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- RESEARCH ARTICLE -

Using Response Surface Methodology for Amperometric Glucose Biosensor Construction

Gul Ozyilmaz*, Ali Tuncay Özyılmaz, Seda Ağçam

Mustafa Kemal University, Faculty of Arts & Sciences, Department of Chemistry, Hatay/ TURKEY

Abstract

In this study, construction of amperometric glucose biosensor was carried out by immobilizing of glucose oxidase (GOD) on platinum electrode with 0.09 cm^2 surface area which coated with polypyrrole (PPy) by cyclic voltammetry technique. Because measured current values in the presence of glucose would be affected from the electrode preparing and working conditions, experimental parameters should be optimized by response surface methodology (RSM). To this, State Ease Design Expert 8.0.7.1. (Serial Number:0021-6578) programe was used. PPy synthesis conditions of pyrrole (Py) monomer concentration and scan rate were optimized according to current response in presence of glucose. Optimal Py monomer concentration and scan rate for PPy synthesis were determined as 10 mM and 50 mV/s, respectively. Immobilization parameters such as concentrations of chitosan, GOD and glutaraldehyde (GAL) also were optimized by RSM as 1.0 %, 4 mg/ml and 0.0625 %, respectively. The digital photos of electrodes at each stage were obtained. All electrodes well characterized in absence and in the presence of glucose by cyclic voltammetry and impedance techniques and it was observed that electrodes were sensitive to glucose molecule. Finally the effect of working pH and applied potential on the current response was investigated by RSM. The highest current response was obsreved when pH of glucose solution and applied potential were 6.0 and 0.8, respectively.

Keywords:

Response surface methodology, Pt electrode, Polypyrrole, amperometric biosensor, glucose oxidase

Article history:

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* *Corresponding Author: Gül Özyılmaz, e-mail: gozyilmaz@gmail.com*

Introduction

The determination of glucose concentration is very important in food, fermentation products as well as biological fluids and diagnosis of diabetics (Dong et al. 2013). There is an increase in the development of glucose sensing devices including biosensors for monitoring the glucose level. Because of good sensitivity, low detection limit, operational simplicity, low cost and easy in use, amperometric bisensors, which are based on electron transfer between an electrode and immobilized glucose oxidase, have attracted substantial interest. An increase in the studies about producing selective, sensitive, safe and low cost glucose biosensors. Specific properties of an amperometric electrodes depend on kinetics of electron transfer process between electrode surface and active site of immobilized enzyme at proper potential range. Precendition for development of biosensor at high sensitivity and fast response is to provide rapid electron tranfer from biocomponent to the electrode. To this, conductive polymers which have redox center are widely used (Bal 2012). There are several studies about biosensors which are prepared using conductive polymers such as polypyrrole (Sung et al. 2003, Ozyilmaz et al. 2011, Chen et al. 2005, Ma et al. 2003), polyaniline (Borole et al. 2004, Zejun et al. 2014, Jie et al. 2015), polythiophen (Kawai et al. 1990), polyparaphenylene (Ivory et al. 1979), poly(N-methylpyrrole) (Miyamoto et al. 1990, Ozyilmaz et al. 2017). Among them, polypyrrole is mostly used conductive polymer due to easy preparation, stability and high conductivity (Ahuja et al. 2007). Although current response will be proportional to glucose concentration, its magnitude will change depending on biosensor construction and working conditions. So, optimization of these parameters is needed to enhance biosensor quality. In general, optimization is required to improve efficiency, quality of production and also decrease in cost, resource and time. Traditionally, classical optimization has been carried out by monitoring the influence of one factor at a time on an experimental response. While only one parameter is changed, others are kept at a constant level. This optimization technique is called one-variable-at-a-time. Its major disadvantage is that it does not include the interactive effects among the variables studied (Bezerra et al. 2008). Response surface methodology (RSM), which is a collection of mathematical and statistical techniques, is the mostly used to overcome this problem. By RSM, the effect of variables can be investigated simultaneously with minimum number of experiments. There are few study related with RSM application for optimization of biosensor construction parameter in the literature (Gouda et al. 2001, Urkut et al. 2011, Zhang et al. 2011, Ebrahimi et al. 2011, Kergavarat et al. 2012, Haghighi et al. 2012, Mirmoghtadaie et al. 2013, Chaichi & Ehsani, 2016).

The aim of this work is to optimize the biosensor construction parameters of glucose sensitive polypyrrole (PPy) based amperometric bisensor by RSM. Firstly, PPy synthesis conditions such as pyrrole monomer concentration and scan rate were optimized. Results were evaluated according to measured current values in 1 mM glucose solution. RSM studies were carried ot by using State Ease Design Expert 8.0.7.1. (Serial Number:0021-6578). Model analysis ANOVA analysis and surface diagrams were obtained by evaluation of current data. Enzyme electrode sensitivity was shown by cyclic voltammetry and impedance spectroscopy techniques.

Material and Methods

Aspergillus niger origin GOD (EC 1.1.3.4), pyrrole, chitosan (Chi), glucose anhydrous, glutaraldehyde (GAL) were purchased from Sigma. Design Expert 8.0.7.1 programme (Serial no:0021-6578) was purchased from State Ease USA. Pyrrole was used after distillation and was

stored in dark when not in use. All other reagents were of analytical grade and used without further purification.

Preparation of enzyme electrodes

Enzyme electrode was prepared including three steps according to our earlier study (Ozyilmaz et al. 2017). Firstly, PPy film was synthesized on Pt electrode (Pt/PPy). Secondly, Pt/PPy electrode was immersed in GOD contained Chi solution for 6 seconds (Pt/PPy/Chi-GO) and electrode was dried for 2 hour open to atmosphere. Lastly, Pt/PPy/Chi-GOD electrode was interacted with GAL solution for 10 sec for crosslinking between amine groups of enzyme and Chi to hinder GOD leakage. Glucose sensitive electrode preparation steps are given in Figure 1.

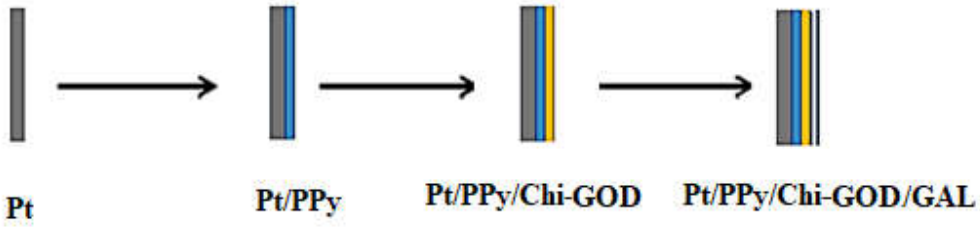

Figure 1. Glucose sensitive electrode preparation steps

PPy film was achieved in a single compartment cell with three electrode configurations. The reference electrode was an Ag/AgCl (3 M KCl) electrode and the counter electrode was a platinum plate with a surface area of 0.25 cm^2. Ivium PocketStat and CHI 660b model electrochemical analyzers were employed in electrochemical experiments. All of the potential values were referred to the Ag/AgCl (3 M KCl) electrode. PPy film was synthesized onto Pt electrode with 0.25 cm^2 surface area by cyclic voltammetry technique in pyrrole monomer containing 0.15 M sodium oxalate electrolyte solution at potential range between -0.5 and +1.8V applying 26 segment. Potential range was chosen after preliminary studies.

After PPy synthesis, Pt/PPy electrode was immersed into GOD containing Chi solution for 15 s (Pt/PPy/Chi-GOD). After 1 h, Pt/PPy/Chi-GOD electrode was immersed into GAL solution for 5 sec. Electrodes were kept at 4 °C until it was used.

Amperometric current measurements

Electrochemical experiments were performed in a single compartment cell with three electrode configurations. The reference electrode was an Ag/AgCl (3 M KCl) electrode and the counter electrode was a platinum plate with a surface area of 0.25 cm^2. CHI 660b model electrochemical analyzer (serial number: A1420) was employed in electrochemical experiments. All of the potential values were referred to the Ag/AgCl (3 M KCl) electrode. The current value depending on H_2O_2 oxidation which was produced by enzyme activity on the enzyme electrode was monitored chronoamperometrically at constant potential. The chronoamperometric measurements were performed at room temperature in steady state conditions in potassium phosphate buffer solution while each measurement was lasted 120 s. And then the same measurement was carried out in 1 mM of glucose solution. Net current value was calculated by subtracting the current value of buffer solution from that of glucose solution.

Optimization of Py monomer concentration and scan rate by RSM

Optimal Py concentration and scan rate for glucose biosensor construction was investigated using Response Surface Methodology (RSM). To this, Design Expert 8.0.7.1 (Serial No: 0021-6578) which was purchased from State Ease. Optimal design was used 2 factor 3 levels of Py concentration (10, 25 and 50 mM) and scan rate (20, 50 and 100 mV/s). 16 sets of experiments were constructed by the programme which were given in Table 1.

Table 1. Experimental design constructed for PPy synthesis to determine optimal Py concentration and scan rate by RSM

Working Sets	Pyrrole Concentration (mM)	Scan Rate (mV/s)
1	10	20
2	10	20
3	10	20
4	10	50
5	10	100
6	25	20
7	25	50
8	25	50
9	25	50
10	25	50
11	25	100
12	50	20
13	50	50
14	50	50
15	50	100
16	50	100

16 electrodes with PPy layer obtained according to Table 1 were constructed and all PPy coated electrodes were used to prepare enzyme electrode with 0.5% Chi, 2 mg/ml of GOD and 0.05% GAL. Net current values of all alactrodes were measured chronoamperometrically and results were evaluated by Design Expert.

The effects of Chi, GOD and GAL concentration on current response were investigated by RSM applying Box-Behnken Design. The low and high values of investigated parameters were chosen by preliminary studies and were given in Table 2. Pt electrodes were coated by PPy at optimum monomer concentration and scan rate. According to Table 2, 17 set of experimental work was formed by programme and current responses were measured for each electrode.

Table 2. Chi, GOD and GAL concentrations of used in Box-Behnken Design for optimization of biosensor construction parameters.

		Codes of variables		
		Low	**Medium**	**High**
Variables	**Symbols**	-1	0	+1
C_{Chi} (%)	A	0,25	0,625	1,000
C_{GOD} (mg/ml)	B	1,00	2,50	4,000
C_{GAL} (%)	C	0,025	1,00	0,0625

Electrochemical characterization of enzyme electrode

To show the sensitivity of enzyme electrode to glucose, electrochemical characterization was carried out. To this, cyclic voltammetry and impedance analysis of enzyme electrode were carried out in the absence and in the presence of glucose. CV analysis was achieved by applying potential range between -0.5 and 1.8 V at 100 mV/s scan rate. In case of impedance analysis, Nyquist diagrams were obtained by applying 10^{-3}-10^{5} frequency range and 7 mV amplitude. The effect of pH and applied potential was investigated by RSM using optimal design.

Results and Discussion

Optimization of Py concetration and scan rate by RSM

PPy films were synthesized onto Pt electrode according to working sets which are constructed by Design Expert programme as given in Table 1. GOD was immobilized onto PPy films and thus 16 enzyme electrodes were constructed. Current responses in 1 mM of glucose solution were measured for each electrode. Responses were evaluated by the programme and model analysis were carried out depending on Py concentration (C_{Py}) and scan rate (SR). The fit model should meet several requirement such as low model p-value (<0.05), high lack of fit p-value (>0.05) and high R^2 value. From the this point of view, quadratic model found the most suitable to predict the current value depending on studied parameters as C_{Py} and SR because low model p-value and high lack of fit model p-value. As seen in the Table 3, quadratic model fitted best to predict current value at 1 mM glucose solution as a function of C_{Py} and SR.

Table 3. Model analysis of RSM study for C_{Py} and SR (Current responses were obtained in 1 mM of glucose concentration)

Sources	Sequential p-value	Lack of fit p-value		R^2_{adj}	Pred-R^2
Linear	0,0486	0,0037	0,2755		-0.0119
2FI	0,2199	0,0037	0,3113	-0,0077	
Quadratic	0,0014	0,1020	0,7766	0,5868	suggested
Cubic	0,0712	0,2870	0,8557	0,0138	

Current response depending on C_{Py} and scan rate SR can be predicted according to equation given below:

$$\mathbf{I} = 11.01458 - 0.53034 x C_{Py} + 0.17197 x SR + 1.20052.10^{-3} x C_{Py} x SR + 6.0839.10^{-3} x C_{Py}^2 - 1.57328.10^{-3} x SR$$

The surface (3D) diagram for current response depending on C_{Py} and SR was given in Figure 2.

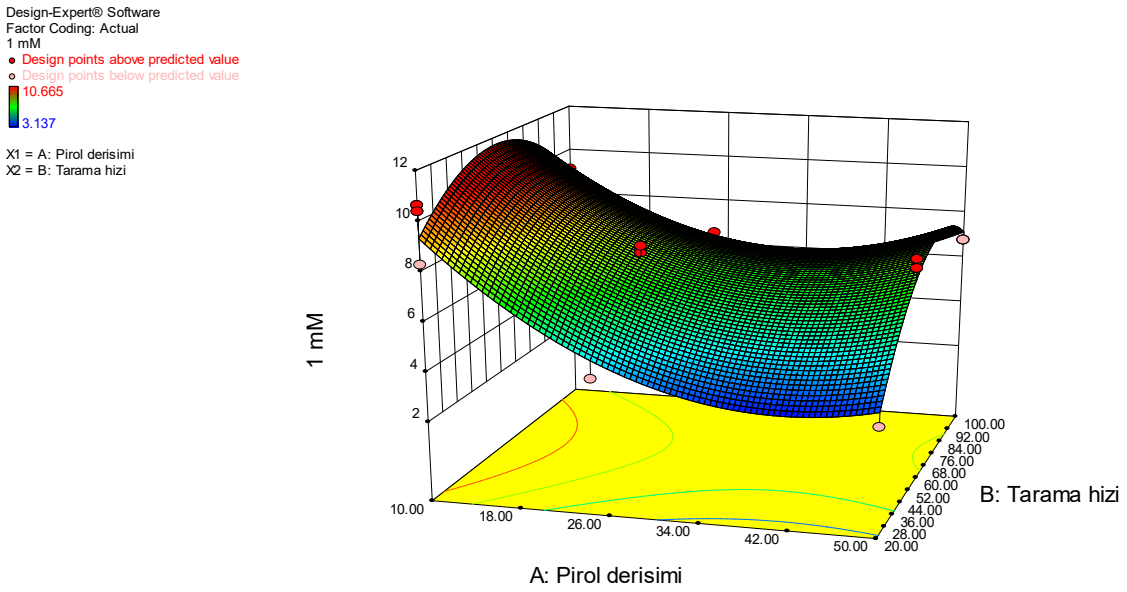

Figure 2. Surface diagram for current response depending on CPy and SR

As seen in Figure 2, RSM study gave important results that the effects of simultaneous change of CPy and SR and possible response at every possible point could be obtained. Current response decreased by increase in monomer concentration at PPy synthesis while, high current values were observed when PPy synthesis was carried out applying 50 mV/s scan rate. So, optimal PPy synthesis conditions were chosen as 10 mM pyrrole concentration and 50 mV/s scan rate. Xu et al. (2012) constructed a glucose biosensor by GOD adsorbtion onto PPy and Pt nanoparticle modified electrode and they found optimal conditions as 200 mM pyrrole and 50 mV/s scan rate. As a result of data evaluation, ANOVA analysis was carried out by Design Expert Programe for quadratic model (Table 4).

It is clearly seen in Table 3 that, the effect of C_{Py} on current response increadibly higher than due to higher sum of squares. If p-value of prob>F less than 0.0500 indicate model terms are significant. In this case, all model terms, A, B, AB, A^2 and B^2 are significant model terms. The "Lack of Fit F-value" of 3.04 implies the Lack of Fit is not significant relative to the pure error. There is a 10.2% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good; the model is wanted to fit.

Table 4. ANOVA analysis for C_{Py} and SR optimization study (Quadratic model).

ANOVA for Response Surface Quadratic Model
Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	52.00	5,00	14885,00	16011,00	0.0007	significant
A-(C_{Py})	29.79	1,00	29.79	32.73	0.0002	
B-(SR)	24563,00	1,00	24563,00	41395,00	0.0470	
AB	19480,00	1,00	19480,00	41826,00	0.0334	
A^2	15.90	1,00	15.90	17.47	0.0019	
B^2	17.42	1,00	17.42	19.14	0.0014	
Residual	41921,00	10,00	0.91			
Lack of Fit	42125,00	3,00	26299,00	41732,00	0.1020	not significant
Pure Error	34759,00	7,00	0.56			
Cor Total	61.10	15,00				

The 67 times magnified digital images of electrodes obtained at different stage were given in Figure 3.

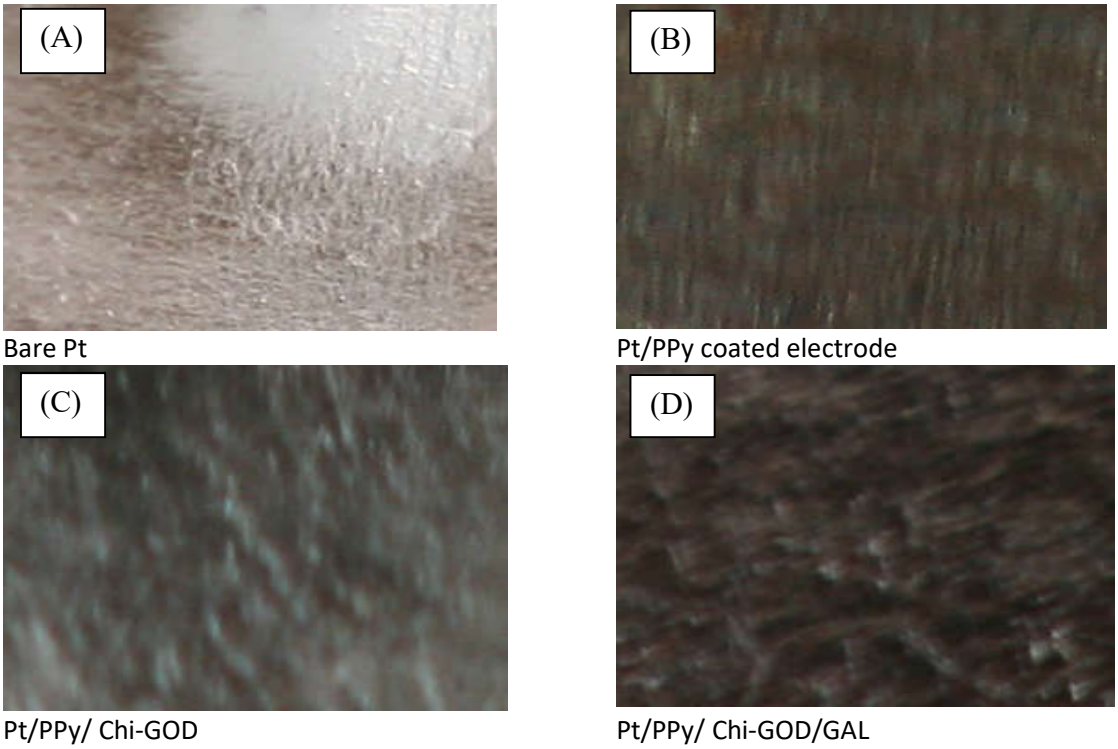

Figure 3. Digital photos of electrode surface at different preparing stages (67 times magnituted).

The colour of Pt/PPy electrode was turned dark blue (B) while bare Pt electrode was metallic bright gray (A). After enzyme immobilization, Pt/PPy/Chi-GOD (C) surface was dark gray and finally, the colour of Pt/PPy/Chi-GAL /GAL (D) electrode turned to brown probably due to magenta coloured schiff base formation between aldehyde groups of GAL and amine groups of enzyme and Chi matrix (Ozyilmaz et al. 2005). After each interaction on the surface of the Pt electrode, the surface shows a different colour transformation which may be considered the interaction took place.

As the highest net current response was obtained at a 10 mM Py concentration and a scan rate of 50 mV /s, these conditions were considered as optimum values. The CV curve of film growth during PPy synthesis at the optimum parameters is shown in Figure 4.

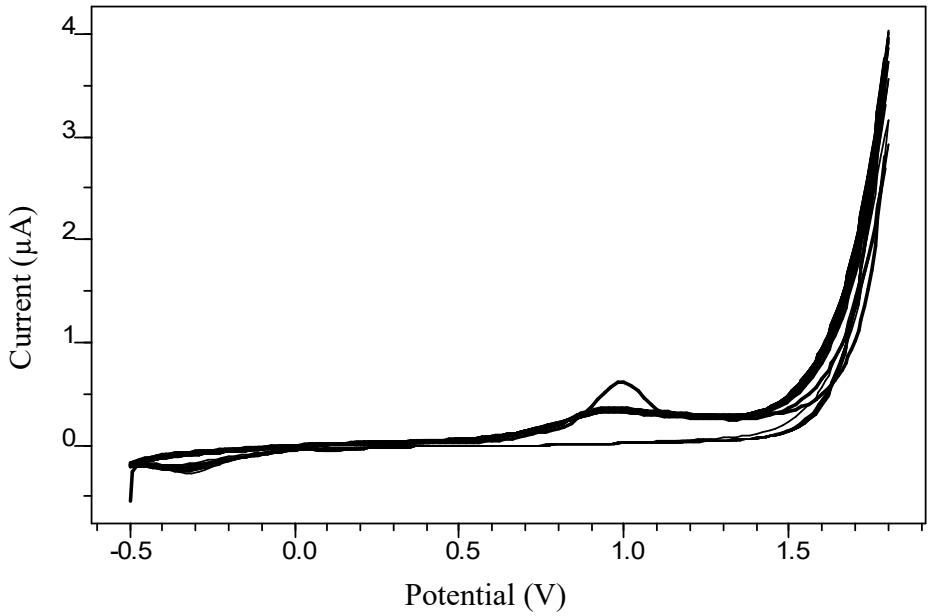

Figure 4. CV curve of synthesized PPy on Pt electrode at optimal monomer concentration (10 mM) and scan rate (50 mV/s)

When the CV curve given in Figure 4. was examined, it was seen that the downward-looking peak in the potential range of -0.5 to -0.2 V represented the reduction occurring in the polymer film. The straight line above, corresponding to the same peak, also indicated that the passive layer on the surface occured. The peak occurring at the potential range of 0.8-1.1 V corresponded to monomer oxidation.

Optimization of Chi, GOD and GAL concentrations at construction of enzyme electrodes by RSM

At this stage, RSM was applied to optimize Chi, GOD and GAL concentrations for enzyme immobilization on the PPy layer formed at the optimum pyrrole concentration (10 mM) and scan rate (50 mV/s) previously determined. Box-Behnken design was used applying high and low values for Chi as 0.25 and 1.00 %, for GOD as 1.0 and 4.0 mg/mL, for GAL as 0.025 and 0.100 %, respectively. 17 of working set was generated by programme and thus 17 electrodes were

constructed. Each electrode was incubated in 50 mM phosphate buffer at a working potential of 0.8 V until steady state currents were obtained, then the net current values in 1 mM glucose solution were measured. Surface diagrams for current responses depending on studied parameters were given in Figure 5.

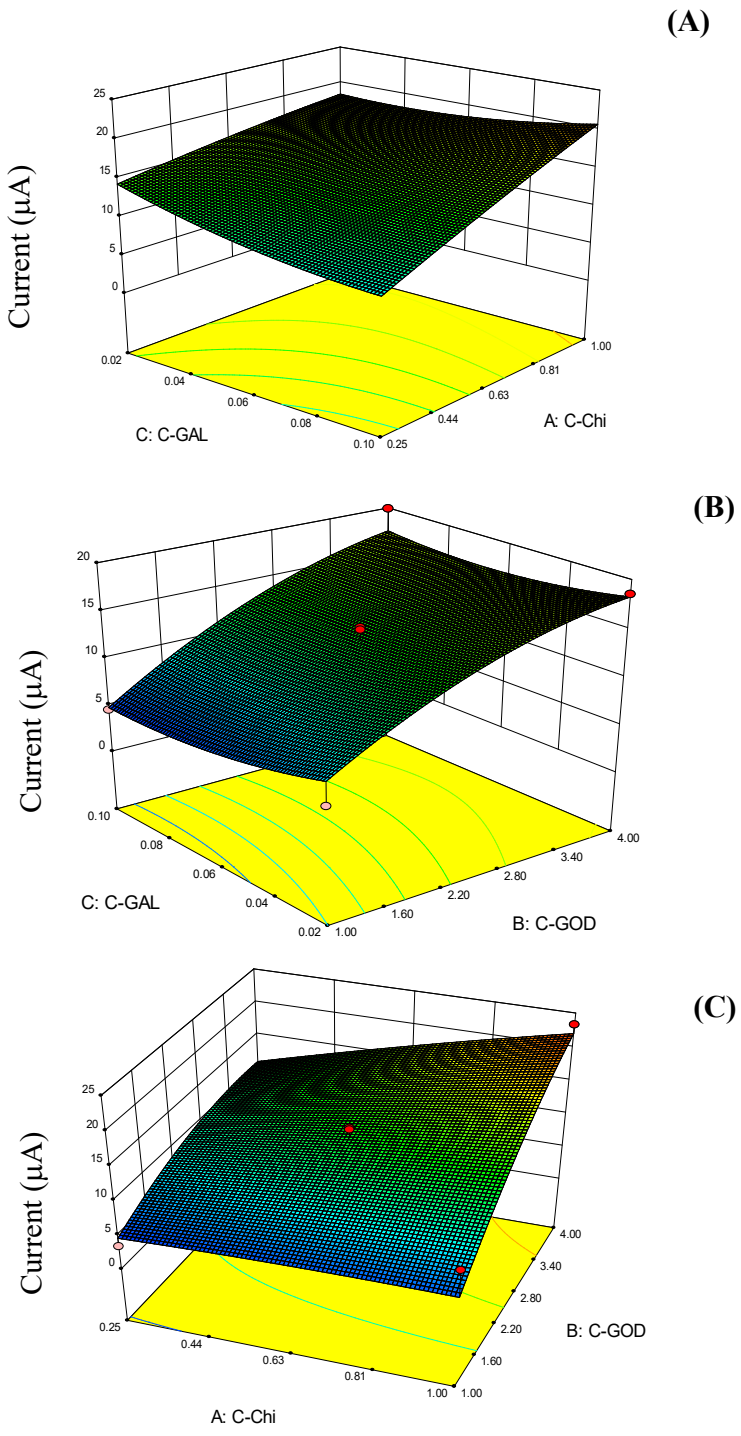

Figure 5. Surface diagrams of current responses depending on Chi, GOD and GAL concentration obtained by Box-Behnken Design

As shown in Figure 5 (A), it is clear that the Chi variable determined the response surface. As the Chi concentration increased, the current values increased and no significant effect of GAL concentration was observed. As seen in Figure 5 (B), the current response increases with the GOD concentration. For stationary GOD concentration, there is no significant effect of GAL concentration. As shown in Figure 5 (C), as the GOD concentration increases, the current response values increase. In addition, as the Chi concentration increased, the increase in net current values due to the increase in GOD concentration also increased. The highest current response was obtained for 1.0% Chi and 4 mg / ml GOD concentration.

The highest current value was observed when using enzyme electrode prepared with 1% Chi, 0.0625% GAL and 4 mg / ml GOD.

Characterization of Enzyme Electrodes

CV Analysis of enzyme electrode

The cyclic voltammogram curve (CV) obtained for Pt / PPy / Chi-GOD/ GAL electrode with a scan rate of 50 m /s in buffer solution (glucose free) and in glucose solution is given in Figure 6. The cyclic voltammetry technique is a technique used to observe the oxidation and reduction events occurring at the electrode surface at the metal / solution interface.

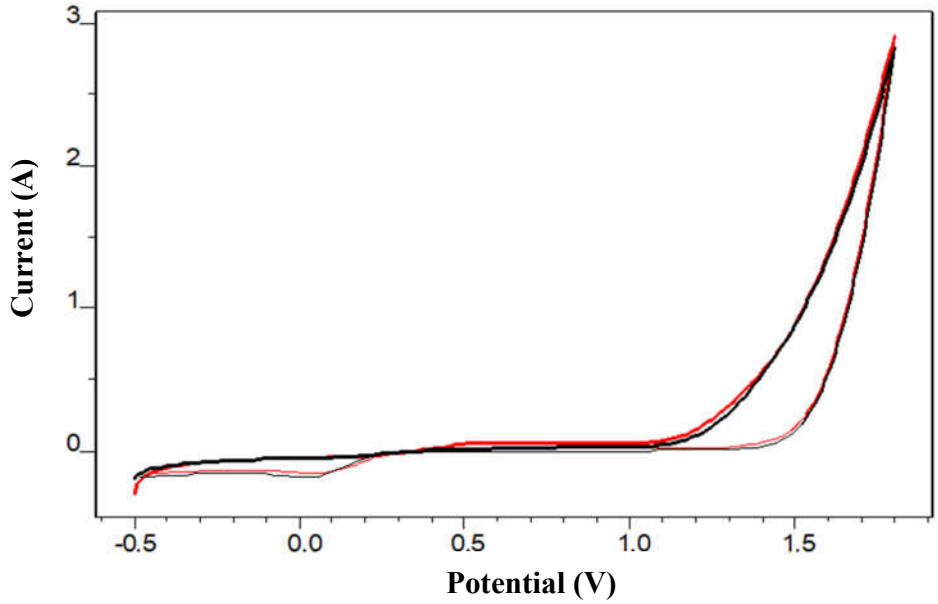

Figure 6. Cyclic voltammograms of enzyme electrode in the buffered glucose free (▪) and glucose (▪) solution (Scan rate: 50 mV/s)

The current increase after 0.45 V in the glucose buffer solution compared to the current values obtained in the glucose free buffer solution at -0.5 V and 1.1 V potential range during the curve anodic scan as shown in Figure 6 shows that the glucose-originated hydrogen peroxide fragment the reactions come to a conclusion.

Impedance analysis of enzyme electrode

The impedance measurement as Nyquist diagrams of the Pt / PPy / Chi-GOD / GAL electrode in glucose-free and glucose-containing buffer solutions is given in Figure 7. The semi circle part of Nyquist diagram corresponding to uncharged region at the metal/solution interface extending from high frequency to low frequency region is due to resistance which origin from difficulty of electron transfer at the electrode surface. The smaller diameter of the semi circle region corresponding to the charge transfer resistance in the high frequency region at the metal/solution interface, the easier the electron transfer for possible electrochemical reactions (Özyılmaz et al. 2014).

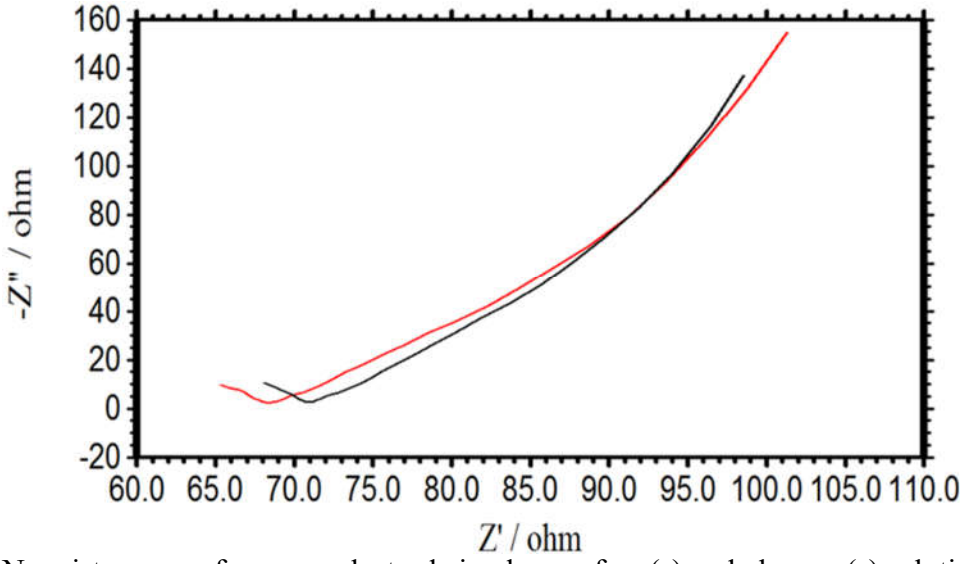

Figure 7. Nyquist curves of enzyme electrode in glucose free (▪) and glucose (▪) solution

As seen in Figure 7, the smaller diameter of the partial half region which is corresponding to the charge transfer resistance observed in the high frequency region in the Nyquist curves obtained for the glucose-containing buffer solution indicates that the charge transfer is better.

Investigation of the effects of the working pH and applied potential on current response by RSM

RSM was used for enzyme electrode which was constructed at optimal conditions investigation the working pH and applied potential. Optimal design was used for five different pHs (4, 5, 6, 7 and 8) and potentials (0.4, 0.5, 0.6, 0.7 and 0.8) and 16 set of study was constructed by the programe. Current response was measured for each working set results were given in Table 5.

Table 5. Investigation of the effect of pH and applied potential on current response by RSM using optimal design.

Working Set	Applied Potential (V)	pH	Current Response (µA)
1	0.40	4	6.90
2	0.60	5	20.53
3	0.60	4	13.45
4	0.50	7	16.94
5	0.40	8	16.43
6	0.60	5	22.88
7	0.80	6	25.32
8	0.80	8	9.69
9	0.60	8	20.37
10	0.50	7	18.29
11	0.70	7	25.73
12	0.40	5	2.97
13	0.60	5	18.13
14	0.80	6	27.07
15	0.80	6	21.04
16	0.40	5	2.41

As seen in Table 5, the highest current response was observed at pH 6 by applying 0.8 V of potential. Surface diagram was given in Figure 8. As seen in Figure 8, the effect on current response is higher than pH value. When potential was small current response was observed; as potential increased, current response appears to increased. The working pH affected current response because GOD activity changed depending on pH of solution. Optimal pH of immobilized GOD is reported as 6.0 in our previous study (Ozyilmaz et al 2005). The biosensor response increased with increasing potential below 0.60 V. This indicated that the biosensor response was controlled by the kinetics of enzyme catalysis reaction and electrochemical process. When the potential is over 0.60 V, the response changes slightly. This indicates that the biosensor response was controlled by the diffusion of the substrate and the product (Shan et al.2008). RSM study gave important results that the effects of simultaneous change of pH and potential and possible response at every possible point could be obtained. For example, at the 0.4V potential there was no any significant change at

the current response, but, the effect of pH on current increased prominently by increasing potential value.

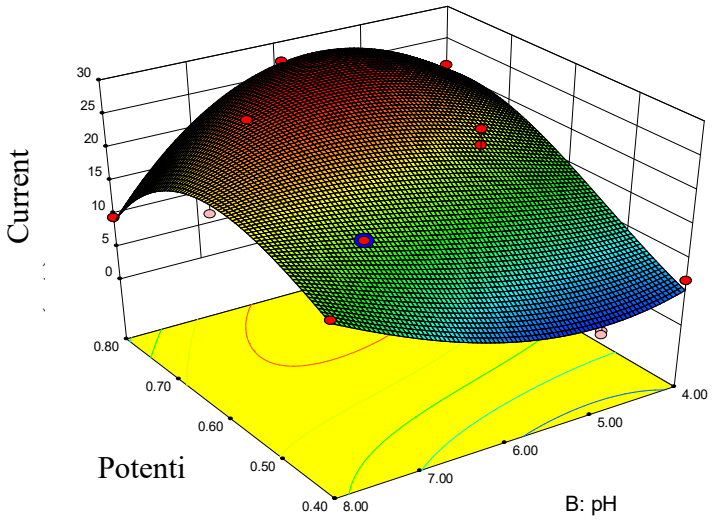

Figure 8. Surface diagram obtained from RSM study for current response depending on working pH and applied potential.

Conclusion

A glucose sensitive biosensor was constructed by GOD immobilizing onto PPy coated Pt electrode. Enzyme immobilization was carried out by immersing PPy coated electrode to GOD containing Chi solution afterwards reacted with GAL solution. Enzyme electrode was used to measure current response depending on glucose molecule at the constant potential. Because current responses would change due to construction parameters of electrodes as well as working conditions, all parameters were optimized in terms of RSM in detail. It was concluded that, according to 3 different RSM studies, pyrrole concentration, GOD concentration and applied potential were the most efficient parameters for the stage of PPy synthesis, GOD immobilization and measurement of current response studies, respectively.

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