide, H_2O_2 as shown in Figure 1.8.

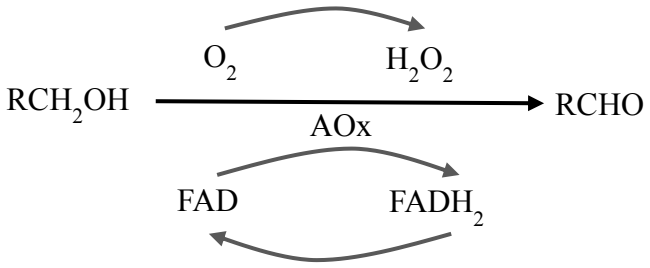

Figure 1.8. Representation of the reaction mechanism of alcohol oxidase

1.2 Conjugated Polymers

After the discovery of polyacetylene via Ziegler Natta catalyzed polymerization by Hideki Shirakawa, Alan MacDiarmid, and Alan J. Heeger, polymer and conductivity terms were begun to be used together. This discovery did not only bring Nobel Prize to these three scientists in 2000, but also became a milestone for many scientists in search for a new field. [28] Some common conjugated polymers are given in Figure 1.9. Since conjugated polymers contain π bonding, that causes electron delocalization along the polymer chain. And charge mobility along the polymer chain provides various electronic properties such as conductivity. Besides doping process can be defined as charge transfer from π-system or to π system. This doping process can increase conductivity of conjugated polymers.

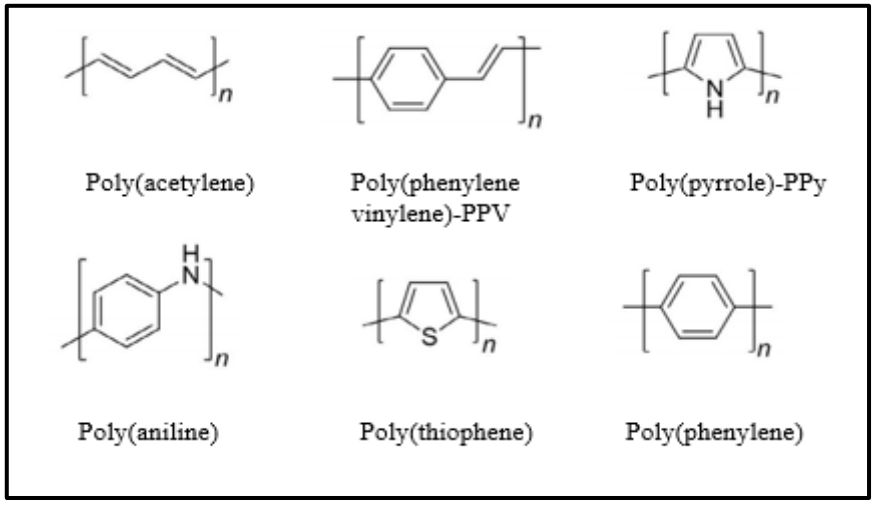

Figure 1.9. Some common conjugated polymers

Conjugated polymers can be obtained in both electrochemical and chemical methods. Chemical synthesis of conjugated polymers is performed in presence of various condition such as pressure, heating, catalyst, or light. Although yield of chemical synthesis of conjugated polymers is high, impurities can occur in product. Electrochemical polymerization is based on redox reaction by applying voltage.

Main advantage of electrochemical polymerization is to control of the film thickness and the morphology.

Conjugated polymers attracted scientists' attention and opened many research areas due to their superior properties. They have thermal stability and low-cost fabrication. They also have unique mechanical and optical properties. Apart from these advantages, their conductivity, biocompatibility and processability enable their use in various areas such as electrochromic devices, biosensor application, drug delivery, and light emitting diodes.[28]

1.2.1 Conjugated Polymers (CP) in Biosensor Design

Biosensor application is one the most common area where conjugated polymers (CPs) are used due their specific properties. Their extended π- orbital system allows electrons to move along the polymer chain. They have structures that can be easily modified for biosensors applications. In biosensor studies, stability is one the most desired the properties and conjugated polymers have excellent environmental and thermal stability. [29]

Another important advantage of CPs is that they are biocompatible. Their modified structures enable the simple and straightforward binding of the enzyme or other biomolecules in biosensor design. Also, their adjustable film thickness and controllable charge distribution makes CPs advantageous in preparation of suitable immobilized biomolecule. [29]

1.3 Carbon Nanotubes in Biosensor Design

Carbon nanotubes (CNTs), which are one dimensional allotropes of carbon, are cylindrical graphenes. They can be synthesized with various methods such as laser ablation, chemical vapor deposition and arc-discharge. There are two types of carbon nanotubes: single walled carbon nanotubes (SWCNTs) and multiwalled carbon

nanotubes (MWCNTs). While SWCNTs contain single layer of graphene sheet, MWCNTs contain two or more graphene sheets surrounding each other. (Figure 1.10 and Figure 1.11)

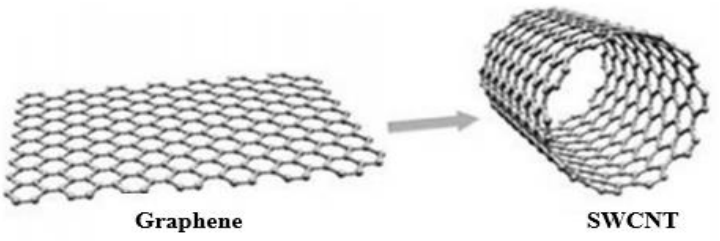

Figure 1.10. Single-walled carbon nanotube (SWCNT)[30]

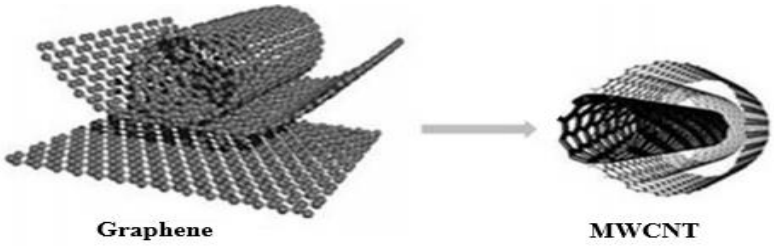

Figure 1.11. Multi-walled carbon nanotube (MWCNT)[30]

CNTs have unique properties and are used in various applications such as energy storage, biomedical application, and electronic application. Apart from other applications, CNTs have excellent features for sensor design. Carbon nanotubes have excellent conductivity properties, and this increase the effective surface area. Also, CNTs are biocompatible material. Their ability to be functionalized provide ease of use in biosensor applications. Carbon nanotube-based biosensors show high sensitivity and fast response. Besides having a low cost, ease of operation makes CNTs one of the greatest alternative materials in biosensor applications. [31]

1.4 Aim of Thesis

Aim of this thesis is to construct a novel biosensor design based on conjugated polymer and multiwalled carbon nanotubes for ethanol detection. For that purpose, a bare graphite electrode modified with PF-g-PEG and MWCNTs was used. Alcohol oxidase enzyme was immobilized on the modified surface. MWCNTs are deposited on a bare surface by drop casting. After that, monomer was electrochemically polymerized on MWCNT coated electrode. Later, alcohol oxidase enzyme was immobilized via physical adsorption technique using glutaraldehyde. Conjugated polymer was chosen due to its electroactive and hydrophilic nature. It provided suitable enzyme immobilization. MWCNT was used as the supporting materials to enhance the conductivity and charge transfer. Biosensor response was achieved with amperometric detection at -0.7 V potential. To improve the biosensor's performance, optimization studies were carried out. After finding the optimum values, calibration curve was constructed, and analytical parameters were determined. To examine morphological changes on modified surface, scanning electron microscopy technique was used. To test reliability of the proposed sensor, real sample applicability of developed sensor was investigated.

CHAPTER 2

PF-g-PEG/MWCNT/AOx BIOSENSOR

2.1 Experimental Studies

2.1.1 Materials

Alcohol oxidase (AOx, E.C. 1.1.3.13) (35 Units/mg protein) from Pichia pastoris, glutaraldehyde (%50 wt. in H_2O), multi- walled carbon nanotube (carbon > 95 %, O.D. x L 6-9 nm x 5 μm) and boron trifluoride diethyl etherate ($BF_3.O(C_2H_5)_2$) were obtained from Sigma-Aldrich. Dichloromethane (DCM), acetonitrile (ACN), ethanol (as a substrate), methanol and 2-propanol were purchased from Merck (Darmstadt, Germany). Poly(ethylene glycol) monomethyl ether ((PEG) Mn~2,000, Aldrich) N, N'-dicyclohexylcarbodiimide (DCC, 99%, Sigma-Aldrich) and 4-dimethyl-aminopyridine (DMAP, 99%, Sigma-Aldrich) were used as received. All chemicals were of analytical reagent grade. [32]

2.1.2 Instrumentation

Electropolymerization was achieved with GAMRY Reference 600 (GAMRY Instruments Inc., Pennsylvania, USA). A PalmSens potentiostat (Palm Instruments, Houten, The Netherlands) was used for amperometric measurements. Both electrochemical and amperometric experiments were carried out with a conventional three-electrode system with the enzyme electrode as the working electrode, a platinum wire as the counter electrode and a silver wire as the pseudo reference electrode. As a bare transducer surface a graphite electrode (3.0 mm diameter) was

used as the working electrode, platinum electrode and silver wire were used as the counter and reference electrodes, respectively. Three repeatable signals were used to evaluate amperometric measurements and reported as ±SD. For surface morphology characterization of the biosensor, scanning electron microscope (SEM) (JEOL JSM–6400 model) was used. ^{1}H NMR spectra were recorded using an Agilent VNMRS 500 MHz (Santa Clara, CA, USA) and chemical shifts were recorded in ppm using tetramethyl silane as the internal standard. All measurements were performed at room temperature. [32]

2.1.3 Synthesis of Monomer (Fluorene functional poly(ethylene glycol))

The monomer, fluorene functionalized poly(ethylene glycol) was synthesized by Prof. Dr. Yusuf Yağcı and his group from İstanbul Technical University.

In a round bottom flask equipped with a magnetic stirrer, fluorene-9-carboxylic acid (2.5 eq), PEG (Mn: 2000 g/mol, 1.0 eq) and DMAP (1.0 eq) was added and dissolved in CH_2Cl_2. Next, N, N'-dicyclohexylcarbodiimide (1 eq) dissolved in CH_2Cl_2 was slowly added to the mixture. The reaction mixture was stirred for 24 hours. The precipitate was filtered off and the residue was twice precipitated in cold diethyl ether. The precipitate was filtered off and the residue was twice precipitated into cold diethyl ether to give the yellowish product. Yield was found to be 79.8% determined by ^{1}H-NMR analysis by comparing the aromatic protons of the fluorene group with the protons of the PEG chain. Pathway of synthesis is shown in Figure 2.1. [32]

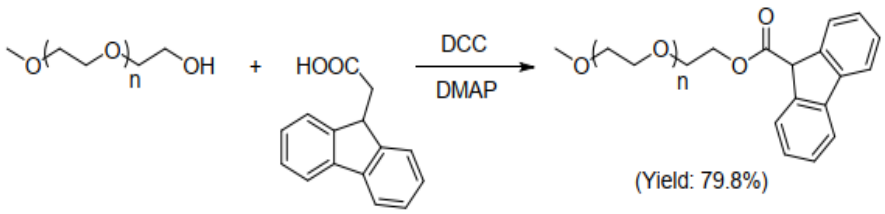

Figure 2.1. Synthesis of the fluorene functionalized poly(ethylene glycol) (PEG-FL) monomer

2.1.4 Electropolymerization of PEG-FL

Electrochemical polymerization was performed on MWCNTs coated electrode surface using cyclic voltammetry technique. PEG-FL macromonomer (5 mg) was dissolved in 1 mL of ACN: DCM (95:5) in 0.1 M $NaClO_4$/$LiClO_4$/BFEE electrolyte-solvent system by scanning between 0.0 V and + 1.8 V potential as shown is Figure 2.2. Scan rate is 100 $mV.s^{-1}$. Figure shows that increasing current density proves polymerization of monomer successfully on carbon nanotube coated graphite electrode. Electropolymerization was performed in a smaller cell (2 mL). After electropolymerization was completed, electrode was washed with distilled water to get rid of impurities. [32]

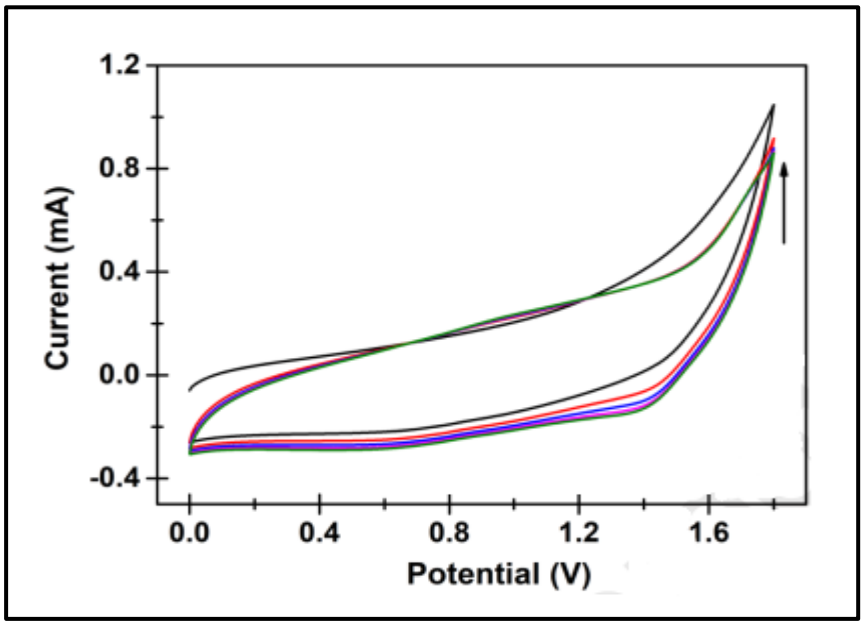

Figure 2.2. Cyclic voltammogram of PEG-FL on graphite electrode (up to 5 cycles)

2.1.5 Biosensor Fabrication

Before experiments graphite electrodes were polished with emery paper, then rinsed thoroughly with distilled water and dried. For the preparation of a MWCNTs/PF-g-PEG/AOx electrode, first 0.25 mg of MWCNT was dispersed in 5.0 mL of DMF by ultrasonication for 1 hour. Then 10 µL of the corresponding solution was cast on the cleaned graphite electrode surface. The carbon nanotube modified electrode was dried at room temperature for 1 hour. MWCNT modified electrode was made ready for electropolymerization process. PEG-FL monomer (5 mg/mL) was polymerized in the presence of ACN: DCM (95:5) in 0.1 M NaClO4/LiClO4 containing borontrifluoride diethyl etherate (BFEE) by scanning between 0.0 V and + 1.8 V (scan rate: 100 mV/s). [32] After electropolymerization, polymer modified MWCNT coated electrode was rinsed with distilled water to get rid of impurities. For enzyme immobilization step, 4.0 µL of AOx solution and glutaraldehyde (GA) (4.0 µL, %1 in H_2O) was immobilized on the modified electrode surface to obtain final biosensor for ethanol sensing. The prepared electrode was left to dry for 2 hours at room temperature. Before using it, the electrodes were washed with distilled water to get rid of unbound enzyme molecules. [32] In Figure 2.3, procedure of modified biosensor was represented schematically.

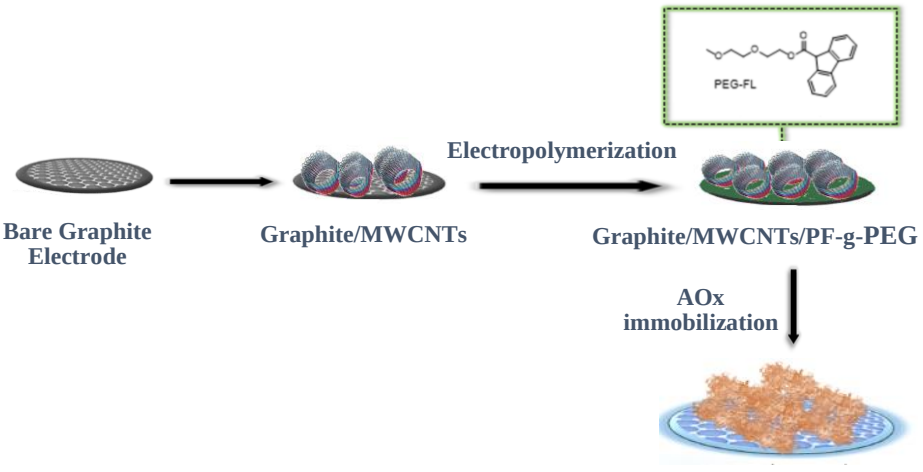

Figure 2.3. Representation of MWCNTs/ PF-g- PEG/AOx biosensor

2.1.6 Amperometric Measurements

Amperometric measurements were performed in a reaction cell containing 10 mL PBS solution (50 mM). Three electrodes were inserted into the reaction cell that are connected to potentiostat and response of the modified biosensor was monitored by applying a constant potential (at –0.7 V). Response signal is based on oxygen consumption from oxidation of short chain alcohol by alcohol oxidase enzyme. [33] After each measurement, electrodes were washed with distilled water and the buffer solution was refreshed for new measurements. (Figure 2.4)

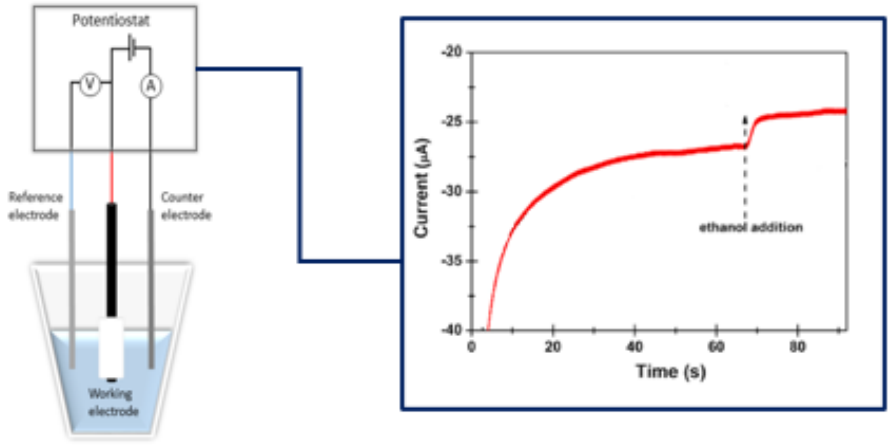

Figure 2.4. Representation of amperometric measurement

2.1.7 Optimization Studies of the Constructed Biosensor

To develop a stable and sensitive biosensor, parameters that affect the performance of MWCNTs/PF-g- PEG/AOx sensor should be optimized. Therefore, response of the modified sensor was investigated using different cycle number, amount of enzyme, and pH.

2.1.8 Characterization

2.1.8.1 Analytical Characterization of Proposed Biosensor

After optimization studies were performed, analytical parameters were examined. Firstly, calibration curve towards ethanol was constructed and analytical parameters such as limit of detection (LOD), dynamic range and sensitivity were determined.,

To test the precision of the proposed biosensor, 10 sequential measurements were recorded. Standard deviation and relative standard deviation (RSD) were calculated accordingly.

2.1.8.2 Electrochemical and Surface Characterization of Biosensor

Cyclic voltammetry was carried out in 5.0 mM $Fe(CN)_6^{3-/4-}$ solution containing 0.1 M KCl to evaluate the electroactive surface area. Cyclic voltammograms were obtained at a scan rate of 100 mV/s. [32] The average value of effective surface area of the modified sensor was determined according to Randles-Sevcik equation. [34]:

$$Ip = (2.69 \times 10^5)\, n^{3/2}\, ACD^{1/2}\, \nu^{1/2}$$

In equation, Ip is peak current and n is number of electrons in redox reaction. While A (cm^2) is area of electrode and D (cm^2s^{-1}) is diffusion coefficient in solution. C ($mol.cm^{-3}$) is concentration of molecule in solution and lastly ν ($V.s^{-1}$) is the scan rate.

Besides, morphological characterization of different electrodes was investigated using scanning electron microscopy (SEM). Scanning electron microscopy images of polymer coated surface, multiwalled carbon nanotube/polymer coated surface and carbon nanotube/polymer/enzyme coated surface were recorded.

2.1.9 Sample Application

Proposed biosensor was tested for commercial beverages that contain ethanol to investigate accuracy and the precision. According to linear range of modified biosensor, ethanol contents of rum, raki and vodka were scaled down by dilution. Samples were added into the cell and the amperometric signal was recorded for each sample. The concentration obtained through the constructed biosensor was compared to the concentration of ethanol given in the product labels.

2.2 Results and Discussion

2.2.1 Optimization Studies of Biosensor Parameters

All parameters that are thought to be affecting the performance of the biosensor were optimized in MWCNT/PF-g-PEG/AOx system.

2.2.1.1 Optimization of Multiwalled Carbon Nanotube Amount

Amount of carbon nanotubes used in the fabricated system was optimized by preparing different concentration of carbon nanotube solution. Different amounts of multiwalled carbon nanotubes solutions (0.10 mg, 0.25 mg, 0.50 mg, and 0.75 mg) were prepared in 5.0 mL DMF. 10 μL of these solutions were cast to four different electrodes by keeping all the other parameters constant. The most stable and highest signal was achieved in solution of 0.25 mg/5 mL DMF.

2.2.1.2 Optimization of Polymer Film Thickness

Effect of polymer thickness was investigated in terms of varying the number of cycles used during polymerizations on multiwalled carbon nanotube coated electrode surface. PF-g-PEG monomer was electropolymerized with different cycle numbers

(15, 20, 30 and 40) onto MWCNT coated electrodes. The average currents obtained different number of cycles reported in Figure 2.5. As can be seen, the best signal was achieved with 30 scans. Optimization of polymer film thickness is very important in such sensing systems because polymer thickness directly affects the binding of enzyme to the electrode surface. While a very thick polymeric film may not allow the binding of enzyme on the electrode surface, a very thin polymeric film may not provide sufficient electron transfer between the electrode and enzyme. Hence, to create appropriate surface area for enzyme immobilization, the polymer matrix thickness was fixed to 30 cycles.

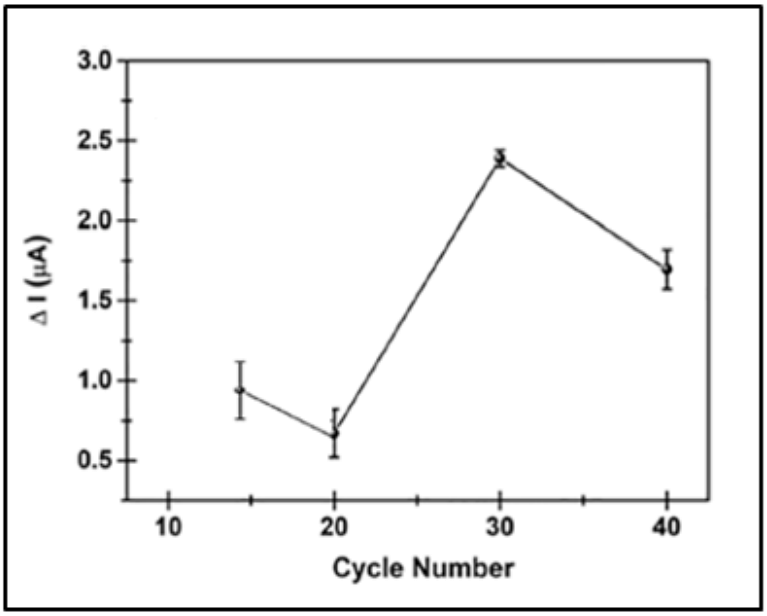

Figure 2.5. Effect of cycle number on constructed biosensor response (in 50 mM PBS, pH 7.0, 25 ºC)

2.2.1.3 Optimization of Enzyme Amount

Alcohol oxidase (AOx) amount was also optimized since it directly affects the sensor's performance. Excess amount of enzyme may exceed loading capacity of enzyme. On the other hand, small amount of enzyme immobilization may not cause low diffusion in enzymatic surface. To optimize the enzyme amount, different

electrodes were prepared by adjusting the concentration of enzyme (0.5 µL, 2 µL, 4 µL, 6 µL and 8 µL of AOx) while keeping the other parameters constant. Amperometric results showed than 4 µL (6.4 U) of AOx amount provided the most stable and highest response as shown in Figure 2.6.

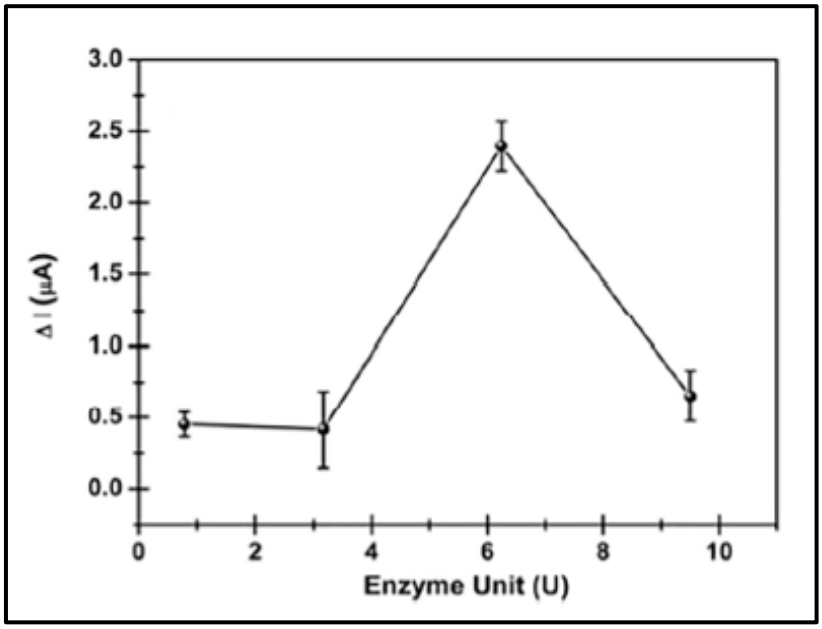

Figure 2.6. Effect of enzyme amount on constructed biosensor response (in 50 mM PBS, pH 7.0, 25 ^{o}C)

2.2.1.4 Optimization of pH

pH of working medium for the fabricated biosensor was optimized to achieve the most sensitive and stable system. For this purpose, 50 mM buffer solutions were prepared in pH range of 3.0 – 10.0 (NaOAc buffer at pH 3.0 and 5.0, PBS buffer at pH 7.0 and Tris buffer at pH 8.0 and 10.0) and the sensor response was recorded at these different pH solutions. As shown in Figure 2.7., maximum and most stable current was obtained at pH 7.0 medium.

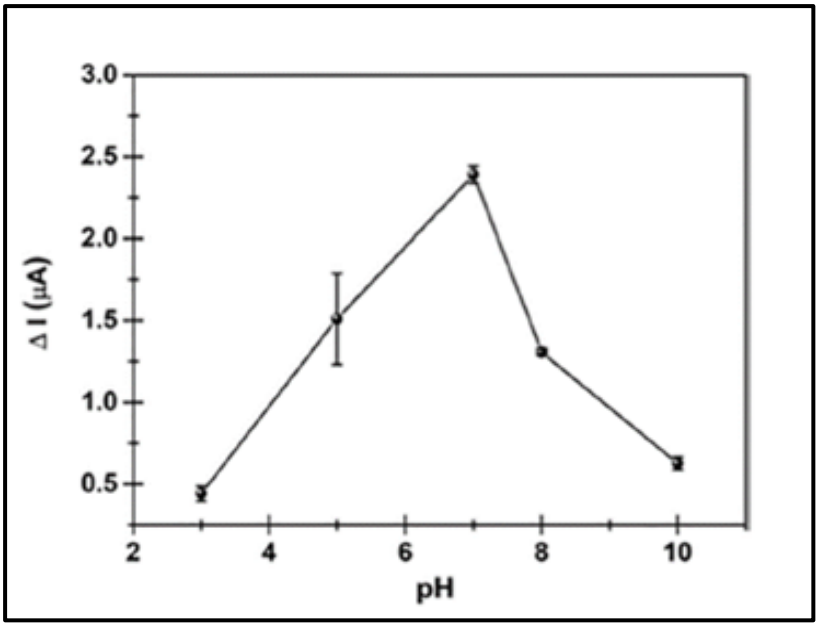

Figure 2.7. Effect of pH on constructed biosensor response (at -0.7 V, 25 ^{o}C)

2.2.1.5 Effect of Combination of Polymer and Carbon Nanotube

To obtain the highest sensor response, effect of polymer and multiwalled carbon nanotube combination was also examined. Under optimized conditions, different combined electrodes (PF-g-PEG/AOx and MWCNT/PF-g-PEG/AOx) were prepared. Amperometric responses of different modified electrodes were shown in Figure 2.8. MWCNT/PF-g-PEG/AOx) biosensor system has higher amperometric signal and short response time towards ethanol. It is seen that MWCNT and CP combination increased stability of the sensor and provided faster electron transfer than CP/AOx.

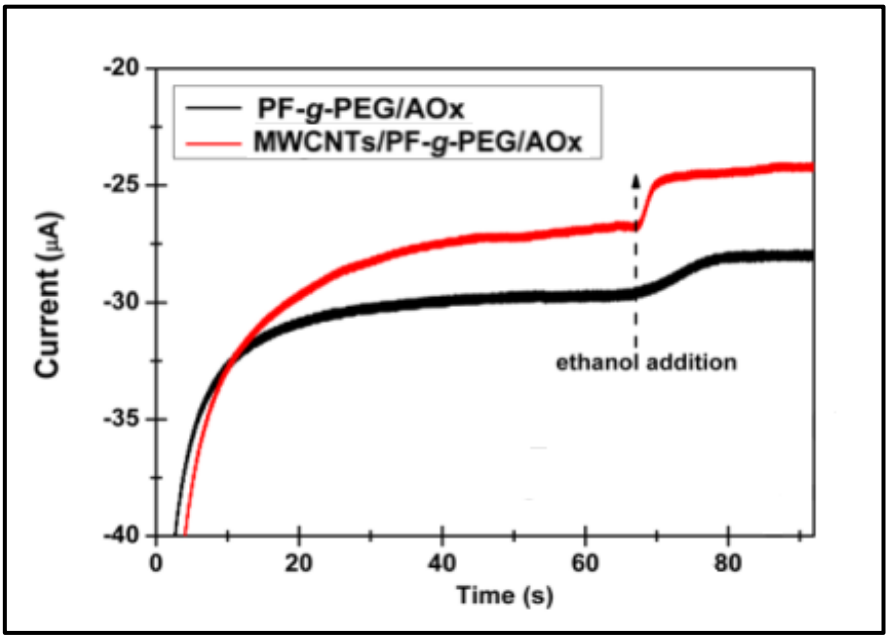

Figure 2.8. Comparison of the amperometric responses of the alcohol sensors for 4.25 mM ethanol

In Figure 2.9 amperometric measurements of these two modified electrodes were given at different ethanol concentrations. PF-g-PEG/AOx modified electrodes response towards ethanol with low sensitivity and longer response time. Modification of multiwalled carbon nanotube increased the stability of proposed sensor and decreased the response time in comparison with PF-g-PEG/AOx sensor. Besides this modification increased the sensitivity of the sensor. As a result, MWCNT/CP/AOx fabricated system gave rise to more precise and increased amperometric signal in comparison to CP/AOx system

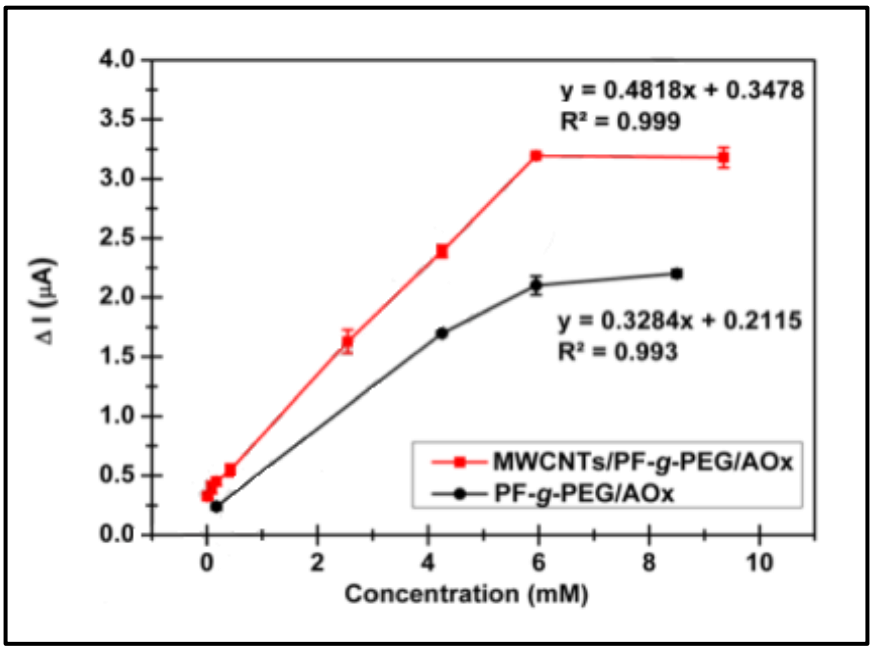

Figure 2.9. Calibration curve of the biosensors (MWCNTs/PF-g-PEG/AOx and PF-g-PEG/AOx to increasing ethanol concentrations in 50 mM pH 7.0 PBS

2.2.2 Investigation of Interference

One of the important desired features of the biosensor was its specificity towards ethanol. To investigate the effect of different interferants on the proposed biosensor system, 0.1 mM of common substances (ethanol, urea, glucose, ascorbic acid, and citric acid) were injected 50 mM buffer solution at ph 7.0, and their behavior was investigated. Results showed that the proposed biosensor did not show a notable response in the presence of interferants. (Figure 2.10)

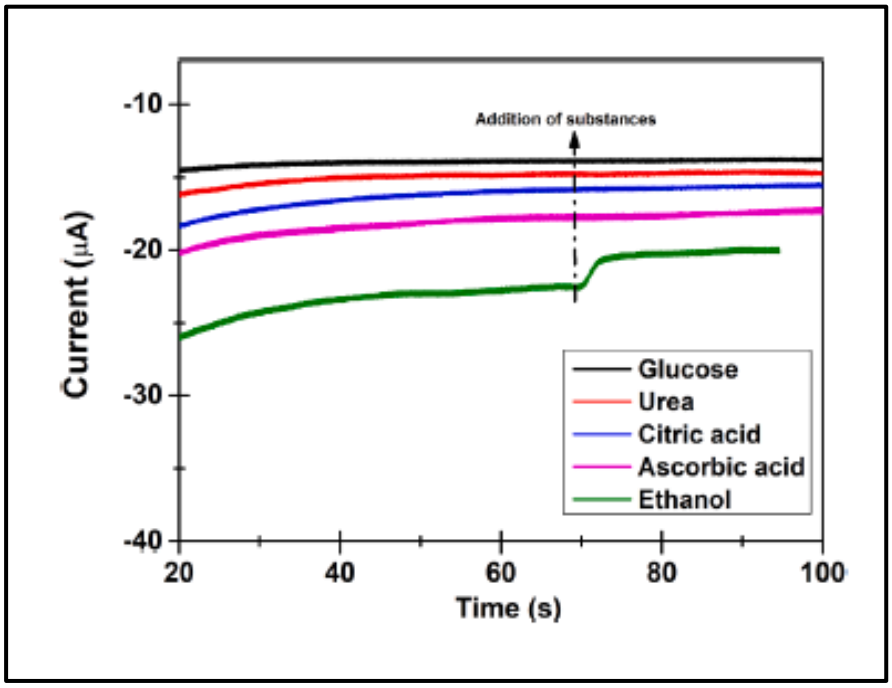

Figure 2.10. Interference effect of interferants on biosensor response (in 50 mM PBS pH 7.0, -0.7 V)

2.2.3 Investigation of Substrate Specificity

To investigate the substrate selectivity of the fabricated biosensor, various alcohols (methanol, ethanol, and 2-propanol) were investigated. 4.25 mM of alcohol solutions were injected to the buffer solution. Response of the methanol was accepted as 100 % (as reference). The responses of the biosensor to other alcohol solutions showed good correlation. (Figure 2.11) Results revealed that fabricated biosensor can analyze ethanol content correctly.

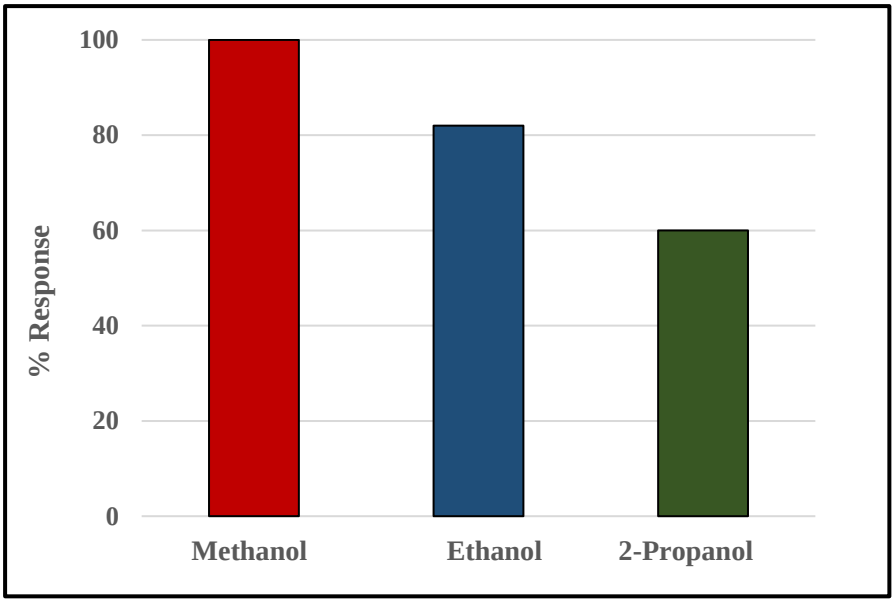

Figure 2.11 Comparison of derivatives of alcohol using proposed biosensor (50 mM PBS, pH 7.0, 25 ^{o}C)

2.2.4 Characterization

2.2.4.1 Analytical Characterization of Proposed Biosensor

Linear relationship between the change in current and concentration of ethanol was achieved in the range of 0.0085 – 5.95 mM, and that range was used as the dynamic linear range in further analytical characterization. (Figure 2.12)

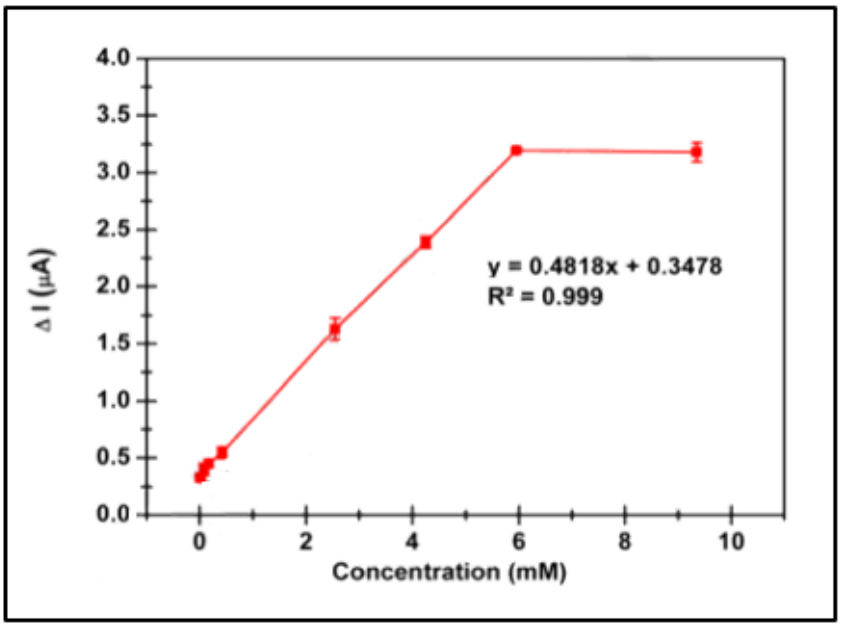

Figure 2.12. Calibration curve of proposed biosensor (50 mM PBS, ph 7.0)

Represented equation for calibration curve is $y = 0.4818x + 0.3478$ with $R^2 = 0.999$. When concentration of ethanol exceeds 5.95 mM, saturation was observed, and a deviation from linearity was achieved. The lowest concentration for detection which is the limit of detection was found as 0.11 mM. Also, sensitivity value was calculated as 7.99 $\mu A\ mM^{-1}\ cm^{-2}$. Obtained results are compared with other ethanol sensing studies, and it is clearly seen that fabricated MWCNT/PF-g-PEG/AOx system has wider dynamic range and low detection limit. (Table 2.1)

Biosensors	Linear Range (mM)	LOD (mM)	Reference
Hydrogel/Platinum electrode/AOx	0.02-3.75	NR	**[35]**
PNR/carbon film electrode	0 – 0.8	0.044	**[36]**
DPP/Mercury Electrode	0.2 – 2.0	4.3	**[37]**
SPCE/MWCNT/AuNP/PNR/AOx/GA	0.178 – 1.0	0.05	**[38]**
PMCCH/AOx	NR	8.3	**[39]**
MWCNTs/PF-g-PEG/AOx	**0.0085 – 5.95**	**0.11**	**This Work**

Table 2.1. Comparison of analytical parameters of ethanol biosensor in literature

To examine the precision of the proposed biosensor, 10 sequential measurements were recorded. Standard deviation (SD) and relative standard deviation (RSD) for these 10 consecutive measurements were found as ± 0.09 and 3.91% respectively.

Km^{app} for the enzyme in the proposed biosensor system was calculated using Lineweaver-Burk plot as 0.015 mM. These results confirm the high affinity of immobilized alcohol oxidase towards to ethanol.

2.2.4.2 Surface Characterization of Biosensor

Effective surface areas of modified electrodes (MWCNT/PF-g-PEG/AOx and MWCNT/PF-g-PEG and bare electrode) were calculated using Randles-Sevcik equation. Experiments were performed in 50 mM $Fe(CN)_6^{-3/-4}$ solution that is containing 0.1 M KCl. Cyclic voltammograms were achieved at 100 mV/s scan rate (Figure 2.13) and peak currents were found as 0.43 cm^2 and 0.193 cm^2 for MWCNT/PF-g-PEG and MWCNT/PF-g-PEG/AOx, respectively. Immobilized enzyme decreased the current and electroactive surface area due to its adsorption onto the probe.

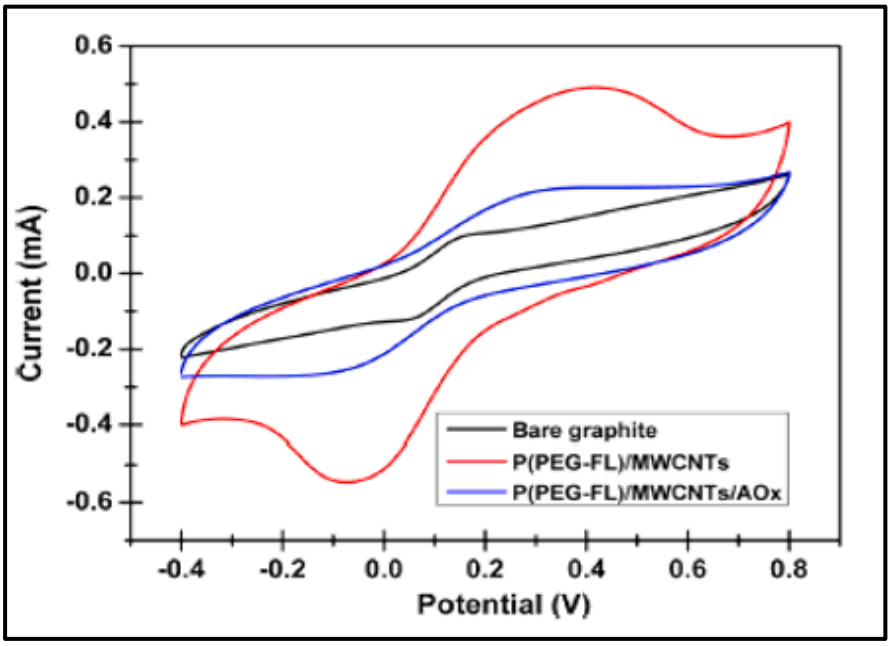

Figure 2.13 Cyclic voltammogram of bare graphite electrode, MWCNTs/ PF-g-PEG and MWCNTs/PF-g-PEG/AOx in 5mM $Fe(CN)_6^{3-/4-}$

To analyze the changes in morphological surface, scanning electron microscopy (SEM) technique was used. (Figure 2.14) MWCNT/PF-g-PEG image is more homogeneous to compare with PF-g-PEG film. Immobilization of the enzyme on modified MWCNT/PF-g-PEG surface made surface more uniform indicating the coverage of the active surface by the enzyme.

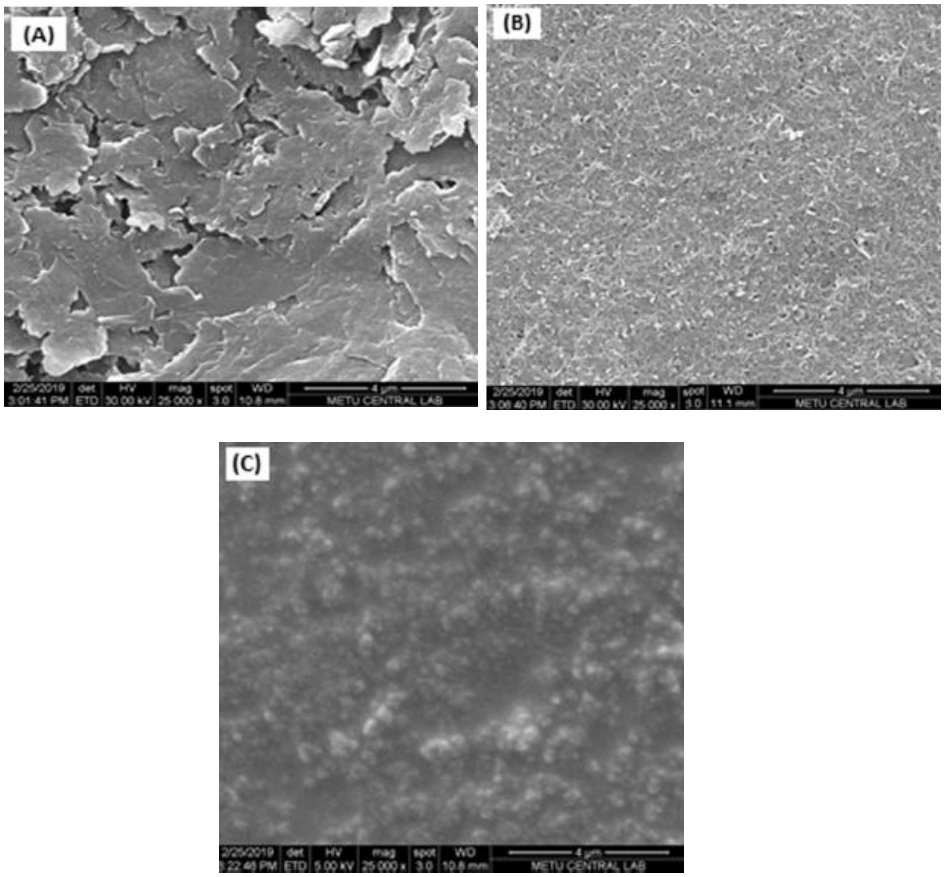

Figure 2.14. SEM images of A) PF-g-PEG B) MWCNT/PF-g-PEG C) MWCNT/PF-g-PEG/AOx

2.2.5 Applicability of Biosensor

To test the accuracy of the proposed biosensor, commercial samples were analyzed using proposed biosensor by injecting appropriate volume for each sample. Ethanol concentration in each sample was determined using previously constructed the calibration. Experimentally obtained ethanol concentrations were compared with the concentration given in the product labels of beverages. (Table 2.2) Results proved that the fabricated biosensor can be used to detect ethanol content accurately in real samples.

Sample	Product Label (%)	MWCNTs/PF-g-PEG/AOx (%)	Percent Error (%)
Rum	37.5	35.04	6.56
Raki	45.0	42.07	6.51
Vodka	37.5	39.25	4.66

Table 2.2. Ethanol detection in beverages using proposed biosensor

CHAPTER 3

CONCLUSION

In this thesis study a novel amperometric biosensor based on conjugated polymer and multiwalled carbon nanotube was designed for detection of alcohol. Multiwalled carbon nanotube solution was cast on cleaned bare graphite electrode surface. Then, poly(ethylene glycol) with fluorene functionality (PEG-FL) monomer was synthesized in one step procedure and electrochemically polymerized with cyclic voltammetry technique onto multiwalled carbon nanotube modified graphite electrode surface. Combination of carbon nanotube and monomer on graphite surface enabled the suitable immobilization of alcohol oxidase enzyme (AOx). Enzyme was immobilized onto the modified surface with physical adsorption technique. Synthesized conjugated polymer; PF-g-PEG, increased the interaction with enzyme due to hydrophilic structure of monomer and increased the effective surface area. Apart from the conjugated polymer, a multiwalled CNT was inserted to sensing system since MWCNT provided fast electron transfer and chemical stability in the constructed system.

Amperometric biosensor response was investigated at -0.7 V in a buffer solution (pH 7). As a result of enzymatic reaction between alcohol oxidase and the substrate, changes in current due to change oxygen level were monitored in terms of the signal of biosensor. To obtain the best sensor response, parameters that affect the biosensor response were optimized. After finding the optimum conditions, a calibration curve was constructed for ethanol detection. Then, kinetic, and analytical characterization of the proposed biosensor were completed. The constructed biosensor was found to have linear dynamic range between 0.0085 mM and 5.95 mM with a detection limit of 0.11 mM and sensitivity of 7.99 $\mu A\ mM^{-1}cm^{-2}$. Compared to similar studies reported in the literature, proposed biosensor has good kinetic parameters, a high

dynamic range, and a low limit of detection. Besides, to show the specificity of the proposed biosensor, different interferants were tested, and fabricated biosensor was found to be very specific for alcohol. Apart from the interference effect to investigate the surface morphology, scanning electron microscopy (SEM) was used. Finally, fabricated biosensor was used in the quantification of alcohol in several beverages and revealed high accuracy.

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