AMPEROMETRIC CHOLESTEROL AND ALCOHOL BIOSENSORS BASED ON CONDUCTING POLYMERS

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AMPEROMETRIC CHOLESTEROL AND ALCOHOL BIOSENSORS BASED ON CONDUCTING POLYMERS

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ABSTRACT

AMPEROMETRIC CHOLESTEROL AND ALCOHOL BIOSENSORS BASED ON CONDUCTING POLYMERS

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Cholesterol and ethanol biosensors based on conducting polypyrrole (PPy), poly(3,4-ethylenedioxythiophene) (PEDOT) and poly(3,4-ethylenedioxypyrrole) (PEDOP) were constructed. Cholesterol oxidase (ChOx, from *Pseudomonas fluorescens*) and alcohol oxidase (AlcOx, from *Pichia pastoris*) were physically entrapped during electropolymerization of the monomers (Py, EDOT, EDOP) in phosphate buffer containing sodium dodecylsulfate (SDS) as the supporting electrolyte. The amperometric responses of the enzyme electrodes were measured monitoring oxidation current of H_2O_2 at +0.7 V in the absence of a mediator. Kinetic parameters, such as K_m and I_{max}, operational and storage stabilities, effects of pH and temperature were determined for all entrapment supports. Based on Michaelis-Menten (K_m) constants, it can be interpreted that both enzymes immobilized in PEDOT showed the highest affinities towards their substrates. Before testing the alcohol biosensors on alcoholic beverages,

effects of interferents (glucose, acetic acid, citric acid, L-ascorbic acid) which might be present in beverages were determined. The alcohol content of the distilled beverages (vodka, dry cin, whisky, rakı) was measured with these biosensors. A good match with the chromatography results (done by the companies) was observed.

Keywords: Amperometric Biosensors, Conducting Polymers, Enzyme Electrodes, Cholesterol Biosensors, Alcohol Biosensors

ÖZ

İLETKEN POLİMER TABANLI
AMPEROMETRİK KOLESTEROL VE ALKOL BİOSENSÖRLERİ

Türkarslan, Özlem
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Polipirol (PPy), poli(3,4-etilendioksitiyofen) (PEDOT) ve poli(3,4-etilendioksipirol) iletken polimer matrislerinde *Pseudomonas fluorescens*'tan elde edilen kolesterol oksidaz (ChOx) ve *Pichia pastoris*'ten elde edilen alkol oksidaz (AlcOx) enzimleri fiziksel yolla tutuklanmıştır. Destek elektrolit olarak sodium dodesilsulfatın, monomerin ve enzimin bulunduğu fosfat tampon çözeltisine gerilim uygulanmasıyla monomerler elektrot yüzeyinde polimerleşirken enzimler de hapsolmuştur. Enzim elektrotlarının amperometrik cevapları medyatörsüz ortamda H_2O_2'nin yükseltgenme akımının izlenmesiyle elde edilmiştir. K_m, I_{max} gibi kinetik parametreler, kullanım kararlılığı ve raf ömrü tayinleri, pH ve sıcaklıktaki değişimlerin etkileri tüm tutuklama matrisleri için incelenmiştir. K_m değerlerine dayanılarak PEDOT matrisinde her iki enzimin de sübstratlarına karşı daha ilgili olduğu kanısına varılmıştır. Alkol biosensörleri piyasada bulunan çeşitli alkollü içkilerde sınamadan önce içkilerde bulunabilen ve

sonuçları etkileyebileceği düşünülen glükoz, asetik asit, sitrik asit ve L-askorbik asit içeren ortamlarda test edilmiştir. Daha sonra bu biosensörlerle votka, cin, viski, rakı gibi distillenmiş içkilerde bulunan alkol miktarı tayin edilmeye çalışılmıştır. Bulunan sonuçlar şişelerin üzerinde yazılı çeşitli kromatografik yöntemlerle belirlenmiş sonuçlarla uyum içerisindedir.

Anahtar sözcükler: Amperometrik biosensörler, İletken polimerler, Enzim Elektrotları, Kolesterol biosensörleri, Alkol Biosensörleri

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LIST OF ABBREVIATIONS

Py: Pyrrole

EDOT: 3,4-Ethylenedioxythiophene

EDOP: 3,4-Ethylenedioxypyrrole

PPy: Polypyrrole

PEDOT: Poly(3,4-ethylenedioxythiophene)

PEDOP: Poly(3,4-ethylenedioxypyrrole)

SDS: Sodium dodecylsulfate

ChOx: Cholesterol oxidase

AlcOx: Alcohol oxidase

CHAPTER I

INTRODUCTION

1.1 Conducting Polymers

Conducting polymers (CPs) are the resonance stabilized π-conjugated organic polymers. They are also named as "synthetic metals" since they mimic the electrical, electronic, magnetic and optical properties of metals retaining the ease of chemical and physical modification associated with ordinary polymers.

An intelligent material can be defined as a material capable of recognizing appropriate environmental stimuli, processing the information arising from stimuli and responding to it in an appropriate manner and time frame. The intelligent material systems and structures sense or recognize the stimuli, process the information, convert or store energy, and then actuate or generate response. Conducting polymers are sensitive to numerous stimuli and can be made to respond. Additionally, they can store information and energy and are capable of performing intelligent functions. Hence, they are worldwide used in construction or improvement of intelligent materials systems or structures by many research groups as well as companies (Wallace, et al. 2009). Their unique and practical advantage is the behavior manipulation in situ using appropriate stimuli (Table 1.1).

Table 1. 1 Property changes typically observed upon electrical stimulation to switch CPs between oxidized and reduced states (Wallace, et al. 2009)

Property	Typical change	Potential application
Conductivity	From 10^{-7} to 10^{3} S/cm	Electronic components, sensors
Volume	10%	Electromechanical actuators
Color	300 nm shift in absorbance band	Displays, smart windows
Mechanical	Ductile to brittle transition	-
Ion permeability	From 0 to 10^{-8} mol $cm^{-2}s^{-1}$ in solution	Membranes

1.1.1 Historical Review of Conducting Polymers

Polyacetylene was synthesized in 1958 by Natta and co-workers as a black powder possessing semi-conductor properties depending on how the polymer was processed. The scientific curiosity was elucidated in 1967 by Hideki Shirakawa's group; they succeeded in synthesizing polyacetylene directly in the form of a thin silvery semiconductor film using Zieglar-Natta catalyst nearly a thousand times greater than that usually needed. Upon treating with halogens the conductivity drastically increased. It is discovered that polyacetylene can be converted from an insulator to a semiconductor, to a full metal depending on the concentration of dopant. On the other hand, in University of Pennsylvania Alan Heeger, physicist, and Alan MacDiarmid, chemist, have been working on $(SN)_x$ having strong electronic properties and they have discovered that bromine addition increased the conductivity tenfold. Actually, the inorganic sulfur nitride polymer discovered in 1973 showed properties very close to those of metal; however its explosive nature

prevented it from becoming commercially important (Walatka, Labes and Perlstein 1973). These three scientists cooperated and the Nobel Prize for the year 2000 went to them “for the discovery and development of electrically conducting polymers” (Shirakawa 2001), (MacDiarmid 2002), (Heeger 2001).

Although polyacetylene exhibits very high conductivity in its doped state, it is not stable in open air. Hence, in 1980s polyheterocycles, which were more air stable than polyacetylene, due to lower oxidation potential, were developed. Even if none has exhibited higher conductivity than the parent organic conducting polymer, these polymers have been helpful in designing new functionalized monomers that are soluble and stable (Pratt 2009).

In Figure 1.1 common conducting polymers are shown.

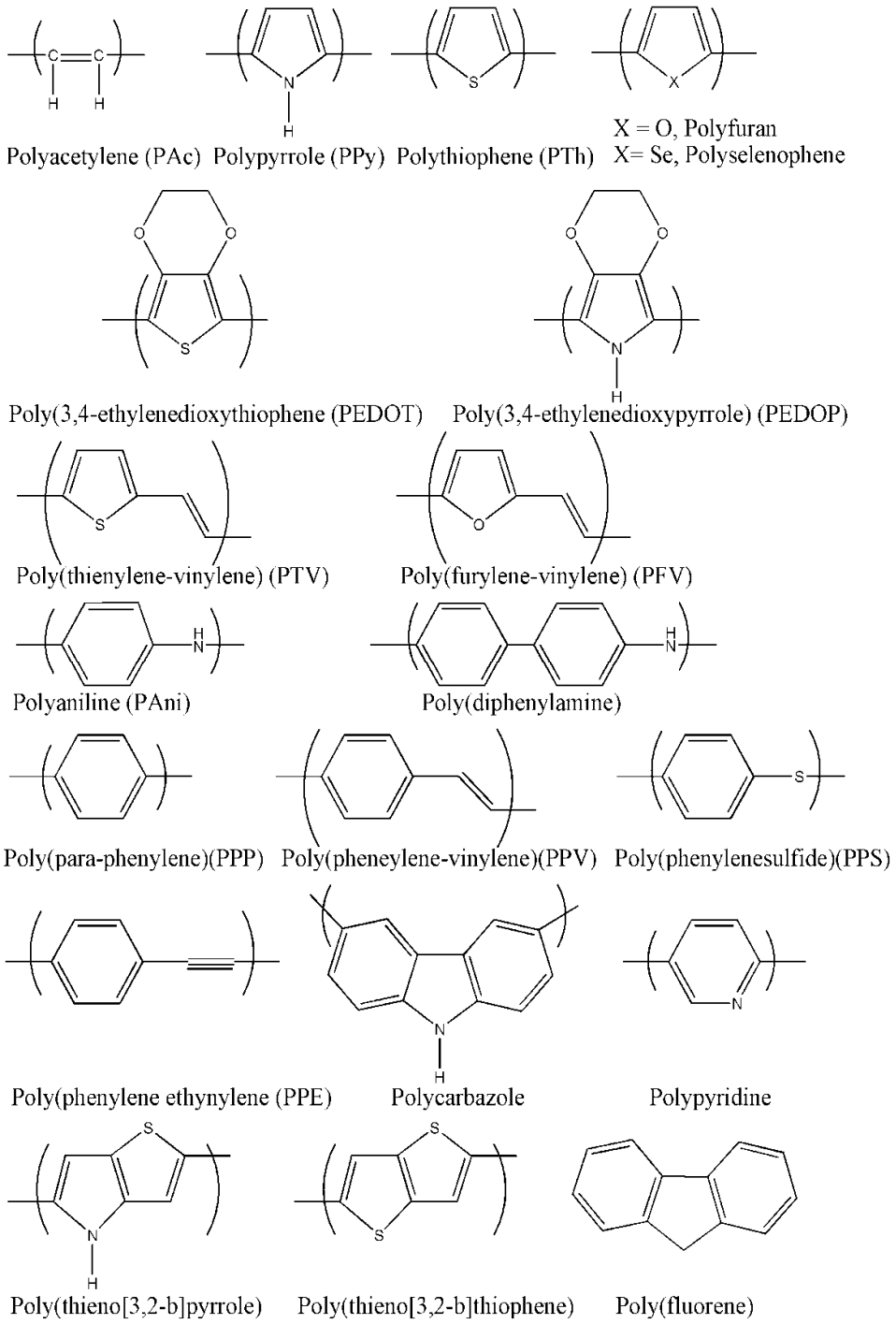


Figure 1. 1 Typical conducting polymer structures

1.1.2 Conduction Mechanism in Conducting Polymers

In conjugated polymers the electronic configuration is different since the chemical bonding leads to one unpaired electron (π-electron) per carbon atom. Moreover, the π-bonding in which the orbitals of successive carbon atoms overlap results in electron delocalization. This electronic delocalization provides the "highway" for charge mobility along the backbone of the polymer chain. The electrical conductivity results from the existence of doping induced charge carriers and their motion along the bonded π-"highway" (Heeger 2001).

1.1.2.1 Conductivity

The movement of charge carriers such as electrons and holes through a medium (metal, polymer, etc.) under influence of an electric field is named as electronic conduction. Conductivities of some metals and polymers are displayed in Figure 1.2.

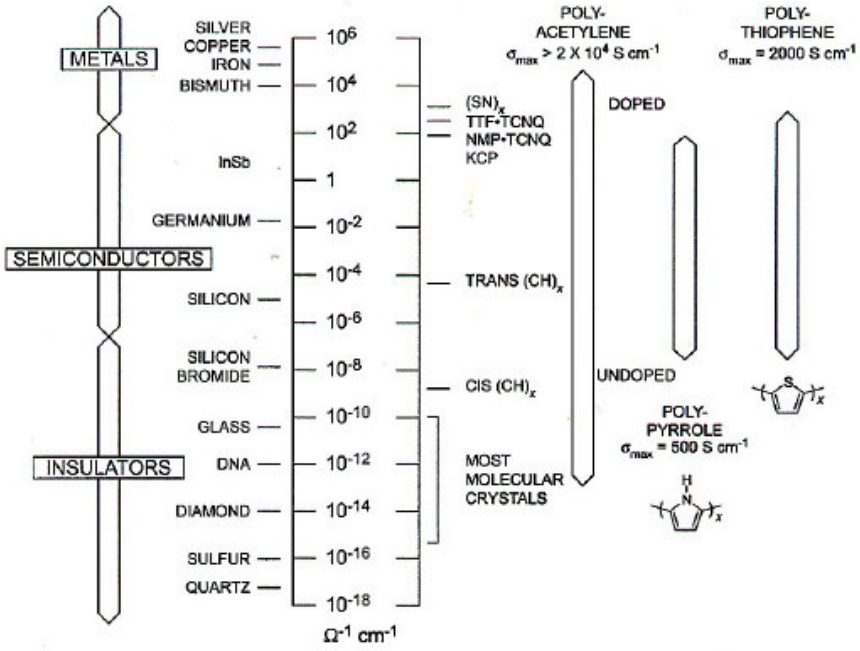


Figure 1. 2 Conductivities of some metals, semiconductors and insulators

1.1.2.2 Band Theory

When molecular orbitals are formed from two atoms, each type of atomic orbitals gives rise to two molecular orbitals. When *n* atoms are used, the same approach results in *n* molecular orbitals. Since the number of atoms is large, the number of orbitals and energy levels with closely spaced energies is also large. The result is a band of orbitals of similar energy, rather than the discrete energy levels of small molecules. These bands contain the electrons from the atoms. The highest energy band containing electrons is called the "valence band"; the next higher empty band is called the "conduction band". In a material with filled valence band and a large energy difference between the highest valence band and the lowest

conduction band, this "band gap" (E_g) prevents motion of the electrons and the material is an insulator, with the electrons restricted in their motion. In a material with partially filled orbitals, the valence band-conduction band distinction is blurred and very little energy is required to move some electrons to higher energy levels within the band. As a result, they are free to move throughout the matrix, as are the holes (electron vacancies) left behind in the occupied portion of the band. This material is "conductor" of electricity because the electrons and the holes are both free to move. A "semiconductor" has much higher conductivity than an insulator and much lower conductivity than a conductor (Miessler and Tarr 1999). Figure 1.3 shows the band structures of insulators, semiconductors and conductors.

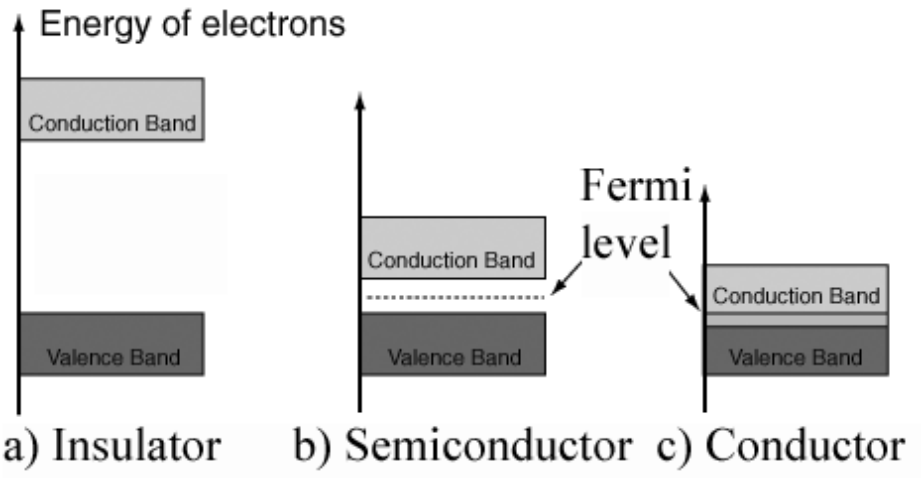


Figure 1. 3 Band structures of a) an insulator, b) a semiconductor and c) a conductor (Hyperphysics 2009)

"Fermi level" (E_f) is the energy at which electrons are equally likely to be in each of the two levels and is near the middle of the band gap. In a

semiconductor, this level may be raised or lowered upon “n-type doping” or “p-type doping”, respectively (Miessler and Tarr 1999). P-type doping creates an abundance of holes, whereas in n-type doping an abundance of mobile or “carrier” electrons in the material is produced as shown in Figure 1.4.

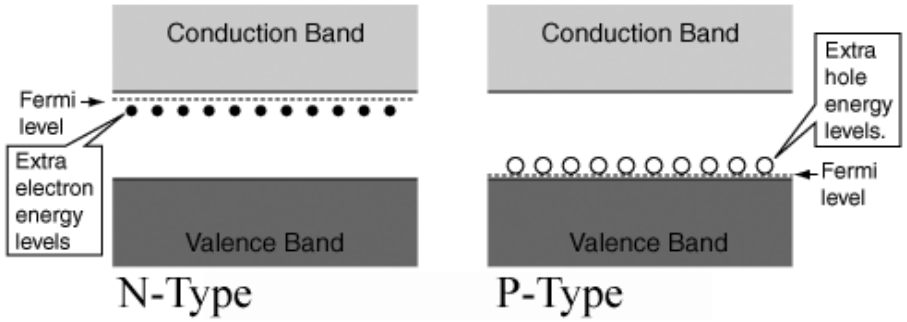


Figure 1. 4 Bands for n-type and p-type semiconductors (Hyperphysics 2009)

1.1.2.3 Doping

The electrical conduction properties of elemental semiconductors, such as silicon, can be controlled via addition of foreign atoms. The host semiconductor can be made “n type” or “p type” depending on the nature of the added dopant atoms, i.e. whether the latter has an excess or deficit of electrons. New dopant energy levels are introduced into the band gap and conduction is facilitated. The conductivity level attained strongly depends on dopant concentration. Since doping results in excess generation of electrons or holes, conductivity is enhanced. However, the doping mechanism in macromolecules differs considerably from that observed for

elemental semiconductors. Doping levels attained in conjugated polymers can be as large as 10 mole percent. Additionally, there is a charge transfer between the incorporated dopant atom and the polymer chain, hence the latter is partially oxidized or reduced (Lyons 1994).

The partial oxidation of the polymer chain is termed “p-doping”. The process involves removal of electrons to form a positively charged repeat unit:

$$(P)_x + xyA^- \longrightarrow \left[(P^{y+})A_y^-\right]_x + xye^-$$

where P represents the basic monomeric repeat unit in the polymer. The partial reduction of the polymer chain is called “n-doping”:

$$(P)_x + xye^- + xyM^+ \longrightarrow \left[M_y^+ (P^{y-})\right]_x$$

The partial oxidation or deduction of the backbone can be achieved either electrochemically via potential application or chemically using oxidizing/reducing agent in gas or solution phase (Lyons 1994).

Initially, the high conductivity increase observed upon doping organic polymers was thought to result from the formation of unfilled electronic bands. It was simply assumed that upon p-type or n-type doping, electrons were respectively removed from the top of the valence band or added to the bottom of the conductance band, analogous to inorganic semicondcutors. However, this assumption was challenged by the discovery of polyacetylene, polyparaphenylene and polypyrrole: The conductivities of these polymers are not associated with unpaired electrons but rather with spinless charge carriers (Bredas and Street 1985).

As shown in Figure 1.5 a, in a polymer a vertical ionization process $E_{IP\text{-}V}$ (a process whereby an electron is removed from a molecule in its ground or excited sate so rapidly that a positive ion is produced without change in the positions of the atoms (IUPAC 1997)) creates a hole on top of the valence band. Around the charge created a local distortion of the lattice

takes place. The localized electronic states in the gap due to upward shift Δε of the HOMO and the downward shift of the LUMO appear (Figure 1.5 b). Upon electron removal (oxidation) the ionization energy is lowered by an amount Δε. Polaron, a radical ion associated with a lattice distortion, is created and the presence of localized electronic states in the gap referred to as polaron states. A second electron removal forms bipolaron. A bipolaron is defined as a pair of like charges associated with a strong local lattice distortion. The electronic band structure corresponding to the presence of one bipolaron is depicted in Figure 1.6, the electronic states appearing in the gap for a bipolaron is further away from the band edges than for a polaron (Bredas and Street 1985).

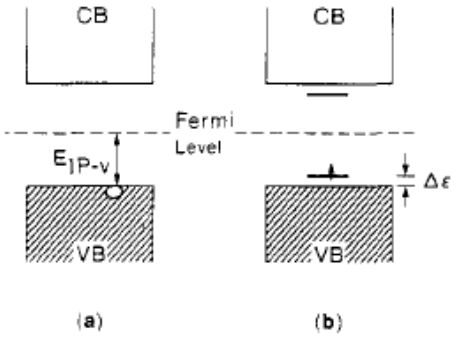


Figure 1. 5 Illustration of the band structure of a polymeric chain in the case of (a) a vertical ionization process and (b) the formation of a polaron. The chemical potential, or Fermi level, is taken as reference level (Bredas and Street 1985)

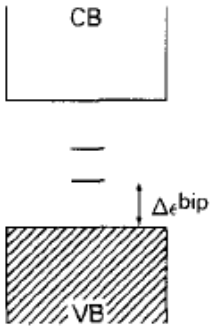


Figure 1. 6 Band structure of a polymer chain containing one bipolaron (Bredas and Street 1985)

As seen in Figure 1.7, in the undoped state, the PPy band gap is 3.2 eV. Taking an electron out of the chain leads to the formation of a polaron whose associated quinoid-like geometry relaxation extends over about four pyrrole rings. When a second electron is taken out of the chain, the energetically favorable species is a bipolaron which also extends over four pyrrole rings. The geometry relaxation is stronger than in the polaron case. Further doping results in overlapping between the bipolaron sates and formation of bipolaron bands. The band gap is widened due to the fact that the bipolaron states coming in the gap are taken from the valence band and conductance band edges. This evolution is supported by ESR measurements: at low doping the signal grows indicating polaron formation with spin ½, at intermediate doping the signal saturates and then decreases as a result of bipolaron formation, at high doping no signal is observed demonstrating that the charge carriers in that regime are spinless (Bredas and Street 1985). Oxidation states are shown in Figure 1.8.

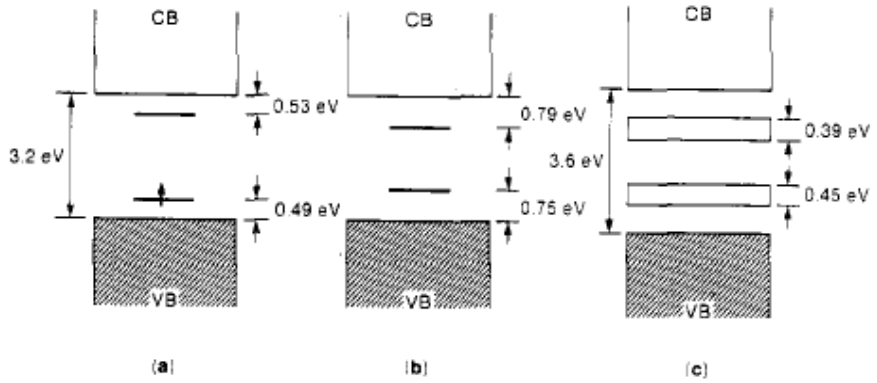


Figure 1. 7 Evolution of the polypyrrole band structure upon doping: (a) low doping level, polaron formation; (b) moderate doping level, bipolaron formation; (c) high (33 mol %) doping level, formation of bipolaron bands (Bredas and Street 1985)

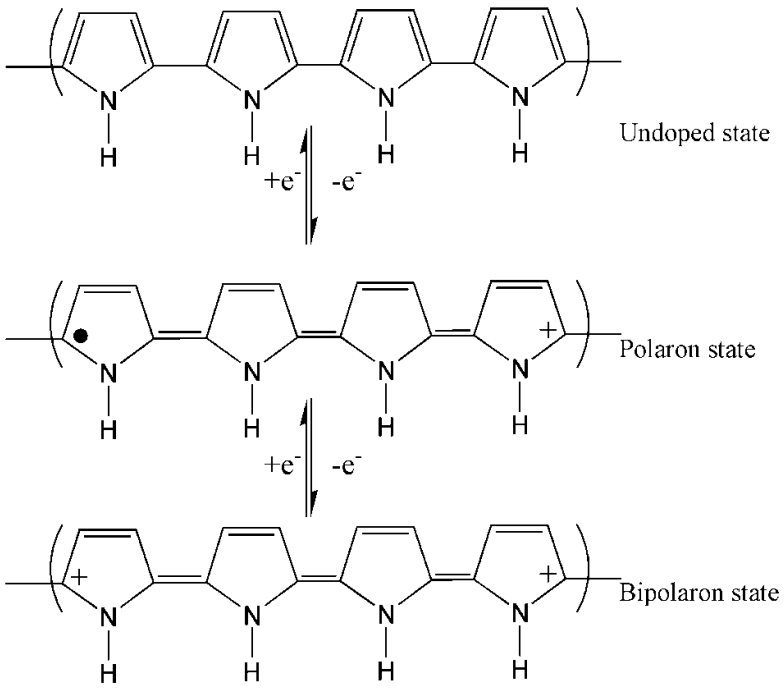
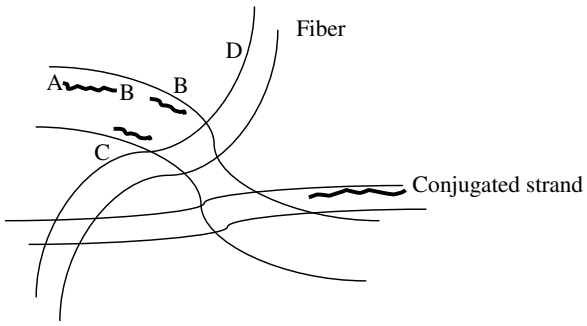


Figure 1. 8 Oxidation states of polypyrrole

Doping agents or dopants are either strong reducing agents or strong oxidizing agents. They may be neutral molecules and compounds or salts which can be easily form ions, inorganic/organic dopants or polymeric dopants. The nature of dopants plays an important role in the stability and physical properties of polymer films (Kumar and Sharma 1998).

1.1.2.4 Hopping

Charge carriers (solitons, polarons, bipolarons) are defects that are delocalized over a number of repeat units on the polymer chain. In conducting polymer matrices there can be a carrier transport within a conjugated strand, from strand to strand, and if the polymer morphology is fibrous, from fiber to fiber as illustrated in Figure 1.9. The intrinsic conductivity refers to conduction processes within a strand (Lyons 1994). It is governed by the physics of conjugated double bonds, in other words it can be improved by ensuring that the polymer chains are well-aligned and contain minimum defects.



[AB] Intrachain

[BC] Interchain

[CD] Interfiber

[AD] Macroscopic charge transfer

Figure 1. 9 Charge transport processes (microscopic and macroscopic) in conjugated polymers

1.1.3 Synthesis of Conducting Polymers

Conducting polymers can be synthesized using standard methods of polymerization including conventional as well as specific routes such as Witting, Horner and Grignard reactions, polycondensation processes and metal or enzyme catalyzed reactions (Kumar and Sharma 1998).

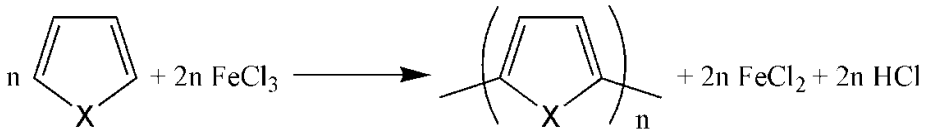
The following polymerization techniques can be applied (Kumar and Sharma 1998):

1. Chemical polymerization
2. Electrochemical polymerization
3. Photochemical polymerization
4. Metathesis polymerization
5. Concentrated emulsion polymerization
6. Inclusion polymerization

7. Solid-state polymerization
8. Plasma polymerization
9. Pyrolysis
10. Soluble precursor polymer preparation

1.1.3.1 Chemical Polymerization

Chemical polymerization (oxidative coupling) is the most useful technique to prepare large amounts of conducting polymers. The monomers can be polymerized using $FeCl_3$, one of the most common oxidizing agents as indicated below (Toshima and Hara 1995):



X = N-H, S, O

Chemical polymerization mechanism was investigated in 1990s and a feasible polymerization mechanism for 3-alkyl thiophenes was developed on the basis of crystal structure of $FeCl_3$ and quantum chemical computations of thiophene derivatives. The polymerization is hypothesized to proceed through a radical mechanism rather than a radical cation mechanism (Niemi, Knuuttila and Österholm 1992). According to Niemi et. al. in solid $FeCl_3$, the iron (III) ions are mostly hidden within the crystal and chemically inert, additionally each chloride ion is coordinated to two iron (III) ions. On the other hand, at the surface some chloride ions are coordinated to only one iron (III) ion; consequently each iron (III) at the surface of the crystal has one unshared chloride ion and one free orbital. Hence, the active sites in polymerization are the iron (III) ions at the surface

of the crystal with strong Lewis acid character because of the one free orbital. The chloride ion that is no longer coordinated to the iron (III) ion capture a proton from the radical cation forming HCl molecule. The suggested mechanism is shown in Figure 1.10.

Solution or spin casting as well as vacuum deposition can be used to obtain polymer films after chemical polymerization if conducting polymers synthesized are soluble in common solvents.

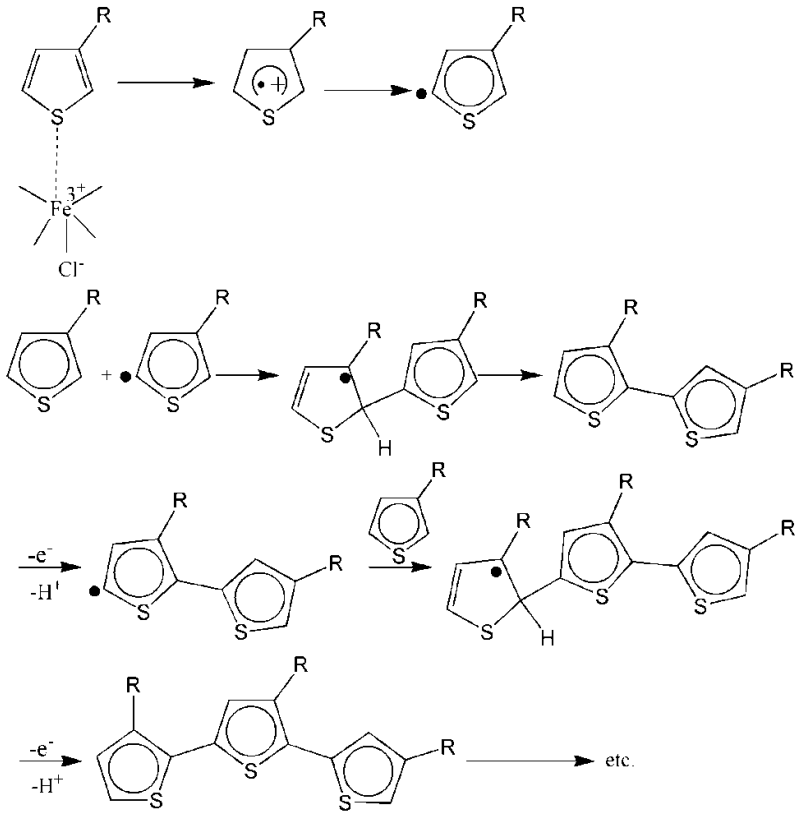


Figure 1. 10 Chemical polymerization via radical mechanism

1.1.3.2 Electrochemical Polymerization

Electrochemical polymerization is carried out in a single or dual compartment cell by adopting a standard three electrode configuration in an appropriate solvent containing both supporting electrolyte and monomer. Electrochemical polymerization can be performed either potentiometrically to obtain thin films or galvanostatically to obtain thick films. Polymer films thickness or geometry can be controlled via monitoring charge passed. Furthermore, during synthesis the conducting polymer is simultaneously doped (Kumar and Sharma 1998).

The electroactive monomer can be anodically electropolymerized in the absence of a catalyst onto the electrode surface enabling in situ characterization of the polymer by electrochemical and/or spectroscopic techniques. On the other hand, cathodic synthesis is also possible; however, the polymer produced in its neutral insulating form which leads rapidly to a passivation of the electrode and limits attainable film thickness (Roncali 1992).

Figure 1.11 represents the mechanism proposed for the eelctropolymerization of cylopentadienyls. In the first electrochemical step (E) the monomer is oxidized to its radical cation. Since the electron-transfer reaction is much faster than the diffusion of the monomer from the bulk solution, radicals are highly concentrated near the electrode surface. The second step (chemical (C)) involves the coupling of two radicals to produce a dihydro dimer dication which leads to a dimer after loss of two protons and re-aromatization. However, radical cation-radical cation is also possible (Figure 1.12). Due to the applied potential, the dimer, which is more easily oxidized than the monomer, becomes radical and undergoes a further coupling with a monomeric radical. Electropolymerization proceeds then through successive electrochemical and chemical steps according to a

general E(CE)n scheme until the oligomer becomes insoluble in the electrolytic medium and coated on the electrode surface (Roncali 1992).

Electrosynthesis conditions, such as solvent, supporting electrolyte, monomer concentration, cell geometry, nature and shape of the electrodes, temperature and applied electrical conditions, determine the structure and properties of the resulting polymer.

The increase in conductivity is correlated to decrease in oxidation potential and to bathochromic shift of the absorption maximum. Those changes are indicative of an extension of conjugation. The mean effective conjugation length along the polymer chain is determined by two parameters: the stereoregularity of the polymer, i.e. the ratio of α-β' and α-α' linkages (Figure 1.13), and the planarity of the conjugated π-system. The α-β' linkages result in branching and also generate distortions in adjacent chains modifying electronic distribution. These linkage errors increase morphological disorder and decrease the conductivity.

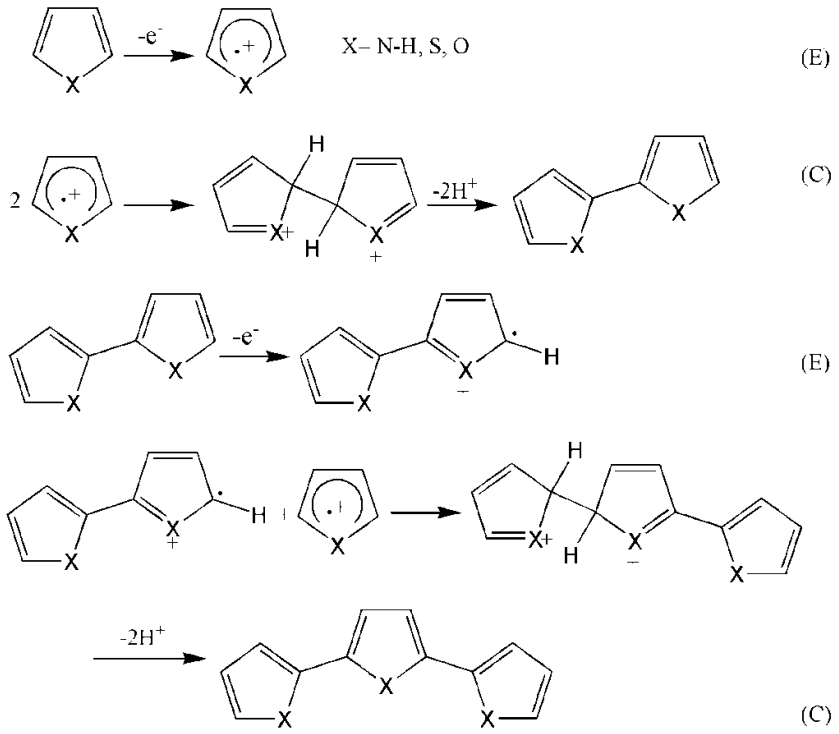


Figure 1. 11 Electropolymerization mechanism of cyclopentadienyls

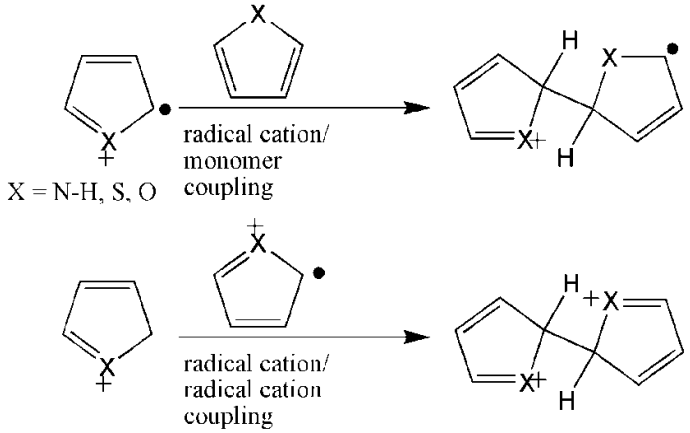


Figure 1. 12 Radical-cation/monomer and radical-cation/radical-cation couplings

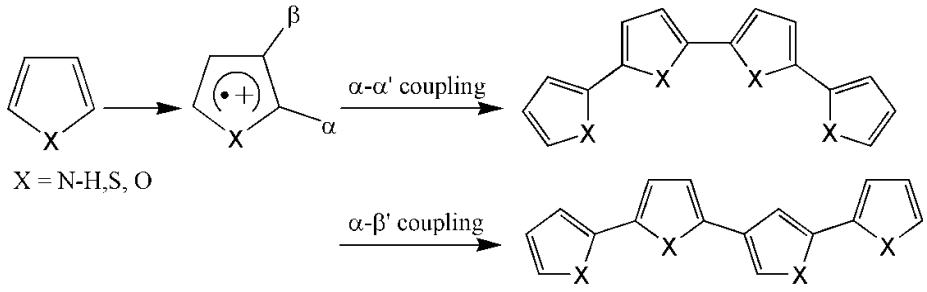


Figure 1. 13 Competitive reactions pathways in the electropolymerization of cyclopentadienyls

1.1.4 Applications of Conducting Polymers

Applications utilizing the polymers' inherent conductivity

Conducting polymers are investigated for shielding of electromagnetic interference (EMI) since they are strong absorbers of electromagnetic radiation over a wide frequency range (Geetha, et al. 2009). The semiconductor polymers are also utilized in antistatic applications (Kim, et al. 2008). Additionally, they are used in plastic chips (Ziemelis 1998).

Applications in energy storage and conversion

Conducting polymers have potential applications in energy storage (Liu and Lee 2008) and supercapacitor productions (Subramania and Devi 2008).

Applications in polymer photovoltaics

Photosensitive conducting polymers are used in solar cells since they can be manufactured very cheaply and can be applied to very large areas (Hoppe and Sariciftci 2008).

Applications in display technologies

Conducting polymers with appropriate derivatizations are utilized in polymer light-emitting diodes that emit the primary colors: red, green, blue (Dai 2002).

Applications in electrochromics

Another interesting application that uses the dynamic properties of thin conducting polymer films is electrochromic devices based on color change upon potential application (Mortimer, Dyer and Reynolds 2006).

Applications in electromechanical actuators

More futuristic applications of conducting polymers include electromechanical applications, in other words artificial muscles in view of the fact that they can change their physical dimensions as a response to electrical signal (NASA 2009).

Applications in separation technologies

The dynamic character of conducting polymers is used in membrane technology; they can be stimulated in situ using small electrical pulses to trigger the transport of ions (Wang, et al. 1990).

Applications in controlled-release devices

Conducting polymers are also ideal hosts for the controlled release of chemical substances including therapeutic drugs (Lira and Cordoba de Torresi 2008) by incorporating target species as the dopant and using the redox chemistry to release the target species at the desired time.

Applications in corrosion protection

Conducting polymer coatings protect metals from corrosion (Armelin, et al. 2008).

Applications in chemical sensors and biosensors

Sensing surfaces are capable of interacting with ions, proteins, vapors that are either electron donors or acceptors etc. The electrical signal can be change in current density, resistance, capacitance etc. (Rahman, et al. 2008).

Applications in micro-electromechanical systems (MEMS)

Conducting polymers can be potentially used in MEMS which are sensor and actuator elements linked by a signal transduction or processing unit (Nisar, et al. 2008).

1.1.5 Characterization of Conducting Polymers

Electrical changes accompanying electrical stimuli are investigated via electrochemical methods. The cyclic voltammetry (CV), the most common method, records a continuous current corresponding to the sequential oxidation-reduction of different oligomeric/polymeric species in the sample. The electrochemical quartz crystal microbalance (EQCM) in situ monitor changes in polymer mass during redox process. Electromechanical analysis (EMA) screen volume changes during cyclic potential steps to determine stress-strain behavior. The molecular interaction capabilities of the polymers are determined via chromatography in which the polymer is used as the stationary phase and a series of molecular probes is utilized. Using dynamic contact angle (DCA) analyses polymer-solvent interaction is determined. Scanning probe microscopy (SPM) allows topographical mapping of the polymer surface. UV-Visible spectroscopy is a useful tool to conduct spectroelectrochemical studies upon potential applications for conducting polymer films deposited on ITO glass. Fourier transform infrared spectroscopy (FTIR) and Raman spectroscopy are utilized to identify and track changes in specific chemical groups. In electron spin resonance (ESR) spectroscopy, absorption of microwaves by molecules causes unpaired electrons to change spin. Consequently, it is used to identify and quantify the presence of polaron charge carriers (Wallace, et al. 2009).

1.1.6 Polypyrroles and Polythiophenes

The heteroaromatic polypyrrole and polythiophene have the disadvantage of being insoluble and infusible. In order to overcome these problems, alkyl, alkoxy substituted derivatives of these polymers have been synthesized. By controlling main-chain architecture (e.g. regioregularity) and pendant group chemistry (e.g. water-soluble sulfonates), a broad variety of properties were made available from the parent systems.

During the second half of the 1980s, scientists at the Bayer AG research laboratories in Germany developed a new polythiophene derivative, poly(3,4-ethylenedioxythiophene), abbreviated as PEDT or PEDOT. Its 3- and 4- positions are blocked to prevent undesired α,β'- and β,β'-couplings within the polymer backbone. This new monomer has lower oxidation potential compared to thiophene and its polymer has lower band gap compared to the parent polymer. Thin PEDOT films are transparent and stable at oxidized state, and blue at neutral state. A water-soluble polyelectrolyte system containing PEDOT and a water-soluble polyelectrolyte, poly(styrene sulfonic acid) (PSS), as the charge-balancing dopant was also developed (Groenendaal, Jonas, et al. 2000), (Groenendaal, Zotti, et al. 2003).

Combining the electron-rich character of polypyrroles with these 3,4-dioxy substitution concepts, 3,4-ethylenedioxypyrrole was synthesized. EDOP oxidizes more easily than EDOT and pyrrole, confirming its electron-rich character and allowing electropolymerization to proceed under quite mild conditions. During the polymerization, an electroactive polymer film forms quickly on the electrode surface. The film is red at neutral sate whereas nearly transparent at oxidized form. PEDOP-PSS blend was prepared by Kodak (Gaupp, et al. 2000), (Zong and Reynolds 2001), (Walczak and Reynolds 2006).

Aqueous-compatible conducting polymers are polymers that are able to undergo electrochemical switching in aqueous media. Polypyrrole, poly(3,4-ethylenedioxythiophene) and poly(3,4-ethylenedioxypyrrole) have aqueous compatibility and hence potential biocompatibility. They can be electropolymerized in aqueous solutions with suitable oxidative potential with concurrent film formation and high electroactivities and conductivities are observed in aqua (Thomas, et al. 2000).

1.2 Enzymes

1.2.1 Enzymes as Proteins

The 20 different amino acids (Figure 1.14) found in living organisms are the building blocks of peptides/proteins and they play important roles in metabolism. Peptides and proteins are macromolecules made up from long chains of amino acids and they are joined head-to-tail via peptide bonds (Figure 1.15). Shorter chains of up to a few hundred amino acids are referred to as peptides; on the other hand, proteins may consist of thousands of amino acids. The sequence of the amino acids within the molecule is essential for the structure and function of peptides/proteins in biological process (Manz, Pamme and Iossifidis 2004).

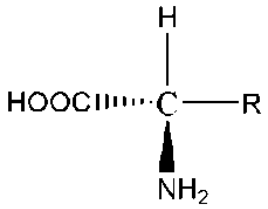


Figure 1. 14 General structure of an amino acid; the substituent group (R) varies from one amino acid to another

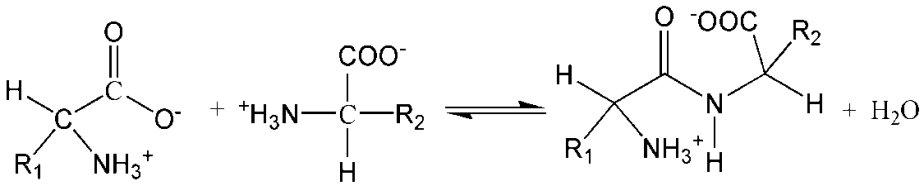


Figure 1. 15 Peptide bond formation from two amino acids

The C-N bond cannot rotate due to its partial double character (Figure 1.16) giving to the peptide unit NH-CO rigidity. However, the bonds to the neighboring α-C atoms can rotate within steric constraints (Figure 1.17) allowing to folding of proteins (Manz, Pamme and Iossifidis 2004).

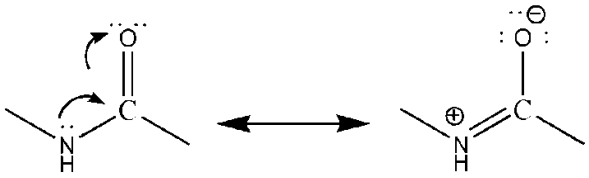


Figure 1. 16 Double bond character of the C-N bond in peptide

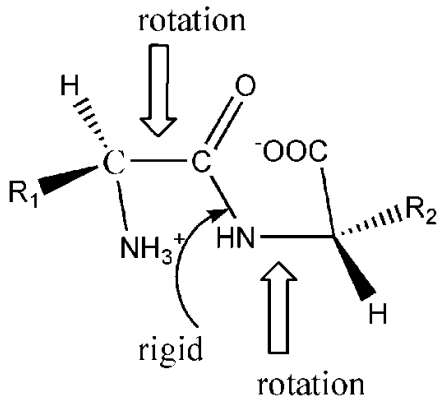


Figure 1. 17 The C-N bond is rigid due to the partial double bond character; rotation is possible within steric constraints around the bonds to the α-C atoms

The three- dimensional structure of a protein is very well defined and essential for it to function. Proteins are found in all forms of living organisms and perform a wide variety of tasks.

There are mainly two types of proteins:

1. Fibrous, elongated proteins which are insoluble in water and provide structural support.

2. Globular, spherical proteins which are water soluble and have specific functions in the immune system and metabolism.

Globular proteins have compact structure with very characteristic grooves and peaks on their surface. Analogous to a key fitting into a lock, other molecules fit into these grooves and peaks. Enzymes and antibodies are example of such specific proteins (Manz, Pamme and Iossifidis 2004).

1.2.2 Zwitterionic Character

Since amino acids contain both acidic and basic functional groups, they are amphoteric. The carboxyl group of an amino acid has a pK_a between 1.8 and 2.5, whereas the amino group has a pK_a between 8.7 and 10.7. As seen in Figure 1.18 at acidic pH's the amino acid is positively charged; on the other hand, at basic pH's it becomes negatively charged. However, at the pH found under physiological conditions, pH 6 to 7, the amino group is ionized to NH_3^+ and the carboxyl group is ionized to COO^-. Hence, the amino acid is zwitterionic (Manz, Pamme and Iossifidis 2004).

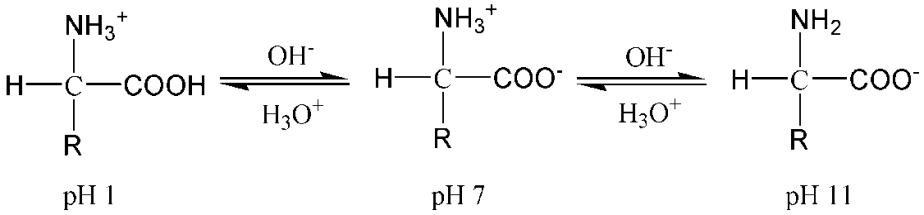


Figure 1. 18 Charge of an amino acid at different pH values; zwitterionic character at pH 7, positive charge at low pH and negative charge at high pH

For every amino acid, there is a specific pH value at which it exhibits no net charge. This is called the isoelectric point, pI. At this point, an amino acid remains stationary in an applied electric field. The isoelectric point can be estimated via the Henderson-Hasselbalch equation:

$pI = ½ (pK_{a,i} + pK_{a,j})$

where $pK_{a,i}$ and $pK_{a,j}$ are the PK_a values of the amino group and the carboxylic acid group, respectively (Manz, Pamme and Iossifidis 2004).

At a neutral pH, most proteins have both positive and negative charges available along the amino acid chain. The opposite charges attract

each other, while like charges repel. Although, the force is not very strong, these repulsion/attraction forces play a significant role in maintaining overall three-dimensional structure of the protein (Mathewson 1998).

1.2. 3 General Properties of Enzymes

Enzymes are biological catalysts that speed biochemical reactions without being permanently changed. Every enzyme is very specific in its action and can increase the rate of one particular reaction or one type of reaction. The name of an enzyme is often formed by adding *ase* to the name of its substrate (Mader 1996). Figure 1.19 compares energy of activation (E_a) when an enzyme is not present to when an enzyme is present; illustrating that enzyme lowers the amount of energy required for activation.

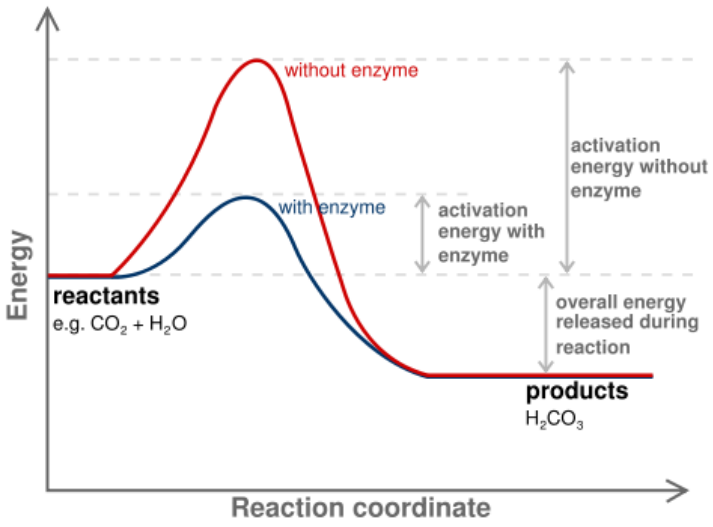


Figure 1. 19 Enzymes, as all catalysts, increase the reaction rate lowering energy of activation (Wikipedia 2009)

Enzyme catalyzed biochemical reactions take place in a specific region of the protein called active site. The characteristics of active sites can be summarized as follows:

1. The active site constitutes a small portion of the overall protein structure
2. The active site is a three-dimensional niche in the protein
3. The specificity of the enzyme depends on the arrangement of atoms in the active site
4. The substrate-enzyme binding process involves a relatively small amount of energy

The mechanism by which the active site takes part in the reaction was first postulated by Emil Fischer in 1860. The specificity of the reaction, according to Fischer, was the result of the "lock and key" fit of the enzyme and substrate. However, in 1962 the "induced fit model" by Daniel Koshland was proposed because the enzyme is induced to undergo a structural rearrangement upon substrate binding to accommodate it more perfectly (Carr and Bowers 1980). The change in shape of the active site facilitates the reaction. After the reaction has been completed, the products are released, and the active site returns to its original state. Only a small amount of enzyme is actually needed in a cell since enzymes are repeatedly used (Mader 1996). Figure 1.20 depicts an enzymatic reaction.

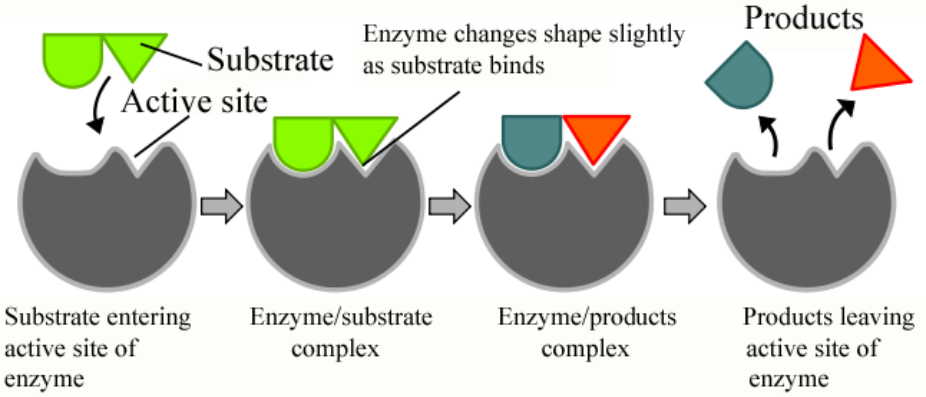


Figure 1. 20 Diagrams to show the induced fit hypothesis of enzyme reaction (Wikipedia 2009)

The decrease in the rate of a reaction brought about by the addition of a substance, inhibitor, is called inhibition. In competitive inhibition (Figure 1.21), another molecule is so close in shape to the enzyme's substrate inhibits the reaction. In noncompetitive inhibition, a molecule binds to an enzyme, but not at the active site, leading to a shift in the three-dimensional structure of the enzyme (Mader 1996).

Denaturation is defined as the breakdown of the numerous interactions which maintain the biologically active conformation. Due to cooperative nature of the forces which sustain the ordered structure, denaturation generally results in essentially random conformation (Carr and Bowers 1980).

Many enzymes require a nonprotein "cofactor" to assist them in carrying out their function. Some cofactors are ions; magnesium (Mg^{2+}), potassium (K^{+}) and calcium (Ca^{2+}) are often involved in enzymatic reactions. Some other cofactors, called "coenzymes", are organic molecules that bind to enzymes and serve as carriers for chemical groups or electrons (Mader 1996).

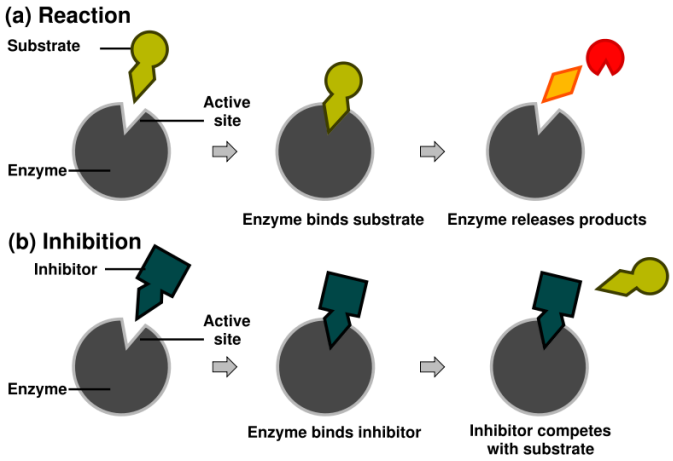


Figure 1. 21 Competitive inhibitors bind reversibly to the enzyme, preventing the binding of substrate (Wikipedia 2009)

1.2.4 Enzyme Classification

The nomenclature was determined by the Enzyme Commission in 1961 (with the latest update in 1992), hence all enzymes are assigned with an *EC* number. Enzymes are classified into six different groups according to the reaction being catalyzed. The systematic name of the enzyme consists of two parts: the first part describes the substrate(s) and the second portion defines the type of reaction catalyzed (Carr and Bowers 1980).

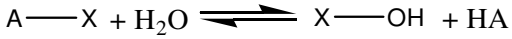
EC 1 ***Oxidoreductases*** catalyze oxidation-reduction reactions; they are also called "oxidases", "dehydrogenases", or "reductases". An electron donor/acceptor is required to complete the reaction (Carr and Bowers 1980).

These are the enzymes concerned with biological redox reactions, and therefore with respiration and fermentation processes. The class includes not only the dehydrogenases and oxidases (1.1 to 1.10) but also peroxidases (1.11), which use H_2O_2 as the oxidant, the hydroxylases

(1.99.1), which introduce hydroxyl groups and the oxygenases (1.99.2), which introduce molecular O_2 in place of a double bond in the substrate. Sub-group 1.1 contains enzymes which convert "CHOH" to "C=O" groups in many alcohols, sugar alcohols, hydroxyacids, sugars and hydroxy-steroids (Dixon and Webb 1964).

EC 2 ***Transferases*** catalyze functional group transfers; these are of the general form (Carr and Bowers 1980):

EC 3 ***Hydrolases*** catalyze hydrolysis; these are of the general form (Carr and Bowers 1980):



EC 4 ***Lyases*** catalyze lysis reactions, in other words non-hydrolytic removal of functional groups from substrates, often creating a double bond in the product; or the reverse reaction, i.e., addition of function groups across a double bond (Carr and Bowers 1980).

EC 5 ***Isomerases*** catalyze molecular isomerization, including racemizations and cis-trans isomerizations (Carr and Bowers 1980).

EC 6 ***Ligases*** catalyze ligation, synthesis reaction involving condensation of two molecules with the hydrolysis of a pyrophosphate bond (Carr and Bowers 1980).

EC numbers are four digits, for example a.b.c.d, where "a" is the class, "b" is the subclass, "c" is the sub-subclass, and "d" is the sub-sub-subclass. The "b" and "c" digits describe the reaction, while the "d" digit is used to distinguish between different enzymes of the same function based on the actual substrate in the reaction (ocw.mit.edu 2009). To illustrate, the EC number for glucose oxidase, which binds glucose and aids breaking it down to its metabolites, is 1.1.3.4 (IUBMB 2009):

EC 1 The enzyme is an oxidoreductase

EC 1.1 The enzyme acts on CH-OH groups of donors

EC 1.1.3 The enzyme uses oxygen as acceptor

EC 1.1.3.4 The enzyme is called glucose oxidase

1.2.5 Enzyme Activity

Enzyme activity is defined as measure of the ability of an enzyme to catalyze a specific reaction and expressed in term of units per milligram of enzyme or molecules of substrate transformed per minute per molecule of enzyme (Medical Dictionary 2009).

1.2.6 Factors Affecting Enzymatic Speed

Rates of enzymatic reactions depend on amount of enzyme and substrate as well as temperature and pH in absence of inhibitors. To achieve maximum product per unit time substrate should fill active sites most of the time.

To study the effect of enzyme concentration on the reaction rate the substrate is kept in excess amount. Hence, the order of reaction becomes zero, in other words the reaction becomes independent of substrate concentration. Figure 1.22 depicts that any change in the amount of product formed over a specified period of time is dependent upon the level of enzyme present.

When the amount of enzyme is kept constant and the substrate concentration is gradually increased, the reaction velocity will increase until it reaches a maximum (Figure 1.23).

A higher temperature generally results in an increase in enzyme activity. As the temperature rises, the movement of both enzyme and substrate increases, and there are more effective collisions between them. If the temperature rises beyond a certain point, the enzyme is denaturated and

no enzyme activity is observed (Mader 1996). At that temperature, the energy introduced to the system begins to overcome the energy of the active forces holding the enzyme in its 3D form. For the majority of the commercial enzymes, the optimal temperature range is between 40 ºC and 60 ºC (Mathewson 1998). Effect of temperature on reaction rate is shown in Figure 1.24.

The ability of the amino acids at the active site of an enzyme to interact with the substrate depends on their electrostatic state, i.e. whether they are properly charged or uncharged, as well as their spatial orientation. Enzymes, with some exceptions, generally work in the pH range of 6.0-8.0. If the pH is not right, the charge on one or all of the required amino acids is such that the substrate can neither bind nor react to produce product. In addition, the static forces holding the amino acid chain may be altered and the chain may unfold (Mathewson 1998). Each enzyme has an optimal pH that helps maintain its normal configuration (Figure 1.25) (Mader 1996).

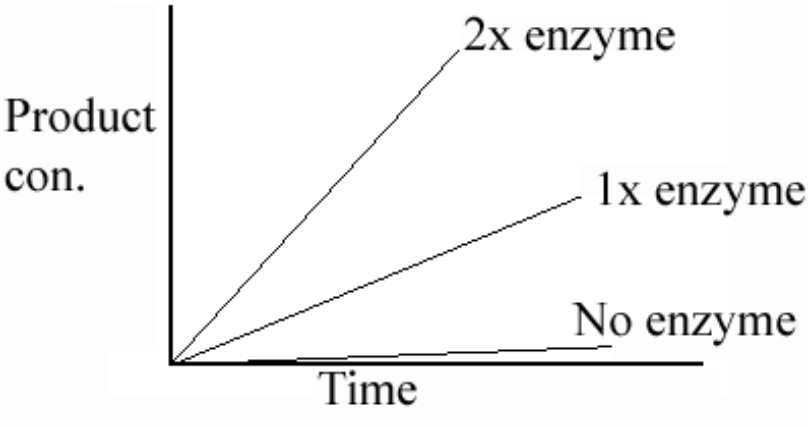


Figure 1. 22 "Zero order" reaction rate is independent of substrate concentration (Worthington Biochemical Corporation 2009)

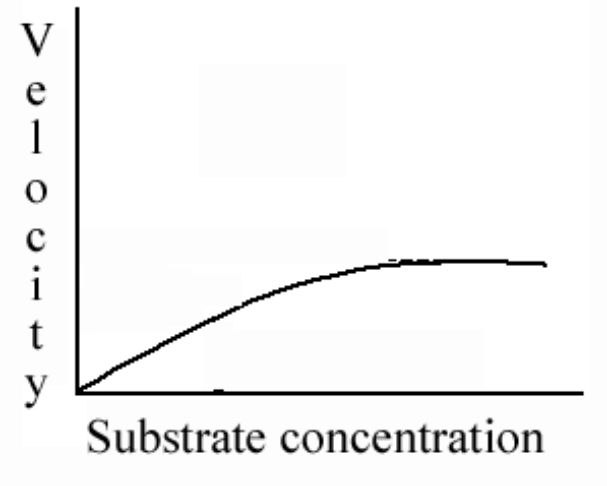


Figure 1. 23 Effect of substrate concentration (Worthington Biochemical Corporation 2009)

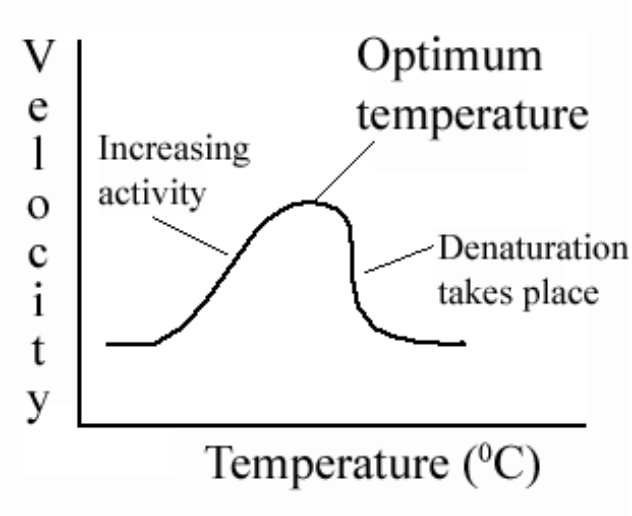


Figure 1. 24 Effect of temperature on reaction rate (Worthington Biochemical Corporation 2009)

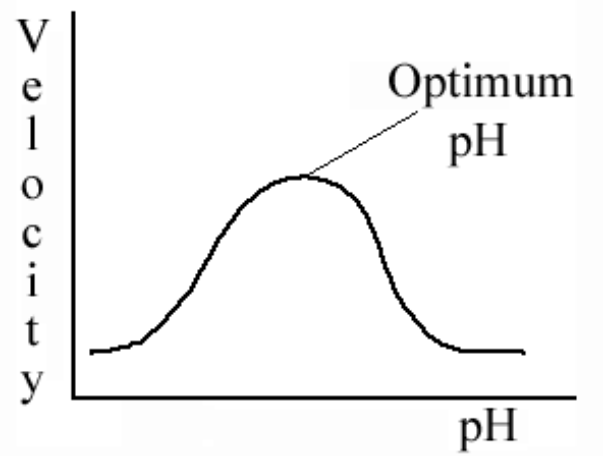


Figure 1. 25 Effect of pH on reaction rate (Worthington Biochemical Corporation 2009)

1.2.7 Basic Enzyme Kinetics

The kinetic feature that most distinguishes enzyme-catalyzed reactions from simple chemical reactions is that they show saturation. Nearly all enzyme catalyzed reactions show first-order dependence of rate on substrate concentration at very low concentrations, but instead of increasing indefinitely as the concentration increases, the rate approaches a limit at which there is no dependence of rate on concentration and the reaction is zero order with respect to substrate. Figure 1.26 illustrates this behavior (Cornish-Bowden and Wharton 1988).

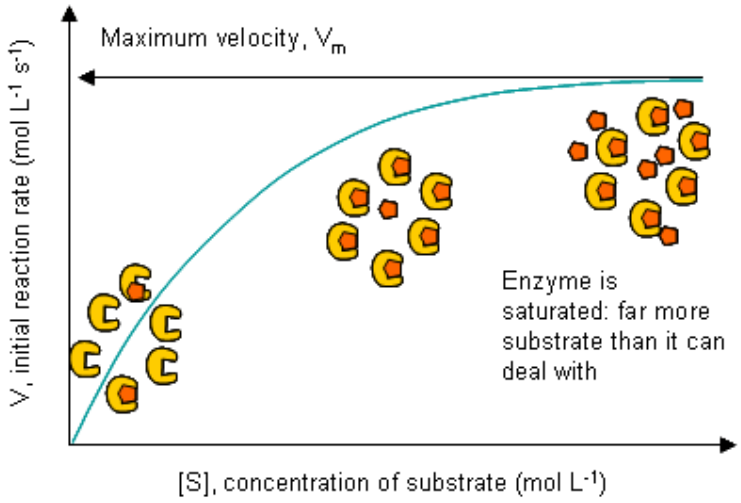


Figure 1. 26 Dependence of rate on substrate concentration for a typical enzyme catalyzed reaction (Enzymes 2009)

In 1913 Michaelis and Menten proposed a mechanism to explain these observations. Their mechanism supposes that the first step in the reaction is the binding of the substrate "S" to the enzyme "E" to form an "enzyme-substrate complex" (ES) which then reacts to give the product "P" with the regeneration of the free enzyme:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \qquad (1)$$

"ES" can be denoted as "X".

Since $[E] + [X] = [E]_0$ and $[S] + [P] = [S]_0$, there are two independent rate equations for this mechanism:

$$\frac{d[X]}{dt} = k_1[E][S] - (k_2 + k_3)[X] \qquad (2)$$

$$\frac{d[P]}{dt} = k_3[X] \qquad (3)$$

These two equations cannot be solved to obtain analytical expressions for [E], [S], [X] and [P] as function of time.

Since enzymatic reactions are generally studied with enzyme concentrations much lower than the concentrations of substrates, it is a good approximation to assume that the enzymatic reaction is in a steady state in which d[X]/dt = 0. By introducing the equation for the conservation of enzyme, $[E] = [E]_0 - [X]$, in equation 2, we obtain

$$[X] = \frac{k_1[E]_0[S]}{k_1[S] + k_2 + k_3} \qquad (4)$$

Substituting this expression in equation 3 yields

$$\frac{d[P]}{dt} = \frac{k_3[E]_0}{1 + (k_2 + k_3)/k_1[S]} \qquad (5)$$

The steady-state equation for the overall reaction is frequently written

$$v = \frac{k_{cat}[E]_0}{1 + K_m/[S]} \qquad (6)$$

Where "k_{cat}" is the "turnover number", in this case "k_3", and "K_m" is the "Michaelis constant" in this case "$(k_2+k_3)/k_1$". The turnover number is the number of product molecules produced per enzyme molecule (strictly per catalytic site) per second (Silbey and Alberty 2001).

The equation 6 can be written as shown:

$$v = \frac{v_{\max}[S]}{K_m + [S]} \qquad (7)$$

The most natural way of plotting steady-state kinetic data is to plot the rate against the substrate concentration as in Figure 1.27. However, determination of kinetic parameters is difficult since the line is curved. To transform the Michaelis-Menten equation into the equation for a straight line there are three ways. The most popular is plotting the inverse of

velocity versus the inverse of substrate concentration (Lineweaver-Burk plot) as illustrated in Figure 1.28 (Cornish-Bowden and Wharton 1988). This is obtained by taking reciprocals of both sides of equation 7.

$$\frac{1}{v} = \frac{1}{v_{\max}} + \frac{K_m}{v_{\max}} \frac{1}{[S]} \qquad (8)$$

The reaction rate is the number of reactions per second catalyzed per mole of the enzyme. Since the reaction rate asymptotically increases with increasing substrate concentration approaching the maximum rate V_{max}, there is not a clearly-defined substrate concentration at which the enzyme can be said to be saturated with substrate. A more appropriate measure is to characterize an enzyme is the substrate concentration at which the reaction rate reaches half of its maximum value ($V_{max}/2$). This concentration is equal to the Michaelis constant, K_m (Wikipedia 2009).

A small K_m indicates that the enzyme requires only a small amount of substrate to become saturated, and vice versa. Hence, the maximum velocity is reached at relatively low substrate concentrations. K_m and affinity are inversely proportional, in other words an enzyme with small K_m shows high affinity towards its substrate.

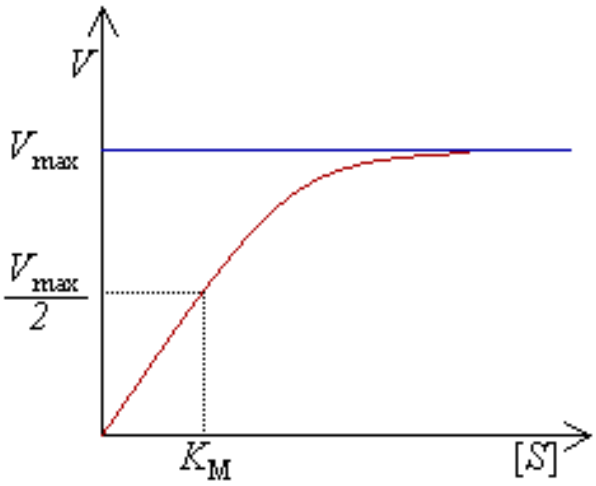


Figure 1. 27 Michaelis-Menten plot relating the reaction rate v to the substrate concentration (Wikipedia 2009)

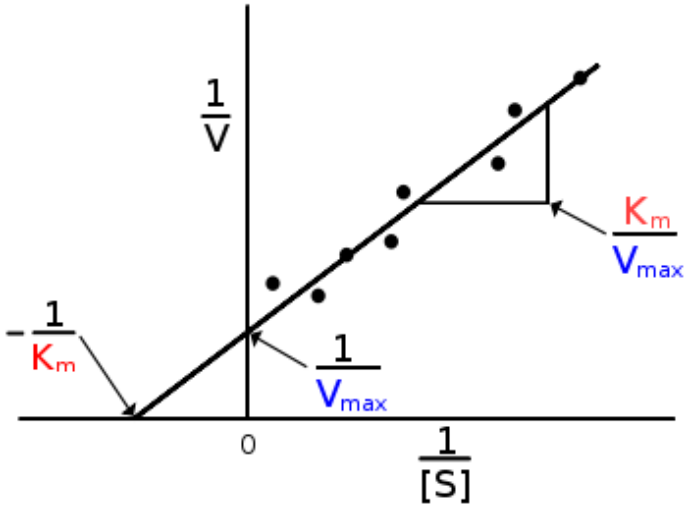


Figure 1. 28 Lineweaver-Burk plot relating the 1/v to 1/substrate concentration (Wikipedia 2009)

1.2.8 Enzyme Immobilization

Enzymes and other biological agents can be physically localized in or on a variety of insoluble matrices with the concomitant retention of biological activity.

Advantages of enzyme immobilization can be summarized as follows:

- Enzyme stability increases
- The enzyme support system can be easily removed from solution without contamination by the contents of the reaction mixture
- A single aliquot of enzyme can be repetitively used to achieve more analyses than could be performed with the same amount of enzyme in solution
- It is possible to prepare unstable, sensitive or expensive reagents using an immobilized enzyme

In enzyme immobilization, retention of the activity as well as stability of the immobilized enzyme should be considered. It is well-known that there is a loss of enzyme activity in the immobilization process. Since the activity retention is related to the severity of the protein modification in addition to the interaction between enzyme and matrix, immobilization procedure should be carefully chosen. Several types of stability can be checked after immobilization. Thermal and pH stabilities reflect the ability of the enzyme-support conjugate to withstand higher temperatures or pHs at acidic/alkaline sides before denaturation occurs. Storage stability is the ability of the preparation to retain its activity under some specified storage conditions and can provide information about the shelf life. The operational stability is not only a function of the enzyme, but also a function of the

carrier durability and inhibitor concentrations in the analyte solution (Carr and Bowers 1980).

1.2.9 Immobilization Methods

Immobilization methods are schematized in Figure 1.29.

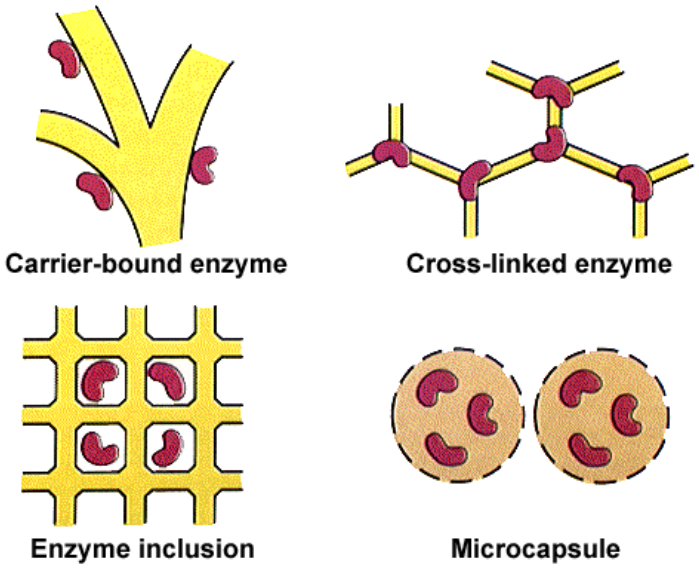


Figure 1. 29 Physical and chemical enzyme immobilization methods (UFL 2009)

Physical Methods

Physical methods include any methods which do not involve the formation of covalent bonds. They can be categorized in three (Carr and Bowers 1980):

- Adsorption of enzyme onto water-insoluble matrix

- Entrapment of enzyme inside water-insoluble polymer lattice
- Entrapment of enzyme within a semipermeable microcapsule

Chemical Methods

Chemical methods involve the formation of at least one covalent bond between the enzyme and a functionalized insoluble carrier, or between two protein molecules. Chemical methods can be classified in two (Carr and Bowers 1980):

- Attachment of enzyme to derivatized water-insoluble matrix
- Intermolecular crosslinking of enzyme molecules

Hybrid Methods

Hybrid methods involve both physical and chemical modification of the protein (Carr and Bowers 1980).

1.2.9.1 Enzyme Immobilization via Adsorption

Enzyme immobilization via adsorption onto a water-insoluble material is the simplest method to obtain enzyme-support conjugate. Basically, it consists of placing an aqueous solution of enzyme in contact with an active material for some period of time after which the excess enzyme is washed off the insoluble matrix (Carr and Bowers 1980). Numerous surface-active materials have been used, such as ion-exchange resins (Kurota, Kamata and Yamauchi 1990), acrylate resins (Chen, et al. 2007), functionalized magnetic beads (Bayramoğlu, Yılmaz, et al. 2008), hydrogel beads (Kara, et al. 2005), epoxy supports (Mateo, et al. 2000), activated montmorillonite (Sanjay and Sugunan 2008) and alumina (Yang, Shihui and Zhang 2008). Among the immobilization techniques, adsorption is the simplest and cheapest one with retention of catalytic activity. However, adsorption is generally not very strong and some of the adsorbed protein desorbs during washing and other operation steps. Hence, an

electrostatic interaction between the enzyme and support required (Bayramoğlu, Yalçın and Arıca 2005) or the support should be covered with a dialysis membrane (Odacı, et al. 2008). Additionally, the adsorbent should not adsorb the reaction product or enzyme inhibitors.

1.2.9.2 Enzyme Immobilization via Gel Entrapment

The use of physical entrapment of an enzyme within the lattices of synthetic polymers was first employed in 1963 (Bernfeld and Wan 1963). Because of their size, enzymes which are entrapped into the matrix during the crosslinking process should not diffuse out, while appropriately sized substrate and product molecules can (Carr and Bowers 1980). The most popular matrices are silica-gel (Chen, Kenausis and Heller 1998), sol-gel (Nguyen-Ngoc and Tran-Minh 2007), sol-gel organic-inorganic composite, polyacrylamide gel (Gonzalez-Saiz and Pizarro 2001), hydro-gel (Jeon, Malmstadt and Schmidt 2006) and polymeric network (Yapar, et al. 2009). Gels provide mechanical stability and aqueous environment while enabling a low resistance path to the pore for ionic currents and diffusing analytes (Jeon, Malmstadt and Schmidt 2006).

1.2.9.3 Enzyme Immobilization via Intermolecular Crosslinking

The preparation of insoluble enzyme derivatives through the use of low molecular weight bifunctional reagents such as gluteraldehyde to crosslink proteins is used. The main drawback of the method is lack of selectivity, in other words it is difficult to control intramolecular molecular crosslinking while obtaining a high degree of intermolecular crosslinking (Carr and Bowers 1980). Enzymes may be crosslinked or enzymes may be

covalently binded to a biological support via crosslinking (Gyurcsanyi, Vagföldi and Nagy 1999).

1.2.9.4 Enzyme Immobilization via Covalent Attachment

The most popular covalent attachment technique involves the reaction of an aqueous solution of enzyme with an activated, functionalized, water insoluble support. There are essentially three steps in an immobilization scheme: activation of the support, enzyme coupling, and removal of loosely bound enzyme (Carr and Bowers 1980). Enzymes can be immobilized onto a functionalized polymeric support via gluteraldehyde induced covalent attachment (Badea, Curulli and Palleschi 2003). Stabilization of enzyme is also possible via both protein and surface modification (Grazu, et al. 2005).

Additionally, "self-assembly" is a widely used biomolecule immobilization technique via covalent bond formation. "Self-assembly" can be defined as the autonomous organization of components into patterns or structures without human intervention (Whitesides and Gryzbowski 2002). To illustrate, alkane thiols can self-assemble on gold substrates thanks to their aurophilicity (Ozin and Arsenault 2005). SAMs of thiol and amine/carboxyl capped molecules can be formed on a gold substrate in addition amine/carboxyl parts can make covalent bonds with enzymes, in other words they can be tuned to suit various biosensor applications and are suitable for immobilization of enzymes (Solna and Skladal 2005) (Delvaux, Demoustier-Champagne and Walcarius 2004). The main advantage lies in the close proximity of immobilized biomolecules to the electrode surface. Nevertheless, the self-assembly methods require perfect gold surfaces with complicated cleaning procedures to prevent any pinhole defects. Moreover, the formation of a covalently grafted protein layer necessitates an additional

chemical activation step and SAMs based on long alkyl chains can lead to nonspecific hydrophobic adsorption of contaminants (Cosnier 2005).

1.2.10 Enzyme Immobilization via Electrochemical Polymerization

Biomolecule immobilization via conventional procedures, such as adsorption, entrapment in gels or membranes, crosslinking and covalent binding suffer from low reproducibility and poor spatially controlled deposition. Advantages and disadvantages are listed in Table 1.2 (Gerard, Chaubey and Malhotra 2002).

The electrochemical method involves the physical entrapment of biomolecules in organic polymers during their electrogeneration on an electrode surface. The polymer formation is carried out by controlled potential electrolysis of an aqueous solution containing monomers, biomolecules and supporting electrolytes. One major advantage of electrochemical deposition procedures over more conventional methods is the possibility to precisely and reproducibly electrogenerate the polymer coating over small electrode surfaces of complex geometry (Cosnier 1999). Additionally, enzyme immobilization during electropolymerization is a one-step procedure with easy control over the properties of the polymeric coating such as morphology and thickness via monitoring charge deposition. Moreover, biomolecule activity is not altered (Cosnier 2005). Electrically conducting polymers have considerable flexibility in the available chemical structure, which can be modified as required via monomer modeling (Gerard, Chaubey and Malhotra 2002). The pioneering work was done in 1980s: Glucose oxidase was entrapped in polypyrrole matrix electrochemically deposited on platinum or glassy carbon electrodes from an aqueous solution containing pyrrole via application of a potential less than 1.0 V (Foulds and Lowe 1986), (Umana and Waller 1986) .

The main disadvantage of that method is the poor water-solubility or water-insolubility of monomers making physical incorporation of biomolecules difficult in growing polymers. Hence, among the conducting polymers polypyrrole and its derivatives, such as copolymers (Kıralp, Toppare and Yağcı 2003) (Yıldız, et al. 2005) and composites (Mailley, et al. 2004) (Retama, Cabarcos, et al. 2004), play a leading role due to their versatile applicability thanks to partial aqueous solubility of pyrrole allowing electrodeposition in single step. Additionally, in recent years the thiophene derivative, water soluble 3,4-ethylenedioxythiophene, has been also used as immobilization matrix (Kros, et al. 2001), (Fabiano, et al. 2002).

Table 1. 2 Conventional immobilization procedures

Method	Advantages	Disadvantages
Physical 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 confinement of biocatalyst near transducer Low cost	High diffusion barrier
Crosslinking	Loss of biocatalyst at minimum Moderate cost	Harsh treatment of biocatalyst by toxic chemicals
Covalent bonding	Low diffusional resistance Stable under adverse conditions	Harsh treatment by toxic chemicals Matrix not regenerable

1.3 Biosensors

A biosensor can be defined as a device consisting of a biological recognition system and a transducer (Figure 1.30). A biosensor includes two steps: a recognition step and a transducing step. In the recognition step the biological element can recognize the analyte either in solution or in the

atmosphere. Bioreceptors can be classified according to the biorecognition elements embedded; bioreceptors may be biological molecular species (e.g. antibodies, enzymes, proteins, or nucleic acids) or living biological systems (e.g cells, tissue, or whole organisms) that utilize a biochemical mechanism for recognition (Vo-Dinh and Cullum 2000). The transduction element converts the analyte-receptor binding event into a quantitative optical or electrical signal. The signal can be a change in (a) the resonance unit (surface plasmon resonance), (b) the optical properties (UV-Vis-IR absorption), (c) the mass (piezoelectric biosensors), (d) the electrical properties (Sadana 2003).

The first demonstration of enzyme integration into an electrode was performed in the early 1960s (Clark and Lyons 1962), and a term "enzyme electrode" was derived. The enzyme electrode can be defined as a miniature chemical transducer which functions by combining an electrochemical procedure with immobilized enzyme activity (Updike and Hicks 1967).

Sensors need to be specific, sensitive, stable, easy to use, portable and inexpensive. Hence, several factors should be considered before choosing an appropriate immobilization technique aiming on the preservation of a maximum of enzyme activity and stability, a sufficient enzyme loading on the sensor surface, and a proper design of the sensor architecture to enable a productive communication between the biocatalytic recognition process and the transducer surface (Borgmann, et al. 2005). The possible immobilization techniques are schematized in Figure 1.31.

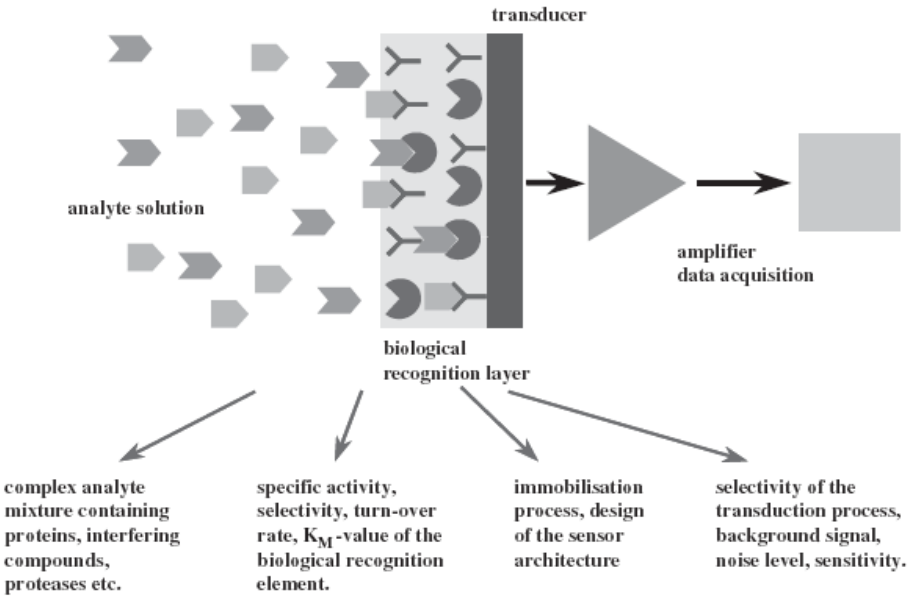


Figure 1. 30 Schematic representation of a biosensor and the factors defining the sensor signal (Borgmann, et al. 2005)

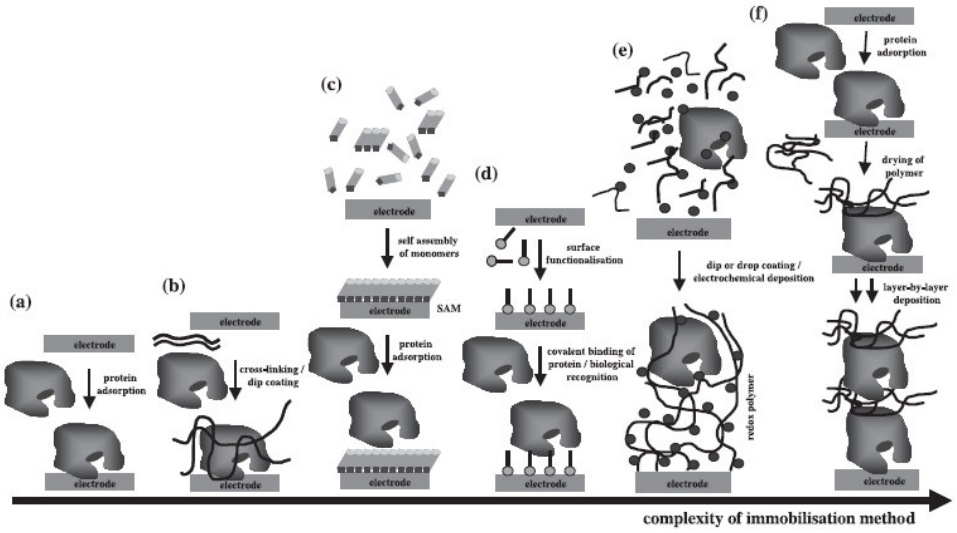


Figure 1. 31 Schematic representation of protein immobilization techniques for the modification of transducer surfaces: (a) adsorption of proteins, (b) cross-linking of proteins, (c) formation of self assembled monolayers (SAMs) and protein attachment on SAM, (d) covalent attachment of proteins to the electrode surface or binding of proteins via biological recognition, (e) formation of redox polymers with entrapped proteins by means of dip or drop coating or electrodeposition, (f) layer-by-layer deposition (Borgmann, et al. 2005)

1.3.1 Biosensors Based on Conducting Polymers

Thanks to their bulkiness, biomolecule immobilization in or on electrosynthesized polymers is carried out following various strategies involving affinity interactions, electrostatic adsorption or incorporation, chemical grafting or entrapment process during the electrochemical growth of the polymer (Cosnier 2005).

Electrochemically deposited polymer films used for biomolecule immobilization are conducting polymers such as polyacetylene (Varfolomeyev and Bachurin 1984), polythiophene (Hiller, et al. 1996), polypyrrole (Foulds and Lowe 1986) (Umana and Waller 1986), polyaniline (Laska, Wlodarczyk and Zaborska 1999) and polyindole (Pandey 1988).

Although the most used conducting polymer, polypyrrole is a good immobilization matrix, its derivatives are also widely used to enhance its properties and biocompatibility. To illustrate, gold-polypyrrole nanocomposites (Njagi and Andreescu 2007), layer-by-layer assembled carbon nanotube-polypyrrole (Shirsat, Too and Wallace 2008), polypyrrole-polystyrenesulfonate embedded in polyacrylamide microgels (Retama, Cabarcos, et al. 2004), (Chen, Chen. and Xue 2008) polypyrrole/alginate films to construct glucose sensor, and polypyrrole doped with polyvinyl sulfonate (Kharat, et al. 2009), polypyrrole/polynaphtol bipolymeric layer (Massafera and Cordoba de Torresi 2009) to detect urea were recently used. The other most used conducting polymer is polyaniline. For instance, polyaniline nanofibers (Zhao, Wu and Cai 2009) to immobilize glucose oxidase, electroactive nanostructured membranes made with polyaniline and silver nanoparticles stabilized in polyvinyl alcohol (Crespilho, et al. 2009) as urea biosensor matrices, ordered mesoporous polyaniline film (Xu, Zhu and Hu 2007) to construct hydrogen peroxide biosensor were used and trypsin was lately entrapped in polyaniline assembled on the Fe_3O_4/CNT composite (Wang, et al. 2008). On the other hand, polythiophenes, especially poly(3-hexylthiophene) and poly(3,4-ethylenedioxythiophene) were used as immobilization matrices. Langmuir-Blodgett films of poly(3-hexylthiophene) were utilized to construct glucose biosensor (Singhal, et al. 2004) and galactose biosensor (Sharma, et al. 2004), alternatively poly(3,4-ethylenedioxythiophene) was used to physically entrap tyrosinase (Vedrine, Fabiano ve Tran-Minh 2003).

Even though electrochemical synthesis allows the direct deposition of the polymer on the electrode surface while simultaneously trapping protein molecules, it is also possible to modify polymer to bind protein molecules (Gerard, Chaubey and Malhotra 2002). Although, biomolecules are generally physically entrapped in polypyrrole and PEDOT during electrodeposition thanks to the water solubility of their monomers, immobilization through adsorption or via chemical bonding is also feasible. On the other hand, biomolecules are immobilized in polyanilines and polythiophenes via either adsorption or chemical modification.

Conducting polymers are also known to be compatible with biological molecules in neutral aqueous solutions (Gerard, Chaubey and Malhotra 2002), thus they are widely used with modification or without modification.

1.3.2 Amperometric Biosensors

Electrochemical techniques are frequently used in transduction of chemical information into an understandable signal. Voltammetry, amperometry, potentiometry and impedometry are commonly utilized in sensing. *Voltammetry* refers to the measurement of current resulting from potential application, whereas in *amperometry*, a uniform potential is applied and the change in current is monitored as a function of time. On the other hand, *potentiometry* is based on the potential measurement under no current flow; the potential developed is the result of change in free energy. In the case of *impedometry*, the approach is to perturb the cell with an alternating potential in small magnitude and to observe the way in which the system follows the perturbation at steady state (Rahman, et al. 2008). Amperometry is extensively used in biosensor applications where an oxidation-reduction reaction is involved.

Amperometric biosensors measure the current produced during the oxidation or reduction of a product or reactant at a constant applied potential. The most important factor affecting the functioning of amperometric biosensors is the electron transfer between catalytic molecule, usually oxidase or dehydrogenase, and the electrode surface most often involving mediation or conducting polymer (Gerard, Chaubey and Malhotra 2002).

Oxidoreductases produce a flow of electrons directly to the electrode in the process of turning a substrate into a product. Those enzymes contain various active sites, including flavin adeneine dinucleotides (FADs), metal centers that allow them to take part in oxidation-reduction reactions. The active sites are capable of existing at multiple oxidation states, permitting the enzymes to gain electrons from their substrate, followed by transfer of those electrons to an electrode via some intermediary. Since, oxidoreductases use oxygen in the reaction and produces H_2O_2 as side product either oxygen depletion using a Clark electrode or production of H_2O_2 can be monitored (Figure 1.32). Additionally mediated sensing employing charge mediator sites within the sensor to shuttle electrons is also used (Figure 1.33).

As seen in Figure 1.32, the substrate reacts with the immobilized enzyme and reduces its active site by transferring electrons. The reduced enzyme then reacts with molecular oxygen present in the medium and returns to its oxidized state while generating hydrogen peroxide. H_2O_2 subsequently diffuses to electrode surface where it is oxidized generating electrons. The current change created by these electrons is measured and correlated with amount of substrate. The drawback is the variable oxygen amount in body for *in-vivo* applications (Sirkar December 2000), (Taylor May 1998).

Mediated sensing (Figure 1.33) is predicated on a signal transducer other than hydrogen peroxide that serves to “shuttle” electrons from the

enzyme-substrate reaction to the electrode surface. Charge mediators, often referred as redox polymers, may be free or immobilized. In this manner, electrons may be transferred at a higher rate such that oxygen interference can be minimized. The possible leaching of mediator *in-vivo* applications is the disadvantage.

A commercially available glucose biosensor is available: A drop of blood is applied to a disposable electrode strip, which can be inserted into the device. The electrical current is read after few seconds and the signal is converted to a glucose concentration, which is then displayed on the instrument (Manz, Pamme and Iossifidis 2004).

1.3.3 Amperometric Biosensors Based on Conducting Polymers

Conducting polymers have the ability to efficiently transfer electric charge produced by the biochemical reaction to electronic circuit. They provide good detectability and fast response as the redox reaction of the substrate, catalyzed by an appropriate enzyme, taking place in the bulk of polymer layer (Gerard, Chaubey and Malhotra 2002). Hence, they are widely used in amperometric sensor applications. To illustrate, single-walled carbon nanotube/polypyrrole composite (Cosnier, Ionescu and Holzinger 2008), poly(3-thiophene acetic acid) matrix (Nien, Chen ve Ho 2009), poly(N-3-aminopropyl pyrrole-co-pyrrole) film (Bisht, Takashima and Kaneto 2005) and poly(3,4-ethylenedioxythiophene)-poly(styrene sulfonic acid) film were recently used for amperometric glucose, cholesterol, urea, and alcohol sensing.

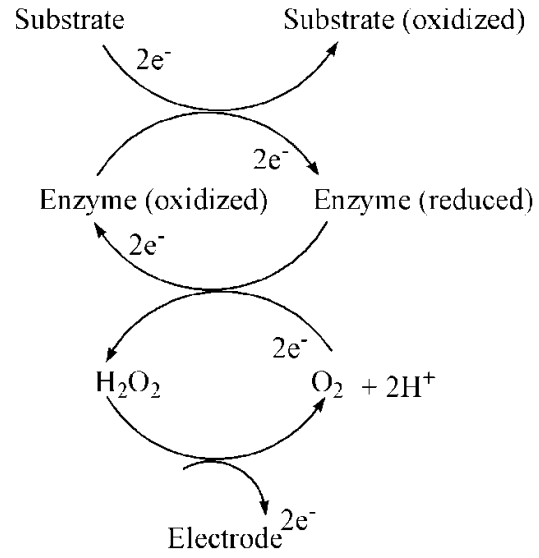


Figure 1. 32 Operating principle of the amperometric detection without mediator

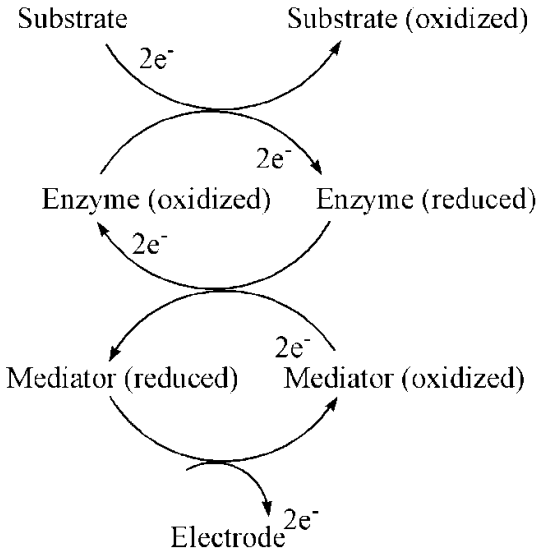


Figure 1. 33 Operating principle of the amperometric detection with mediator

1.3.4 Applications of Biosensors

A wide variety of applications for qualitative and quantitative analysis is listed in Table 1.3. Only a few biosensors have been made commercially available, such as a blood glucose sensor for home monitoring diabetics (Manz, Pamme and Iossifidis 2004).

Table 1. 3 Biosensor applications

Field	Applications
Health care	Markers of diseases Monitoring of administered drugs Diagnosis of infectious diseases Analysis of glucose/urea/cholesterol, etc. and hormone levels
Environmental	Water and soil analysis Detection of pesticides and other toxic substances Industrial effluent control
Agriculture	Pesticides, crop diseases
Food control	Food freshness Determination of fruit ripeness by glucose content Quantification of cholesterol in butter Pathogenic organisms like E. coli
Process control	Fermentation monitoring
Microbiology	Bacterial and viral analysis

1.4 Cholesterol Biosensors

Steroids are lipids with ring structures. Although they have similar backbones, their functions in the body vary with different attached groups. Cholesterol, found in animal plasma membranes, is the precursor of several other steroids, including the vertebrate and sex hormones. Cholesterol is manufactured in the liver and small intestine, as well as derived from dietary

sources. Accumulation of cholesterol under the inner linings of arteries may lead to atherosclerosis (Mader 1996). Cholesterol determination in blood is clinically important in the diagnosis and prevention of cardiovascular diseases.

The first enzymatic determination of total serum cholesterol was done by Allain et. al., in their work, cholesterol esters were first hydrolyzed to free cholesterol by cholesterol ester hydrolase, and then this free cholesterol was oxidized to cholest-4-en-3-one by cholesterol oxidase accompanied with the production of hydrogen peroxide. Hydrogen peroxide reacts with 4-aminoantipyrine and phenol in the presence of a peroxidase yielding a chromogen which absorbs at 500 nm (Allain, et al. 1974). Since 70% of the cholesterol present in serum samples are esterified (only 30% is free), a typical assay for total serum cholesterol usually begins with the incubation of serum with cholesterol esterase in order to afford free cholesterol amenable to oxidation with cholesterol oxidase (MacLachlan, et al. 2000).

Several enzymatic cholesterol biosensors have been designed based on different analytical methods. The starting point is the immobilization of the enzymes, such as cholesterol oxidase and cholesterol esterase and then enzyme-substrate interactions are detected by several techniques including spectrophotometry (Çırpan, et al. 2003), (Singh, Chaubey and Malhotra 2003), (Kumar, Pandey and Brantley 2006), surface plasmon resonance (Arya, Solanki, et al. 2007), (Arya, Prusty, et al. 2007), (Solanki, Arya, et al. 2007), quartz crystal microbalance (Chong, et al. 2002), and electrochemistry (Vidal, Espuelas and Castillo 2004), (Devadoss, et al. 2005) (Özer, et al. 2007).

Chronoamperometry is the preferred technique in electrochemical detection systems for cholesterol since it allows monitoring either oxygen consumption or hydrogen peroxide production, which is shown in Figure 1.34. In recent years, various enzyme immobilization matrices were used in

construction of amperometric cholesterol biosensors, such as self assembled monolayers (Zhou, et al. 2006), lipid bilayer membranes (Devadoss, et al. 2005), sol-gel/chitosan hybrid composite films on multiwalled carbon nanotubes (Tan, et al. 2005), polypyrrole (Singh, Chaubey and Malhotra 2004), PPy/Prussian blue layers (Vidal, Espuelas and Garcia-Ruiz, et al. 2004), PPy-hydrogel membrane (Brahim, Narinesingh and Guiseppi-Elie 2001), diaminonaphthalene isomers (Garcia-Ruiz, et al. 2004), polyaniline (Singh, Solanki, et al. 2006), poly(aniline-co-pyrrole) film (Solanki, Singh, et al. 2007) and polyaniline Langmuir-Blodgett film (Matharu, et al. 2007).

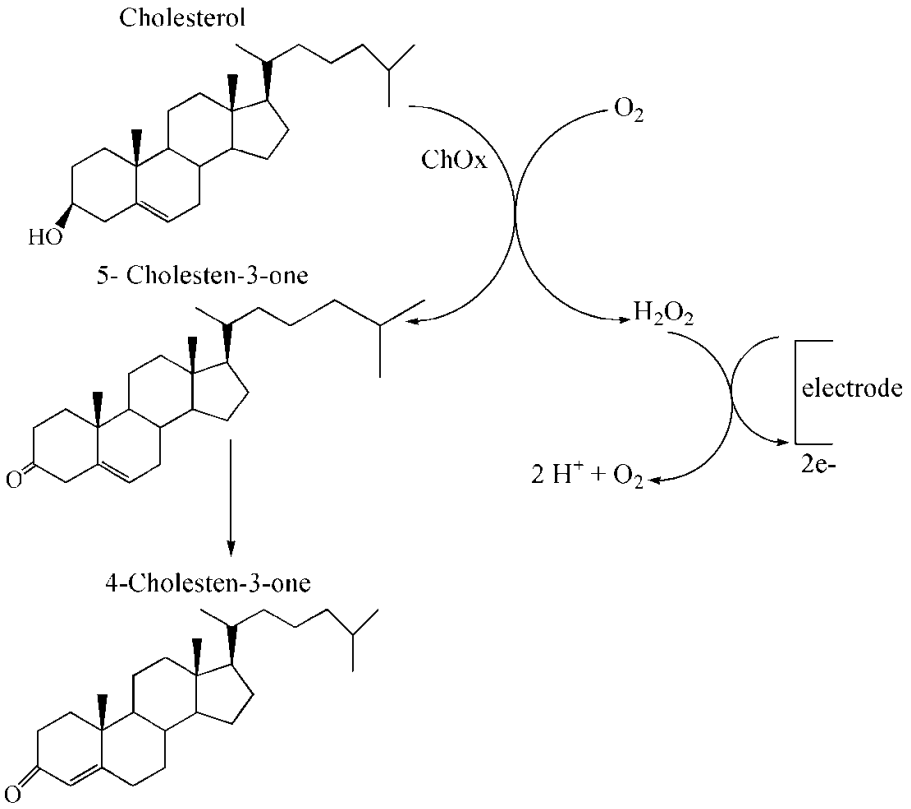


Figure 1. 34 Mechanism for non-mediated amperometric cholesterol sensing based on monitoring hydrogen peroxide production

1.5 Alcohol Biosensors

Ethanol can be made by the fermentation of sugars, and it is also of all alcoholic beverages. Fermentation is usually carried out by adding yeast to a mixture of sugars and water. Yeasts contain enzymes that promote a long series of reactions that ultimately convert a simple sugar to ethanol and carbon dioxide:

$$C_6H_{12}O_6 \xrightarrow[95\%\text{ yield}]{\text{yeast}} 2\ C_2H_5OH + 2\ CO_2$$

Fermentation alone does not produce beverages with ethanol content greater than 12-15% because the enzymes of the yeasts are deactivated at higher concentrations. To produce beverages of higher alcohol content the aqueous solution must be distilled. The flavors of the various distilled liquors result from other organic compounds that distill with the alcohol and water. Ethanol is a hypnotic, depresses activity in the upper brain even though it gives the illusion of being a stimulant. Ethanol is also toxic, but it is much less toxic than methanol. Abuse of ethanol is a major drug problem in most countries (Solomons and Fryhle 2000).

Alcohol oxidase catalyzes biological oxidation of short chain; primary, aliphatic alcohols to the respective aldehydes while molecular oxygen is reduced to hydrogen peroxide. Enzymatic assay of alcohol oxidase is described below:

$$\text{Methanol} + O_2 \xrightarrow{\text{AlcOx}} \text{Methanal} + H_2O_2$$

$$H_2O_2 + \text{ABTS} \xrightarrow{\text{POD}} 2H_2O + \text{oxidized ABTS}$$

AlcOx: Alcohol oxidase
POD: Peroxidase
ABTS: 2,2'-Azino-bis(3-Ethylbenzthiazoline-6-sulfonic acid)

The absorbance is measured at 405 nm and the intensity is correlated with the amount of product (Sigma-Aldrich 2009).

The enzyme has the highest affinity for methanol with the affinity decreasing with increasing chain length of the alkyl (R) group. Alcohol oxidase plays a major role in the metabolism of methanol resulting in the formation of formaldehyde and has been detected in several genera of yeasts, such as *Candida*, *Pichia*, and *Hansenula*, that utilize methanol as a sole carbon and energy source.

Alcohol oxidase can be used in alcohol detection in blood or food analysis. AlcOx based biosensors are straightforward since they function (using molecular oxygen as the cofactor) without any addition of cofactor such as β-nicotinamide adenine dinucleotide or flavin adenine dinucleotide (NADH or FADH). As shown in Figure 1.35, the enzyme requires molecular oxygen to oxidize ethanol to acetaldehyde, and product formation is amperometrically detected.

In recent years, various immobilization matrices were utilized in amperometric alcohol biosensors based on AlcOx, such as cellulose membrane (Murtinho, et al. 1998) chitosan immobilized eggshell membrane (Wen, et al. 2007), polyvinylferrocenium matrix (Gülce, et al. 2002), graphite-teflon composite (De Prada, et al. 2003), overoxidized polypyrrole (Carelli, et al. 2006).

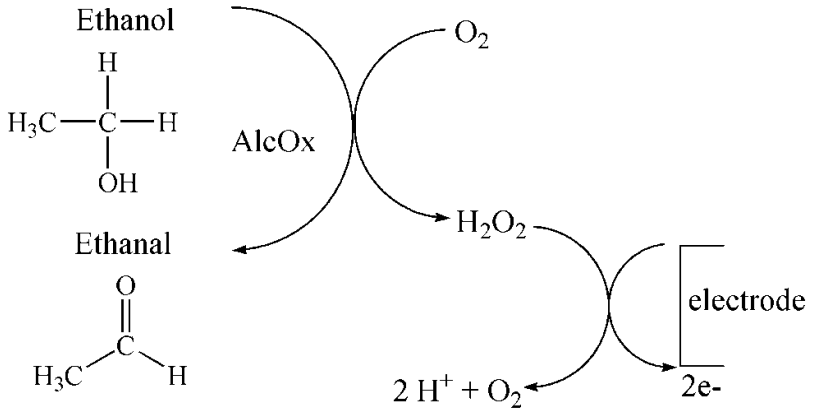


Figure 1. 35 Mechanism for non-mediated amperometric cholesterol sensing based on monitoring hydrogen peroxide production

1.6 Aim of the Study

This work aims to describe construction of cholesterol and alcohol biosensors based on three different conducting polymers, polypyrrole, poly(3,4-ethylenedioxythiophene), poly(3,4-ethylenedioxypyrrole), and then compare the biosensor properties. In this study, via application of small potentials into an aqueous medium containing monomer, surfactant (acting also as supporting electrolyte) and enzyme, conducting polymers bearing the enzymes were electrodeposited on the working electrode of any size and geometry. This technique permits control the film thickness as well as the spatial distribution of the immobilized enzymes. Oxidation current of enzymatically produced hydrogen peroxide was measured at +0.7 V without using a mediator. The stability and optimization experiments were carried out and the alcohol biosensors were tested on distilled alcoholic beverages.

CHAPTER II

EXPERIMENTAL

2.1 Materials

Pyrrole (Py), 3,4-ethylenedioxythiophene (EDOT), cholesterol oxidase (ChOx) [E.C.1.1.3.6] (26.4 U/mg protein) from *Pseudomonas fluorescens*, cholesterol, alcohol oxidase [E.C. 1.1.3.13] (35 U/mg protein) from *Pichia pastoris*, sodium dodecyl sulfate (SDS) and Triton X-100 were purchased from Sigma-Aldrich and used with no further purification. 3,4-Ethylenedioxypyrrole (EDOP) (in 2% THF) (Aldrich) was polymerized after evaporating tetrahydrofuran. Ethanol (Merck), used as solvent, was of analytical grade and utilized as received for preparing the cholesterol stock solution (0.05 M). Ethanol (v/v ≥99.5), used in analysis, was also supplied from Merck and utilized without dilution. Phosphate buffer (pH 7) for the electrosynthesis was prepared by dissolving 0.025 moles of Na_2HPO_4 (Fisher Scientific Company) and NaH_2PO_4 (Fischer Scientific Company) in one liter distilled water. On the other hand, the phosphate buffer (pH 7) utilized in the amperometric measurements consisted of 0.04 M Na_2HPO_4, 0.04 M KH_2PO_4 (Merck) and 0.1 M KCl (Fisher Scientific Company) to provide ionic conductivity. Glucose, glacial acetic acid, citric acid and L-ascorbic acid, used in interference studies, were purchased from Aldrich.

2.2 Equipment

All electrosyntheses and amperometric studies were carried out with Ivium CompactStat model (the Netherlands) potentiostat in a three electrode cell.

2.2.1 Potentiostat

A potentiostat is an electronic device that maintains the potential of a working electrode at a constant level relative to a reference electrode (Skoog D. A. et al, 1997). The Ivium potentiostat used is a portable device composed of two modules: The "Plus" module increases the power of the "CompactStat".

2.2.2 Electrolysis Cell

Constant potential electrolyses and all analytical measurements were carried out in the cell furnished with Ag/AgCl reference electrode (silver wire dipped in 4 M KCl saturated with silver chloride, Fischer Scientific Company), platinum (Pt, Aldrich) plate working and auxiliary electrodes with 0.12 cm^2 area each. The only role of reference electrode is to act as reference in measuring and controlling the working electrode potential and at no point does it pass any current. On the other hand the auxiliary electrode passes all the current needed to balance the current observed at the working electrode.

2.3 Method: Chronoamperometry

Chronoamperometry is an electrochemical technique in which the potential of the working electrode is stepped, and the resulting current from Faradaic processes occurring at the electrode is monitored as a function of time. During the thesis studies, in all electrosyntheses as well as electrochemical analyses, constant potential was applied to the system. The potential of the working electrode was adjusted to a desired value and kept constant while current was allowed to vary. Control on potential prevents formation of unwanted side products additionally enables reproducible and precise formation of polymeric films with controlled thickness and morphology. This method is also preferred in amperometric detection of redox species since it allows monitoring either oxygen consumption or hydrogen peroxide production at the reduction potential of molecular oxygen.

2.4 Enzyme Electrode Preparation

Cholesterol oxidase and alcohol oxidase were immobilized in three different conducting polymer matrices via constant potential application. These polymers, whose monomers are partially water soluble, were polypyrrole (PPy), poly(3,4-ethylenedioxythiophene) (PEDOT) and poly(3,4-ethylenedioxypyrrole) (PEDOP). Enzymes were physically entrapped during electropolymerization of monomers dissolved in phosphate buffer containing SDS (Figure 2.1). SDS was used not only as the supporting electrolyte in all syntheses but also as the ionic surfactant.

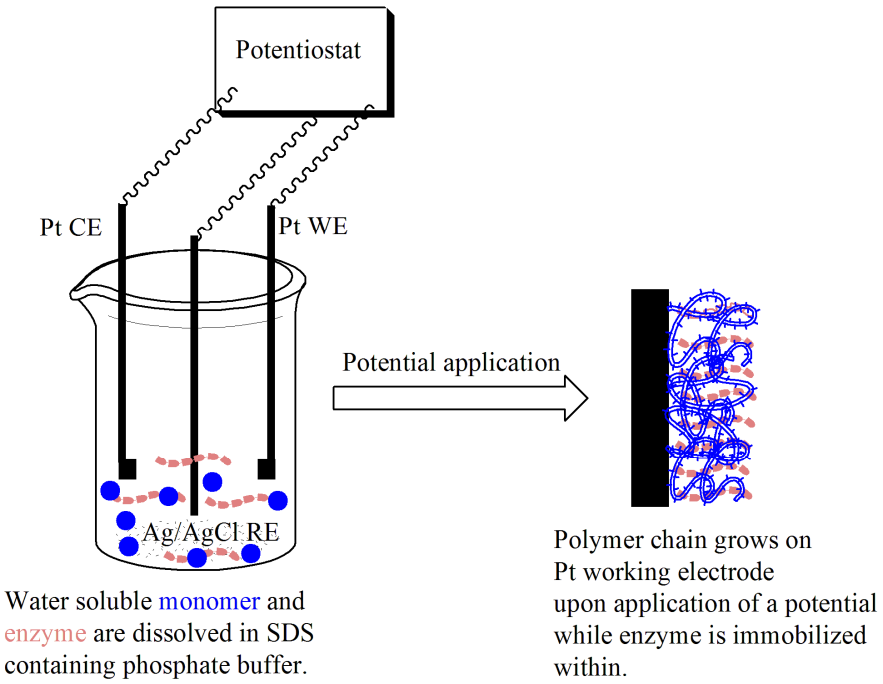


Figure 2. 1 Enzyme immobilization

The effect of film thickness on the oxidation current of hydrogen peroxide was initially studied. Deciding on the optimal thickness is important since very thin polymeric films may be unable to protect the enzyme from the environmental effects, on the other hand very thick films may complicate the diffusion process between solution and entrapment support, and as a result the substrate may not associate with the recognition element. The polymeric film thickness was controlled by fixing the charge. For all the subsequent experiments the charge loaded on the PPy, PEDOT and PEDOP enzyme electrodes were 0.0856 C, 0.0428 C and 0.0856 C, respectively. First, Pt electrodes were coated with PPy depositing 1Q (0.0428 C-charge deposited in a minute), 2Q (0.0856 C) and 3Q (0.128 C), and then the experiments were carried out adding 5 mL H_2O_2 (0.222 mM in

a total volume of 15 mL) to medium. The current responses were found as 24 μA/cm^2, 49 μA/cm^2 and 40 μA/cm^2 for 1Q, 2Q and 3Q PPy electrodes respectively. Hence, throughout the studies, 2Q PPy electrodes were fabricated. In the case of PEDOT, only 1Q (0.0428 C) deposited electrode was utilized since the polymeric films with higher thicknesses were instantly peeled off from the surfaces of the Pt electrodes. For 1Q PEDOT electrode, the amplitude of the response was calculated as 21 μA/cm^2. Since responses obtained for the 2Q and 3Q electrodes were 1.2 times greater than the one observed for the 1Q electrode, in all subsequent experiments 2Q PEDOP electrodes were used and any peeling off was observed. In summary, in all of the subsequent experiments 2Q PPy and 2Q PEDOP (nearly 50 μm thick) and 1Q PEDOT (nearly 20 μm thick) electrodes were utilized. The thickness of the polymer films were estimated using the charge required for the film coating on the electrode surfaces. These values were checked with a micrometer after peeling off the polymer film from the electrode surface. The enzyme electrodes were stored at 4 °C in phosphate buffer prior to use.

2.4.1 Construction of Cholesterol Biosensors

PPy enzyme electrodes were prepared via application of +1.0 V vs reference electrode in the electrolysis cell described previously containing 0.042 M Py, 0.1 M SDS and 0.72 mg of protein dissolved in 10 mL of phosphate buffer. In the case of PEDOT matrix, 0.028 M EDOT, 0.05 M SDS, 0.36 mg of protein and 10 mL of phosphate buffer were used and +0.8 V were applied. SDS is not only the supporting electrolyte for the electrosynthesis but also it enhances solubility of Py and EDOT in aqueous solutions thanks to its ionic surfactant property.

In the case of PEDOP enzyme electrodes a different procedure was applied. 500 μL EDOP was put in volumetric flask and THF was evaporated under vacuum, while 0.1 M SDS dissolved saline phosphate buffer (10 mL) was deoxygenated under argon atmosphere in another flask. This solution was mixed with EDOP under inert atmosphere, and then was continuously stirred for one hour to obtain a dark blue dispersion containing oligomeric EDOP. After dissolving 0.72 mg of protein, this mixture was transferred to the electrolysis cell and via application of +0.6 V at 24 °C the enzyme was immobilized in PEDOP matrix on the Pt electrodes.

2.4.2 Construction of Alcohol Biosensors

Alcohol oxidase was also immobilized in the conducting polymer matrices via constant potential electrolysis. For the PPy enzyme electrodes construction, +1.0 V was applied to a medium containing 0.042 M Py, 0.1 M SDS, 5 μL AlcOx (0.27 mg protein) dissolved phosphate buffer. In the case of PEDOT/AlcOx electrodes 0.028 M EDOT was polymerized via application of +0.8 V in the presence of 0.05 M SDS, 5 μL enzyme (0.27 mg protein) and phosphate buffer. On the other hand, during physical entrapment of the enzyme (0.27 mg protein) in PEDOP matrix a different method was used since EDOP is available in THF. After evaporating THF, EDOP was mixed with SDS dissolved deoxygenated buffer, the mixture was continuously stirred at room temperature until dark blue colored dispersion containing oligomeric EDOP was obtained, and then +0.6 V were applied to prepare the electrodes.

2.5 Amperometric Biosensor Measurements

Amperometric response studies were carried out in the saline phosphate buffer in open atmosphere while continuously and mildly stirring. Operational, storage, pH and temperature stabilities were determined via application of +0.7 V with respect to Ag/AgCl electrode to detect oxidation current of H_2O_2. After the background current reached stable value, cholesterol solution or ethanol was added to the medium, the current immediately increased (1-3 seconds) and reached a steady state (almost at the end of 60-150 seconds) depending on the type of sensor and the amount of substrate added, and the resulting current difference was recorded (Figure 2.2). The enzyme electrodes were tested towards various concentrations of substrate to determine K_m, I_{max} and sensitivity values; whereas in the stability experiments, the same amount of substrate solution was used for each biosensor. All the experiments except for temperature optimization were carried out at 24 °C.

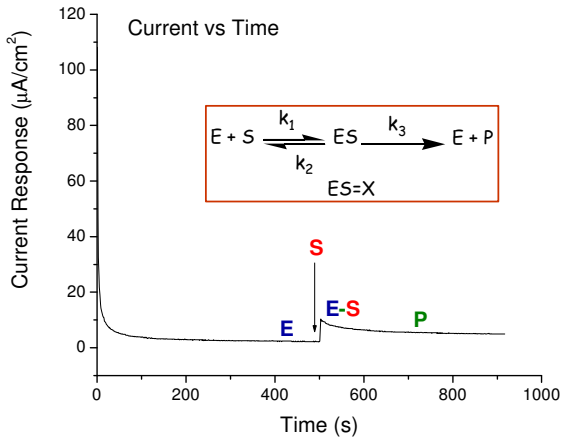


Figure 2. 2 A typical current response vs time graph observed in amperometric measurements

2.5.1 Determination of Kinetic Parameters

In the equilibrium model of Michaelis-Menten, the substrate binding step is assumed to be fast relative to the rate of breakdown of the enzyme-substrate complex. Therefore, this reaction is assumed to be at equilibrium, and the equilibrium constant for the complex, K_m, is a measure of the affinity of enzyme for its substrate and corresponds to substrate concentration at ½ V_{max} (Marangoni A. G., 2003). In the case of amperometric sensors, K_m and I_{max} (instead of V_{max}) were determined from the Lineweaver-Burk plot by plotting "1/current response vs 1/substrate concentration". After linear regression, an equation of type $y=A+Bx$ was obtained. Inverse of A gives I_{max}, where at that point x is zero, whereas K_m was calculated as $B(A)^{-1}$, where at that point y is zero (Figure 2.3).

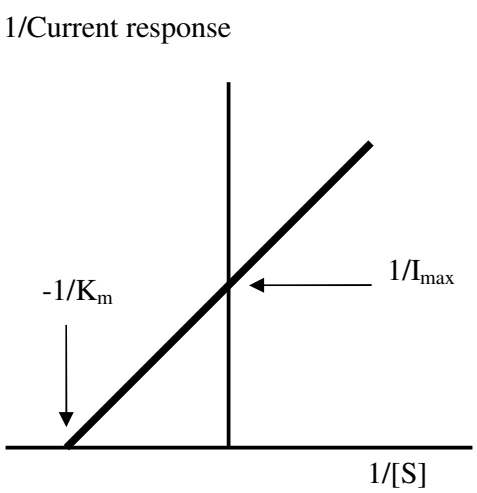


Figure 2. 3 A typical Lineweaver-Burk plot

2.5.2 Stability Experiments

The response time and reproducibility based on daily use and shelf life are the important parameters when testing the analytical performance of a biosensor. The biomolecules have limited stabilities, especially when removed from their native microdomains; in addition, their stabilities and performances decrease upon immobilization (Lundgren S. J. et. al, 1996). Thus, the stability experiments were carried out. The operational stabilities of the enzyme electrodes in terms of repetitive use were obtained running several measurements on the same day. To determine the storage stability or shelf life, the same electrode was used for consecutive 28 days.

2.5.3 Optimization Experiments

Optimum pH values should be determined in enzyme assays since pH changes in the medium (especially high and low pH) cause denaturation. The pH optimization experiments were carried out with freshly prepared enzyme electrodes. In addition, the same electrodes were used two or three times in either alkaline or acidic medium. The effect of temperature between 10 ºC and 50 ºC were investigated via checking the current responses of the freshly prepared electrodes. Since ethanol was used as solvent and also as analyte, the enzyme electrode activities were not checked at higher temperatures to prevent vaporization.

2.6 Analysis

2.6.1 Preparation of Cholesterol Solution

Cholesterol stock solution (0.05M) was prepared dissolving 0.387 g of cholesterol in 20 mL of ethanol at room temperature via gently mixing with constant speed to obtain a clear solution. The stock solution was stored at +4 ºC in dark and consumed in 10 days. Triton X-100, the nonionic surfactant providing solubility of cholesterol in aqueous solutions was added to the substrate solutions just before the injection. Since high Triton X-100 concentration can inhibit the ChOx activity, the ratio added was set as 1% (v/v) (Tan X. et al, 2005).

2.6.2 Cholesterol Analysis

Kinetic parameters K_m and I_{max} for the ChOx biosensor were found from the Lineweaver-Burk plot at constant temperature (24 ºC) and pH (pH 7) while varying the substrate concentration. In the stability and optimization experiements, substrate solutions of 16 mM, 13 mM and 3.4 mM in 10 mL were used for PPy, PEDOT and PEDOP electrodes, respectively.

2.7 Alcohol Analysis

The kinetic parameters were determined by testing the electrodes on various ethanol concentrations; on the other hand in the stability experiments the same amount of substrate was consumed for each biosensor (500 µL, 0.817 M).

CHAPTER III

RESULTS AND DISCUSSION

3.1 Cholesterol Oxidase Biosensors

3.1.1 Enzyme Loading Studies

Several enzyme electrodes based on PPy, PEDOT and PEDOP containing different amounts of cholesterol oxidase were prepared, and their amperometric responses were checked in the presence of 10 mM cholesterol to decide on the optimal amount of enzyme loading. In Figure 3.1 the current response versus enzyme loading is illustrated. As shown in the graph the magnitude of the response depends on the amount of enzyme immobilized in the polymer matrix; the current responses rise with increasing enzyme loading and then reach saturation. Since the maximum responses were obtained with 0.72 mg protein/10 mL, 0.36 mg protein/10 mL and 0.72 mg protein/10 mL for PPy, PEDOT and PEDOP enzyme electrodes, respectively; throughout the study these amounts were added to the polymerization medium (10 mL) to construct the enzyme electrodes (Türkarslan, Kıralp Kayahan and Toppare 2009) (Türkarslan, Kıralp Kayahan and Toppare 2009).

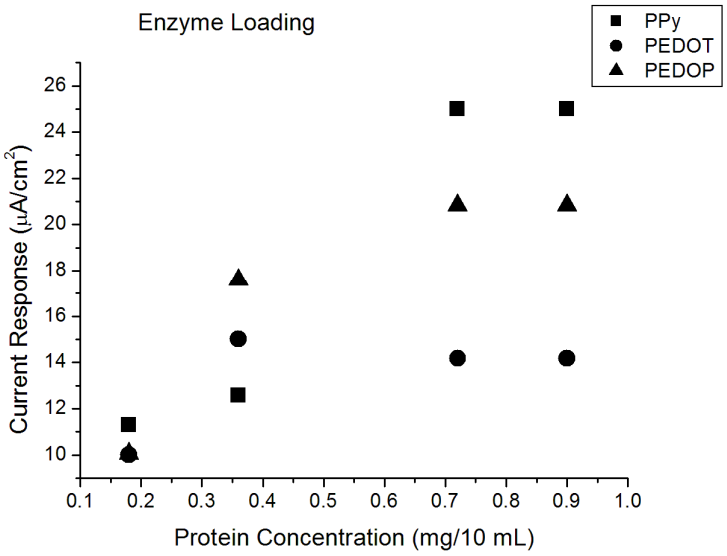


Figure 3. 1 Current responses of the enzyme electrodes containing different amounts of ChOx in the presence of 10 mM cholesterol solution

3.1.2 Determination of Kinetic Parameters

Calibration graphs are shown in Figures 3.2 a, 3.3 a and 3.4 a revealing an increasing current response with the increasing substrate concentration. Lineweaver-Burk plots are given in Figures 3.2 b, 3.3 b and 3.4 b, where the kinetic parameters are calculated. The important parameters obtained for the enzyme electrodes are summarized in Table 3.1. The lowest K_m and I_{max} values were calculated for the PEDOT/ChOx. Since K_m is inversely proportional to the affinity of the enzyme for its substrate, in PEDOT matrix more ChOx is associated with cholesterol; however the intensity of the response was lower compared to the other conducting polymeric matrices, which can be interpreted as less product formation. Sensitivities are calculated as 6.7 $\mu AmM^{-1}cm^{-2}$, 10 $\mu AmM^{-1}cm^{-2}$ and 14.3

$\mu AmM^{-1}cm^{-2}$ for PPy, PEDOP and PEDOT enzyme electrodes, respectively by dividing I_{max} to K_m. As regards to minimum detectable substrate concentrations 0.6 mM, 0.4 mM and 0.2 mM, the current responses were 3.8 $\mu A/cm^2$, 3.8 $\mu A/cm^2$ and 2.5 $\mu A/cm^2$, respectively for PPy, PEDOP and PEDOT based biosensors. It can be concluded that, with small K_m value and comparatively high sensitivity, the PEDOT enzyme electrode reveals better biosensor characteristics (Türkarslan, Kıralp Kayahan and Toppare 2009) (Türkarslan, Kıralp Kayahan and Toppare 2009). A comparison with the other conducting polymer based amperometric cholesterol biosensors is given in Table 3.2.

Table 3. 1 Kinetic parameters for the cholesterol biosensors

Matrices	**K_m (mM)**	**I_{max} (μAcm^{-2})**	**Minimum Detection (mM)**	**Sensitivity ($\mu AmM^{-1}cm^{-2}$)**
PPy	7.9	52.6	0.6	6.7
PEDOP	3.4	34	0.4	10
PEDOT	1.3	17.9	0.2	14.3

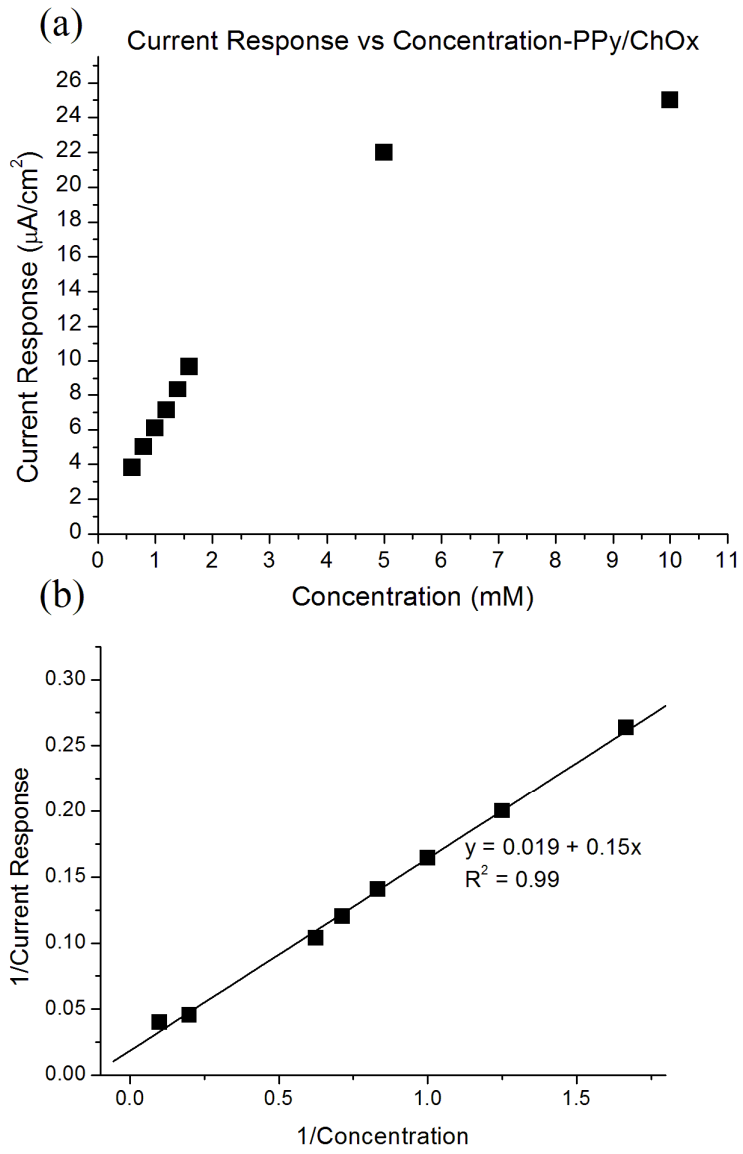


Figure 3. 2 (a) Current response vs concentration. (b) 1/Current response vs 1/concentration for the PPy/ChOx enzyme electrode (pH 7; 24 °C)

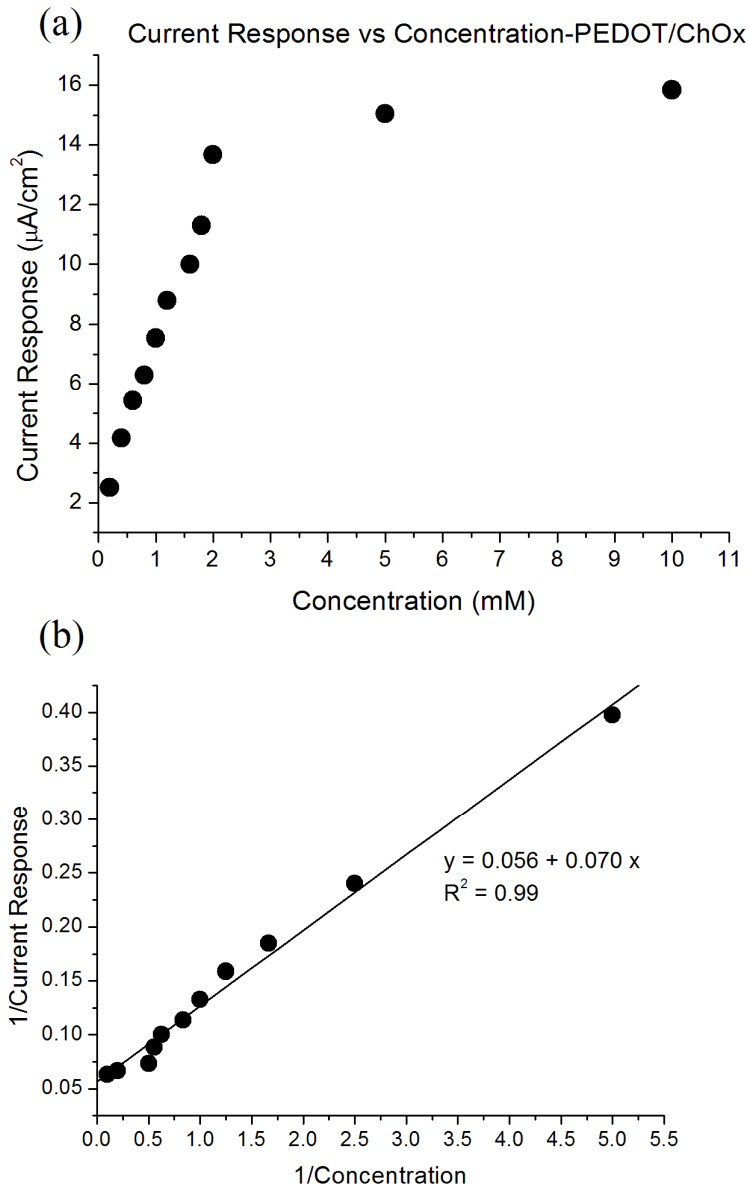


Figure 3. 3 (a) Current response vs concentration. (b) 1/Current response vs 1/concentration for the PEDOT/ChOx enzyme electrode (pH 7; 24 °C)

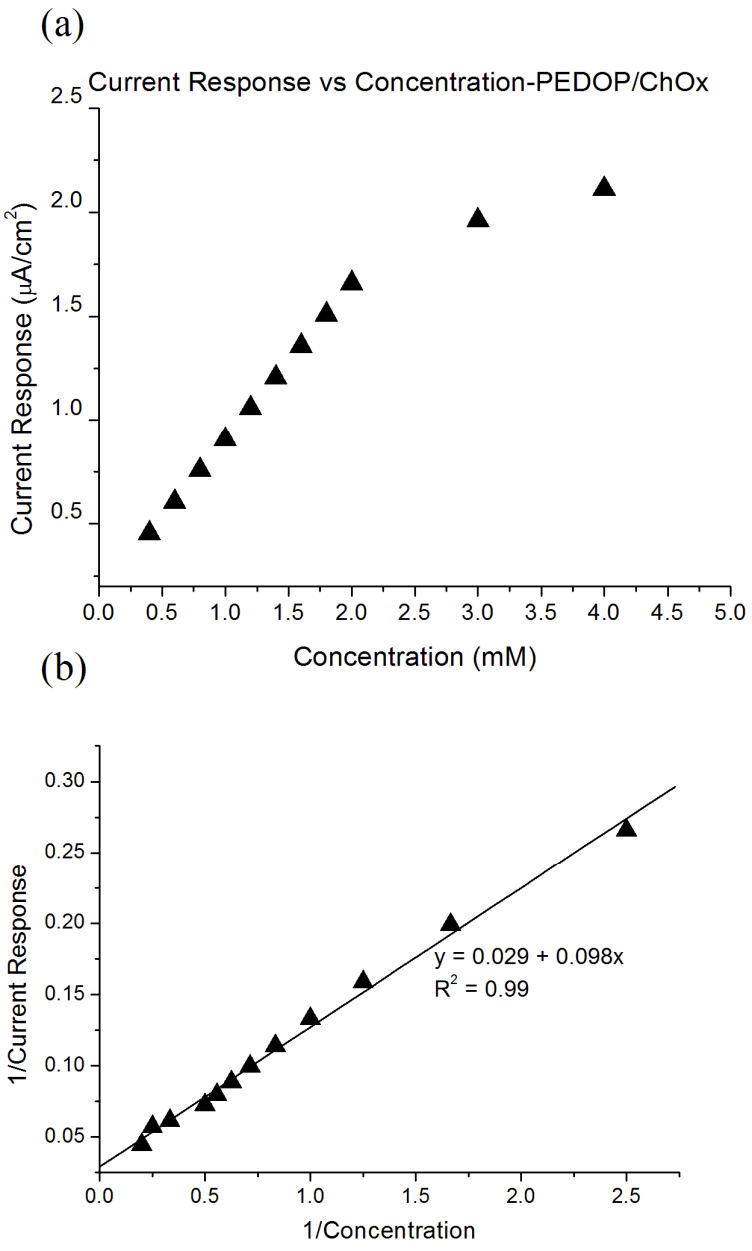


Figure 3. 4 (a) Current response vs concentration. (b) 1/Current response vs 1/concentration for the PEDOP/ChOx enzyme electrode (pH 7; 24 °C)

Table 3. 2 Comparison between conducting polymer based amperometric biosensors

Immob. Matrix	**Sensing Element**	**Immob. Method.**	**Km**	**Ref.**
PPy	ChOx	Electrochem. entrapment	0.59	(Vidal J. et al., 2003)
PEDOT	ChOx	Electrochem. entrapment	1.3	Present Work
PAni	ChOx/ChEt /HRP	Covalent attachment	1.94	(Singh S., et al. 2007)
PEDOP	ChOx	Electrochem. entrapment	3.4	Present Work
P(Ani-co-Py)	ChOx	Covalent attachment	4	(Solanki P. R., et al. 2007)
PPy	ChOx	Electrochem. entrapment	7.9	Present Work
PPy	ChOx/ChEt	Covalent attachment	9.8	(Singh S. et al., 2000)

3.1.3 Operational and Storage Stabilities

Operational and storage stabilities are shown in Figures 3.5 and 3.6, respectively. The operational stabilities were determined by running several measurements on the same day. Between each subsequent measurement electrodes stayed at 4 ºC in the buffer solution for 30 minutes. As illustrated in Figure 3.5, 60%, 35% and 67% activity losses are observed at the 10^{th} use

for ChOx entrapped in PPy, PEDOT and PEDOP matrices, respectively. In the case of PEDOP/ChOx, the first three measurements reveal the same response; however, it lost nearly 70% of its activity with the subsequent use. The shelf lives of the enzyme electrodes were determined by recording the amperometric response during 28 days. On the 28^{th} day, the relative activities were 10%, 40% and 80% for the PPy, PEDOT and PEDOP electrodes. Compared to PPy, PEDOT and PEDOP are better supports for ChOx, since the PEDOT/ChOx can be used for 20 days with only 20% loss alternatively the current responses of the PEDOP/ChOx electrode remain constant for 20 days, and then an activity loss of 20% was observed on the 20^{th} day. These long lasting sensor abilities of PEDOT and PEDOP based enzyme electrodes may be attributed to the structure of the polymers where 3- and 4- positions of the thiophene/pyrrole moieties are not available for further polymerization through those cites. Aqueous compatibility of the polymer may also be responsible in protecting the enzyme from the environmental effects. The conformational changes due to chain rotations and diffusional effects may result in the fluctuations (Türkarslan, Kıralp Kayahan and Toppare 2009) (Türkarslan, Kıralp Kayahan and Toppare 2009).

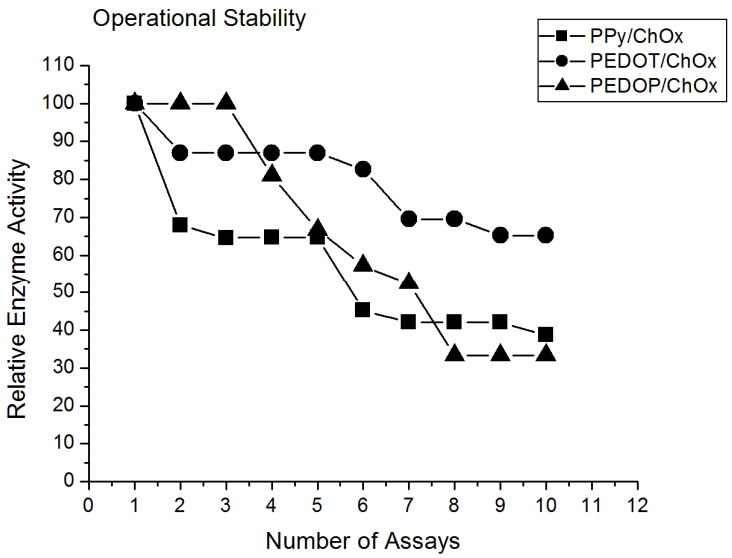


Figure 3. 5 Operational stabilities of the cholesterol biosensors

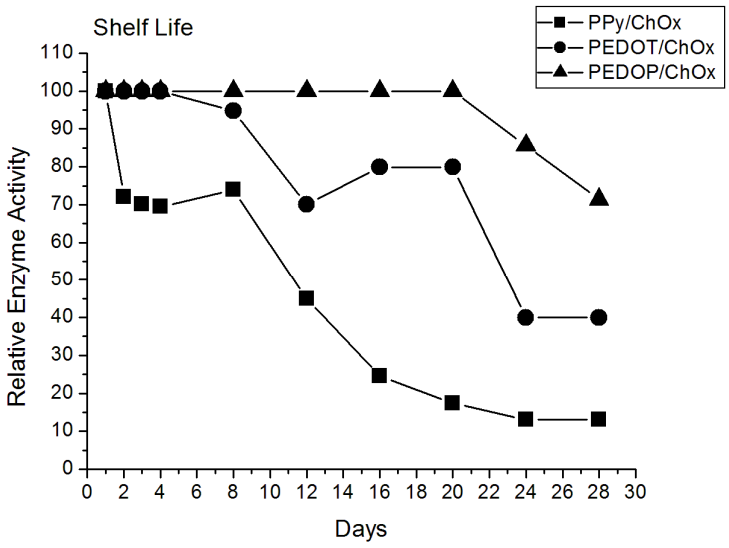


Figure 3. 6 Shelf lives of the cholesterol biosensors

3.1.4 pH Optimization

An enzyme has a characteristic pH optimum to correctly function, for this reason its bioactivity seriously depends on the pH of solution. Since high basicity or acidity result in enzyme denaturation, the optimum pH conditions should be determined. The experiments were carried out with freshly prepared enzyme electrodes and the effect of the pH on current response of the biosensors is illustrated in Figure 3.7. The maximum enzyme activities were observed at pH 7 for all proving that they can be used in blood cholesterol determination. However, as seen in the graph highly acidic or alkaline media give rise to the loss of activity. It can be concluded that PEDOT matrix demonstrates a better protection for ChOx from pH changes; for this reason a broader curve was obtained (Türkarslan, Kıralp Kayahan and Toppare 2009) (Türkarslan, Kıralp Kayahan and Toppare 2009).

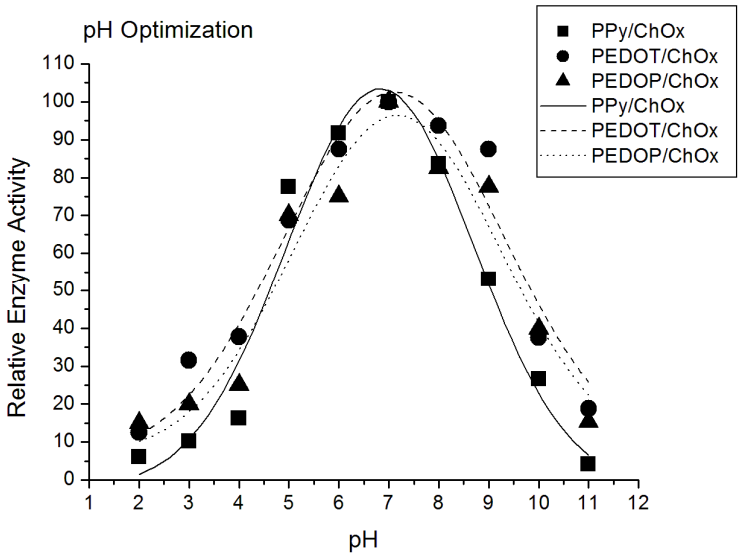


Figure 3. 7 Effect of pH on the cholesterol biosensors

3.1.5 Temperature Optimization

The enzyme activity strongly depends on temperature since very high or low values may inactivate the enzyme. The thermal stabilities of the freshly prepared enzyme electrodes are amperometrically monitored at different temperatures between 10 and 50 Cº. As illustrated in Figure 3.8, current responses gradually increase with increasing temperature and reach a maximum at 40 °C. Although with increasing temperature the reaction rate increases, the responses drop at higher temperatures. The possible reasons might be the enzyme denaturation, ethanol evaporation and decrease in dissolved oxygen (Türkarslan, Kıralp Kayahan and Toppare 2009) (Türkarslan, Kıralp Kayahan and Toppare 2009).

Activation energies are also calculated from the Arrhenius equation:

$I(T) = I_0 \exp(-E_a/RT)$

$\text{Ln } I = \text{Ln } I_0 - E_a/R\ (1/T)$.

"Ln I (current response) vs 1/T graphs" (Figure 3.9) are plotted and after doing linear regressions, equations of type "y = A + Bx" were obtained, and then activation energies were calculated from "E_a=BxR". The activation energies for the enzymatic reactions in PPy, PEDOP and PEDOT matrices were found as 39.5 kJ/mol, 31.3 kJ/mol and 27.7 kJ/mol, respectively. The smaller E_a means that the entrapped enzyme in PEDOT matrix possesses higher enzyme activity and the sensor exhibits higher affinity towards its substrate which is in agreement with a lower K_m value (Türkarslan, Kıralp Kayahan and Toppare 2009) (Türkarslan, Kıralp Kayahan and Toppare 2009).

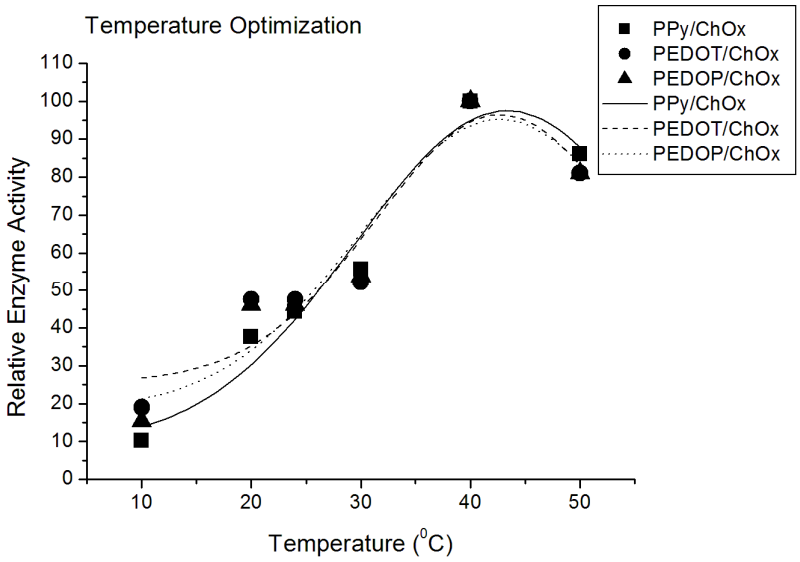


Figure 3. 8 Effect of temperature changes on the cholesterol biosensors

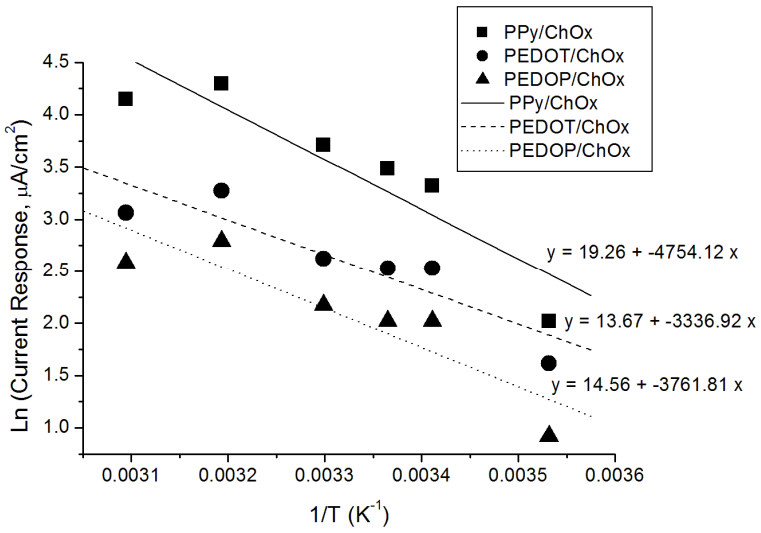


Figure 3. 9 Determination of the activation energy for the cholesterol biosensors

3.2 Alcohol Oxidase Biosensors

3.2.1 Determination of Kinetic Parameters

Figures 3.10 a, 3.11 a and 3.12 a show the profile of current response versus ethanol concentration. Initially current responses increase with the increasing substrate concentration, and then they reach steady state. Kinetic parameters K_m and I_{max} for the AlcOx biosensors were calculated from Lineweaver-Burk plots (Figures 3.10 b, 3.11 b, 3.12 b) at constant temperature (24 ºC) and pH (pH 7). The important parameters obtained for the enzyme electrodes are summarized in Table 3.3. As K_m is inversely proportional to the affinity of the enzyme for its substrate, in PEDOT matrix more AlcOx is associated with ethanol; however I_{max} is lower compared to the other enzyme electrodes, which can be interpreted as less ethanal formation. Although K_m and I_{max} values are different for all enzyme electrodes, the sensitivities calculated by dividing I_{max} to K_m are the same for the PPy and PEDOP enzyme electrodes and slightly higher sensitivity is observed for the PEDOT/AlcOx. Even though K_m and minimum detectable concentrations (given in molar) are comparatively higher than the ones reported in the literature (given in milimolar), the biosensors constructed are able to work in acceptable range with high current responses proving the product formation in appreciable amounts (Türkarslan, Böyükbayram and Toppare 2010).

Table 3. 3 Kinetic parameters for the ethanol biosensors

Matrices	**K_m (M)**	**I_{max} (μAcm^{-2})**	**Minimum Detection (M)**	**Sensitivity ($\mu AmM^{-1}cm^{-2}$)**
PPy	12.3	263.2	0.17	21.4
PEDOP	7.8	166.7	0.17	21.4
PEDOT	6	133.3	0.17	22.2

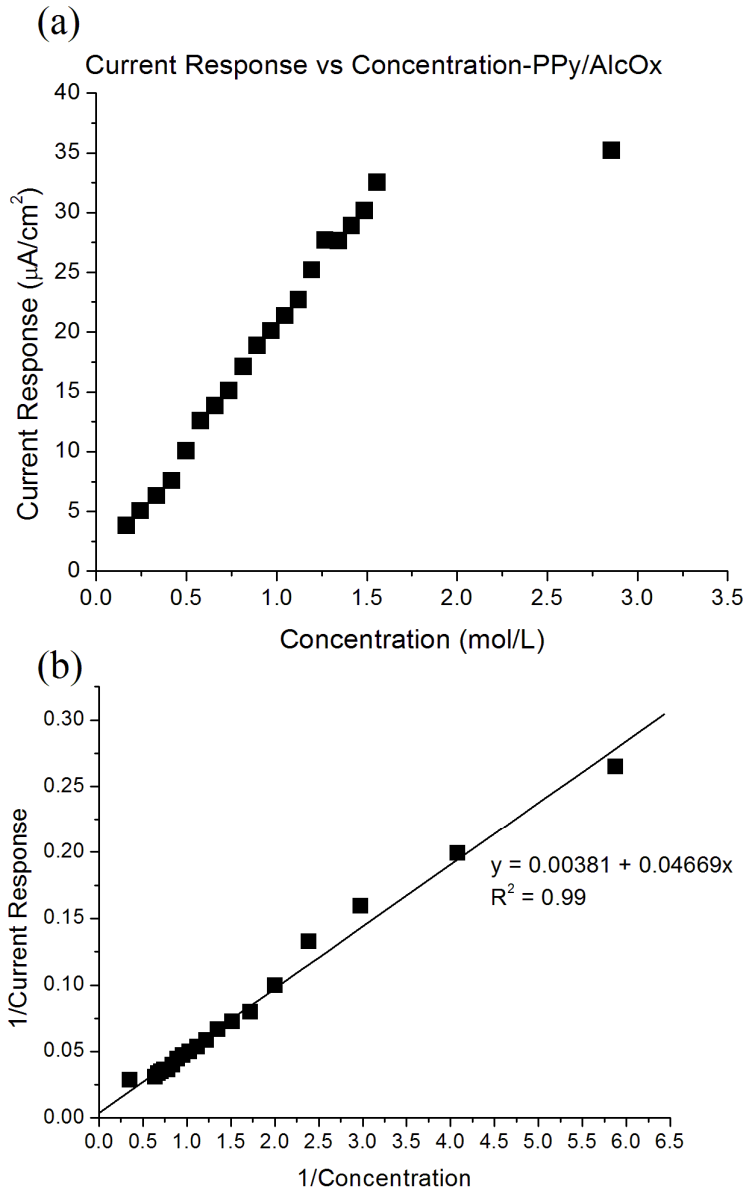


Figure 3. 10 (a) Current response vs concentration (b) 1/Current response vs 1/concentration for the PPy/AlcOx enzyme electrode (pH 7; 24 °C)

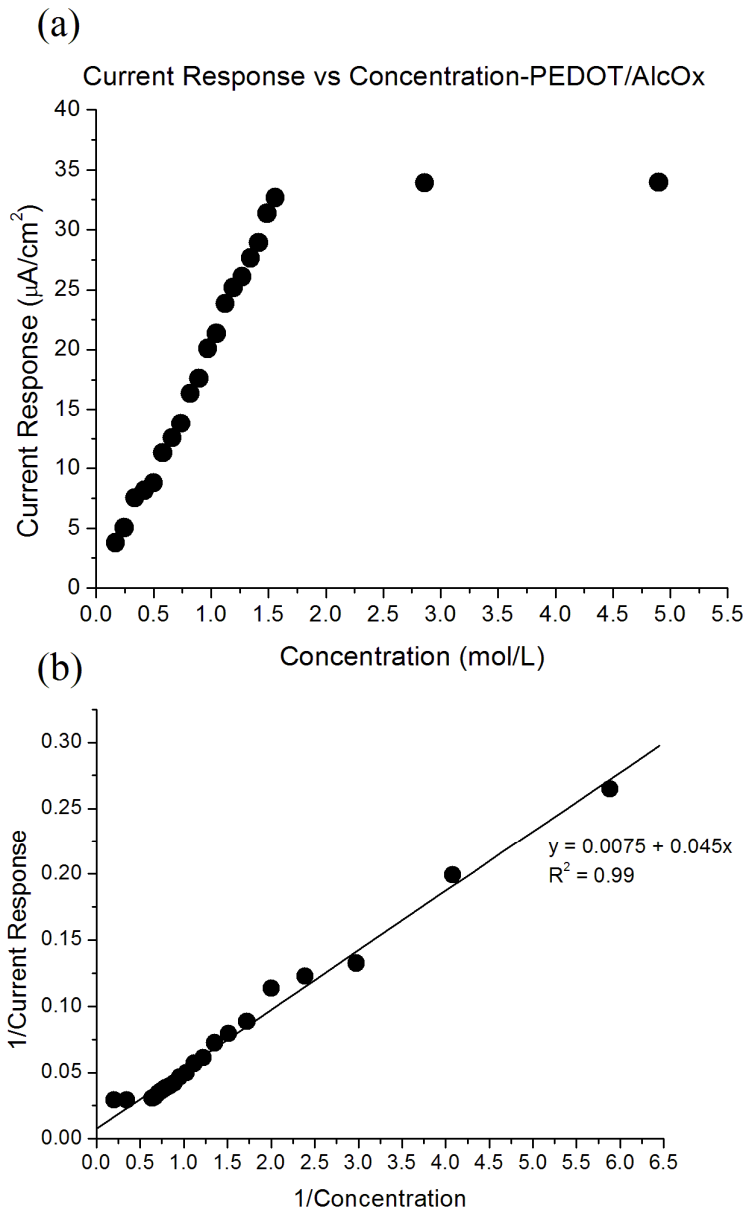


Figure 3. 11 (a) Current response vs concentration (b) 1/Current response vs 1/concentration for the PEDOT/AlcOx enzyme electrode (pH 7; 24 °C)

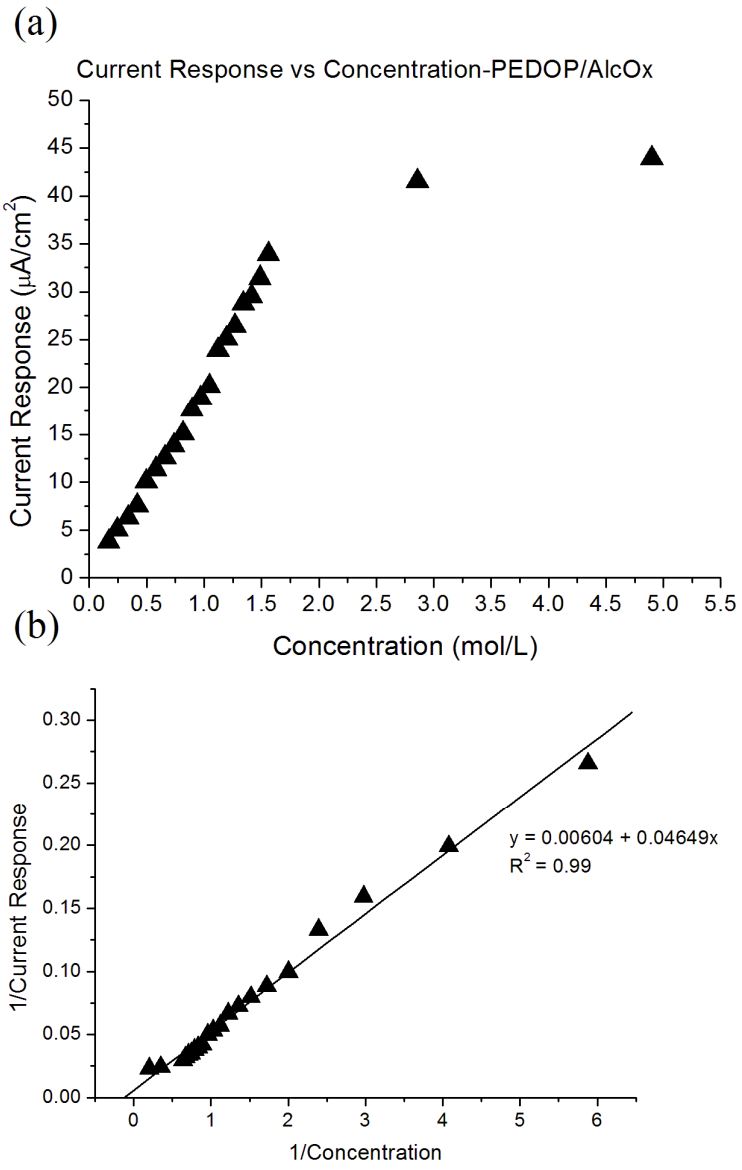


Figure 3. 12 (a) Current response vs concentration. (b) 1/Current response vs 1/concentration for the PEDOP/ChOx enzyme electrode (pH 7; 24 °C)

3.2.2 Operational and Storage Stabilities

Operational and storage stabilities are shown in Figures 3.13 and 3.14 respectively. The operational stabilities of the AlcOx immobilized in different matrices were determined running several measurements on the same day. The relative enzyme activities were calculated as 30%, 22% and 12% for the PEDOP, PPy and PEDOT based biosensors at the 15^{th} use respectively. The storage stabilities of the biosensors were established monitoring the amperometric response during 28 consecutive days. On the 28^{th} day, only 20% of the activity was lost for the PEDOP enzyme electrode, whereas the PPy and PEDOT enzyme electrodes show 50% and 23% activity respectively. For both stability experiments the best responses were observed for the AlcOx immobilized in PEDOP matrix. It can be stated that compare to the thiophene polymers, the N-H functionality of the polypyrrole derivatives increase aqueous compatibility enhancing electrochemical stability (Türkarslan, Böyükbayram and Toppare 2010).

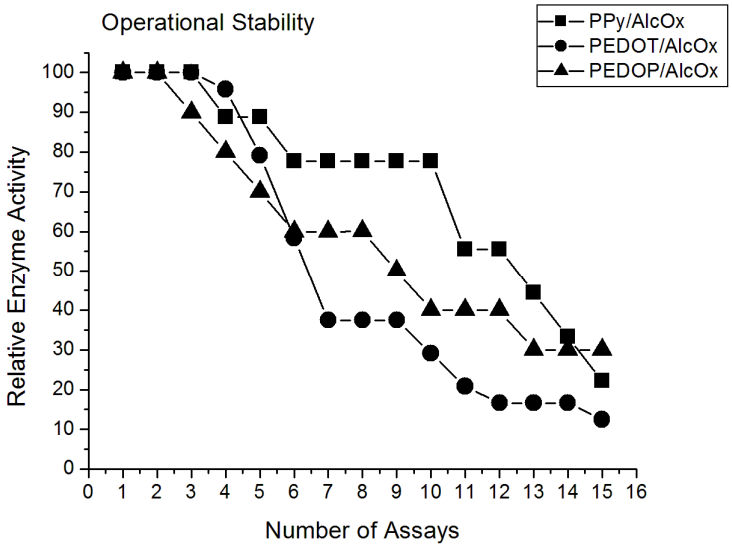


Figure 3. 13 Operational stabilities of the ethanol biosensors

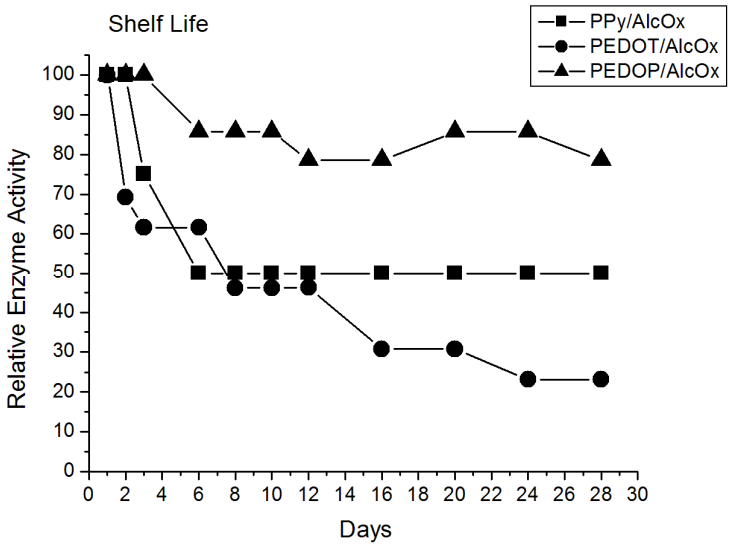


Figure 3. 14 Shelf lives of the ethanol biosensors

3.2.3 pH Optimization

The optimum pH values were determined since pH changes in working environment, especially extremely acidic or basic media, cause denaturation. The effect of pH on the amperometric response of the enzyme electrodes were studied in a range from 6 to 8 (Figure 3.15). The maximal responses were obtained at pH 7 for the biosensors; however the PEDOP matrix protects AlcOx better than the other supports against pH changes (Türkarslan, Böyükbayram and Toppare 2010).

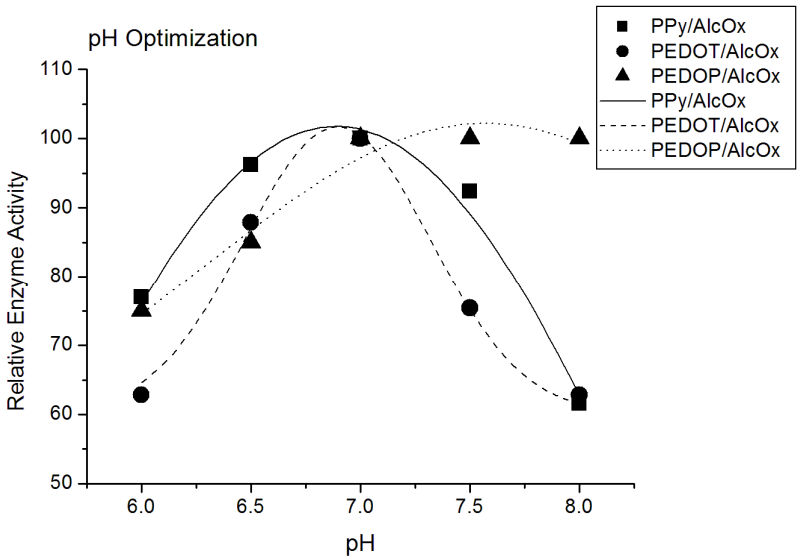


Figure 3. 15 Effect of pH on the ethanol biosensors

3.2.4 Temperature Optimization

The effect of temperature changes on the activities is shown in Figure 3.16. The thermal stabilities of the freshly prepared enzyme electrodes were checked at five different temperatures between 10 ºC and 50 ºC. Since ethanol is quite volatile (bp: 78.3 ºC) the range is not extended to higher temperatures. A similar trend was observed for all the biosensors with a maximum at 40 ºC. Although with increasing temperature the reaction rate increases, the responses drop at higher temperatures. The possible reasons might be the denaturation of the enzyme, the evaporation of ethanol and the decrease in dissolved oxygen. The activation energies of the enzymatic reactions were calculated as 25.1 kJ mol^{-1}, 24.3 kJ mol^{-1} and 23.5 kJ mol^{-1} for the PPy, PEDOP and PEDOT enzyme electrodes, respectively from "Ln current response vs 1/T graphs" (Figure 3.17). The smaller E_a means that the enzyme entrapped in PEDOT matrix shows higher enzyme activity and affinity towards its substrate, which is in agreement with a lower K_m value (Türkarslan, Böyükbayram and Toppare 2010)

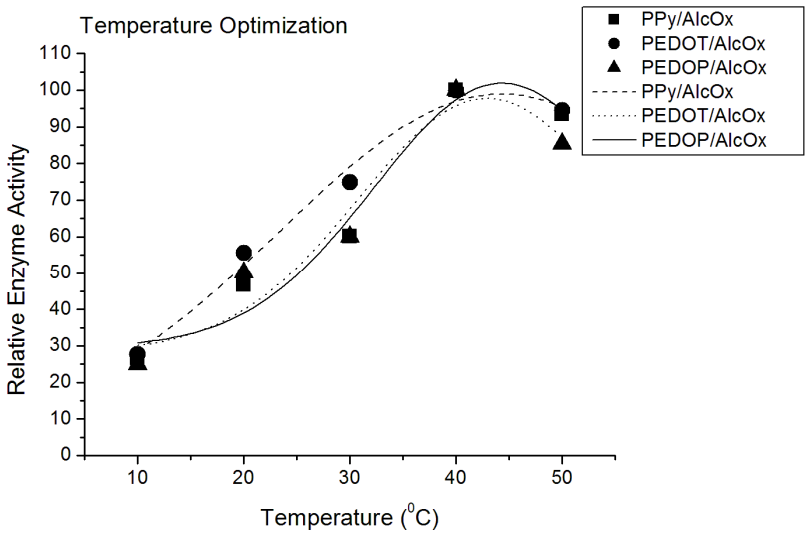


Figure 3. 16 Effect of temperature changes on the ethanol biosensors

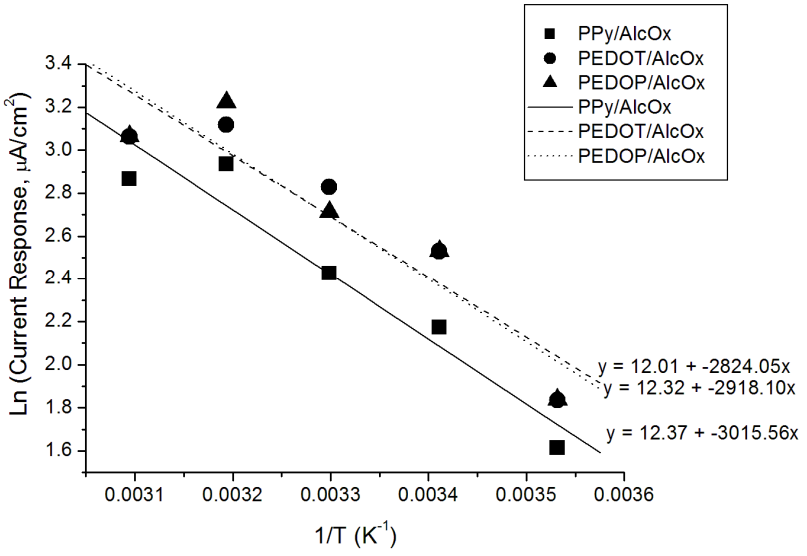


Figure 3. 17 Determination of the activation energy for the ethanol biosensors

3.2.5 Determination of Immobilized Alcohol Oxidase

Enzyme contents of the PPy/AlcOx and the PEDOT/AlcOx electrodes were determined via Lowry method (Lowry, et al. 1951). However, measurements in the PEDOP/AlcOx sensor were not successful due to dark interfering color of the immobilization solution. Protein standard solutions were prepared in the conventional way of Lowry using bovine serum albumin. Aliquots taken from electrolysis solutions before and after electrolysis were added to the usual Lowry coloring solutions to determine protein contents of immobilization solutions. Same determinations were repeated after the electrodeposition. The difference in protein content of the same solution before and after electropolymerization is calculated to determine the amount of enzyme loading. Immobilized alcohol oxidase amounts were found as 0.075 and 0.058 mg for PPy/AlcOx and PEDOT/AlcOx enzyme electrodes, respectively. The higher I_{max} value of PPy/AlcOx electrode is consistent with the higher amount of entrapped enzyme in this electrode (Türkarslan, Böyükbayram and Toppare 2010).

3.2.6 Interference Studies

The interference of some common ingredients present in alcoholic beverages was tested. Sensitivities were calculated from Lineweaver-Burk plots in the presence of 2 mM of interferent (glucose, acetic acid, citric acid, L-ascorbic acid) and the percent deviations from the sensitivity values are shown in Table 3.4. Depending on the type of ingredient and polymeric matrix, the interferents affect the results between 2 and 8% (Türkarslan, Böyükbayram and Toppare 2010).

Table 3. 4 Interference studies

Electrodes	Sensitivity	Sensitivity in the Presence of Interferents			
		Glucose (2mM)	**Acetic Acid (2mM)**	**Citric Acid (2mM)**	**L-Ascorbic Acid (2mM)**
PPy/AlcOx	21.4	22.9	22.4	23.0	22.6
	%Deviation:	*7.0%*	*4.7%*	*7.7%*	*5.6%*
PEDOT/AlcOx	22.2	22.7	22.9	23.0	22.7
	%Deviation:	*2.3%*	*3.2%*	*3.6%*	*2.3%*
PEDOP/AlcOx	21.4	22.5	22.2	22.2	22.4
	%Deviation:	*4.7%*	*3.3%*	*3.3%*	*4.2%*

3.2.7 Testing on Alcoholic Beverages

The biosensors were tested on commercially available, distilled alcoholic beverages containing at least 40% of ethanol. Russian vodka, British dry cin, Scotch whisky and Turkish 'rakı' (a traditional, Mediterranean alcoholic beverage made of grape and aniseed) were chosen as the analytes. It was found that aniseed exhibits an interference of 20% bias in the activity. Hence a type of rakı without aniseed was used in the following studies and its ethanol content was determined by HPLC analysis at METU Central Laboratory. Aliquots of 0.5 mL and 1 mL were injected to the reaction cell and current changes were evaluated according to calibration plots. Table 3.5 depicts the analytical results in terms of precision and accuracy. As seen in the table, percent errors are not very

significant and a good match with the chromatography results (reported by the companies) was observed (Türkarslan, Böyükbayram and Toppare 2010).

Table 3. 5 Tests on alcoholic beverages

Electrodes	**Vodka Brand B Alc. 40%**	**Cin Brand B Alc.40%**	**Whisky Brand JW Alc.40%**	**Rakı Brand B (no aniseed) Alc. 56%**
PPy/AlcOx	40.2±3.1% *%E=0.4%*	39.1±3.5% *%E=2.3%*	40.5±4.8% *%E=1.2%*	55.7±3.9% *%E=0.53%*
PEDOT/AlcOx	40.1±3.1% *%E 0.3%*	40.6±3.5% *%E=1.5%*	41.0±6.0% *%E=2.4%*	56.5±2.7% *%E=0.89%*
PEDOP/AlcOx	39.6±3.4% *%E=1.0%*	39.7±3.4% *%E=0.8%*	39.4±3.9% *%E=1.5%*	55.2±2.5% *%E=1.43%*

CHAPTER IV

CONCLUSION

The redox enzymes, cholesterol oxidase from *Pseudomonas fluorescens* and alcohol oxidase from *Pichia Pastoris* were immobilized in three conducting polymeric matrices where the monomers are partially water soluble. The polymers were grown on Pt working electrodes in the presence of phosphate buffer, enzyme, water soluble monomers, pyrrole, 3,4-ethylenedioxythiophene, 3,4-ethylenedioxypyrrole, and sodium dodecyl sulfate. These redox enzymes, cholesterol oxidase and alcohol oxidase, catalyzing oxidation of cholesterol and alcohol to the respective aldehydes, were immobilized in PEDOP for the first time to construct biosensors. After calculating kinetic parameters, optimization experiments were carried out. Operational and storage stabilities, pH and temperature dependencies were determined. The enzymes immobilized in PEDOT matrix had higher sensitivities, small K_m and E_a, which could be interpreted in terms of high affinity towards the substrates; cholesterol and ethanol. Moreover, thanks to its structural order PEDOT and PEDOP matrices revealed better stabilities and protected the enzyme from environmental changes, such as pH and temperature. Before testing the AlcOx electrodes on alcoholic beverages effect of interferents (glucose, acetic acid, citric acid, L-ascorbic acid) which might be present in liquors were determined. The results showed that

these ingredients exhibit interference between 2% and 8% on the sensitivity. The alcohol contents of four different types of distilled beverages were measured with the sensors constructed within acceptable error ranges.

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