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DIRECT COUPLING OF GLUCOSE OXIDASE TO PLATINUM AND POSSIBLE USE FOR *IN VIVO* GLUCOSE DETERMINATION

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Glucose oxidase was attached to platinum-platinum oxide screens via alkylamine silane-glutaraldehyde coupling. The amount of immobilized enzyme was equivalent to 0.0031 μg of soluble glucose oxidase per cm^2 of screen surface. The platinum-silane-glutaraldehyde-enzyme screens were tested potentiometrically in buffered glucose solutions, with respect to a Ag/AgCl reference electrode. The results were expressed as the difference in potential for the enzyme screens placed in buffer containing glucose and placed in plain buffer. This difference in potential was related linearily to the logarithm of the glucose concentration over the range 5–150 mg glucose/100 ml. The source of the potential may be due to the decomposition of hydrogen peroxide produced by the glucose oxidase catalyzed oxidation of glucose. The approach is being studied for possible development of an implantable sensor for continuous *in vivo* monitoring of glucose levels.

INTRODUCTION

The measurement of blood glucose concentrations following administration of known amounts of carbohydrate is the standard procedure used in the diagnosis and control of diabetes in humans (1). The glucose determinations are done *in vitro* using a variety of analytical techniques (2). Recently a proecedure was described for continuous withdrawal of blood and continuous analysis for glucose, using a withdrawal pump and a polarographically-operated glucose oxidase electrode (3). Although several approaches for the development of a miniature intracorporeal sensor for the continuous *in vivo* measurement of blood or tissue glucose concentrations have been described, all of these approaches so far have been limited by problems of selectivity, size, or catalyst life (4–6).

In a previous study (7), we immobilized glucose oxidase and catalase in polyacrylamide gel around a platinum screen and found that the potential

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difference between the enzyme electrode and a Ag/AgCl reference electrode was proportional to the logarithm of the glucose concentration over the range 3–40 mg glucose/100 ml at 37°C in pH 7.3 phosphate buffer. Since the potentiometric approach operates without any applied voltage and the electrodes can be made very small, we decided to investigate further to see if a similar potentiometric output would arise with glucose oxidase attached directly to the platinum screen. This paper describes the attachment of glucose oxidase to platinum and the initial potentiometric evaluation. The general approach consisted of treating platinum with an alkylamine silane to which the enzyme could be coupled. The ability of alkylamine silanes to bind covalently to platinum was suggested by H. Weetall (personal communication, 1976) and was reported by Lenhard and Murray (9) a year later. The covalent coupling of enzymes to immobilized alkylamine silanes has been summarized by Weetall and Filbert (10).

MATERIALS AND METHODS

Materials

Platinum screens, 1.5 cm by 1.5 cm of 45-mesh 0.2-mm diam wire, were purchased from Matthey Bishop (Malvern, Pa.). Lyophilized glucose oxidase (EC 1.1.3.4, about 100 U/mg dry weight) was used as received from Worthington Biochemical Corporation (Freehold, N.J.). γ-Aminopropyltriethoxy silane was obtained from Pierce Chemical Company (Rockford, Ill.) and bovine serum albumin (fraction V) and glutaraldehyde (grade II) were from Sigma Chemical Company (St. Louis, Mo.). The glutaraldehyde was purified by treatment with activated charcoal prior to use (8). All other chemicals were reagent grade.

Immobilization of Enzyme

The platinum screens were pretreated to clean and mildly oxidize the surface of the metal. The screens were placed in boiling 1 N sodium hydroxide to remove any oil film or previously attached silane, rinsed with water, and soaked several hours in 15% nitric acid to promote very mild oxidation of the surface. Some of the screens were soaked overnight in 15% nitric acid and then oxidized electrochemically, using the general scheme outlined by Lenhard and Murray (9). For this oxidation the screens were used as the working electrode, and a plain platinum disk 1 cm in diameter was used as the counter electrode. Both electrodes were placed in 0.5 N sulfuric acid, and a voltage was applied using a Princeton Applied Research Model

170 Electrochemical System. The applied voltage was varied such that the working electrode, with respect to a standard calomel reference electrode, was cycled twice from +1.90 V to −0.45 V to +1.90 V at a rate of 50 mV/s. Then it was held 5 min at +1.90 V for mild oxidation of the surface. The pretreated platinum screens were rinsed several times with distilled water, vacuum dried overnight at 50°C, and stored dry or in toluene until used.

Two variations were used for the silanization procedure. In one variation the pretreated platinum screens were held for 24 h in refluxing toluene contaiing 3% (w/v) γ-aminopropyltriethoxy silane and then rinsed with toluene and acetone and air dried several hours at 115°C. Two of these alkylamine silanized screens served as controls. In the second variation the screens were held for 30 min in benzene containing 10% (v/v) γ-aminopropyltriethoxy silane at room temperature and then rinsed with benzene and vacuum dried at 50°C. In both cases the reagents were dried either by treatment with type 4A molecular sieves or by distillation after addition of metallic sodium.

The alkylamine silanized screens were activated by incubation for 1 h at 0°C with 2.5% glutaraldehyde in 0.05 M sodium phosphate buffer of pH 7.0. The resulting glutaraldehyde-coupled screens were washed 4–6 times with distilled water and immediately placed in 3.0 ml of 1% glucose oxidase in 0.05 M phosphate buffer of pH 7.0 for 4 h at 0°C. With several screens 1% bovine serum albumin was used in place of the glucose oxidase solution. In either case the starting solution for protein immobilization contained 60 mg protein per g of support. The enzyme or albumin coupled screens were washed 4 times with 0.1 M sodium phosphate buffer of pH 7.4, followed by incubation in 2N sodium chloride for 15 min at 0°C to displace any weakly adsorbed enzyme. The enzyme screens were washed again four times with buffer and stored in the buffer until tested for enzyme activity or for potentiometric response to glucose.

Testing Methods

Screens that had received only nitric acid pretreatment and 24-h silanization prior to enzyme immobilization were used to obtain the potentiometric test results. Assay of these screens after enzyme immobilization showed no detectable enzyme activity, using the assay procedure described below, but with a much less sensitive spectrophotometer. Additional screens received the voltammetric cycling and 1.90-V electrochemical pretreatment described above, to increase the number of oxide binding sites; this was followed by the 30-min silanization and enzyme immobilization, and was used to obtain the immobilized enzyme activity results.

Enzyme activity was measured by the *o*-dianisidine-peroxidase method at pH 6.0 using a Cary 14 spectrophotometer (11). For the soluble enzyme the blank was prepared by mixing all of the reagents except the glucose oxidase. For the assay of the activity of the immobilized enzyme, eight enzyme screens were placed in 25 mM glucose at time zero. Aliquots were taken at 0 and 5 min and mixed immediately with the dianisidine-peroxidase reagents; the absorbance was read at 460 nm against the zero time glucose solution. No drift was observed in the spectrophotometer readings. In calculating the units of activity, the same rate of change of absorbance equivalent to a unit of activity of soluble enzyme was used to calculate the units of activity of immobilized enzyme. Essentially no change in absorbance was observed when plain platinum control screens were tested.

For the potentiometric testing, an enzyme screen and a Ag/AgCl reference electrode were placed in 50 ml of 0.1 M sodium phosphate buffer pH 7.4 at 37°C and connected to a recording Keithley Model 610C electrometer of 10^{13} ohms resistance. After baseline stabilization was obtained, increments of D-glucose solution were added to give glucose concentrations of 5.0, 9.9, 49, 98, 242, and 472 mg/100 ml; and the new steady-state potential of the enzyme screen, relative to the Ag/AgCl reference electrode, was noted. In determining the amount of baseline drift over this series of concentrations, the 472 mg/100 ml solution was aspirated from the test beaker and replaced with 50 ml of buffer; and the potential was compared with that at the start of the glucose additions. The drift was 3 mV. All tests were made with a mixture of 12% oxygen and 88% nitrogen bubbled through the glucose solution, since previous work had shown that oxygen was not rate limiting for acrylamide-glucose-oxidase-platinum electrodes with greater amounts of enzyme present (7). The baseline potentials of +365 to +380 mV became less positive as glucose was added. The data were reported as the potential differences between the potential with glucose present and with glucose absent, the latter being the baseline potential.

RESULTS

Enzyme Activity

The eight immobilized enzyme screens, tested as a group on the same day as the immobilization was carried out, gave a total glucose oxidase activity of 0.0093 U, or 0.0011 U per screen. This was equivalent to 0.014 μg of glucose oxidase per screen (0.0018 U/g of support or 0.00024 U/cm^2 of support surface area) based on the measured soluble enzyme activity of 84.2 U/mg of dry enzyme. Therefore, much less than 1%

of the enzyme was coupled to the screens. Such a small immobilization efficiency might be attributed to adsorption rather than covalent coupling; however, as discussed below, there is no evidence for adsorption being the mechanism here. The enzyme screens were stored overnight in pH 6.0 phosphate buffer at 4°C. The next day the screens were washed several times with pH 7.4 phosphate buffer and again assayed for activity; the total activity of 0.00074 U represented only 8% of the activity measured the previous day.

Potentiometric Response to Glucose

The potentiometric responses for five enzyme screens, three albumin screens, and two silanized control screens are shown in Fig. 1. The results for

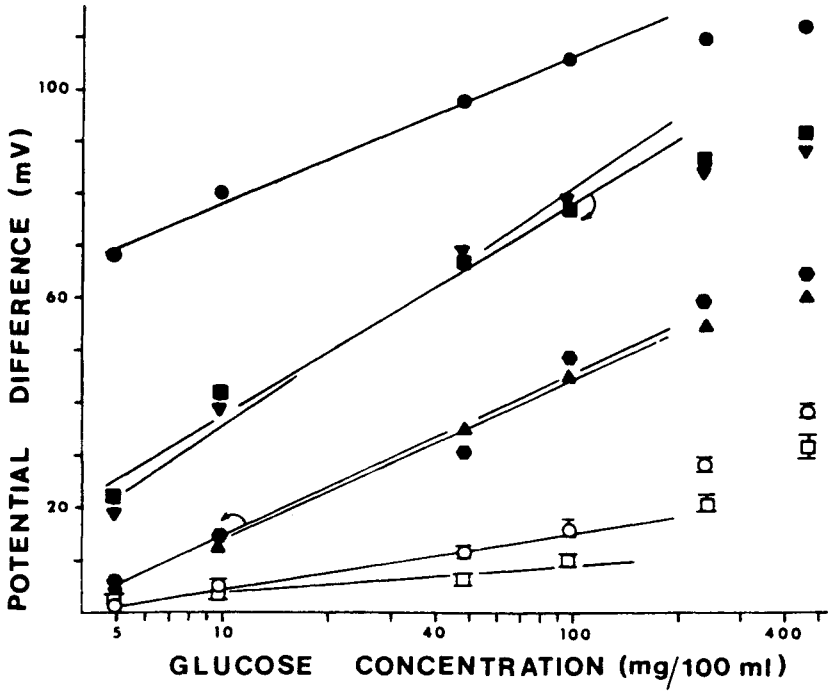

FIG. 1. Potentiometric response of various modified platinum screen electrodes to glucose. Potentials were measured with respect to a Ag/AgCl reference electrode. The potential difference shown is the measured potential less the potential at zero glucose concentration; all measurements were done using 0.1 M phosphate buffered glucose, pH 7.4, at 37°C. Solid symbols are for five different glucose oxidase screens. Open symbols show the mean and 1 SD for three bovine serum albumin screens (□), and two silanized screens (○). Lines fitted by linear least square using data from 5 to 98 mg/100 ml.

each of the five glucose oxidase screens are shown to indicate the good internal consistency yet wide variation between screens. From Fig. 1 it is evident that the platinum-silane-glutaraldehyde-glucose-oxidase screens gave significantly larger potential differences than did the platinum-silane-glutaraldehyde-albumin or platinum-silane control screens. Since tests on nearly similar screens showed very small but measurable immobilized glucose oxidase activity, the increased potential difference with enzyme screens was attributed to the presence of immobilized glucose oxidase.

In order to see if the enzyme could be removed from the screens by repeated washings and incubation in glucose, three enzyme screens were washed and tested for potentiometric response in glucose solutions immediately after the enzyme was attached. The same screens were then washed for about 1 h with 7.4 phosphate buffer, retested for potentiometric response in glucose, repeatedly washed with pH 7.4 buffer, refrigerated overnight in pH 6.0 buffer, and then washed with pH 7.4 buffer and retested the next day for potentiometric response in glucose. The results are summarized in Table 1. The three enzyme screens gave very uniform results, similar to those represented by the solid triangles and solid hexagons of Fig. 1. The results of the second test (Table 1) for potentiometric response to glucose were significantly higher than those of the first test, as determined by a paired t-test at each concentration ($p < 0.02$ for glucose concentrations of 49 mg/100 ml or larger; $p < 0.05$ for glucose concentrations of 5 and 9.9 mg/100 ml). Washing the enzyme screens in solutions of high ionic strength also did not appear to remove the enzyme, as evidenced by no

TABLE 1. **Repeated Testing of Potentiometric Response of Three Enzyme Screens**

	Mean potential difference (mV ± SD)[a]					
	Glucose concentrations (mg/100 ml)					
Testing sequence	5.0	9.9	49	98	242	472
A. Test immediately after making	1 (1.0)	3 (1.5)	15 (1.5)	23 (1.7)	32 (1.1)	39 (0)
B. Wash 1 h and retest	5 (0.6)	10 (1.5)	26 (1.0)	36 (1.7)	47 (2.9)	54 (3.2)
C. Wash, store 16 h at 4°, retest	7 (1.5)	14 (2.3)	33 (2.5)	42 (2.6)	54 (3.2)	61 (2.5)
D. Wash 1 h and retest	7 (1.5)	16 (1.1)	34 (3.6)	44 (4.0)	55 (3.5)	63 (4.6)

[a]SD is shown in parenthesis.

change in the potential difference at 472 mg glucose/100 ml before and after washing two enzyme screens in 4 M sodium chloride.

DISCUSSION

The results of this study suggest strongly that small amounts of glucose oxidase can be attached covalently to platinum through alkylamine silane-glutaraldehyde coupling. According to Lenhard and Murray (9) and to Untereker et al. (12), the silane binds covalently to surface hydroxyl groups and therefore is more likely to bind to platinum oxide, rather than to bare platinum metal. This would also be expected based on the use of alkylamine silane to couple enzymes to nickel–nickel oxide (13) and to other metal–metal oxide films. In the present study the formation of platinum oxide on the surface of the screens was carried out in two ways: electrochemically and by nitric acid treatment. No attempt was made to control or vary the degree of completeness of the surface oxidation, although this obviously is an aspect that needs to be investigated, since it should influence the number of silane groups and thus the enzyme loading of the screens. In addition, the degree of surface oxidation of the platinum may influence the magnitude of the potentiometric response to glucose; this point was not studied in the present work.

There was no indication that the enzyme was adsorbed rather than covalently attached to the screens, since washing with a high ionic strength solution of sodium chloride was done before the screens were assayed, and enzyme activity was found after the washing. In addition, a similar high ionic strength wash gave no change in the potentiometric response for the enzyme screens in glucose. The major decrease in immobilized enzyme activity after about 24 h could have been caused by several factors, including enzyme deactivation by hydrogen peroxide, uncoupling of the silane from the platinum oxide, loss of flavin cofactors, or other processes of enzyme denaturation. Additional work is needed to define the effect such a large decrease in enzyme activity would have on the potentiometric response of the screens to glucose. In our earlier studies with acrylamide gel enzyme screens (7), the magnitude of the potentiometric responses varied with the glucose oxidase loading, but only at low loadings; so that the relationship of the potentiometric response to glucose oxidase loading needs to be more clearly defined.

The potentiometric response of the enzyme screens, prepared by alkylamine silane-glutaraldehyde coupling, was similar to the response obtained previously (7) with the enzyme trapped in polyacrylamide gel around platinum screens. In both cases the potential difference was related

linearly to the logarithm of the glucose concentration; however, the linearity extended over a wider range of glucose concentrations in the present covalent coupled study (5–150 mg/100 ml) as compared to the earlier gel entrapment study (3–40 mg/100 ml). The source of the potentiometric response appeared to be the hydrogen peroxide produced as a result of the glucose oxidase catalyzed oxidation of β-D-glucose (7). In the previous work the magnitude of the potentiometric response could be varied by changing the relative proportions of glucose oxidase and catalase coentrapped in the gel matrix.

In addition, a similar linear response for the potential difference versus the logarithm of the glucose concentration could be obtained using hydrogen peroxide and a plain platinum screen (7). However, the detailed mechanism whereby the hydrogen peroxide in the microenvironment of the metal surface generated a unique potential is not known. The structure and composition of the platinum–platinum oxide surface may be a factor. This is suggested by the work of Anson (14), who showed that the reversible electrochemical oxidation–reduction of Fe(II)–Fe(III) on platinum electrodes was influenced markedly by both the oxide film and the presence of finely divided platinum metal on the electrode surface. Since platinum is a good catalyst for the decomposition of hydrogen peroxide, the potentiometric response may arise from this decomposition reaction.

Some support for this idea is based on the observation that the decomposition of hydrogen peroxide to oxygen and water requires the transfer of two electrons. In addition, the average slope of 35.4 mV per factor of ten in concentration for the five enzyme screens in Fig. 1 is close to the expected Nernst equation value of 30.7 for a two-electron transfer. In other words, the potentiometric response for the enzyme screens of Fig. 1 appears to result from a reversible reaction involving the transfer of two electrons; and the decomposition of hydrogen peroxide to oxygen and water may be the pertinent two-electron transfer reaction.

The data for the silane-glutaraldehyde-albumin and silane control screens in Fig. 1 show that there is also a nonselective portion of the potentiometric response, but that this nonselective part is small. In addition, the slopes of 11.0 mV per factor of 10 in concentration for the silane-glutaraldehyde-albumin screens and of 4.8 mV per factor of 10 in concentration for the silane screens in Fig. 1 differ significantly from the slopes for the enzyme screens. Thus this small nonselective response probably is caused by a different mechanism than that responsible for the selective response with the enzyme screens.

So far, only a few materials that might be present in blood or tissue fluids have been tested for possible interference. The results of the interference studies were generally favorable. They will be reported elsewhere, along with data on alternative methods of enzyme immobilization.

The results in Table 1 show that the potentiometric response changed between the initial test and one made after washing the enzyme screens for 1 h in buffer. Several processes that might have occurred during the washing include the removal of loosely held silane-glutaraldehyde-enzyme, elution of some of the flavin cofactor, or other changes that in general would have given lower activity of the immobilized glucose oxidase. However, a loss in enzyme activity does not appear to explain this increase in potentiometric response after washing and testing. In our previous work with the gel entrapped enzyme, we did not observe a decrease in potentiometric response with addition of enzyme, but at low enzyme loadings saw an increased response with more enzyme. Another possible explanation may be that the washing allowed time for unreacted aldehyde groups on silane-glutaraldehyde appendages to attach to nearby glucose oxidase molecules and give multipoint attachment of the enzyme. This should have resulted in a tighter diffusional barrier and therefore a higher hydrogen peroxide concentration at the platinum surface.

The wide variation among the five enzyme screens (solid symbols) in Fig. 1 may be due to different degrees of enzyme loading or to different catalytic activities of the platinum surfaces. The explanation for this variation will require considerably more study; however, the good linearity and narrow range of slopes suggests strongly that this work should be continued to see if this approach could lead to a glucose sensor for *in vivo* use.

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