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**ELECTRICAL COMMUNICATION BETWEEN GLUCOSE
OXIDASE AND ELECTRODES BASED ON POLY (VINYL
IMIDAZOLE) COMPLEX OF Os(bpy)₂ Cl₂**

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Abstract

Glucose oxidase was electrically 'wired' to glassy carbon electrodes by redox hydrogels based on poly (N-vinyl imidazole) (PVI) complexed with Os(bpy)₂Cl₂ (PVI-Os). The hydrogels were formed by crosslinking PVI-Os and glucose oxidase with poly(ethylene glycol) diglycidyl ether (PEGDGE). Glucose was electrooxidized on the PVI-Os based 'wired' enzyme electrodes at 350 mV vs SCE. The dependence of the electrooxidation current on pH, ionic strength, film thickness, weight fraction of the enzyme in the redox hydrogel and oxygen partial pressure for electrodes rotating at 1000 rpm is described.

Introduction

Polymeric redox mediators have been used for the transport of electrons between active sites of redox enzymes and electrodes.¹⁻¹¹ In complexes between high molecular weight redox polymers and redox enzymes, electrons are transferred efficiently from the substrate reduced redox active site of the enzyme to redox centers of the polymers.^{7,12}

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Our research focuses on water soluble redox polymers that are crosslinked on electrode surfaces to form 3-dimensional redox hydrogel, to the polymer skeletons of which enzymes are covalently bound and in which segments of the polymer may also form complexes with the enzymes. Redox polymers based on poly (vinyl pyridine) complexed with Os (bpy)₂Cl^{+1/2+} (bpy = 2,2' bipyridine) have been used to 'wire' i.e. to electrochemically connect enzymes to electrodes. By quarternizing part of the free pyridine rings on the polymer with bromoethylamine we formed earlier a water soluble, PEGDGE crosslinked redox polyamine, POsEA.^{4,5}

Electrooxidation of ascorbate, urate, acetaminophen and other readily electrooxidizable substrates often interferes with assays of analytes such as glucose. The problem of the resulting poor specificity for glucose can be alleviated by operating the electrodes at less oxidizing potentials, where the rate of electrooxidation of interferences can be slower. Alternatively, membranes can be placed on electrodes to exclude anionic interferences or large interference molecules. An example of such a membrane is Nafion.¹³ Recently we showed that interference by ascorbate, urate and acetaminophen can be altogether eliminated by their oxidation in a crosslinked overlayer of horseradish peroxidase by in situ generated or externally added H₂O₂.¹⁴

A goal of our research in glucose sensors is to develop a sensor that measures glucose levels accurately in whole blood samples and when implanted in the human body. Specific research objectives include improvement of the selectivity towards glucose, enhancement of the stability, enhanced current outputs, biocompatibility and insensitivity towards oxygen partial pressure. The last four issues are addressed elsewhere.^{15,16} The redox potentials of the PVI-Os hydrogels that are the subject of this paper are more reducing than the redox potential of POsEA, and their use may alleviate interference by electrooxidizable species.

Experimental Section

Chemicals : 2-Bromoethylamine hydrobromide (Aldrich), poly (ethylene glycol) diglycidyl ether (Polysciences, PEG400), K_2OsCl_6 (Johnson Matthey) and N-vinyl imidazole (VI) (Aldrich) were used as received. Glucose oxidase (D-glucose : oxygen reductase, EC 1.11.1.7) from *Aspergillus niger* was purchased from Sigma.

Synthesis of PVI : PVI was synthesized from N-vinyl imidazole (VI) by the method of Chapiro and Mankowski.¹⁷ A 7M aqueous solution of VI was purged with nitrogen for 30 min, and the bottle sealed. The solution was subjected to 2.5 MRad gamma radiation from a Co^{60} source. The polymer was precipitated from acetone, redissolved in methanol and reprecipitated from ethyl ether. The filtered precipitate was a pale yellow solid. Its molecular weight was determined by HPLC analysis using a Synchrom Catsec 300 column with 0.1% trifluoroacetic acid and 0.2 M NaCl as the eluent to be about 40kDa (M_n). The flow rate in the molecular weight determination was 0.4ml/min and poly (2-vinylpyridine) was used as the standard.

Redox polymers PVI-Os, PVI-Os-NH₂ : Osmium derivatized polymers were prepared by a method similar to that of Forster and Vos.¹⁸ Os (bpy)₂Cl₂ (0.456g, 0.8mmoles) and poly (N-vinyl imidazole) (0.380g, 4.0mmoles of imidazole groups) were refluxed in 100ml of ethanol under a nitrogen atmosphere for 7 days . The polymer was precipitated in ether, and dried at 70°C for 24 hr. This polymer was designated as PVI-Os. (Figure 1)

PVI-Os was quarternized with ethylamine functions by a method similar to that of Gregg and Heller.⁶ PVI-Os (500mg) and bromoethylamine hydrobromide (0.88g, 4.3mmoles) were reacted in an 18mL ethylene glycol : 30 mL DMF mixture at 60° C for 24 hr. After the solvent was evaporated, the polymer was dissolved in

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methanol and precipitated by adding it dropwise to a rapidly stirred solution of ethylether. It was then dissolved in water and stirred with 10 g of ion exchange (BioRad AG 1X-4, chloride form) beads for 12 h. This polymer is designated as PVI-Os-NH₂. (Figure 1)

Electrodes : The electrodes were glassy carbon discs, 3mm in diameter (V-10 grade vitreous carbon from Atomergic). Each glassy carbon rod was encased in a teflon cylinder with a deaerated slow setting epoxy (Armstrong) and the cylinder was fitted on an AFMSRX rotator (Pine Instruments).

All electrodes were prepared by polishing successively with four grades of alumina (20, 5, 1, 0.3 micron) with sonication and thorough washing between the polishing steps. Background scans were taken at 100mV/s in STD buffer to ensure that the voltammograms were featureless. The STD buffer is a solution of 0.15 M NaCl buffered with phosphate (33mM, pH 7.4) and was the buffer used in all electrochemical experiments. The electrodes were then washed and stored in a dessicator until use.

The electrodes were modified by syringing an appropriate volume of a 5 mg/mL solution of the polymer onto the electrode surface (0.071cm²). Then an aliquot of 5 mg/mL solution of glucose oxidase (10mM HEPES, pH 8.1) was added to the electrode, followed by an appropriate volume of a 1mg/mL solution of PEGDGE. The solutions were then mixed on the surface of the electrode and the resulting film allowed to cure for at least 24 hours under vaccum. Figure 2 shows the reactions of the crosslinker, PEGDGE with amines and with imidazole functions.¹⁹ The nitrogen of the imidazole group, the amine functions in the case of PVI-Os-NH₂ and amine functions on the lysine groups on the enzyme participate in the crosslinking.

Electrochemistry : The electrochemical experiments were performed with a Princeton Applied Research 173 potentiostat, a 175 PAR universal programmer equipped with a model 179 digital coulometer. Signals were recorded on an X-Y-Y' Kipp and Zonen recorder. The water jacketed cell used was thermostatted at 21.4°C. The cell had an aqueous saturated calomel electrode as reference (SCE) and all potentials are reported with respect to this electrode. A platinum wire encased in a heat shrinkable sleeve with a frit was used as the counter electrode. Unless otherwise noted, the steady state current was monitored with the electrodes poised at 0.350 V, where the current no longer varied with potential. The electrodes were rotated at 1000 rpm.

Results and Discussion

Cyclic voltametry of PVI-Os-NH₂ :

The cyclic voltammograms of electrodes coated with PEGDGE crosslinked PVI-Os-NH₂ films had the classical symmetrical shape characteristic of reversible oxidation and reduction of a surface bound species. Their half-wave potential was 190mV vs. SCE. This potential varied by about +/- 10mV with the amount of crosslinker in the film which ranged from 3wt% to 21wt%. At 12wt% PEGDGE the redox potential was 187mV and the peak separation was 10mV.

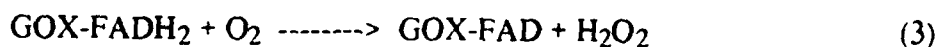
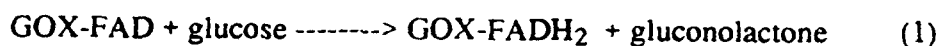
Glucose response of PVI-Os and PVI-Os-NH₂ glucose electrodes :

Figure 3 shows the variation of the current density with glucose concentration of PEGDGE crosslinked PVI-Os and PVI-Os-NH₂ glucose electrodes. The electrodes

were prepared by immobilizing 7µg redox polymer and 3µg GOX with 0.6µg PEGDGE. Evidently, both PVI-Os or PVI-Os-NH₂ effectively 'wire' glucose oxidase.

Oxygen competition : The steady state glucose response curves were measured in argon saturated and in oxygen saturated solutions for PVI-Os-NH₂. (30 wt% GOX, 6 wt% PEGDGE) (Figure 4a). Figure 4b shows the fraction of the retained current in the O₂ saturated solution with the electrode rotating at 1000 rpm, to emphasize the oxygen effect which would be very small in a stagnant solution, where the reaction of FADH₂ centers with O₂ would be severely mass transport limited because of the low solubility of O₂ in water.

At low and moderate glucose concentrations, the electrode current decreased substantially in O₂ saturated solutions because O₂ competes for the FADH₂ electrons with the Os^{III} centers (reactions (2) and (3)).⁵



The loss in current under oxygen was higher at lower glucose concentrations. Apparently at high glucose concentrations the glucose flux maintains the enzyme, even in the presence of O₂, in the reduced form, which transfers electrons via the redox polymer to the electrode.

pH dependence : Figure 5 shows the pH dependence of the currents of an electrode under argon at 100 mM glucose. (Henceforth, currents at 100mM glucose will be referred to as *j*_{max}. In Figure 5, the current is normalized with respect to the current at pH 9.0 where it reaches a maximum). The current varies only within +/-

10% through the 7.3 to 10.0 pH range. Below pH 7.3, the current decreases rapidly, and is practically nil at pH 3.4. POsEA based glucose electrodes show a similar dependence on pH.⁶

The driving potential difference for electron transfer reaction from the FADH₂ centers of the enzymes to the redox polymer increases with pH, where the enzyme's redox potential becomes more reducing, while the redox polymer potential does not change with pH.⁶ The rate constant for electron transfer between the active center of GOX and ferrocene based diffusing mediators also decreases with decreasing pH.²⁰ A second cause for the lesser current at low pH is the absence of electrostatic attraction leading to complex formation between PVI-Os-NH₂ and GOX. The pI of GOX is ca. 4.0. The polycationic polymer complexes the enzyme that is a polyanion at pH 7.0. At pH 3.0, the enzyme, like the redox polymer, is a polycation and the two repel each other.

Ionic strength dependence: Figure 6 shows the dependence of the current at 100mM glucose on the ionic strength. The currents shown are normalized with respect to the current at 150 mM NaCl. The current decreases as the ionic strength is increased, dropping to almost nil at 1M NaCl concentration.

The cause of this drop is probably due to the coiling of the redox polymer segments at high ionic strength. At low ionic strength the segments are stretched by repulsive forces between the cationic sites. When the positive charges are screened by counterions, i.e. anions, the segments assume an entropically favoured coiled configuration, i.e. they ball up. The balled up segments no longer fold along the surface contours of the enzyme and the redox centers of the enzyme and the polymer are too far apart for efficient electron transfer between the two.¹

Thickness dependence : The increase in current density upon increasing the surface coverage by Os centers, i.e. polymer film thickness, is shown in Figure 7. The surface coverage by osmium centers was varied between 1.77×10^{-9} to 1.855×10^{-7} mol/cm² by varying the amount of total material applied to the electrode surface. The surface coverage was determined by integration of the voltammogram, i.e. of the current from the potential where electrooxidation starts, through the potential where it is complete. The films in these experiments contained 38wt % GOX and 10 wt % PEGDGE.

The current density increased approximately linearly with increasing thickness even for the highest thickness considered here, even though the rate of increase was less at high thickness. We conclude that the electroactive portion of the film, the reaction layer, extends through the entire film thickness. Again, the result is similar to that observed for POsEA.⁵ This suggests that the diffusion coefficient for electron transfer in the redox polymer film is large enough for electrons from polymer redox centers near active sites of enzymes to be transported efficiently to the electrode, even when the enzyme molecules are near the solution side of the electrodes.

Enzyme loading dependence

a) **Constant polymer loading :** Glucose response curves were measured for a series of electrodes where the enzyme loading was varied from 20% to 65%. The polymer loading was kept constant at $70\mu\text{g}/\text{cm}^2$ and 10 wt% of PEGDGE was used to crosslink the films (Figure 8). Except for one data point, at 65 wt% enzyme loading, the data points at different enzyme loadings show that j_{max} increases initially with increasing enzyme loading, peaks around 40 wt% GOX and then decreases upon further GOX loading. The trend is similar to that observed for POsEA.⁵

IDA measurements show that in crosslinked POsEA based hydrogels with glucose oxidase, D_e decreases upon increasing the enzyme loading.²¹ It is conceivable that at enzyme loadings greater than 40%, the diffusion of electrons through the redox polymer becomes rate limiting. It is also possible that as the enzyme content is increased beyond 40wt% not all the enzyme is in electrical contact with the redox polymer, i.e. not all the enzyme is 'wired'. A third possibility is that the decline in current when an excess of enzyme is loaded into the polymer derives from loss of material that is rotating at 1000 rpm. The enzyme is not as readily crosslinked as the polymer by PEGDGE as the polymer, and when the enzyme loading increases, more material may dissolve from the electrode surface.

h) Constant enzyme loading : Glucose response curves were measured for a series of electrodes where the enzyme loading was kept constant at $14.7\mu\text{g}/\text{cm}^2$ and the polymer to enzyme weight ratio was increased from 1 to 10 (Figure 9). The purpose of this experiment was to determine the maximum current that can be obtained from a fixed amount of enzyme on the electrode since, at high polymer loading, we are presumably 'wiring' the enzyme with the maximum efficiency.

In this experiment j_{max} exhibited sigmoidal behaviour, increasing upon addition of polymer at low P/E ratios, and then becoming constant at P/E values near 10. If one assumes that the current is proportional to the rate of electron transfer between the enzyme and the polymer, i.e. that the electrodes are kinetically limited by the rate of electron transfer, then $j_{\text{max}} \propto C_{\text{enz}} * C_{\text{O}_2}$. For a fixed amount of GOX on the electrode, increasing the amount of polymer increases the product, but once all the enzyme is electrically 'wired', the product can no longer increase. It should also be noted that for $14.7\text{mg}/\text{cm}^2$ of GOX on the electrode surface, if all the enzyme were active and 'wired' by the redox polymer, the current output would approach $4\text{mA}/\text{cm}^2$. We observe, however, a maximum current density of $200\mu\text{A}/\text{cm}^2$, about 5% of the

calculated value. Reasons for the lower than theoretical current may include inactive enzyme, loss of enzyme from the electrode and the presence of non-'wired' enzyme molecules.

Conclusions

The PVI based complex of $[\text{Os}(\text{bpy})_2\text{Cl}]^{+2+}$ can be used as a molecular 'wire' to electrochemically connect glucose oxidase to electrodes. PVI-Os-NH₂ based glucose electrodes rotating at 1000 rpm exhibit characteristics similar to glucose electrodes based on POsEA. The glucose response of these electrodes is a function of pH, ionic strength, thickness, enzyme loading and O₂ partial pressure. The dependence of current on these factors mirrors that of POsEA, but glucose is electrooxidized at a potential 100mV more reducing than in electrodes based on POsEA.

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Figure Captions

Figure 1 Structures of PVI-Os (top) and PVI-Os-NH₂ (bottom).

Figure 2 Structure of PEGDGE (top), its reaction with imidazole (center) and with an amine (bottom)

Figure 3 Steady state glucose electrooxidation current densities for PVI-Os and PVI-Os-NH₂. The films contain 30 wt % GOX and 6 wt.% PEGDGE. Argon atmosphere, pH 7.4, 1000 rpm, 0.35V (SCE). The total polymer + enzyme loading is 140 $\mu\text{g}/\text{cm}^2$.

Figure 4 a) Steady state electrooxidation current densities for PVI-Os-NH₂ wired GOX electrodes. Electrode conditions as in Figure 3.
b) Glucose concentration dependence of the fraction of the current retained under O₂.

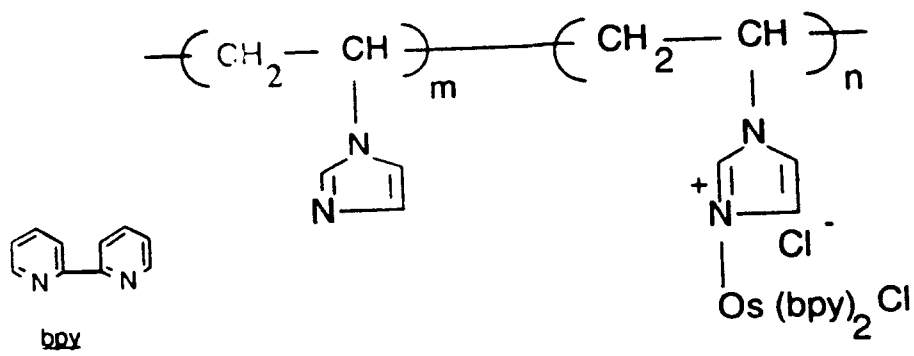
Figure 5 Dependence of the steady state current on pH, normalized with respect to the current at pH 9.0. Electrode conditions as in Figure 3.

Figure 6 Dependence of the steady state current on ionic strength. The currents are normalized with respect to the current at 0.15M NaCl. Electrode conditions as in Figure 3.

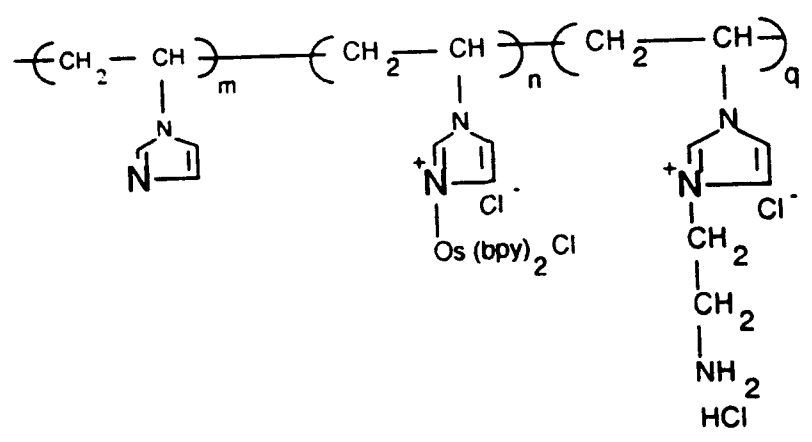
Figure 7 Dependence of the steady state current density on the thickness of the polymer films. Electrodes with 38wt. % GOX and 10 wt. % PEGDGE. [Glucose] = 100 mM; Electrode conditions as in Figure 3.

Fig. 8 Dependence of the steady state current density on the GOX loading. The PVI-Os-NH₂ loading was held constant at 70 μg/cm². [Glucose] = 100 mM; Electrode conditions as in Figure 3.

Fig. 9 Dependence of the steady state current density on the redox polymer loading. The GOX loading was held constant at 14.2 μg/cm². [Glucose] = 100mM. Electrode conditions as in Figure 3.

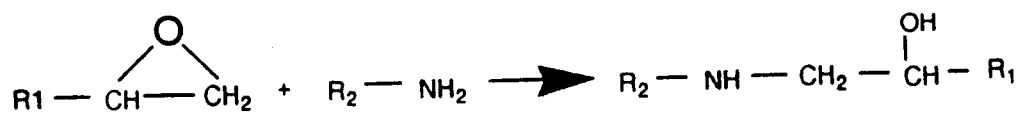
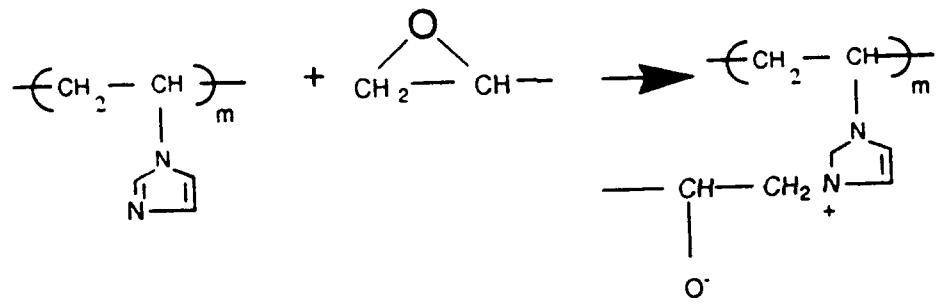
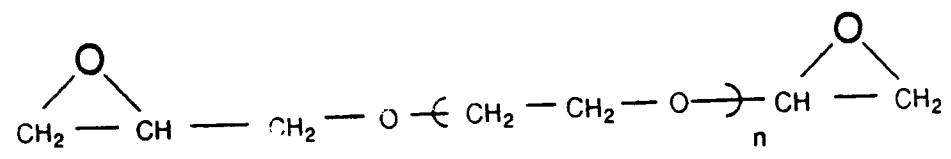


$$n / m+n = 1/5$$



$$n / m+n+q = 1/5$$

$$q / m+n+q = 2/5$$



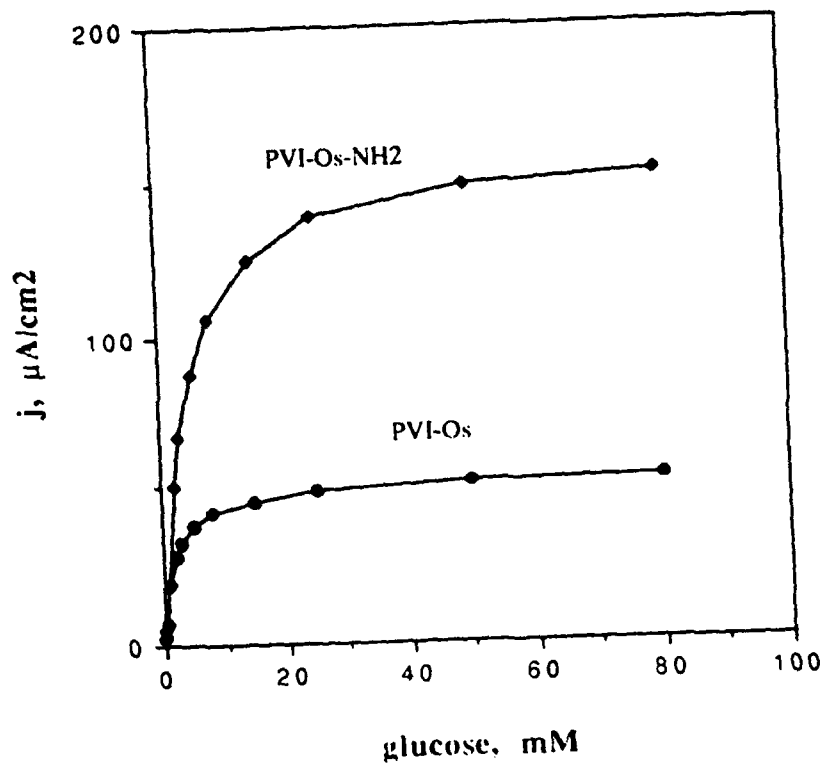


Figure 4a

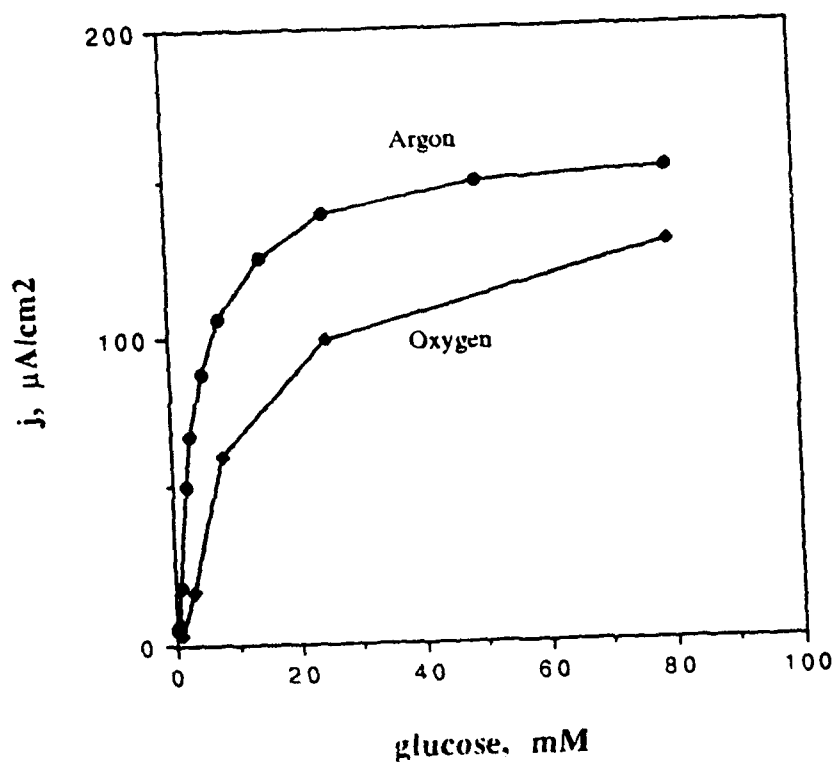


Figure 4b

