SPECTROPHOTOMETRIC AND AMPEROMETRIC BIOSENSORS BASED ON CONDUCTING POLYMERS OF 1-BENZYL-2,5-DI(THIOPHEN-2-YL)-1H-PYRROLE AND (4,7-DITHIEN-2-YL-2,1,3-BENZOTHIADIAZOLE)

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

SİMLA TANRIVERDİ

IN PARTIAL FULFILLMENT OF THE REQUIREMENT
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
POLYMER SCIENCE AND TECHNOLOGY

NOVEMBER 2012

Approval of the thesis:

SPECTROPHOTOMETRIC AND AMPEROMETRIC BIOSENSORS BASED ON CONDUCTING POLYMERS OF 1-BENZYL-2,5-DI(THIOPHEN-2-YL)-1H-PYRROLE AND (4,7-DITHIEN-2-YL-2,1,3-BENZOTHIADIAZOLE)

submitted by **SİMLA TANRIVERDİ** in partial fulfillment of the requirements for the degree of **Master of Science in Polymer Science and Technology Department, Middle East Technical University** by,

Prof. Dr. Canan ÖZGEN _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Teoman TİNÇER _____
Head of Department, **Polymer Science and Tech. Dept.**

Prof. Dr. Levent TOPPARE _____
Supervisor, **Chemistry Dept., METU**

Prof. Dr. Suna TİMUR _____
Co-advisor, **Biochemistry Dept., EGE UNI**

Examining Committee Members:

Prof. Dr. Leyla ARAS _____
Chemistry Dept., METU

Prof. Dr. Levent TOPPARE _____
Chemistry Dept., METU

Assoc. Prof. Dr. Ali ÇIRPAN _____
Chemistry Dept., METU

Assist. Prof. Dr. Elif BÖYÜKBAYRAM _____
Chemistry Dept., KARABÜK UNI

Dr. Yusuf NUR _____
Chemistry Dept., MUSTAFA KEMAL UNI

Date: 30.11.2012

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.

Name, Last Name: Simla Tanrıverdi

Signature:

ABSTRACT

SPECTROPHOTOMETRIC AND AMPEROMETRIC BIOSENSORS BASED ON CONDUCTING POLYMERS OF 1-BENZYL-2,5-DI(THIOPHEN-2-YL)-1H-PYRROLE AND (4,7-DITHIEN-2-YL-2,1,3-BENZOTHIADIAZOLE)

Tanrıverdi, Simla

M.Sc., Department of Polymer Science and Technology

Supervisor: Prof. Dr. Levent TOPPARE

Co-supervisor: Prof. Dr. Suna TİMUR

November 2012, 49 pages

In this thesis, two different biosensors based on conducting polymers of (4,7-dithien-2-yl-2,1,3-benzothiadiazole) (TBTD) and 1-benzyl-2,5 di(thiophene-2-yl)-1H-Pyrrole (SNBS) were prepared. Electrochemical technique was used for polymerization of conducting polymers and two different immobilization techniques were used for immobilizing enzyme into the conducting polymer matrices. The proposed biosensors were characterized and optimized. Optimum pH, thickness, enzyme amount were determined and linearity, repeatability, operational stability experiments were performed. In the first part of the thesis a redox enzyme alcohol oxidase was immobilized in TBTD/EDOT bilayer and PEDOT to construct an amperometric biosensor. AOX is responsible for the oxidation of low molecular weight alcohols to the corresponding aldehyde, using molecular oxygen as the electron acceptor. Using entrapment method, alcohol oxidase enzyme was immobilized in PEDOT matrix on platinum disk electrodes. Then, enzyme

was immobilized in poly(4,7-dithien-2-yl-2,1,3-benzothiadiazole) (TBTD)-EDOT matrix. Finally these two were compared. Proposed biosensors were characterized using ethanol as the substrate and the responses were measured. Kinetic parameters, I_{max}, K_m and sensitivity were determined for the biosensors with and without TBTD. Optimization studies for TBTD thickness, PEDOT amount and pH were carried out. The biosensing capacity was discussed for TBTD matrix. In the second part of the thesis a second biosensor was designed through electrochemical polymerization of 1-benzyl-2,5 di(thiophene-2-yl)-1H-Pyrrole (SNBS). Invertase enzyme immobilized SNBS generates a spectrophotometric biosensor. Kinetic parameters, Michaelis-Menten constant, K_m, maximum reaction rate, V_{max} were investigated. Operational stability, pH and temperature optimization of the enzyme electrodes were also examined.

Keywords: Electrochemical Polymerization, Conducting Polymers, Conducting Polymers, Electrochemical Biosensors, Alcohol Oxidase, Invertase, Amperometric Biosensors

ÖZ

1-BENZİL-2,5-DI(TİYOFEN-2-L)-1H-PİROL VE (4,7-DITİEN-2-L-2,1,3-BENZOTİADIAZOL) İLETKEN POLİMERLERİ BAZLI SPEKTROFOTOMETRİK VE AMPEROMETRİK BİYOSENSÖRLER

Tanrıverdi, Simla

Yüksek Lisans, PST Bölümü

Tez Yöneticisi: Prof. Dr. Levent TOPPARE

Ortak Tez Yöneticisi: Prof. Dr. Suna TİMUR

Kasım 2012, 49 sayfa

Bu tezde, poli 1-benzil-2,5-di(tiyofen-2-l)-1H-pirol) poli(SNBS) ve poli(4,7-ditien-2-l-2,1,3-benzotiadiazol) poli(TBTD) iletken polimerleri bazlı biyosensörler hazırlanmıştır. İletken polimerlerin sentezi için elektrokimyasal polimerleştirme tekniği kullanılmıştır. Spektrofotometrik ve amperometrik yöntemler kullanılarak hazırlanan biyosensörler optimize ve karakterize edilmiştir. Uygun çalışma pH değeri, sıcaklığı, iletken polimer kalınlığı, biyolojik madde miktarı gibi parametreler optimize edilmiş, tekrarlanabilirlik, lineer çalışma aralığı, ve kararlılıkları belirlenmiştir. Tezin ilk kısmında alkol oksidaz enzimi kullanılarak amperometrik bir biyosensör hazırlanmıştır. AOX, moleküler oksijenin elektron akseptörü olarak kullanılmasıyla, düşük molekül ağırlıklı alkollerin, ilgili aldehite yükseltgenmesi için kullanılmıştır. Alkol oksidaz enzimi platin elektrot yüzeyine, farklı polimer matrislerinde tutuklanmış, elde edilen biyosensörlerde, etanol sübstratı kullanılarak,

optimizasyon analitik karakterizasyon çalışmaları tamamlanmıştır. Alkol oksidaz enzimi, tutuklama yöntemi kullanılarak, poli(4,7-ditien-2-l-2,1,3-benzotiadiazol) (TBTD)-EDOT matrisinde tutuklanmıştır. Enzimin bu matriste tutuklanması platin disk elektrotları üzerinde gerçekleştirilmiştir. Enzimin PEDOT ve TBTD-PEDOT matrislerinde tutuklanması sonucu elde edilen değerler karşılaştırılmıştır. Bu çalışmalarda sabit potansiyelde dönüştürücü ile elektrik sinyaline dönüştürülen fiziksel özellikteki değişim esas alınmıştır. Elektrodun ilk tabakasında TBTD polimerizasyonu olurken, ikinci tabakada EDOT polimerizasyonu gerçekleştirilmiş ve alkol oksidaz enzimi tutuklanmıştır. pH optimizasyonu, yük determinasyonu, tarama sayısı optimizasyonu çalışılmış, V_{max}, K_m değerleri hesaplanmıştır. Daha sonra yalnız PEDOT polimerizasyonu ile oluşturulan elektrotta alkol oksidaz enzimi tutuklanmış ve çalışmalar karşılaştırılmıştır. Tezin ikinci kısmında ise elektrokimyasal yöntem kullanılarak poli 1-benzil-2,5-di(tiyofen-2-l)-1H-pirol) poli(SNBS) iletken kopolimerinde invertaz enzimi tutuklanmıştır. SDS (sodyum dodesil fosfat) destekleyici elektrolit olarak kullanılmış, 1 volt potansiyel uygulanarak tutuklama matrisleri hazırlanmış, daha sonra enzimin pH, sıcaklık, kinetik parametreleri (V_{max} ve K_m değerleri) ve raf ömrü değerleri hesaplanmıştır. İnvertaz enziminin polipirolde ve kopolimerde tutuklanması sonucu elde edilen değerler karşılaştırılmıştır.

Anahtar Kelimeler: Elektrokimyasal polimerleştirme, İletken Polimerler, Elektrokimyasal Biyosensörler, Alkol Oksidaz, İnvertaz, Amperometrik Biyosensörler

Dedicated to my parents, my sister and my brother.

ACKNOWLEDGMENTS

I would like to express my greatest appreciations to my supervisor Prof. Dr. Levent Toppare, for his everlasting encouragement, guidance and valuable advices.

I gratefully thank to my co-advisor, Assoc. Prof. Dr. Suna Timur, for her support, motivation and patience during my studies.

I would like to give endless thanks to Prof. Dr. Leyla Aras, for her everlasting positive energy and being there whenever I lose my courage during my studies and leading the way for life.

I owe great thanks to Dr. Senem Kıralp Kayahan,, MSc. Sevinç Tunçağıl and Dr. Özlem Türkarslan, for their moral support, help and valuable discussions.

I would like to express my special thanks to my best friends Esin İbibikcan, Melis Baltalı, Ceren Yapça and Begül Yeşilöz for their eternal support and precious friendship.

I want to give special thanks to Aytek Ay for his valuable moral support and encouragement.

I am thankful to my friends Zeynep Ergül, Selin Çelebi, Merve Çay, Ece Salıcı, Samed Atak, Huriye Erdoğan, Serhat Güler, Bahar Şanlı and Hava Zekiye Akpınar for making the time valuable during my master.

I also want to thank to every member in Toppare Research Group for their friendship.

I would like to express my appreciation to the academic staff of METU Department of Polymer Science and Chemistry and Department of Chemistry for their professional support and guidance.

I also would like to express my endless thanks to my father Ahmet Tanrıverdi, my mother Hülya Tanrıverdi who made everything possible for me and my sister Serap Tanrıverdi and my brother Efe Tanrıverdi for their moral support, encouragement and for always being there for me whenever I need.

TABLE OF CONTENTS

ABSTRACT iv
ÖZ vi
TABLE OF CONTENTS x
LIST OF TABLES xii
LIST OF FIGURES xiii
ABBREVIATIONS xiv
CHAPTERS 1
1. INTRODUCTION 1
1.1. Biosensors 1
1.1.1. Electrochemical biosensors 4
1.1.1.1. Enzyme-based electrochemical biosensors 6
1.2. Conducting polymers in biosensors 10
2. EXPERIMENTAL 15
2.1. Reagents 15
2.2. Instrumentation 16
2.2.1. Amperometric measurements 16
2.2.2. Spectrophotometric measurements 16
2.2.3. Cyclic voltammetry measurements 16
2.2.4. Surface characterization 17
2.3. Experimental procedures 17
2.3.1. Synthesis of 4,7-dithien-2-yl-2,1,3-benzothiadiazole [TBTD] 17
2.3.1.1. Preparation of amperometric alcohol biosensor based on poly (TBTD) 18
2.3.1.2. Amperometric biosensor measurements 18
2.3.1.3. Effect of electropolymerization time 19
2.3.1.4. Optimization of TBTD thickness 19
2.3.1.5. Optimization of PEDOT amount 19
2.3.1.6. pH optimization of the biosensors 20

2.3.1.7. Analytical characterization of biosensors 20
2.3.2. Synthesis of 1-benzyl-2,5-di(thiophene-2-yl)-1H-pyrrole [SNBS]... 21
2.3.2.1. Synthesis of copolymer of SNBS with pyrrole 22
2.3.2.2. Immobilization of invertase in polymer matrice 22
2.3.2.3. Determination of invertase activity 23
2.3.2.4. Determination of kinetic parameters 24
2.3.2.5. Determination of optimum pH and temperature values 24
2.3.2.6. Operational stability 25
3. RESULTS AND DISCUSSION 26
3.1. Amperometric alcohol biosensor based on poly(TBTD) 26
3.1.1. Effect of electropolymerization time 26
3.1.2. Optimization of TBTD thickness 28
3.1.3. Optimization of PEDOT amount 29
3.1.4. pH optimization of the biosensors 29
3.1.5. Analytical approach for poly(TBTD) and AOX based biosensor . 30
3.2. Spectrophotometric invertase biosensor based on poly(SNBS), invertase……. 33
3.2.1. Morphologies of the immobilization matrices 33
3.2.2. Kinetic parameters of immobilized invertase 35
3.2.3. Effect of pH on enzyme activity 36
3.2.4. Effect of temperature on enzyme activity 37
3.2.5. Operational stability of the enzyme electrode 38
4. CONCLUSION 40
5. REFERENCES 42

LIST OF TABLES

TABLES

Table 1. Immobilization methods via their advantages and disadvantages 4
Table 2. Relation between deposited charges and measured signals..... 29
Table 3. Kinetic parameters for AOX biosensor 32
Table 4. Kinetic parameters for invertase biosensor..... 36

LIST OF FIGURES

FIGURES

Figure 1. Schematic representation of a simple biosensor 2
Figure 2. Glucose oxidase reaction mechanism 9
Figure 3. Invertase reaction mechanism 10
Figure 4. Conducting polymer based biosensors 12
Figure 5. Electron transfer in CP based biosensors 142
Figure 6. Immobilization of mediators in the polymerization solution 13
Figure 7. Electrochemical cell with three electrode system 14
Figure 8. Synthesis of (4,7-dithien-2-yl-2,1,3-benzothiadiazole) [TBTD] 17
Figure 9. Synthesis of 1-Benzyl-2,5 di(thiophene-2-yl)-1H-pyrrole [SNBS] 21
Figure 10. Method for copolymerization of SNBS 22
Figure 11. Nelson method 24
Figure 12. a) Cyclic voltammograms of (B) bare Pt electrode and (A) after electrochemical polymerization of TBTD .....
b) Polymerization of TBTD 27
Figure 13. Optimization of TBTD thickness according to effect of number of scan on the biosensor response 28
Figure 14. Effect of pH on AOX immobilized TBTD/PEDOT electrodes 30
Figure 15. Calibration curves for A: TBTD/PEDOT and B: PEDOT matrice 31
Figure 16. SEM image of polymer 1 with invertase, K_m 15 mM 34
Figure 17. SEM image of polymer 2 with invertase, K_m 40 mM 34
Figure 18. SEM image of poly(SNBS) with invertase, K_m 65 mM 35
Figure 19. pH stability of enzyme immobilized in copolymer of SNBS 37
Figure 20. Temperature stability of enzyme immobilized in copolymer of SNBS ... 38
Figure 21. Operational stability of enzyme immobilized in copolymer of SNBS 39

ABBREVIATIONS

CP	Conducting polymer
SNBS	1-benzyl-2,5 di(thiophene-2-yl)-1H-pyrrole
TBTD	(4,7-dithien-2-yl-2,1,3-benzothiadiazole)
poly(SNBS)	polymer of 1-benzyl-2,5 di(thiophene-2-yl)-1H-pyrrole
poly(TBTD)	polymer of (4,7-dithien-2-yl-2,1,3-benzothiadiazole)
EtOH	Ethanol
V_{max}	Maximum reaction rate
K_m	Michelis Menten constant
SEM	Scanning electron microscopy
CVs	Cyclic voltammograms
SCE	Saturated calomel electrode

CHAPTER 1

INTRODUCTION

1.1 Biosensors

A device having a biological sensing element integrated with a transducer is named as a biosensor. An electronic signal that is in proportion to the concentration of a specific chemical is produced.

Biosensors have two main components consisting of a bioreceptor and a transducer. The bioreceptor is the biomolecule that is used for the recognition of the target analyte meanwhile the transducer converts that information into a measurable signal, such as an electrical signal [1,2]. In medical, nourishment and environmental monitoring, biosensors constitute the largest category of usage areas enabling the detection of chemical and biological components. They provide some advantages including low cost and rapid response, also specificity, sensitivity and user-friendly operation [3,4].

A bioreceptor functions as a biochemical transducer and an immobilized sensitive biological element that recognizes the analyte. Although tissues, antigens, antibodies, bacteria and organelles are used as biocomponents, enzymes are the most commonly used biosensing elements. These all elements have short lifetime in solution phase. Thus, a suitable matrix is used as a fixing environment [2,5].

A transducer provides the conversion of a biological signal to an electronic signal. The conversion of an electrical device reacts in a way that a signal can be reinforced, electronically stored and displayed [2]. Most commonly used transducers are

amperometric, potentiometric and electrochemical ones [6].

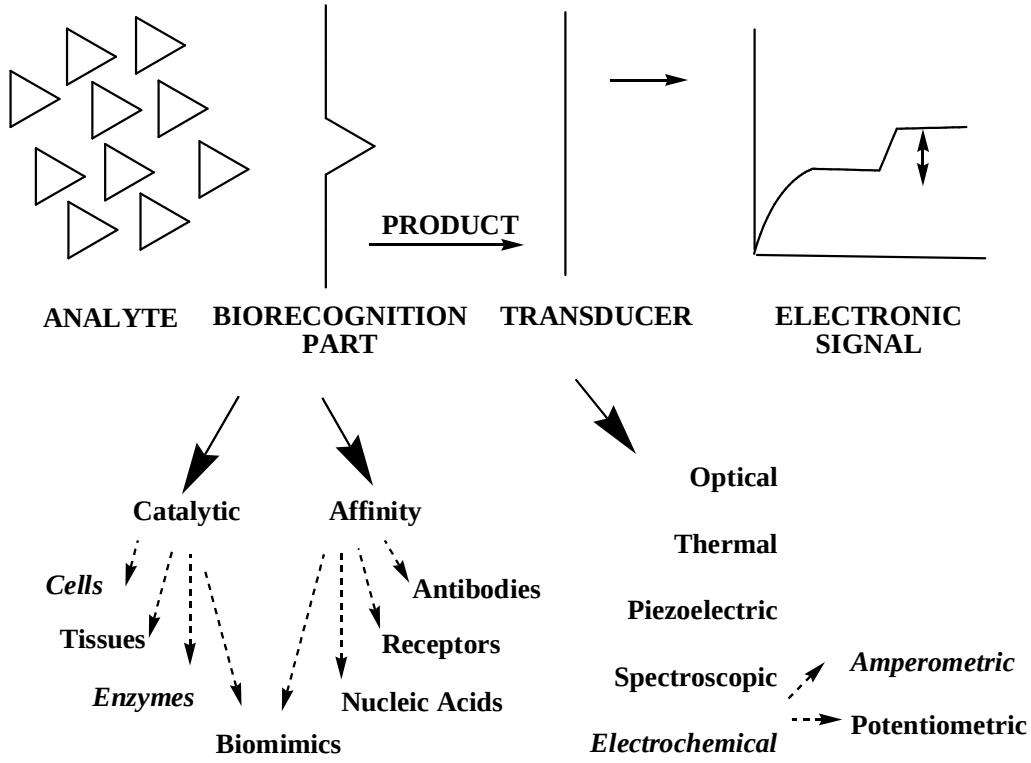


Figure 1. Schematic representation of a simple biosensor [2,13]

Immobilization Methods and Enzyme Immobilization in Biosensors

Immobilization of enzymes is an important and effective technique. This technique is the attachment of enzyme to a solid matrix. Protein becomes immobilized by chemical binding or by physical retention in immobilization [7,8]. Insolubility of immobilized enzymes allows their reusability, ease at separation from the reaction media and controlled product formation. They also do not lose much of their efficiency during the reaction process. Immobilization provides efficient use of enzymes in food technology, biomedicine, biotechnology and analytical chemistry [9]. Immobilization also controls the termination process of reactions (more

rapid terminations) [10,11,12].

There are several available methods for immobilization of biomolecules. Major commonly used biosensor immobilization techniques targeting to design and develop specific sensors are physical adsorption, entrapment; inter molecular cross-linking or covalent binding and encapsulation [13].

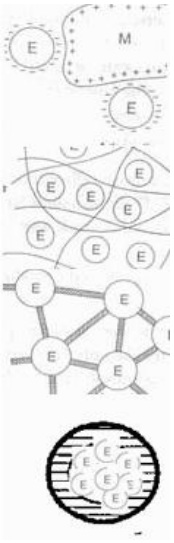
In physical adsorption process, static interactions occur between the oxidized polymer matrix and the total negative enzyme charge [14]. Cellulose, silica gel, collagen and glass are well known substrates to adsorb enzymes. There occur binding forces due to hydrogen bonds, Van der Waal's forces, electron transition complex formations and multiple salt linkages [13].

Entrapment of enzymes is the simplest and most controlled method of localizing biologically active molecules in described environment on the electrodes [15]. Monomer is electrochemically oxidized in the presence of enzyme to form a polymer that combines with the distributed enzyme molecules during its growth process [14].

Covalent bonding or cross linking of receptors activated bifunctional or multifunctional reagents like NH_2, COOH or spacers, such as glutaraldehyde and multilayers. These reagents can bind biomolecules to solid supports. Covalent bonding is carried out through functional group in the enzyme that are not required for its catalytic activity [13,16].

Table 1. Immobilization Methods via Their Advantages and Disadvantages

Method	Advantages	Disadvantages
Adsorption	"No modification of biocatalyst. Matrix can be regenerated. Low cost"	"Binding forces are susceptible to change in pH, temperature and ionic strength"
Entrapment	"Only physical immobilization of biocatalyst near transducer. Low cost"	"High diffusion barrier, substrate accessibility to the enzyme is low"
Crosslinking	"Loss of biocatalyst is minimum. Low diffusional resistance. Stable under adverse conditions. Moderate cost"	"Produces very little of immobilized enzyme that has high intrinsic activity"
Encapsulation	"Provides a larger biocatalyst loading, protected against contamination and biodegradation"	"Only small substrate molecules are utilized with the intact membrane"



1.1.1 Electrochemical Biosensors

Electrochemistry is a useful way to study redox chemistry related with biological redox reactions. Small biomolecules can transfer electrons to an electrode. As a result redox behaviors of biomolecules can be studied electrochemically [17].

Electrochemical biosensor is defined as the class of biosensors that has an electrochemical transducer. An electrode, modified with a biological film, is considered as the transducer in electrochemical biosensors [16,18].

Depending on the receptor type or transduction process, biosensors can be divided into several categories. Enzymatic biosensors, genosensors or immunosensors are classes of biosensors linking to receptor type. On the other hand, according to transduction process, biosensors can be divided into categories such as electrochemical, optical, thermal or calorimetric biosensors [19]. Biocatalytic

reactions with electrochemical transduction of the recognition incident in enzyme electrodes have significant attention [20].

Electrochemical biosensor can be grouped as amperometric, potentiometric and impedimetric sensors depending on the type of transducer. Due to good selectivity, sensitivity, specificity, speed, reproducibility, miniature size, rapid response and low cost, amperometric biosensors are very selective, useful and most commonly used in biosensor applications [6,21].

Amperometric biosensors are based on the measurement of the current resulting from the electrochemical oxidation or reduction of electroactive species. Due to biological activity, either oxygen is consumed or hydrogen peroxide is produced. This change is measured by an electrode versus a Ag/AgCl reference electrode [22]. A constant potential is applied to a Pt, Au or C based working electrode with respect to a reference electrode [16]. Amperometric detection has many advantages due to their rapid response, high selectivity, sensitivity, small size and reproducibility [21].

Amperometry measures the current generated via the oxidation or reduction process of a product at a constant applied potential. In this process, electron transfer between electrode surface usually containing a conducting polymer and the catalytic molecule usually oxidase or dehydrogenase plays an important role and affects the functioning of amperometric biosensor. Redox enzymes can be covalently bound to functional groups or can be entrapped within conducting polymer layers [2].

First biosensor for direct electrochemical detection of the substrate of the enzyme reaction was demonstrated by Clark. Amperometric detection was usually obtained at platinum electrodes [23].

Potentiometry is a rarely used method in biosensor applications. Potentiometric biosensors are based on membrane ion, gas sensing electrodes, thermistors or conductive sensing devices. For biosensors with the response that is not rapid enough, the rate of potential change should be considered as analytical signal instead of steady state potential values for substrate quantification [24]. In potentiometric devices biorecognition process is converted into a potential signal related with the

usage of ion selective electrodes (ISE) whereas in amperometric biosensors a constant potential application is needed to monitor the current due to oxidation or reduction of electroactive species [6].

Impedimetry is a characterization method for enzyme based impedimetric biosensors and impedance spectroscopy studies the electrical properties of surface-modified electrodes [25].

There exist two categories of electrochemical biosensors including biological recognition part as biocatalytic recognition and bioaffinity recognition element whereas biocatalytic based biosensors are widely studied. Enzymes are the most commonly used biocatalysts although tissues, microorganisms as bacteria and cell organelles are used as biocatalysts as well [26].

1.1.1.1 Enzyme-based electrochemical biosensors

Enzymes are effective biological catalysts that are used in industrial processes and catalyze the reactions essential for life. They speed up biochemical reactions and weaken the chemical bonds by lowering the activation energy. Enzymes are very sensitive substances as they can be affected by temperature and pH [27,28,29]. The rate is very high at first and its variation is not linear with time and that makes it hard to obtain reliable data for the values of the kinetic constants [30].

In enzyme electrodes the bioreceptor is enzyme and it measures the concentration of substrate whereas the transducer is a proper electrode. The substrate is transformed into a reaction product with the enzymatic reaction that is perceivable by a transducer. On electrode surface analytical power of electrochemical devices and the individuality of the enzyme for its substrate come together. Enzyme electrodes have been shown to be extremely useful to control a wide variety of substrates of analytical importance in environmental, clinical and food samples [31].

Enzyme electrodes that are used to measure the concentration of substrate are often based on enzymes using an electron acceptor serving as an electrochemical mediator [32]. It is assumed that there is no mass transfer across the interfacial surface of electrode that is in contact with an enzymatic layer. Enzyme coated surface is in a solution that contains the substrate. Migration of substrate from solution to the enzymatic layer occurs during reaction and it is converted to reaction products [33].

Enzyme-based electrochemical biosensors have been used widely in our life, such as health care, food safety and environmental monitoring. Among these health care is the most important area in biosensor applications. For the sake of example, monitoring blood glucose levels and diabetics by glucose biosensors are the most primacy and critical ones [34].

Alcohol oxidase

Many analytical methods including calorimetry, refractometry, chromatographic and spectroscopic techniques have been used to determine the amount of aliphatic alcohols such as ethanol and methanol. Unfortunately, these techniques are not only time consuming but also require expensive instrumentation and trained operators. All these handicaps can be overcome with the use of biosensors that are very attractive alternatives for the basic, quick and possible on-line detection of many pollutants [35,36].

Quantitative determination of alcohols takes an important role in clinical analysis for life since human health is seriously affected by alcohol ingestion or inhalation even causing death. Also it gives terrible effects to skin when used as a solvent or disinfectant in cosmetics. As a result, accurate quantization of ethanol concentration is very important and critical for health. Moreover, food and alcoholic beverages such as wine, beer and liquor quality can be controlled by determination of ethanol [35,37,38].

Alcohol oxidase (AOX; Alcohol: O_2 oxidoreductase, EC 1.1.3.13) is an oligomeric enzyme that consists of eight identical sub-units, each containing a strongly bound cofactor, flavin adenine dinucleotide molecule [36,39]. Alcohol oxidase catalyzes the oxidation of low molecular weight alcohols to their corresponding aldehydes, using molecular oxygen as the electron acceptor. Alcohol oxidase was used as the biosensing material in electrochemical biosensors [40,41,42].

$RCH_2OH + O_2 + AOX \rightarrow RCHO + H_2O_2$ [35].

Alcohol oxidase uses only molecular oxygen (O_2) as the cofactor and oxidation of ethanol to acetaldehyde and hydrogen peroxide comprises O_2 in the reaction. Consequently, the catalytic reaction can be easily followed amperometrically [43,44].

Glucose oxidase

Diabetes mellitus is a serious disease and metabolic defect beginning with insulin lack due to the pathological inability of pancreas. Body cells need energy and for that reason, they require insulin to absorb glucose. Diabetic cells suffer from the lack of glucose (blood sugar) while glucose levels build up in the blood. For the treatment and control of diabetes mellitus, the amount of blood sugar level has to be monitored and controlled properly [45,46].

Glucose oxidase (GOx) is an ideal enzyme and has played a leading role in glucose biosensing due to its reliability, good stability, and practical usage, low cost and high specific activity. GOx is extracted from *Aspergillus* and it contains two tightly bound flavine adenine dinucleotide redox centers that catalyze the electron transfer from glucose to gluconolactone and hydrogen peroxide. [45,47].

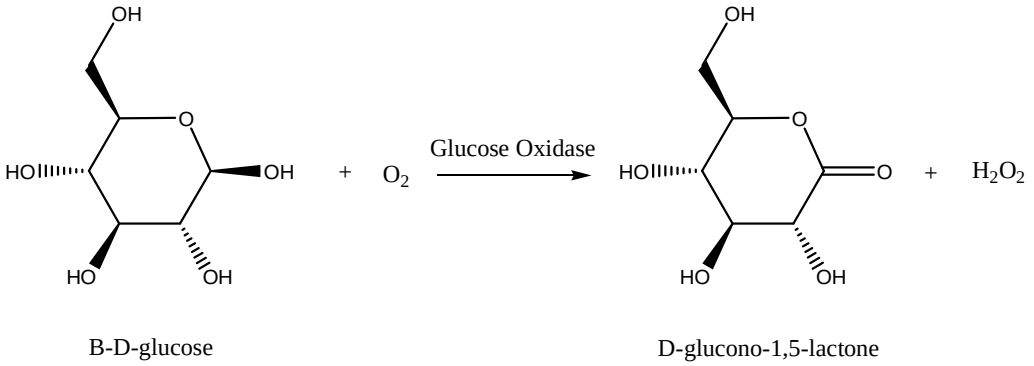


Figure 2. Glucose oxidase reaction mechanism

The amperometric glucose biosensor is a widely used biosensor and it is proper to biochemical analysis due to several advantages, such as simplicity in preparation and rapid response [48].

Invertase

Invertase (1, 2-β-d-fructofuranosidase fructohydrolase), (E.C.3.2.1.26) is an essential enzyme for food industry and has an important role in biological systems. This enzyme is responsible for catalyzing the hydrolysis of sucrose to glucose and fructose. The main usage of invertase is to produce invert sugar for manufacturing confectionary products, as it prevents the production of colored byproducts during the hydrolysis process. Also it lowers the degree of crystallization. Being colorless and having a lower crystallinity than sucrose at high concentrations are advantages of invert sugar [49, 50].

Sucrose is an important product of photosynthesis. Before use of sucrose, it is effected with invertase and invertase catalyses the hydrolysis of sucrose into D-glucose and D-fructose. This case supply carbon and energy to plant cell [51].

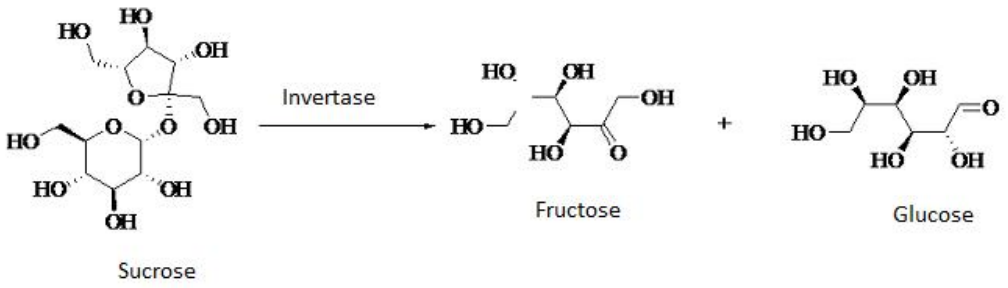


Figure 3. Invertase reaction mechanism

Invertase is isolated and refined from diverse plants, such as beetroot [52], carrot [53], potato [54], etc. Guar gum biopolymer composite is also used in immobilization of invertase [55].

2. Conducting Polymers in Biosensors

In the last two decades, optically transparent and electrically conductive materials have attracted great attention [56,57] as these materials' usefulness and importance have been revealed in devices and applications [58] such as solar cells [59], optical displays [60], batteries [61], light emitting diodes [62], enzyme immobilization matrices [49], electrochromic devices [63], chemical sensors and arrays [64], photovoltaics [65], ion exchange membrane in fuel cells [66], drug release systems [67]. Conducting polymers are very stable, show resistance to corrosion and attach to a metal surface easily. Due to conducting polymers' unique conjugated π-electron system, they show interesting chemical and physical properties [68].

Conducting polymers (CP) are novel materials with a short history of research that dates back to 1960s and still at this time little was understood about the polymers and the discovery was primarily lost. In 1977, first intrinsically conducting polymer was recognized by Alan Mac Diarmid, Hideki Shirakawa and Alan Heeger who reported a considerable increase in the conductivity of polyacetylene doped with iodine.

Conducting polymer developments for different applications have embraced much attention. PPy, polythiophene (PT), polyaniline (PANI) and poly (3,4-ethylenedioxythiophene) (PEDOT) were developed in the 1980s that exhibit good conductivities, stabilities and ease of synthesis [69, 70, 71].

A p- and n-dopable, stable, low band gap polymer was previously electrochemically synthesized from the monomer; 4,7-dithien-2-yl-2,1,3-benzothiadiazole. The polymer has a remarkable level of stability to number of switched cycles, to over-oxidation, a good memory effect, and a high switching ability with a 65 % transmittance (T) [72].

Also an asymmetric monomer with both thiophene and EDOT donor groups have also been studied as an alternative to copolymer studies. Properties of such polymer revealed were even better than its corresponding copolymer in terms of switching time and contrast [73].

Recently, conducting polymers, which are excellent materials for immobilization of biomolecules and rapid electron transfer for the production of influential biosensors, have attracted much interest in the development of biosensors. They are suitable matrices for the entrapment of enzymes. CPs have proper flexibility in the available chemical structure, that can be modified as required. They can easily bind to protein molecules. Moreover, conducting polymers allow rapid electron transfer. They contain conjugated π electron backbones that are responsible for free movement of electrons along the lattice, electrical conductivity, low energy optical transitions, high electron transfer and low ionization potential. Another advantage offered by conducting polymers is that the electrochemical synthesis allows the direct deposition of the polymer on the electrode surface meanwhile entrapping the protein molecules [74, 2].

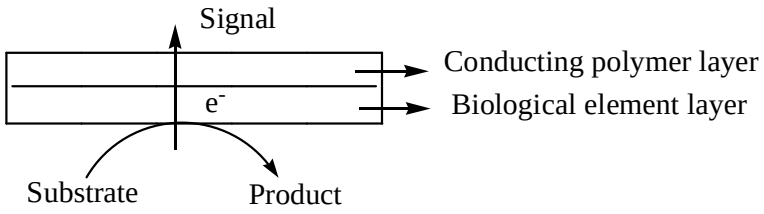


Figure 4. Conducting polymer based biosensors

The electrochemical formation of polymer layers with thickness control and observing the enzyme activity in potentiostatic or galvanostatic conditions offer a good deal of advantages over conventional procedures in designing biosensors. These advantages are in arrangement of complete coverage of the active surface, greater control over film thickness and greater reproducibility. The major advantages of the electrochemical immobilization techniques are: it is a one step, fast than in all immobilization procedures, immobilized enzyme is spatially controlled irrespective of geometry, dimension and shape of the electrode [75, 76].

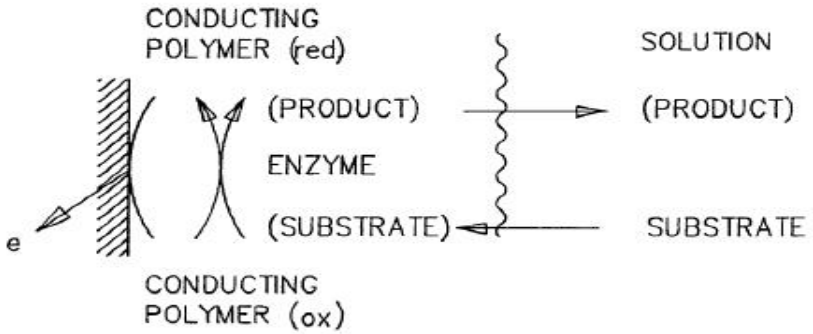


Figure 5. Electron transfer in CP based biosensors

Umana, Waller and Bartlett were the pioneers of manufacturing biosensors by entrapment of biomolecules in electropolymerized films which is the most popular approach [77]. Monomer is electrochemically oxidized in the presence of enzyme to form a polymer during immobilization by enzyme entrapment. This method is the most obvious method of immobilization covering advantages of simplicity, reproducibility and the possibility of immobilizing mediators [77].

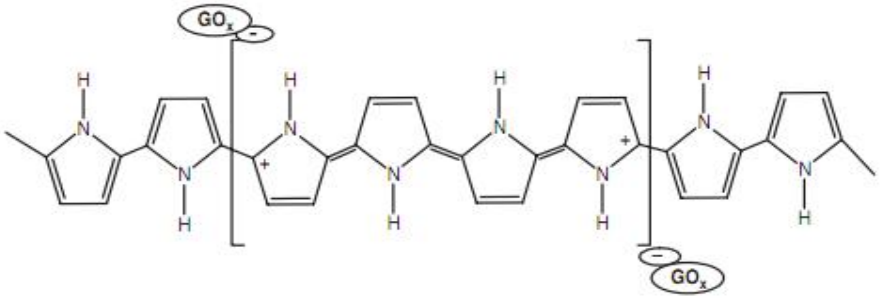


Figure 6. Immobilization of mediators in the polymerization solution

Electrochemical synthesis is the most preferred method to prepare electrically conducting polymers due to its simplicity and reproducibility. The main advantage of electrochemical polymerization is that the reactions take place at room temperature which is very important for the biological material. The thickness of the film can be controlled via varying the potential or current with time [2].

A standard electrochemical technique needs a three electrode system that involves a cell containing a working electrode, a counter electrode and a reference electrode. Working electrode is coated with a biocatalyst. The analyte is oxidized on the working electrode and electrons are transferred to the electrode. A foil of platinum, gold, nickel is generally used as the counter electrode. Common reference electrodes for an aqueous system are SCE, silver electrode and mercurous sulfate [77].

Various electrodes (platinum, glassy carbon, graphite, carbon paste) can be used in this three electrode system.

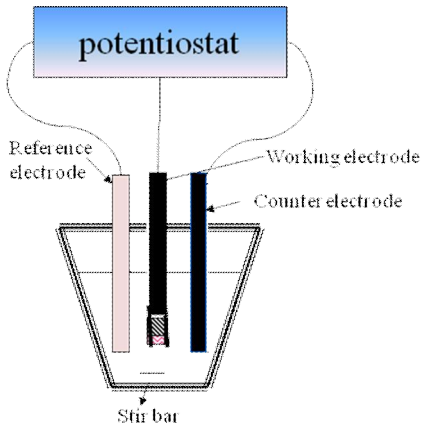


Figure 7. Electrochemical cell with three electrode system

CHAPTER 2

EXPERIMENTAL

2.1 Reagents

Invertase (EC 3.2.1.26) Type V was purchased from Sigma and used as received without further purification. Pyrrole, supplied from Merck, was distilled before use and stored at 4◦C. In order to prepare Nelson reagent, sodium carbonate, sodium potassium tartarate, sodium bicarbonate, sodium sulfate, copper sulfate pentahydrate, ammonium heptamolybdatetetrahydrate ($(NH_4)_6Mo_7O_{24}.4H_2O$) and sodium arsenate ($Na_2HAsO_4.7H_2O$) were provided from Aldrich. Alcohol oxidase, (AOX, E.C. 1.1.3.13 Pichia pastoris,), ethanol, sodium dodecylsulfate (SDS) were purchased from Sigma (St. Louis, USA) and used with no further purification and acetonitrile was purchased from Merck (Darmstadt, Germany). 3,4-Ethylenedioxythiophene was obtained from Aldrich. Ethanol was purchased from Sigma. Na_2HPO_4 and NaH_2PO_4 were purchased from Fisher Scientific Company. All other chemicals were of analytical grade and purchased either from Merck (Darmstadt, Germany) or from Sigma (St. Louis, USA).

2.2 Instrumentation

2.2.1 Amperometric measurements

Chronoamperometry measurements were carried out with a Radiometer electrochemical measurement unit (Lyon, France). A platinum electrode (Metrohm, Switzerland) was used as the auxiliary electrode and an Ag/AgCl (3 M KCl saturated with AgCl as an inner solution, Radiometer Analytical) as the reference electrode. Electrochemical measurements were performed with Ivium CompactStat (The Netherlands) potentiostat in a cell equipped with Ag/AgCl reference electrode, platinum (Pt) foil working and counter electrodes with 2.01 mm^2 area.

2.2.2. Spectrophotometric measurements

Shimadzu UV-160-A model spectrophotometer, Potentioscan Wenking POS-73 and ST-88 potentiostats were used for characterization.

2.2.3. Cyclic Voltammetry measurements

Palm Instrument (PalmSens, Houten, The Netherlands) with three electrode configurations was used for cyclic voltammetry experiments.

2.2.4. Surface Characterization

Scanning electron microscope (SEM) (JEOL JSM-6400) was used for surface imaging of the enzyme electrodes.

2.3 Experimental Procedures

2.3.1 Synthesis of 4,7-dithien-2-yl-2,1,3-benzothiadiazole [TBTD]

The monomer, TBTD, was synthesized from 4,7-dibromo-2,1,3-benzothiadiazole and tributyl(thien-2-yl)stannane. The coupling reaction of 4,7-dibromo-2,1,3-benzothiadiazole with tributyl(thien-2-yl)stannane in the presence of catalytic bis (triphenylphosphine) dichloropalladium(II) [$PdCl_2$-$(PPh_3)_2$] and THF gave the dinitro compound in 82% yield. The synthesis of monomer is described in Figure 8 [78].

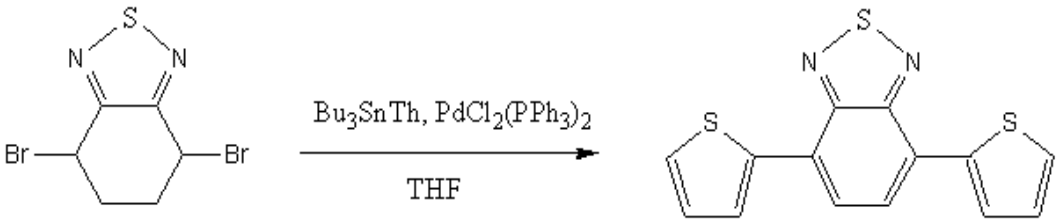


Figure 8. Synthesis of (4,7-dithien-2-yl-2,1,3-benzothiadiazole) [TBTD]

2.3.1.1 Preparation of amperometric alcohol biosensor based on *poly(TBTD)*, Alcohol Oxidase

Electropolymerization of TBTD was carried out via cyclic voltammetry (CV) [42]. Conducting polymer was coated on the platinum electrode (2.01 mm^2) surface through running 15 cycles between -0.4 V and 1.6 V. The polymerization was achieved in 10 ml acetonitrile solution containing 0.01 M TBTD monomer and 0.1 M Bu_4NClO_4. Onto conducting polymer coated surface, alcohol oxidase was entrapped by polymerizing EDOT with constant potential electrolysis at 0.9 V. Electropolymerization was achieved in the presence of 0.05 M sodium dodecylsulphate (SDS), 30 µl EDOT, 15 µl alcohol oxidase and 10 ml pH buffer. Initially, SDS was dissolved in a phosphate buffer (pH 7) consisting of 0.2 M Na_2HPO_4 and 0.2 M NaH_2PO_4, until no turbidity. Then enzyme and EDOT was added to the medium.

2.3.1.2 Amperometric biosensor measurements

Amperometric measurements were carried out at optimized conditions in the electrochemical cell with a three electrode configuration with a Ag/AgCl reference electrode, platinum working and counter electrodes. The saline phosphate buffer (pH 7) was utilized in the amperometric measurements to provide ionic conductivity. Electrochemical cell contains 10 ml buffer solution and continuous stirring was provided with a magnetic stirrer at 210 rpm. All the experiments were carried out at except for temperature optimization were carried out at 24 ^{0}C. The substrate was added to the electrochemical cell. The biosensor responses were determined via application of -0.7 V with respect to Ag/AgCl electrode to detect the oxygen consumption due to biological activity of immobilized alcohol oxidase. After a while, background current reached a stable value. Then ethanol solution was added as the substrate. A current response was observed immediately in 2-3 seconds. Then a steady state was observed and the resulting current difference was recorded. For the

determinations of K_m and I_{max}, various concentrations of ethanol solution were used.

2.3.1.3 Effect of electropolymerization time

One of the advantages of electropolymerization is the ability of controlling the film thickness by regulating the amount of charge passed. The most proper electrochemical method for characterization is cyclic voltammetry [80,81]. Charges related with the scan number were also calculated for conducting polymer. Cyclic voltammograms before and after the electropolymerization of TBTD on the platinum electrode were calculated.

2.3.1.4 Optimization of TBTD thickness

Electropolymerization time is directly related with the thickness of the polymer on the Pt electrodes and it affects both the rate of growth and the quality of the conducting polymer films produced [82]. The thickness can be measured in terms of charge, obtained with the number of scans. To optimize the thickness of TBTD on the electrode, four different electrodes were prepared with different thicknesses, the optimum thickness of TBTD was provided and further experiments were executed with that number of cycles.

2.3.1.5. Optimization of PEDOT amount

PEDOT amount was controlled by determining the charge at which the maximum current response was observed at a constant potential. PEDOT thickness was given with respect to charge passing during polymerization. Consequently, immobilized enzyme amount also increased with increasing PEDOT thickness. When the surface of the electrode was too thick, substrate may not reach the active site of enzyme

resulting in the decrease in the response. As the electropolymerization time increased, respective charge value also increased. Three different thickness of PEDOT electrodes were prepared depositing 0.5Q (5×10-3C), 1Q (1×10-2 C) and 2Q (2×10-2 C).

2.3.1.6. pH optimization of the biosensors

Enzyme activity highly depends on the pH of the medium since extreme pH conditions cause enzyme denaturation. As a result optimum pH values should be determined. To see the effect of pH on the biosensor responses, pH was adjusted between 6.0 and 8.0 using phosphate buffer (50 mM) and sodium acetate buffers (50 mM). The current density was adjusted as 100 % to the maximum response pH, and further experiments were conducted relative to this value.

2.3.1.7. Analytical characterization of biosensors

The analytical characteristics of the biosensors in terms of linear dynamic ranges and the equations were obtained based on optimized conditions for immobilized AOX in PEDOT on TBTD coated surface and also for only PEDOT matrix. Calibration curves were plotted for current density versus substrate concentration (where y is the sensor response in terms of current density (nA/cm^2) and x is the substrate concentration in mM).

Repeatability of the biosensors was estimated by repetitive measurements with their substrates. Furthermore, the standard deviation and coefficient of the variation were calculated.

Kinetic parameters K_m and I_{max} for the enzyme biosensors were found from the Lineweaver-Burk plots (1/current response vs 1/concentration). For the two

biosensors, I_{max}, K_m and sensitivity (I_{max}/K_m) were calculated.

2.3.2 Synthesis of 1-Benzyl-2,5 di(thiophene-2-yl)-1H-Pyrrole [SNBS]

For synthesizing the monomer; 1-benzyl-2,5 di(thiophene-2-yl)-1H-pyrrole [SNBS], a round-bottomed flask equipped with an argon inlet and magnetic stirrer was fitted with 1,4-di(2-thienyl)-1,4-butanedione, benzylamine, propionic acid and toluene. The resultant mixture was stirred and refluxed for 24 h at 110 °C under argon. After flash column chromatography at first, than evaporation of toluene, SNBS monomer was obtained as pale yellow powder (mp: 98 ◦C). The synthetic route of the monomer is shown in Figure 9 [79].

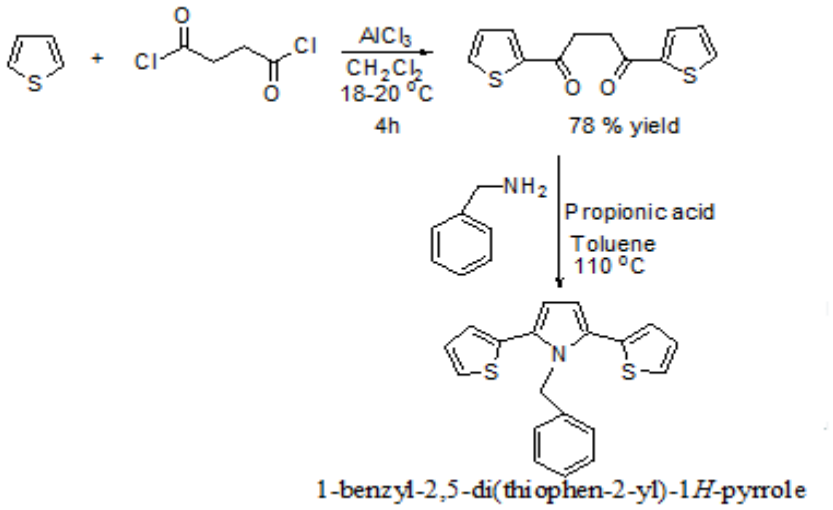


Figure 9. Synthesis of 1-Benzyl-2,5 di(thiophene-2-yl)-1H-Pyrrole [SNBS]

2.3.2.1. Synthesis of copolymer of SNBS with Pyrrole

Working electrode was coated with SNBS. Solvent was evaporated at first. Than the electrode was immersed into a three electrode electrochemical cell that contains 10 ml acetate buffer, 0.05 M SDS and 100 µl pyrrole. Electropolymerization (30 min.) was performed by applying +1.0 V vs. Ag wire pseudo reference electrode. The resulting copolymer can be seen in Fig. 10 below.

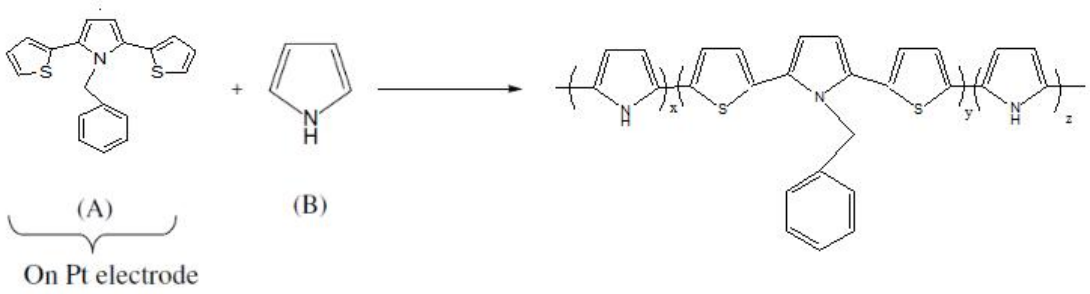


Figure 10. Method for copolymerization of SNBS

2.3.2.2. Immobilization of Invertase in polymer matrix

Immobilization of invertase in polymer matrix was performed by electropolymerization of the SNBS with pyrrole in a typical three electrode cell with constant potential electrolysis at room temperature. Electrolysis medium that contains 1 mg/ml invertase, 1 mg/ml sodiumdodecyl sulfate (SDS) and 100µl pyrrole in 10 ml acetate buffer (pH 5.1) was prepared. Immobilization was carried out

at a constant potential of +1.0 V for 30 min at room temperature using SNBS coated Pt

foil as the working electrode. Counter electrode was platinum and reference electrode was a Ag wire. Immobilization of enzyme was carried out on bare and polymer coated electrodes. Enzyme electrodes were washed with distilled water after electrolysis to remove both the unbound enzyme and the excess supporting electrolyte. Electrode was kept in acetate buffer (pH 5.1) at 4°C when not in use.

2.3.2.3. Determination of Invertase activity

Nelson's method was used to determine the immobilized invertase activity [83]. Nelson reagent prepared with a ratio of 1:25 reagent A and reagent B. sodium carbonate (25 g), sodium potassium tartarate (25 g), sodium bicarbonate (20 g) and sodium sulfate (200 g) were dissolved in distilled water to obtain Reagent A. Reagent B was prepared from 15% (w/v) copper sulfate ($CuSO_4.5H_2O$) with few drops of concentrated sulfuric acid. Also, Arsenomolibdate reagent was prepared with dissolving 3 g $Na_2HAsO_4.7H_2O$ and 25 g $(NH_4)_6Mo_7O_{24}.4H_2O$ in 500 ml distilled water.

Different concentrations of sucrose solutions were prepared in acetate buffer solution (pH 5.1) for 10 min at 25 ºC. Than enzyme electrode was placed in test tubes containing sucrose solutions for specific reaction times (2, 4 and 6 min). After removing the electrode, 1 ml sample was taken and 1 ml Nelson's reagent was added to terminate the reaction. The sample was then placed in boiling water bath for 20 min. than cooled to room temperature. Then 1 ml arsenomolybdate reagent was added to each test tube. Finally 7 ml distilled water added again to every test tube. After vortexing, absorbance values for the blank and the substrate solutions were determined at 540 nm. One unit of invertase activity was described as the amount of enzyme required to release 1 µmol glucose from sucrose per minute at pH 5.1 and 25 ºC. Nelson method is described in Figure 11 below.

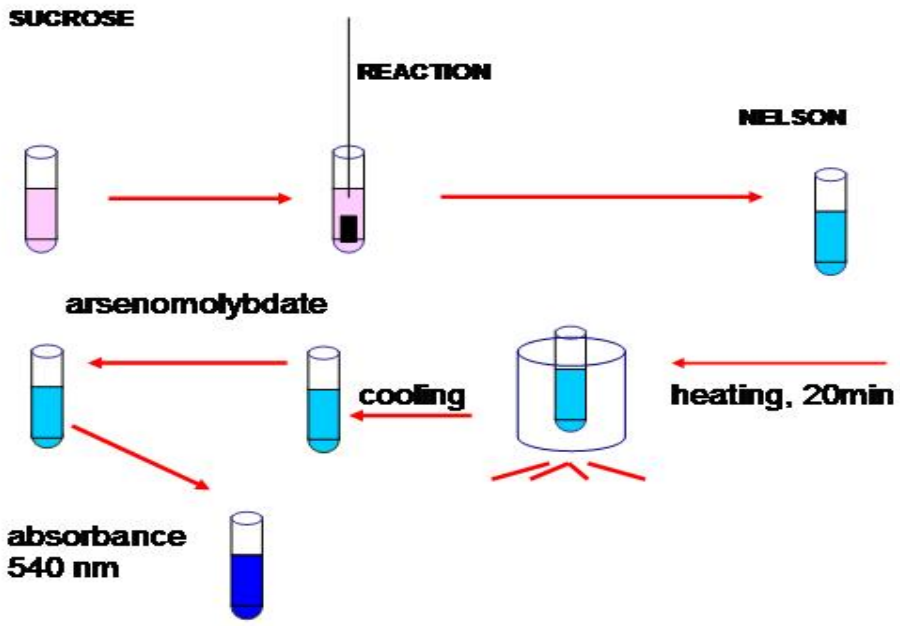


Figure 11. Nelson method

2.3.2.4. Determination of Kinetic Parameters

In order to determine maximum speed of the enzymatic reaction (V_{max}) and the affinity between substrate and enzyme named as Michaelis–Menten constant (K_m) for immobilized enzyme, activity assay was performed for different concentrations of sucrose.

2.3.2.5. Determination of Optimum pH and Temperature values

Solutions with pH value changing between 2.0 and 11.0 were prepared at a constant sucrose concentration to define the optimum pH value.

Optimum temperature values were determined by changing incubation temperature between 10 ºC and 80 ºC at a constant concentration of sucrose solutions which were

prepared in acetate buffer solution (pH 5.1).

The remaining of the both procedures was the same as invertase activity determinations.

2.3.2.6. Operational Stability

The way of determining the operational stability of immobilized enzymes proceeded at a constant concentration of sucrose, optimum pH and temperature conditions. Repeated enzyme activity measurements were done in order to determine the stability of electrodes. Operational stability of immobilized enzymes was tested by performing 40 activity assays in one day.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Amperometric alcohol biosensor based on poly(TBTD)

The redox enzyme alcohol oxidase was immobilized in TBTD/PEDOT and PEDOT to construct an amperometric biosensor. Effect of polymerization time, conducting polymer effect, effect of thickness studies were performed and these studies reveal that conducting polymer based biosensor has better efficiencies than the biosensor without TBTD.

3.1.1 Effect of electropolymerization time

Electropolymerization has the advantages such as using different monomers or supporting electrolytes and the control of film thickness by arranging the amount of charge passed. The most proper electrochemical method for characterization is cyclic voltammetry. Cyclic voltammograms before and after the electropolymerization of TBTD on the platinum electrode were shown in Figure 12a and polymerization of TBTD is given in Figure 12b. TBTD was well characterized in previous studies [27].

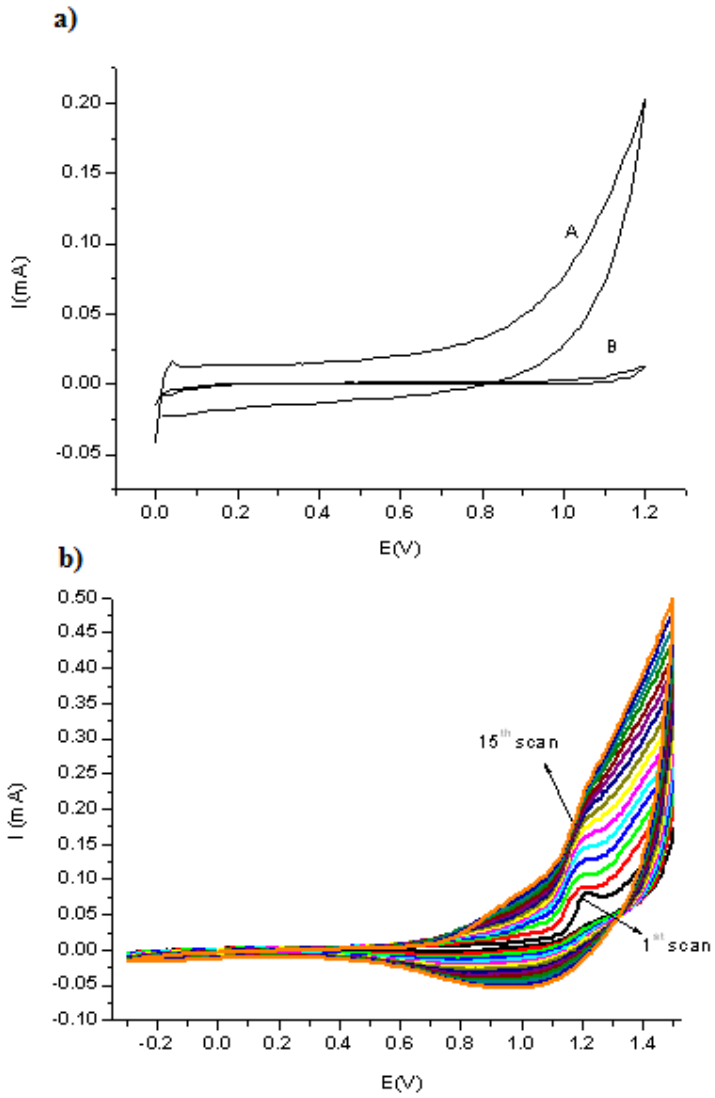


Figure 12. **a) Cyclic voltammograms of (B) bare Pt electrode and (A) after electrochemical polymerization of TBTD**

b) Polymerization of TBTD.

3.1.2 Optimization of TBTD thickness

Electropolymerization time specifies the thickness of the polymer on the electrode. The total charges involved in the film formation and the scan numbers were measured after 5, 10, 15, and 20 min of electropolymerization.

The thickness can be measured in terms of charge, obtained with the number of scans. To optimize the thickness of TBTD on the electrode, four different electrodes were prepared with different thicknesses. As shown in Figure 12, the optimum thickness of TBTD was provided with 15 scans. Further experiments were executed with 15 cycle polymerization of TBTD which refers to 96 mC (ca.2 µm).

Electropolymerization time is precisely correlated with the thickness of the polymer on the electrode. The thickness can be determined with different number of scans and can be measured in terms of charge during the deposition of conducting polymer.

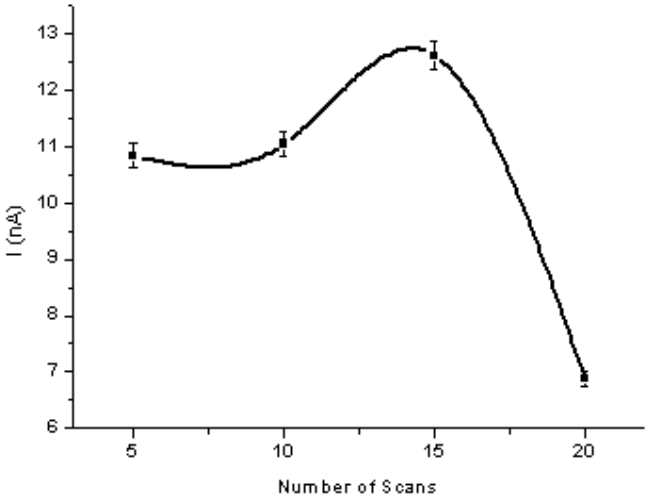


Figure 13. Optimization of TBTD thickness according to effect of number of scan on the biosensor response

3.1.3 Optimization of PEDOT amount

As shown in Table 2, it is clear that when the working electrode was coated using 1.00×10^{-2} coulombs during electropolymerization, biosensor activity was obtained in the maximum performance. Therefore, 1Q PEDOT was used in all of the following experiments.

Table 2. Kinetic parameters for AOX biosensor

Deposited Charges (C)	Biosensing Response (nA)
0.5Q (5×10^{-3} C)	0.05 nA
1Q (1.00×10^{-2} C)	110 nA
2Q (2.00×10^{-2} C)	0.21 nA

3.1.4 pH Optimization of the Biosensors

The effect of pH on biosensing response was optimized by arranging the pH between 6.0 and 8.0 by using phosphate buffer (50mM). The biosensing response of the sensor at pH between 6.0 and 8.0 was shown in Figure 14. pH 7.0 was chosen as the optimum pH due to its maximum biosensing response. Further experiments were conducted with this pH value.

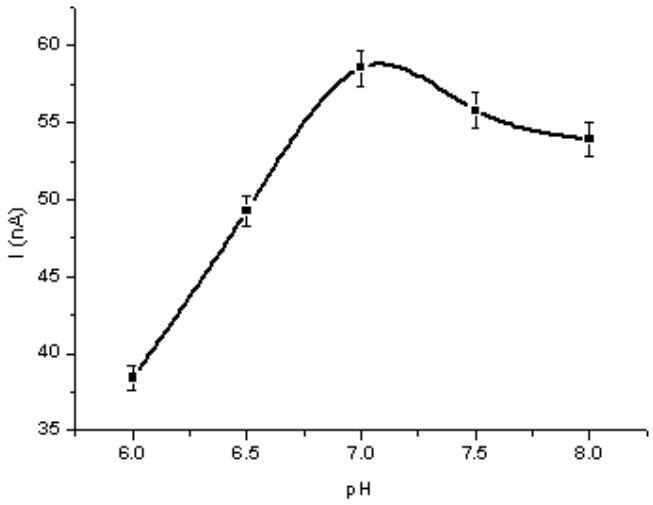


Figure 14. Effect of pH on AOX immobilized TBTD/PEDOT electrodes

3.1.5 Analytical approach for poly(TBTD) and AOX based biosensor

Alcohol oxidase biosensor was prepared as mentioned in the experimental part to analyze the analytical characteristics based on optimized conditions. As the substrate concentration increased, there was also an increase in amperometric current signal. Linear dynamic ranges and the equations were obtained based on optimized conditions for immobilized AOX in PEDOT on TBTD coated surface and also for PEDOT matrix. For the biosensing system including TBTD/PEDOT matrix, a linear calibration graph was obtained for current density versus substrate concentration between 0.02 and 1.7 mM ethanol. A linear relation was defined by the equation of $y = 42.066x + 10.058$ ($r^2 = 0.9921$) (y is the sensor response in current density (nA/cm^2) and x is the substrate concentration in mM).

The other type of biosensing system including PEDOT was also prepared and a linear calibration graph was obtained for current density versus substrate concentration between 0.02 and 1.7 mM ethanol. A linear relation was defined by the equation of $y = 10.733x + 2.8104$ ($r^2 = 0.9915$) (y is the sensor response in current density ($nA/cm^{2)}$ and x is the substrate concentration in mM) (Fig 15).

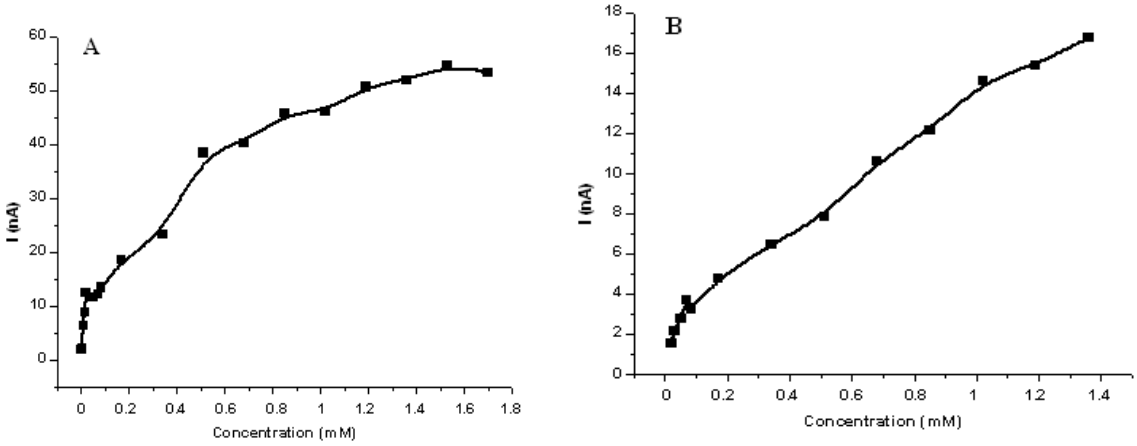


Figure 15. Calibration curves for A: TBTD/PEDOT and B: PEDOT matrice

From the Lineweaver-Burk plots (1/current response vs 1/concentration), kinetic parameters K_m and I_{max} for the enzyme biosensors were found. I_{max}, K_m and sensitivity (I_{max}/K_m) were calculated for the two biosensors. Results are given in Table 3.

Table 3. Kinetic parameters for AOX biosensor

	Imax(nA)	K_m (mM)	Imax/ K_m (nA/mM)
PEDOT/AOX	12	0.14	85.71
TBTD/PEDOT/AOX	55	0.27	203.70

As seen from the results, I_{max} increased when TBTD conducting polymer was present on the electrode surface. When the surface was modified with conducting polymer, the signal increased almost 5 times.

K_m is a measure of the affinity of enzyme for its substrate and it is inversely proportional to the affinity. K_m was also altered due to a change of electron transport to the electrode surface.

It can easily be seen from the results that I_{max}, K_m and sensitivity increased when the conducting polymer TBTD was coated on the Pt electrode surface. TBTD behaves as mediator and that increases the signal.

3.2 Spectrophotometric invertase biosensor based on *poly(SNBS)*, Invertase

The sucrose enzyme invertase was immobilized in SNBS/pyrrole matrix and Polypyrrole matrix to construct a spectrophotometric biosensor. Electropolymerization of the SNBS with pyrrole was performed in a typical three electrode cell by applying constant potential electrolysis at room temperature.
Determination of invertase activity, determination of Kinetic parameters, determination of optimum pH and temperature values and operational stability studies were performed and that studies reveal that conducting polymer based biosensor has better efficiencies than the biosensor without SNBS.

3.2.1 Morphologies of the immobilization matrices

Scanning electron microscopy is a technique that is used to study the surface characteristics of the films before and after the enzyme immobilization. With the use of this method morphologies of enzyme electrodes were identified. This indicates the most certain and comparative information on the enzyme matrices. The films were washed before analysis to remove unbound enzymes. Morphology of polymer matrix was detected and compared with some other studies performed with other polymers. As it is seen from Fig. 16 the morphology of poly(SNBS) is more compact compared to the other studies with different polymers. Also, SEM morphologies show that there are much more pores in poly(SNBS) meaning that more product rise to the surface. Therefore, amount of measured product increases with the raise of porosity number proportionally. This finding agrees with the kinetic parameters which show that the affinity of enzyme in poly(SNBS) is lower. The enzyme substrate complex can leave immediately, max velocity is high while affinity is low and that increases the amount of consisted product.

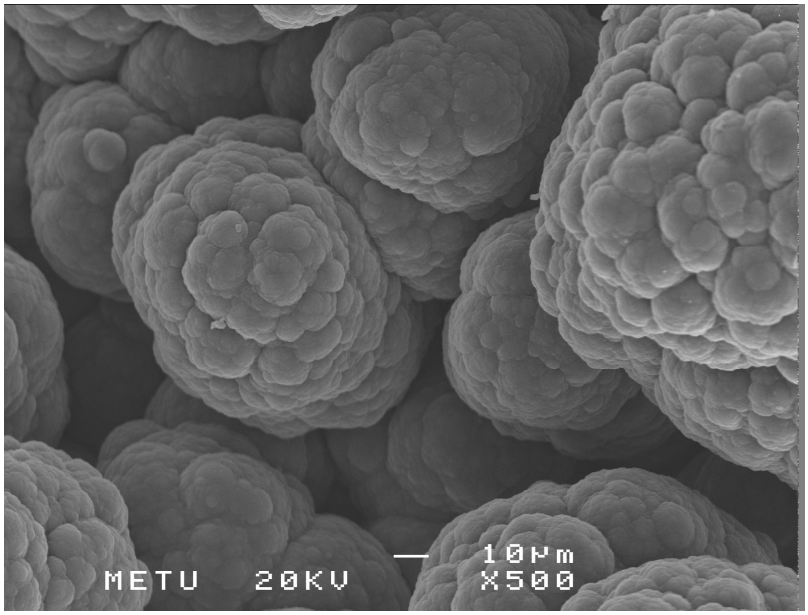


Figure 16. SEM image of polymer 1 with invertase, K_m 15 mM [49].

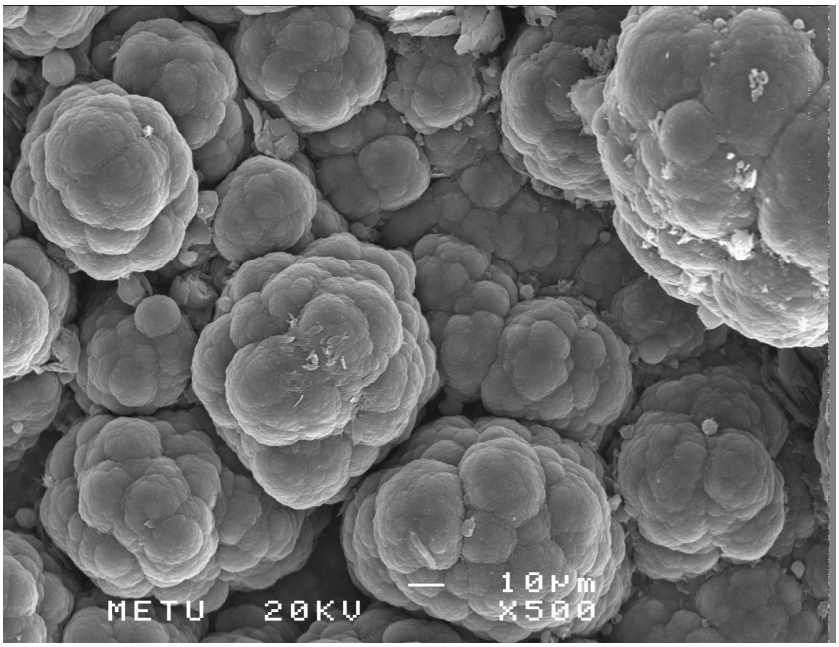


Figure 17. SEM image of polymer 2 with invertase, K_m 40 mM [9].

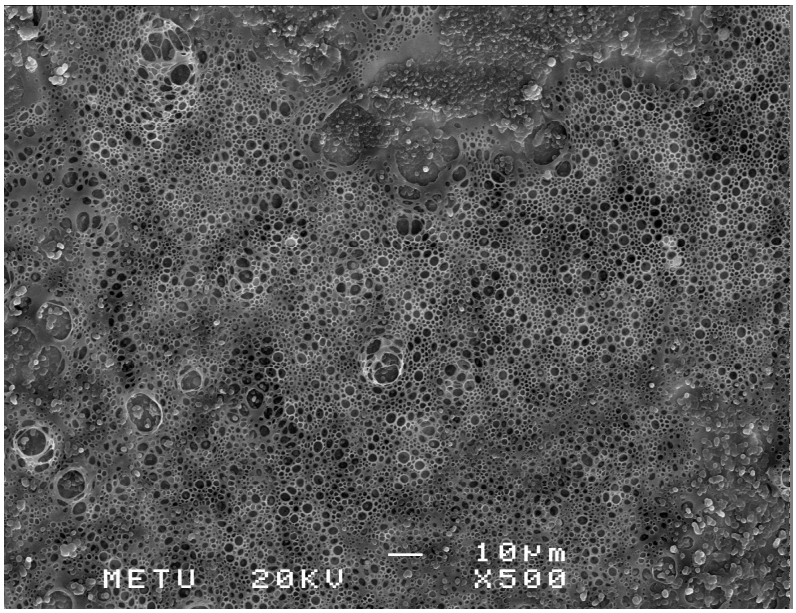


Figure 18. SEM image of poly(SNBS)with invertase, K_m 65 mM [84].

3.2.2 Kinetic parameters of immobilized invertase

The substrate concentration is increased until a constant rate of product formation is achieved so as to determine the maximum velocity of an enzymatic reaction, This is identified as V_{max}, the maximum velocity, of the enzyme. Half of the maximum velocity used for determining the substrate concentration is called K_m which shows the affinity between the substrate and enzyme. There is an opposite relation between the affinity and the K_m.

The maximum reaction rate, V_{max}, and Michealis–Menten constant, K_m were determined from Lineweaver–Burk plots. Kinetic parameters for immobilized invertase activity are given in Table 4. The results show that there is an increase in K_m values when invertase immobilized in copolymer matrix compared with that of the invertase immobilized in polypyrrole matrix. Thereby, the interaction between the substrate and the enzyme is stronger for PPy than the one for the SNBS polymer since lower K_m means that the affinity between enzyme and substrate is higher.

Moreover, there is an increase in V_{max} values. At this point it can be concluded that whenever the enzyme and substrate come together, they produce the product immediately and leave each other.

Table 4. Kinetic Parameters for Invertase biosensor

	V_{max} (µmol/min Electrode)	K_m (mM)
PPy / Invertase	0,3	2,8
Copolymer /Invertase	9,5	65

3.2.3 Effect of pH on enzyme activity

As long as enzymes are very sensitive molecules, they can easily be denaturized when they are removed from their natural medium. Therefore, the pH of the immobilization is very important. During the immobilization of invertase in conducting copolymer matrix, protons are released due to pyrrole polymerization giving rise to the decrease of the pH of the medium. A buffer solution must be used in order in order to prevent denaturation of the enzyme.

Since the isoelectric point of invertase is pH 4.6, acetate buffer with pH 5.1 was used to maintain the enzyme with a negative charge. Therefore, enzyme can easily diffuse to the surface of the electrode. pH behavior with respect to enzyme activity is shown

in Fig. 17. The maximum activity of polymer was observed at pH 5. Between the pH 5 and pH 7, there is a decrease because enzyme is in loose microenvironment and can easily feel the environmental changes around isoelectric point. As a result, the sudden drop of pH is quite normal due to the isoelectronic point.

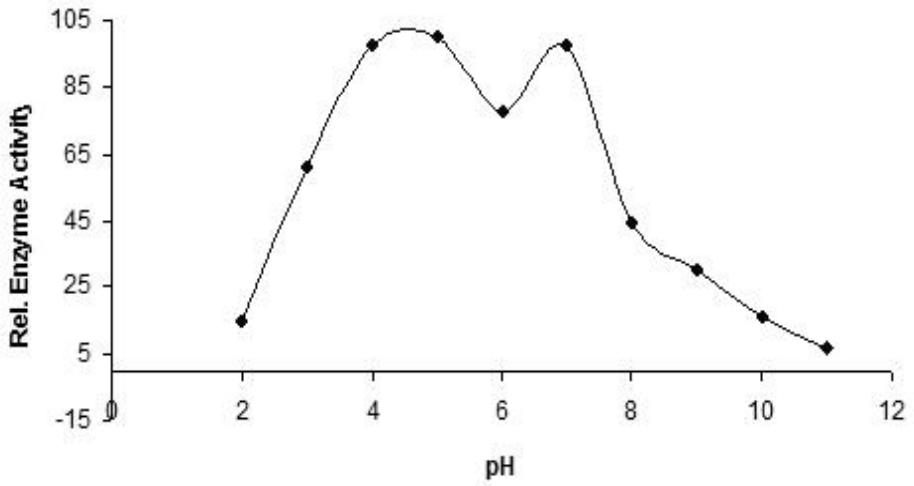


Figure 19. pH stability of enzyme immobilized in copolymer of SNBS.

3.2.4 Effect of temperature on enzyme activity

Enzymes are sensitive to temperature changes. The effect of temperature on enzyme activity was studied and illustrated in Figure 18.

The maximum enzyme activity was found to be at 40 ºC. Also the electrode can be used in a wider temperature range. After 70 ºC, the response of invertase in polymer matrix decreases and denatures completely at 80 ºC. Enzyme preserves is activity with only 20% lost in the interval of 10 ºC and 60 ºC.

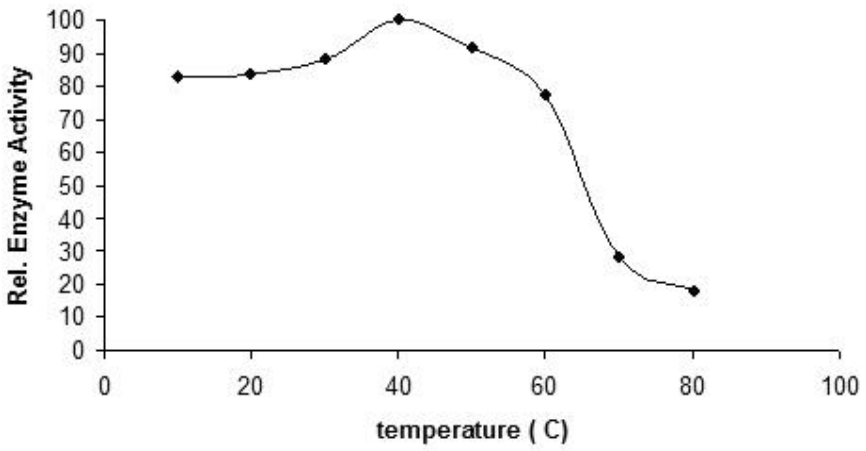


Figure 20. Temperature stability of enzyme immobilized in copolymer of SNBS.

3.2.5 Operational stability of the enzyme electrode

Operational stability studies were performed with taking forty measurements for immobilized invertase per one day. Enzyme activity is very good as shown in Figure 19. The changes are due to enzymes' being very mobile. Polymer matrix reveals 80 % activity even after 40 measurements. Experiments for operational stability of immobilized enzyme display very small losses in activity and have a great stability where only a 20 % of activity loss was observed.

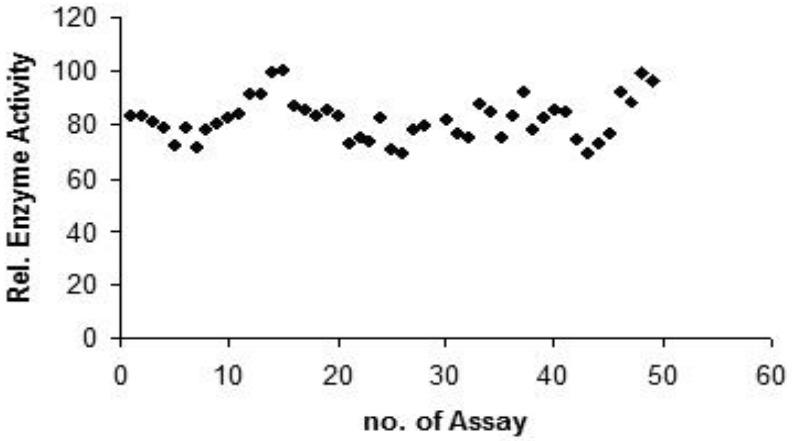


Figure 21. Operational stability of enzyme immobilized in copolymer of SNBS.

CHAPTER 4

CONCLUSION

In this thesis, two different biosensors based on conducting polymers of (4,7-dithien-2-yl-2,1,3-benzothiadiazole) (TBTD) and 1-benzyl-2,5 di(thiophene-2-yl)-1H-Pyrrole (SNBS) were prepared.

Electrochemical technique was used for the synthesis of conducting polymers. Two different immobilization techniques were used for immobilizing enzyme onto the conducting polymer matrices. The proposed biosensors were characterized and optimized. Optimum pH, thickness, enzyme amount were determined and linearity, repeatability, operational stability experiments were performed.

In the first part of the thesis the redox enzyme, alcohol oxidase was immobilized in TBTD/EDOT bilayer and PEDOT to construct an amperometric biosensor. Kinetic parameters, I_{max}, K_m and sensitivity were calculated as 12 nA, 0.14 mM and 85.71 nA/mM for AOX immobilized in PEDOT while they were found as 55 nA, 0.27 mM and 203.70 nA/mM for AOX immobilized in TBTD/EDOT. Optimization of TBTD thickness, optimization of PEDOT amount and optimum pH were also determined. The biosensor was well characterized analytically both in the presence and absence of TBTD. TBTD effect was well seen with these studies hence biosensor coated by TBTD has better efficiency than the one without TBTD. This biosensor provides a bioactive surface showing better current responses. The study shows that TBTD coated biosensor has better efficiencies.

In the second part of the thesis Invertase enzyme immobilized in SNBS copolymer constructs a spectrophotometric biosensor. Kinetic parameters, pH, temperature dependencies and operational stability optimizations were determined. Immobilization of invertase was achieved by electrochemical polymerization. Kinetic parameters (V_{max}, K_m) and optimum parameters such as pH, temperature and operational stability were investigated for immobilized invertase electrode of polymer.

The immobilization was carried out using sodium dodecylsulfate (SDS) as the supporting electrolyte and pyrrole as the comonomer for the synthesis of immobilization matrices. The copolymer reveal good results in terms of V_{max}, K_m values, optimum pH, optimum temperature and operational stability. K_m value is increased and determined as 65 mM meaning that lower K_m means that the affinity between enzyme and substrate is higher. The interaction between the substrate and the enzyme is stronger for PPy than the one for the SNBS polymer. V_{max} value is determined as 9,5 μmol/min.Electrode meaning that whenever the enzyme and substrate come together, they produce the product immediately and leave each other. Invertase reveals maximum activity at 40 ºC and; pH 5 for polymer matrix respectively. Furthermore, a different morphology in the presence of invertase was observed for the matrice. The parameters show that this new conducting polymer of 1-benzyl-2,5 di(thiophene-2-yl)-1H-pyrrole (SNBS) can be used successfully as the immobilization matrices for invertase.

Conducting polymers have attracted much attention due to providing suitable matrices for biological materials. Various numbers of papers on the advantages of using CPs for novel catalytic surfaces have been published. The use of conducting polymers with particular properties with the immobilized biological systems enables to develop novel biomicroelectronic devices. In the future, biosensors based on CPs would be gradually more miniaturized due to the flexibility of electrodeposition with in micro or nano order. Moreover, it can be possible to obtain microbial or enzymatic sensors in required scope with the appropriate immobilization method.

REFERENCES

[1] T. Vo-Dinh, B. Cullum, Biosensors and biochips: Advances in biological and medical diagnostics, Journal of Analytical Chemistry, 366 (2000) 540.

[2] M. Gerard, A. Chaubey, B.D. Malhotra, Application of conducting polymers to biosensors, Biosensors and Bioelectronics, 17 (2002) 345-359.

[3] S. Tuncagil, S. Kıralp, G. Bayramoglu, M.Y. Arıca, L. Toppare, L-Dopa synthesis using tyrosinase immobilized on magnetic beads, Journal of Molecular Catalysis B: Enzymatic, 58 (2009) 187.

[4] S. B. Adeloju and G. G. Wallace, Conducting polymers and the bioanalytical sciences: New Tools for biomolecular communications, Analyst, 21 (1996) 699-703.

[5] A. Sassolas, L.J. Blum, B.D. Leca-Bouvier, Immobilization strategies to develop enzymatic biosensors, Biotechnology Advances, xxx (2011) xxx.

[6] J. Wang, Electrochemical biosensors: Towards point-of-care cancer diagnostics, Biosensors and Bioelectronics, 21 (2006) 1887.

[7] S. Ahmad, A. Anwar, M. Saleemuddin, Immobilization and stabilization of invertase on cajanus cajan lectin support, 79 (2001) 121-127.

[8] S. Tuncagil, S. Varis, L. Toppare, Design of a biosensor based on 1-(4-nitrophenyl)-2,5-di(2-thienyl)-1*H* pyrrole, Journal of Molecular Catalysis B: Enzymatic, 64 (2010) 195-199.

[9] S. Celebi, E. Ibibikcan, S. Kıralp Kayahan, B. Yigitsoy, L. Toppare, Immobilization of invertase in copolymer of 2,5-di(thiophen-2-yl)-1-p-tolyl-1H pyrrole with pyrrole, Journal of Macromolecular Science, Part A, 46 (2009) 739-744.

[10] L. Gómez, H.L. Ramírez, M.L. Villalonga, J. Hernández,, R. Villalonga, Immobilization of chitosan-modified invertase on alginate-coated chitin support via polyelectrolyte complex formation, Enzyme microbial technology, 38 (2006) 22-27.

[11] Q. Shi, P. Wang, Y. Jiang J.Kan Biocatalysis and Biotransformation. 27 (2009) 54-59.

[12] S.H. Lim, J. Wei, J. Lin, Q. Li, J.K. You, A glucose biosensor based on electrodeposition of palladium nanoparticles and glucose oxidase onto nafion-

solubilized carbon nanotube electrode, Biosensors and Bioelectronics, 20 (2005 2341-2346.

[13] S.K. Sharma, N. Sehgal, A. Kumar, Biomolecules for development of biosensors and their applications, Current Applied Physics, 3 (2003) 307–316.

[14] J.C. Vidal, E. Garcia Ruiz, J.R. Castillo, Recent Advances in electropolymerized conducting polymers in amperometric biosensors, Microchim. Acta, 143 (2003) 93-111.

[15] A.L. Sharma, R. Singhal, A. Kumar, R. Krishan, K. Pande, B.D. Malhotra, Immobilization of glucose oxidase onto electrochemically prepared poly(aniline-co-fluoroaniline) films, Inc. J Appl Polym Sci 91 (2004) 3999-4006.

[16] D.R. Thevenot, K. Toth, R.A. Durst, G.S. Wilson, Electrochemical biosensors: recommended definitions and classification, Biosensors & Bioelectronics, 16 (2001) 121–131.

[17] T. Ikeda, K. Kano, An electrochemical approach to the studies of biological redox reactions and their applications to biosensors, bioreactors and biofuel cells, Journal of Bioscience and Bioengineering, 92 (2001) 9-18.

[18] A. Ramanavičius, A. Ramanavičienė, A. Malinauskas, Electrochemical sensors based on conducting polymer—polypyrrole, Electrochimica Acta 51 (2006) 6025–6037.

[19] Z. Zhao, W. Lei, X. Zhang, B. Wang, H. Jiang, ZnO-Based Amperometric enzyme biosensors, Sensors, 10 (2010) 1216-1231.

[20] J. Wang, Electroanalysis and Biosensors, Analytical Chemistry, 67 (1995) 487-492.

[21] J. Wang, Amperometric biosensors for clinical and therapeutic drug monitoring: a review, Journal of Pharmaceutical and Biomedical Analysis, 19 (1999) 47.

[22] T. Hamano, Y. Mitsuhashi, N. Kojima, N. Aoki, M. Semma, Y. Ito, Y. Oji, Enzymic method for the amperometric determination of nicotinic acid in meat products, Analyst, 120 (1995) 135-138.

[23] A.A. Kayakin, O.V. Gitelmacher, E.E. Kayakina, Prussian Blue-Based First-Generation Biosensor. A Sensitive Amperometric Electrode for Glucose, Analytical Chemistry, 67 (1995) 2419-2423.

[24] M. Trojanowicz, T.K. Krawczyk, Electrochemical Biosensors Based on

Enzymes Immobilized in Electropolymerized Films, Mikrochimica Acta, 121 (1995) 167-181.

[25] J.G. Guan, Y.Q. Miao, Q.J. Zhang, Impedimetric Biosensors[, Journal of Bioscience and Bioengineering, 97 (2004) 219-226.

[26] T. Palmer, Understanding Enzymes, Prentice Hall London, Book: 4th ed. 1995

[27] C. Ko, W. Chen, C. Tsai, W. Jane, C. Liu, J. Tu, Paenibacillus campinasensis BL11: A wood material-utilizing bacterial strain isolated from black liquor, Bioresource Technology, 98 (2007) 2727-2733.

[28] G. Sanjay, S. Sugunan, Invertase immobilised on montmorillonite: reusability enhancement and reduction in leaching, Catalysis Communications, 6 (2005) 81-86.

[29] Z. Balcı, U. Akbulut, L. Toppare, S. Alkan, U. Bakir, Y. Yagci, Immobilization of yeast cells in several conducting polymer matrices. Journal of Macromolecular Science-Pure and Applied Chemistry, 39(3) (2002) 183-197.

[30] S. B. Adeloju and G. G. Wallace, Conducting polymers and the bioanalytical sciences: New Tools for Biomolecular Communications, *Analyst*, 21 (1996) 699-703.

[31] M. Trojanowicz, Application of conducting polymer in chemical analysis, Microchim Acta, 143 (2003) 75-91.

[32] R. Monosik, M. Stredansky, J. Tkac, E. Sturdik, Application of Enzyme Biosensors in Analysis of Food and Beverages, Food Anal. Methods, DOI 10.1007/s12161-011-9222-4.

[33] TM. Canh Biosensors. London: Chapman & Hall; 1993.p. 46.

[34] Z. Zhao, H. Jiang, Enzyme-based Electrochemical Biosensors, Biosensors, Source: Biosensors, Book edited by: Pier Andrea Serra, ISBN 978-953-7619-99-2, pp. 302, (2010) 1-22.

[35] M. Hnaien, F. Lagarde, N. Jaffrezic-Renault, A rapid and sensitive alcohol oxidase/catalase conductometric biosensor for alcohol determination, Talanta, 81 (2010) 222-227.

[36] A.M. Azevedo, D. Miguel, F. Prazeres, J.M.S. Cabral, L.P. Fonseca, Ethanol biosensors based on alcohol oxidase, Biosensors and Bioelectronics 21 (2005) 235–247.

[37] T. Zima, L. Fialov, O. Mestek, M. Janebov, J. Crkovska, I. Malbohan, S. Stipek, L. Mikulfkova, P. Popov, Oxidative stress, metabolism of ethanol and

alcohol-related diseases, Journal of biomedical science, 8 (2001) 59-70.

[38] M.G. Neuman, J.A. Haber, I.M. Malkiewicz, R.G. Cameron, G.G. Katz, N.H. Shear, Ethanol signals for apoptosis in cultured skin cells, Alcohol 26 (2002) 179–190.

[39] H.B. Yıldız, L. Toppare, Biosensing approach for alcohol determination using immobilized alcohol oxidase, Biosensors and Bioelectronics, 21 (2006) 2306-2310.

[40] M. M. Barsan, C. M.A. Brett, An alcohol oxidase biosensor using PNR redox mediator at carbon film electrodes, *Talanta*, 74 (2008) 1505-1510.

[41] H. Gulce, A. Gulce, M. Kavanoz, H. Coskun, A. Yıldız, A new amperometric enzyme electrode for alcohol determination, Biosensors and Bioelectronics 17 (2002) 517-521.

[42] O. Smutok, B. Ngounou, H. Pavlishko, G. Gaydaa, M. Gonchar, W. Schuhmann, A reagentless bienzyme amperometric biosensor based on alcohol oxidase/peroxidase and an Os-complex modified electrodeposition paint, Sensors and Actuators B: Chemical, 113 (2006) 590-598.

[43] H. Zhou, H. Liu, Q.S. Li, H.J. Zhao, W. Li, Alcohol enzyme electrode, *Fenxi-Huaxue*, 21 (1993) 1471.

[44] A. G. Prada, N. Pen˜a, M.L. Mena, A.J. Reviejo, J.M. Pingarro´n, Graphite–Teflon composite bienzyme amperometric biosensors for monitoring of alcohols, Biosensors and Bioelectronics. 18 (2003) 1279.

[45] X. Jiang, Y. Wu, X. Mao, X. Cui, L. Zhu, Amperometric glucose biosensor based on integration of glucose oxidase with platinum nanoparticles/ordered mesoporous carbon nanocomposite, Sensors and Actuators B: Chemical, 153 (2011) 158-163.

[46] F. Mizutani, S. Yabuki, Rapid determination of glucose and sucrose by an amperometric glucosesensing electrode combined with an invertase/mutarotase attached measuring cell, Biosensors & Bioelectronics, 12 (1997) 1013-1020.

[47] J. Liu, X. Xu, L. Tang, G. Zeng, Determination of trace mercury in compost extract by inhibition based glucose oxidase biosensor, Transactions of Nanferrous Metals Societyof China, 19 (2009) 235-240.

[48] G. Fortter, E. Brassard, D. Belanger, Fast and easy preparation of an amperometric glucose biosensor. Biotechnology Techniques, 2 (1988) 177.

[49] S. Tuncagil, S. Kıralp, S. Varis, L. Toppare, Immobilization of invertase on a conducting polymer of 1-(4-nitrophenyl)-2,5-di(2-thienyl)-1H-pyrrole, Reactive & Functional Polymers 68 (2008) 710–717.

[50] M. Rebroš, M. Rosenberg, Z. Mlichová, Ľ. Kristofikova, Hydrolysis of sucrose by invertase entrapped in polyvinyl alcohol hydrogel capsules, Food Chemistry, 102 (2007) 784-787.

[51] C.C Liu, L.C. Huang, C.T. Chang, H.Y. Sung, Purification and characterization of soluble invertases from suspension-cultured bamboo (Bambusa edulis) cells, Food Chemistry, 96 (2006) 621-631.

[52] R.A. Leigh, T. Rees, W.A. Fuller, J. Banfield, The Location of Acid Invertase Activity and Sucrose in the Vacuoles of Storage Roots of Beetroot (Beta vulgaris), 178 (1979) 539-547.

[53] H.S. Lee, A. Sturm, Purification and Characterization of Neutral and Alkaline Invertase from Carrot, Plant Physiol. 112 (1996) 1513-1522.

[54] C. M. Endo, A. Kobayashi, T. Noda, S. Takigawa, H. Yamauchi, M. Mori, Changes in sugar content and activity of vacuolar acid invertase during low-temperature storage of potato tubers from six Japanese cultivars, J Plant Res, 117 (2004) 131–137.

[55] D. Bagal, M.S. Karve, Entrapment of plant invertase within novel composite of agarose–guar gum biopolymer membrane, Analytica Chimica Acta, 555 (2006) 316-321.

[56] R. H. Friend, R. W. Gymer, A. B. Holmes, J. H. Burroughes, R. N. Marks, C. Taliani, D. D. C. Bradley, D. A. Dos Santos, J. L. BreÂdas, M. LoÈgdlund & W. R. Salaneck, Electroluminescence in conjugated polymers, Nature, 397 (1999) 121-128.

[57] Y. Xia, J. Luo, X. Deng,X. Li, D. Li, X. Zhu, W. Yang, Y. Cao, Novel Random Low-Band-Gap Fluorene-Based Copolymers for Deep Red/Near Infrared Light-Emitting Diodes and Bulk Heterojunction Photovoltaic Cells, Macromolecular Chemistry and Physics, 207 (2006) 511-520.

[58] S. Jin, T.H. Tiefel, R. Wolfe, R.C. Sherwood, J.J. Mottine, Optically transparent, electrically conductive composite medium, Science, 255 (1992) 446-448.

[59] A. Balan, D. Baran, N.S. Sarıciftci, L. Toppare, Electrochromic device and bulk heterojunction solar cell applications of poly 4,7-bis(2,3-dihydrothieno[3,4-

b][1,4] dioxin-5-yl)-2-dodecyl-2H-benzo[1,2,3]triazole (PBEBT), Solar Energy Materials & Solar Cells, 94 (2010) 1797-1802.

[60] J.C. Gustafsson, O. Inganas, A.M. Anderson, Conductive polyheterocycles as electrode materials in solid state electrochromic devices, Synthetic Metals, 62 (1994) 17-21.

[61] K. Gurunathan, D.P. Amalnerkar, D.C. Trivedi, Synthesis and characterization of conducting polymer composite (PAn/TiO_2) for cathode material in rechargeable battery, Materials Letters, 57 (2003) 1642-1648.

[62] G. Gustafsson, Y. Cao, G.M. Treacy, F. Klavetter, N. Colaneri, A.J. Heeger, Flexible light-emitting diodes made from soluble conducting polymers, Letters to Nature, 357 (1992) 477-479.

[63] Y. Coskun, A. Cirpan, L. Toppare, Conducting polymers of terepthalic acid bis-(2-thiophen-3-yl-ethyl) ester and their electrochromic properties, Polymer., 45 (2004) 4989–4995.

[64] U. Lange, N.V. Roznyatovskaya, V.M. Mirsky, Conducting polymers in chemical sensors and arrays, Analytica Chimica Acta., 614 (2008) 1-26.

[65] F. Zhang, M. Johansson, M.R. Andersson, J.C. Hummelen, O. Inganas, Polymer Photovoltaic Cells with Conducting Polymer Anodes, Advanced Materials, 14 (2002) 662-665.

[66] K.D. Kreuer, On the development of proton conducting polymer membranesfor hydrogen and methanol fuel cells, Journal of Membrane Science, 185 (2001) 29-39.

[67] S. Geetha, C.R.K. Rao, M. Vijayan, D.C. Trivedi, Biosensing and drug delivery by polypyrrole, Analytica Chimica Acta, 568 (2006) 119-125.

[68] Y. Xiao, C.M. Li, S. Yu, Q. Zhou, V.S. Lee, S.M. Moochhala, Synthesis and characterization of p-toluenesulfonate incorporated poly(3,4-ethylenedioxythiophene), Talanta, 72 (2007) 532-538.

[69] N.K. Guimard, N. Gomez, C.E. Schmidt, Conducting polymers in biomedical engineering, Progress in Polymer Science, 32 (2007) 876-921.

[70] G.G. Wallace, G.M. Spinks, L.A.P. Kane-Maguire, P.R. Teasdale, Conductive Electroactive Polymers: Intelligent Materials Systems, 2nd ed., CRC, London, 2003, p.8-10

[71] D. Giri, K. Kundu, Evolution of the electronic structure of cyclic polythiophene upon bipolaron doping, Am Inst Physics, 105 (1996) 11075–80.

[72] O. Atwani, C. Baristiran, A. Erden, G. Sonmez, A stable, low band gap electroactive polymer: Poly(4,7-dithien-2-yl-2,1,3-benzothiadiazole), Synthetic Metals, 158 (2008) 83-89.

[73] M. Sendur, A. Balan, D. Baran, B. Karabay, L. Toppare, Combination of donor characters in a donor–acceptor–donor (DAD) type polymer containing benzothiadiazole as the acceptor unit, Organic Electronics, 11 (2010) 1877-1885.

[74] E. Bakker, M. Telting-Diaz, Electrochemical sensors, Analytical Chemistry, 74 (2002) 2781.

[75] F. Palmisano, D. Centonze, P.G. Zambonin, An in situ electro synthesized amperometric biosensor based on lactate oxidase immobilized in a poly-o-phenylenediamine film: determination of lactate in serum by flow injection analysis, Biosensensors and Bioelectronics, 9 (1994) 471–479.

[76] G.E.D. Benedett, F. Palmisano, P.G. Zambonin, Flow-through tyrosinase enzyme reactor based on reticulated vitreous carbon functionalized by an electrochemically synthesized film. Analytica Chimica Acta, 326 (1996) 149–154.

[77] T. Ahuja, I.A. Mir, D. Kumar, Rajesh, Biomolecular immobilization on conducting polymers for biosensing applications, Biomaterials, 28 (2007) 791-805.

[78] C. Kitamura, S. Tanaka, Y. Yamashita, Design of narrow-bandgap Polymers. Syntheses and properties of monomers and polymers containing aromatic-donor

and o-quinoid-acceptor units, Chemistry of Materials, 8 (1996) 570-578.

[79] S. Tarkuc, E. Sahmetlioglu, C. Tanyeli, I.M. Akhmedov, L. Toppare, Electrochromic properties of a soluble conducting polymer of 1-benzyl-2,5-di(thiophene-2-yl)-1H-pyrrole, Sensors and Actuators B, 121 (2007) 622–628.

[80] M. Dietrich, J. Heinze, Poly(4,4′-dimethoxybithiophene) A new conducting polymer with extraordinary redox and optical properties, Synthetic Metals, 41 (1991) 503-506.

[81] X.S. Wang, X.Q. Feng, X.Q., Journal of Materials Science & Technology, 21 (2002) 715-717.

[82] S. Tuncagil, D. Odaci, S. Varis, S. Timur, L. Toppare, Electrochemical polymerization of 1-(4-nitrophenyl)-2,5-di(2-thienyl)-1 H-pyrrole as a novel immobilization platform for microbial sensing, Bioelectrochemistry, 76 (2009)169.

[83] N.Nelson, A photometric adaptation of the somogyi method for the determination of glucose, College of Medicine, University of Cincinnati, February 3, 1944.

[84] S. Tanrıverdi, S. Tuncagil, L. Toppare, A New Amperometric Alcohol Oxidase Biosensor Based on Conducting Polymer of (4,7-Dithien-2-yl-2, 1,3-benzothiadiazole), Journal of Macromolecular Science, Part A: Pure and Applied Chemistry, 49 (2012)185-190.