In the name of God

Razi University
Faculty of Chemistry
Department of Applied Chemistry

M.Sc.Thesis

Title of the Thesis:
Laccase immobilization on the electrode surface to design a biosensor for the detection of phenolic compound such as catechol

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Abstract

Biosensors based on the coupling of a biological entity with a suitable transducer offer an effective route for detection of phenolic compounds. Phenol and phenolic compounds are among the most toxic environmental pollutants. Laccases are multi-copper oxidases that can oxide phenol and phenolic compounds.

A method is described for construction of an electrochemical biosensor for detection of phenolic compounds based on covalent immobilization of laccase (Lac) onto poly aniline (PANI) electrodeposited onto a glassy carbon electrode (GCE) via glutaraldehyde coupling. The modified electrode was characterized by voltammetry, Fourier transform infrared (FTIR) spectroscopy and atomic force microscopy (AFM) techniques. The results indicated that laccase was immobilized onto modified GC electrode by the covalent interaction between laccase and terminal functional groups of the glutaraldehyde. The laccase immobilized modified electrode showed a direct electron transfer reaction between laccase and the electrode. Linear range, sensitivity, and detection limit for this biosensor were 3.2×10^{-6} to 19.6×10^{-6} M, 0.7067 μA/μM, 2.07×10^{-6} M respectively.

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List of abbreviations

Abbreviation	Meaning
ISFETs	ion-selective field-effect transistors
MOS	metal/oxide/semiconductor
ENFET	Enzyme Field Effect Transistor
HrP	Horseradish peroxidase
Lac	laccase
PANI	polyaniline
ES	emeraldine salts
EB	emeraldine base
GA	glutaraldehyde
BSA	bovine serum albumin
R	reduced form
O	oxidase form
FTIR	Fourier transforms infrared spectroscopy
AFM	Atomic force microscopy
CV	Cyclic voltammetry
GCE	glassy carbon electrode
[S]	substrate concentration
V_o	initial rate
E_q	A quasireversible electrochemical process
C_i	An irreversible chemical reaction

CHAPTER ONE

INTRODUCTION

1.1. Sensors and Biosensors

Sensors are the devices, which are composed of an active sensing material with a signal transducer. The role of these two important components in sensors is to transmit the signal without any amplification from a selective compound or from a change in a reaction. These devices produce any one of the signals as electrical, thermal or optical output signals which could be converted in to digital signals for further processing. Among these, electrochemical sensors have more advantage over the others because; in these, the electrodes can sense the materials which are present within the host without doing any damage to the host system. On the other hand, sensors can be broadly classified in to two categories as chemical sensors and biosensors [1]. According an IUPAC nomenclature, biosensor is a device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals [2]. As bio-components, an enzyme, antibody, nucleic acid, lectine, hormone, cell structure or tissue can be used. Its role is to interact specifically with the target analyte and the result of biochemical reaction is consequently transformed through transducer to measurable signal [3]. The transducing systems can be electrochemical, optical, piezoelectric, thermometric, ion-sensitive, magnetic or acoustic one. Very important part of a biosensor fabrication is the immobilization of bio-component [4].

1.2. Bioreceptors

1.2.1. Enzymes

Enzymes are often used as biomaterials for the development of biosensors. These biosensors utilize enzymes (Table 1.1) which are specific for the desired molecules and catalyze generation of the product, which is then directly determined using one of the transducers mentioned above. Many factors have influence on the performance of enzyme-based biosensors, such an enzyme loading, the use of a suitable pH, temperature and in some cases a cofactor can help to retain the abilities of the enzyme. Another factor that can affect the electrode performance is the type of immobilization method used to retain the enzyme as well as the thickness of the enzyme layer on the sensor [4].

Table 1.1 Enzyme categories and their functions which are used for selective detection of their competent substrates as analytes by biosensor.

Enzyme category	Functions
Oxidoreductases	Oxidation/reduction reactions
Transferases	Transfer of molecular groups from one molecule to another
Hydrolases	Hydrolytic cleavage
Lyases	Cleavage of C-C, C-O, C-N bonds by other means than oxidation or hydrolysis
Isomerases	Intramolecular rearrangement
Ligases	Joining of two molecules

1.2.2. Antibodies

An antibody is a complex biomolecule, made up of hundreds of individual amino acids arranged in a highly ordered sequence. An antigen-specific antibody fits its unique antigen in a highly specific way. An antigen-specific antibody fits its unique antigen in a highly specific way. This unique property of antibodies is crucial to their usefulness in

immunosensors where only the specific analyte of interest, the antigen, fits into the antibody binding site. Antibodies, with their characteristics of high affinity and excellent selectivity, are ideal analytical tools. Antibody-based biosensors are also called immunosensors [4].

1.2.3. Nucleic acids

Biosensors based on DNA, RNA and peptide nucleic acid gain their high sensitivity and selectivity from the very strong base pair affinity between complementary sections of lined up nucleotide strands [4].

1.2.4. Cells

These bioreceptors are either based on biorecognition by an entire cell/microorganism or a specific cellular component that is capable of specific binding to certain species. One of the major advantages resulting from using this class of bioreceptors is that the detection limits can be very low because of signal amplification. Many biosensors developed with these types of bioreceptors rely on their catalytic or pseudocatalytic properties [4].

1.3. Transducers (Types of biosensors)

Transducer is an analytical tool which provides an output quantity having a given relationship to the input quantity [2]. Biosensors can be classified according the transduction methods they utilize. Most forms of transduction can be categorized in one of five main classes: electrochemical, electrical, optical, piezoelectric (mass detection methods) and thermal detection [4].

1.3.1. Electrochemical

Electrochemical biosensors are the most commonly used class of biosensors. These are based on the fact that during a bio-interaction process, electrochemical species such as

electrons are consumed or generated producing an electrochemical signal which can in turn be measured by an electrochemical detector. Electrochemical biosensors have been widely accepted in biosensing devices [1].

1.3.1.1. Amperometric

Amperometric biosensors are the most widespread class of biosensors. Amperometric biosensors measure the changes in the current on the working electrode due to direct oxidation of the products of a biochemical reaction. Amperometric techniques are linearly dependent on analyte concentration and give anormal dynamic range and a response to errors in the measurement of current [1]. Most of biochemicals can now be detected and quantified amperometrically by their enzyme-catalyzed electro-oxidation or electroreduction, or their enzyme-catalyzed hydrolysis/ phosphorylation followed by electro-oxidation/ electroreduction, or their involvement in a bioaffinity reaction enabling electro-oxidation/ electroreduction. Amperometric biosensors are very sensitive and more suitable for mass production than the potentiometric ones [4].

1.3.1.2. Potentiometric

Potentiometric biosensors consist of measurement of potentials at the working electrode with respect to the reference electrode. They function under equilibrium conditions and monitor the accumulation of charge, at zero current, created by selective binding at the electrode surface. For example, ISE detect ions such as Na^{+}, K^{+}, Ca^{2+}, H^{+} or NH^{4+} in complex biological matrices by sensing changes in electrode potential when the ions bind to an appropriate ion exchange membrane. Nearly all potentiometric sensors, including glass electrodes, metal oxide based sensors as well as ion-selective electrodes, are

commercially available. Moreover, they can be easily mass-fabricated in the miniature formats using advanced modern silicon or thick-film technologies [1].

1.3.2. Electrical

1.3.2.1. Conductometric (Impedimetric)

When ions or electrons are produced during the course of biochemical reaction, the overall conductivity or resistivity of the solution is changing. The measured parameter when using this transducer is the electrical conductance/resistance of the solution. Conductometric biosensors measure the changes in the conductance between a pair of metal electrodes as a consequence of the biological component. Conductance measurements have relatively low sensitivity [1].

1.3.2.2. Ion-sensitive

Biosensors based on ion-selective field-effect transistors (ISFETs) earlier considered as a category of potentiometric sensor, are now, according to the last IUPAC technical report on electrochemical biosensors, separated into the fourth class of electrochemical sensors. ISFET is a classical metal/oxide/semiconductor (MOS) field-effect transistor with a gate formed by a separated reference electrode and attached to the gate area via an aqueous solution. These semiconductor FETs have an ionsensitive surface. The surface electrical potential changes due to the interaction between ions and the semiconductor. This change in the potential can be subsequently measured. ISFET can be constructed by covering the sensor electrode with a selectively permeable polymer layer, through which ions may diffuse and cause a change in the FET surface potential. This type of biosensor is also called an ENFET (Enzyme Field Effect Transistor) [4].

1.3.3. Optical

Optical biosensors are based on the measurement of light absorbed or emitted as a consequence of a biochemical reaction. In such a biosensor, the light waves are guided by means of optical fibers to suitable detectors. They can be used for measurement of pH, O_2 or CO_2 etc. A commercial optical biosensor, which is the hybrid electrochemical/optical LAPS (light addressable potentiometric sensor) was developed by the company Molecular Devices in Palo Alto, USA [1].

1.3.4. Piezoelectric (mass-sensitive)

These biosensors are based on the coupling of the bioelement with a piezoelectric component, usually a quartz-crystal coated with gold electrodes. Many types of materials (quartz, tourmaline, lithium niobate or tantalate, oriented zinc oxide or aluminium nitride) exhibit the piezoelectric effect. However, the properties of quartz are the main reason for its common usage for analytical applications. Piezoelectric transducers allow label-free detection of molecules. These crystals can be made to vibrate at a specific frequency with the application of an electrical signal of a specific frequency. Based on this, the frequency of oscillation is dependent on the electrical frequency applied to the crystal as well as the crystal's mass. With increasing of the mass due to binding of molecules, the oscillation frequency of the crystal is changed and the resulting change can be measured electrically and finally used to determine the additional mass (either positive or negative one) of the crystal (mass-sensitive techniques) [1].

1.3.5. Calorimetric (thermometric)

Calorimetric biosensors detect an analyte on the basis of the heat evolved due to the biochemical reaction of the analyte with a suitable enzyme. Recently, integrated circuit

temperature sensitive structures have been modified with enzymes. Different substrates, enzymes, vitamins and antigens have been determined using thermometric biosensors. The measurement of the temperature is via a thermistor, and such devices are called as enzyme thermistors. Thermal biosensors do not require frequent recalibration and are insensitive to the optical and electrochemical properties of the sample. The most commonly used approach in the thermal enzyme probes was related to the enzyme directly attached to the thermistor. It was observed that the majority of the heat evolved in the enzymatic reaction was lost to the surrounding solution without being detected by thermistor resulting in the decrease in sensitivity of the biosensor. Calorimetric biosensors were used for food, cosmetics, pharmaceutical and other component analysis [1, 5].

1.4. Phenolic compounds

1.4.1. The importance of detection of phenolic compounds

Phenolic compounds are important contaminants in food and environmental matrices. Many of them are very toxic, showing harmful effects on plants, animals, and human health. Therefore, the identification and quantification of these compounds are important for environmental monitoring. The commonly used techniques for determination of phenolic compounds are spectrophotometry, chromatography, and capillary electrophoresis. However, these methods are time-consuming and the equipment are expensive. Therefore, there is an interest in developing simple, sensitive, and effective analytical techniques for their determination. Among them, electrochemical biosensors have been shown to be very simple and sensitive tools for phenolic compounds assay [6,7].

Biosensors can make ideal sensing systems to monitor the effects of pollution on the environment, due to their biological base, ability to operate in complex matrices, short response time and small size. The determination of phenol and its derivative compounds is

of the environmental greatness, since these species are released into the environment by a large number of industries, *e.g.* the manufacture of plastics, dyes, drugs, antioxidants and waste waters from pulp and paper production. This group of biosensors is of great interest because of their application in food and pharmaceutical industry.

1.4.2. Catechol

Catechol (1,2- dihydroxybenzene) is one of the most important phenolic compounds which occurs naturally in fruits and vegetables and can be released to the environment during its manufacture and use. It is also detected at low levels in, groundwater, drinking-water, soil samples and in wastewaters from coal conversion. Catechol has a great importance in both biological and environmental analysis fields and this due to its excellent electrochemical activity and can be used for the characterization of different analytical methods. Different analytical methods were used for the determination of catechol such as spectrophotometry [8, 9] and high-performance liquid chromatography [10]. The electrochemical determination of catechol was also studied using enzymatic [11-17] or non-enzymatic electrodes [18- 26]. The direct electrochemical determination of catechol based on the oxidation of catechol at the electrode surface to *o*-quinone [27].

Among enzymes, laccases and tyrosinases [28] or horse-radish peroxidase [29] as well as polyphenol oxidase are groups of enzymes that catalyze the transformation of a large number of phenolic compounds. The mechanism for tyrosinase, laccase and peroxidase in the electrochemical biosensors are different. Enzyme molecules are reduced by phenolic compounds after they were oxidized by oxygen (for tyrosinase and laccase) or hydrogen peroxide (for peroxidase) on the surface to the electrode. The tyrosinase biosensors are applicable to the monitoring of phenolic compounds with at least one free *ortho*-position. On the other hand, the laccase biosensor can detect phenolic compounds with free *para*-