Celal Bayar University Journal of Science

A Novel Biosensor Based on Laccase for The Detection of Catechol

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Received: 18 October 2019
Accepted:17 March 2020
DOI: 10.18466/cbayarfbe.634500

Abstract

Phenolic compounds are broadly used in petrol industrial production processes and thus they are hazardous for ground and surface water and environment. Many of them have harmful effects on plants, animals and human health. Laccases can oxide phenol and phenolic compounds. Biosensors provide ideal sensing systems to determine phenolic compounds. In the present paper, it was developed novel sensor surface with modified laccase on gold electrode which could recognize the phenolic compound catechol. The electrode arrays integrated microfluidics have been fabricated and characterized in order to create a sensor chip which is easy, rapid, and cheaper to produce also have good electrochemical sensing properties. This gold electrode was used to obtain laccase active surface. Laccase was covalently bounded to self-assembled monolayer on gold electrode via glutaraldehyde reactions. Cyclic voltammetry and amperometry activity assays showed that the detection of catechol with laccase modified gold surface. All of the study was carried out using automated biosensor device. Linear range for this biosensor was 0.025 mM to 0.8 mM.

Keywords: Catechol, electrochemical biosensor, laccase immobilization

1. Introduction

Biosensors are of great importance in the bioprocesses, diagnosis, agricultural applications, food industry and environmental imaging. The market share of commercially important biosensors in these areas is expressed in billions of dollars. In this respect, research and development of new sensor devices is of great importance. Electrochemical sensors are the most widely used biosensor group because of their selectivity, rapid analysis, high sensitivity, ease of working with solutions and miniaturization [1-4]. The design of an electrochemical sensor includes many parameters such as electrode design, surface chemistry, recognition element immobilization to the electrode surface and optimum experimental conditions that need to be considered carefully.

Phenolic compounds occupy a large part in nature. They are found in vegetables and fruits and add properties to the products prepared with them; olive-like and they have antioxidant properties. Phenolic compounds are widely used in the production processes of paints, pesticides, surfactants, resins, and plastics in petroleum and pharmaceutical industries. Opposite to their positive properties, phenolic compounds have pollutant properties in nature and water resources that are emerging as by-products of the resin production, pharmaceutical, paint, textile and petrochemical industries. Some phenolic compounds have the ability to mimic endocrine hormones and poses a threat to health [5]. It is possible to identify and quantify phenolic compounds by conventional methods such as spectroscopy and chromatography [6, 7] but these are time-consuming and expensive methods. Recently, biosensor applications have attracted attention for the determination of phenolic compounds.

The laccase enzyme (polyphenoloxidase; EC 1.10.3.2) is a member of the multi-copper-containing family of oxidase enzymes and is capable of oxidizing many organic substrates to reduce molecular oxygen to water. They are produced by a wide variety of organisms such as bacteria, fungi and plants. Laccases can also oxidize both phenolic and non-phenolic compounds and many environmental pollutant compounds. Due to these oxidation ability of laccases, they are preferred in biosensors. Electrochemical sensors are commonly used in the determination of phenolic compounds due to their stability and reproducibility. In order to use the laccase enzyme in the biosensor, immobilization is required for the enzyme to be stable and reusable. Covalent binding

[8-12], physical adsorption [13-16] and entrapmenti in a matrix [17-20] are the immobilization techniques. The method of enzyme binding to the electrode surface is the first step in effective enzyme biosensor due to a critical factor for electron transfer between the enzyme and the electrode surface. The electrode surface modificaiton is carried out with nanomaterials, conductive polymers and self-assembled monolayers (SAM) [21-25].

In this study, a novel biosensor based on laccase enzyme was developed for the detection of phenolic compound catechol. Laccase was covalently bounded to self-assembled monolayer on gold electrode via glutaraldehyde reactions. Modifications and enzyme immobilization on sensor chips integrated into the microfluidic system were carried out quickly and effectively in biosensor device.

2. Materials and Methods

2.1. Materials and Instrumentations

Phosphate buffered saline (PBS, 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4) tablets, laccase (EC=1.10.3.2, from *Trametes versicolor*), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) liquid substrate system, 6-amino-1-hexanethiol (AHT), potassium hexacyanoferrate, glutaraldehyde 25%, tris-base, potassium chloride (KCI), ethanol, 1,2-dihydroxybenzene (catechol) were purchased from Sigma-Aldrich. Ultrapure water (18 MΩ cm^{-1}) was obtained from a Milli-Q water system.

MiSens biosensor device was used in electrochemical analysis and assays [26-28]. The biosensor device is an integrated and automated electrochemical biosensor with microfluidic system including tubing connected sample pick up needle and sample/reagent carousel, includes biochip docking station electronic parts and software developed by BILGEM Research Center (BILGEM-TUBITAK, Kocaeli, Turkey). The electrodes were designed on glass plates (10 x 20 mm^2) with a laser-shaped stainless steel thin metal mask. Gold metal coated on glass plates using e-beam evaporator. Biochips includes Au electrode arrays that consist of shared reference/counter electrodes and 8 working electrodes (d =1 mm) has been fabricated and used for the assays (~ 7-10 µL capacity flow cell on the electrode array).

2.2. Modification on The Surface of Electrodes

Before the modification, plasma cleaning was applied on the electrode arrays for a clean sensor surface. A self-assembled monolayer (SAM) was then applied by immersing the sensor chips in ethanolic solution of 2 mM 6-amino-1-hexanethiol (AHT) for overnight (Figure 1). Later they were rinsed with ethanol and water, dryed with nitrogen stream and were vacuum packed. The functionalised biochips were stored at +4°C until used. After the surface modification, the biochip was inserted into the cassette and placed to the device. It was integrated the electronic and fluidic connections and to created a microfluidics system.

2.3. Immobilization of Laccase onto Surface

For the immobilization of enzyme, firstly 6-amino-1-hexanethiol (AHT) functionalised chip surface was activated with 0.5% (w/v) glutaraldehyde (GA) prepared in water. GA (200 µL, 50 µL/min), later laccase solution (100 µg/mL, 200 µL, 25 µL/min) were injected to sensor surfaces (Figure 1). Phosphate buffered saline (PBS, 10 mM, pH 7.4) used for washing buffer and this buffer continuously flowed (100 µL/min) over the sensor surfaces between the injections. Non-reacted aldehyde groups were capped with ethanolamine solution pH 8.5 (1 M, 200 µL, 50 µL/min). Determination of enzyme activity was carried out using substrate solution ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid).

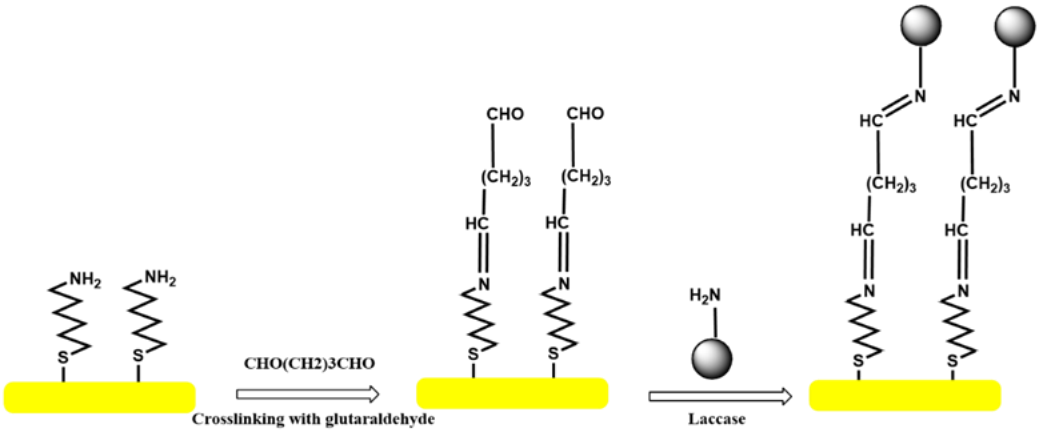

Figure 1. Preparing of the laccase modified biosensor chip

2.4. Detection of catechol and electrochemical measurements

Catechol stock solutions (6 mM) in ethanol were prepared and stored at -20°C until used. Phenolic compound catechol was prepared at varying concentrations (0.025 - 2.0 mM) in PBS buffer solution (10 mM, pH 7.4) and the was measured on laccase immobilized biosensor electrodes.
Cyclic voltammetry (CV) and amperometric measurements were also performed with biosensor device. CV was performed using potassium ferrocyanide solution (1 mM $K_4[Fe(CN)_6]$ / KCI at 100 mV/s scan rate). Amperometric signal was measured using substrate ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) with a fixed potential of -0.1 V All measurements was carried out during buffer injection and resulted in a real-time amperometric reading.

3. Results and Discussion

3.1. Biosensor Surface Modification

To coating of self-assembled monolayer (SAM) on the gold chip surface, the chips were incubated with 2 mM 6-amino-1-hexanethiol (AHT) solution for overnight. Baregold electrode and AHT coupled electrode were investigated by cyclic voltammetry using potassium ferrocyanide ($K_4[Fe(CN)_6]$) solution as a redox marker in MiSens biosensor device (scan rate: 100 mV/s).

In voltammogram Figure 2, the redox area between the positive and negative potentials of the bare gold electrode, the oxidation and reduction peaks of potassium ferrocyanide are larger than the redox area of the AHT coated chip. After the coating of chip with AHT, a dramatic decrease in redox current was observed.

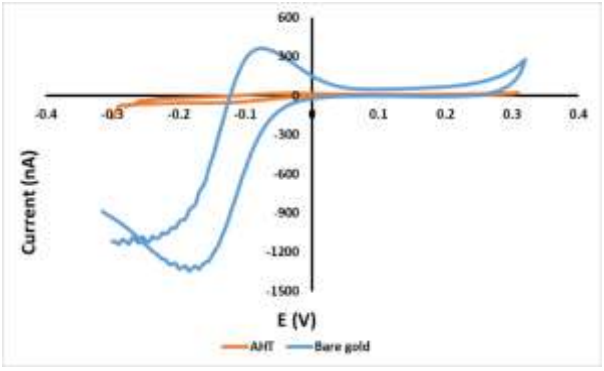

Figure 2. The cyclic voltammetry of bare gold and AHT coated gold electrode arrays (1 mM $K_4[Fe(CN)_6]$/KCI at 100 mV/s scan rate).

It is important that providing high quality packaging in alkanethiol for a good dense and uniform SAM surface. Because the electron tunnel must be formed to enable the transfer of electrons between the electrode and a donor or acceptor [29]. It was observed that a quality packaging achieved using AHT (Fig 1). 6-amino-1-hexanethiol was binded on the gold surface by the strong affinity of a thiol group to gold and thus the amino group ready for enzyme immobilization.

3.2. Activity assay of immobilized laccase

Laccase was immobilized onto AHT coated chips. To binding of laccase on the surface, sensor chip with the SAM of AHT was then placed in the flow cell of the biosensor device, and glutaraldehyde solution was injected (0.5% w/v, 50 µL/min) and then enzyme was injected (100 µg/ml, 200 µL, 25 µL/min). An CHO group binds to NH_2 group of AHT and the other CHO group binds to amino group of enzyme with acetal or semi-acetal formation between amino (NH_2) groups and aldehyde groups (CHO) on the surface. Enzyme activity was determined by amperometric measurement using ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) substrate solution. In the MiSens biosensor device, Real-time measurement was performed to obtain electrochemical signal by amperometric method, current was measured continuously while applying potential to electrode surface (Real−time Electrochemical Profiling; REP™). A change in catalytic current was observed by injection of ABTS solution (50 µL/min) into the system by reaction of the bound laccase enzyme on the chip surface. While applying potential (-0.1 V) to the electrode surface the current was continuously measured. Initial current measurements during PBS buffer injection were taken and this signal was recorded as a baseline (Figure 3). It was recorded the maximum current value in 170. seconds, average 0.029 ± 0.001 µA current signal.

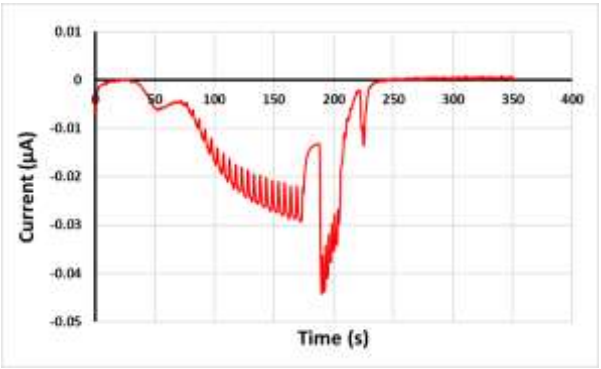

Figure 3. Sensor response of current vs time with respect to ABTS against -0.1V potential.

3.3. Detection of catechol

The determination of catechol on the laccase immobilized sensor surface was performed by cyclic voltammetry (CV) and amperometric measurement of the catalytic signal formed by the reaction of the enzyme with the catechol. Firstly the laccase immobilized sensor surface was investigated by cyclic voltammetry using catechol solution (400 µM) and also CV measurement was performed for PBS solution. The electrochemical behavior of catechol was characterized by cyclic voltammetry. Comparing the redox peak potentials for laccase immobilized and non-immobilized electrodes, laccase biosensor was the highest current (Figure 4), therefore it was the highest catalytic ability for catechol oxidation. Catechol oxidized to o-quinone on the surface of the biosensor. Amperometric measurement was performed at -0.1V, as shown in CV graphs, with reduction peak.

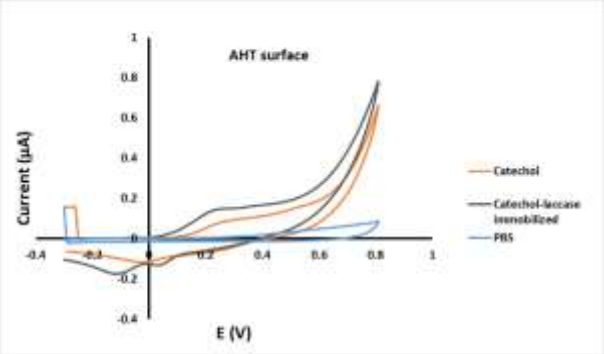

Figure 4. The cyclic voltammetry of laccase immobilized and non-immobilized gold electrode arrays (400 µM catechol solution and PBS at 100 mV/s scan rate).

In the catechol determination study, catechol was prepared in different concentrations (0.025-2.0 mM in PBS buffer solution) and amperometric measurement was performed on the laccase modified chips with the subsequent injection catechol (250 µL) with 50 µL/min fluid flow with a fixed potential of -0.1V. Amperometric signal was measured in real time, an increase in the catalytic current was observed. The initial current is a background measurement taken by injection of PBS solution. Then, as the catechol solution flowed through the sensor, a change in the current was observed by the reaction between the bound enzyme laccase and the catechol on the chip surface (Figure 5) and the subtraction between the current obtained during catechol and buffer was used as sensor response.

The amperometric response of the assay was calculated the maximum current obtained 150 s after the catechol injection was used as the sensor response. The catalytic current proportional increased on increasing the concentration of catechol in the range from 0.025 mM to 0.8 mM with a correlation coefficient of R^2 0.91 and LOD of 0.015 mM (Figure 6).

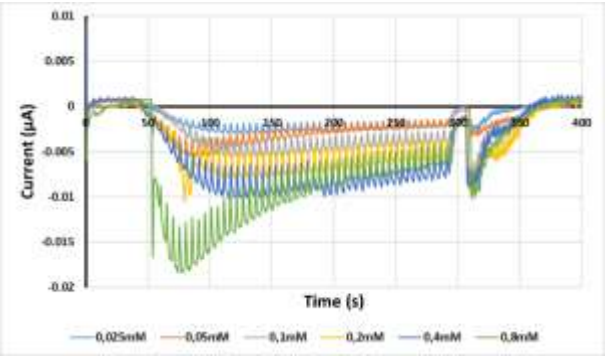

Figure 5. The raw data of the real-time responses of current vs time with respect to catechol concentrations against -0.1V potential.

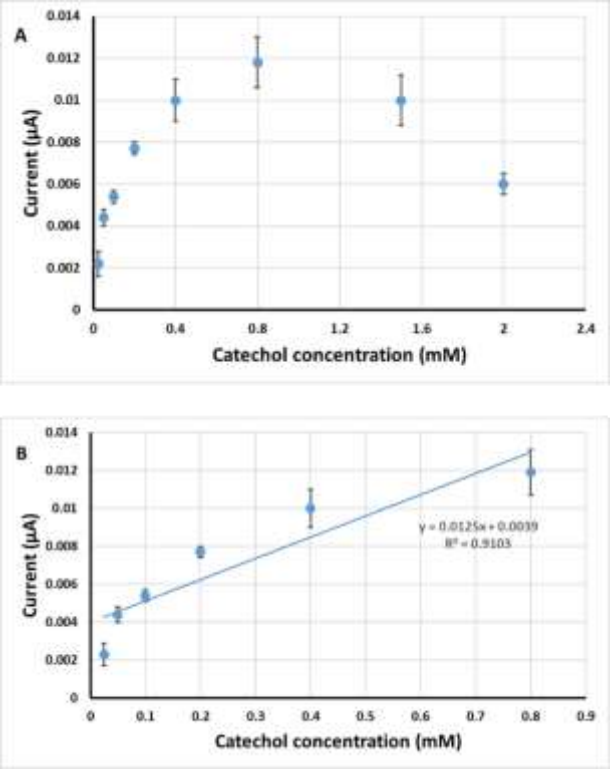

Figure 6. Calibration curve (A) and linear calibration curve (B) for the catechol detection assay.

Phenolic compounds are widely used in the production processes of paints, pesticides, surfactants, resins and plastics in the petroleum and pharmaceutical industries. As a result, they pose a danger to the surrounding water resources. Because these compounds have toxic effects on plants, animals and human health. Catechols (1,2-dihydroxy benzene; catechol), one of these phenolics are used in the industrial synthesis of rubbers, paints, plastics and in the pharmaceutical and cosmetic fields.

For these reasons, the determination of catechol is important using biosensors.

In the literature, there is no study on laccase immobilization and catechol detection studies on 6-amino-1-hexanethiol (AHT) surface in microfluidic system, the other studies were carried out in static medium. Protein immobilization step after sensor surface modification with AHT takes approximately 30 minutes on the sensor device however, these steps are carried out in conventional assays times for hours. In the literature, the conventional phenolic detection assays in require long incubation times for the chemicals and protein immobilization [22-25, 30, 31], whereas during microfluidic flow in the biosensor system the mass transfer effects are minimized and a rapid reaction occurs. Another importance of this study is that all experiments are carried out in a microfluidic system. In amperometric biosensors, electron transfer process is a major issue that will occur at the electrode surface and the redox center within the enzyme is provided by the most suitable electron transfer way. Transfer problem occurs when amperometric measurement method is used in static environment [32,33]. The automated electrochemical biosensor with a microfluidic and automatic injection system was useful tools due to their sensitivity and fast response. Laccase immobilization and catechol detection steps were carried out quickly and enabled easy modification of electrode surface with using glutaraldehyde in a short period of time.

4. Conclusion

In this work, it was developed a novel sensor surface modified with 6-amino-1-hexanethiol on gold electrode which could detection of catechol. It was formed self-assembled monolayer on gold electrode via 6-amino-1-hexanethiol and then the laccase was immobilized with glutaraldehyde. All the experiments were carried out first time using automated biosensor device, during microfluidic flow. Cyclic voltammetry and amperometry activity assays showed that the detection of catechol with laccase modified gold surface. This could lead to further studies for developing of high performance biosensors for the specific detection of catechol and other phenolic compounds based on lacccase immobilized surfaces.

Acknowledgement

The author would like to thanks the Gebze Technical University (GTU) for the provided financial support (Grant no: BAP 2018-A105-23). We gratefully acknowledge to Dr. Yıldız Uludag and Dr. Aylin Ersoy from BILGEM-TUBITAK for their contribution to the fabrication of the biochip and the sensing platform.

Ethics

There are no ethical issues after the publication of this manuscript.

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