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Construction of An Amperometric Cholesterol Biosensor Based on DTP(aryl)aniline Conducting Polymer Bound Cholesterol Oxidase

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Abstract: In this study, an amperometric cholesterol biosensor was constructed based on cholesterol oxidase immobilized on a conducting 4-(4H-dithienol[3,2-b:2′,3′-d]pyrrole-4)aniline polymer, (DTP(aryl)aniline). Glassy carbon electrodes were covered with P(DTP(aryl)aniline) which is used for the wiring of enzyme to the electrode surface by using electro-polymerization. The electron transfer was successfully made by the bio-catalytic activity and possession of the unique morphology of the polymer allowed efficient immobilization of the cholesterol oxidase enzyme. Analytical performances; linear range, detection limit, limit of quantification and the Michaelis-Menten constant (K_m) of biosensor electrodes were obtained 2.0 μM–23.7 μM, 0.27 μM, 0.82 μM, 17,81 μM respectively. Biosensor optimization parameters: optimum pH, optimum temperature, stability test and response time were evaluated. The real sample and recovery studies were also performed in order to show applicability of the biosensing electrodes.

Keywords: Cholesterol biosensor · Cholesterol Oxidase · DTP Conducting polymer.

1 Introduction

Biosensors are technology-based devices with a wide range of use such as; health, food and the environmental monitoring [1]. According to the recent reports, researches on biosensors studies are mainly based on early detection of diseases, freshness/safety of foods and detection of environmental pollutants [2]. Biosensors used in these studies are classified as enzymatic biosensors, immunosensors, genosensors and bacterial biosensors according to biomaterials [3]. In analytical detection, enzyme-based biosensors are the most reported biosensors in the literature [4] compared to other systems and are successfully used in real-time [4b,5] sensitive and selective detection [6].

Determination of the cholesterol level, one of the key biochemical metabolites, is of great importance for human health [4a]. It is an important metabolite that causes disorders such as high cholesterol level, human cardiovascular disease, hypertension, arteriosclerosis, myocardial infarction [7]. Different analytical methods for the determination of cholesterol have been reported such as spectrophotometric [8], HPLC [9], colorimetric [10] and molecular imprinting [11]. However, these methods have some disadvantages, such as lack of selectivity, high cost, long analysis time and pre-treatment requirement [11]. In addition to that, these methods are not suitable to perform on-site measurement or long-term screening [4c,12]. However, electrochemical cholesterol biosensors, mostly based on amperometric detection and oxidoreductase enzymes, have become a serious alternative to these problems [4a,13]. Cholesterol oxidase (ChOx) is a Flavin-enzyme (contain FAD, prosthetic group) that catalyzes the biochemical degradation of cholesterol in the presence of oxygen yielding cholest-4-en-3-one and hydrogen peroxide;

$$\text{Cholesterol} + O_2 + \text{ChOx} \rightleftharpoons \text{Cholest-4-en-3-one} + H_2O_2$$

After catalytic reaction of the enzyme, the hydrogen peroxide which is the product of the enzymatic reaction is determined at a specific potential [14]. A sensitive cholesterol biosensor based on a ChOx attached to the conducting polymer was reported Dervisevic et al., 2016 [7c]. Moonla et al., was developed a polymeric and nanomaterial based composite matrix for hosting the ChOx and utilized for the amperometric cholesterol detection

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[15]. Another sensitive and selective amperometric biosensor was reported by Kaur et al., (2016) [4c] for point of care diagnosis of cholesterol. In addition, important review articles on this topic have been also published in the literature [4a].

Conducting polymers have been reported in many studies [7b, 13a, 14, 16] with features such as easy synthesis, wide application range, good environmental stability and long-lasting electrical conductivity. In the field of continuously growing conductive polymers, the synthesis and application of conductive polymers with π-conjugated systems has become a huge interest in recent years [17]. These polymers exhibit very good optical and electronic properties, [18] low cost synthesis, their ability to be used in flexible large devices, and their ability to combine with inorganic semiconductors [19]. According to recent reports, conductive polymers with dithienopyrrole (DTP) have attracted researchers' interest in conductive polymers with a π-conjugated system [3a, 20]. Dithienopyrrole (DTP) is a term used to describe contiguous ring systems consisting of two thiophene rings fused to the pyrrole ring. Compounds with π-conjugated systems are reported in many investigations due to their properties in optics, electronics, applications in photovoltaic devices, light emitting diodes, biosensors, field effect transistors [21].

Herein, we report an electrochemical cholesterol biosensor using cholesterol oxidase based on DTP(aryl)aniline as a polymeric mediator and immobilization matrix onto the glassy carbon electrode. The biosensors were utilized for analysis of cholesterol from serum samples. The performance parameters; detection limits, linear ranges, temperature, pH, stability, reusability and of the biosensors were optimized and results were compared with previously reported cholesterol biosensors.

2 Experimental Parts

2.1 Materials

Cholesterol and Cholesterol oxidase (E.C. 1.1.3.6) were purchased from Sigma-Aldrich. Dichloromethane, toluene, n-hexane, ethyl acetate, hydrogene peroxide (30 %), methanol, glutaraldehyde (25 %), $NaH_2PO_4 \cdot H_2O$, $Na_2HPO_4 \cdot 2\,H_2O$ were analytical grade and purchased from Merck (Germany). 1,3 diaminopropane, silica gel HF (254), DMSO, Triton X-100, acetone, potassium carbonate, Acetonitrile (ACN), Dichloromethane (DCM), tetrabutylammonium hexafluorophosphate ($TBAPF_6$), and 3,3-dibromo-2,2-dithiophene were purchased from Sigma-Aldrich (USA). All other chemicals used in this study were analytical grade and all of them were used without purification. All solutions were prepared using ultrapure water from the Millipore-Milli-Q system.

2.2 Apparatus

All Electrochemical measurements were performed using a PalmSens EmStat (Palm Instruments, The Netherlands) analyzer and a BASC C3 cell stand. The working electrode is a glassy carbon electrode (BAS MF 2012), the auxiliary electrode is a platinum wire (BASI MW-1032) and the reference electrode is Ag/AgCl (3 M KCl) reference electrode (BASF MF-2052)–2060). In addition, Ag/Ag+electrode (BASI MW-1085) was used as the reference electrode in the electro-polymerization experiments. Experiments were carried out at room temperature with a conventional three-electrode system. All electrochemical measurements were performed in a reaction cell, including 10 mL of reaction solution.

2.3 Preparation of Materials

2.3.1 Synthesis and Characterization of 4-(4H-dithiol [3,2-b:2′,3′-d] Pyrrol-4-yl) Aniline

The chemical synthesis of DTP(aryl)aniline was followed by our previous study (Udum et al., 2014) [18]. After synthesis of polymer, ^{1}H-NMR, ^{13}C-NMR and IR (cm^{-1}) characterizations were performed as; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.75 (d, J = 8.2 Hz, =CH, 2 H), 7.08 (d, J = 5.2 Hz, =CH, 2 H), 7.14 (d, J = 5.2 Hz, =CH, 2 H), 7.80 (d, J = 8.6 Hz, =CH, 2 H), 3.84–3.78 (bs, NH_2, 2 H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 148.47, 145.78, 124.32, 120.23, 115.78, 114.75, 112.06. IR (cm^{-1}): 3737.3, 3622.2, 3366.7, 3057.3, 3024.9, 2924.1, 2856.7, 2337.6, 1707.9, 1600.0, 1571.2, 1495.6, 1448.8, 1398.4, 1304.9.

2.3.2 Preparation of Enzyme Electrode

Prior to any modifications, mechanical cleaning of GCEs was done by using alumina slurry (0,05 μm) and sonicated for 2 minutes in Milli-Q water then ethanol. Between each step electrodes rinsed with Milli-Q water. After that, electrochemical cleaning was employed for all electrodes in 0.08 M H_2SO_4 applying the potential between −1.5 V–1.5 V at a scan rate of 100 mV/s. The different polymer coating was formed on the working electrode using 5-10-15-20-30-50 voltammetric cycles in 0.1 M $TBAPF_6$ containing ACN/DCN medium (data not shown). The highest current response was obtained from the electrode having 15 cycles and it was selected as optimum. The ChOx biosensing electrode was prepared according to following procedure as illustrated in Figure 1. The ChOx solution (1.0 mg/mL in 10 mM sodium phosphate buffer, pH 7.5) was prepared spread over the surface of the electrode and then a glutaraldehyde solution (5.0 μL, 1.0 % in phosphate buffer, pH 7.5) prepared at specific ratios was added to polymer and enzyme coated GCE. Subsequently, the electrodes were allowed to incubate at ambient conditions for 6 h at 30 °C. Then the electrodes were rinsed with Milli-Q water.

2.4 Instrumentations

Initially, the unmodified GCE (without any biological components) and further modifications were electro-

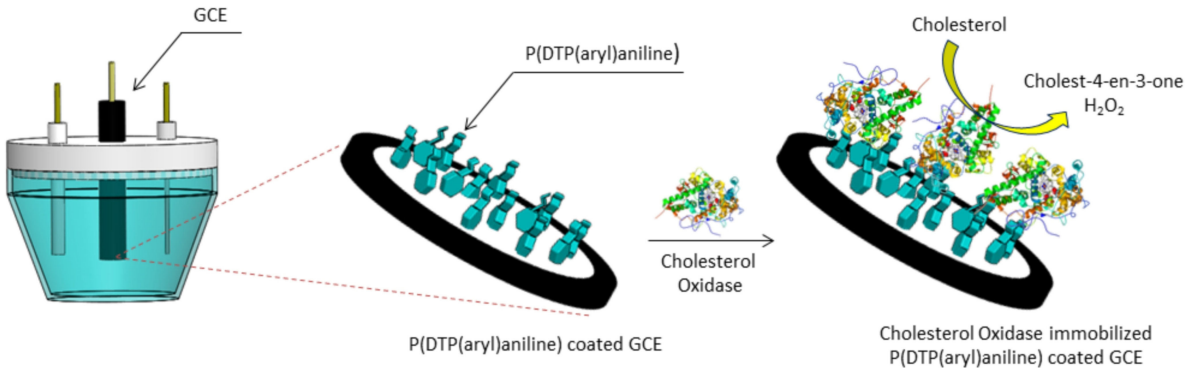

Fig. 1. Preparation of GCE/P(DTP(aryl)aniline)/ChOx electrodes.

chemically analyzed by the cyclic voltammetry (CV) method. The amperometric measurements were performed under constant stirring and room temperature conditions. After each measurement, electrodes were washed with distilled water and incubated in a 10 mL PBS (10 mM pH 7.5) until a new steady state was reached. Cholesterol detection mechanism of the proposed biosensor was dependent on the current values obtained from the biosensor electrodes were recorded as microamperes (μA) followed by the degradation of hydrogen peroxide at the applied potential (−0.7 V) due to the biological activity of ChOx. For the determination of optimal biosensor conditions, 1 μM cholesterol standard solution was used as substrate in all electrochemical measurements. Standard deviations and coefficient of variation of the obtained biosensors and average values were calculated by applying the 5 subsequent measurements for each different sample.

The cholesterol standard solution (0.05 M) was prepared by dissolving 0.387 g cholesterol in 20 mL of ethanol. The prepared stock solution was stored in the refrigerator at +4 °C for 10 days. To prepare the cholesterol solution, 1 ml of stock solution was taken before the experiment, diluted with ethanol in a 25 ml flask, and 1 % Triton X-100 was added.

3 Results and Discussion

3.1 Determination of Experimental Variables

3.1.1 Electro-polymerization of 4-(4H-ditiyol[3,2-b:2′,3′-d] Pirol-4-il) Aniline

The electro-polymerization of 4- (4H-dithiol [3,2-b:2′,3′-d] pyrrol-4-yl) aniline, 4 mg of monomer and 0.387 g of TBAPF6 was added to ACN/DCM (1:2) solution. 15 cycles of cyclic voltammetry were applied in a potential range between 0.0 V to 1.5 V at a scan rate of 0.1 V/s. A reversible redox couples were well observed during the monomer oxidation and the reduction around 1.1 V and 0.5 V in the voltammogram (Figure 2A). The aniline present in the monomeric structure has an electron donating tendency and increases the stability of the aromatic structure by giving electrons to the pyrrole group in the ring structure. The potential energy of the chemical structure is reduced by electron delocalization and becomes more stable.

In order to optimize the polymer thickness of DTP(aryl)aniline, amperometric measurements were performed using 1 μM cholesterol standard solution with enzyme electrodes having different polymer coating cycles. The maximum amperometric response of 0.016 μA was obtained from the biosensing electrode with DTP(aryl)aniline 15 cycles. A further increase on the electropolymerization cycles up to 50 cycles, caused a decrease in the current response was observed. At the cycles less than 15, a gradual increase in the amperometric response was clearly observed up to 15 cycles. However the insufficient polymer thickness for electron transfer not allowed to sufficient number of electrons reached to the electrode. This shows that the polymer film has optimum thickness and conductivity for electron transfer (Figure 2B).

After construction of the GCE/P(DTP(aryl)aniline)/ChOx biosensing electrodes, CV characterization was performed in 5 mM $[Fe(CN)6]^{3+/4-}$ between −0.6 V–0.8 V (shown in Figure 2C). The reversible CV of bare GCE was obtained (represented with black line) and anodic (Ipa) and cathodic (Ipc) peak potentials were recorded at a scan rate of 100 mV/s. The decrease in the Epc and Epa were observed after modification with polymer GCE/P(DTP(aryl)aniline) (red line). A further decrease was observed on the peaks after immobilization of a GCE/P(DTP(aryl)aniline)/ChOx (shown in green line) indicate the formation of the layers. The negative effects on the peak potentials, indicating that the blocking the electrical contact with the electrode attributed the biological materials are cross-linked and well oriented in the electrode architecture.

Figure 2D shows an electrochemical impedance spectroscopy (EIS) graph of bare GCE electrode, GCE modified with P(DTP(Aryl)Aniline) and GCE modified P(DTP(Aryl)Aniline)/ChOx. The diameter of the semicircles obtained from EIS graphs are equal to the charge transfer resistance (R_{ct}) controlling the electron transitions. The charge transfer resistance is obtained by fitting

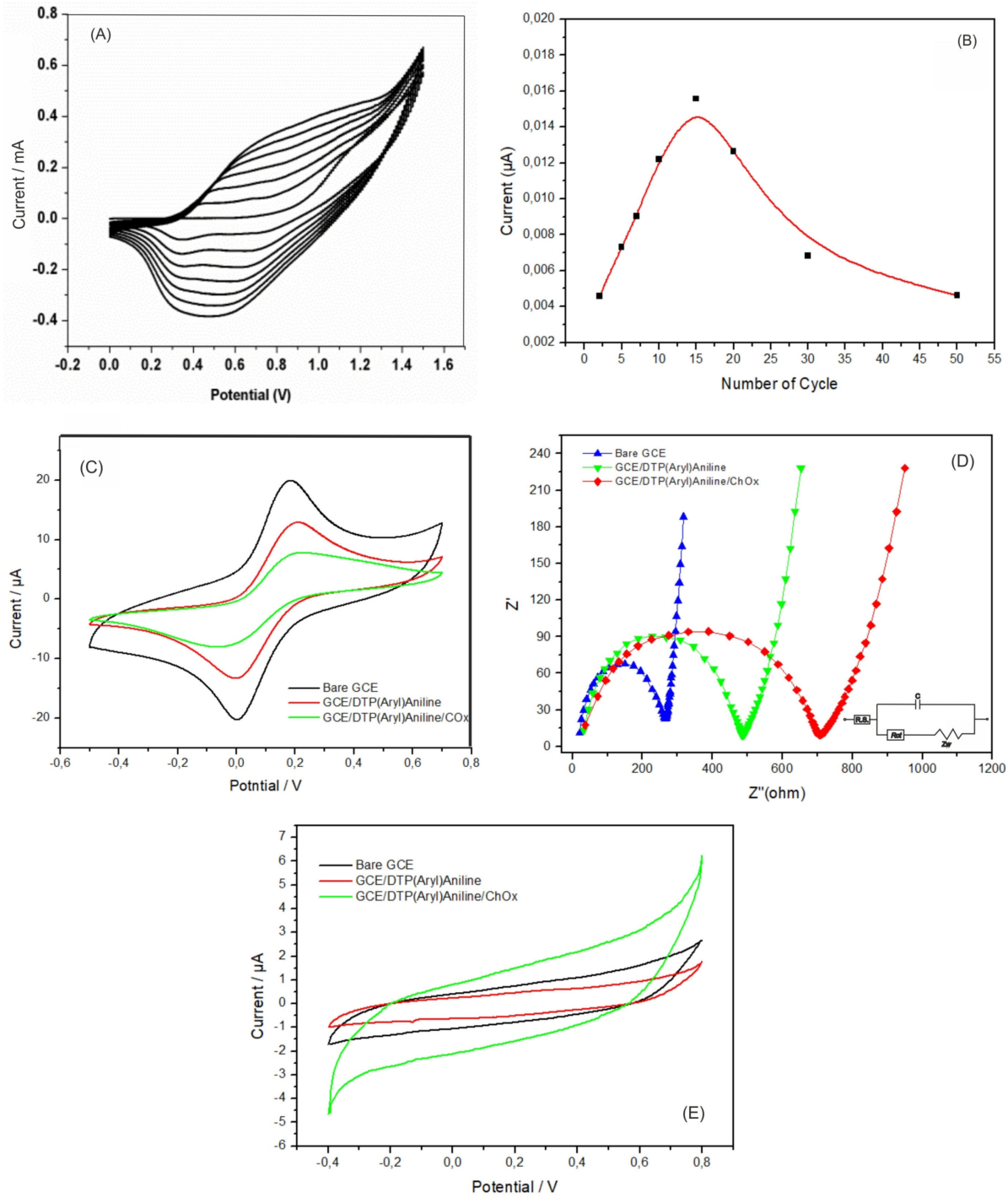

Fig. 2. A) Cyclic voltammetry (CV) of electro-polymerization of DTP(aryl)aniline in TBAPF6/DCN/ACN at a scan rate of 100 mV/s vs Ag/Ag^{+} under nitrogen. B) Amperoteric response of the enzyme electrode coated with DTP(aryl)aniline polymer having different electoropolymerization cycles (3, 5, 7, 10, 15, 20, 30 and 50). C) Cyclic voltammetry of Bare GCE, DTP(aryl)aniline coated GCE and ChOx enzyme immobilized electrode in in 5 mM $[Fe(CN)6]^{3+/4-}$ at a scan rate of 100 mV/s. D) EIS graph of bare GCE and stepwise attachment of components DTP(aryl)aniline and DTP(aryl)aniline/ChOx the inset shows the circuit diagram of the system. E) The CV comparison of catalytic characteristics for bare GCE, GCE/P(DTP(aryl)aniline, and GCE/P(DTP(aryl)aniline/ChOx in the 1 µM cholesterol standard solution at a scan rate of 100 mV/s.

the EIS graphs on a suitable equivalent circuit for each electrode (represented in the inset of Figure 2E). In the circuit diagram, *R.S.* is describing the resistance of the electrolyte and *C* is the capacitance of the bioactive layer. R_{ct} describes the charge transfer resistance at the electrode surface, and Z_w represents the Warburg

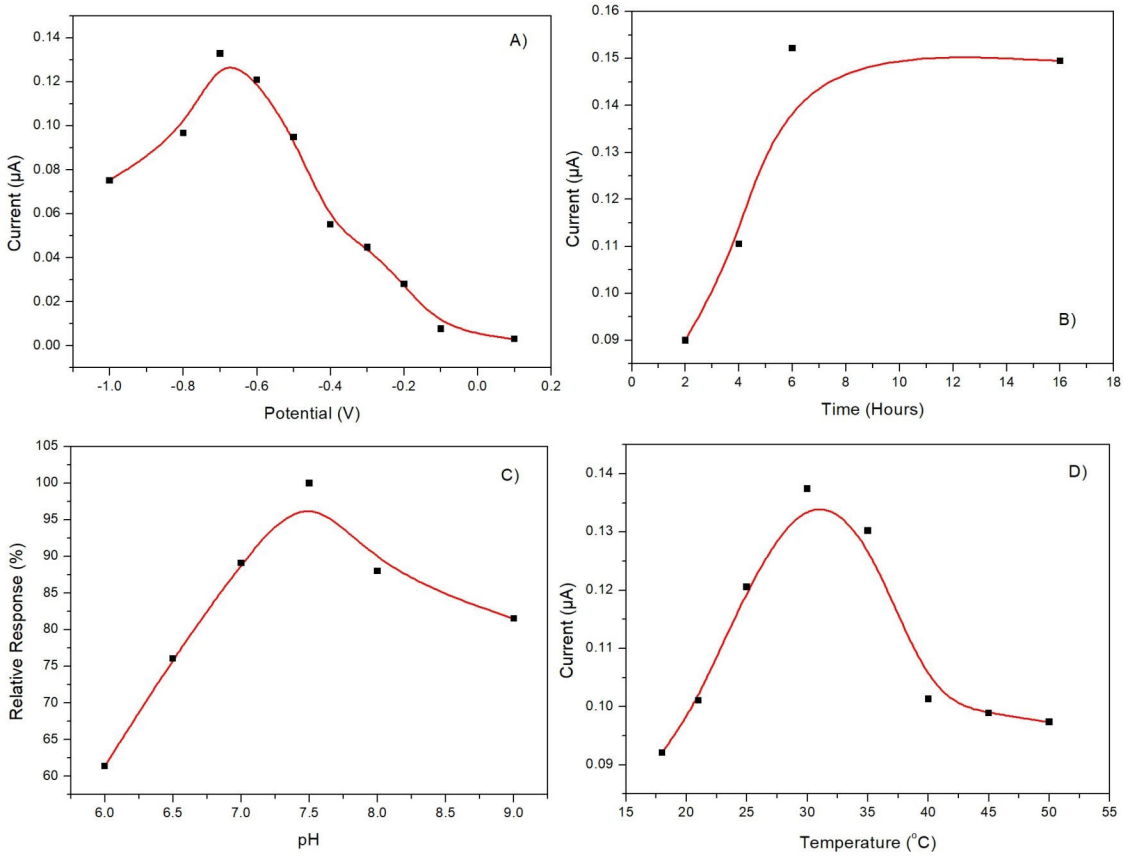

Fig. 3. A) The applied potential of GCE/ P(DTP(aryl)aniline)/ChOx electrode performed in pH 7.5 PBS. B) The optimum immobilization time for the ChOx enzyme obtained in pH 7.5 PBS. C) The optimum pH and D) the optimum temperature graphs of the GCE/p(DTP-Aryl-aniline)/ChOx obtained at an applied potential of −0.7 V in pH 7.5 PBS.

impedance. The lowest R_{ct} value (117 ohms) was obtained from the bare GCE (unmodified) electrode represented with a blue line in Figure 2D). After modification with the polymer (DTP (Aryl) Aniline, 15 cycles), it was observed that the Rct value increased to 223 ohms, indicating that the electrode surface was covered with the polymer. A further increase on the R_{ct} value was observed (350 ohms) after immobilization of ChOx proves that the enzyme was successfully attached on the electrode surface.

Electrochemical layer by layer characterizations of bare GCE, GCE/P(DTP(aryl)aniline and GCE/P(DTP(aryl)aniline/ChOx coated electrodes were carried out by CV, as shown in Figure 2E. CVs were performed with 1 μM cholesterol standard solution as a model reversible redox couple at potential range of −0.4 to 0.8 V versus Ag/AgCl at a scan rate 100 mV/s. As shown in Figure 2E black line, the response of no any redox couple observed in bare GCE. After electropolyerization with P(DTP(aryl)aniline (15 cycle) almost same CV characteristics were observed leading no any catalytic activity take place at the electrode surface. For further characterization of ChOx immobilized GCE/P(DTP(aryl)aniline electrode was performed and enhanced oxidation and reduction peaks were observed attributed to catalytic activity between ChOx and cholesterol. This result confirmed that the enzyme exists on the surface of the electrode-polymer film.

In order to determine the optimum applied potential, enzyme electrodes were tested with different applied potentials in a range of −1.0 V to 0.1 V at a constant scan rate of 100 mV/s. The maximum current response for the GCE/P(DTP(aryl)aniline)/ChOx electrode was obtained at -0.7 V. The increase in the applied potential resulted a sharp decrease in current response which clearly seen in the Figure 3A. Based on the results −0.7 V was chosen as the optimum potential and applied to the all amperometric measurements.

The immobilization time plays an important role in the electrode construction to get maximum performance from an enzyme electrode. Factors such as pH, temperature and concentration may affect the duration of immobilization and may lead to the formation of electrodes in different structures. For this reason, the optimal immobilization time for ChOx was studied at different time intervals from 2 hours to 16 hours under the same immobilization conditions; pH, concentration and temperature (Figure 3B). Same amount of ChOx (1.0 mg/1 mL) was added to the GCE/P(DTP(aryl)aniline) electrodes and incubated at different time periods. The current response was increased up to 6 h incubation time and then reached a

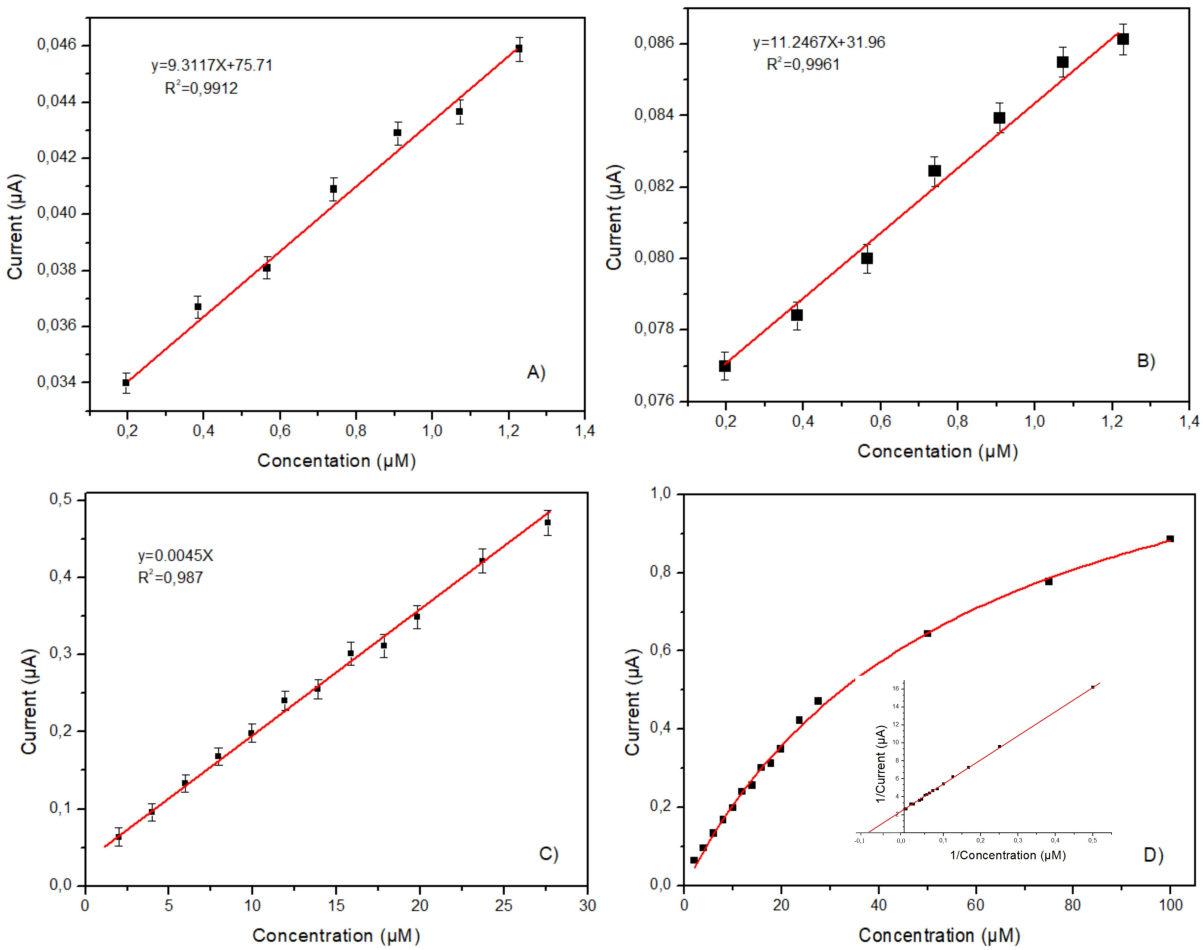

Fig. 4. A) The amperometric response of unmodified GCE upon additions of 0.05 mM H_2O_2 in 10 mL pH 7.5 PBS at −0.7 V B) The amperometric response of P(DTP(aryl)aniline) modified GCE to the additions of 0.05 mM H_2O_2 in 10 mL pH 7.5 PBS at −0.7 V. C) The calibration curve of GCE/ P(DTP(aryl)aniline)/ChOx biosensor obtained in 10 mL pH 7.5 PBS at -0.7 V applied potential. D) Lineweiver-burk plot of cholesterol biosensor.

plateau. As seen in the graph, further increase in the time the current response not effected and 6 h was selected as anoptimum enzyme immobilization time.

The effect of pH on the biosensor GCE/P(DTP(aryl)aniline)/ChOx response was investigated by testing the current response to successful injections of 10 µL cholesterol into the reaction cell containing PBS in the range of pH 5.0–9.0. The current response of the biosensor was gradually increased with increasing in the pH up to 7.5 shown in Figure 3C). Since the maximum current response has the reached the current response tended to decrease at pH values higher than 7.5. The pH 7.5 was chosen as the optimum pH for all ChOx based experiments were conducted with this pH value and at room temperature.

The optimum temperature studies of the GCE/ P(DTP(aryl)aniline)/ChOx biosensor were performed in PBS solution having a different temperatures ranging from 18.0 to 50.0 °C at an applied potential of −0.7 V. Figure 3D) shows that the maximum current response of 0.14 µA of the cholesterol biosensor was obtained at a temperature of 30 °C. Thus, this temperature was selected as optimal for all electrochemical measurements.

3.2 Amperometric Responses of Enzyme Electrode

In amperometric cholesterol biosensor studies hydrogen peroxide plays an important role on the basis of oxidation on the working electrode. Thus, the sensitivity of the non-modified GCE and P(DTP(aryl)aniline) modified GCE to the addition of H_2O_2 was studied. The same protocol was followed for both electrodes where measurements performed upon successful addition of 10 µL H_2O_2 (0.05 mM) after electrodes reaching the steady-state. When graphs are examined in Figure 4A) and B), it is calculated that the unmodified electrode gave a sensitivity of 9,3117 µA/µM, while the modified electrode gave a sensitivity of 11,246 µA/µM. The P(DTP(aryl)aniline) modified electrode appears to have a higher sensitivity than the bare electrode.

The analytical characteristics of the biosensors; linear range and linear equations, were studied under optimum

Table 1. Comparison of the analytical performance of the Cholesterol biosensor.

Electrode	RT (s)	LR (μM)	DL (μM)	Km (μM)	Sensitivity (μA/μM)	Ref.
GCE/p DTP(aryl)aniline/ChOx	2	2.0–27.6	0.174	17.81	0.0045	This work
CNT-Pt/ChOx/sol-gel	<20	4.0–100.0	1.4	–	1.4	[7a]
PTBA/FAD/apo-ChOx	2	0.8–4.8	0.22	–	0.21	[7c]
PABA/FAD/apo-ChOx	2	0.8–5.6	0.32		0.22	[7c]
Poly(CBNP)/graphite/ChOx	–	2.5–27.5	0.40	37.3	1.49	[13a]
GCE/GR-CS/ChOx	–	5.0–1.0 mM	0.71	17.39	0.715	[23]
MWCNT/sol-gel/Chitosan/ChOx	13	4 μM–0.7 mM	1	410	1.55	[24]
Pt/Pt/PPy-ChOx + FcMC	8.7	0–3.0 mM	12.4	–	88.51	[16a]
G/PVP/PANI-ChOx	–	50–10 mM	1	–	34.77	[25]
GCE/PTH/ChOx/HRP	–	25–125	6.3	–	0.18	[6b]
ChOx/Nano-CdS/ITO	20	2-500 mg dL^{-1}	1.87	220	–	[26]
ChOx/AuNPs/TGHs/TGPHs	7	0.05–590	0.017	210	–	[27]
ChOx/PtPd-CS-GS/GCE	7	2.2–520	0.75	110	–	[28]

GCE, glassy carbon electrode; RT, response time; LR, Linear Range; DL, Detection Limit

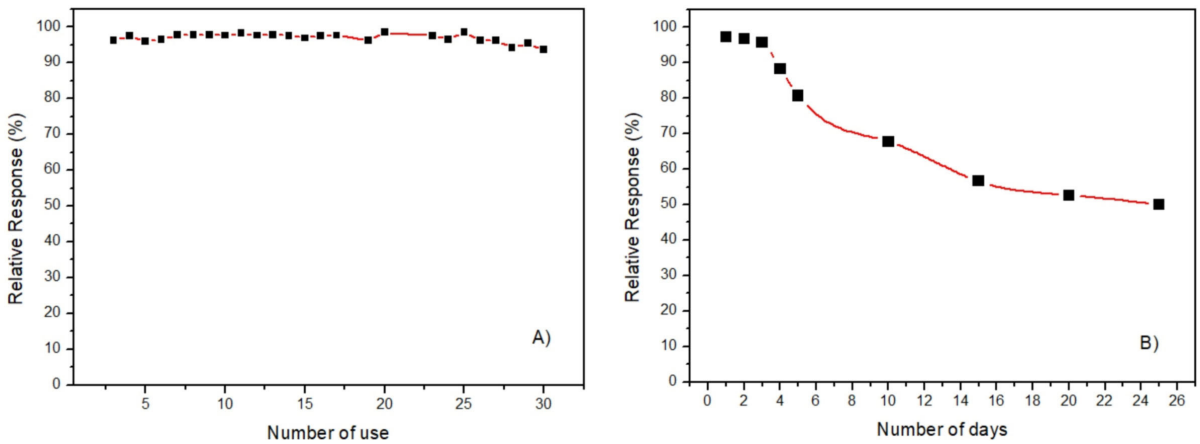

Fig. 5. A) The Reusability of the biosensing electrodes upon addition of cholesterol in 10 mM PBS pH 7.5, at an applied potential of −0.7 V. B) The amperometric responses of biosensing electrodes obtained for long time period of 10 days in 10 mM PBS pH 7.5, at an applied potential of −0.7 V.

conditions. The calibration curves of the biosensors were plotted for the current response versus cholesterol concentration.

The analytical performances of cholesterol biosensor were tested under optimized conditions by using same cholesterol standards in the range of 0.10 μM–30.0 μM. Different biosensor electrodes were evaluated upon addition of different cholesterol levels and they showed similar characteristics in the calibration curves. The GCE/P(DTP(aryl)aniline)/ChOx biosensor showed a linear range between 2 μM–27.6 μM and the regression equation was calculated y (μA) = 0.0045[Cholesterol μM] with a regression coefficient of (R) 0.987 (Figure 4C). The biosensor reached steady-state response in ~2 s. The detection limit (LOD) of the prepared GCE/P(DTP(aryl)aniline)/ChOx biosensor was calculated (S/N = 3) as 0.174 μM and the limit of quantification (LOQ) were found as 0.82 μM.

The amperometric responses of the enzyme in the biosensor to different cholesterol concentrations, the rate of substrate degradation and the amount of product formed are very important in the analytical performance of the biosensor. According to Michaelis-Menten kinetics, the rate of the reaction is increase by increasing the concentration of the substrate while keeping the amount of enzyme in the medium constant and eventually reaches a plateau [22]. To calculate the Michaelis-Menten constant (Km) (Figure 4D), a Line weaver Burk plot was drawn (inset of Figure 4D). The equation was obtained and the Km value was calculated as 17.81 μM. The enzyme activity of the cholesterol oxidase biosensor, which has a very low Km value, is attributed to high compatibility, fast electron transfer and well construction of the enzyme electrode. The corresponding Km value of the proposed biosensor is much lower compared to the previously reported studies in the lirterature (Table 1).

In order to determine the reusability, repeated measurements were performed to observe the changes occurring in the sensor response and the results calculated as a percentage (Figure 5A). Operational stability of biosensor was tested for 1 μM cholesterol (n = 30) using same biosensing electrode in 10 mM PBS pH 7.5 at an applied

Table 2. Real sample and recovery of Cholesterol biosensor.

Samples	Serum [Cholesterol] (μM)	Added [Cholesterol] (μM)	Detected [Cholesterol] (μM)	Recovery (%)	Error (%)
Sample1	2.5	0.5	2.51 ± 0.011	100.4	2.2
Sample2	2.5	1.0	3.55 ± 0.042	101.6	5.0
Sample3	2.5	10.0	12.6 ± 0.045	105.0	6.0

Recovery(%) = [C (found)/C (added) + C (normal cholesterol)]*100

potential of −0.7 V. A coefficient of variation was calculated as 4.91 %.

The longtime stability of the fabricated cholesterol biosensor was evaluated by regular amperometric measurements over a 25 day period. Same biosensing electrode was used for the measurements of stability tests were performed in 10 mM PBS pH 7.5 at an applied potential of -0.7 V. The electrode was stored in 10 mM PBS pH 7.5 at a temperature of 4 °C when not in use. Figure 5B shows that the catalytic current response was obtained from the GCE/ P(DTP(aryl)aniline)/ChOx biosensor which retained 95 % of initial activity within the first 3 days. After this point, the current response tends to decrease slowly and the biosensor lost its 45 % of initial activity after 25 days. The maximum performance of the biosensing electrode during the first three days was obtained by preserving the maximum interaction between the electrode, the polymer and the enzyme. The decrease from this point can be explained by the loss of wiring ability in the polymer interface with the electrode surface. In addition, the time dependent decrease of enzyme activity has also been a significant factor in this result.

Analytical comparison with previously reported cholesterol biosensors shows the performance characteristic of the proposed GCE/P(DTP(aryl)aniline)/ChOx biosensor clearly (Table 1). It is understood that the GCE/ P(DTP(aryl)aniline)/ChOx biosensor is highly sensitive for the cholesterol compared to the other biosensors.

3.3 Interference, Real Sample and Recovery Measurements

To assess the specificity of the proposed biosensors, different possible interfering substances were employed. For GCE/P(DTP(aryl)aniline)/ChOx biosensor, the interference effects of glucose, uric acid, and lactic acid were performed using amperometric measurements in 10 mM pH 7.5 PBS at an applied potential of −0.7 V. Results were recorded as current responses and converted in percentage. The maximum percent current responses obtained from cholesterol additions (as % 100) and current responses of other interfering substances (glucose, uric acid, and lactic acid) were calculated as 2 %, 1 %, and 2 %, respectively, compared to cholesterol.

Real sample studies were performed to show the applicability of the designed biosensor. The human serum including different amount of cholesterol concentrations were tested using the GCE/P(DTP(aryl)aniline)/ChOx biosensors. Before the measurements, sample pretreatments (dilutions) were applied to fit linear ranges of the cholesterol biosensors. All electrochemical measurements repeated five times and average data were represented in the Table 2. The biosensor showed very good performance for the cholesterol sensing with less than 5 % error. Among the different assays the relative standard deviations (RSD) were calculated for each biosensor. The average relative standard deviations for ChOx modified electrodes were calculated as 1.534 %. The recovery of the cholesterol biosensor was evaluated by adding 0.5, 1.0 and 10.0 μM cholesterol to the serum samples already containing 2.5 μM of cholesterol. Calculations for the recovery results were done by using the equation given under the Table 2. The biosensor was shown excellent recovery for low amount of cholesterol samples. For the higher amount of cholesterol the recovery was decreased but still in the acceptable range.

4 Conclusions

In this work, amperometric cholesterol biosensor was constructed using aniline functionalized conducting polymer immobilizing the cholesterol oxidase. DTP(aryl)aniline was covered to the GCE surface by using electrochemical polymerization and ChOx was covalently immobilized onto electrodes and stabilized via the cross linker (glutaraldehyde). The optimization of biosensors construction parameters; immobilization time, optimum pH, optimum temperature and polymer thickness were evaluated. Biosensor was shown successful performance for reusability and longtime stability indicating the well orientation of the enzyme and biocompatible character of the polymer. A wide linear range between 2.0–23.7 μM was obtained with a very low detection limit 0.17 μM. The real sample tests show that cholesterol biosensor can practically be used for cholesterol sensing successfully.

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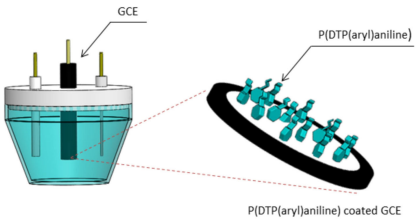

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