Electrochemical Characterization of Cholesterol Biosensor Formed by Polymer Film Based on o-Phenylenediamine and Benzoquinone

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Abstract

Aim: The detection of cholesterol concentration in the blood is an important parameter in the diagnosis and follow-up of many diseases, including cardiovascular and hepatobiliary diseases. Nowadays, various methods have been used for the routine analysis of cholesterol, including spectrophotometric, high-performance liquid chromatography (HPLC), and amperometric methods. Since biosensors have advantages such as high sensitivity, fast response, low cost, small size, continuous online detection, and reproducible results, it is aimed to develop a biosensor method for cholesterol determination.

Methods: Polymer film immobilization was performed on the gold electrode surface electrochemically in an acetonitrile-water medium containing o-phenylenediamine and benzoquinone. Cholesterol oxidase (ChOx, E.C. 1.1.3.6) enzyme was immobilized on the polymer film-immobilized electrode surface by the self-forming monolayer method. Optimization and characterization studies were carried out for the determination of cholesterol with the prepared electrodes.

Results: The determination of cholesterol was performed via monitoring the oxidation current of enzymatically produced H_2O_2 at 0.70 V Ag/AgCl. Optimum biosensor conditions were determined for phosphate buffer at 0.1M, pH 7.0, and 30°C for the Au/oPD-BQ/ChOx enzyme electrode. The linear working range is 9.8×10^{-6}-1.1×10^{-2} mM, and the cholesterol detection limit is 9.8×10^{-6} mM. The analysis of total cholesterol in solutions was performed using the proposed Au/oPD-BQ/ChOx electrode.

Conclusion: A convenient, simple, reproducible, and stable method was developed for the determination of cholesterol by immobilizing cholesterol oxidase on the prepared electrode surface through a polymer film. The sensor we designed can be expanded to improve the detection of many analytes in the clinic.

Keywords: Benzoquinone, cholesterol, biosensor, o-phenylenediamine, polymer film.

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O-Fenilendiamin ve Benzokinon Bazlı Polimer Filmle Oluşturulan Kolesterol Biyosensörünün Elektrokimyasal Karakterizasyonu

Öz

Amaç: Kandaki kolesterol konsantrasyonunun tespiti, kardiyovasküler, hepatobiliyer hastalıklar dahil olmak üzere birçok hastalığın tanı ve izleminde önemli bir parametredir. Günümüzde rutin olarak kolesterol analizi için spektrofotometrik, HPLC ve amperometrik yöntemler gibi çeşitli yöntemler kullanılmaktadır. Biyosensörlerin yüksek hassasiyet, hızlı yanıt, düşük maliyet, küçük boyut, sürekli çevrimiçi algılama ve tekrarlanabilir sonuçlar gibi avantajları olduğu için kolesterol tayinine yönelik bir biyosensör yöntemi geliştirilmesi amaçlanmıştır.

Yöntem: Altın elektrot yüzeyine elektrokimyasal olarak o-fenilendiamin ve benzokinon içeren asetonitril-su ortamından polimer film immobilizasyonu yapıldı. Polimer film immobilize elektrot yüzeyine kendiliğinden oluşan tek tabaka yöntemiyle kolesterol oksidaz (ChOx,E.C 1.1.3.6) enzimi immobilize edildi. Hazırlanan elektrotlarla kolesterol tayini için optimizasyon ve karakterizasyon çalışmaları yapıldı.

Bulgular: Kolesterol tayini, bienzimatik olarak 0.70 V Ag/AgCl'de enzimatik olarak üretilen H_2O_2'nin oksidasyon akımının izlenmesi yoluyla gerçekleştirilmiştir. Au/oPD-BQ/ChOx enzim elektrotu için fosfat tamponu, 0.1M, pH 7.0 ve 30°C, optimum biyosensör koşulları olarak belirlendi. Doğrusal çalışma aralığı 9.8×10^{-6}-1.1×10^{-2} mM'dir. Kolesterol tespit limiti 9.8×10^{-6} mM'dir.

Sonuç: Hazırlanan elektrot yüzeyine polimer film aracılığıyla kolesterol oksidaz immobilize edilmesiyle kolesterol tespiti için uygun, basit, tekrarlanabilir ve stabil bir yöntem geliştirilmiştir. Tasarladığımız sensör klinikte birçok analitin tespitinde geliştirilmek üzere yaygınlaştırılabilir.

Anahtar Sözcükler: Benzokinon, biyosensör, kolesterol, o-fenilendiamin, polimer film.

Introduction

The assessment of metabolites such as lactate, cholesterol, glucose, and urea in whole blood has great importance for clinical diagnostics. Cholesterol can be synthesized in every cell in both animals and humans. Cholesterol and its derivatives have important biochemical roles such as the synthesis of bile acids, signal transduction, and sex hormones. It is abundant in nervous tissues, the brain, skin, and liver. Therefore, its detection is important for the diagnosis of many diseases such as hypertension, atherosclerosis, and coronary heart disease[1-3].

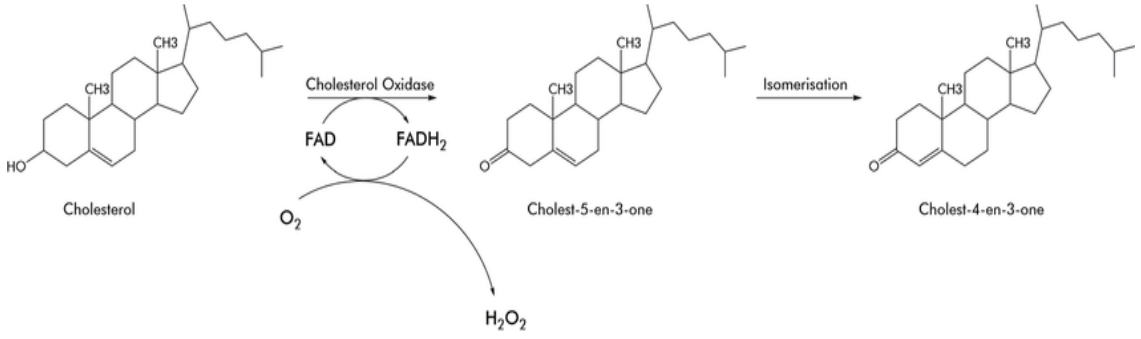
In cholesterol measurement, gas chromatography (GC), high-performance liquid chromatography (HPLC), colorimetry, and spectrophotometry are often used[4]. Among these methods, they have some detectable problems, such as a lack of selectivity, the requirement of pre-treatment of the samples, the necessity of a large amount of serum, and the long response time[5]. Another important application for cholesterol measurement is the electrochemical approach. This method offers high performance detection in analyte measurement as well as simplicity[6]. Amperometric biosensors are the most commonly used electrochemical transducers due to their low cost[7]. Contrarily, enzymatic methods are more advanced than chemical methods

in serum samples for the detection of cholesterol due to their excellent specificity and non-interfering effect[8].

A bioactive layer in a biosensor for biological identification can be built by immobilizing specific biochemical recognition material, such as antibodies and enzymes, on a special matrix, as an example of a self-assembled monolayer (SAM), sol-gel deposited thin films, hydrogel, lipid bilayer membranes, conducting polymers, and many composite matrix[9]. Self-assembled monolayer (SAM) modified electrode was recently exhibited for surface protection and used in the construction of chemical biosensors. It has aroused much interest because of its simple preparation method and ability to provide high stability electrode[10]. To improve the selectivity of the biosensor, in recent years, non-conductive polymers such as o-phenylenediamine (oPD) are intensively used as a matrix environment[11]. Some types of these polymers are thought to be an advantage not to pass, especially for large molecules as the surface electrode. The prepared film layer was used both as a selectively permeable membrane and as a matrix for enzyme immobilization. The determination of relevant substrates without the need for an additional film layer is considered to be easy[12]. Several studies have shown that the PoPD films have an influence on the relieving effect of electroactive destructive species, preventing poisoning of electrodes, and creating a matrix for the immobilization of enzymes[13].

The most important and widely used enzyme on the basis of cholesterol measurement is cholesterol oxidase (ChOx)[14]. ChOx is a FAD (flavin-adenine-dinucleotide)-containing flavoenzyme[15]. FAD catalyzes the dehydrogenation of C(3)-OH in the cholestan system[16]. The cholesterol concentration is conventionally measured using an amperometric method by oxidation at the working electrode. The reaction in a cholesterol biosensor that has an amporometric technique used to determine the cholesterol concentration is as follows:

Scheme 1. Pathway of cholesterol oxidase enzyme reaction[17]



Cholesterol oxidase (EC 1.1.3.6, 3β-hydroxysterol oxidase) are FAD dependent (favin adenine dinucleotide) enzyme, which oxidizes cholesterol to form cholest-4-en-3-one (cholestenone) and H_2O_2 (Scheme 1). In this study, we aimed to produce an amperometric cholesterol biosensor by

cholesterol oxidase-mediated reaction using the mediating agent benzoquinone (BQ) and poly o-phenylenediamine (PoPD) matrix.

Materials and Methods

Chemicals

All solutions were purchased from Randox Laborotaries Ltd., UK. All biochemical solutions were freshly prepared before the experiment. %94 acetonitryle and %6 distilled water containing 0.1M oPD and 0.04M ferrozine were used to prepare the Au/PoPD matrix. For determining cholesterol, 0.11 U/ml cholesterol oxidase was used. Stock cholesterol ($5x10^{-5}$ M) was diluted x100 fold.

Apparatus

To perform the electrochemical measurements, a gold working electrode (Basi) was combined with the reference Ag/AgCl electrode and the auxiliary Au/Pd (98:2%) electrode. PalmSens potentiostat (Holland) to measure the electrochemical potential.

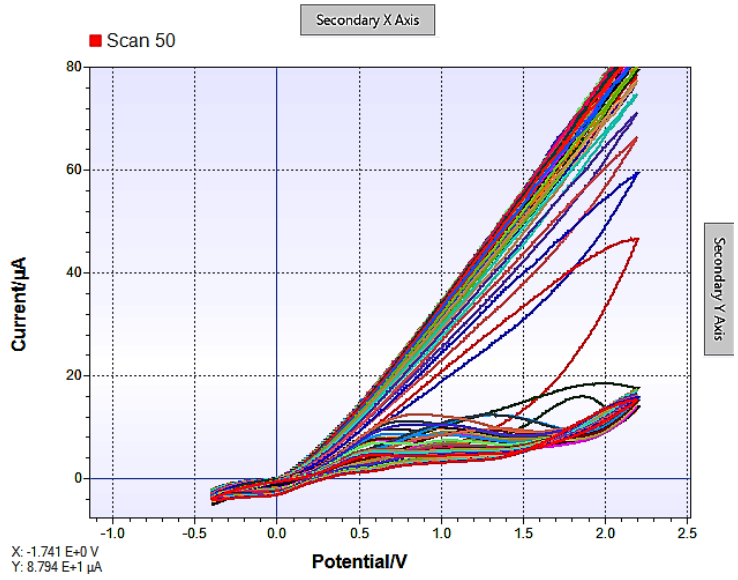
Preparation of the Biosensor

Prior to the establishment of the bioactive layer, the gold working electrode surface was polished with alumina slurries on microfiber to obtain a clean surface. The clean gold working electrode was rinsed two times with distile water to remove the alumina slurries. The electrode was then sonicated in pure ethanol in order to remove undispersed absorbable particules and rinsed two times with distile water[18]. For the next step, the immobilization of cholesterol oxidase, the gold electrode was first covered with oPD and ferrozine by electropolymerization (Fig. 1). The current was applied at a potential of (-0.4)-(2.2) V with 0.025 V/s scanning rate, having 50 cycles through 2 hours. We combined the Au electrode with the reference Ag/AgCl electrode. In preparation of the enzyme electrode for the determination of cholesterol, cholesterol oxidase was immobilized on a pre-made Au/PoPD electrode. For this purpose, 250 µl cholesterol was laid in an eppendorf tube. Lastly, for enzyme trapping, immerse in the crosslinking agent glutaraldehyde (2.5%) applied on the gold working electrodes last layer for 1 h at 4 °C.

Detection of Interference in a Biosensor

To confirm the selectivity of the designed cholesterol oxidase-based biosensor, a possible interference effect was investigated. $1x10^{-2}$ mM glucose solution and $1x10^{-2}$ mM ascorbic acid solutions were added separately to the phosphate buffered reaction medium containing $9.8x10^{-6}$ mM cholesterol and studied. It was observed that there was no change in the current difference. This result proves that the selectivity of the biosensor is acceptable.

Figure 1. The cyclic voltammograms observed during the o-PD film formation (0,1 M o-PD and 0.04 M Ferrozine containing %94 Acetonitrile-%6 distilled water media)

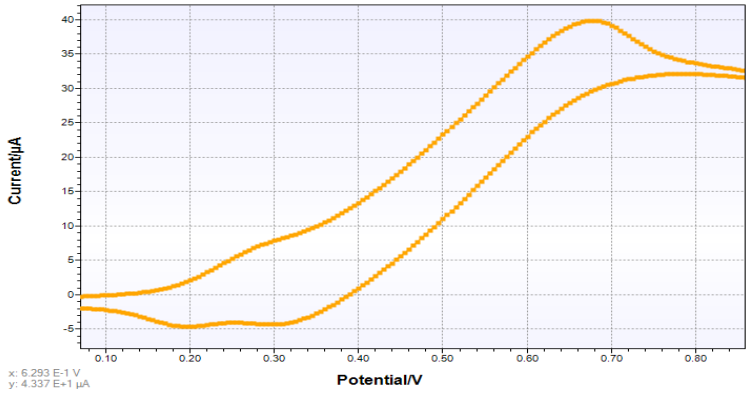


Results

Immobilization

Potential range of bare electrodes Cyclic voltammograms were performed at a voltage between −0.4 V and 0.6 V. When the bioactive layer formed on the electrode, the anodic peak current was found at 0.19 V. The cyclic voltammograms were obtained with the electrode shown in Fig. 2. Cyclic voltammograms showed that immobilization of cholesterol and the cholesterol oxidase enzyme brought about prominent oxidation and reduction peaks. In this study, as a result of the immobilization method with electropolymerization, a stable polymer layer was formed on the surface, preventing the separation of bioreceptors from the surface. In this way, high stability was achieved in the long-term use of cholesterol oxidase.

Figure 2. Biosensors produce cyclic voltammograms at different potential ranges in a phosphate buffer solution containing 0.1M $K_4Fe(CN)_6$ at a 0.1 Vs^{-1} scan rate.



Electrochemical Characterization of the Cholesterol Biosensor

Cholesterol biosensors were used to investigate the electrochemical characteristics of biosensors with a cyclic voltammogram. The cyclic voltammograms were utilized for the determination of the potential point of 0.7 V in the study.

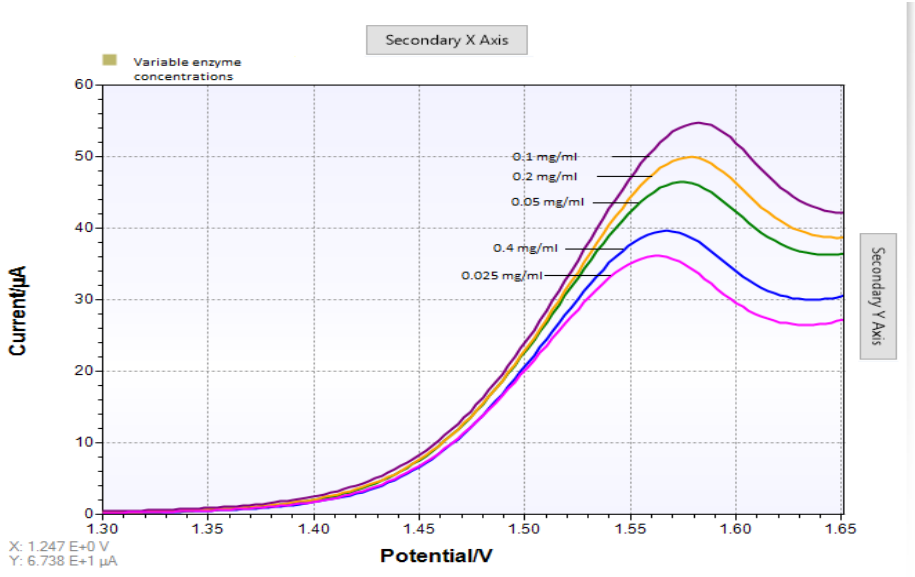
Optimization of the Bioactive Layer of the Biosensor

Biologically active layers are vital to the functionality of the biosensor because they act as an interface for the recognition and measurement of the analyte. These layers allow for the recognition and selectivity of specific analytes. In this aim for the biosensor's bioactive layer constituent optimization, enzyme concentration effects, mediator complex concentration effects, and polymerization time effects on the biosensor response were investigated.

Effect of the Enzyme Concentration

Enzyme concentration is the key factor for the sensitivity of the biosensor because the biosensor's potentiometric differentiation responses depend on the activity of the enzyme. To determine the effect of the activity of the enzyme on the biosensor response, the variable enzyme concentrations (0.025, 0.05, 0.1, 0.2, and 0.4 mg/mL) were used. According to the experiment results, when the biosensor bioactive layer contained concentrations of 0.1 mg/mL cholesterol oxidase, a smooth calibration curve was obtained (Fig. 3).

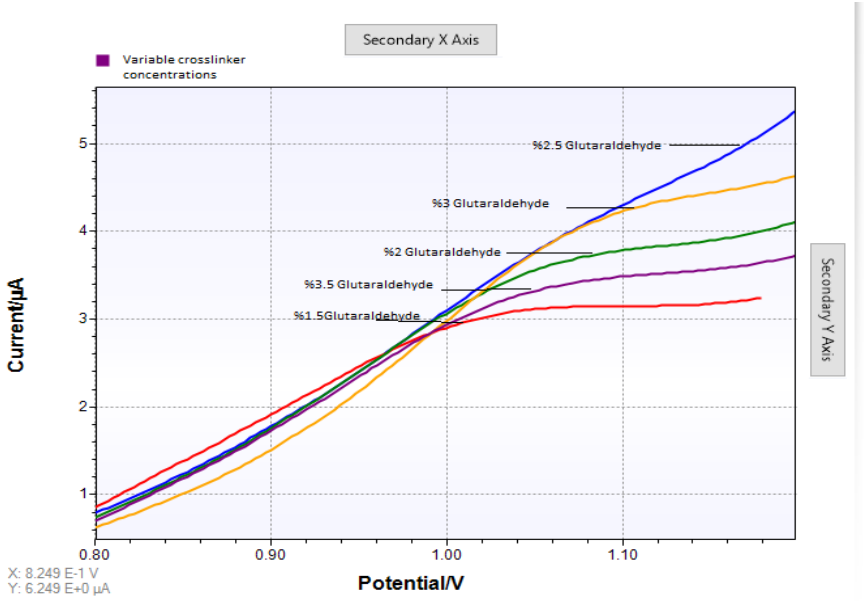
Figure 3. Variable enzyme concentrations observed in the response of the biosensor



The Cross-Linkers Concentration Effects

For the determination of the cross-linker concentration effect on biosensor response, 1.5%, 2.0%, 2.5%, 3.0%, and 3.5% glutaraldehyde concentrations were used. According to experiment results, the optimum value was obtained at 2.5% (Fig. 4).

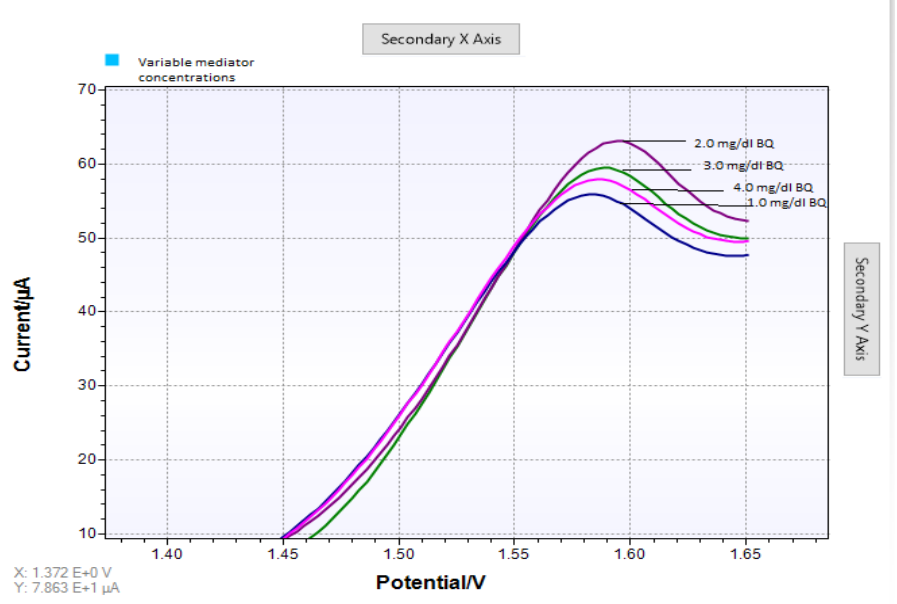
Figure 4. Different cross-linker (glutaraldehyde) concentrations were observed in the response of the biosensor



Mediator Concentration

Mediators are compounds that catalyze redox reactions in biological systems, i.e., participate in oxidation or reduction reactions through electron transfer. In the mediator concentration effect investigation about the biosensor response, 1.0 mg/dL, 2.0 mg/dL, 3.0 mg/dL, and 4.0 mg/dL benzoquinone were used in the preparation of the biosensor buffer. According to the experiment results, 2.0 mg/dL has the most effective and useful results (Fig. 5).

Figure 5. Different mediator concentrations (BQ) observed in the response of the biosensor

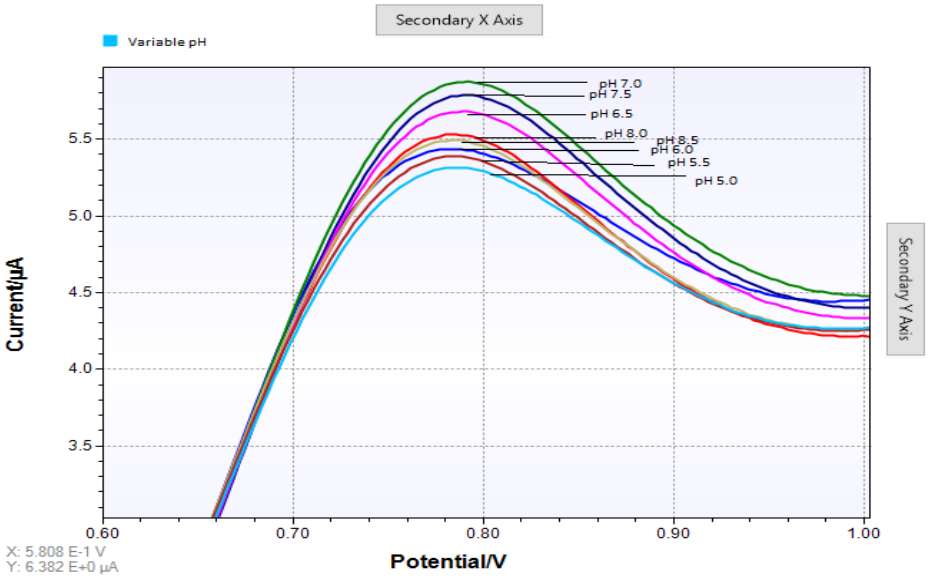


Biosensors Working Conditions Optimization

The pH Effect on the Biosensor Response

The enzymes need a suitable pH medium for the best responses to be obtained. For the detection of the pH on the biosensor response, we used different buffers at different pHs. The buffers are 50 mM concentrations of acetate (pH 5.0–5.5), phosphate (pH 6.0–6.5–7.0–7.5), and Tris-HCl (pH 8.0–8.5). Due to the 100% activity rate, the optimum pH value was 7.0. Above and below pH 7.0 cause decreases in the biosensor response (Fig. 6).

Figure 6. Different pH concentrations observed in the response of the biosensor



Temperature Effect

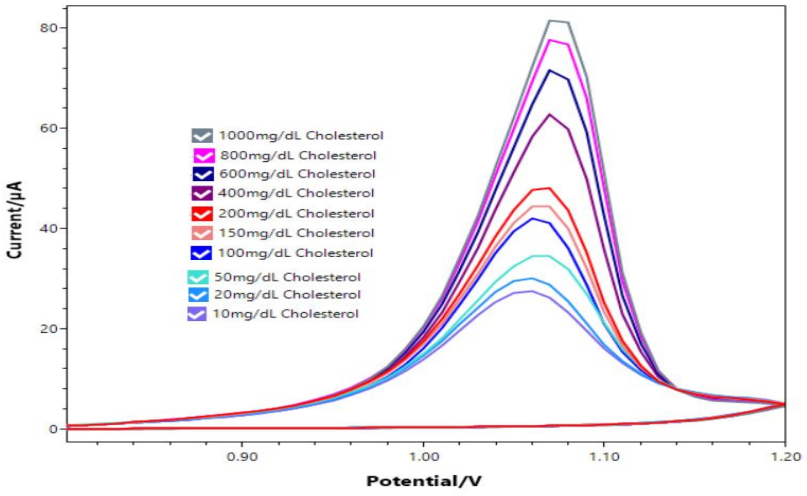
To determine the temperature effect on the biosensor response, variable temperatures (20–55 °C) were examined. The biosensors' working temperature was detected at 35 °C. The biosensor response directly increased with temperature until 35 °C, but further increases in temperature caused a decrease in the biosensor response.

Analytical Characteristics of the Biosensor

Biosensor Response Linear Detection Range

The "linear detection range" refers to the interval between the minimum and maximum measurement values that a biosensor can measure. A linear calibration curve was obtained at concentrations between 10 mg/dL and 1000 mg/dL cholesterol under the optimum experimental conditions. Detection and quantification limits were determined on smooth calibration curves for a 200 mg/dL cholesterol concentration (n = 10).

Figure 7. Biosensor response linear detection range



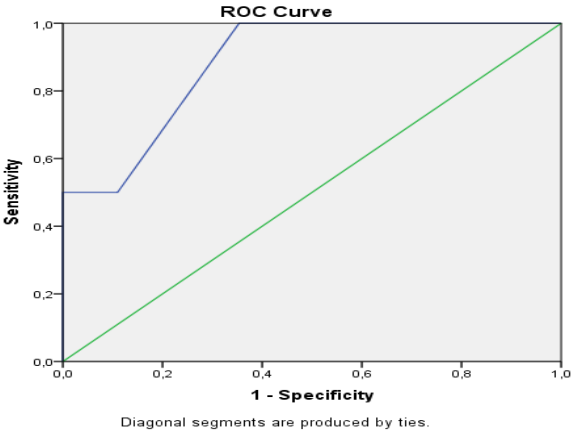
Repeatability and Reproducibility

Repeatability is a measure of the closeness of the measurement results of a method in a short time interval. The biosensor repeatability experiments were studied with the same cholesterol concentration (200 mg/dL). The standard deviation of the cholesterol sample with a concentration of 200 mg/dL obtained after 15 consecutive measurements is 3.6%, and the coefficient of variation is 9%. Reproducibility is a measure of the closeness of a method's measurement results on different days. According to the experiments, when the working electrode stores at +4°C, it can be used for 16 days or 340 voltammograms.

ROC Analyses

The ROC curve is used to determine the discriminatory power of the test, to determine the appropriate positivity threshold, to monitor the quality of laboratory results, to compare the diagnostic performance of two or more diagnostic or laboratory tests[19]. As a result of the analysis we made here, we determined that the area we obtained in the roc curve is 88.4%. If the area obtained in the ROC curve analysis exceeds 50%, it means that the test is successful.

Figure 8. Biosensor tests ROC curve



Discussion

Cholesterol determination aims to obtain information about a person's cardiovascular health by measuring cholesterol levels in the blood. Cholesterol is a type of fat essential to the body and plays a role in making cell membranes, hormones, and other important compounds. But high cholesterol levels can increase the risk of heart disease. Clinically, it is an important tool for risk assessment, taking appropriate precautions, establishing a treatment strategy, and monitoring cardiac diseases.

When the bioactive layer components in cholesterol biosensors are examined, it is seen that many agents such as indium-tin-oxide, polypyrrole, and gold nanoparticle coated formulations are used (Table 1). The o-phenylenediamine used in this study is a phenol-derived chemical compound. This compound, in the form of colorless crystals, was used in the immobilization of cholesterol oxidase and, consequently, in the determination and measurement of cholesterol. Different concentrations of the cholesterol oxidase enzyme were prepared, and the optimum concentration obtained for the sensor was 0.1 mg/mL. Crosslinkers are also important in that they can bind biological compounds used in biosensors to the active layer without changing their properties. It can also increase the reusability and lifetime of the biosensor by increasing the stability of the biological compounds in the active layer. In the case where the 2.5% glutaraldehyde concentration increased, two conditions caused the signal to decrease. The first of these is the saturation of the sensor, and the other is the increase in the thickness of the active layer. In both cases, the signal is low, as it will be very difficult to reach the analyte. The benzoquinone concentration above 2 mg/dL caused low signal formation as it saturated the rate of oxidation in the system and caused the oxidation of the analyte to decrease. Another reason for the signal drop is that it increases the adsorption of the biological matrix to the electrode surface. This, in turn, reduces the sensitivity of the electrode by binding to the electrode surface of the biological matrix. As reported in the literature, the appropriate pH range for cholesterol oxidase is between 6.5 and 7.5. As can be seen in Figure 6, a signal decrease is observed due to a loss of activity at pH values above 7.5. The

structure of cholesterol oxidase includes three disulfide bridges. These disulfide bridges are critical to the stability and activity of the enzyme. pH values higher than 7.5 can cause the breakdown of disulfide bridges and change the enzyme structure, resulting in a loss of activity. In acidic conditions, amino acids can become positively charged by gaining protons and affect the activity of the enzyme by changing the electrostatic interactions between the charged residues in the active site of the enzyme. In addition, a pH value below 6.5 for cholesterol oxidase inhibits the function of its cofactor.

Table 1. Comparison of cholesterol biosensors

Method/Enzyme/Mediator/ The study potential/ type of immobilization	**Working electrode and modification**	**Linear range**	**Reproducibility**	**Buffer/pH/ temparature**	**Reference**
Electrochemical (LSV)/ChOx/(-0,5)-(0,9) V, covalent binding optical/ ChOx/ o-dianisidine 500 nm	Covalent binding of cholesterol oxidase with glutaraldehyde to the polyaniline sheet prepared on indium-tin-oxide (ITO) coated glass plates	25-400 mg/dL	-/-	-/-/35 °C	Matharu et al.[20]
Spectrophotometry/ChOx /o-dianisidine/ 500 nm/ Covalent Binding	Cholesterol oxidase (ChOx), covalent immobilization of N-(2-aminoethyl)-3-aminopropyl-trimethoxysilane (AEAPTS) in monolayer (SAM) on indium-tin oxide (ITO) coated glass plates	50-500 mg/dl^{-1}	-/-	50 mM/ Phosphate buffer/ 7,0/ 50 °C	Arya et al.[21]
Amperometric/ChOx//To Ag/AgCl against/ 0,7 V/ Arrest	Immobilization of cholesterol oxidase in electropolymerized polypyrrole (PPy) film	1,35 $\mu mol/l^{-1}$/43,99 nA l $mmol^{-1}$	Pt/PPy-ChOx/oPPD/7,0 s/ BSS % 0,70 (n=10)	0.05 M/ Phosphate buffer /7,0/-	Rappolt et al.[22]
DPV(differential pulse) / nonenzymatic/ PVIM-(Co_5POM)-MNC composite	A flexible sensor developed by coating PVIM–Co5POM/ MNC on Whatman filter paper	1 fM–5 mM	-	0.1M phosphate buffer (pH 7.4) and 1mM $K_4Fe(CN)_6$	Thakur et al.[23]
ECL/ AuNPs/ L-cys-C60/ChOx	C60 was functionalized with L-cysteine (L-cys) to adsorb gold colloidal nanoparticles (AuNPs) to obtain a modified Lcys-C60 composite on the surface of glassy carbon electrodes. Cholesterol oxidase (ChOx) was then dripped onto the surface of the modified electrode.	1.7×10^{-9} - 0.30×10^{-3}	The relative standard deviation (RSD) was found to be 1% under continuousscans for 20 cycles	PBS 0.050 M, pH 7.4	Ou et al.[24]

Conclusions

Cholesterol biosensors are economical, less time-consuming, sensitive, and practical. The enzymatic biosensor studies have been known to be very sensitive, specific, simple, and useful methods, in which the immobilized forms of the enzymes are generally used. Since biosensor technology provides economical, practical, specific, and sensitive results for the determination of cholesterol, it has been improved very efficiently. The developed cholesterol biosensor may be a promising candidate for application in clinical biochemistry laboratories.

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