Glucose Electrode Based on Immobilized Glucose Oxidase on a Tungsten-tungsten Oxide Electrode

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Um eletrodo para determinação de glicose foi desenvolvido. O eletrodo para glicose foi construído imobilizando glicose oxidase sobre uma membrana de acetato de celulose com glutaraldeído e montada sobre um eletrodo de tungstênio - óxido de tungstênio. Os íons H_3O^+ produzidos na reação enzimática foram detectados no eletrodo de tungstênio. Os efeitos da posição da camada de enzima, quantidade de enzima, pH, capacidade tamponante e interferentes sobre a resposta do eletrodo foram estudados. Para diferentes valores de pH, a resposta do eletrodo é linear nas seguintes faixas de concentração: 2.81×10^{-4} - 2.04×10^{-3} M (pH=4,7); 2.40×10^{-4} - 1.66×10^{-4} M (pH=5,5); 2.48×10^{-4} - 2.04×10^{-3} M (pH=6,00); 1.95×10^{-4} - 1.59×10^{-3} M (pH=6,5); 2.20×10^{-4} - 1.86×10^{-3} M (pH=6,8); 1.68×10^{-4} - 2.04×10^{-3} M (pH=7,3) and 2.37×10^{-4} - 1.86×10^{-3} M (pH=7,6) com inclinação de 56,2; 51,3; 47,7; 41,5; 33,9; 22,8; e 25,6 mV/década, respectivamente. A aplicação deste biossensor na determinação de glicose em um produto farmacêutico foi realizada.

An electrode for the determination of glucose is described. The glucose electrode is constructed by immobilization of glucose oxidase on a cellulose acetate membrane with glutaraldehyde and mounting over the tip of a tungsten-tungsten oxide electrode with an O-ring. This biosensor is based on the detection of H_3O^+ by the tungsten electrode, produced by the enzymatic reaction. The effects of the position of the immobilized enzyme layer, enzyme amount, pH, buffer capacity and interferents on the response of the electrode are studied. For different pH values, the electrode response is linear in the following concentration ranges: 2.81×10^{-4} - 2.04×10^{-3} M (pH=4.7); 2.40×10^{-4} - 1.66×10^{-4} M (pH=5.5); 2.48×10^{-4} - 2.04×10^{-3} M (pH=6.00); 1.95×10^{-4} - 1.59×10^{-3} M (pH=6.5); 2.20×10^{-4} - 1.86×10^{-3} M (pH=6.8); 1.68×10^{-4} - 2.04×10^{-3} M (pH=7.3) and 2.37×10^{-4} - 1.86×10^{-3} M (pH=7.6) with slope of 56.2, 51.3, 47.7, 41.5, 33.9, 22.8 and 25.6 mV/decade, respectively. Application of this biosensor for glucose determination in a pharmaceutical product is reported.

Key words: glucose determination; glucose electrode; tungsten-tungsten oxide; potentiometric determination.

Introduction

All enzymatic reactions in which acids or bases are generated can be monitored through a pH electrode. The well known glass pH electrode has been used for the construction of biosensors for glucose¹, urea^{1,4}, penicillin^{1,2,5,6}, and acetylcholine esters². In addition to conventional glass pH electrodes, several metal-metal oxide electrodes have been used as pH sensors. Of those, the most used are the antimony & palladium^{8,9}, niobium¹⁰, copper¹¹, tantalum¹², stainless

steel¹³ and tungsten¹⁴ electrodes. Some of these electrodes have also been used as potentiometric biosensors for urea: antimony¹⁵, titanium covered with¹⁶ IrO_2 , palladium¹⁷, and tungsten¹⁸. Penicillin was determined by immobilization of penicillinase on a thin film antimony electrode¹⁹. Trypsin^{20,21} and chymotrypsin²⁰ were immobilized on titanium electrodes for the determination of trypsin inhibitor²¹ and esters of amino acids²⁰.

In the present paper, a biosensor is described for the determination of glucose. This glucose electrode was con-

structed by chemically immobilizing glucose oxidase over the tip of a tungsten - tungsten oxide electrode.

Glucose oxidase [E.C.1.1.3.4] reversibly catalyzes the hydrolysis of glucose to H_2O_2 and gluconic acid (Eq. 1):

The hydronium ion generated in the dissociation of gluconic acid is sensed by the electrode described, the steady state potential of which is proportional to the activity of H_3O^+ ions, i.e. the concentration of glucose in the solution.

Experimental

Apparatus. Potentiometric measurements were made with an Orion EA™ 940 pH/mV meter connected to a strip-chart recorder ECB model RB201. An electrode of tungsten - tungsten oxide covered with a thin film of glucose oxidase on a cellulose acetate membrane (Spectra/por 2) and an Ag/AgCl saturated electrode were used as indicator and reference electrodes, respectively.

During the measurements the solutions were stirred using a Fisaton Model 702 magnetic stirrer. Measurements were carried out at 25°C.

Reagents. Analytical grade reagents were used to prepare buffers and standard glucose solutions. Phosphate buffers, glucose oxidase, glucose and other carbohydrates were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Phosphate buffers of various pH values (4.7, 5.5, 6.0, 6.5, 6.8, 7.3 and 7.5) were prepared at 0.2M ionic strength and used to prepare reference and interferent solutions.

The enzyme used was glucose oxidase [E.C.1.1.3.4.], type VII S from *Aspergillus niger*, with 150 units/mg protein.

Preparation of enzyme membranes. The Spectra/por 2^R molecular porous dialysis membrane (MW CO 12,000-14,000) from Spectrum Medical Industries, INC (Los Angeles, CA, USA) was washed with water overnight before use. Five microliters of enzyme solution containing 0.20 mg of glucose oxidase (30 units) dissolved in 0.2M phosphate buffer, pH 6.88, were transferred to the membrane with the help of a microsyringe. Three microliters 1% w/v bovine serum albumin were then added to it and the resultant material was mixed for 30s. Finally, 2 microliters of 2.5 % w/v glutaraldehyde solution were added and gently mixed for a few seconds. The resulting membrane was allowed to dry in a desiccator for 3 - 4h and afterwards washed with 0.1M glycine in phosphate buffer solution for 5min, and then in phosphate buffer before being placed on the tungsten - tungsten oxide electrode.

Preparation of enzyme electrode. The enzyme electrode was constructed as described elsewhere²². A 5mm diameter metal rod was sealed in a 10mm diameter glass tube; its surface was polished with 600 sandpaper and washed with distilled water. This electrode was used without further preparation due the formation of a thin oxide film. Then, the membrane with immobilized enzyme was fixed at the bottom

of the tungsten electrode with an O-ring. These membranes were placed with either inner or outer enzyme layer with regard to tungsten electrode.

Between measurements the biosensor was kept in a refrigerator at 4°C in a 0.2M phosphate buffer solution (pH 6.88).

Results and Discussion

Several parameters were investigated to evaluate the performance of the glucose electrode in terms of reproducibility, response time, recovery time, slope of calibration curve, selectivity and sample analysis.

Analytical Characteristics of the Tungsten-Tungsten Oxide. Several procedures of metal oxidation were investigated. Of these, the surface treatment with sandpaper was the best. A typical calibration curve for this electrode is presented in Figure 1. The electrode response was linear (correlation coefficient of 0.999) in the pH range of 5.0–7.5 with a super-nernstian response of -67.4 mV/decade. The response time for the indicator electrode was approximately 1 min for a pH change of one unit.

Figure 1. Potential vs. pH for tungsten - tungsten oxide electrode in 0.2M phosphate buffer, at 25°C.

Effect of the amount of enzyme. The effect of different amounts of immobilized glucose oxidase on both the response and stability of the glucose electrode was studied. The response of the electrode increases with increasing amounts used up to 30 units, then levels off between 31 and 55 units. Additionally, the stability of the electrode was not affected by the amount of the immobilized enzyme.

Effect of pH and Buffer Capacity. The effect of pH and the buffer capacity of the systems used on the performance of various enzyme electrodes is well documented by Guilbault *et al.*^{23,24} The characteristics of the electrodes were examined at pH 4.7, 5.5, 6.0, 6.5, 6.8, 7.3 and 7.5. Typical results of these measurements are shown in Figure 2 for the glucose electrode with inner and outer enzyme layers in 0.2 M phosphate buffer solution. The plots slope (mV/decade) vs. pH represent a mean of three determinations.

Maximum activity was obtained at pH 5 - 5.6, and it decreased rapidly after pH 6.0, and was completely lost at pH 9.0.

Figure 2: Effect of pH and position of the immobilized enzyme layer inner (-x-x-) and outer (-.-) in 0.2 M phosphate buffer.

The influence of buffer capacity on the glucose biosensor response with inner enzyme position (30 units), at pH 5.5 and 25°C, is shown in Table 1. The best performance of this electrode was found in the 0.2M phosphate buffer. Consequently, all studies were carried out with the 0.2 M phosphate buffer at pH 5.5.

Analytical Characteristics of the Glucose Electrode. Typical analytical curves for the glucose enzyme electrode are presented in Fig. 3. Table 2 shows the characteristics of the electrodes in 0.2 M phosphate buffer at 25°C for pH 4.7-7.6. As it can be seen from this Table, an increase in the pH of the phosphate buffer from 4.7 to 7.6 results in an increase in the response of the electrode up to pH 5.5 and then a decrease in its response. The best linear response (2.40×10^{-4} - 1.66×10^{-3} M) was obtained in pH 5.5 with a correlation coefficient of 0.993 and slope of 51.3 mV/decade, for the inner enzyme layer immobilized onto the tungsten - tungsten oxide electrode. The levelling off the response at higher glucose concentrations (upper limit) can be explained by the inhibition of the enzymatic reaction, due the excess of hy-

Figure 3. typical analytical curves for the glucose biosensor in 0.2 m phosphate buffer with position of the immobilized enzyme layer: outer (-.-) and inner (-x-x-), at 25°C.

Table 1. Effect of buffer capacity on the glucose biosensor response with inner enzymew layer membrane (30 U), at pH 5.5 and 25°C.

Buffer Capacity/mol.l-1	linear range/mol.l-1	slope/ mV/dec	correlation coefficient
0.05	2.82×10^{-4} - 1.86×10^{-3}	36.3	0.9489
0.10	2.82×10^{-4} - 1.66×10^{-3}	29.9	0.9278
0.20	2.40×10^{-4} - 1.66×10^{-3}	51.3	0.9931
0.50	2.82×10^{-4} - 1.66×10^{-3}	58.6	0.9636

* Each results is an average of three measurements.

drogen peroxide production (Eq. 1) that decreases the diffusion of substrate (glucose) into the enzymatic membrane layer. In addition, the weak acid formed in the enzymic reaction (gluconic acid, $pK_a = 3.77$)²⁵ decreases the pH at the surface of the pH sensor (tungsten-tungsten oxide), leading to a change in the analytical curve.

Table 2. Effect of pH and Enzyme Layer Position (30 U of Enzyme) on the Glucose Response in 0.2 M Phosphate buffer, at 25°C.*

pH	inner enzyme layer		
	slope/ mV/decade	concentration range/mol.l ⁻¹	correlation coefficient
4.7	56.2	2.81×10^{-4} - 2.04×10^{-3}	0.9949
5.5	51.3	2.40×10^{-4} - 1.66×10^{-3}	0.9931
6.0	47.7	2.48×10^{-4} - 2.04×10^{-3}	0.9931
6.5	41.5	1.95×10^{-4} - 1.59×10^{-3}	0.9941
6.8	33.9	2.20×10^{-4} - 1.86×10^{-3}	0.9966
7.3	22.9	1.68×10^{-4} - 2.04×10^{-3}	0.9852
7.6	25.6	2.37×10^{-4} - 1.86×10^{-3}	0.9929

* These results are a mean of three measurements

ph	outer enzyme layer		
	slope/ mV/decade	concentration range/mol.l ⁻¹	correlation coefficient
4.7	54.6	1.99×10^{-4} - 9.52×10^{-4}	0.9946
5.5	55.6	1.67×10^{-4} - 1.41×10^{-3}	0.9866
6.0	36.5	1.76×10^{-4} - 9.77×10^{-4}	0.9950
6.5	31.9	1.81×10^{-4} - 8.64×10^{-4}	0.9884
6.8	25.5	1.53×10^{-4} - 1.03×10^{-3}	0.9954
7.3	18.2	1.85×10^{-4} - 9.77×10^{-4}	0.9914
7.6	20.2	1.57×10^{-4} - 9.77×10^{-4}	0.9886

Table 3. Determination of glucose in pharmaceutical product using glucose electrode compared with spectrophotometric method (Somogyi - Nelson Method²⁶).

Replicate	glucose spectrophotometry	%W/V Potentiometry	Relative Error %
1	4.93	4.79	0.14
2	5.02	4.97	0.05
3	4.81	4.92	0.10
4	5.00	5.10	0.10
5	4.98	4.80	0.18
$\bar{X}$	4.90	4.92	
S^2	0.0156	0.0165	
$F = S_B^2 / S_A^2 = 0.0156 / 0.0165 = 1.0596$			
$F_{0.05/44} = 6.39$			

This electrode presents a useful life time of 1 month (about 1000 determinations), without significant loss of enzyme activity and with a detection limit of 2×10^{-4} M.

Interference studies were performed by testing the response of the glucose electrode to several 1.0×10^{-2} M solutions of substances such as sucrose, fructose, galactose, raffinose, ribose, xylose, mannose, sorbitol, mannitol, sorbose, maltose, arabinose, aspartame, saccharin and cyclamate in the presence of a 1.0×10^{-3} M glucose solution. None of these substances interferes in the response of the biosensor. The reproducibility of the glucose electrode response was tested by measuring eight times the potential response for 4.6×10^{-4} M glucose in 0.2 M phosphate buffer solution at pH 5.5. The electrode showed a small variation in the measured values, with a standard deviation of 7.8%.

The response time of the electrode was 4 min with a complete baseline recovery of 8×10 min, depending on the concentration of glucose.

Table 3 presents the results of analysis of five replicates of a pharmaceutical product (5% Glucose solution - Sandex) with the respective variances (S_A , S_B).

The results of the direct potentiometric analysis (Table 3) are in good agreement with those obtained by spectrophotometry²⁶, since the value of F obtained for 95% confidence level is lower than the critical value of 6.39 ($F_{0.05/4,4}$). This results show that the proposed method can be satisfactorily applied to the determination of glucose in pharmaceutical products or in not too complex sample since high ionic strength media can impair the determination. Furthermore, the proposed method is reliable, simple, fast, without matrix effect.

The results obtained illustrate the potential of the method for the determination of glucose with a limit of detection of $36 \mu\text{g ml}^{-1}$. Although this electrode is less sensitive than the amperometric biosensor²⁷, it is cheaper, easily prepared and has the same lifetime and selectivity of that electrode.

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