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Optimized fabrication of newly cholesterol biosensor based on nanocellulose

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ABSTRACT

A novel and sensitive electrochemical cholesterol biosensor was developed based on immobilization cholesterol oxidase (ChOx) on the polyaniline/crystalline nanocellulose/ionic liquid modified Screen-Printed Electrode (PANi/CNC/IL/SPE). A thin layer of ionic liquid (IL) was spin coated on the modified electrode to enhance the electron transferring. Crystalline nanocellulose was prepared from Semantan bamboo (*Gigantochloa scortechinii*) via acid hydrolysis and it was used to synthesize a nanocomposite of PANi/CNC via in situ oxidative polymerization process. FESEM and TEM images showed high porosity of the nanostructure with no phase separation, revealing the homogenous polymerization of the monomer on the surface of the crystalline cellulose. Research surface methodology (RSM) was carried out to optimize the parameters and conditions leading to maximize the performance and sensitivity of biosensors. The PANi/CNC/IL/GLU/ChOx-modified electrode showed a high sensitivity value of 35.19 μA mM/cm^{-2} at optimized conditions. The proposed biosensor exhibited a dynamic linear range of 1 μM to 12 mM ($R^2 = 0.99083$) with the low Limit of Detection of 0.48 μM for cholesterol determination. An acceptable reproducibility (RSDs ≤3.76%) and repeatability (RSDs ≤3.31%) with the minimal interference from the coexisting electroactive compounds such as ascorbic acid, uric acid and glucose was observed for proposed biosensor.

1. Introduction

The cholesterol screening is an important blood test that has been found to rely heavily on spectroscopies methods [1], while these techniques usually suffer of complexities, time-consumption, lack of specificity and selectivity, and exorbitant cost for high technology equipment purchases [2]. As a result, the development of an efficient, sensitive and low-cost cholesterol sensor for clinical analysis/diagnosis and also for measuring cholesterol in food is great importance. Of late, electrochemical biosensor has been more preferential for its rapid and real-time analysis owing to wide range of selection, and cost effectiveness [3–5].

Furthermore, previous studies have shown that the enzymatic biosensor displays a notable selectivity and specification features compared to the chemical method [4–7]. Electrochemical techniques operate by detecting oxygen consumption or production rate of hydrogen peroxide through the enzymatic reaction [8]. In the presence of oxygen, enzyme catalyzes the oxidation of cholesterol to cholest-4-en-3-on and H_2O_2 as a by-product which H_2O_2 would be electrochemically quantified at a favorable potential without any interference effect [8]. However usually redox reaction of H_2O_2 need a high-applied potential that may cause interference problem [9]. Various researches on fabricating a nanostructure material have been done to accelerate redox reaction of H_2O_2 on the surface of electrode without any over potential applied [9,10]. In addition, the immobilization of enzymes on the electrode surface is a critical step to preserve the enzymatic activity. Consequently, to have a stable and long-life biosensor, a suitable material and media necessary to hold the biomolecule and maintain the natural condition of enzymes without any denaturation [11,12].

Providing an appropriate platform for the enzyme immobilization is still a big challenge, however, nanoscale dimensions, high porosity and surface area are very important characteristics for the immobilization of enzymes [10]. Crystalline nanocellulose (CNC) is one of the most interesting macromolecules and nature-based nanomaterials that have been viewed as appropriate materials for enzyme loading. Crystalline nanocellulose (CNC) is a derivative of cellulose that is formed through an acid hydrolysis process creating crystals in a nanostructure [13,14]. The physical properties of CNC such as its dimensions, morphologies, and degree of crystallinity vary with various factors, such as cellulose source, reaction time and temperature, experimental technique,

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and types of acid used for hydrolysis [14,15]. Several studies have successfully produced CNC from various sources such as oil palm trunk [16], raw garlic residue [17], grain straw [18], sugarcane [19], coconut fiber [20]. In current research, we prepared CNC from Semantan bamboo (*Gigantochloa scortechinii*) collected from Pahang, Malaysia via acid hydrolysis. Hydroxyl groups that cover the surface of cellulose allow cellulose to react well with a variety of materials including conducting polymers [21,22]. Composite formation of cellulose and conducting polymers (CPs) is an important way to modify the crystals and fibers cellulose properties for biosensing application.

Conducting polymers (CPs) have shown good compatibility with biomolecules and been viewed as suitable metrics for enzyme loading [8,23]. Among the various CPs, PANi, and PPy are being widely explored for biosensing applications due to their excellent electrochemical properties. They are demonstrated to function as good-performance electron-transfer mediators in redox and enzymatic reactions, and to be suitable metrics for biomolecules immobilization [24–26]. However, despite the great properties, PANi has major processing limitations and inferior dispersibility and solubility in most solvents. In addition, the electron transfer rates and conductivity of PANi in solutions with a high value of pH is low which has limited its performance in biosensors [27,28].

Nanocomposite preparation by addition of nanoparticles, inorganic materials, graphene or biopolymers to PANi can overcome these limitations. Ruecha et al. [4] developed a PANi based enzymatic cholesterol sensor with sensitivity of 34.77 μA mM^{-1} cm^{-2} using graphene and polyvinylpyrrolidone (PVP). In another research, Prussian blue nanoparticles (PBNPs) was used to modify screen-printed electrodes (SPEs) by inkjet printing method and a sensitivity of 0.2 μA mM^{-1} cm^{-2} was observed for cholesterol sensor [29]. In current research, crystalline nano-cellulose (CNC) has been introduced into the polymer by polymerization of aniline on the surface of CNC. It has been shown that crystalline nano-cellulose displays improved yield compared to the cellulose fibers which is the result of nanoscale dimensions, high specific strength and modulus, low density, and high surface area of cellulose nanostructure. These advantages of CNC will improve the electron transfer properties and enhancing the mediator performance in biosensor [30]. It has been shown the modification of substrate by nanomaterial will improve the immobilization process and recognition capacity toward the target material owing to the nano scale and larger surface area of sensing area [31]. Different nano-materials using nanoparticles, inorganic materials, or biopolymers have been synthesized and exploited in a sensing platform [30].

In our research, to enhance ionic conductivity and electron transferring of modified electrode, ionic liquid (IL) was introduced to the nanocomposite. Ionic liquid is a highly efficient and green medium for electrochemical applications owing to its unique properties, such as wide potential window, low toxicity, high ionic conductivity, and outstanding thermal and chemical stability [32]. Not only that, IL has reportedly shown good compatibility with biomaterials such as proteins and enzymes make it promising material to be used in biosensor application [33,34]. The current study was aimed to develop a sensitive enzymatic cholesterol biosensor using the PANi/CNC nanocomposite followed by coating a thin layer of IL. The synergistic effect of CNC and conducting polymers on the electrochemical properties along with high porosity of nanocomposite has made this material a good candidate for hosting enzymes and biomolecules. Additionally, the sensitivity of the biosensor has been enhanced through the introduction of IL to the substrate. To the best of our knowledge, this is the first report on the preparation of CNC from Semantan bamboo for biosensor applications.

2. Experimental details

2.1. Materials and characterization

Aniline (Ani), ammonium persulfate (APS), hydrochloric acid (HCl), Triton-X, sodium hydroxide (NaOH), glutaraldehyde (GLU), uric acid (UA), ascorbic acid (AA) were purchased from R&M Chemical. Phosphate buffer solution (PBS) was prepared from potassium dihydrogen phosphate (KH_2PO_4) and di-potassium hydrogen phosphate (K_2HPO_4) that were purchased from Systerm. The pH was adjusted using H_3PO_4 and NaOH solutions. Potassium chloride (KCl) was provided from Merck. Potassium hexacyanoferrate (III) ($K_2Fe(CN)_6$) was from AnalaR. Cholesterol oxidase (ChOx) from *Streptomyces* sp. was provided from Sigma Aldrich. Highly purified cholesterol with molecular weight of 386.7 was from Calbiochem and 1-n-butyl-3-methylimidazolium chloride ([BMIM]Cl) was purchased from Alfa Aesar. All chemicals were of analytical reagent grade and used without further purification except aniline. Aniline monomer was doubly distilled before use and deionized water (DIW) was used throughout all experimental works. Screen printed carbon electrode (SPE) was purchased from DropSens.

The electrochemical measurements were conducted using potentiostat Autolab 204 from Metrohm connected to a PC and controlled by Nova software version 1.11. The FE-SEM analysis was done by using JEOL JSM-7600F Field Emission Scanning Electron Microscope. The surface of samples onto the aluminium stub was coated by a thin layer of gold to prevent charging. The TEM analysis was done using Hitachi H-7100 TEM.

2.2. Preparation of solutions

The ionic liquid solution was prepared by dissolving 0.017 g of 1-butyl-3-methylimidazolium chloride ([BMIM] Cl) in 100 μL DMF. The cholesterol stock solution of 10 mM was prepared by dissolving 0.040 g of highly purified cholesterol in 1 mL Triton X-100 at 60 °C under continuous stirring followed by addition of 9 mL PBS (pH 7.0). The v/v ratio of Triton X-100 to PBS was 1:9. The stock solution stored at 4 °C and was further diluted using PBS to make different concentrations of the analyte (cholesterol). The ChOx solution was freshly prepared by dissolving 1 mg of enzyme in 0.5 mL PBS solution (50 mM, pH 7.0). The solution was kept at 4 °C.

2.3. Crystalline nanocellulose preparation from Semantan bamboo (Gigantochloa Scortechinii) pulp

Cellulose nanocrystal was prepared according to the literature reported from our research group earlier [35]. Briefly, the bleached Semantan bamboo (*Gigantochloa scortechinii*) pulp from Pahang, Malaysia was hydrolysed by sulfuric acid followed by centrifugation at 6000 rpm for 30 min. The suspension was washed six times to remove the surplus sulfuric acid and dried using a vacuum freeze dryer.

2.4. Preparation of PANi/CNC nanocomposite

The solution of aniline in 1 M HCl was mixed with the solution of CNC in DIW and stirred at 0–5 °C for 20 min. The PANi/CNC nanocomposite was prepared with Ani/CNC molar ratio of (0.16/0.84). The solution of APS was added dropwise to the mixture in which the molar ratio of APS and aniline was always maintained at 1:1. The mixture was kept under stirring for 45 min and was allowed to rest at room temperature for 18 h. The green emeraldine polyaniline precipitate was filtered and washed with distilled water at least 3 times by centrifugation till the supernatant become clear at 4500 rpm. The precipitate was dried in the oven at 60 °C for 6 h.

2.5. Preparation of modified electrode for cholesterol biosensor

In order to prepare suspension, 1 mg powder of PANi/CNC nanocomposite in 2 mL DIW was bath sonicated for 30 min. The suspension was drop casted on the screen-printed electrode (SPE) and it was dried at room temperature. After drop casting of PANi/CNC nanocomposite suspension on the modified electrode, 4 μL IL solution was layered on the PANi/CNC-modified electrode by spin coating for 300 s at 3000 rpm.

Next, GLU solution was drop casted on the PANi/CNC/IL-modified electrode until it dried at room temperature. Lastly, the immobilization of enzyme was done by drop casting of ChOx solution on the PANi/CNC/IL/GLU-modified electrode and drying at room temperature. The amounts of PANi/CNC nanocomposite suspension, IL, GLU and ChOx was determined and optimized by RSM. Scheme 1 shows the schematic diagram of cholesterol biosensor fabrication by electrode modification.

2.6. Statistical analysis

In this study, response surface methodology (RSM) was used to find the best conditions for the cholesterol biosensor fabrication to obtain the highest response. Changing one factor at a time (OFAT) and keeping the other parameters constant is one of the conventional methods of optimization, which only provide information related to that specific parameter. However, this weakness can be encountered by using a statistical technique that is currently used in many studies and is based on design of experiments (DOE). RSM is an efficient method to evaluate the effects of multiple parameters, alone or in combination, on the response. Throughout the analytical measurement, Design Expert Software, version 6.07 (State Ease Inc., Statistic made Easy Minneapolis, MN, USA) was used to generate the model. All the measurements were done in PBS containing 0.1 mM cholesterol.

The parameters selected for the cholesterol biosensor fabrication were amount of PANi/CNC nanocomposite sample, glutaraldehyde as crosslinking agent, ionic liquid, enzyme loading, pH and buffer capacity. The response was the current peak obtained from differential pulse voltammetry (DPV). Since there are many parameters to consider, the screening design was done to find the significant parameters. Plackett–Burman (PB) design was employed to screen the parameters by building-up 12 experiments for six variables which were represented at two levels, maximum and minimum. The PB experimental design matrix for six variables is shown in Table 1. From the data obtained by the screening process significant parameters (enzyme, buffer capacity, glutaraldehyde and PANi/CNC nanocomposite) were determined and used for further optimization using response surface methodology based on central composite rotatable design (CCRD). The CCRD design comprised 27 experiments with 16 factorial points, 8 axial points and 3 centre points. The CCRD design and the response are shown in Table 2. The range and coded levels of the variables are presented in Table 3. High and low levels of each variable were coded as +2 and –2, respectively, and the centre values were coded as zero.

Table 1
Plackett–Burman experimental design matrix for six variables.

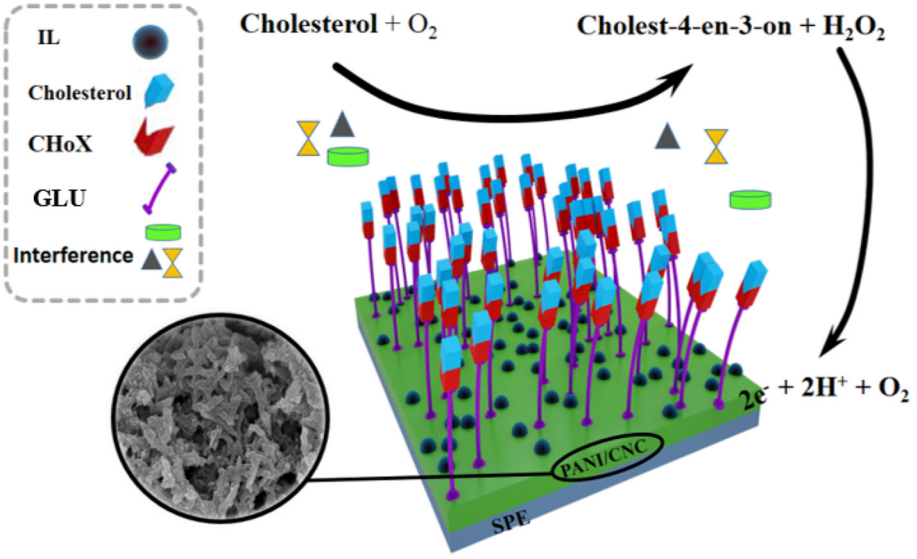
Exp. no.	Variables					
	Enzyme (μL)	pH	Buffer capacity (mM)	Ionic liquid (μL)	GLU (μL)	Sample (μL)
1	15	5.5	150	0	0	5
2	1	8.5	150	0	12	5
3	15	8.5	150	0	12	20
4	15	5.5	150	8	0	20
5	1	8.5	150	8	0	20
6	1	5.5	150	8	12	5
7	1	8.5	20	0	0	20
8	15	8.5	20	8	12	5
9	15	8.5	20	8	0	5
10	1	5.5	20	0	0	5
11	1	5.5	20	8	12	20
12	15	5.5	20	0	12	20

The data were fitted to various models (linear, two factorial, quadratic, and cubic). Subsequent analysis of variance (ANOVA) was performed to evaluate and identify a good fitted model of the experimental data. After predicting optimized variable sets by the software, the experiments were carried out under the recommended conditions and the resulting responses were compared to the predicted values by RSM. In this study, all the variables (enzyme, buffer capacity, glutaraldehyde, and sample) were set to "in range" and the current response was set to maximum.

3. Results and discussion

3.1. Characterization

The electrochemical properties of modified electrodes were studied using cyclic voltammetry (CV), and the voltammograms are shown in Fig. 1. Fig. 1 compares the cyclic voltammograms of the bare SPE, the modified electrode with pure PANi, PANi/CNC nanocomposite, PANi/CNC/IL nanocomposite and PANI/CNC/IL/GLU/ChOx in the presence of 0.1 mM cholesterol. The cyclic voltammogram of the bare SPE showed no anodic peak; meanwhile the adsorption of PANi on SPE electrode displayed two anodic peaks at the potentials of −0.16 V and 0.76 V. The first anodic peak at −0.16 V was corresponding to conversion of fully reduced leucoemeraldine base to the partially-oxidized emeraldine. The second peak at 0.76 V was associated to oxidation of emeraldine to the fully oxidized pernigraniline form [36]. Besides the van der Waals force, the dispersion interactions between the π-electrons of the aromatic



Scheme 1. Illustration of the preparation process of the modified electrode.

Table 2
Central composite rotatable design (CCRD) matrix and actual responses for fabrication of cholesterol biosensor.

Exp. no.	X_1; enzyme amount	X_2; buffer capacity	X_3; GLU amount	X_4; PANi/CNC nanocomposite	Current (μA)	
					Predicted	Actual
1	0	0	0	−2	0.82	0.77
2	−1	−1	−1	−1	0.89	1.16
3	+1	−1	−1	−1	0.79	0.81
4	−1	+1	−1	−1	0.80	0.74
5	+1	+1	−1	−1	0.84	0.90
6	−1	−1	+1	−1	1.22	1.17
7	+1	−1	+1	−1	0.77	0.79
8	−1	+1	+1	−1	0.89	0.87
9	+1	+1	+1	−1	1.24	1.21
10	0	0	1	0	0.57	0.57
11	0	−2	0	0	0.49	0.48
12	−2	0	0	0	0.75	0.72
13	0	0	0	0	0.94	0.88
14	0	0	0	0	0.89	0.72
15	0	0	0	0	0.59	0.64
16	+2	0	0	0	0.86	0.89
17	0	+2	0	0	0.92	0.95
18	0	0	+2	0	0.88	0.99
19	−1	−1	−1	+1	0.32	0.28
20	+1	−1	−1	+1	0.8	0.86
21	−1	+1	−1	+1	0.87	0.86
22	+1	+1	−1	+1	0.72	0.72
23	−1	−1	+1	+1	0.68	0.60
24	+1	−1	+1	+1	0.84	0.86
25	−1	+1	+1	+1	0.92	0.84
26	+1	+1	+1	+1	0.98	1.04
27	0	0	0	+2	0.71	0.70

ring and those of the graphene layers may play important roles on the physisorption of PAni and PAni/CNC on the surface of SPE.

The voltammogram of PANi/CNC nanocomposite modified electrode showed an increase of anodic peaks at the potentials of −0.09 V and 0.76 V as a result of the PANi protonation in the presence of CNC. In the presence of cellulose, the negative charges from carboxyl groups, sulfonic acid, and hydroxyl that cover the surface of cellulose will accumulate and form the charge barrier. To compensate the excess charges, the composite might undergo protonation and proton concentration in the composite increased as compared to the external solution. Therefore, PANi in the composite was protonated and conducted even in neutral solutions [37]. The drastic increase of anodic peak might also result from higher surface area and porosity of PANi/CNC nanocomposite that facilitated electron transfer and improved the electrochemical properties of the modified electrode [35]. Coating of a thin layer of IL on the PANi/CNC modified electrode showed an improved current peak, confirming that the presence of IL offers an excellent ionic conductivity that facilitated the electron transfer. Ion liquids have reportedly shown good compatibility with biomaterials such as proteins, enzymes, and living cells. It has been shown that the electrostatic interaction between ILs and the enzyme creates a hydrogen bond, producing a high kinetic barrier for biomaterial unfolding. Thus, the enzyme structure is well protected from disruption by its surroundings [33].

To study the effect of proposed biosensor toward cholesterol detection, the cholesterol oxidase (ChOx) was immobilized on the modified electrode supported by glutaraldehyde as the crosslink agent. The

Table 3
Variables and their levels employed on the central composite design.

Variables		Unit	Coded level of variables				
			−2	−1	0	+1	+2
X_1	Enzyme	μL	1.00	4.50	8.00	11.50	15.00
X_2	Buffer capacity	mM	100.00	112.50	125.00	137.50	150.00
X_3	Glutaraldehyde	μL	1.00	3.75	6.50	9.25	12.00
X_4	Sample amount	μL	5.00	8.75	12.50	16.25	20.00

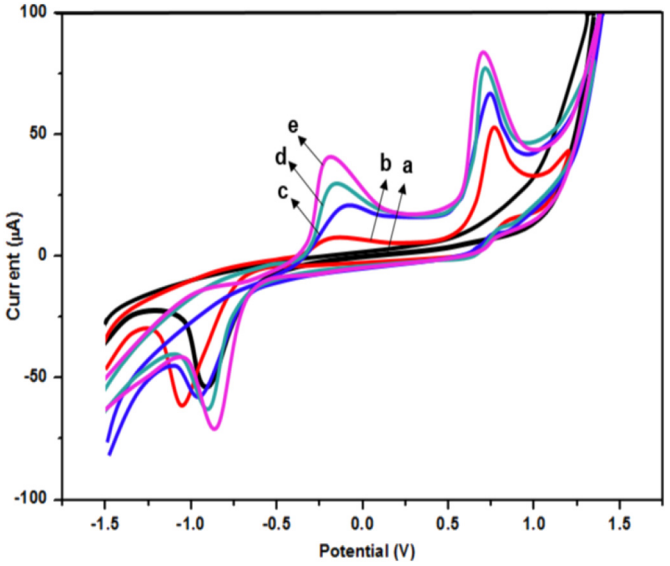


Fig. 1. Cyclic voltammograms of a) bare SPE, b) pure PANi, c) PANi/CNC, d) PANi/CNC/IL-modified electrodes in PBS with a pH of 7.0 and e) PANi/CNC/IL/GLU/ChOx-modified electrode in 1.0 M cholesterol solution. PANi/CNC was prepared from solution containing Ani/CNC with mass ratio of 0.84/0.16.

PANI/CNC/IL/GLU/ChOx modified electrode showed a drastic increase of redox peaks as a result of chemical reaction between the enzyme and cholesterol, accompanied by stoichiometric production of hydrogen peroxide.

FESEM and TEM characterizations were done to study the surface morphology of PANi, CNC and PANi/CNC nanocomposite and the images have been presented in Fig. 2. Fig. 2a shows rod like structure of CNC with a diameter in the range of 10–20 nm that is in good agreement with others [38]. The end of the crystal particles seems a little sharp and pointed due to eroding during acid hydrolysis. Same shape and feature was observed for CNC prepared from *Bambusa vulgaris* [39]. Kaushik et al. [40] have shown that the geometrical dimension of cellulose differently depends on the source of cellulose and the preparation condition. PANi images are shown in Fig. 2b displaying a granular structure with micrometer-sized. Agglomerations with no uniformity of PANi structure were observed in some parts that is in good agreement with others [41]. The images of PANi/CNC nanocomposite show a fibrous structure with high porosity owing to the presence of CNC. In the presence of higher amount of aniline intensive polymerization associated with agglomeration was observed in some parts of the nanocomposite.

3.2. Optimization of analytical condition

3.2.1. Screening process

The Plackett–Burman design was used to screen the variables that are usually effective on the cholesterol biosensor performance. Several variables may affect the response of the system studied, and it is practically unmanageable to recognize and control the small contributions from each one. Therefore, it is essential to select those variables with main effects. In our research six parameters of pH, buffer capacity, amounts of enzymes, glutaraldehyde, ionic liquid, and PANi/CNC nanocomposite were considered for biosensor fabrication and the significant factors were determined by analysis of variance (ANOVA) and presented in Table 4.

From the screening process, it has been identified that the amount of IL and pH variables are not as significant as other parameters. This screening test was supported by other works in which cholesterol sensors have operated best at the pH around 7.0 to 7.5 [8,42] The optimum amount of IL was manually obtained using OFAT method and DPV technique. Based on the differential pulse voltammograms no significant

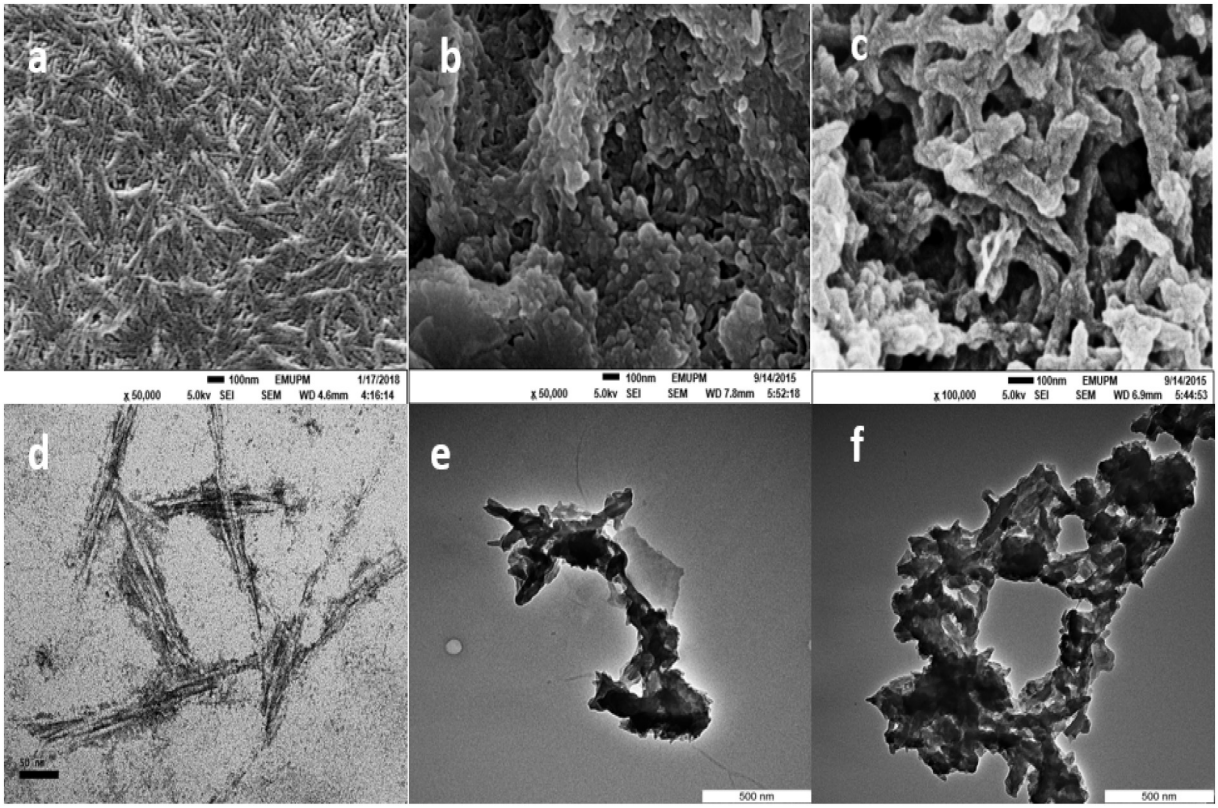


Fig. 2. FESEM and TEM micrographs of a,d) CNC, b,e) pure PANi and c,f) PANi/CNC nanocomposite with mass ratio of Ani/CNC (0.84/0.16).

changes in anodic current were observed for electrodes prepared from different amounts of IL. This result confirmed the data presented via the screening process indicating that a thin layer of IL (4 μL) could improve electron transfer and the sensitivity of the cholesterol biosensor. Thus, 4 μL of IL was selected as the fixed value for the addition of IL in the fabrication of the cholesterol sensor.

Table 4 presents the analysis of variance (ANOVA) for the generated PB factorial model. ANOVA allows us to investigate the significance of parameters affecting the current response by identifying the sequential F-test. The desired result for this test is when the Prob > F (P-value) is <0.05 [43]. The P-value is defined as the probability of F-value calculated from the data exceeds a theoretical value. By that, by increasing F-value the P-value will decrease. It was shown that the computed F-value of the model is 46.88, implying the model is significant. The P-value of the model was very low value (0.0011) and all the significant variables exhibited a low P-value (<0.05). The coefficient of determination, $R^2 = 0.988$, was close to 1 indicating the model fitted the data well.

3.2.2. Response surface methodology

Four significant variables determined by PB design including; buffer capacity, sample amount, enzyme amount, and GLU were selected for further optimization using central composite rotatable design (CCRD). ANOVA of the model developed for cholesterol biosensor fabrication is presented in Table 5. Fig. 3 shows the predicted response values versus actual values obtained for cholesterol biosensor fabrication. The coefficient of determination (R^2) of the model is 0.8573 demonstrating that the model describes 85.73% of the response variability.

The data was fitted to various models and their subsequent ANOVA showed that the fabrication of cholesterol biosensor was most suitably described by a cubic model with a R^2 of 0.8573. Based on Table 5, the F-value of 5.20 indicates that the model is significant. There is only a 0.34% chance that a "model F-value" this large could occur due to noise. In general, a model considered to be good if the calculated F-value is several times greater than the tabulated value [44]. The P-value of 0.0034 (<0.05) indicates that the model terms are significant.

Table 4
Analysis of variance for the Plackett–Burman design.

Source	Sum of squares	df	Mean square	F-value	P-value
Model	0.048630	7	0.006947	46.88	0.0011
Enzyme amount, X_1	0.007574	1	0.007574	51.11	0.0020
Buffer capacity, X_2	0.017170	1	0.017170	115.88	0.0004
GLU amount, X_3	0.013260	1	0.013260	89.46	0.0007
Sample amount, X_4	0.001773	1	0.001773	11.96	0.0258
X_1X_3	0.005017	1	0.005017	33.86	0.0043
X_2X_3	0.002811	1	0.002811	18.97	0.0121
X_3X_4	0.010000	1	0.010010	67.56	0.0012
Residual	0.000593	4	0.000148		
Corrected total	0.049220	11			

Table 5
Analysis of variance (ANOVA) and R-squared (R^2) of cholesterol biosensor fabrication (cubic model).

Source	Sum of squares	df	Mean square	F-value	P-value
Model	0.94	14	0.067	5.20	0.0034[a]
Residual	0.16	12	0.013	–	–
Lack of fit	0.056	10	0.005	0.11	0.9941[b]
Pure error	0.100	2	0.050	–	–
Corrected total	1.10	26	–	–	–
R-squared		0.8586	Standard deviation		0.11
Adequate precision		10.833	PRESS[c]		0.48

[a] Model F-value is significant at "Prob>F" less than 0.05.
[b] Lack of Fit value is not significant relative to pure error.
[c] PRESS is Predicted Residual Error of Sum of Squares.

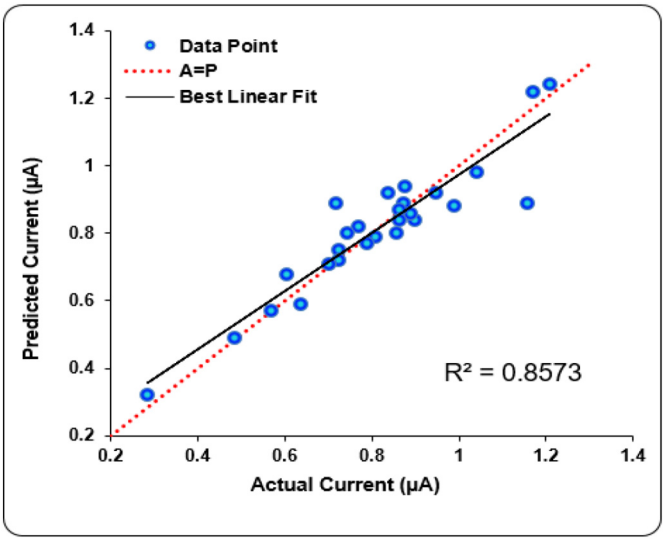


Fig. 3. Scatter plot of predicted versus actual current value from central composite design for cholesterol biosensor fabrication.

The P-value can be used to verify the significance of each variable, in which the interaction strength between each independent variable can be identified [45]. The $R^2 = 0.8586$ is acceptable for a biological system. It has been proposed that a well-fitted model should have $R^2 > 0.80$ to be a good fit model. The high value for R^2 may vary from field to field [46]. The $R^2 = 0.8586$ suggests that the regression models explains the system well. Hence, the response surface model developed in this study for fabrication of cholesterol biosensor was considered to be satisfactory. In addition, the F-value of 0.11 for the lack of fit implies it is not significant relative to the pure error, indicating that the model fits the data well. The model also presented the adequate precision measured the signal to noise ratio. The ratio >4 is appropriate. Therefore, the ratio of 10.833 showed an acceptable signal. This confirmed that the proposed model could be selected to navigate the design space.

From the results obtained in Table 6, all the linear coefficients terms were significant ("Prob > F" <0.05) except for GLU amount (X_3) in which the P-value was slightly higher than 0.05. It can be seen that the amount of enzyme and the nanocomposite sample exhibited a low P-value and this shows that both of these parameters gave a strong influence on the current response. Among the interaction coefficients terms, the interaction between GLU and sample amount (X_3X_4) showed the most significant interaction with the P-value of 0.033, and the interaction between amount of enzyme and buffer capacity (X_1X_2) shows otherwise with the P-value of 0.5546. The final reduced model to predict the optimum conditions for cholesterol biosensor fabrication is shown in Eq. (1).

$$\begin{aligned} Response = {} & +0.8926 - 0.07836\,X_1 + 0.06218\,X_2 + 0.08387\,X_3 \\ & + 0.1468\,X_4 - 0.0423\,X_1^2 - 0.04506\,X_2^2 - 0.01729\,X_1X_2 \\ & - 0.06291\,X_1X_4 - 0.04889\,X_2X_3 - 0.06853\,X_3X_4 \\ & - 0.03249\;X_3 3 - 0.0331\,X_4 3 - 0.08771\,X_1X_2X_4 \\ & + 0.5551\,X_2X_3X_4 \end{aligned}$$

where X_1 is the enzyme amount, X_2 is the buffer capacity, X_3 is the GLU amount, and X_4 is the PANi/CNC nanocomposite amount. Based on the Equation, X_2, X_3, X_4, and $X_2X_3X_4$ terms exhibited the positive value attribute to the synergistic effect, while other terms exhibited the negative value due to antagonistic effects. A synergistic effect between two or more factors happens in which an increase in one variable value is caused by an increase in value of other factors. On the contrary, the antagonistic effect occurs when an increase in the variable value achieved by reducing the value of other variables [47]. It was observed that only enzyme amount (X_1) has a negative effect while the other parameters provide a positive effect. This may be due to the current response was undesirably affected by the presence of higher amount of ChOx enzyme. Indeed, despite the negative value, the amount of enzyme has one of the biggest effects on the current response, followed by the amount of PANi/CNC nanocomposite sample with the estimated effect of 13.32. The obtained model was employed to study the effect of the four significant parameters and their interactions on the current response.

3.2.3. Response surface analysis

From the response surfaces, effects of interaction of individual variables on the current response can be understood and studied in further detail. Each response surface plotted for current response illustrated the different combinations of two variables at one time while maintaining the other variables at the centre point values (coded level: 0). The combination of these variables was described in the form of 3D graphic plots and shown in Fig. 4.

The effects of buffer capacity and the amount of enzyme on the current response are presented in Fig. 4a. The result showed that high buffer capacity and low amount of enzyme improved the current response. A stable high current response can be observed in the lower region of the enzyme amount (1.0 μL to 8.0 μL) and high region of buffer capacity (137.5 mM to 150.0 mM). High buffer capacity contained high concentrations of the weak acid and conjugate base. It seems that by increasing the concentration of $H_2PO_4^-$ and $HPO4^{2-}$ in the electrolyte, a higher concentration gradient was achieved. This phenomenon could enhance the diffusion of cholesterol toward the surface of electrode and increased the reaction between the cholesterol and enzyme. However, an optimum enzyme amount was clearly observed at 4.5 μL. At any given buffer capacity from 100 to 150 mM, the current response increased with increasing of enzyme amount to 4.5 μL. Further increase in enzyme amount resulted in a decrease in the current response. This observation was similar to other studies in which high current response was observed using low amount of enzyme [8,29]. This might be due to the fact that high amount of enzyme results in an increase background noise and adsorption onto the channel walls [42]. It was reported by other researchers that a high response could be achieved even with low enzyme and high substrate amounts [48]. The low current response with no significant effect confirmed that the interaction of X_1X_2 should have a high P-value (0.5546). The effect of enzyme and PANi/CNC nanocomposite sample amount on the current response is presented in Fig. 4b. It was observed that by increase of PANi/CNC sample amount, the current response increased drastically with the decreasing of enzyme amount. An increase of PANi/CNC nanocomposite amount provided more nanostructure matrices that enhanced the electron communication between the enzyme active site and the electrode. The addition of CNC inside the nanocomposite provides a high surface area and

Table 6
Analysis of variance and regression coefficients of cholesterol biosensor fabrication (cubic model).

Source	Coefficient estimate	Sum of squares	df	Mean square	F-value	P-value
Enzyme amount, X_1	−0.078	0.0147	1	0.0147	11.38	0.0055
Buffer capacity, X_2	0.062	0.0928	1	0.0928	7.17	0.0201
GLU amount, X_3	0.084	0.0563	1	0.0563	4.35	0.0591
Sample amount, X^4	0.15	0.1725	1	0.1725	13.32	0.0033
X_1^2	−0.042	0.0458	1	0.0458	3.54	0.0844
X_2^2	−0.045	0.052	1	0.0520	4.01	0.0682
X_1X_2	−0.017	0.0048	1	0.0048	0.37	0.5546
X_1X_4	−0.063	0.0633	1	0.0633	4.89	0.0471
X_2X_3	−0.049	0.0383	1	0.0383	2.95	0.1113
X_3X_4	−0.069	0.0751	1	0.0751	5.80	0.0330
X_3^3	−0.032	0.0507	1	0.0507	3.91	0.0713
X_4^3	−0.033	0.0526	1	0.0526	4.06	0.0668
$X_1X_2X_4$	−0.088	0.1231	1	0.1231	9.51	0.0095
$X_2X_3X_4$	0.056	0.0493	1	0.0493	3.81	0.0748

*Sum of square and mean square is in picoAmpire.

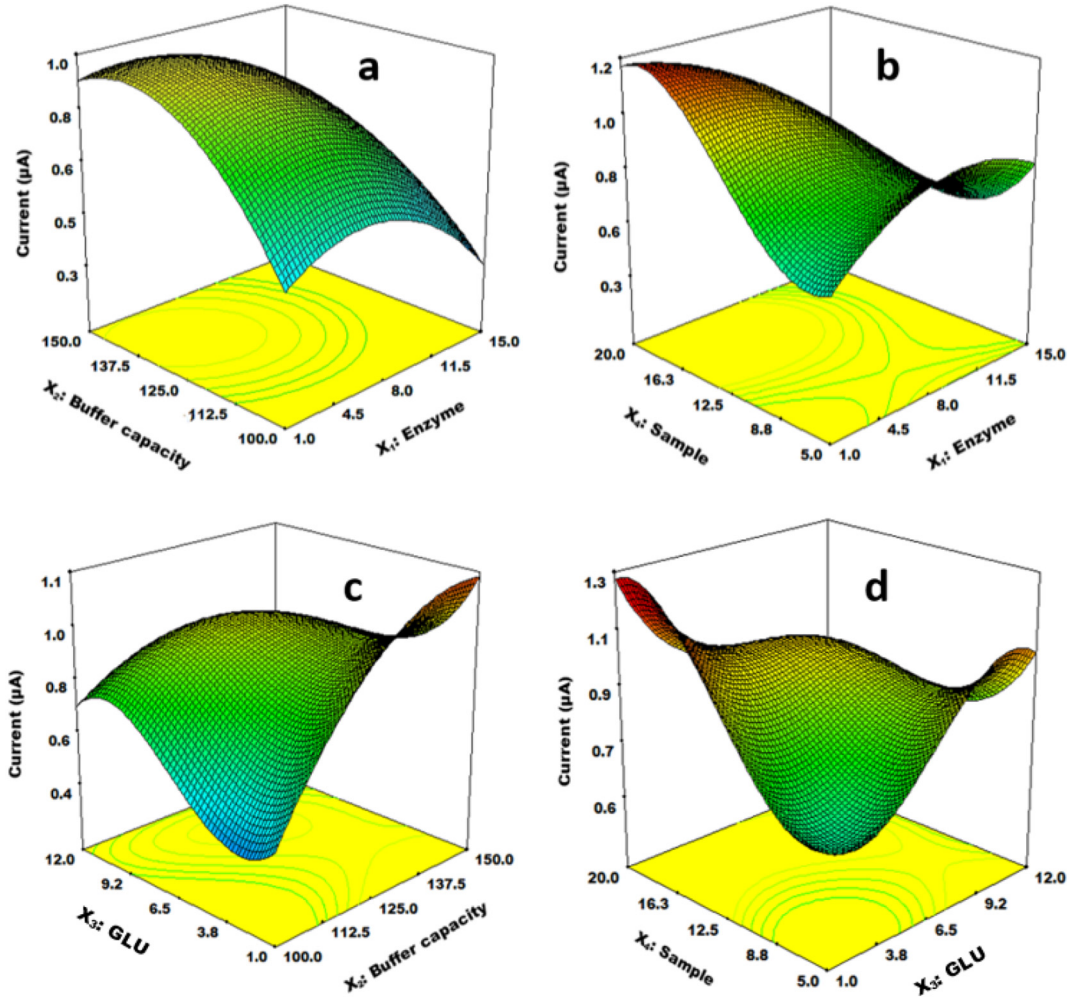


Fig. 4. Response surface plot of the combined effect of a) enzyme amount (μL) and buffer capacity (mM) (X_1X_2), b) nanocomposite sample (μL) and enzyme amount (μL) (X_1X_4), c) GLU (μL) and buffer capacity (mM) (X_2X_3), d) GLU (μL) and sample amount (μL) (X_3X_4) on the current response.

porosity which facilitates the enzyme immobilization on the surface of modified electrode [37]. A high current response was obtained at low amount of the enzyme (1.0 μL to 3.3 μL) and high amount of the sample (18.0 μL to 20.0 μL).

The effects of buffer capacity and the amount of GLU on the current response are presented in Fig. 4c. At any given GLU amount, the current response increased with increasing of buffer capacity. However, the optimum GLU amount was clearly observed at 4.7 μL where there was no significant change of current response at higher amount of GLU. As it was mentioned before, higher ions concentration gradient was achieved at higher buffer capacity which could enhance cholesterol diffusion and its reaction with enzyme. The effect of GLU amount and PANi/CNC nanocomposite sample on the current response is presented in Fig. 4d. The 3D plot clearly shows that the current response increases with increasing of both nanocomposite sample and GLU amounts. However, it was observed that with further increase of GLU amount from 8.3 μL, the current response decreased with an increase of sample amount. Same behaviour also was observed by other study in which with further increasing of glutaraldehyde concentration, the enzyme activity decreased gradually [49]. The addition of GLU in the fabrication of cholesterol biosensor did improve the current response and it was predictable in the development of biosensors, in which most of the matrices require cross-linkers to hold the enzyme. However, when the amount of glutaraldehyde solution is in excess, the enzyme molecules formed a multi-point binding with the carrier, leading some spatial structural obstacles to inactivate the enzyme [49]. It can be summarized that both GLU and sample amount have significant effect toward each other.

3.2.4. Optimization by response surface methodology

The optimum conditions predicted by RSM model with the highest response were chosen for fabrication of the biosensor. A new set of experiments were carried out under the suggested conditions and the resulting responses were compared to the predicted values by RSM model. The experiment with the highest current response and a closer experimental data toward the predicted value with a low Relative Standard Error (RSE) value was chosen as the optimum value for the fabrication of the cholesterol sensor. The optimum conditions predicted by RSM model are as follows: modified electrode prepared from Ani/CNC with mass ratio of 0.84/0.16 followed by spin coating of 4 μL IL, drop cast of 11.50 μL GLU and immobilization of 5.34 μL ChOx. The phosphate buffer solution was prepared with pH 7.0 and buffer capacity of 105.44 mM. Optimized parameters predicted by RSM were then validated with a series of experiments to generate the actual current response. The predicted current of model (1.29 μA) was in excellent accordance with experimental value (1.37, 1.31 and 1.19 μA).

3.3. Analytical performance of PANi/CNC/IL/GLU/ChOx based cholesterol biosensor

The performance of the prepared biosensor toward cholesterol was evaluated by some criteria and characteristics such as, sensitivity,

stability, repeatability, linearity, and selectivity. To investigate the electrocatalytic behaviour of nanocomposite toward the electrochemical reaction of hydrogen peroxide, the modified electrode was characterized by DPV technique ranging from −1 to +1 V at a scan rate of 10 mV/s. The sensitivity of the proposed biosensor was determined in different concentrations of cholesterol ranging from 1 μm to 12 mM in buffer solution. The cholesterol solution was stirred before use to obtain a homogenous solution. All experiments were done in triplicate and the calibration curve of current response against cholesterol concentration was plotted and shown in the Fig. 5.

As it can be seen in Fig. 5A, the biosensor exhibited a rapid and sensitive response to the changes of cholesterol concentrations with the linear range of 0.001 mM to 12.0 mM and coefficient of determination, R^2, of 0.9908. There are not significant changes in the current response when cholesterol concentration was higher than 12 mM, that could be due to the saturation of active sites of ChOx [50]. The current cholesterol biosensor based on PANi/CNC/IL/GLU/ChOx-modified electrode exhibited a wider linear range from 1 μM to 12 mM compared to the others [50,51].

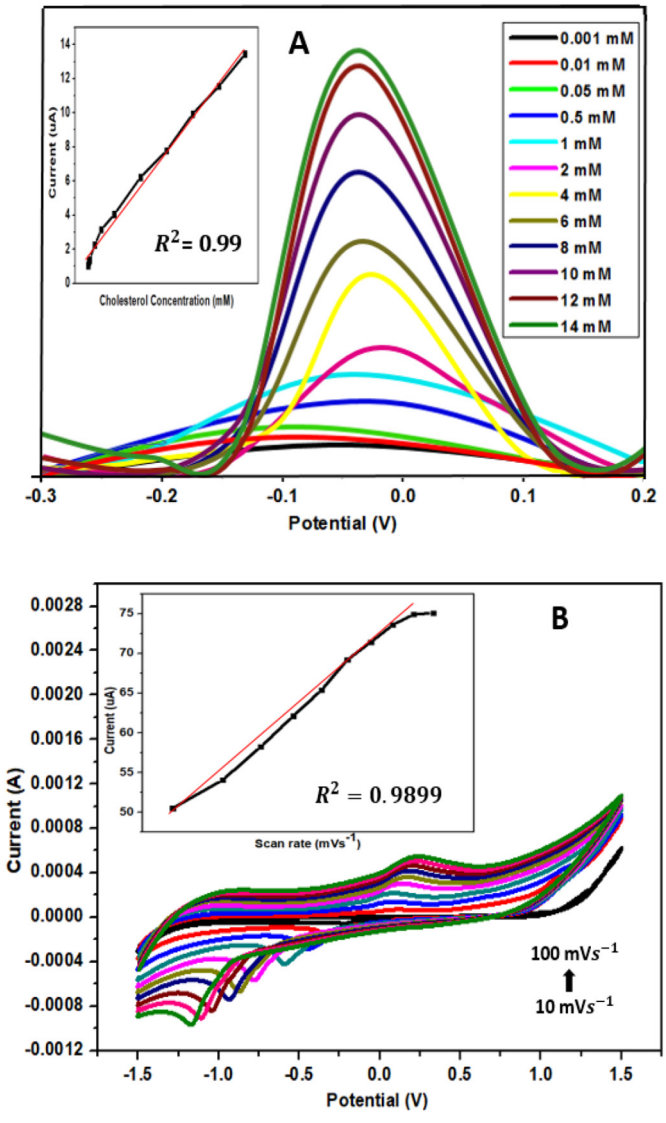


Fig. 5. A) Differential pulse voltammograms of PANi/CNC/IL/GLU/ChOx-modified electrode in various concentration of cholesterol in buffer solution pH 7. Inset: calibration curve of current against cholesterol concentration from 0.1 μM to 14 mM. B) Cyclic voltammograms of PANi/CNC modified electrode at different scan rates (10 to 100 mV/s) in 4 mM $K_3Fe(CN)_6$ in 1 M KCl solution. Inset is the plot of oxidation peak current against the square root of scan rates ($\upsilon^{1/2}$) from 10 to 100 mV/s.

In order to have sensitivity per area, the electroactive surface area (A) of PANi/CNC-modified electrode was calculated by using Randles–Sevcik equation;

$$i_p = 268,600\, n^{3/2} A D^{1/2} C \upsilon^{1/2}$$

where Ip is maximum current (ampere), n is number of electrons transferred (in this work n is 1), D is the diffusion coefficient of a known electrolyte such as potassium ferrocyanide, A is the electrode area (cm^2), C is concentration (mol cm^{-3}) and υ is scan rate (mV/s) [52]. The solution of 4 mM potassium ferrocyanide ($K_3Fe(CN)_6$) in 1.0 M KCl was used to perform the CV on the PANi/CNC-modified electrode from the potential of −1.5 V to 1.5 V with the scan rate varying from 10 to 100 mV/s. Diffusion coefficient ($cm^2\ s^{-1}$) of $Fe(CN)_6]^{3-/4-}$ solution is 6.3×10^{-10} $cm^2\ s^{-1}$.

As can be seen from the Fig. 5B, the cyclic voltammograms show well-defined anodic and cathodic peaks at all scan rates. The increasing in current peak with scan rate represents the excellent electroactive properties of PANi/CNC by the diffusional control process of $[Fe(CN)_6]^{3-/4-}$ ions on the electrode surface. The anodic and cathodic peak currents increased linearly along with the increasing square root of scan rates ($\upsilon^{1/2}$), suggesting an electrochemical diffusion-controlled process in the electrode/electrolyte interface. The electroactive surface area and sensitivity per active surface area of modified electrode were calculated to be 0.029 cm^2 and 35.19 μA mM/cm^{-2}, respectively. The sensitivity measured for PANi/CNC/IL/GLU/ChOx–modified electrode has been compared with others and the results are shown in Table 7. This comparison clearly shows that this cholesterol biosensor exhibited a high and favorable sensitivity compared to other ChOx based sensors [47,49,53]. The limit of detection (LOD) and limit of quantification (LOQ) are two critical characteristics in validation method. The LOD and LOQ were calculated as the 3 and 10 times the standard deviation of the blank divided by the slope of the calibration line, respectively [54]. In this research, the LOD and LOQ obtained for the cholesterol biosensor are 0.48 μM and 0.6 μM, respectively. Table 7 shows some electrochemical performance characteristics comparison of PANi/CNC/IL/GLU/ChOx-modified electrode and other modified electrodes from the previous studies for cholesterol detection. In general, PANi/CNC/IL/GLU/ChOx-modified electrode based cholesterol biosensor exhibited a wide linear range and a relatively high electrochemical sensitivity which are promising for the clinical diagnostics of total cholesterol in blood and food.

3.4. Reproducibility and repeatability

In this study, five PANi/CNC/IL/GLU/ChOx-modified electrodes were prepared from same composition and condition and then they were used to measure 1.0 mM cholesterol in buffer solution. The relative standard deviation (RSD) for reproducibility of the developed electrode was calculated to be 3.76%. In order to obtain the repeatability of the modified electrode, one PANi/CNC/IL/GLU/ChOx-modified electrode was used to run five consecutive measurements in the 1.0 mM cholesterol solution. A repeatable current response was observed with a low value of RSD of 3.31%. A small value of RSD indicates a good precision of the developed modified electrode.

3.5. Interference and long-term stability test

In the evaluation of biosensors performance, qualification of its selectivity is one of the important functions. In purpose of selectivity evaluation of the prepared sensor, the influence of some possible interfering substances such as glucose, ascorbic acid (AA), and uric acid (UA), was examined in 1.0 M cholesterol solution. The same amount of 1.0 mM of glucose, (ascorbic acid (AA), and uric acid (UA) was mixed with 1.0 mM cholesterol and the anti-interference ability of the biosensor was investigated. The influence of the above-mentioned species on the current response can be seen in Fig. 6A and it was shown that no obvious changes of current were observed for glucose, AA, and UA. An

Table 7
Comparison of data for proposed cholesterol biosensor from other studies.

Modified electrode	Detection method	Linear range (mM)	Sensitivity (μA mM/cm^{-2})	LOD (mM)	Shelf life (days)	References
G/PVP/PANi/ChOx	Amperometry	0.05–10	34.77	0.001	14	[4]
PBNPs-ChOx/SPE	Amperometry	0–15	2.10	0.2	–	[29]
Ti/NPAu/ChOx–HRP–ChE	CV	0.97–7.80	29.33	0.01	60	[53]
CSNFs/AuNPs/ChOx	Amperometry	0.001–0.045	1.02	0.0005	25	[55]
$NiFe_2O_4$/CuO/FeO-CH/ChOx	DPV	0.13–12.95	16.54	0.81	90	[56]
Si/Ag/ZnO NRs	Amperometry	0.01–16.00	74.10	1.5×10^{-6}	45	[57]
PANi/CNC/IL/GLU/ChOx	DPV	0.001–12.00	35.19	0.00048	19	This work

G: graphene, PVP: polyvinylpyrrolidone, PANi: polyaniline, PBNP: Prussian blue nanoparticles, SPE: screen-printed electrodes Ti: titanium, NPAu: gold nanoparticle, Che: cholesterol esterase, HRP: horseradish peroxidase, CSNF: chitosan nanofibers, AuNP: gold nanoparticle, $NiFe_2O_4$: Nickel ferrite, CuO: Copper (II) oxide, FeO: Iron (II) Oxide, Ch: chitosan, Si/Ag: silver electrode, ZnO NRs: zinc oxide nanorods.

increase of current response was observed with the addition of 1 mM cholesterol to the previous cholesterol solution confirming a high sensitivity, and selectivity for the proposed biosensor toward the target analyte. The stability test was conducted by storing the PANi/CNC/IL/GLU/ChOx-modified electrode at 4 °C in a close container over 20 days. The

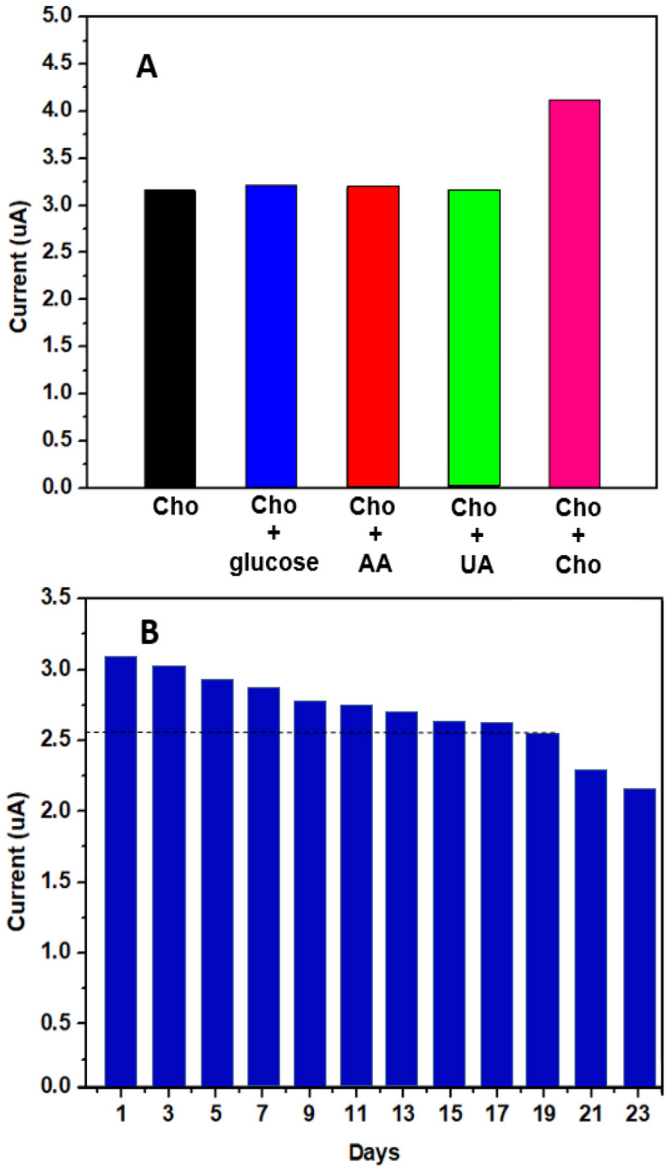


Fig. 6. A) Current response of PANi/CNC/IL/GLU/ChOx modified electrode for A) interference study of cholesterol solution (1 mM) mixed with 1 mM of glucose, AA, UA and cholesterol in 0.5 M PBS of pH 7. B) Stability study of cholesterol solution during 23 days.

biosensor's activity was measured in cholesterol sample of 1.0 mM every two days and it was observed that the current response of biosensor retained 84% of the original response at 19th day (Fig. 6B). The reduction of current response might due to denaturation of the immobilized enzyme [58]. Based on the House et al. study [59], the stability of the enzymatic sensor can be improved by letting the crosslinking process of glutaraldehyde and cholesterol oxidase completed at least 24-hours at room temperature. The stability of biosensor also can be improved by soaking the modified electrode in purified water for 20 min to remove the excess free enzyme.

4. Conclusion

In this research, a nanocomposite of CNC from Semantan bamboo (*Gigantochloa scortechinii*), IL, and PAni were introduced on the SPE to conveniently construct a new enzymatic biosensor. The synergistic effect of structural properties of CNC and electrochemical properties of IL/CPs on the electrocatalytic properties of nanocomposite along with high porosity have made this material a good candidate for hosting enzymes and biomolecules. The images of FESEM and TEM showed nanostructure of composite with no phase separation revealing homogenous polymerization of monomer on the surface of nanocellulose. The PANi/CNC/IL/GLU/ChOx-modified electrode was able to monitor cholesterol in the range of 0.001–12 mM with LOD of 0.5 mM displaying a fast response and a high sensitivity. The electron transferring between enzyme and surface of electrode was fascinated by nanocomposite so that the selective determination of cholesterol with minimal interference was realized. The proposed biosensor also presented satisfactory results related to the operational repeatability, reproducibility and stability suggesting its great promise for cholesterol detection in the field of clinical diagnostics and food industries.

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