BIOACTIVE SURFACE DESIGN BASED ON CONDUCTING POLYMERS AND APPLICATIONS TO BIOSENSORS

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

BY

FULYA EKİZ

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOTECHNOLOGY

JUNE 2012

Approval of the thesis:

BIOACTIVE SURFACE DESIGN BASED ON CONDUCTING POLYMERS AND APPLICATIONS TO BIOSENSORS

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ABSTRACT

BIOACTIVE SURFACE DESIGN BASED ON CONDUCTING POLYMERS AND APPLICATIONS TO BIOSENSORS

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June 2012, 88 pages

An underlying idea of joining the recognition features of biological macromolecules to the sensitivity of electrochemical devices has brought the concept of biosensors as remarkable analytical tools for monitoring desired analytes in different technological areas. Over other methods, biosensors have some advantages including high selectivity, sensitivity, simplicity and this leads to solutions for some problems met in the measurement of some analytes. In this context, conducting polymers are excellent alternatives with their biocompatibility and ease of applicability for an efficient immobilization of biomolecules in preparing biosensors. Using several materials and arranging the surface properties of the electrodes, more efficient and seminal designs can be achieved. In this thesis, it is aimed to create new direct biosensors systems for the detection of several analytes such as glucose and pesticides thought to be harmful to the environment. Recently synthesized conducting polymers (polyTBT); (poly(2-dodecyl-4,7-di(thiophen-2-yl)-2*H*-benzo[*d*][1,2,3]triazole) and (poly(TBT_6-NH_2); poly(6-(4,7-di(thiophen-2-yl)-2*H*-benzo[*d*][1,2,3]triazol-2-yl)hexan-1-amine) were utilized as a matrices for biomolecule immobilization. After successful electrochemical deposition the

polymers on the graphite electrode surfaces, immobilization of glucose oxidase (GOx) and choline oxidase (ChO) were carried out. Amperometric measurements were recorded by monitoring oxygen consumption in the presence of substrates at -0.7 V. The optimized biosensors showed a very good linearity with rapid response times and low detection limits (LOD) to glucose and choline. Also, kinetic parameters, operational and storage stabilities were determined. Finally, designed biosensor systems were applied for glucose and pesticide detection in different media.

Keywords: Conjugated Polymers, Biosensors, Glucose Oxidase, Choline Oxidase, Conducting Polymer Based Electrochemical Biosensors.

ÖZ

İLETKEN POLİMERE DAYALI BİYOAKTİF YÜZEY DİZAYNI VE BİYOSENSÖR UYGULAMALARI

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Haziran 2012, 88 sayfa

İstenilen analitlerin tayinlerinde, sıklıkla kullanılan biyolojik makromoleküllerle elektrokimyasal cihazların duyarlılığının birleştirilmesiyle biyosensörler ortaya çıkmış ve farklı teknolojik alanlarda kullanılan önemli bir analitik tayin metodu haline gelmiştir. Diğer tekniklerden üstün olarak biyosensörler, yüksek seçicilikleri, duyarlılıkları, sadelikleri ile bazı analitlerin ölçümlerinde karşılaşılan sorunlarda avantaj sağlamaktadır. Bu bağlamda, biyosensör hazırlamada yüzey modifikasyonu için iletken polimerler mükemmel birer alternatiftirler. Kolay uygulanabilirlikleri ve biyouyumluluklarıyla biyomoleküllerin verimli immobilizasyonlarının gerçekleştirilmesini sağlamaktadırlar. Bu çalışmada, çevreye ve sağlığa zararlı oldukları düşünülen çeşitli pestisitlerin ve glukozun tayini için doğrudan biyosensör sistemleri hazırlanması hedeflenmiştir. Sentezlenen iletken polimerler (poliTBT); (poli(2-dodesil-4,7-di(tiyofen-2-il)-2*H*-benzo[*d*][1,2,3]triazol) ve (poli(TBT_6-NH_2); poli(6-(4,7-di(tiyofen-2-il)-2*H*-benzo[*d*][1,2,3]triazol-2-il)hegzan-1-amin) biyomolekül immobilizasyonu için matris olarak kullanışmıştır. Polimerlerin grafit elektrot yüzeyleri üzerinde başarılı bir şekilde biriktirilmesinin ardından, glukoz

oksidaz (GOx) ve kolin oksidaz (ChO) enzimlerinin immobilizasyonu gerçekleştirildi. Amperometrik ölçümler, substurat varlığında -0.7 V uygulaması altında oksijen tükenişinin takibi ile gerçekleştirildi. Optimize edilmiş biyosensörler, kısa cevap verme süreleri ve düşük tespit sınırları ile glukoz ve kolin substuratları için çok iyi lineerlik göstermiştir. Bunların yanısıra, biyosensörlerin çeşitli kinetik parametreleri, operasyonel ve depo kararlılıkları tayin edilmiştir. Son olarak, tasarlanan biyosensör sistemleri farklı ortamlarda glukoz ve pestisit tayini için kullanılmıştır.

Anahtar kelimeler: Konjüge Polimerler, Biyosensörler, Glukoz Oksidaz, Kolin Oksidaz, Konjüge Polimer Esaslı Elektrokimyasal Biyosensörler.

Dedicated to my precious parents and husband.

ACKNOWLEDGMENTS

I would like to express my sincere thanks and my greatest appreciations to my supervisor Prof. Dr. Levent Toppare for his guidance, support, encouragement, patience, valuable advices and beyond everything being a father for us.

I would like to express my deepest sense of gratitude to my co-advisor, Prof. Dr. Suna Timur, for providing me the opportunity to develop my studies at Department of Biochemistry, Ege University. I gratefully thank her for her extraordinary support, endless helps, valuable ideas, motivation and patience besides her kind friendship.

I owe great thanks to Merve Yüksel, Mehriban Akın, Caner Geyik, Dr. Dilek Odacı Demirkol, Çağlar Özdemir, Eylül Başkurt, Murat Karadağ for providing an orientation to biosensors and evaluation as well as for sharing their knowledge on biosensors and their help.

I would like to thank Prof. Dr. Müfit Bahadır and Dr. Marit Kolb from Technische Universität Braunschweig for their valuable collaboration, partnership and providing me great research facilities in Germany.

I would like to thank all Toppare Research Group and Timur Research Group members for their cooperation and kind friendship.

I am thankful to my friends sister Hava, Abidin, Sema, Bilge for their real friendship.

I am thankful to my friends Özge, Melis, Gözde, Gönül, Seda, Naime, Şerife, Ayda, Buket, Eda for a wonderful research environment.

Words fail to express my eternal gratitude to my beloved parents for being my family, their guidance, believing in me and giving me endless support during all my life. Without them, my life would not have been possible. I am the luckiest person in the world being your daughter.

I would like to express my profound gratitude to my husband, Zafer Kanık, for every single thing that we share, for his continuous and great love, for being always at my side, loving me and encouraging me. I am the luckiest person in the world being your wife and I will always love you.

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LIST OF ABBREVIATIONS

ACN Acetonitrile

Au NPs Gold Nanoparticles

BSA Bovine Serum Albumin

CE Counter Electrode

ChO Choline Oxidase

CLEA Cross-Linked Enzyme Aggregate

CLECs Cross-Linked Enzyme Crystals

CNT Carbon Nanotube

CP Conducting Polymer

CV Cyclic Voltammetry

DCM Dichloromethane

DMF Dimethylformamide

FAD Flavin Adenine Dinucleotide

GA Glutaraldehyde

GC Gas Chromatography

Glc Glucose

GOx Glucose Oxidase

HPLC High Performance Liquid Chromatography

Ile Isoleucine

ITO Indium Tin Oxide

$LiClO_4$ Lithium Percholorate

LOD Limit of Detection

MWNTs Multi-Wall Carbon Nanotubes

$NaClO_4$ Sodium Perchlorate

n-BuLi n-Butyllithium

PA Polyacetylene

PANI Polyaniline

PEDOT Poly(3,4-Ethylenedioxythiophene)

PF Polyfuran

PPy Polypyrrole

PTh Polythiophene

RE Reference Electrode

RSD Relative Standard Deviation

SAM Self-Assembled Monolayer

SCE Standard Calomel Electrode

SD Standard Deviation

SEM Scanning Electron Microscopy

$TBAPF_6$ Tetrabutylammonium Hexafluorophosphate

TBT 2-Dodecyl-4,7-Di(Thiophen-2-yl)-2*H*-benzo[*d*][1,2,3]Triazole

TBT_6-$NH_2$6-(4,7-Di(Thiophen-2-yl)-2*H*-Benzo[*d*][1,2,3]Triazol-2-Yl)Hexan-1-Amine

THF Tetrahydrofuran

WE Working Electrode

XPS X-Ray Photoelectron Spectroscopy

CHAPTER 1

INTRODUCTION

1.1 Conducting Polymers

The first report introducing the electrical conductivity of conjugated polymers was revealed by Shirakawa, Heeger and MacDiarmid in 1977 [1,2]. After this discovery, conjugated polymers appeared as an attractive field of study in many academic and industrial researches. Hence, conducting polymers (CP) are drawing great deal of attention in various application areas such as electrochromic devices [3], light-emitting displays [4], solar cells [5], drug release systems [6] and biosensors [7-9]. Their electronic, structural and optical properties with easy synthesis and good stability have been the driving force for many research fields and make them applicable in electronic, optoelectronic and biotechnological applications [10,11].

Along with this prominent discovery, it was found out that conjugated polymers can act as metals upon doping. Hence, they are named as synthetic metals as a new class of materials. Conducting polymers have conjugated π-electron backbones [12]. High degree of overlapping of repeating units' π orbitals leads the free movement of the electrons throughout the chain. Thus, the generated charged species upon oxidation (p-doping) or reduction (n-doping) can move along the conjugated backbone which, then, yields the electron transport along the chain and so do electrical conductivity [13].

Conducting polymers can be generated via chemical or electrochemical polymerization. In general, electrochemical polymerization is favorable because of

its simplicity, easy thickness and morphology control and purity of the prepared polymer compared to the chemical polymerization technique. Electropolymerization is achieved, generally, in an electrolyte solution via anodic oxidation of the corresponding monomer and finalized with the deposition of the conduction polymer on a substrate material. Various techniques can be preferred for electropolymerization such as potentiostatic (constant-potential), potentiodynamic (potential scanning, via cyclic voltammetry) and galvanostatic (constant-current) techniques [14].

Various types of conducting polymers were synthesized including different structures in their backbone such as thiophene and pyrrole [15,16]. (Figure 1.1)

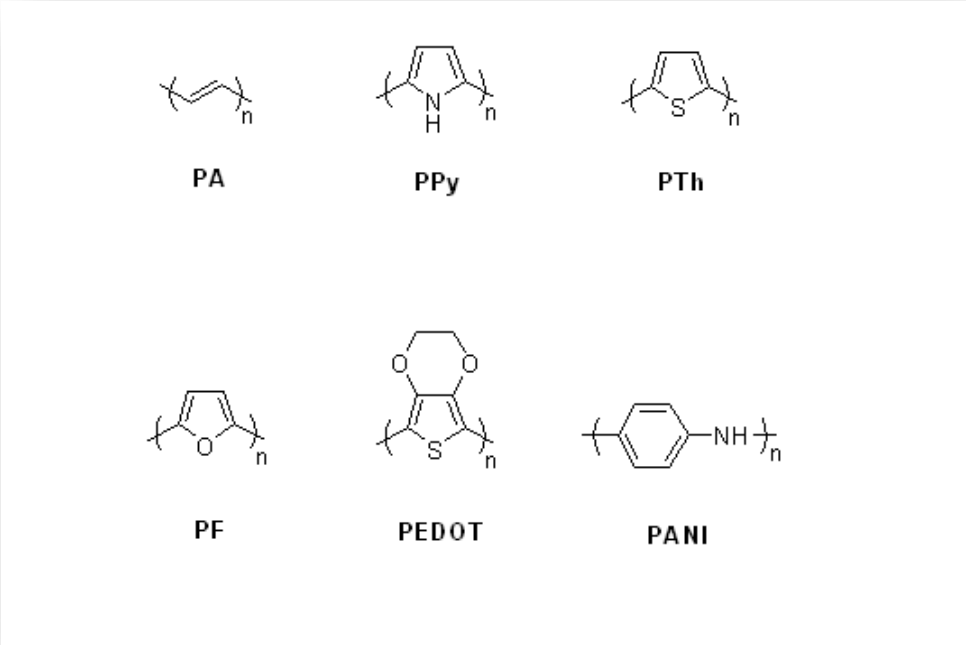


Figure 1. 1 Common organic conducting polymers.

Conducting polymers with their distinct properties as multipurpose materials have large scope of applications. Taking the advantages of homogeneous and manageable film character, ability of modification of physical and optical properties, stability and biocompatibility, reproducible and easy production, conducting polymers are effectively used in many biotechnological applications such as biosensors.

1.2 Biosensors

A biosensor is a type of analytical device which consists of a biological recognition element either merged within a transducer or closely connected to it, in order to make a quantitative determination of a specific analyte. [17,18] The goal is to transform selective information corresponding to the presence of an anaylte into a digital, meaningful signal. The aim in biosensor design is to detect an analyte of interest in a sample matrix directly. Hence, a biosensor is designed to be specific, selective, sensitive, rapid, simple, handy and easy to construct. These pioneering miniature systems have attracted attention recently in terms of their sensitivity, selectivity and stability.

Biosensor has two main compartments: a biological detection element and a transducer connected to a processing element. (Figure 1.2)

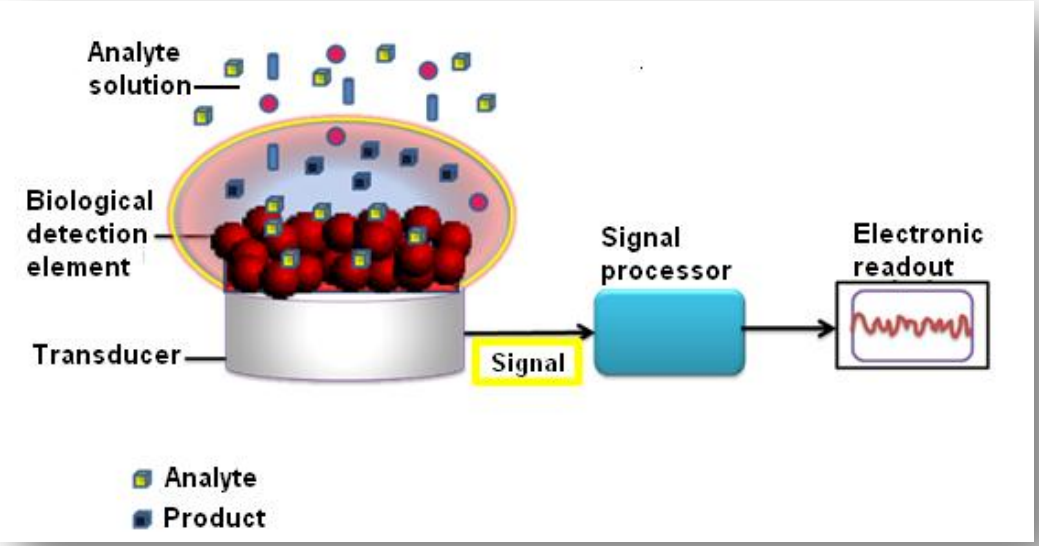


Figure 1. 2 Simple representation of a biosensor and its working principle.

The biological element functions as a bioreceptor which can be enzyme, antibody, microorganism or DNA probe which recognizes its analyte such as enzyme substrate, antigen or complementary DNA fragment. The transducer is utilized to convert the biochemical signal due to the biochemical interaction of the analyte with the recognition element into an electronic signal. With the choice of transducer, the detection method is determined. Electrochemical transducers are preferred due to their low-cost availabilities, simple and small-scale designs.

For a reliable detection, sensors have to be specific to the analyte of interest, sensitive, rapid and inexpensive.

Biosensors are categorized in accordance with biological element type and transducer type. (Figure 1.3)

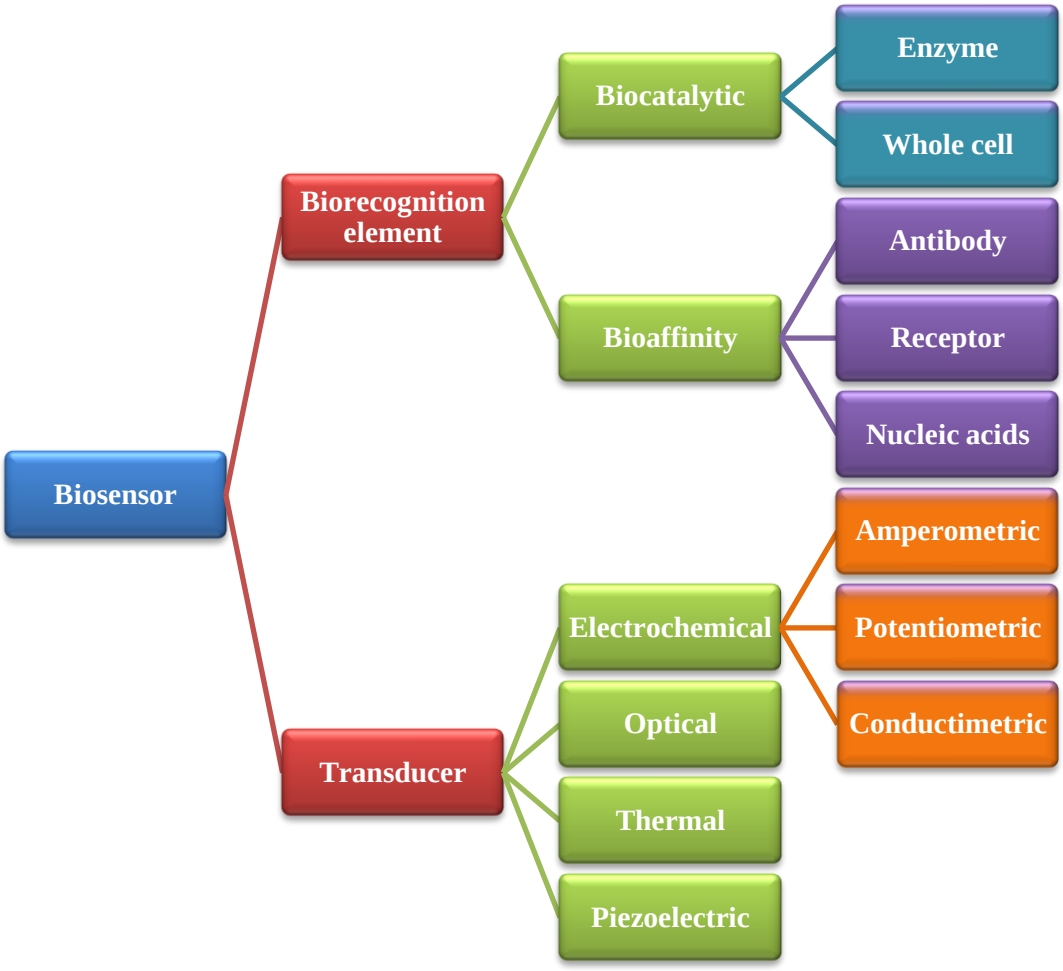


Figure 1. 3 Biosensor types.

The first example of biosensor was elucidated by Clark and Lyons in 1962 who introduced enzyme glucose oxidase as the biocomponent [19]. They utilized a semipermeable membrane to fix the enzyme on an amperometric oxygen electrode surface. The main purpose was to determine the glucose concentration in a sample

directly. After the first demonstration of the biosensor, enzymes appear as a significant tool and are widely used in biosensing studies [20,21,22].

Since the incorporated biomolecule is susceptible to environment and vulnerable to extreme conditions; pH and temperature, fixation of the biocomponent is decisive in biosensor construction. Mainly, biological compounds like enzymes, whole cells etc. are not stable in various media and have very short lifetime. After the long and expensive isolation and purification processes, biological materials have really short lifetime in solution phase during the sensing steps. Moreover, once they are introduced in a medium, it is hard to isolate and reuse them. Hence, it is important to immobilize them in an appropriate insoluble matrix in order to prolong the stability and lifetime of these biomolecules during the sensing applications [23]. Therefore, after immobilization of the biological component, it needs to completely retain its biological activity and stability. This depends on the technique chosen for immobilization, surface characteristics, porosity and hydrophilicity of the chosen matrix [18]. Moreover, the matrix needs to be biocompatible and inert in order to maintain the activity of the biomolecules [24]. The aim of immobilization is to achieve a close contact between biological component and transducer surface while preserving its activity with the concomitant stability. Furthermore, immobilization of the biomolecules such as enzyme brings many advantages. These advantages can be summarized as follows:

- Operational stability of enzyme is enhanced.
- The immobilized enzyme can be easily and practically removed from the contaminated media and separated from the product.
- Substantial use of the constructed system is possible.
- With a single aliquot of enzyme, repeated use can be possible which reduces costs.
- The shelf life of the biosensor is prolonged.
- Time, material, labor force and cost of operation can be reduced.

1.2.1 Immobilization Methods

As far as enzyme-based biosensors are considered, the enzyme immobilization step can be regarded as the most fundamentally crucial issue for the successful construction of the biosensor. Therefore, as well as the choice of a suitable matrix, the immobilization technique is also important for the successful immobilization of enzymes for the preparation of durable and functioning biosensors. For an efficient and stable immobilization, the activity and accessibility of the enzyme should be considered after the immobilization. Thus, compare to free enzymes, immobilized enzymes should be durable and resistive to environmental conditions.

A variety of methods can be used in the immobilization of enzymes. Since the most crucial step is the immobilization of the enzyme in biosensor construction, the most suitable one should be chosen. The method should be appropriate for both enzyme and the matrix. The selection of the appropriate method depends on the nature of the biological material, transducer type, detection mode, physical and chemical properties of the operation conditions. Therefore, for successful biosensor architecture, the immobilization should promote the electrochemical connection between the enzyme and the transducer surface. Absolutely, the performance, sensitivity, response time and lifetime of the biosensor are affected and closely related with the choice of immobilization technique. In the case of enzyme denaturation or conformational change because of the immobilization, modification on the active site occurs and sensitivity decreases [25]. Hence, the choice of immobilization way is the key step in fabrication of the biosensors.

The interaction between the support (transducer surface) and the enzyme can be weak or robust according to the purpose. These interactions can be broadly classified in terms of physical and chemical interaction between support and enzyme. Commonly used immobilization techniques are summarized as follows.

1.2.1.1 Physical adsorption

Enzymes can be spontaneously adsorbed on various electrode surfaces by miscellaneous interactions. Physical methods do not include the formation of covalent bonds. The adsorption of the enzyme occurs essentially on account of the electrostatic, hydrophobic interactions or hydrogen bond formations [26]. These interactions are closely related with the ionic strength, pH etc. and binding can be affected by changing conditions; thus, the stability and performance of the sensing layer are profoundly affected with these conditions. Among all the immobilization methods, adsorption is the most simple and cheap one. However, since the interactions between the support and protein are weak and susceptible to the conditions, it may bring the leaching of the enzyme during the operational steps or shortening the lifetime and stability of the obtained biosensor [27]. A representative scheme is shown in Figure 1.4. A way to overcome these problems is to cover the sensing layer with a dialysis membrane to prevent desorption and leaching of the biocomponent. A variety of biological materials can be immobilized using this technique. Inorganic materials such as clay [28], conducting polymers [29], nanoparticle containing matrices [30] or layer-by-layer prepared multiwalled carbon nanotubes (CNTs)-nanoparticle matrices [31] can be used as immobilization matrix.

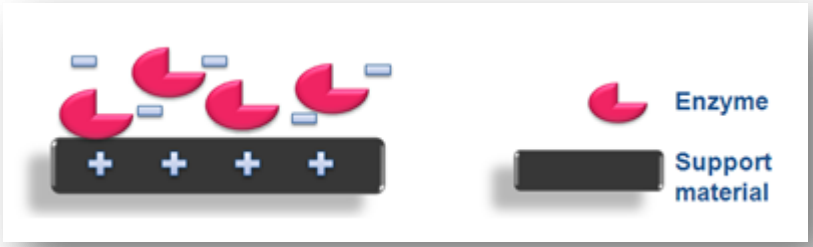


Figure 1. 4 Schematic representation of physical adsorption of enzymes.

1.2.1.2 Entrapment

Enzyme immobilization can be achieved with three-dimensional network formation for the fixation of the enzyme. The biocomponent can be entrapped in a network on the transducer surface. This network can be a polymeric dialysis membrane. This immobilization is quite simple and easy. Enzymes, mediators or additives can be easily entrapped at the same time in the same sensing layer. There is no need to modify the enzyme and it can preserve its biological activity. This prolongs the operational stability. However, diffusion problems and long response time are some drawbacks of this technique. Since the enzymes are caged within a network or a membrane, the focus is directed towards the pore size of the network facilitating the diffusion of the substrates, products and keeping the enzyme efficiently [26]. For entrapment of the biocomponents, dialysis membranes [7], biological polymeric structures like chitosan matrix [32], sol-gel encapsulation [33] can be used. Moreover, the entrapment of the biocomponents can be achieved during the polymerization procedure. In this one-step method, the transducer is dipped into an aqueous solution containing both monomer and enzyme. During the electrogeneration of the polymer, the enzyme molecules can be entrapped in the polymeric matrix on the working electrode (transducer) surface. The polymeric network allows the substrate and product to migrate whereas it does not let the enzyme molecules leach. It is a widely used technique for its availability, simplicity and reproducibility [21,34]. However, the solubility of the monomer in aqueous medium, the durability of the enzyme during electropolymerization under potential application and interference possibility of the supporting electrolyte in the medium are some limitations for this technique. Furthermore, the large size of the enzymes makes the catalytic processes in the network difficult due to the steric hindrance [35]. A representative scheme for entrapment options is shown in Figure 1.5. In entrapment, mostly, conducting polymers are used due to their conductivity, ability to control the thickness and surface morphology, biocompatibility and pore size of

the generated polymeric network. Due to their high conductivity, especially for redox enzymes, the electron transfer from the active site of the enzyme to the transducer is enhanced. Nevertheless, the excess use of enzyme amount in the aqueous solution and accessibility limitations to the trapped enzyme make this method disadvantageous.

Figure 1. 5 Schematic representation of entrapment of enzymes (a) in a dialysis membrane and (b) in a polymeric matrix.

1.2.1.3 Covalent Binding

Covalent binding as an immobilization technique means the attachment of biomolecule onto the support material surface with robust covalent bonds between the free functional groups of the biomolecules and functional pendant groups of the support. (Figure 1.6) As a matter of fact, in order to obtain long lifetime and high stability of enzyme electrodes, covalent binding can be preferred. It reduces the diffusion limitations, leaching out of the enzyme molecules, and enhances the stability of the enzyme [36]. Covalent attachment contains two-step procedure. First

of all, the functionalized support is prepared. This can be achieved via electrogeneration of a functional polymer on the transducer surface [37], formation of self-assembled monolayer (SAM) [38], functionalized nanotubes on the electrode surface [39] or porous organic-inorganic hybrid sol-gel composites [40]. Then, as a second step, biocomponent is introduced to the surface and biomolecule coupling is achieved via covalently binding on the transducer surface using multifunctional reagents. When enzymes are considered, they have free primary amine groups and carboxylic acid groups in their structure. These functional groups can take place in the covalent attachment. A great number of activation methods can be used for attachment, for instance using glutaraldeyhe or carbodiimide. Glutaraldehye is used as the activating agent and allows the binding between amino groups of the support and amino groups of the enzyme [9]. Carbodiimide provides the binding between carboxylic acid of the support and amino groups of the enzyme or vice versa [37]. Compared to other biosensor architectures, covalently bound enzymes are more stable and provide increased sensitivity. Due to the closeness of the enzyme to the transducer, the electron transfer is also enhanced. Moreover, two-step sequential immobilization procedure provides the possibility and advantage of the optimization for each step to get the most sensitive optimal conditions. Unlike entrapment, this method enables the modulation of amount of the enzyme immobilized. On the other hand, excess attachment of the enzyme molecules can cause a denaturation of them; thus, this brings the sensitivity and activity loss.

1.2.1.4 Cross-linking

With this method, enzymes can be intermolecularly crosslinked or covalently bind to a biological support via crosslinking. It is used to attach biomolecules to the support material or within itself. Moreover, it is also used to stabilize the enzymes which are adsorbed on the support [41]. Thus, more compact protein structure is

achieved and the leaching out problems may be limited. However, excess use of bifunctional crosslinkers may cause the extra binding which brings the indiscriminative binding, consequently, activity loss. Inert protein such as bovine serum albumin (BSA) can be used to crosslink the enzymes using glutaraldehyde on the transducer surface [42]. This technique assists a good operational and storage stability; however, it is important to find the optimum amount of crosslinker for the system.

The figure showing both covalent and crosslinking attachment is below. (Figure 1.6)

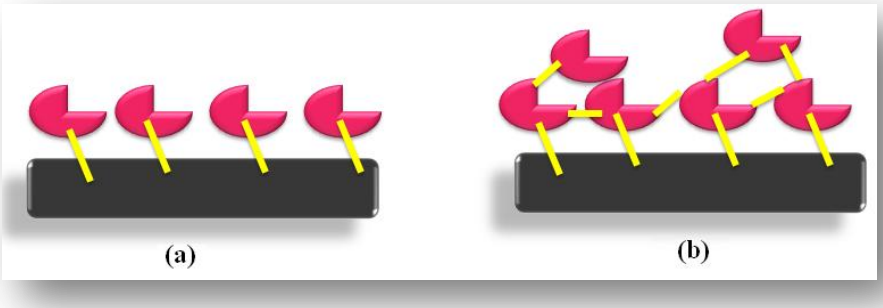


Figure 1. 6 Schematic representation of (a) covalently attached enzyme molecules and (b) crosslinked enzyme molecules.

1.2.2 Electrochemical Transducers

Electrochemical biosensors with electrochemical transducers are widely used tools in biosensing applications. In electrochemical biosensors, an electrode is used as a transduction element. This electrode can be modified according to the desired immobilization technique. Electrochemical transducers stand out with their fast and

simple design, accuracy specificity and selectivity [43]. They can be classified into three groups: amperometric, potentiometric and conductimetric transducers.

1.2.2.1 Amperometric Biosensors

Electrochemical amperometric biosensors are widely used class of electrochemical sensors. In amperometry, at a constant potential application, some redox species are oxidized or reduced so that an electrochemical reaction occurs on the transducer surface. Thus, this reaction results in a current change. The current is monitored as a function of time. The change in the current is directly proportional to the amount of the oxidized or reduced electroactive species. The main factor that influences the results of the amperometric biosensor is the electron transfer between the electroactive species and the transducer surface [18]. This electroactive species is usually an oxidase or dehydrogenase. Those enzymes have cofactors or metals in their active sites promoting the oxidation-reduction reactions; hence, during the electrochemical reactions, generated electrons are transferred to the electrode surface. Due to its high sensitivity and wide linear range, amperometry is the one more attractive among the other electrochemical techniques [44].

Moreover, among all the biosensors, amperometric enzymatic electrodes have a pioneering place. Due to the combination of selectivity and specificity of enzymes in recognition, and sensitivity and rapidness of amperometry in signal processing, amperometric enzymatic biosensor system is the most favored technique.

Fabrication of amperometric biosensors are based on the electroactivity of the substrate or the product of the enzymatic reaction (first generation biosensors). Furthermore, in fabrication, mediators can be used to get more efficient electron transfer during the enzymatic reactions (second generation biosensors). Alternatively, direct electron transfer between enzyme active site and transducer can be achieved in the construction of the biosensors (third generation biosensors).

The applied potential during the amperometric studies have an important place in applications. This potential should not be too high to oxidize all the species other then the desired electroactive species participating in the enzymatic reactions. Hence, it is important to reduce the applied potential. For this purpose, mediators may be preferred to use in essential conditions. However, these mediators can also assist some interfering reactions other than the electron transfer between enzyme and transducer [45]. Moreover, enzyme orientation and immobilization become extremely important for the construction of the biosensor for an efficient electron transfer. The distance between the redox site of the enzyme and electrode surface should be as short as possible to ensure the electron transfer.

Electrochemical amperometric measurements require a three-electrode system including a working electrode, a counter electrode and a reference electrode. The constant potential is applied at a Pt-, Au- or C-working electrode with respect to the reference electrode. Working electrode is the enzyme-modified electrode which is the biosensor. In a batch system, the electrodes are placed into the sample solution to get the output signal during the amperometric measurements.

The enzymatic activity of enzyme-based amperometric biosensors can be followed by either monitoring the consumption of the oxygen or production of the hydrogen peroxide in the system. For example, in a glucose oxidase biosensor, in the case of detection of hydrogen peroxide produced by the enzymatic reaction, oxidation of hydrogen peroxide is achieved on the working electrode surface at 0.7 V (vs. Ag/AgCl reference electrode) which makes the working electrode anode. On the other hand, in the same reaction, oxygen depletion can also be monitored at -0.7 V (vs. Ag/AgCl reference electrode). In this case, oxygen is reduced to hydrogen peroxide; hence, the working electrode becomes cathode. This is a preferred system for the first generation biosensing systems where either oxygen consumption or peroxide liberation is monitored.

1.2.2.1.1 Oxygen Electrodes

During the enzymatic reactions, oxygen consumption can be determined at -0.7 V (vs. Ag/AgCl reference electrode) applied between the working electrode (Pt cathode, for example) and the silver anode. When a potential of -0.7 V is applied to cathode, a current proportional to the oxygen content is generated. During the catalytic reactions of oxidase enzymes, oxygen is consumed. The rate of this reduction is derived from the rate of diffusion of the oxygen from the bulk solution which is based on the concentration of oxygen in the bulk [46]. Therefore, when the catalytic reaction occurs, the depletion of the diffused oxygen concentration can be easily detected via the current change due to the establishment of the new equilibrium [47]. (Figure 1.7)

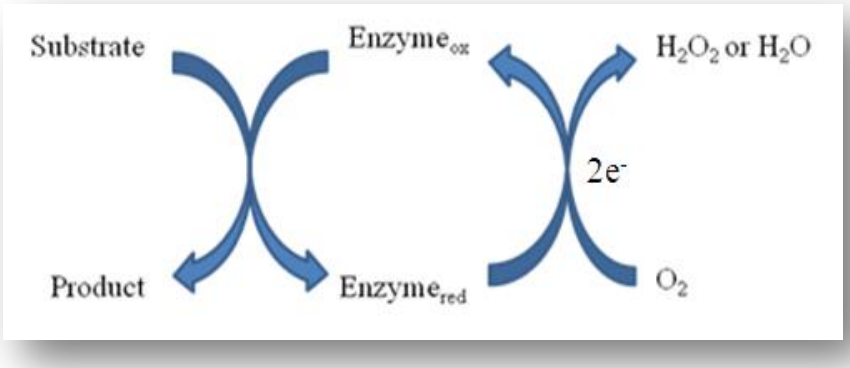


Figure 1. 7 Reaction sequence for an oxidation reaction catalyzed by an oxidase enzyme using molecular oxygen as electron-proton acceptor.

Because the consumed oxygen is proportional with the consumed substrate amount, the concentration of the analyte is proportional to the current change upon

oxygen consumption. Moreover, it is significant to apply low detection potentials in order to overcome the interference problem of the common species showing interference in biosensor systems, such as ascorbic acid and uric acid [48]. This brings the correct measurement with no interference in the measured signal. However, the oxidation of H_2O_2 usually requires a relatively high positive potential (usually over 0.6 V vs. SCE) [49].

1.2.2.1.2 Hydrogen Peroxide Electrodes

During the enzymatic reactions, the produced hydrogen peroxide can be detected directly by applying a potential around 0.7 V (vs. Ag/AgCl reference electrode) to the working electrode. (Figure 1.8) This potential is quite high compared to the oxygen electrode. At high potentials, a great number of electroactive species can interfere; hence, the specificity and selectivity of the enzyme electrode may be damaged. These interferents are generally ascorbic acid, uric acid and paracetamol and they are oxidative species commonly present in the biological samples. In order to overcome this limitation, the working potential should be lower than 0.0 V (vs. Ag/AgCl) or they should be hindered from reaching the electrode surface [45].

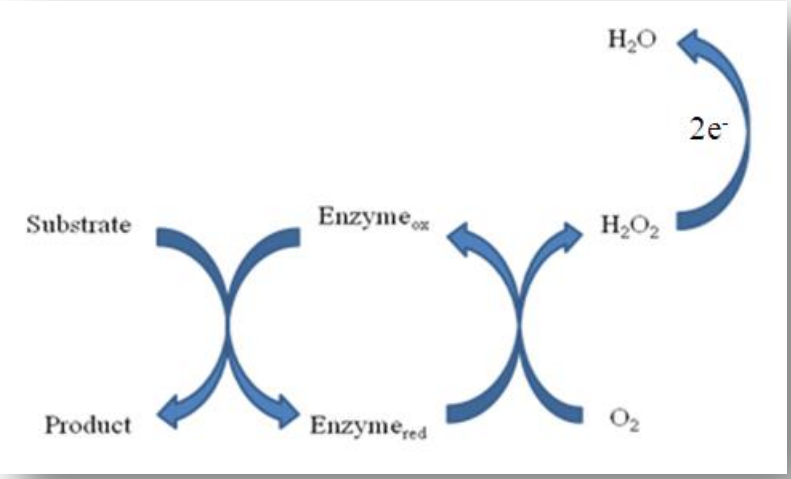


Figure 1. 8 Reaction sequence for an oxidation reaction catalyzed by a hydrogen peroxide producing enzyme.

1.3 Conducting Polymers in Biosensing

Conducting polymers can efficiently be used in various applications due to their ability to transfer electric charge produced by biochemical reactions. Conducting polymers are versatile materials with their unique properties such as conductivity, ability to be functionalized, large surface area etc. [50]. They can be produced easily and rapidly. Moreover, the physical properties such as morphology, thickness and porosity are almost controlled during the generation onto the desired surface. Conducting polymers (CPs) offer an extensive possibility to modify the surface of conventional electrodes supplying fascinating properties [18]. Moreover, providing simplicity and high reproducibility in preparation, easiness in arranging the thickness of the film, compatibility with biological molecules, and possibility to produce at room temperature make electrochemically synthesized CPs charming in

designing biosensors [18,21]. Immobilization of biomolecules onto electropolymerized films is acquiring importance in the fabrication of biosensors rendering as an enzyme immobilization platform [7, 51-53]. Various polymers are notably used in immobilization and biosensor fabrication. Moreover, it was shown that the use of conducting polymers affects the sensitivity, stability and speed of the biosensor considerably.

Due to the well-organized molecular structure and well-defined formation of layers of conducting polymers, they can function as a three dimensional matrix for immobilization of biomolecules [7,9,43]. Moreover, after immobilization and during the biochemical reactions, they transfer the electrical charge and serve as an appropriate microenvironment for the immobilization [54].

Furthermore, conducting polymers can be used also as an entrapment network in biosensor fabrication. They exhibit superior properties as a suitable matrix for entrapment of enzymes [55,56]. During the electrogeneration of the growing polymer, biomolecules are entrapped on the electrode surface. Due to the inertness of the generated polymer and no side reactions, entrapped biomolecules are able to maintain their biological activity. With this one step, rapid and simple procedure, stable and sensitive biosensor systems can be formed [57].

On the other hand, upon demand, the chemical structure of conducting polymers can be modified. Required electronic and mechanical properties can be achieved by modulating and synthesizing the new structures. In addition to synthesis, polymers can be modified with different materials such as dendrimers or amino acids [58]. In the case of hydrophobicity, polymeric material can be modulated by introducing hydrophilic groups in order to get the highest interaction with biomolecules.

It is easy to control the enzyme activity by changing the polymer thickness or surface morphology. Taking the advantage of electrochemically controlled thickness, it is possible to obtain freestanding and homogenous film each time. Thus, the spatial location of biomolecule is facilitated properly on the polymeric structure [59]. Moreover, with the hydrophilic and biocompatible structure of the conducting

polymers, storage stability of the constructed systems is also enhanced [24]. With their versatile applicability, they can be deposited on various surfaces such as Pt-, Au- or C-based electrodes or ITO coated glasses. Whatever the size and shape of the solid substrate is, conducting polymers can be deposited on various surfaces precisely. This brings the high reproducibility of the biological system.

In addition to their physical properties, conducting polymers are also biocompatible with biological molecules so that they can be easily utilized for biochemical reactions and biomolecules can maintain their activity for long duration [60].

1.4 Glucose Biosensors

Recently, biosensors have attracted great interest among researchers as a worthy analytical tool for the detection of desired substances. They have had a considerable place in different technological areas such as clinical and food diagnostics and bioprocess and pollution monitoring due to their usability in rapid detection and monitoring [50,61]. Detection and following up of glucose level in human blood of diabetes is very important in clinical diagnostics. For control of diabetes, glucose level is needed to be monitored several times in short periods. Moreover, it is important to detect the glucose levels in industrial bioprocesses such as food industry.

Glucose oxidase (GOx) is widely used in glucose biosensing. In the presence of molecular oxygen, GOx catalyzes the oxidation of β-D-glucose to hydrogen peroxide and glucono-δ-lactone which is subsequently hydrolyzed into gluconic acid [62]. (Figure 1.9)

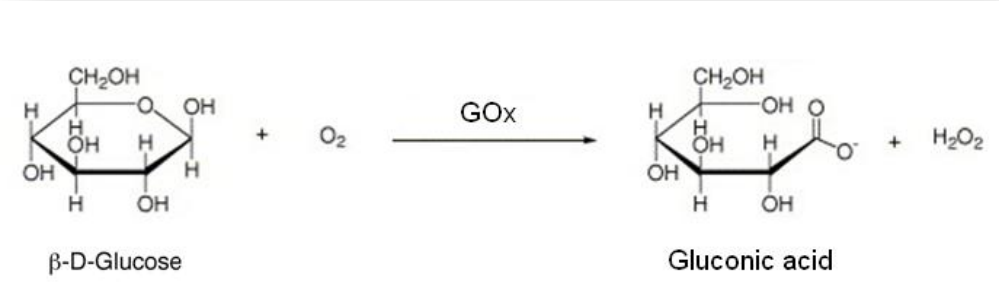


Figure 1. 9 Reaction mechanism of glucose oxidase.

Table 1.1 contains a number of results of the studies on glucose oxidase (GOx) biosensors and various CPs as a comparison [63-69].

Tablo 1. 1 Comparison of biosensor examples from the literature involving glucose oxidase and various conducting polymers as immobilization matrix.

Conducting polymer	**Immob. Tech.**	**K_m/V_{max} or I_{max}**	**Linear Range**	**Sensitivity**	**Ref.**
Polypyrrole/poly(methyl methacrylate (PMMA)-co-thienyl methacrylate (PMTM))	Entrapment	40.0 mM / 1.74 μmol/min cm^2	NR	NR	[63]
Polypyrrole	Entrapment	NR	0-20.0 mM	40.0 nA/mM	[64]

Poly(o-phenylenediamine) (POPD)	Entrapment	NR	8.0-14.0 mM	0.2 - 0.7 μA/mM cm^2	[65]
Polypyrrole	Entrapment	7.01 mM / 120 μA	0.5-10 mM	7.4 mA $M^{/}$ cm^2	[66]
Poly(o-anisidine) (POA)-dodecylbenzene sulphonic acid (DBS)	Covalent binding	3.0 mM / 6.5 μA	0-9 mM	20.0 μA/mM	[67]
PANI nanofibers and Au NPs	Covalent binding	NR	1.0×10^{-6} / 8.0×10^{-4} mol/L	20.0 mg/mL	[68]
Poly(N-(4-(3-thienyl methylene)-oxycarbonyl phenyl) maleimide-co-pyrrole)	Entrapment	4.57 mM / 0.033μmol / (min-electrode)	NR	NR	[69]

NR: Not reported.

In this study, a recently synthesized conducting polymer (poly(2-dodecyl-4,7-di(thiophen-2-yl)-2*H*-benzo[*d*][1,2,3]triazole (polyTBT)) was tested as a platform for biomolecule immobilization. After electrochemical polymerization of the monomer (TBT) on graphite electrodes, immobilization of glucose oxidase was carried out. To improve the interactions between the enzyme and hydrophobic alkyl chain on the polymeric structure, GOx and isoleucine (Ile) amino acid were mixed in sodium phosphate buffer with a high ionic strength. Consequently, cross-linked enzyme crystal (CLEC) like assembles with regular shapes were observed after immobilization. The optimized biosensor showed a very good linearity between 0.05 and $2.5x10^{-3}$ M with a 52 s response time and a detection limit (LOD) of $0.029x10^{-3}$ M to glucose. Also, kinetic parameters, operational and storage stabilities were

determined. K_m and I_{max} values were found as $4.6x10^{-3}$ M and 2.49 μA, respectively. It was also shown that no activity was lost during operational and storage conditions. Finally, proposed system was applied for glucose biomonitoring during fermentation in yeast culture where HPLC was used as the reference method to verify the data obtained by the proposed biosensor. The biosensor is in a great conformity with the reference method. This confirms the reliability and accuracy of this novel biosensor.

1.5 Choline Biosensors

Choline is one of the main precursors of signaling lipids and the neurotransmitter acetylcholine; hence, it exhibits significance for the continuity of cell membranes, lipid metabolism and cholinergic nerve function [70,71]. For detection of choline, choline biodedectors are widely used [72-74]. Choline oxidase (ChO) catalyzes the oxidation of choline to hydrogen peroxide and betaine aldehyde which is then subsequently turned into glycine betaine [75]. (Figure 1.10)

Pesticides (herbicides, fungicides, insecticides) are widely used all over the world. Accumulation of these pesticides in organisms can cause serious diseases. With their high toxicity, they may inhibit the enzymes in the nerve tissue resulting with death of the living organisms. Restricted and controlled use of pesticides is important for both the environment and people. It has become a need to find new, practical, cheap and rapid techniques for detection instead of traditional, expensive and time-consuming instruments, such as mass spectrometry after chromatographic separation (GC-HPLC). For this purpose, using biosensors as “biodetectors” is extremely applicable.

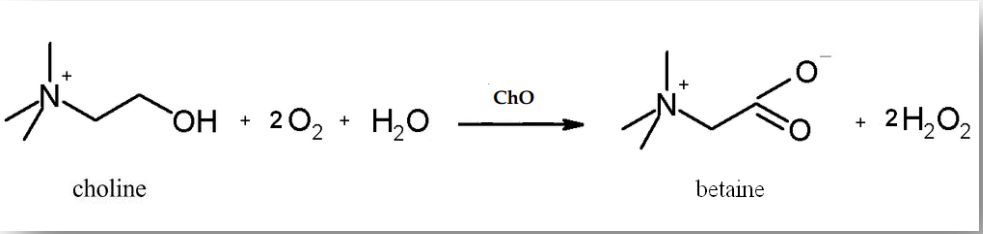


Figure 1. 10 Reaction mechanism of choline oxidase.

Table 1.2 contains a number of results of the studies on choline oxidase biosensors [71, 74, 76-78].

Tablo 1. 2 Comparison of biosensor examples from the literature involving choline oxidase.

Immob. Matrix	**Immob. Tech.**	**K_m or I_{max}**	**Linear Range**	**Sensitivity**	**Ref.**
Prussian Blue (PB)	Adsorption	2.0 mM	$5x10^{-7}$-$5x10^{-3}$M	30200 nA/ mM	[74]
Au nanorods	Cross-linking	NR	$2x10^{-5}$-$4x10^{-4}$M	16 µA/mM cm^2	[71]
Fe_3O_4 nanoparticles	Covalent binding	NR	10^{-9}-10^{-2}M	NR	[76]
PANI-MWNTs	Cross-linking	NR	$1x10^{-6}$-$2x10^{-3}$M	NR	[77]

MWNT-Au nanoparticles	Adsorption	0.42 mM	0.001-0.5 mM	13 μA/mM	[78]

NR: Not reported.

In this study, a recently synthesized conducting polymer (poly(TBT_6-NH_2); poly(6-(4,7-di(thiophen-2-yl)-2*H*-benzo[*d*][1,2,3]triazol-2-yl)hexan-1-amine) was utilized as a matrix for biomolecule immobilization. After successful electrochemical deposition the polymer poly(TBT_6-NH_2) on the graphite electrodes, immobilization of choline oxidase (ChO) was carried out. Due to the free amino functional groups of the polymeric structure, ChO molecules were successfully immobilized onto the polymer surface via covalent binding. Hence, a robust binding between the support and the protein molecules was achieved. The optimized biosensor showed a very good linearity between 0.1 - 10 mM with a 7 s response time and a detection limit (LOD) of 16.8 μM to choline. Also, kinetic parameters, operational and storage stabilities were determined. Finally, designed system was applied for pesticide detection. This showed the well-applicability of the designed biosensor.

CHAPTER 2

EXPERIMENTAL

2.1 Materials

Glucose oxidase (GOx, β-D-glucose: oxygen 1-oxidoreductase, EC 1.1.3.4, 21200 Units/g) from *Aspergillus niger*, choline oxidase (ChO, choline:oxygen 1-oxidoreductase, EC 1.1.3.17, 10 U/mg from *Alcaligenes sp*), choline chloride, isoleucine (Ile), D-glucose, glutaraldehyde (50 wt. % solution in water) and dialysis tubing cellulose membrane (25mmx16mm, cut-off:2000) were purchased from Sigma (St. Louis, USA). Acetonitrile, hydrochloric acid, sodium hydroxide were purchased from Merck (Darmstadt, Germany). Paraoxon-ethyl was purchased from Pestanal, Fluka (Buchs, Switzerland). All chemicals used for the synthesis of monomers and electropolymerization were purchased from Aldrich except tetrahydrofuran (THF) which was purchased from Acros (Geel, Belgium). All other chemicals were analytical grade.

Glucose biosensors were tested in yeast culture medium. *Saccharomyces cerevisiae* H620 was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). For the yeast cultivation, the distillery strain of *Saccharomyces cerevisiae* H620 was subcultured in agar medium containing 4 g/L glucose, 4 g/L yeast extract and 10 g/L malt extract. Yeast cells were inoculated in liquid modified Schatzmann medium. After precultivation, 12.5 mL of fermentation broth were transferred into 62.5 mL of fresh Schatzmann medium where 2.5 g glucose dissolved in 25 ml distilled water containing vitamin and antibiotic solution [79,80]. Cultures were incubated in an orbital shaker (New

Brunswick Scientific, USA) at 30 °C and 200 rpm and samples were collected from fermentation broth during 8 h.

2.2 Instrumentation

2.2.1 Cyclic Voltammetry and Amperometry

For the cyclic voltammograms and amperometric measurements, Palm Instrument (PalmSens, Houten, The Netherlands) and Ivium CompactStat (Ivium Technologies, The Netherlands) were used with a traditional three-electrode configuration. A graphite electrode (Ringsdorff Werke GmbH, Bonn, Germany, type RW001, 3.05 mm diameter and 13 % porosity) as the working, silver (Ag) wire (pseudo reference) as the reference and platinum (Pt) wire electrode as the counter electrode were used during electrochemical measurements and all potentials were reported with respect to Ag wire reference electrode. (Figure 2.1) The reference electrode is used to measure and control the potential on the working electrode and it does not interfere with the current between the working and counter electrodes. On the other hand, the counter electrode has a role in balancing the current observed at the working electrode by passing all the current needed.

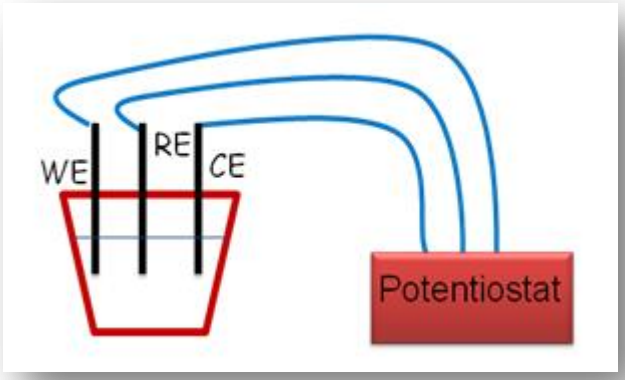


Figure 2. 1 Electrochemical cell with a three electrode configuration**.**

2.2.2 Surface Characterization

2.2.2.1 Scanning Electron Microscopy

JEOL JSM-6400 model scanning electron microscope (SEM) was used for surface imaging of enzymatic biosensors.

2.2.2.2 Fluorescence Microscopy

Epifluorescence microscopy (Olympus, Tokyo, Japan) was used for surface imaging.

2.2.2.3 X-ray Photoelectron Spectroscopy

XPS (X-ray photoelectron spectroscopy) studies were carried out on a PHI 5000 Versa Probe (F ULVAC-PHI, Inc., Japan/USA) model X-ray photoelectron spectrometer instrument with monochromatized Al Kα radiation (1486.6 eV) as an X-ray anode at 24.9 W.

2.2.2.4 Contact Angle

Contact angle measurements of a drop of water (2.0 μL) on the polymer surfaces were carried out using the sessile drop method with a KSV CAM 200 contact angle meter (KSV Instruments, Finland). Recording the drop profile with a CCD camera allowed monitoring the changes in contact angle. All reported data were given as the average of five measurements ± SD. The experiments were conducted at ambient temperature (25 °C).

2.3 Synthesis of the Monomers

2.3.1 Synthesis of 2-Dodecyl-4,7-di(thiophen-2-yl)-2*H*-benzo[*d*][1,2,3]triazole (TBT) Monomer

Synthesis and characterization of the monomer, TBT, were carried out according to a previously described method [63]. Briefly, benzotriazole was alkylated with dodecyl bromide to enhance the solubility. The alkylated benzotriazole was brominated in the presence of hydrobromic acid and bromine. Thiophene was stannylated in the presence of n-BuLi and tributyltin chloride. The

final coupling reaction was achieved by Stille coupling reaction using $Pd(PPh_3)_2Cl_2$ as the catalyst. (Figure 2.2)

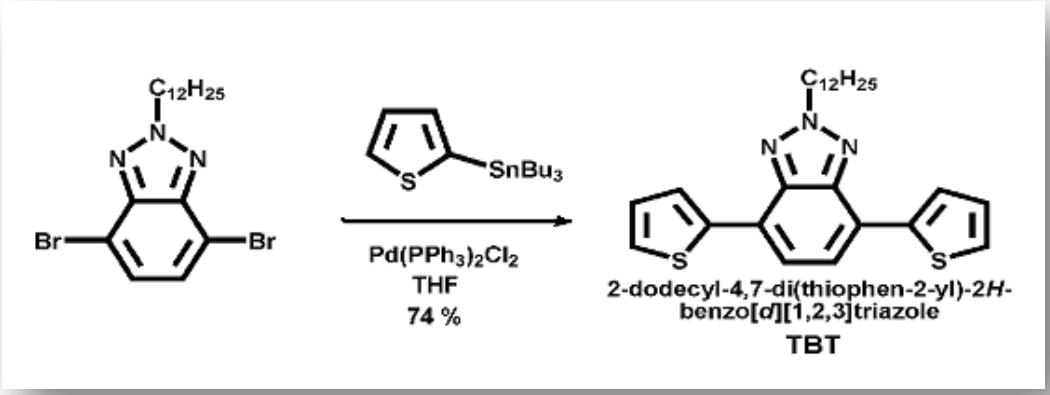


Figure 2. 2 Synthesis of the monomer TBT.

2.3.2 Synthesis of 6-(4,7-Di(thiophen-2-yl)-2*H*-benzo[*d*][1,2,3]triazol-2-yl)hexan-1-amine (TBT_6-NH_2) Monomer

Synthesis and characterization of the monomer, TBT_6-NH_2, were carried out according to a previously described method [63,81]. Benzotriazole (3.0 g, 25.2 mmol) was alkylated with 1,6-dibromohexane (4.5 g, 18.4 mmol) to enhance the solubility and introduce the amine moiety as the pendant group (2.0 g, 71 %). The alkylated benzotriazole was brominated in the presence of hydrobromic acid and bromine (1 g, 68 %). Thiophene was stannylated in the presence of n-BuLi and tributyltin chloride. Coupling was achieved by Stille coupling reaction using $Pd(PPh_3)_2Cl_2$ as the catalyst (75 mg, 74%). Then, the product and potassium phthalimide (138 mg, 0.75 mmol) was stirred in DMF. After purification steps, the

product was compensated with hydrazine for 24 hours and desired monomer was achieved (0.317 g, 96 %). (Figure 2.3)

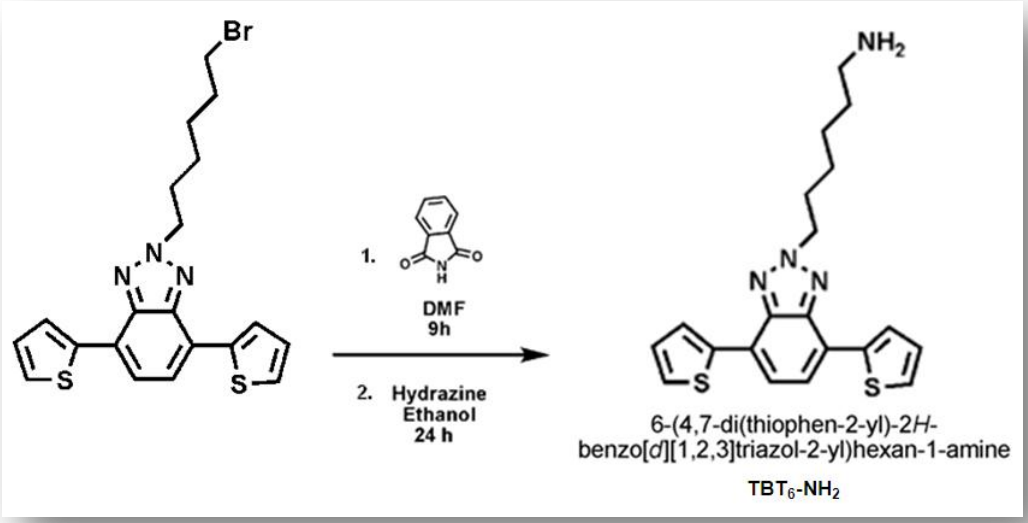


Figure 2. 3 Synthesis of the monomer TBT_6-NH_2.

2.4 Electropolymerization of Monomers

All polymerizations were performed using a three electrode system by applying multiple scan voltammetry at a scan rate of 100 mV/s.

2.4.1 Electropolymerization of TBT

Spectroscopic grade graphite rods were polished on emery paper and washed thoroughly with distilled water prior to the electropolymerization. TBT was

polymerized potentiodynamically on graphite electrodes in acetonitrile (ACN) using 0.1 M tetrabutylammonium hexafluorophosphate ($TBAPF_6$) as the supporting electrolyte with repeated scan interval between -0.5 V and 1.4 V (versus Ag reference electrode).

2.4.2 Electropolymerization of TBT_6-NH_2

Spectroscopic grade graphite rods were polished on emery paper and washed thoroughly with distilled water prior to the electropolymerization. TBT_6-NH_2 was polymerized potentiodynamically on graphite electrodes in 0.1 M DCM/ACN/$NaClO_4$/$LiClO_4$ solvent/electrolyte couple with repeated scan interval between 0.1 V and 1.8 V (versus Ag reference electrode).

2.5 Construction of Biosensors

2.5.1 Construction of Glucose Biosensor

After electropolymerization of TBT and successful conducting polymer deposition of 100 cycles by cyclic voltammetry, for immobilization of enzyme, a proper amount of glucose oxidase (GOx) solution (2.35 mg (49 U) in 5 μL, 250 mM sodium phosphate buffer, pH 7.0) containing isoleucine (Ile) (1.0 mg) was coated over the polymer coated electrode and glutaraldehyde (5 μL, 0.1 %, in 250 mM sodium phosphate buffer, pH 7.0) was then added and the electrode was allowed to stand at ambient conditions to dry for 1 h. Then, the layer was covered with a dialysis membrane which was pre-soaked in water. The membrane was fixed tightly with a silicone rubber O-ring [82].

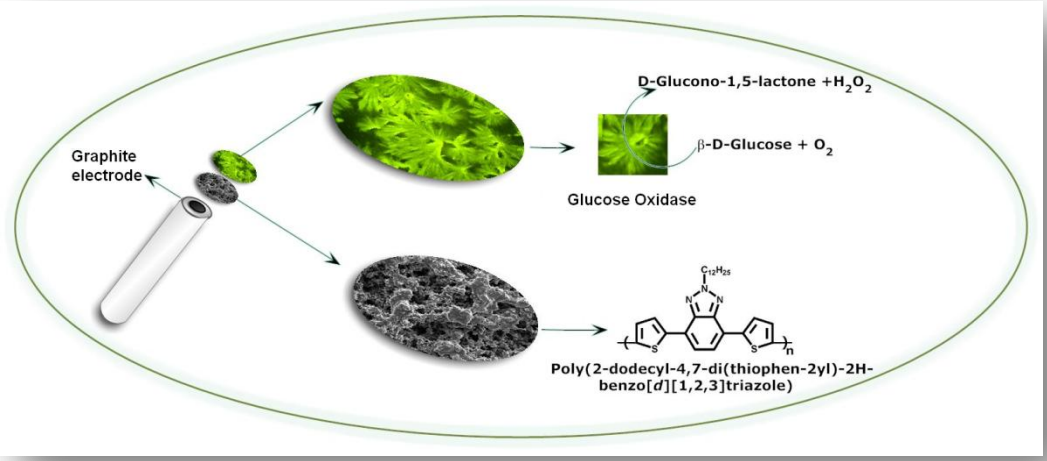


Figure 2. 4 Preparation of poly(TBT)/GOx biosensor.

2.5.2 Construction of Choline Biosensor

After electropolymerization of TBT_6-NH_2 and successful conducting polymer deposition of 50 cycles by cyclic voltammetry, for immobilization of enzyme, a proper amount of choline oxidase (ChO) solution (0.25 mg (3.5 U) in 3 µL, 50 mM sodium phosphate buffer, pH 7.5) was spread over the polymer coated electrode and glutaraldehyde (5 µL, 2.5 %, in 50 mM sodium phosphate buffer, pH 7.5) was then added and the electrode was allowed to stand at ambient conditions to dry for 1.5 h. Its substrate, choline, was prepared daily and stored at +4 °C.

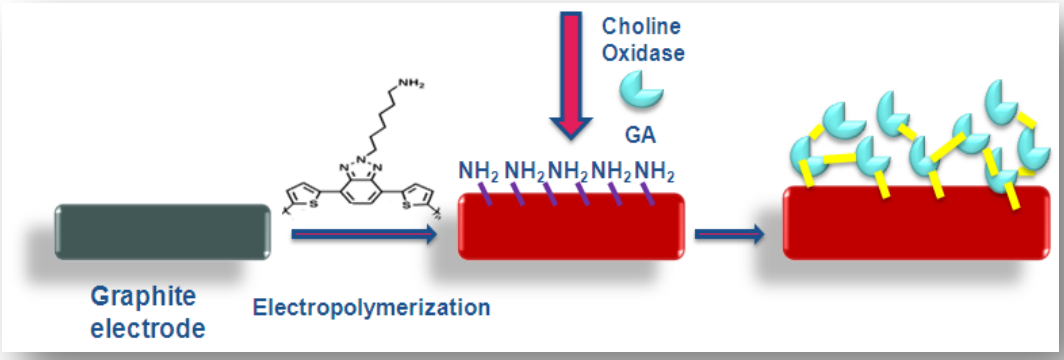


Figure 2. 5 Preparation of poly(TBT_6-NH_2)/ChO biosensor.

2.6 Amperometric Biosensor Measurements

All experiments were carried out at ambient conditions in a vessel (reaction cell) containing 10 mL buffer solution while mildly stirring. Amperometric determination of glucose and choline was performed at ambient conditions by applying a constant potential at -0.7 V (versus Ag reference electrode) and following the oxygen consumption as a result of enzymatic activity in the bioactive surface. The enzyme electrodes were initially equilibrated in buffer and when the background current reached a steady state, substrate solution was added to the electrochemical cell, the current change is monitored and the steady-state current values were recorded. The biosensor responses were registered as the current signal (μA). After each run, buffer solution was refreshed and electrodes were washed with distilled water.

After the optimization studies, the current signal obtained with respect to various standard substrate concentrations were plotted as a calibration curve.

In all amperometric studies, each measurement was carried out three times repetitively, results were recorded and standard deviations were calculated. In the figures, error bars show standard deviation.

2.6.1 Effect of Conducting Polymer Thickness for Biosensors

The effect of conducting polymer thickness on the amperometric responses of prepared biosensors was firstly investigated. It is important to decide the optimum thickness of the polymeric film. The polymeric film thickness was controlled by adjusting the scan number during the electropolymerization. Conducting polymers in different thicknesses were deposited and same amount of enzyme was immobilized on the polymer coated electrodes. Then, amperometric responses were recorded. The highest response belongs to the biosensor with optimum polymer thickness. For further studies, this optimum thickness was used.

2.6.2 Effect of Biomolecule Amount for Biosensors

The effect of biomolecule amount on the amperometric responses was investigated for both biosensors. Biosensors with different amount of enzyme were prepared and amperometric responses were recorded. In all those studies, polymer thickness, pH and crosslinker amount were kept constant and optimum enzyme amounts were detected.

2.6.3 Effect of pH for Biosensors

Different buffers with different pH values were prepared and the responses of the same biosensors were recorded. Various buffer solutions may affect the conformation of the enzymes and so do the activity of them. After determination of the optimum pH of working buffers, amperometric studies were conducted in buffer solutions in that pHs.

2.7 Analytical Characterization of Biosensors

The analytical characteristics of the biosensors were investigated under optimized conditions. Calibration curves were plotted for current (μA) versus substrate concentration (mM). Linearity equations, linear dynamic ranges and limits of detection (LOD) were obtained under the optimized conditions.

2.7.1 Determination of Kinetic Parameters

The kinetic studies of the reaction catalyzed by immobilized enzymes were performed at varying concentrations of substrates by keeping assay conditions constant. K_m is the measure of the affinity of the enzyme toward its substrate and refers to substrate concentration at V_{max} [83]. Analytical parameters, K_m^{app} and I_{max} (instead of V_{max}) values for the amperometric biosensor systems were calculated from the Lineweaver-Burk plot.

2.7.2 Stability Experiments

Response time, operational stability and shelf life are the most important parameters to be considered in enzyme immobilization and biosensors. The operational stability of enzyme electrodes in terms of repetitive uses was studied running several measurements on the same day under optimized conditions. For storage stability or shelf life, the same electrode was tested every other day during a certain period using same amount of substrate and amperometric responses were recorded. Biosensor systems were stored at +4 °C in between consecutive measurements.

2.8 Sample Application

To test the designed biosensors, different sample applications were performed.

2.8.1 Sample Application Using Poly(TBT) Based Biosensor

Glucose biosensor was tested in yeast culture by using the distillery strain of *Saccharomyces cerevisiae* H620. The medium was directly injected to the reaction medium instead of glucose. Results were also compared with the ones obtained by high performance liquid chromatography (HPLC) as the reference method. Yeast cultivations were prepared. Cultures were incubated in an orbital shaker (New Brunswick Scientific, USA) at 30 °C and 200 rpm and samples were collected from fermentation broth during 8 h. HPLC with a refractive index detector (RID) controlled by an HP-Chemstation from Agilent (Karlsruhe, Germany) was used for

independent analysis of the glucose content. HPLC column (5.0 μm, 4.6 id x 250 mm, GL Sciences Inc. Inertsil NH_2, Japan) was used for the chromatographic separation at 30 °C. Injection volume was 20 μL. The mobile phase was H_2SO_4 (5.0 mM) [84]. The flow rate was 0.6 mL/min. Initially standard curve for glucose was plotted (2.5-50 mM for glucose). After dilution with mobile phase and centrifugation, samples were applied to the column and glucose concentrations were calculated using the calibration plot.

2.8.2 Sample Application Using Poly(TBT_6-NH_2) Based Biosensor

Choline biosensor was tested in detection of pesticide, paraoxon-ethyl. Generally, the measurement principle relies on a quantitative measurement of the enzyme activity before and after exposure to a target species.

In detections based on pesticide inhibition, first of all, initial response value was determined during the preliminary study of the biosensor performance. For a proper substrate (choline) concentration, the response of the biosensor was recorded. Then, in different concentrations, pesticide solutions were prepared. The contact of the biosensor with the inhibitor was achieved. The biosensor was incubated in one of those pesticide solutions for a proper time. The inhibition effect of the pesticide on the biosensor was carried out. After incubation, the biosensor was rinsed with distilled water many times to get rid of the pesticide on the surface of the active layer. Lastly, the measurement for substrate was repeated to obtain the reduced response of the biosensor. The response of the biosensor for same amount of substrate was recorded and inhibition effect is followed with respect to the decrease in the signal; hence, the percent inhibition is determined. (Eq.1) The decrease in the response was correlated with the concentration of the pesticide solution. Percent inhibition graph was prepared as a calibration curve. The sensitivity of the designed biosensor toward an inhibitor can be derived from a calibration curve for inhibitor.

1µg/µL pesticide solutions were prepared in methanol as stock solutions. Then, proper concentrations of pesticide solutions were prepared in buffer solutions by dilution.

Inhibition % = $[(I_0-I_i) / I_0] \times 100$ (1)

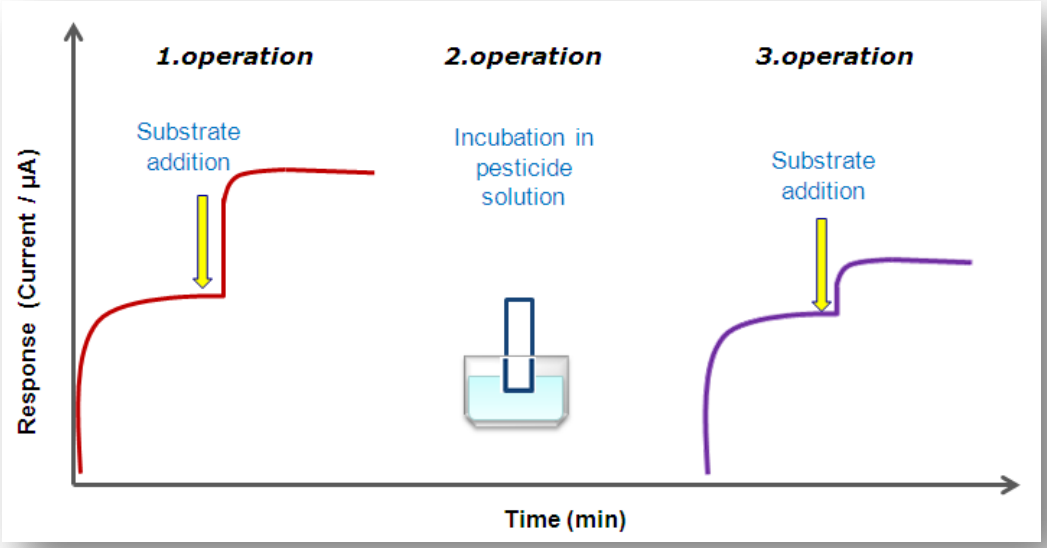


Figure 2. 6 Representation of measurement procedure for pesticide detection.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Glucose Biosensor Based on Poly(TBT) and Glucose Oxidase

Initially, constituents of bioactive layer were optimized to improve the interactions between the enzyme and hydrophobic alkyl chain of the polymer. When the glucose oxidase (GOx) was dissolved in buffer solution (50 mM, sodium phosphate buffer, pH 7) and directly spread over the polymer coated electrode surface, it was not possible to spread it homogeneously due the incompatibility with the hydrophobic polymer. However, when proper amount of an amino acid, isoleucine (Ile), and buffer with higher ionic strength were mixed with the GOx solution, the mixture was easily dispersed on poly(TBT). Hence, Ile and GOx amount, ionic strength of the phosphate buffer used in immobilization and quantity of glutaraldehyde as the crosslinker were optimized accordingly.

3.1.1 Electropolymerization of the Monomer

All polymerizations were performed with multiple scan voltammetry using a three electrode system.

The conducting polymer of 2-dodecyl-4,7-di(thiophen-2-yl)-2*H*-benzo[*d*][1,2,3] triazole) (TBT) was electrochemically synthesized onto the graphite electrode by cyclic voltammetry technique via repeated cycling at the oxidation

potential of the monomer using a scan rate of 0.1 V/s. (Figure 3.1) The electropolymerization of the monomer comprises an E(CE)n (electrochemical, chemical, electrochemical) mechanism where the first step is the formation of the radical cation [85,86]. In the first cycle of the voltammogram, an irreversible monomer oxidation peak at 1.20 V appeared and a reversible polymer redox peak was centered at 1.00 V and 0.75 V versus Ag wire pseudo reference electrode. As seen in Figure 3.1, current increases with increasing number of cycle which proves the electroactivity and formation of the polymer on the graphite surface.

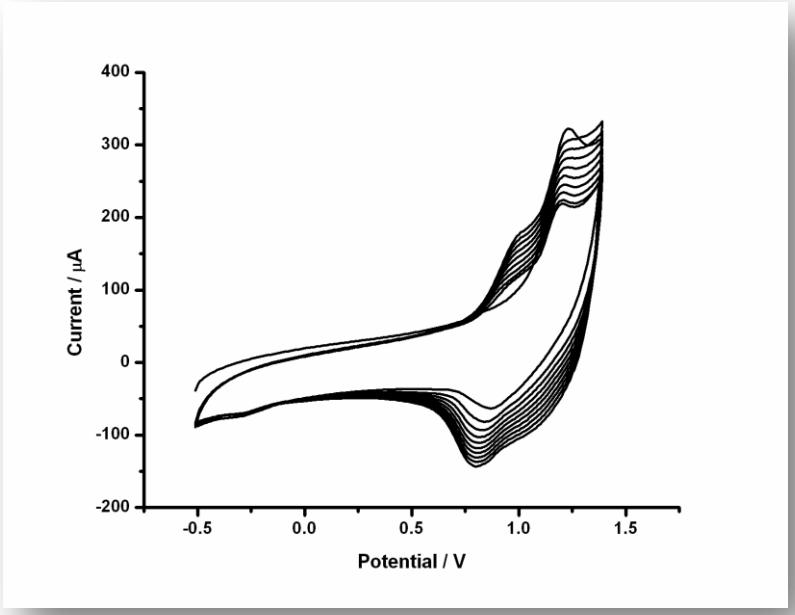


Figure 3.1 Repeated potential-scan electropolymerization of TBT in 0.1 M ACN/$TBAPF_6$ solvent-electrolyte system at a scan rate of 100 mV/s on graphite (up to 10 cycles).

Film formation on the electrode surface, regardless of its shape or size, can be performed with large electrode surfaces with a control of thickness. Moreover, the film thickness can easily be followed by measuring the total charge during the deposition of the conducting polymer [87]. On the other hand, electropolymerization time is quite effective both on the rate of growth and the quality of the conducting polymer films [88]. Proper thickness for polymeric film has high importance in the construction of the biosensors. High thicknesses may cause the degradation or incompact microstructure as immobilization matrix [89]. Hence the quality of the immobilization matrix decreases. According to the experiments and previous works, 63 min of polymerization time (referring to 100 cycles) was chosen and the charge involved in the film formation was measured as 8.37×10^{-4} Coulombs [7,54].

3.1.2 Effect of Biomolecule Amount

The amperometric response was examined as a function of the biomolecule amount. Three different electrodes were prepared with 1.20 mg (25 Unit), 2.35 mg (49 Unit), and 4.70 mg (99 Unit) of GOx (in 5 μL, 50 mM sodium phosphate buffer, pH 7.0, containing 1 mg Ile). The enzyme was immobilized via glutaraldehyde on poly(TBT) modified graphite electrodes. The amount of other components was kept constant. The highest signals were obtained with the biosensor having 2.35 mg (49 Unit) GOx in bioactive matrix while no increase in the higher loads were registered. The relationship between the response of the biosensor and the enzyme amount is exhibited in Figure 3.2.

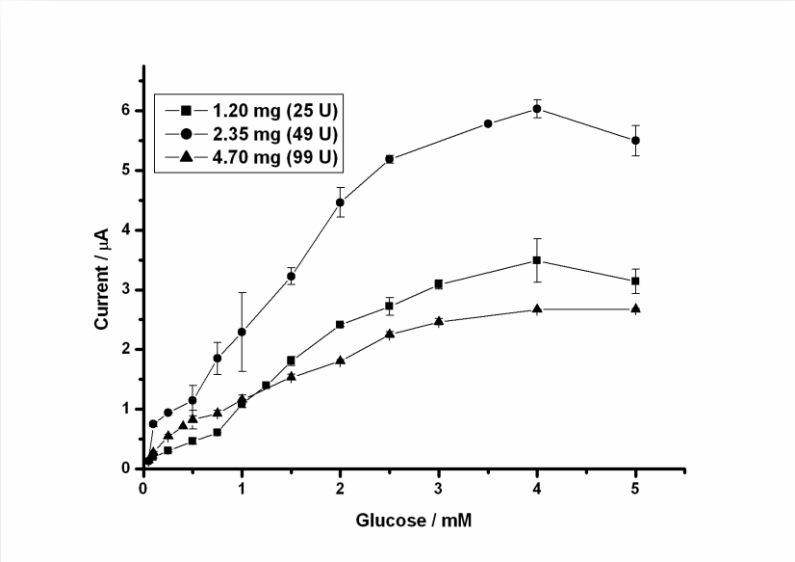
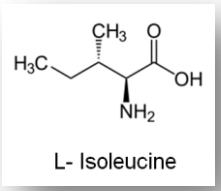


Figure 3. 2 Effect of enzyme amount on the biosensor response (in sodium acetate buffer, 50 mM, pH 4.0, 25°C, -0.7 V). Error bars show standard deviation.

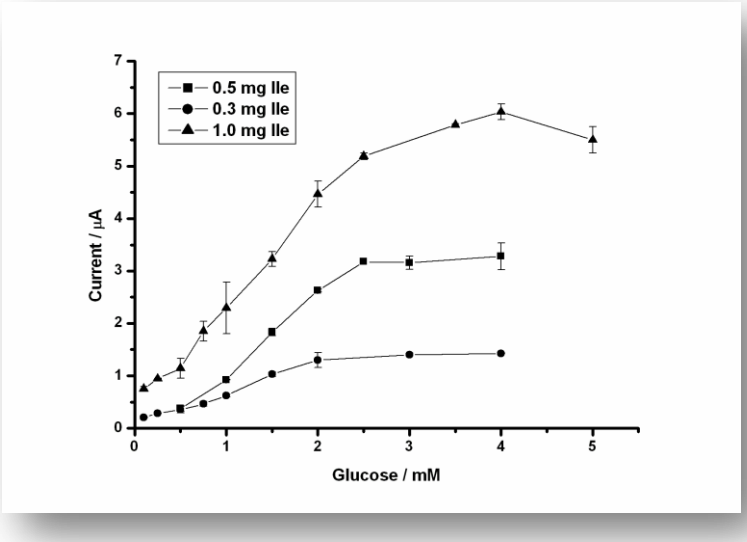
3.1.3 Effect of Isoleucine (Ile) Amount

Isoleucine is a kind of branched-chain amino acid. It bears a branched alkyl chain in its structure. Due to these alkyl chains, it acquires a hydrophobic property. In order to enhance the interaction of enzyme molecules with hydrophobic polymer, Ile is used to make the enzyme solution more hydrophobic. For the optimization of the quantity of Ile, various electrodes containing different Ile amounts between 0.3 mg and 2 mg in bioactive layer were prepared; thereafter sensors were calibrated for glucose. (Figure 3.3) It was observed that in the absence of Ile, it was not possible to obtain a homogeneous dispersion on the polymeric surface. Addition of this hydrophobic amino acid (up to 1 mg) to the enzyme solution caused an increase in

the response whereas at higher amounts, insoluble mixtures were obtained; hence, 1 mg was used as the optimum amount for further steps.



(a)



(b)

Figure 3. 3 (a) Structure of L-Isoleucine and (b) Effect of isoleucine amount in bioactive layer on the biosensor response (in sodium acetate buffer, 50 mM, pH 4.0, 25°C, -0.7 V). Error bars show standard deviation.

3.1.4 Effect of Ionic Strength of the Buffer Used in Preparation of Enzyme Solution

To obtain more compatible protein structure with the hydrophobic surface, ‘‘salting out’’ like conditions, which is the most common method used to precipitate a target protein [90], were applied by increasing the ionic strength of sodium phosphate buffer in the bioactive layer containing the enzyme (2.35 mg, 49 U) and Ile (1 mg). The increase in salt amount in the solution decreases the electrostatic interaction while increasing the hydrophobic ones [68]. It is expected that as the salt concentration of the solution is increased, more of the water becomes associated with the ions. Hence, less water is available to partake in the solvation layer around the protein, which exposes hydrophobic patches on the protein surface. The addition of ions to the enzyme solution generates an electron shielding effect which overrides some activity between water particles and the protein, lessening the solubility as the proteins bind with each other. Proteins may then exhibit improved hydrophobic interactions with the polymer structure and tend to aggregate on the surface. The effect of ionic strength on the current responses was shown in Figure 3.4. In order to detect this effect, various sodium phosphate buffers at pH 7.0 in different concentrations were prepared and used in the biosensor preparation while keeping the other parameters constant. As seen in Figure 3.4, 250 mM sodium phosphate containing buffer gave the best results.

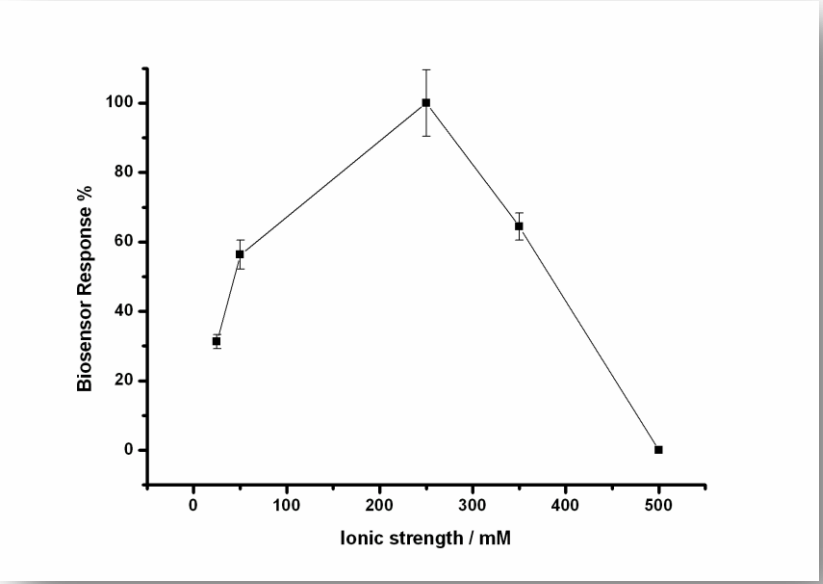


Figure 3. 4 Effect of ionic strength in bioactive layer on the biosensor response (in sodium phosphate buffer, pH 7.0, 25°C, -0.7 V, [Glc]: 1 mM). Error bars show standard deviation.

3.1.5 Effect of Cross-linker (Glutaraldehyde) Amount

After optimizing GOx/Ile mixture in 250 mM sodium phosphate buffer (pH 7.0), different amounts of glutaraldehyde (GA) were then added to enzyme solution to obtain crosslinking between the amino groups in the protein structure. By this way, more compact and stable protein structure might be acquired. While studying the influence of the amount of cross-linker, optimum glutaraldehyde amount was found as 0.1 % (in phosphate buffer, pH 7.0, Figure 3.5) and used in further experimental steps. For higher amounts of GA, enzyme cannot stay in contact with the modified surface due to the excess cross linkages, thus, cannot diffuse into the polymer network. It was also demonstrated that the decrease in enzyme activity with

increasing glutaraldehyde amount showed that the excess linkages between the enzymes are destroying tertiary structure so do the activity of the biomolecules [91].

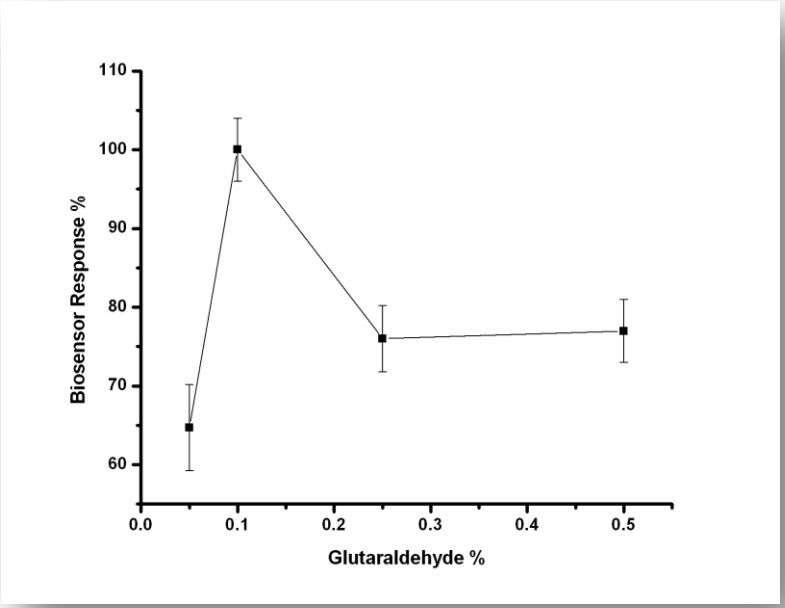


Figure 3. 5 Effect of glutaraldehyde amount on the biosensor response (in sodium acetate buffer, 50 mM, pH 4.0, 25°C, -0.7 V, [Glc]: 1 mM). Error bars show standard deviation.

3.1.6 Effect of pH

The pH dependence of the responses was investigated over a pH range of 3.5-6.5 with sodium acetate and sodium phosphate buffers (50 mM) in the presence of 1 mM glucose. The biosensor revealed the best result at pH 5.5 as given in Figure 3.6. It was also reported that the native enzyme is acidic (with p*I* = 4.2) and shows

activity over a wide range of pH values (pH 3.0–8.0) [92]. For further experiments pH 5.5 sodium acetate buffer was used as the buffer solution.

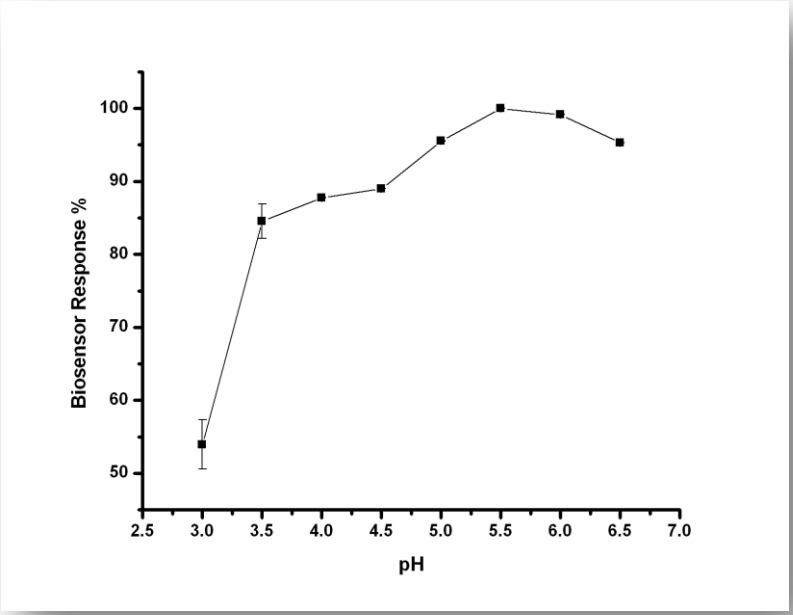
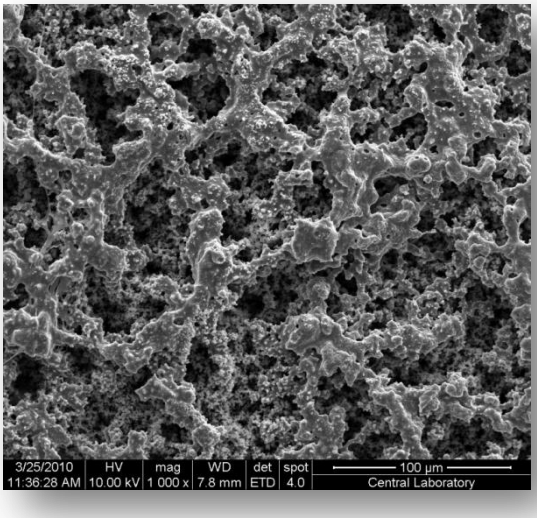


Figure 3. 6 Effect of pH (sodium citrate buffer at pH 3.0; 3.5, sodium acetate buffer at pH 4.0; 4.5; 5.0; 5.5 and sodium phosphate buffer at pH 6.0; 6.5, 25°C, -0.7 V, [Glc]: 1 mM). Error bars show standard deviation.

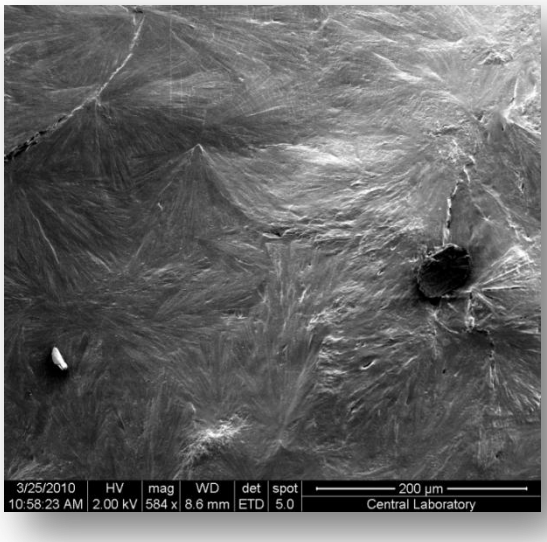
3.1.7 Surface Characterization

3.1.7.1 Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) technique was used to monitor the surface characteristics. Morphology of poly(TBT) was shown in Figure 3.7 (a, b). Figure 3.7,a shows the polymeric layer of poly(TBT) formed as a result of 100 cycle electropolymerization on graphite. The presence of the polymer on the electrode can be revealed from its highly porous and cauliflower-like structure. After immobilization, hydrophobic polymer surface was fully covered by the bioactive layer at the optimized conditions. (Figure 3.7,b) From the micrographs, it is clear that the immobilization of the enzyme on the polymer was successfully achieved.



(a)



(b)

Figure 3. 7 SEM images of poly(TBT) before (a) and after biomolecule immobilization (b) at the optimized conditions.

3.1.7.2 Fluorescence Microscopy

Apart from SEM, poly(TBT) deposition was also monitored by fluorescence microscopy after produced on an ITO glass. Since this valuable polymer itself is fluorescent, it can easily be detected and comfortably seen under the fluorescence microscope [63]. Figure 3.8,a shows the bare matrix prior to immobilization and the one with enzyme molecules on poly(TBT) is shown in Figure 3.8,b. Since GOx itself exhibits the fluorescent property due to the FAD center, it could also be possible to differentiate it on the polymer surface under fluorescence microscopy as indicated in Figure 3.8,b.

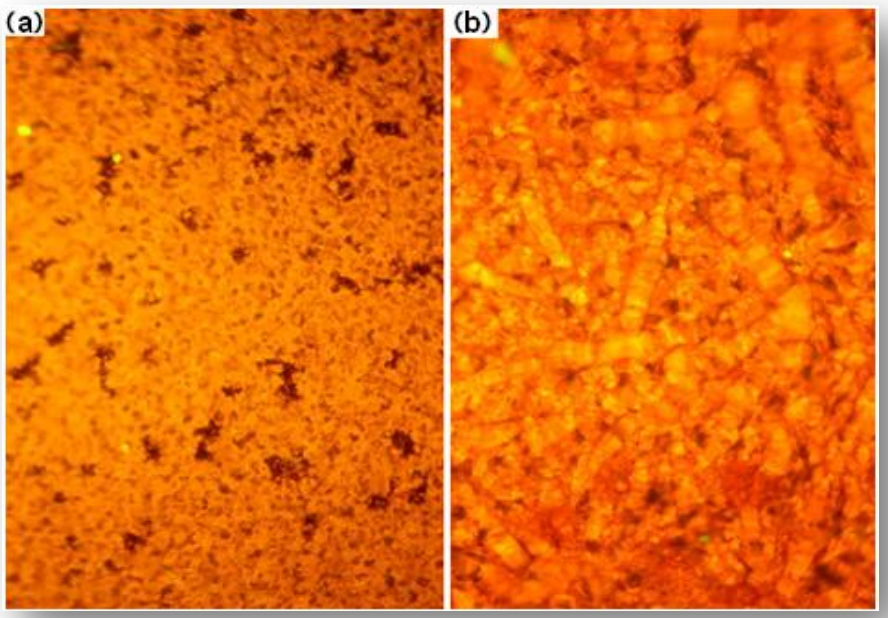
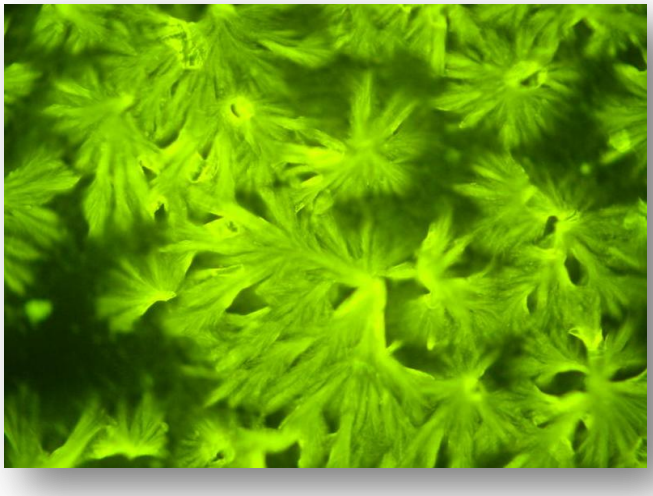


Figure 3. 8 Fluorescence images of TBT polymer (a) and GOx on polymer surface (b) coated on ITO glass (with 200X magnification).

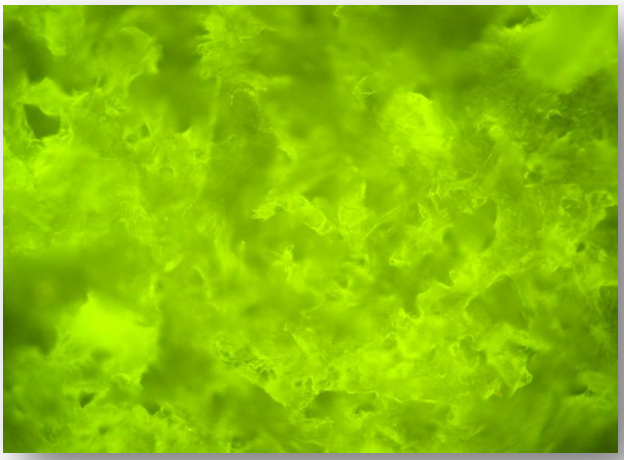
3.1.8 Analytical Characterization of the Biosensor

It is a common fact that as well as the number and the nature of bonds between the enzyme and the carrier, the form of the enzyme affects the stability of immobilized enzyme at unfavorable temperatures and also in the presence of other inactivating agents such as organic solvents [93]. It was proved that the stability increases with crystal formation in enzyme immobilization and the activity is prolonged [94]. CLECs can stay active and stable against harsh environments; hence it is highly desired to form CLECs for biosensor preparation. Crystalline structure is supported with cross-links by multi-attachment of protein to the support by preventing the unfolding of proteins [95]. By this way, 1-100 μm uniform-sized

crystals can be achieved. Moreover, in the same manner, chemical cross-linkers are used to produce cross-linked enzyme aggregates (CLEA) [93]. This extreme stability is an outcome of both high hydrophobic and electrostatic (polar) interactions [95,96]. The raise in these interactions among the protein molecules enhances the formation of crystalline structure. In our case, CLEC-like assembles with regular shapes were observed in the presence of Ile after crosslinking via glutaraldehyde in 250 mM buffer solution. Fluorescence microscopy image of GOx prepared with above mentioned conditions was the evident of CLEC-like structure. (Figure 3. 9) The CLEC-like structure can be confirmed with the change of ionic strength of the buffer used in preparation of enzyme solution. In lower ionic strength, no CLEC-like structure was observed.



(a)



(b)

Figure 3. 9 Fluorescence microscopy image of CLEC-like GOx structure obtained after crosslinked via glutaraldehyde in the presence of Ile in sodium phosphate buffer, (a) 250 mM, (b) 50 mM pH 7.0, pH 7.0, with 200X magnification.

As to the analytical characterization, the dependence of current signals on the glucose concentration was shown in Figure 3.10. A perfect linearity was obtained for 0.1-2.5 mM glucose in 50 mM sodium acetate buffer pH 5.5 (given as inset in Figure 3.10). During the measurements with increasing concentrations of the substrate, after a point, saturation occurs due to the conversion of the free enzyme molecules into the substrate-bound (ES) form. This can be seen in Figure 3.10.

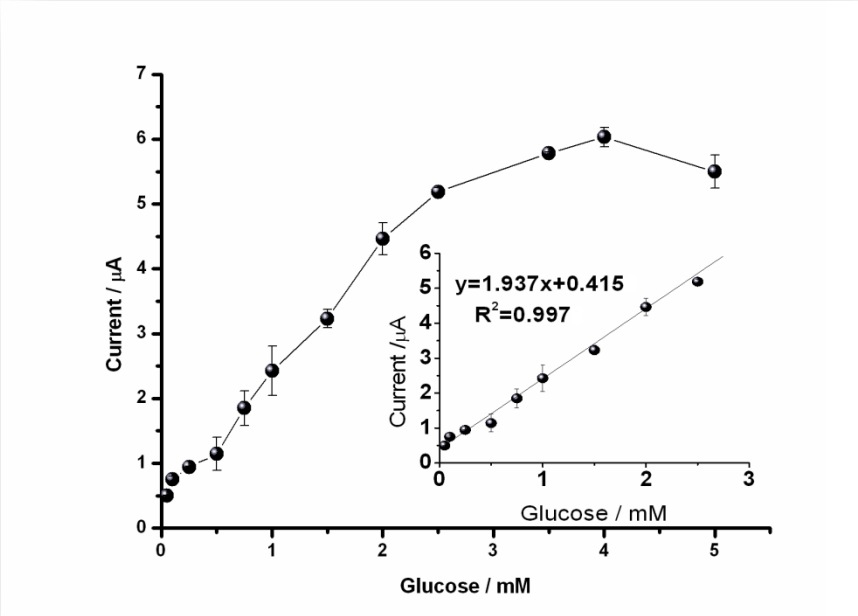


Figure 3. 10 Calibration curve for glucose and linear range (inset) (in sodium acetate buffer, 50 mM, pH 5.5, 25°C, -0.7 V). Error bars show standard deviation.

A typical biosensor response was depicted in Figure 3.11.

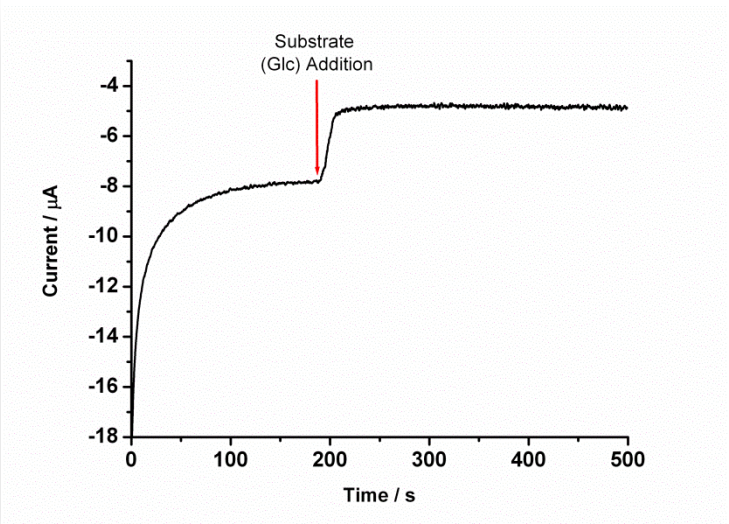


Figure 3. 11 A typical biosensor response to glucose (in sodium acetate buffer, 50 mM, pH 5.5, 25°C, -0.7 V, [Glc]: 1 mM).

On the other hand, the biosensor signals corresponding to 1 mM glucose standard solutions were measured for ten times in order to achieve repeatability of the biosensor response. The standard deviation (SD) and the relative standard deviation (RSD) were calculated as ±0.064 and 4.2 %, respectively. Limit of detection (LOD) was also calculated as 29.3 µM according to S/N=3. In blank experiments, bioactive layer at the optimized conditions as described before was adsorbed on directly graphite surface with no polymer behind the dialysis membrane. However, lower response signals with low repeatability were registered. In this case, linearity was observed between 0.25-1 mM glucose with an equation of

y=0.816x+0.159 and R^2=0.980 and also SD and the RSD were calculated as ±0.279 and 27 %, respectively.

Moreover, stability of the biosensor was also tested. Operational stability of biosensor was investigated for 1 mM glucose under optimized conditions. Signal responses were measured every hour for 8 h and no activity loss was observed. Apart from operational stability, the shelf life of the biosensor was also examined. For a period of 2 weeks no activity loss was observed in the response of the biosensor for 1 mM glucose.

Some characteristic properties including kinetic parameters (K_m^{app}, I_{max}), sensitivity as well as stabilities were shown in Table 3.1. It was found that proposed biosensor obeyed the Michaelis–Menten kinetics and K_m^{app} and I_{max} values were calculated using Lineweaver–Burk plot. The K_m^{app} value of free GOx (from *A. niger*) was previously reported between 33 and 248 mM depending on the conditions used in the activity measurements [97].

Tablo 3. 1 Some characteristics of the proposed biosensor (in 50 mM, pH 5.5 sodium acetate buffer at 25 ^oC, -0.7 V).

Parameter	
K_m^{app}	4.60 mM
I_{max}	2.49 µA
Linear Range	0.05 - 2.50 mM
Sensitivity	0.011 µA/mM
LOD [a)]	0.029 mM
Response time	52 sec
Operational stability	No decrease for 8 hours
Shelf life	No decrease during 15 days

[a)]LOD was calculated according to S/N

For both free and immobilized enzymes compare to previous works where GOx were entrapped on the electrode surface via different CPs, lower K_m value was observed. This may as well be due to the CLEC-like structure on the poly(TBT) matrix that exhibits higher affinity toward the glucose substrate as the other CLEC or CLEA structures do [98].

Furthermore, interference studies were also carried out. For this purpose, interference effect of ascorbic acid, cholesterol, and urea as the possible interfering compounds (between 0.01 and 0.1 M) and Schatzman medium (used in the fermentation experiments as the growth medium) were examined between 10 and 1000 µL. However, no interference was observed at -0.7 V under optimized working conditions. It can be claimed that interference free measurements especially during the sample application could be done via using the proposed biosensor.

3.1.9 Sample Application

Finally, the biosensor was used for monitoring the concentration of the glucose in fermentation medium. During the fermentation, microorganisms consume glucose hence the decrease in the glucose content can be monitored vs. time. For this purpose, samples taken from the *S. cerevisiae* cultivation were analyzed. *S. cerevisiae* was cultivated in Schatzman medium according to literature [80]. During the process, 100-fold dilution of the samples was required to adjust the glucose concentration to keep it within the linear range of the GOx biosensor. On the other hand, HPLC was performed as a reference method for a glucose assay. Both data from biosensor and HPLC are shown in Figure 3.12. As seen, results from biosensor applications are in a great conformity with the reference method.

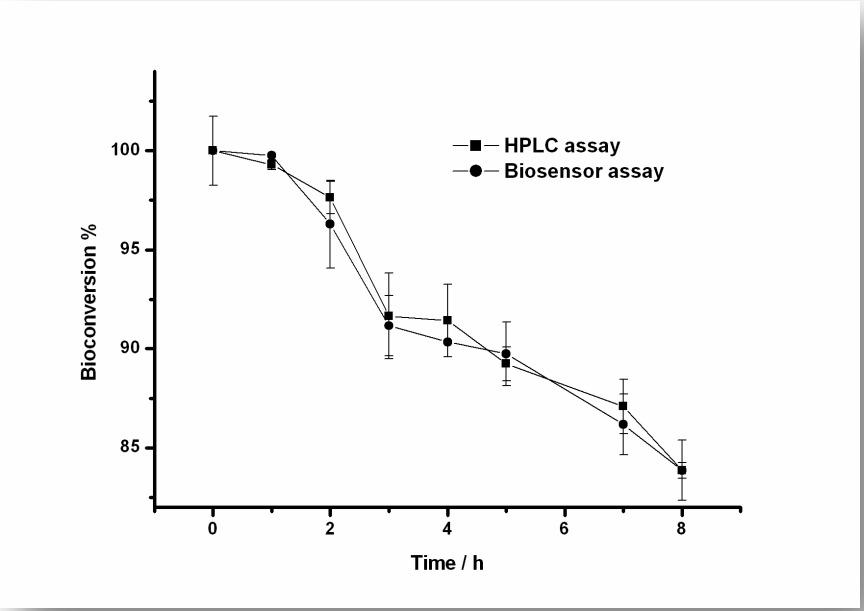


Figure 3. 12 Biomonitoring of time dependent glucose consumption (or bioconversion) in yeast culture in fermentation medium (in sodium acetate buffer, 50 mM, pH 5.5, 25°C, -0.7 V). Error bars show standard deviation.

3.2 Choline Biosensor Based on Poly(TBT_6-NH_2) and Choline Oxidase

Initially, constituents of bioactive layer were optimized to improve the interactions between the enzyme and the polymer. Due to the presence of the functional groups of the polymer, amino groups were used in the covalent linkage of the enzyme. After the electrogeneration of the conducting polymer and determination of optimum thickness, enzyme (ChO) amount, ionic strength of the working buffer, temperature and quantity of glutaraldehyde as crosslinker were optimized accordingly.

3.2.1 Electropolymerization of the Monomer

Electrochemical polymerization of the monomer was potentiodynamically carried out between 0.1 V and 1.8 V (vs. Ag wire pseudo reference electrode) in 0.1 M $NaClO_4$/$LiClO_4$/DCM/ACN/ solvent/electrolyte system at a scan rate of 100 mV/s on graphite. (Figure 3.14) After the polymerization, the surface of the electrode was rinsed with distilled water to remove impurities.

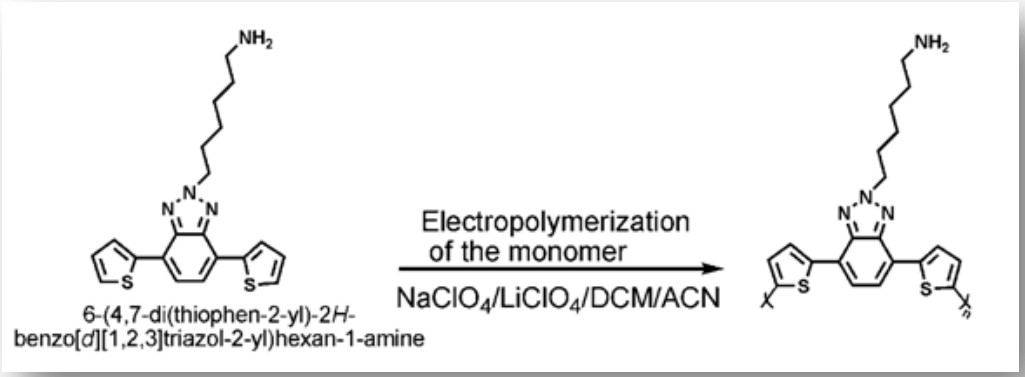


Figure 3. 13 Synthesis of the conducting polymer.

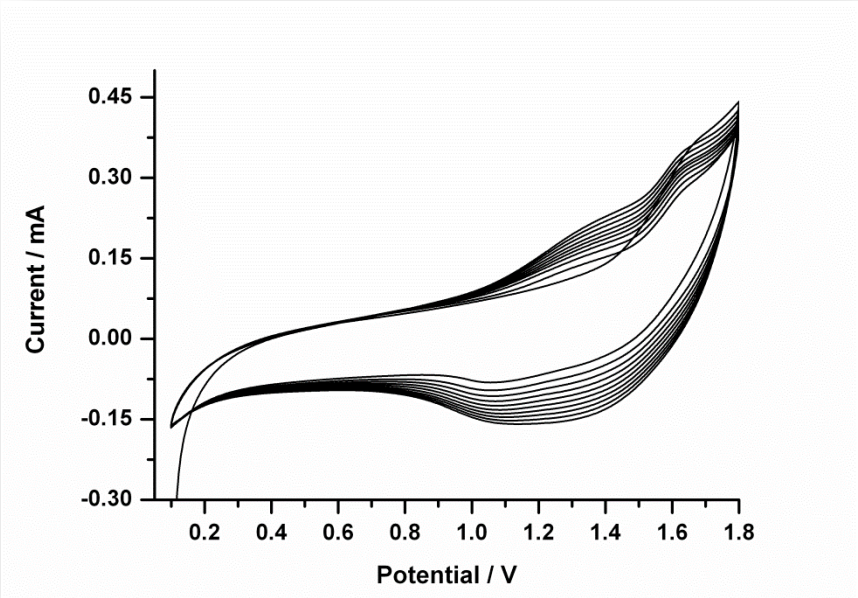


Figure 3. 14 Repeated potential-scan electropolymerization of TBT_6-NH_2 in 0.1 M $NaClO_4$/$LiClO_4$/ACN/DCM solvent-electrolyte system at a scan rate of 100 mV/s on graphite (up to 10 cycles).

3.2.2 Determination of the Working Potential in Amperometric Studies

According to the literature, in most of the amperometric studies using choline oxidase biosensor, potentials in the range of 0.6-0.7 V (vs. Ag/ACl) were chosen as the working potential in order to determine hydrogen peroxide generation in the systems due to the enzymatic activity [78,99,100]. However, it is important to decrease the working potential. Over-potential may cause the oxidation of other electroactive species in the media. Due to the interference risk of some electroactive species, lower potentials are preferred in the amperometric studies to avoid current changes. It is always a need to design functional materials with appropriate matrix

properties together with lowering the operation potential efficiently for the amperometric measurements. To investigate the optimum operation potential, amperometric responses for 10 mM choline were recorded at different potentials. It was found that the maximum signal was recorded at -0.7 V, as expected, as was the case for the interference free measurements. (Figure 3. 15) No meaningful responses were recorded at potentials higher than 0.2 V, such as 0.4 and 0.6 V.

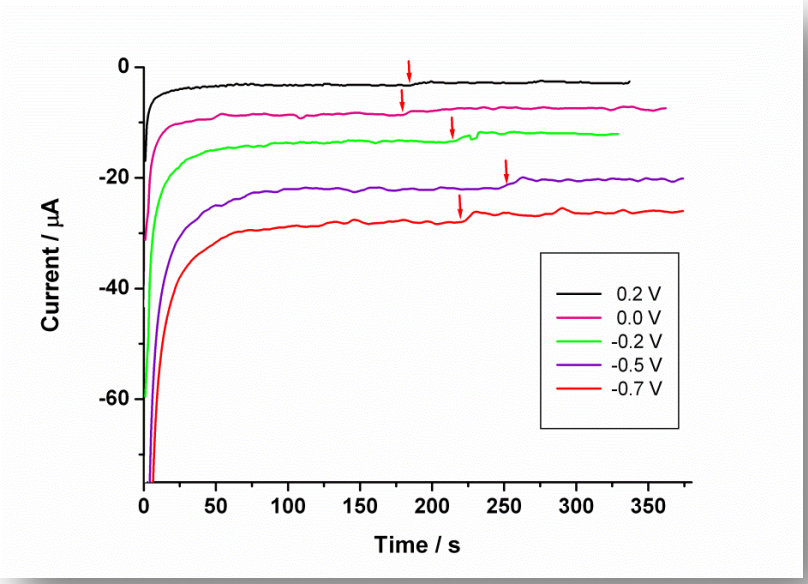


Figure 3. 15 Amperometric responses of immobilized enzyme-conducting polymer coated electrode at different potentials in sodium phosphate buffer (50 mM, pH 7.5, 25°C, [Choline]:10 mM).

Moreover, to diminish the working potential, one of the methods is using mediators [101-104]. In order to see the effect of mediator in a biosensor, cyclic voltammograms of enzyme immobilized-polymer coated electrode with and without

substrate were recorded in the presence of 10 mM $[Fe(CN)_6]^{3-/4-}$ in sodium phosphate buffer at a scan rate of 20 mV/s. As seen in Figure 3. 16, in the system with a mediator, due to the diffusion problems and lack of efficient electron transfer between biolayer and mediator, cyclic voltammogram for the respective biosensor is not well defined. Thus, the results show that mediator use is not eligible for the proposed system.

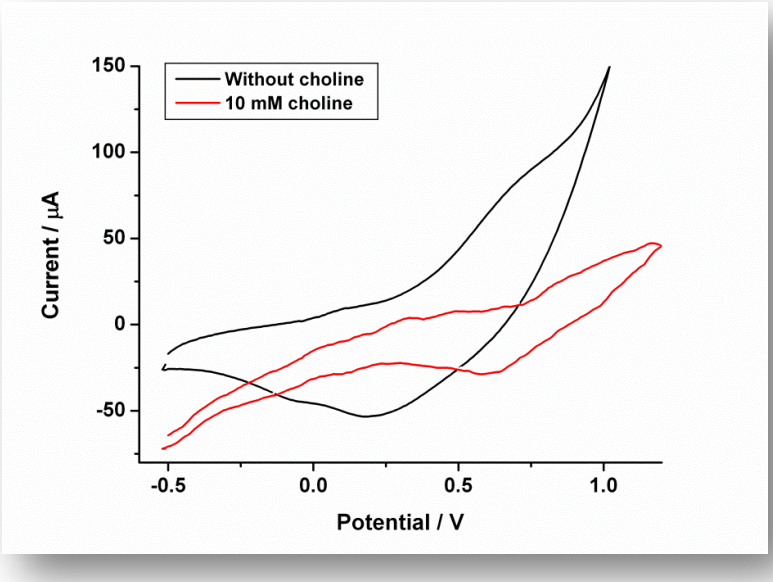


Figure 3. 16 Cyclic voltammograms of immobilized enzyme-conducting polymer coated electrode in 10 mM $[Fe(CN)_6]^{3-/4-}$ containing sodium phosphate buffer (50 mM, pH 7.5; 25°C, 20 mV/s).

3.2.3 Effect of Conducting Polymer Film Thickness

The thickness of the polymer layer can be adjusted with the scan number during the electropolymerization. The thickness of the conducting polymer can be effectively modulated to generate consistent polymeric layers. In order to determine the relationship between biosensor performance and conducting polymer film thickness, electrodes were prepared with different scan numbers, and corresponding biosensor responses were recorded. During these studies, after polymerization, modified graphite electrodes were used for the construction of biosensors as given in immobilization procedure by keeping all other parameters constant. (Figure 3. 17) The decrease in responses could be related to the thickness after 50 scans of electropolymerization. The increase in the thickness of the polymer layer makes the diffusion distance longer for the electroactive groups causing lower charge transfer rates. However, in the low thicknesses, the polymer film may not be thick enough to stabilize the given amount of enzyme. With the lower number of functional groups, enough attachment may not be satisfied and enzyme molecules may not be held on the electrode surface which may bring the leaching of the enzyme molecules. Thus, lower responses were recorded. As seen in Figure 3. 17, 50-scan polymer film revealed the best result; hence, this is chosen as the optimum scan number in electropolymerization. Moreover, when the enzyme solution is directly immobilized onto the bare graphite electrode with no conducting polymer, it is hard to obtain worthy and meaningful signals. The positive effect of the conducting polymer was verified from these studies.

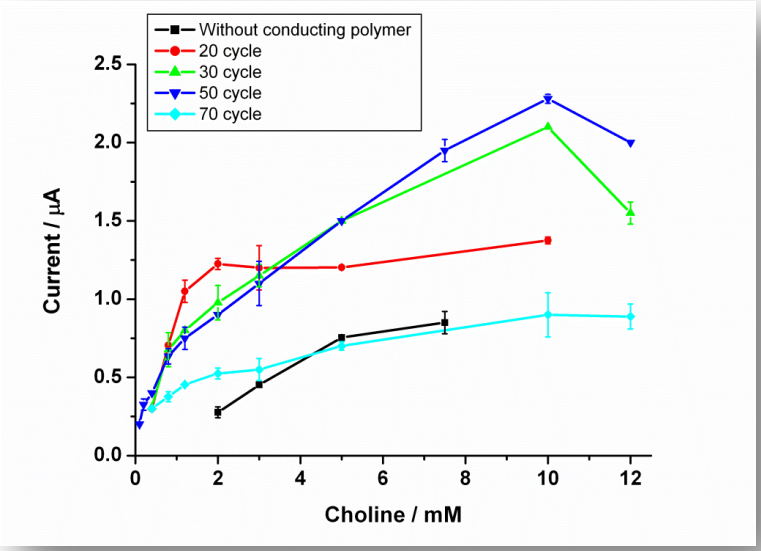


Figure 3. 17 Effect of scan number during the electropolymerization on the biosensor response (in sodium phosphate buffer, 50 mM, pH 7.5, 25°C, -0.7 V). Error bars show standard deviation.

Furthermore, the corresponding polymer was subjected to scan rate variation experiments. By this way, the diffusion property of the thin film on graphite working electrode can be determined. The cyclic voltammograms at different scan rates and current versus scan rate graph showed the linear relationship between scan rates and current. (Figure 3. 18) This reveals that the polymer is well-adhered on the graphite electrode surface and the electrochemical processes on the electrode surface are not diffusion limited [105]. This non-diffusion controlled process is as expected for the immobilized systems [106]. It is clear that the polymer coated electrode surface can efficiently facilitate the electron transfer and improves the electrochemical performance.

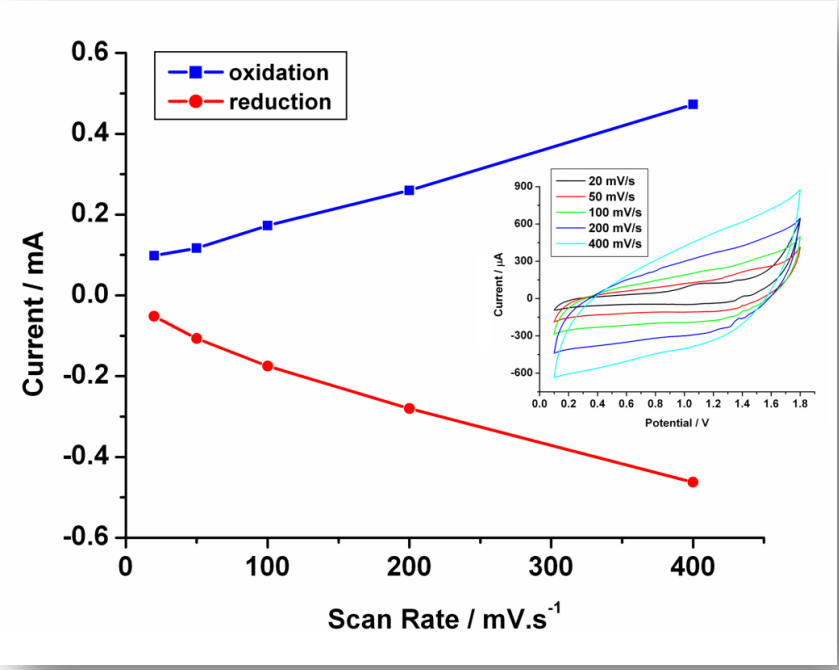


Figure 3. 18 Scan rate dependence of poly(TBT_6-NH_2) film in 0.1 M $NaClO_4$/$LiClO_4$/ACN solvent/electrolyte system at 20, 50, 100, 200 and 400 mV/s.

3.2.4 Electrochemical Behavior of the Biosensor

In order to investigate the electrochemical behaviors of bare and modified graphite electrodes, cyclic voltammetry (CV) technique was used. Enhanced CV responses at the conducting polymer modified electrode confirm the improved electron transfer due to the enhanced conductivity of the matrix [107]. This brings the relative detection sensitivity in the amperometric biosensor measurements due to the improved electron transfer. Moreover, after the modification with biolayer, the modified electrode still preserves its electroactivity. (Figure 3.19)

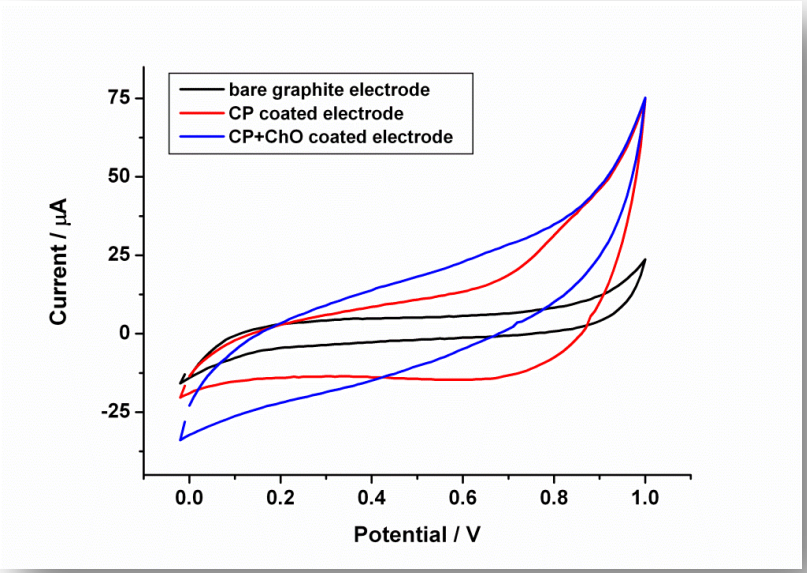


Figure 3. 19 Cyclic voltammograms of bare graphite electrode, 50-cycle conducting polymer (CP) coated graphite electrode and enzyme immobilized-polymer coated electrode (in sodium phosphate buffer, 50 mM, pH 7.5, 25°C, 20 mV/s).

3.2.5 Effect of Biomolecule Amount

Five different electrodes were prepared with 0.070 mg (1.0 Unit), 0.14 mg (2.0 Unit), 0.21 mg (3.0 Unit), 0.25 mg (3.5 Unit) and 0.48 mg (4.0 Unit) of choline oxidase (ChO) (in 3 µL, 50 mM sodium phosphate buffer, pH 7.5). The enzyme was immobilized via glutaraldehyde on poly(TBT_6-NH_2) modified graphite electrodes. The amount of other components was kept constant. The highest signals were obtained by the biosensor with 0.25 mg (3.5 Unit) ChO in bioactive matrix. The relationship between the response of the biosensor and the enzyme amount is exhibited in Figure 3.20. For further experiments, this optimum amount of enzyme was used in the construction of the electrode.

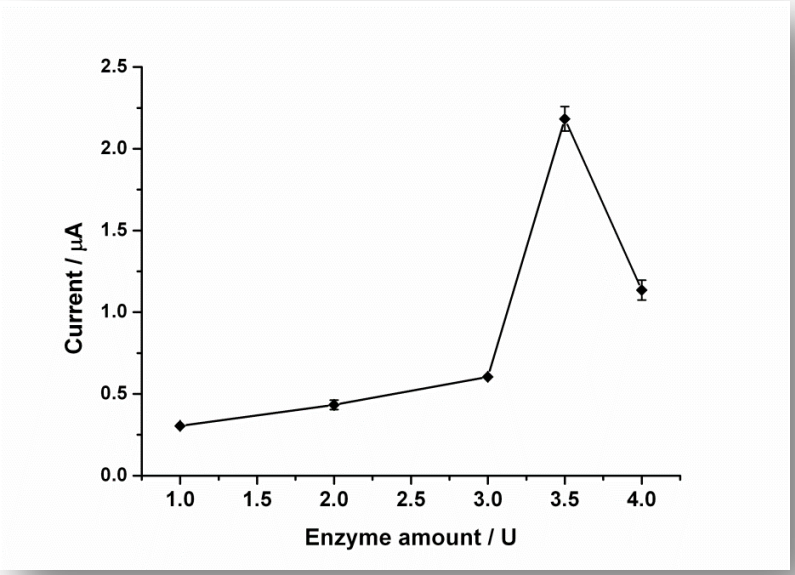


Figure 3. 20 Effect of enzyme amount on the biosensor response (in sodium phosphate buffer, 50 mM, pH 7.5, 25°C, -0.7 V, [Choline]:10 mM). Error bars show standard deviation.

3.2.6 Effect of Cross-linker (Glutaraldehyde) Amount

With the help of glutaraldehyde, the robust linkage between polymeric support and enzyme molecules was overwhelmingly achieved. It is shown that due to the presence of functional groups on the support, biomolecules can be attached on the transducer surface with strong covalent interactions and there is no need to use membrane to keep the bimolecules on the electrode surface. This increases the effectiveness of the immobilization technique.

Cross-linker amount used in the immobilization is also optimized to obtain the ideal crosslinking between the amino groups in the protein structure and amino groups of the polymer. While studying the influence of the amount of cross-linker,

optimum glutaraldehyde amount was found as 2.5 % (in phosphate buffer, pH 7.5, Figure 3.21) and used in further experimental steps.

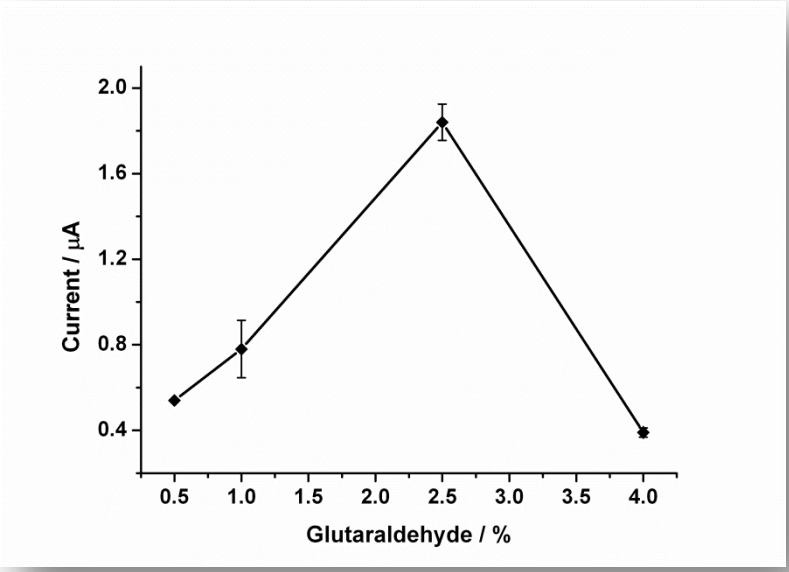


Figure 3. 21 Effect of glutaraldehyde amount on the biosensor response (in sodium phosphate buffer, 50 mM, pH 7.5, 25°C, -0.7 V, [Choline]: 10 mM). Error bars show standard deviation.

3.2.7 Effect of pH

The pH dependence of the biosensor responses to 10 mM was investigated over a pH range of 6.5-8.5. Enzyme molecules contain a great number of acidic and basic groups in their structure. Substantially, these groups are located on the surface of the molecules. With pH change in the environment around them, the charge on these groups will also alter. Hence, it is significant to determine the optimum pH of

the working buffers for immobilized enzymes. Free ChO has an optimum activity at pH 8.0 [108]. The dependency of biosensor response and pH was depicted in Figure 3. 22 by “bell-shaped curve” [106]. The top is the optimum pH for this system and used in further studies. Immobilization brings some changes in the microenvironment of the enzyme active site. Thus, the pH shift is appropriate due to the interactions with the polymer. Additionally, the pH 7.5 is also optimum for sample application studies where most of the pesticides are not stable at high pH values due to the risk of hydrolysis.

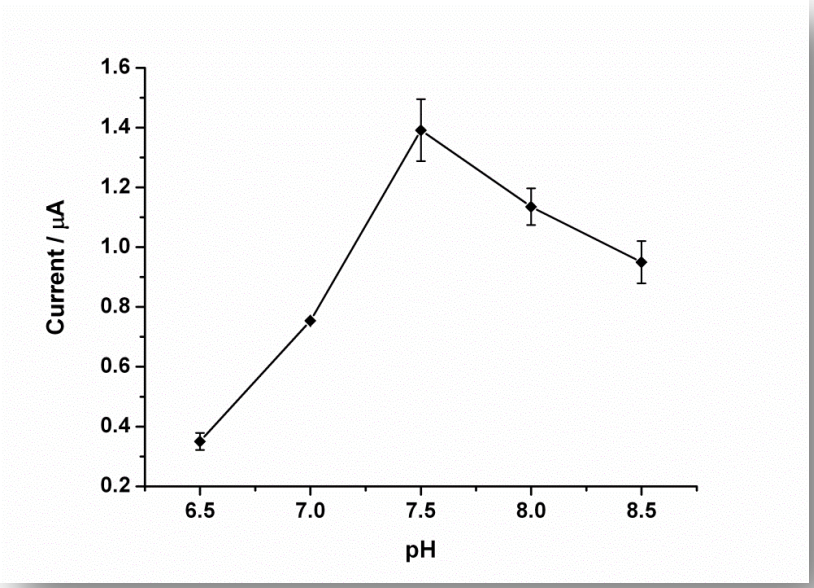


Figure 3. 22 Effect of pH (in sodium phosphate buffer, 50 mM, at pH 6.5, 7.0, 7.5, 8.0, 8.5, 25°C, -0.7 V, [Choline]: 10 mM). Error bars show standard deviation.

Moreover, ionic strength of the buffer used in immobilization was also optimized. It is important to optimize the ionic environment of the biolayer to

maintain more interactions with the polymer. Compare to the previous study, in the lower concentrations of the buffer solution, more interactions were achieved as well as the response. (Figure 3.23) With the increased hydrophilicity of the surface, there is no need to raise the ionic strength of the solution.

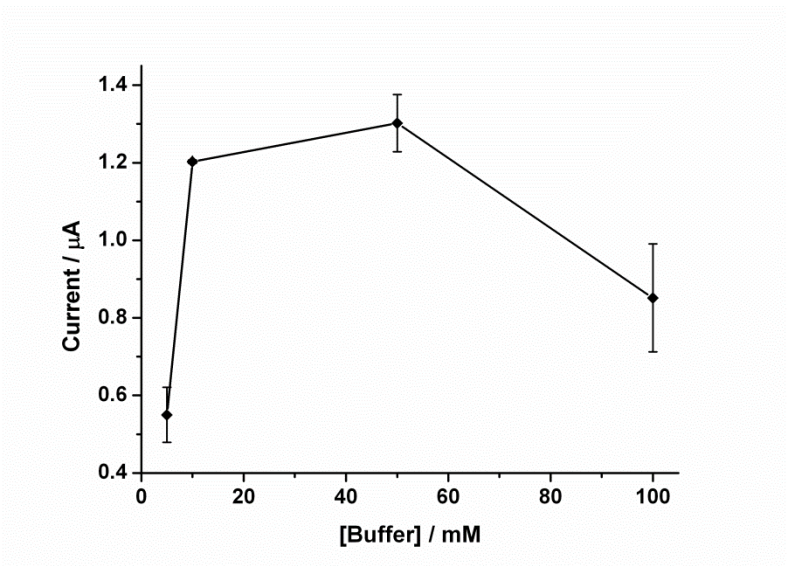


Figure 3. 23 Effect of ionic strength of the working buffer solution (in sodium phosphate buffer, pH 7.5, 25°C, -0.7 V, [Choline]: 10 mM). Error bars show standard deviation.

3.2.8 Effect of Temperature

The effect of temperature on the designed biosensor system was investigated over a temperature range of 20-40 °C. As illustrated in Figure 3.24, the current response has a maximum at 25 °C. After 25 °C, the response of the biosensor

decreases gradually which arises from the thermal denaturation of the enzyme molecules. Moreover, at higher temperatures, there is a risk for working solutions to evaporate; altering the composition and concentration of the buffer. 25 °C was chosen as the optimum and used in further studies.

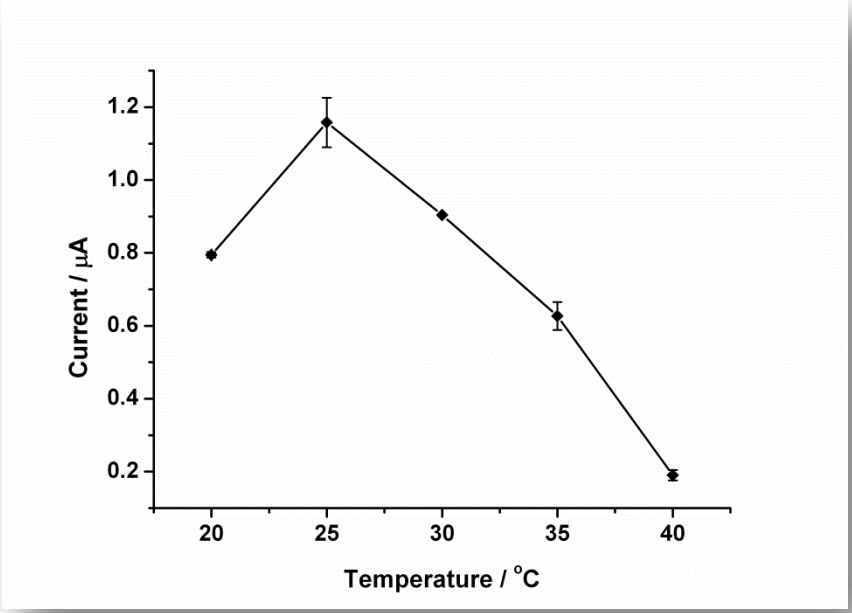
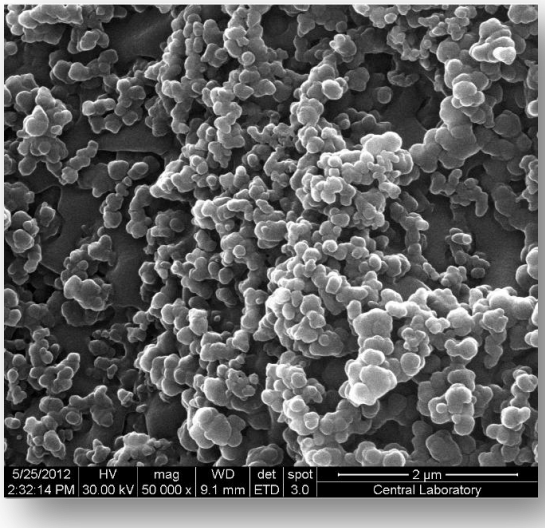


Figure 3. 24 Effect of temperature (in sodium phosphate buffer, 50 mM, pH 7.5, -0.7 V, [Choline]: 10 mM). Error bars show standard deviation.

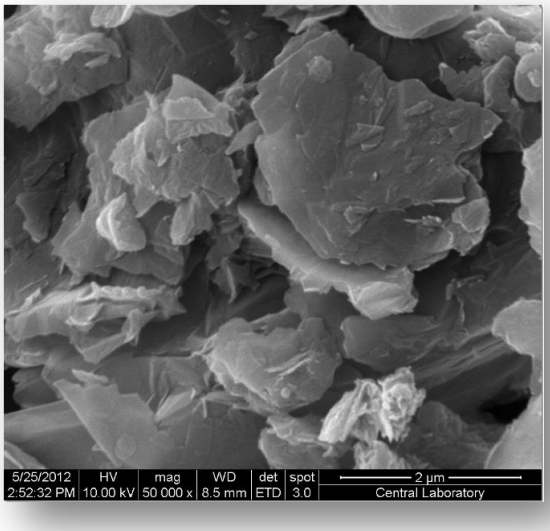
3.2.9 Surface Characterization

3.2.9.1 Scanning Electron Microscopy

The morphology of poly(TBT_6-NH_2) coated and enzyme immobilized-polymer coated electrode surfaces were characterized by SEM as shown in Figure 3.25. Figure 3.25,a shows the typical SEM image of the conducting polymer. It is clearly seen that polymer coated surface reveals a porous, rough and nonuniform morphology on the surface. However, the presence of enzyme confirms the successful immobilization of the enzyme onto the conducting polymer matrix demonstrating that the enzyme molecules could be nicely coated on the conducting polymer. (Figure. 3.25,b) Considering the relatively bigger size of the enzyme molecules and different morphology than the polymer, the penetration of the enzyme into the polymeric film may be thought as hardly possible; hence, the enzyme molecules were utterly bound at the outer part. This situation brings the higher possibility of access of the substrate to the biolayer.



(a)



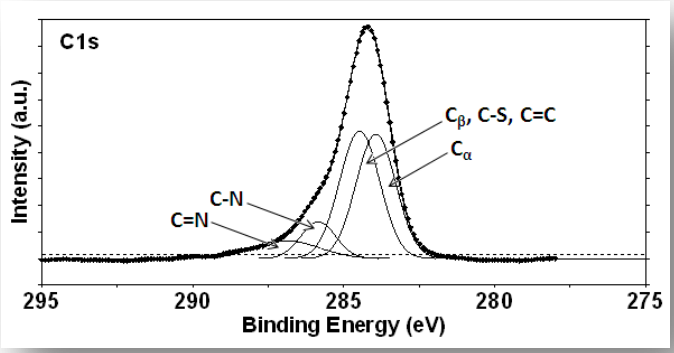
(b)

Figure 3. 25 SEM images of poly(TBT_6-NH_2) before (a) and after biomolecule immobilization (b) at the optimized conditions.

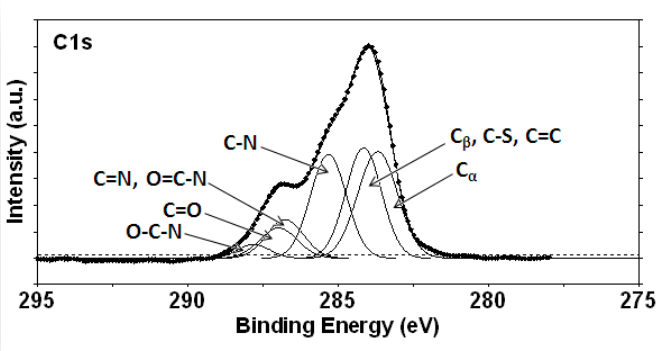
3.2.9.2 X-Ray Photoelectron Spectroscopy

X-ray photoelectron spectroscopy (XPS) experiments were carried out to characterize the modified layers. XPS was used to understand the interactions between the amino groups on the polymer chain and the functional amine groups on the enzyme. The carbon and nitrogen signals were resolved by a fitting program as depicted in Figure 3.26. Specific chemical bonds of the protein or those formed during the protein immobilization were readily detected through XPS analysis. The protein-immobilized surface (Figure 3.26,b) exhibited two signals at 287.0 eV and 287.8 eV (for C=O and O-C-N respectively), indicating the presence of covalent bond referring the linkages with the help of glutaraldehyde [109]. In addition to signals representing aromatic bonds (aromatic and alkyl carbons and C-S in thiophenes at 283.9 eV and 284.5 eV, respectively), C=N (286.8 eV) groups, C-N and characteristic amino group (285.8 eV) [109,110]. No such signal for presence of protein was detected on the untreated polymer coated electrode (Figure 3.26,a) confirming the successful deposition of ChO molecules on the surface of the poly(TBT_6-NH_2).

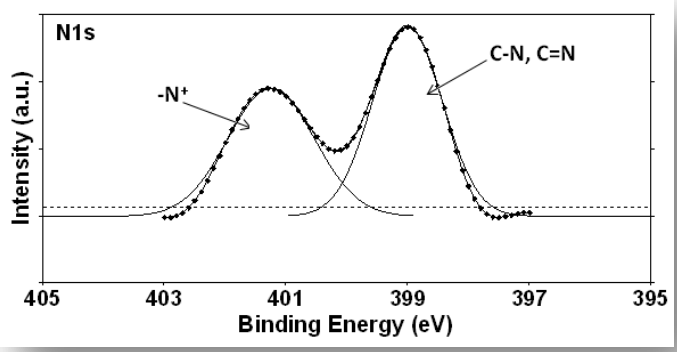
Information regarding the successful attachment of ChO to the polymer surface can also be obtained from the N1s spectra. The nitrogen envelope can be fitted into different components compare to the previously obtained spectrum representing the polymer only. (Figure 3.26,d) The peaks centered at 399.0 eV can be assigned to amine (-NH-) and C=N groups, 401.2 eV corresponds to protonated amine and nitrogens of triazole ring in the polymer backbone [111]. (Figure 3.26,c) However, the decrease in the protonated nitrogens and the appearance of the new groups at 400.5 eV is attributed to the nitrogen of immobilized protein [109]. (Figure 3.26,d)


C1s
Intensity (a.u.)
Cβ, C-S, C=C
Cα
C-N
C=N
295
290
285
280
275
Binding Energy (eV)

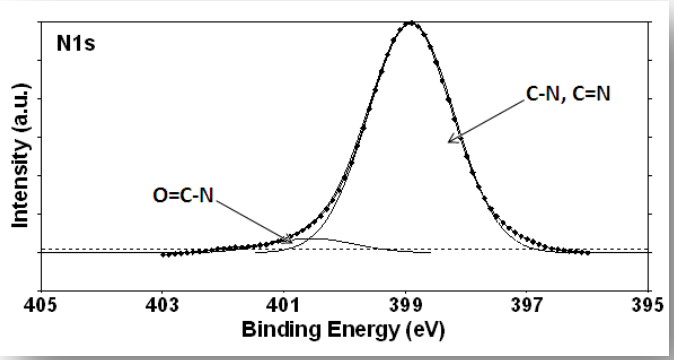
(a)


C1s
Intensity (a.u.)
C-N
Cβ, C-S, C=C
Cα
C=N, O=C-N
C=O
O-C-N
295
290
285
280
275
Binding Energy (eV)

(b)


N1s
Intensity (a.u.)
C-N, C=N
-N+
405
403
401
399
397
395
Binding Energy (eV)

(c)



(d)

Figure 3. 26 C1s and N1s XPS spectra of the polymer (a and c) and protein immobilized onto the polymer (b and d).

3.2.9.3 Contact Angle Measurements

In order to gain information on the changes of hydrophilicity of the surfaces before immobilization and after coupling with the enzyme, contact angle measurements were performed. A more hydrophobic nature of the biomolecule conjugated surface due to the presence of different hydrophobic amino acids was evident through an increase in advancing angle from 72.50° (±0.70) for the initial amine containing functional polymer surface to 89.5.2° (±0.70) for the ChO immobilized polymer.

3.2.10 Analytical Characterization

After optimization studies, amperometric responses of designed biosensor to choline were recorded by successively adding varying concentrations of choline into phosphate buffer solution. Figure 3.27 illustrates the calibration curve for current responses to various choline concentrations. The linear response range was found as 0.1-10 mM. Additionally, the limit of detection (LOD) was measured as 16.8 µM based on S/N=3.

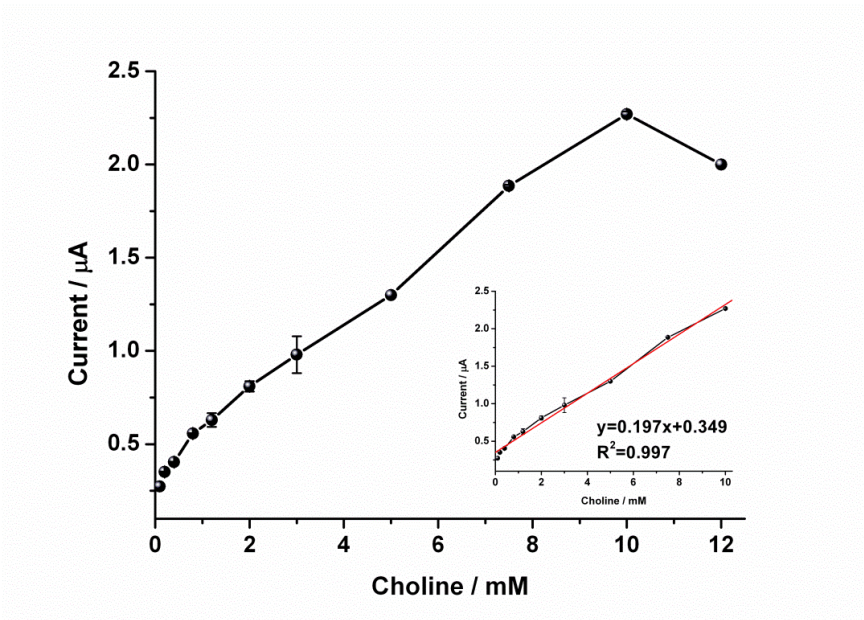


Figure 3. 27 Calibration curve for choline and linear range (inset) (in sodium phosphate buffer, 50 mM, pH 7.5, 25°C, -0.7 V). Error bars show standard deviation.

As depicted in Figure 3.28, the biosensor has a rapid and sensitive response to its substrate and reaches a steady-state equilibrium current in 7 s.

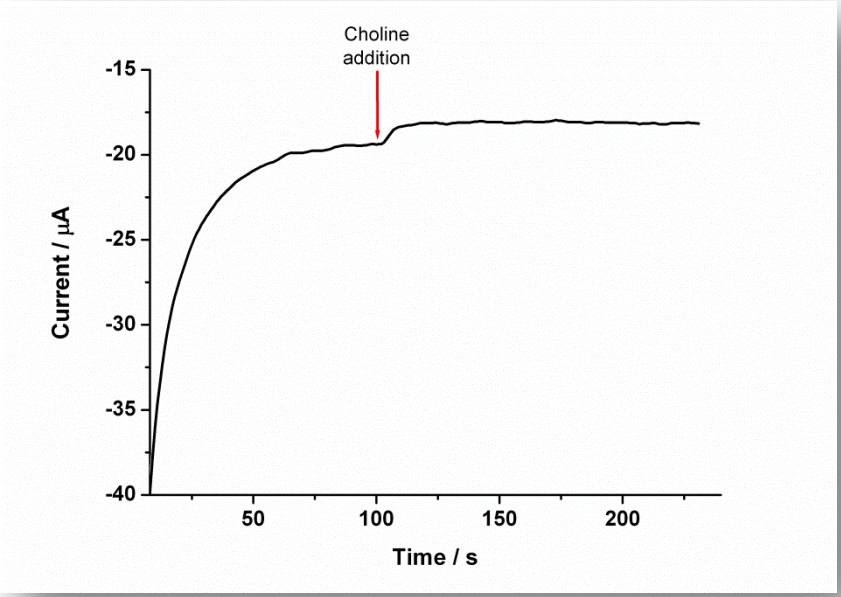


Figure 3. 28 A typical biosensor response to choline (in sodium phosphate buffer, 50 mM, pH 7.5, 25°C, -0.7 V, [Choline]: 8 mM).

The apparent Michaelis-Menten constant, K_m^{app}, shows the enzyme-substrate kinetics and it was calculated from Lineweaver-Burk equation as 5.11 mM. I_{max} is the maximum current measured under substrate saturation which was estimated to be 3.04 µA [112]. The value of K_m for choline is 0.87 mM for free choline oxidase [113]. The K_m^{app} value estimated for the proposed biosensor was higher due to the immobilization of the enzyme molecules. Considering the immobilized enzymes, K_m value is closely related with the diffusion of the materials. When the enzymes are incorporated to the matrices, the diffusion limitation in the biolayer is higher and

mass transfer resistance may occur. As a result, if the immobilization is not achieved efficiently, the enzyme is not well-accessible which complicates the substrate transfer from the bulk. By this way, higher K_m value can be explained for the constructed system.

The analytical performance of the constructed biosensor was given in Table 3.2.

Tablo 3. 2 Some characteristics of the proposed biosensor (in 50 mM, pH 7.5 sodium phosphate buffer at 25 ^{o}C, -0.7 V).

Parameter	
K_m^{app}	5.11 mM
I_{max}	3.04 µA
Linear Range	0.1 - 10 mM
Sensitivity	0.251 µA/mM
LOD [a)]	16.8 µM
Response time	7 sec
Operational stability	No decrease for 12 hours
Shelf life	No decrease during one week

[a)]LOD was calculated according to S/N

To determine the exact and accurate amount of choline in the real samples, the measurements must be interference free. In order to check whether there is an interference effect of some electroactive species such as ascorbic acid and uric acid (between 0.01 and 0.1 M), they were injected in working buffer solution instead of substrate during amperometric measurements. However, no interference was observed at the potential of -0.7 V under optimized working conditions.

On the other hand, the biosensor signals corresponding to 10 mM choline standard solutions were measured for ten times in order to achieve repeatability of

the biosensor response. The standard deviation (SD) and the relative standard deviation (RSD) were calculated as ±0.081 and 3.70 %, respectively.

The long-term stability of the constructed biosensor was evaluated. No activity loss was observed during a period of one week.

3.2.11 Sample Application

The designed biosensor was used in the detection of paraoxon-ethyl. This substance is the oxidation product of the phosphorthionate pesticide parathion and used as insecticide. Inhibition studies were done according to literature data [104,114]. Paraoxon-ethyl has an uncompetitive inhibitory effect on choline oxidase; thus, in the presence of proper amount of its substrate, the inhibitory analysis can be performed [114]. The substrate amount was chosen as 10 mM which is the enzyme-saturated concentration.

In the measurements, the decrease in the responses of the biosensor was in correlation with the paraoxon-ethyl concentrations which cause inhibition of the immobilized choline oxidase. Before the pesticide measurements, the biosensor response to 10 mM choline was recorded. The prepared biosensor was incubated for 5 minutes in paraoxon-ethyl solutions with different concentrations and then, washed many times with distilled water. Percent inhibitions for each concentration were calculated as (% Inhibition) $I\% = (I_0 - I)/ I_0 \times 100$. I_0 is the initial biodedector response to 10 mM choline and I is the response of the biodedector to same amount of substrate after inhibition by paraoxon-ethyl. Percent inhibition versus concentration of paraoxon-ethyl is given in Figure 3.29. The minimum detection value was found as 0.1 μM corresponding to 0.478 μg/L paraoxon-ethyl. The sensitivity of the biosensor was calculated as 24 % per μM. Moreover, after each measurement, the biosensor was rinsed with distilled water several times and the

regeneration was achieved. However, after the 83 % inhibition, the biosensor retained 65.3 % of its initial activity as residual.

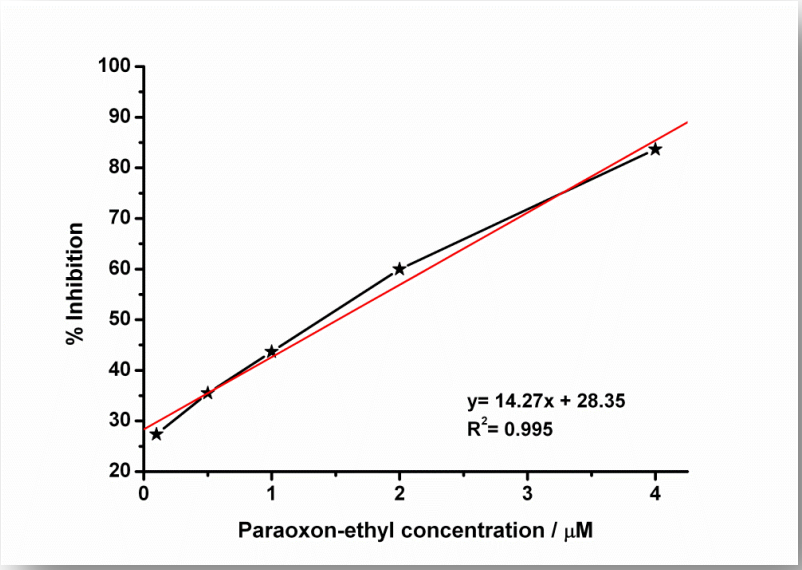


Figure 3. 29 Calibration curve for paraoxon-ethyl detection (in sodium phosphate buffer, 50 mM, pH 7.5; 25°C, -0.7 V, [Choline]: 10 mM).

CHAPTER 4

CONCLUSION

The aim of this study is the construction of two different biosensors for the detection of glucose and paraoxon-ethyl. Two derivatives of bezotriazole containing conducting polymers were designed and used as the immobilization matrices for the enzymes.

Two amperometric biosensors were developed successfully by the formation of cross-linked enzyme molecules. This provides robust, highly active immobilized enzymes with well operational and shelf life stability. Remarkable results were obtained.

The results for glucose biosensor according to this research clearly indicated that even though native GOx has very limited hydrophobic sites; these sites are still available for an interaction with alkyl residues on a hydrophobic carrier. By optimizing ionic strength of enzyme solution for immobilization and using a cross linker, enzyme crystal formation can be enhanced; hence, more stable and active enzyme electrodes were achieved. By compatible results obtained from the biosensor and HPLC, immobilization of GOx enzyme on CP electrodes was proved to be an alternative way for detection of the glucose amount in fermentation medium for yeast cultivation.

The electrochemically polymerized TBT_6-NH_2 performs well as an immobilization matrix for the construction of a choline biosensor. The amperometric biosensor based on conducting polymer was fabricated through the covalent immobilization of the enzyme on the functionalized conducting polymer. The presence of the bonding between the surface and the protein was confirmed by SEM and XPS studies. The fabricated biosensor exhibited well stability and detection.

Moreover, it was successfully applied to pesticide detections. The K_m and I_{max} values can be with various modifications of the immobilization matrix to achieve more accessible enzyme molecules. Thus, this biosensor will be improved in further investigations.

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