ISSN: 2458-8989

Natural and Engineering Sciences

NESciences, 2022, 7(1): 22-33 doi: 10.28978/nesciences.1098655

Optimization of Graphite-Mineral Oil Ratio With Response Surface Methodology in Glucose Oxidase Based Carbon Paste Electrode Design

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Abstract

In this study, construction of amperometric glucose biosensor was carried out by immobilizing of glucose oxidase (GOD) on carbon paste electrode (CPE) which was coated with polyaniline (PANI) by cyclic voltammetry technique. Since the current values to be measured depending on the glucose concentration will be affected by the graphite:mineral oil composition of the electrode, this parametres were optimized by response surface methodology (RSM). For this, State Ease Design Expert 8.0.7.1. (Serial Number: 0021-6578) software software was used applying Optimal Design. By using data obtained, Design Expert software suggested quadratic model to predict current values in terms of working parameters. In study, experimentally measured current values and predicted values by model were considerably found compatable and suitability of model was supported by ANOVA test.

Keywords:
Carbon paste electrode, Response Surface Methodology, Glucose Oxidase, biosensor, polyaniline

Article history:
Received 02 July 2021, Accepted 15 December 2021, Available online 04 April 2022

Introduction

The use of carbon paste electrodes in biosensor design has been increasing in recent years. These electrodes are very advantageous in terms of use, as they can be modified with many different technique. In biosensor applications of carbon paste, which is mainly composed of graphite powder and binder, both the composition of the paste and the CPE surface are modified in various ways. For this purpose, while preparing carbon paste metal or nanometal oxide (Svobodová et al., 2012; Comba et al., 2010), carbonnanotube (Anik & Çevik 2009; Li et al.., 2012) or modification with metallic structures (Meng-Qin et al., 2007; Amiri et al., 2012; Shamsazar et al., 2016) are among the common applications. In addition to these materials, polymeric structures are also suitable composites for immobilization of biological materials (Donmez, 2020; Sadeghi et al., 2015). Due to the redox properties of these materials, their use has become very common in recent years.

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Conductive polymers are excellent materials that can be used to apply biomolecules to the electrode surface in preparing biosensors. Polyaniline (PANI), which has become a popular research subject with the discovery of its electrical conductivity, has a wide variety of and important applications because of its stability in aqueous solutions and atmospheric environment, cheap monomer, easy synthesis, high electrochemical activity. One of the most common causes of food intolerance is lactose intolerance, which occurs after the use of milk and dairy products. Lactose found in milk and dairy products is digested in the small intestine and converted into glucose and galactose. However, if no lactase catalyzes this conversion, or if it is in small amounts, lactose that passes into the large intestine creates a food source for microorganisms and leads to the formation of fatty acids and gases (such as hydrogen, carbon dioxide, and methane) by microorganisms (Delacour et al., 2017). These fatty acids and gases cause abdominal bloating, pain, and eventually diarrhea in people with lactose intolerance. While most newborns have high concentrations of lactase, its concentrations decrease after weaning (Casellas et al., 2009). This occurs to varying degrees in different populations and can cause lactose intolerance.

One of the most important biological components used in the design of amperometric biosensors are enzymes. In biosensor design, enzymes are mostly immobilized on the electrode surface. Immobilization significantly affects interaction with its substrate of the enzyme in the structure of the electrode. Therefore, enzyme immobilization is an important step in biosensor design. The first and most widely used enzyme used in biosensor design is glucose oxidase (Luque et al., 2005; Li & Lin, 2007; Comba et al., 2010; Ozyilmaz et al., 2011; Ozyilmaz et al., 2018; Ozyilmaz et al., 2019).

Optimization is an important work step carried out at the beginning of almost every research in order to increase efficiency in experimental studies, increase quality in production, reduce costs and save time. Traditional test methods require high material cost, long time and resources. In order to investigate the effect of each parameter on the experiment, one parameter at a time is tested, keeping all other parameters constant. In this case, it does not allow examining more than 4 or 5 levels of the parameters at the same time. Therefore; In order to reduce the number of experiments, save time and cost, use resources efficiently, and accelerate research and development activities, it is necessary to design experiments that will measure the examined value of the system. The response surface methodology (RSM) is a very useful technique that meets these goals and can be adapted to many different work areas. RSM provides the factors to be determined in order to find the best response in terms of the process, the factor levels that are effective in the process, the determination of new production conditions in order to provide a product quality above a quality level obtained under current conditions, the definition of the relationship between the response and quantitative factors with a model is to estimate a region (a range of parameters whose effectiveness is investigated) and the optimum point of this region in a design plane consisting of many parameters that are effective on the result in an experimental study. It is decided with the help of regression coefficients how important the effect of a factor or its interaction with other factors has on the values of the response variable (Turan & Altundoğan, 2011).

The aim of this study is to design a glucose sensitive biosensor by immobilization of glucose oxidase enzyme on a polyaniline conductive polymer film synthesized on CPE by cyclic voltammetry technique. Because the current values determined in presence of glucose would be affected from biosensor construction and working conditions, graphite and mineral oil ratio were optimized using response surface methodology.

Materials and Method

Materials

Aniline, sodium oxalate, sodium hydroxide, disodium phosphate, hydrochloric acid were purchased from Merck; glucose, graphite powder (< 20 µm), mineral oil (0.84 g/ml), Glucose oxidase (GOD) from Aspergillus niger (429 U.mg-1, 24 mg. ml-1) were obtained from Sigma.

Method

Preparation of carbon paste electrode

CPE was prepared with graphite powder and mineral oil by throughly hand mixing. A portion of the resulting paste was packed firmly into a insuline syringe. The electrode surface is smoothed and polished with a soft paper before use. A copper wire is placed inside the electrode to provide conductivity.

Optimization Rate of Graphite powder and Mineral oil

In this study, RSM was performed with State Ease Design Expert 8.0.7.1 (Serial number: 0021-6578) software by applying Optimal Design. Factors were chosen as graphite amount (X1) and Mineral oil amount (X2) with three level which were summarized in the Table 1. The lowest and highest values of the levels were determined by preliminary studies so as to create a paste suitable for the study.

Table 1. Levels of factors used in experimental design

Factors	**Name**	**Levels**		
		-1	**0**	**+1**
X_1	Graphite (g)	1.25	1.50	1.75
X_2	Mineral Oil (g)	0.63	0.84	1.05

With the values given in Table 1, 16 study sets were created by the software, and carbon pastes were prepared by mixing graphite and mineral oil in the proportions determined for each set. Using the graphite-mineral oil mixtures obtained for each set, the carbon paste electrodes (CPE) were prepared as described in the previous section. After the electrodes were created, each electrode was used in the enzyme electrode design under the same conditions. First, a PANI film was coated on the CPE surface, then GOD was immobilized on the polymer surface and finally glucose sensitive enzyme electrodes were formed.

Synthesis of PANI film on the CPE surface

PANI was used as a model polymer layer to determine the optimal graphite: mineral oil ratio in the development of a glucose sensitive CPE-based biosensor. To this, 25 mM aniline monomer was prepared in 0.10 M sodium oxalate (Na-Ox) electrolyte solution and PANI was synthesized in 2 step according to preliminary studies. In the first step, 2 segments were applied with 20 mV/s scan rate at -0.2 / 0.85 V potential range, and immediately after, polymer synthesis was completed with 6 segments at 50 mV /s scan rate at -0.2 / 0.7 V potential range. Synthesis of PANI layer was carried out by cyclic voltammetry (CV) technique. Electrochemical studies were carried out in a single compartment cell with three electrode configurations. Ag/AgCl (3 M KCl) electrode was the reference electrode and a platinum plate with a surface area of 0.25 cm2 was the counter electrode. CHI 660b model electrochemical analyzer (serial number: A1420) was used in electrochemical experiments.

Preparation of Enzyme Electrodes

To construct the enzyme electrode, glucose oxidase (GOD) was immobilized by crosslinking onto PANI coated surface of CPE (CPE/PANI). To this, 10 µL of the GOD solution at a concentration of 3 mg / mL and and then 10 µL of GAL solution at a concentration of 0.075% were dropped on the CPE/PANI surface. Glutaraldehyde was used as crosslinking agent. After electrodes washed with distilled water twice, the prepared enzyme electrodes (CPE/PANI/GOD) were dried at room temperature for 90 minutes and kept at 4 °C until it was used.

Current Measurements

The formed electrodes were first kept in pH 6 phosphate buffer until steady-state currents were obtained at 0.7 V potential, then current values were measured in 20 mM glucose solution by chronoamperometric method. The biosensor response was monitored by the chronoamperometric technique at 0.70 V as current value that was measured depending on hydrogen peroxide oxidation which was formed by the GOX activity in the glucose solution. The measurements were performed at room temperature and each measurement was lasted 120 s.

Results and Discussion

In this study, the surface of the CPE was coated with PANI film by CV technique and a glucose sensitive biosensor was constructed by immobilizing GOX on the polymer surface. The current values resulting from the oxidation of hydrogen peroxide, which is produced enzymatically by the obtained biosensor in the presence of glucose, at constant potential by chronoamperometric method, was measured. Current responses depending on the graphite: mineral oil composition of the paste was investigated by RSM. Preparation and use of the biosensor is given schematically in Figure 1.

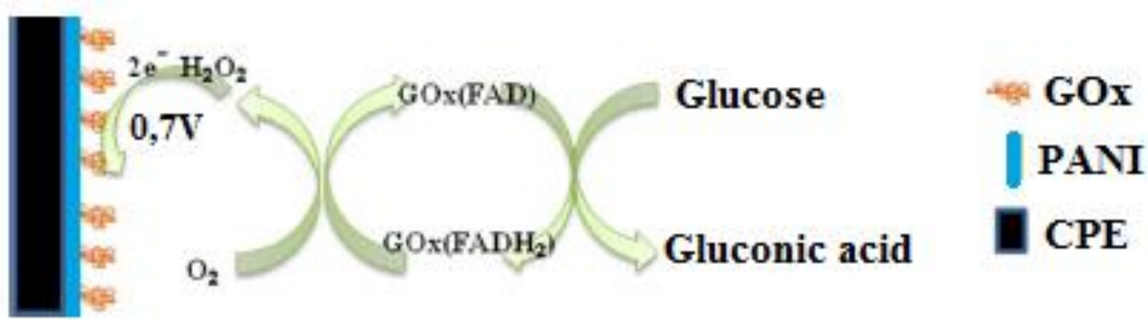

Figure 1. Schematic representation of the preparation and use of the biosensor.

Optimization of Graphite:Mineral oil ratio

In order to determine the optimal graphite:mineral oil ratio with RSM, 16 different experimental sets were created by the software for 3 different graphite and mineral oil amounts. In the experimental sets created by selecting the optimal design, PANI synthesis was first performed on the surface of each of the 16 CPEs, and then 16 glucose-sensitive electrodes were formed by enzyme immobilization. Graphite and mineral oil compositions of the enzyme electrodes prepared, and also current values measured at 20 mM glucose concentration are given in Table 2. When Table 2. was examined, since the current response value of the electrode no. 15 was found to be significantly higher than the other electrodes, the ratio of graphite: mineral oil ratio was chosen as 1.75:0.63.

Table 2. Graphite and mineral oil composition and current values of CPE electrodes.

Experiment	**Graphite (g)**	**Mineral oil (g)**	**I (µA)**
1	1.25	0.63	3.05
2	1.50	0.84	2.44
3	1.75	1.05	3.27
4	1.25	0.63	3.22
5	1.50	1.05	2.19
6	1.50	1.05	2.25
7	1.25	1.05	2.15
8	1.75	0.84	3.54
9	1.50	0.63	3.79
10	1.75	0.84	3.54
11	1.50	0.84	2.88
12	1.50	0.84	3.00
13	1.25	0.84	2.34
14	1.50	0.63	3.60
15	**1.75**	**0.63**	**4.79**
16	1.50	0.84	2.39

When the current values given in Table 2 were analyzed in the Design Expert software, model analysis was carried out for compliance with linear, two-factor interaction (2FI), quadratic and cubic models, and the results were summarized in Table 3. As can be seen from the results in Table 3, the best definition for 20 mM glucose solution can be made with the quadratic model. In the quadratic model, it is seen that the p-value of the model fit (Lack of Fit), the adjusted coefficient of determination (R2adj) and the estimated coefficient of determination (Pred-R2) are higher than the other models, so this model is recommended as statistically significant.

Table 3. Model Fit analyse for CPE electrodes

Source	**Sequential p-value**	**Lack of Fit p-value**	$\mathbf{R^2_{adj}}$	**Pred-$\mathbf{R^2}$**
Linear	< 0.0001	0.0554	0.8081	0.7282
2FI	0.5473	0.0424	0.7985	0.5349
Quadratic	***0.0021***	***0.7444***	***0.9294***	***0.8995***
Cubic	0.6010	0.6174	0.9223	0.8400

In the optimization study of graphite: mineral oil ratio, Design Expert software proposed Equation 1 depending on the real variables to estimate the current responses to be measured for 20 mM glucose.

$$
\begin{aligned}
I\ (\mu A/cm^2) = &+ 14.57296 \\
&- 9.99729 \times \textbf{Graphite} \\
&- 12.00840 \times \textbf{Mineral oil} \\
&- 2.72281 \times \textbf{Graphite} \times \textbf{Mineral oil} \\
&+ 4.96999 \times \textbf{Graphite}^2 \\
&+ 7.66277 \times \textbf{Mineral oil}^2 \qquad \text{(Equation 1)}
\end{aligned}
$$

The effect on the model of the terms used in the proposed mathematical model was examined by analysis of variance (ANOVA) using the least squares method, and the statistical data determined in order to test the conformity of the model for ANOVA values are given in the Table 4.

Table 4. ANOVA analysis for Quadratic Model

Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**
Model	7.57	5	1.51	40.51	< 0.0001 significant
A-Graphite	3.24	1	3.24	86.55	< 0.0001
B-Mineral oil	3.94	1	3.94	105.39	< 0.0001
AB	0.095	1	0.095	2.55	0.1411
A^2	0.37	1	0.37	10.00	0.0101
B^2	0.43	1	0.43	11.40	0.0070
Residual	0.37	10	0.037		
Lack of Fit	0.057	3	0.019	0.42	0.7444 not significant
Pure Eror	0.32	7	0.045		
Cor Total	7.95	15			

Std. Dev	0.19	**R-Squared**	0.9530
Mean	3.02	**Adj R-Squared**	0.9294
C.V.	6.38	**Pred R-Squared**	0.8995
PRESS	0.79	**Adeq Precision**	22.508

For an ideal model, the regression model should be significant and the “lack of fit” should be not significant (Myers & Montgomery, 1995). The proposed model is suitable in this respect.

The importance of each parameter used in the created model was determined with the "F-value" and "p> F value" given in Table 4. As the 'F-value' increases and the 'p> F value' decreases, the importance of the parameters affecting the current response of the biosensor in the created model also increases. In this case, on the current response of the electrode, the effect of the mineral oil used in the paste formation is more than graphite.

However, to test the suitability of the proposed model for the highest current value obtained, the coefficient of determination (R2) calculated by the Design Expert software, the corrected indication coefficient (R2adj), the coefficient of variation (CV), the estimated residual sum of squares error (PRESS), Pred Statistical results such as -R2, sufficient sensitivity (Adeq Precision) were evaluated. R2 shows to what extent the changes in the values of dependent (response) variables can be explained by experimental factors and interactions. The R2 value is always between 0 and 1, and the closer it gets to 1, the better the model's suitability and predictive use. Since the R2 value in this study is 0.9530, it is seen that 95.3% of the changes in the dependent variable are explained by the independent variables.

Since statistically insignificant terms can be used in the model, high R^2 values can be encountered in the evaluation of the created models. For this reason, it is recommended to use R2adj values to evaluate the suitability of the model (Aygün, 2012). In this study, R^2_{adj} value was calculated as 0.9294. The fact that R^2 and R^2_{adj} values are close to each other (2.36%) is proof that the current values estimated by the applied model reflect the real results obtained by experimental studies quite well.

C.V. states what percentage of the standard deviation changes from the mean. Great C.V. values indicate that the data diverged too far from the mean, small C.V. values show that the data have almost the same value as the average (Lazic, 2004). C.V. is 6.38% is a positive situation in terms of using the model for estimation.

The difference between R^2_{adj} and Pred-R^2 values should not be more than 0.2. The proposed model is not suitable if there is a change of more than 20% between the explanatory rates of these specification coefficients. The difference between these two determination coefficients was found to be 0.0299 (2.99%) for the current response, so the model formed is suitable.

In order for the regression model to be suitable, the Adeq Precision value must be greater than 4 (Myers ve Montgomery, 1995). Since Adeq Precision value in this study was calculated as 22.508, the predicted model is also suitable for this criterion.

PRESS value is desired to be lowest compared to other mathematical models. The PRESS value obtained in the ANOVA analysis is suitable in this regard.

As a result of all these statistical evaluations, it was concluded that the model created and the independent variables (graphite and mineral oil amounts) used in the creation of this model can be used in estimating the current value of the biosensor, provided that the range values are taken into account.

The relationship between the estimated results created by using Equation 1 and the actual experimental results is shown in Figure 2. As seen in Figure 2, there is no significant difference between the current responses of the biosensor estimated by the model, depending on the reaction conditions, and the actual current values obtained experimentally. This shows that the model can be used reliably in estimating the current value.

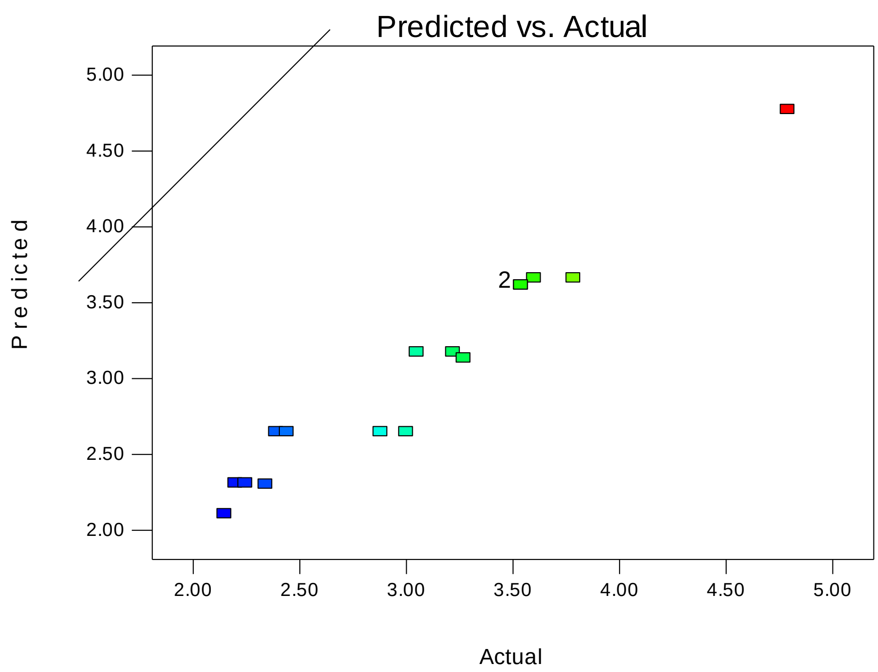

Figure 2. Comparison of experimental results and predicted results

The three-dimensional surface graph created by the Design Expert 8.7.0.1 software in order to examine the effect of the graphite:mineral oil ratio used in the preparation of the carbon paste on the current value of the biosensor is given in Figure 3.

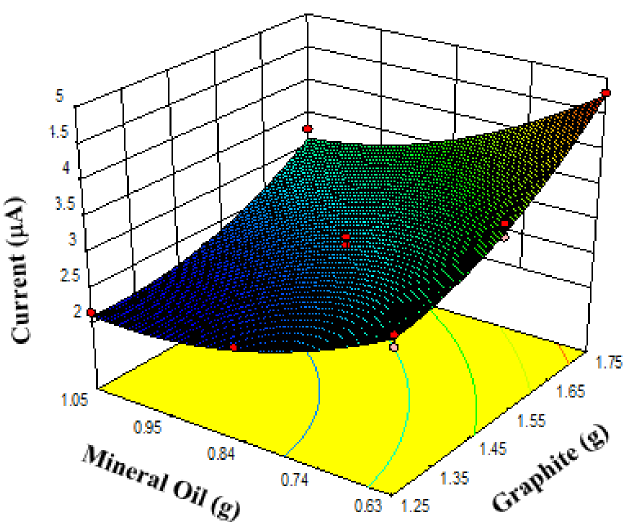

Figure 3. Surface diagrams of the current responses in 20 mM glucose solution of biosensors prepared with different ratios of graphite and mineral oil.

When the surface graph in Figure 3 was examined, it was observed that the measured current value increased as the amount of mineral oil decreased and the amount of graphite increased. When all the results were evaluated, it was seen that the paste prepared with 1.75 g graphite: 0.63 g mineral oil ratios could be used in biosensor design where the highest current value would be observed. From this result, it can be concluded that since mineral oil is not conductive, the conductivity of the electrode formed by using the lowest possible amount of mineral oil is high. It has been reported in the literature that CPEs were prepared in different graphite / mineral oil ratios to design biosensors. For example, for 100 mg of graphite Liu and Ju (Liu & Ju, 2003) used 36 µl of mineral oil, while 50 µl and 80 µl mineral oil was used by Liu et al. (2005) and Shoja et al. (2019), respectively.

In conclusion, carbon paste electrodes can be modified in many different ways and developed using different techniques and materials. In this study, the use of carbon paste electrode prepared with a mixture of graphite and mineral oil in the design of GOX-based amperometric glucose biosensor was investigated. The variation of the current value to be measured depending on the graphite/mineral oil composition of the carbon paste was investigated by RSM. The results showed that the CPE to be formed with the least amount of mineral oil possible, provided the highest glucose-dependent current value to be reached.

Acknowledgements

This study was funded by Scientific Research Units of The University of Mustafa Kemal in Turkey, Project no: 12980

Author Contributions

E.Y.contributed to the experimentation of the study and the writing of the article. G. O. contributed to the planning of the study, rsm analysis and article writing. A.T.O. contributed to conductive polymer synthesis and measurement of current by amperometric glucose biosensor.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author.

Conflict of Interest

The authors declare that they have no competing interests.

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