Immobilized $K_3Fe(CN)_6$ and Glucose Oxidase in Polypyrrole on a Gold Micro-electrode and The it Application as a Glucose Sensor

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The purpose of this research is to prepare a glucose sensor using electrochemical polymerization of pyrrole onto a gold microelectrode in the presence of the enzyme glucose oxidase and ferricyanide at a potential of 0.70 V *versus* Ag/AgCl. The enzyme glucose oxidase and ferricyanide are co-immobilized into the polypyrrole films during the electrochemical polymerization process. The two species compete for incorporation into the films so that on increasing the concentration of enzyme in the growth solution, less ferricyanide is incorporated into the film. The ferricyanide entrapped within the film acts as a redox mediator for the oxidation of the enzyme at 0.45 V *versus* Ag/AgCl. The effects of variation in charge passed during polymerization, pyrrole concentration and ferricyanide loading are studied. Moreover, the determination of a standard amount of glucose of and the stability of the glucose sensor were investigated.

Key word: Polypyrrole, Glucose Sensor, Ferricyanide, Amperometry

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การตรึง $K_3 Fe(CN)_6$ และกลูโคสออกซิเดสในโพลีไพโรล บนอิเล็กโทรดทองขนาดไมโคร และการประยุกต์ใช้เป็นกลูโคสเซนเซอร์

อรวรรณ ชัยลภากุล จิตฤดี พรหมนิล มิทราน โซมาซันดรีม และ มรกต ตันติเจริญ (2543)
วารสารวิจัยวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ***25(1)***

งานวิจัยนี้ได้ใช้เทคนิคทางเคมีไฟฟ้าเตรียมกลูโคสเซนเซอร์ โดยทำการโพลีเมอร์ไรซ์ไพโรลบนอิเล็กโทรดทอง ในสารละลายที่มีเอนไซม์กลูโคสออกซิเดสและเฟอริไซยาไนด์ ที่ศักย์ไฟฟ้า 0.70 โวลต์ เทียบกับขั้วมาตรฐาน Ag/AgCl ขณะที่ทำการโพลีเมอร์ไรซ์เอนไซม์ พบว่ากลูโคสออกซิเดสและเฟอริไซยาไนด์จะเกิดการแข่งขันที่จะถูกตรึงลงไปในฟิล์มไพโรล ดังนั้นเมื่อเพิ่มความเข้มข้นของกลูโคสออกซิเดสในสารละลาย พบว่าจะมีเฟอริไซยาไนด์ตรึงอยู่ในฟิล์มน้อย เฟอริไซยาไนด์มีความสำคัญในการเป็นตัวกลางส่งผ่านอิเล็กตรอนสำหรับปฏิกิริยาออกซิเดชันของเอนไซม์กลูโคสออกซิเดส ที่ศักย์ไฟฟ้า 0.45 โวลต์ เทียบกับขั้วมาตรฐาน Ag/AgCl งานวิจัยนี้จึงได้ทำการศึกษาหาสภาวะที่เหมาะสมที่สุดในการเตรียมกลูโคสเซนเซอร์ ตัวแปรต่างๆ ที่ทำการศึกษาคือ ปริมาณไฟฟ้าที่ใช้สำหรับการเตรียมฟิล์ม ความเข้มข้นของไพโรล และความเข้มข้นของเฟอริไซยาไนด์ นอกจากนี้ได้ทำการทดลองหาปริมาณน้ำตาลมาตรฐานและศึกษาความเสถียรของกลูโคสเซนเซอร์ที่เตรียมได้ด้วย

คำสำคัญ โพลีไพโรล กลูโคสออกซิเดส เฟอริไซยาไนด์ และแอมเพอโรเมทรี

INTRODUCTION

Electropolymerization is an adaptable and easily controlled method for the immobilization of enzymes at an electrode surface. This makes electropolymerization an interesting method for the immobilization of enzymes on microelectrodes, for applications such as glucose sensors.

An amperometric sensor is a device for measuring the current that is generated by an electrochemical reaction at a fixed potential. There are some advantages of the amperometrical sensors that make them very useful in bioprocess control. Amperometric sensors are inexpensive, simple to make and operate, and adaptable to process control instrumentation. The sensors can be often operated in a wide concentration range. However, they can sometimes lack selectivity[1] and can suffer from fouling when used in complex matrices.[2] For an amperometric glucose sensor, the reaction of glucose and oxygen in the presence of the enzyme glucose oxidase (GOD) will generate hydrogen peroxide as shown below:

$$\beta\text{-D-Glucose} + O_2 \xrightarrow{\text{GOD}} \text{gluconic acid} + H_2O_2 \qquad \text{…..…(1)}$$

This is an electroactive species, which is oxidizable at an electrode surface as described by an electrochemical half reaction as follows:

$$H_2O_2 \xrightarrow{0.7\ \text{V } \textit{versus}\ \text{SCE}} 2H^+ + O_2 + 2e^- \qquad \text{…….. (2)}$$

The response to hydrogen peroxide will be proportional to the concentration of glucose. This reaction is monitored amperometrically by measuring the current generated by the increase of hydrogen peroxide,

$$O_2 + 2H^+ + 2e^- \longrightarrow H_2O_2 \qquad \text{…….. (3)}$$

as oxygen is consumed by the enzyme.

The limited solubility of oxygen in solution creates a problem at the high substrate concentrations often encountered in bioprocesses, because it limits the linear range of the sensor. Oxygen can be generated from hydrogen peroxide at the electrode surface, when it is at a positive potential. However, this regenerated oxygen is sometimes not enough to meet the demands of the enzymatic reaction at high substrate concentrations due to loss of oxygen from diffusion.

In this study, preparation of a glucose sensor by immobilization of glucose oxidase and ferricyanide (Ferri) into polypyrrole (PPy) by electrochemical polymerization on a gold (Au)microelectrode was investigated. The glucose sensor is expected to determine the glucose concentration at low potentials (lower than 0.7 V *versus* SCE) in order to avoid interference.

Electron transfer mediators, such as ferri/ferrocyanide ($E° = 0.34$ V *versus* SCE), ferrocene and benzoquinone can sometimes take the place of oxygen. This can sometimes overcome the problem of limited availability of oxygen.[1] Also, the focus of the recent research is the coupling of an electron mediator with a species that electrochemically polymerizes to form a film over the electrode. The film can mediate electron transfer, serve to screen out interference and prevent electrode fouling[3]. Polypyrrole has been a particular choice for

entrapment of protein molecules because films can be grown from aqueous solutions compatible with most biological elements.[4]

EXPERIMENTAL

Chemicals

Glucose oxidase type II derived from *Aspergillus niger*, was used in the amount of 10,000 unit was from Sigma Chemical Company. The 96% pyrrole, received from Fluka Co. Ltd., and freshly distilled before use. The β–D-Glucose purchased from BDH Ltd. was used to prepare the standard solutions of glucose. These solutions were prepared 24 hr before use to ensure that mutarotation between the α and β forms was complete. Other reagents were analytical grade.

Equipment

A potentiostat/galvanostat (Autolab, model PGSTAT10) was used for the electrochemical polymerization and current measurement. Output from the scanning potentiostat/galvanostat was connected to a personal computer and HP Laser Jet 4L printer, supported by GPES 4.4 operating software. The conventional three-electrode system consisted of a gold microelectrode of diameter 180 μm (this number is obtained from electrochemical experiments as explained in section 2.3.1), as silver/silver chloride reference electrode, and platinum wire counter electrode. Electrochemical measurements were made using a Faraday cage for noise prevention and under N_2 gas.

Procedures

Preparation of Gold Microelectrodes

The gold microelectrodes were prepared by sealing fine gold wire into capillary glass rods of diameter of 0.6 mm (Figure 1). The gold microelectrodes were cleaned in a 3:1 H_2O_2 : H_2SO_4 solution. Then they were polished with emery paper and then with aluminium oxide (0.3 μm particle size). Before use, the microelectrodes were pretreated by electro-chemical cleaning in 0.1 M H_2SO_4 by cyclic voltammetry from 0.2 to –1.6 V *versus* silver/ silver chloride, at a scan rate of 0.1 V/sec. The radii were determined from equation(4) [5] by measuring the diffusion-controlled current for the oxidation of $K_3Fe(CN)_6$ in 0.1 M KCl:

$$i_p = (2.69 \times 10^{-5})n^{3/2} AD_0^{1/2} v^{1/2} C_0^{*}, \quad \ldots\ldots(4)$$

where i_p = peak current, n = number of electrons transferred, A = area of electrode [cm^2], D_0 = diffusion coefficient of ferricyanide in 0.1 M KCl (0.673×10^{-5} [cm^2/sec]) [6], v = scan rate [V/sec], and C_0^{*} = concentration of ferricyanide [mol/cm^3].

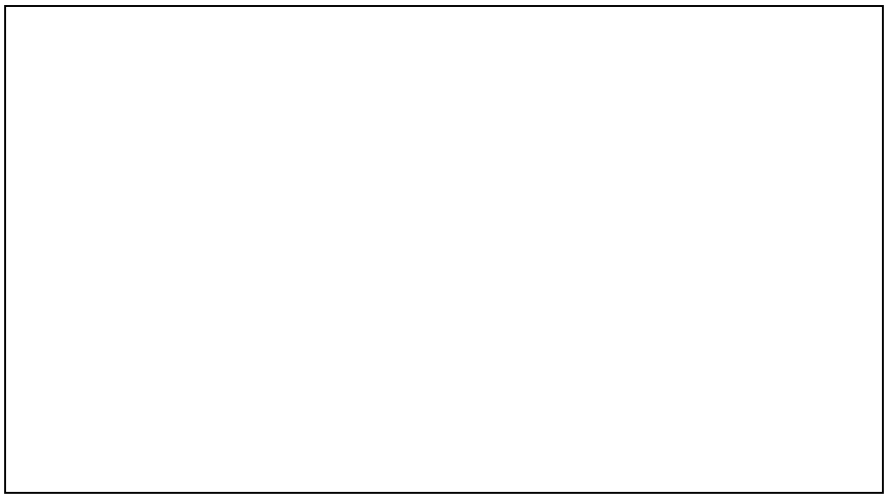

Figure 1. Preparation of a gold microelectrode.

Electrochemical Polymerization of Pyrrole and Immobilization of Glucose Oxidase and Ferricyanide

Electrochemical polymerization of pyrrole onto a gold microelectrode in the presence of the enzyme glucose oxidase and ferricyanide solution was performed at a potential of 0.70 V *versus* silver/silver chloride. Ferricyanide was also used as an electrolyte during the polymerizing process.

Determination of Glucose

The amperometric response to glucose was evaluated using the three-electrode system in 5 ml of 10 mM phosphate buffer pH 7.0. After applying an overpotential, the current background was left to stabilize, and then the glucose solution was added into the measuring cell. The current-time response was recorded as the difference between the stable current (i) after substrate addition and the background current (i_0).

Results and Discussion

Cyclic Voltammetry of Ferricyanide Incorporated in the Polypyrrole Films

Experiments were performed to determine the optimal conditions of the PPy/Ferri/GOD film preparation. Cyclic voltammetry of ferricyanide and the response to glucose of the from. Au/PPy/Ferri/GOD electrode were evaluated as a function of the charge passed, pyrrole concentration and ferricyanide concentration.

Cyclic voltammetry of ferricyanide at PPy and PPy/Ferri modified electrodes at 100 mV/s was performed in phosphate buffer solutions having pH 7.0. (This pH is used because it is similar to physiological conditions.) There was a significant difference between the two electrodes, as shown in Figure 2. Characteristic voltammograms for the redox of ferricyanide in phosphate buffer solution were observed at the PPy/ Ferricyanide electrode. At PPy modified electrodes, no evidence of electron transfer was observed.

Figure 2. Cyclic voltammograms for the Au/Ppy/GOA electrode (solid Lines) and Au/Ppy/Ferri/GOD electrode obtained in phosphate buffer at pH 7 after the electropolymerization. The Scan rate 100 mV/s.

Effect of the Charge Passed during Polymerization on the Enzyme Electrodes

For these experiments, the concentrations of the following compounds were kept constant: glucose oxidase was at 25 units/cm^3, pyrrole was 40 mM and $K_3Fe(CN)_6$ concentration was 50 mM. Cyclic voltammetry was used to investigate the effect of charge passed during film growth on the amount of ferricyanide incorporated. In Figure 3, the charge passed is plotted as a function of anodic peak current (i_{pa}) measured from ferricyanide oxidation. It is found that the amount of ferricyanide incorporated increases linearly with the total charge passed during film growth. This suggests that film thickness increases linearly with charge passed over this range. Based on this information, the film thickness would be estimated by assuming a charge density of 1mC cm^{-2}. This gives an apparent film thickness[7] of 0.22 x 10^{-6} cm.

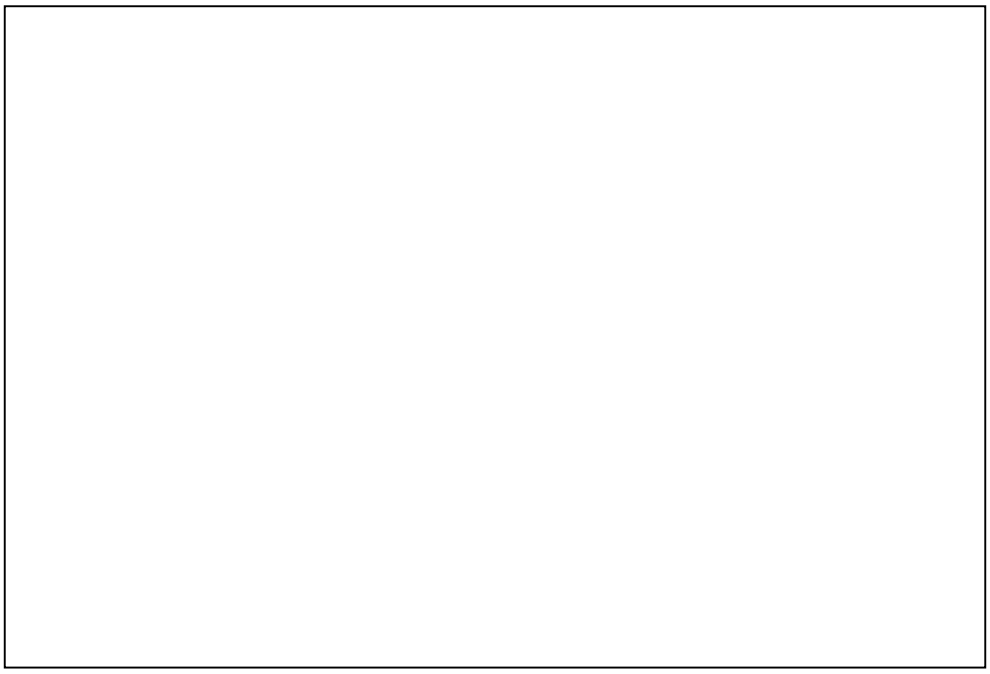

Figure 3. Relationship between charge passed to an Au/Ppy/Ferri/GOD electrode and I_{pa} of ferricyanide incorporated into the polypyrrole films.

The quantity of charge passed should presumably be related to the quantity of enzymes entrapped in the same manner. The effect of charge passed on the amperometric response of an Au/ PPy/Ferri/GOD electrode is studied and the results are shown in Figure 4. The response to glucose at 0.45 V decreases on increasing the charge passed beyond 8 μC. These results can be explained as follows: The average distance, X_r, travelled by a glucose molecule through the film before reacting is given by [8]

$$X_r = \sqrt{D_s K_m / (K_{cat} e_T)}, \qquad \text{……(5)}$$

where D_s is the diffusion coefficient, K_m is the Michaelis constant for the enzyme, K_{cat} is the rate constant for the breakdown of the enzyme-substrate complex and e_T is the total enzyme concentration. When the length X_r is greater than the film thickness, much of the glucose will be lost to the bulk solution before it can react. Hence increasing the film thickness will increase the response, as this enables more glucose to be retained.

However, if the film thickness becomes much greater than X_r, most of the glucose will react only at the outer edge of the film. This means the mediators have a long way to travel before reaching the electrode, so some mediators are lost to the bulk solution. Now, increasing the thickness causes the response to go down. The optimum response will occur when the film thickness and X_r are approxiimatcly, equal. Thus from Figure 4, X_r can be estimated as approximately 0.1 μm.

Thus, for all subsequent experiments the charge passed through the electrodes during polymerization was 8 μC.

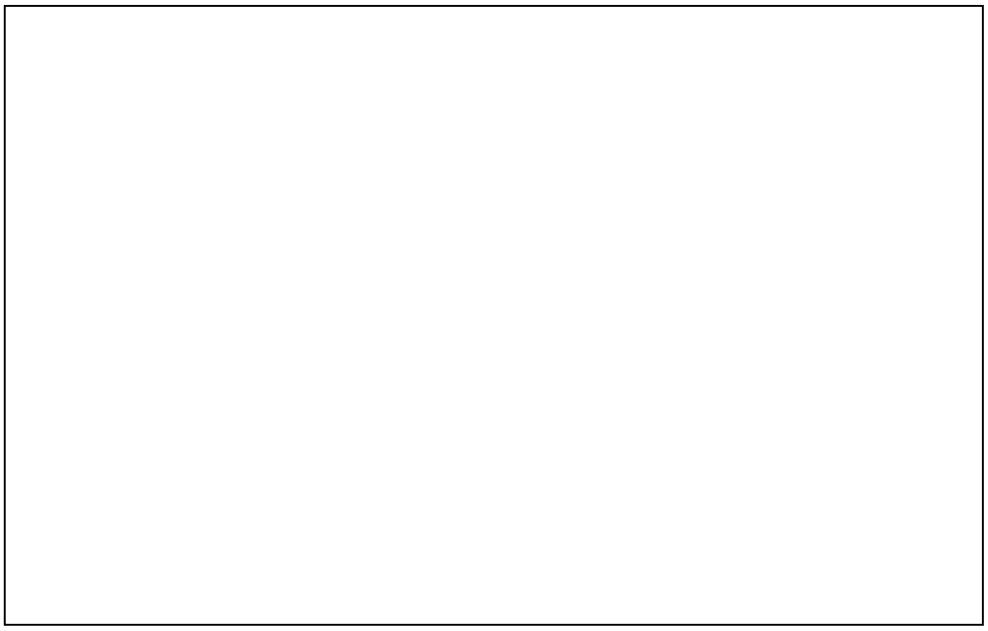

Figure 4. Relationship between the current response to 100 mM glucose solution and charge passed into the 40 mM pyrrole, 25 units/cm^3 GOD and 50 mM ferricyanide solution.

Effect of the Pyrrole Concentration on the Response of the Enzyme Electrodes

We have also investigated the effect of variation of the pyrrole concentration at fixed concentrations of ferricyanide (50 mM) and the enzyme (25 units/cm^3) on the response to glucose at 0.45 V (Figure 5). We find that, at a constant enzyme concentration, the amount of ferricyanide incorporated into the film as determined by cyclic voltammetry increases with increasing pyrrole concentration in the growth solution, up to 40mM. Beyond this, the amount of entrapped ferricyanide decreases.

The effect of the pyrrole concentration used to prepare the Au/PPy/Ferri/GOD electrodes is presented in Figure 6. The total charge passed in modifying the electrodes is 42.5 mC/cm^2, corresponding to a film thickness of about 0.095 μm, assuming the 45 mC/cm^2 correspond[7] to a thickness of 0.1 m. The steady-state current response to 20 mM glucose was recorded at 0.45 V. The optimal concentration of pyrrole was 40 mM as indicated by the maximum current response observed. Thus, for all subsequent experiments a concentration of 40 mM pyrrole was used.

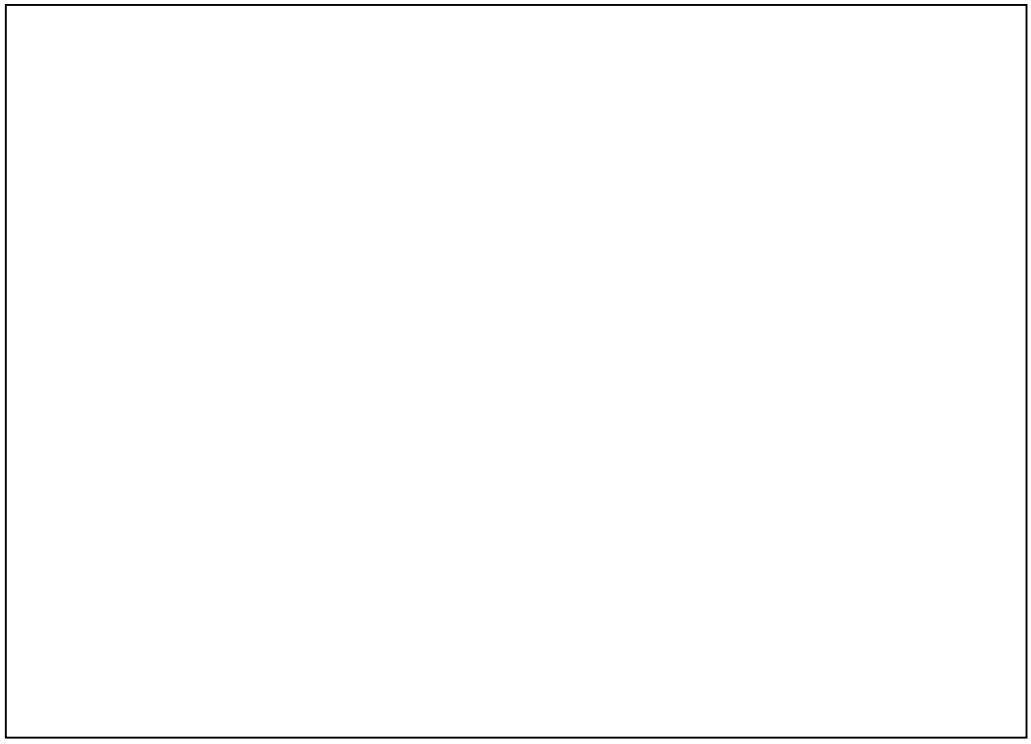

Figure 5. Effect of the pyrrole concentration function on I_{pa} of ferricyanide incorporated in films.

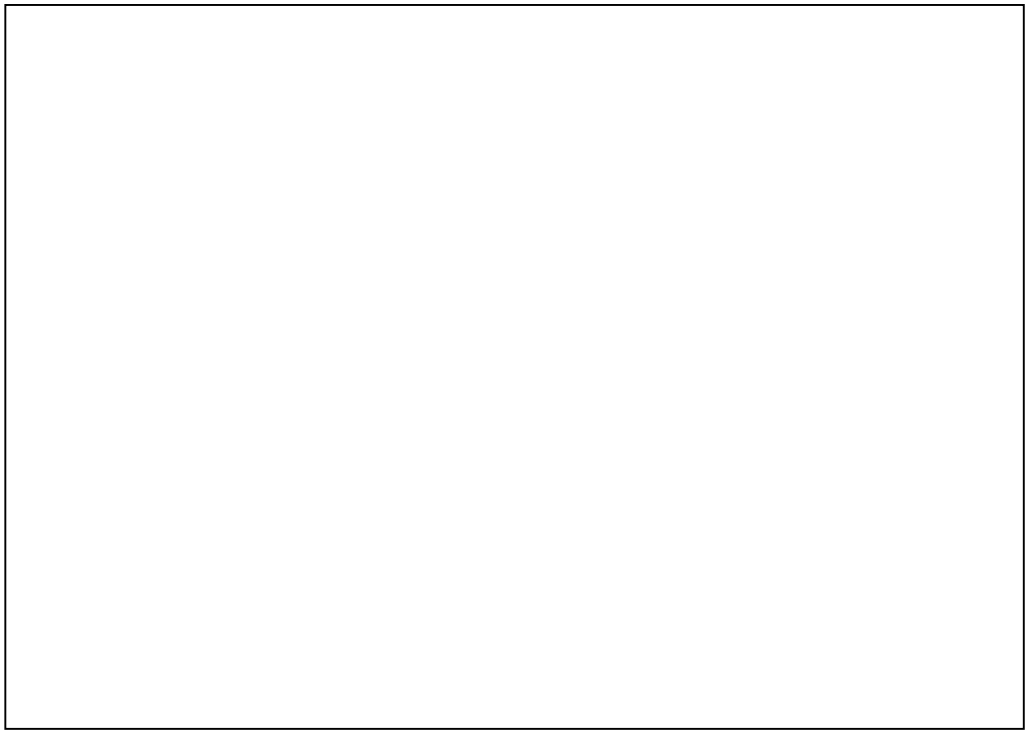

Figure 6. Retationship between pyrrole concentration and current response to 20 mM glucose. The films were grown at 0.7 V from aqueous buffered solutions containing 50 mM ferricyanide, and 25 units/cm^3 GOD, and a varying pyrrole concentration.

Effect of the Ferricyanide Concentration on the Response of the Enzyme Electrodes

Figure 7 shows the correlation between the ferricyanide concentration as a function of and the anodic peak current (i_{pa}) measured from ferricyanide oxidation. We find that the amount of ferricyanide incorporated increases linearly when the ferricyanide concentration increases. We do not obtain a maximum ferricyanide oxidation from Figure 7.

Glucose oxidase is negatively charged at a pH higher than 4.2.[9] Therefore, during electropolymerization at 0.7 V, therefore glucose oxidase can be incorporated into films as a counter ion of polypyrrole [10]. When comparing glucose oxidase to ferricyanide, which also has a negative charge, ferricyanide can diffuse more easierly and faster than glucose oxidase, according to the size of ferricyanide which is smaller than glucose oxidase.

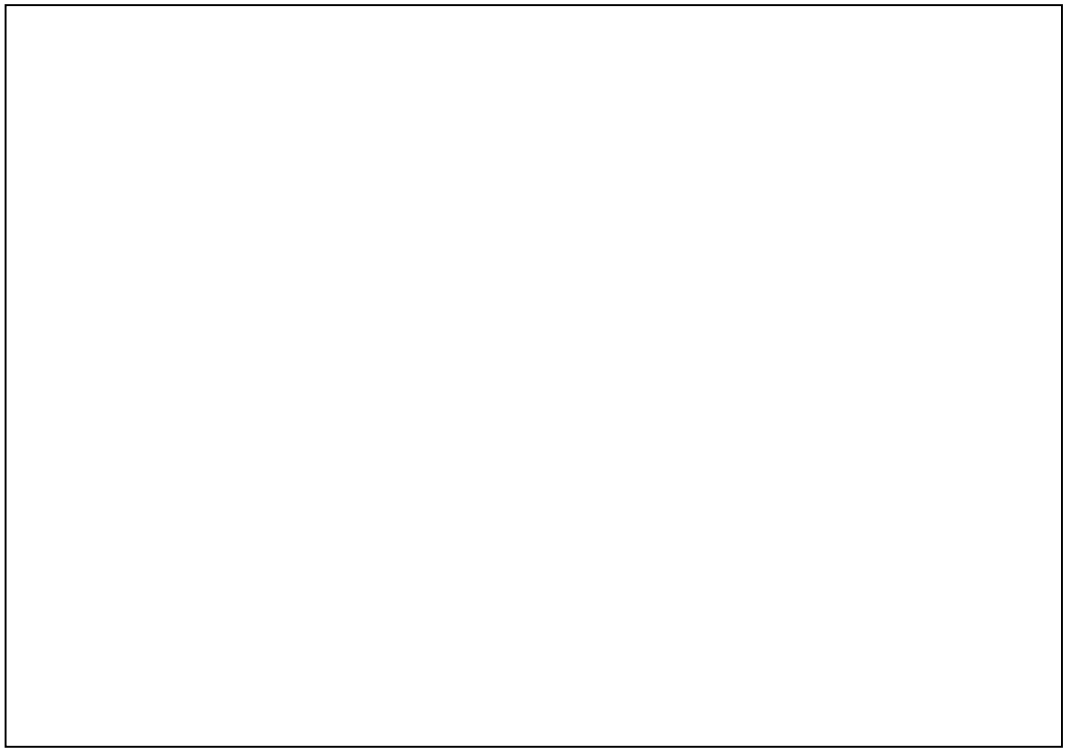

Figure 7. Effect of the ferricyanide concentration function on I_{pa} of ferricyanide incorporated films.

The effect of the ferricyanide concentration on the response of the modified electrode to 20 mM glucose was studied as depicted in Figure 8. We find that with increasing ferricyanide incorporation into the film, the current response of glucose increases until reaching an optimum at 25 mM ferricyanide. Beyond this concentration, the current response of glucose starts to decrease. Thus there appears to be a competition between the enzyme and the ferricyanide for incorporation into the film. This is because both are negative charged and are entrapped into the positively charged polymer by electrostatic attraction. Beyond 25 mM ferricyanide, the quantity of entrapped glucose oxidase decreases so the response to glucose starts to decrease. We can conclude that the polymerization solution should contain 25 mM ferricyanide.

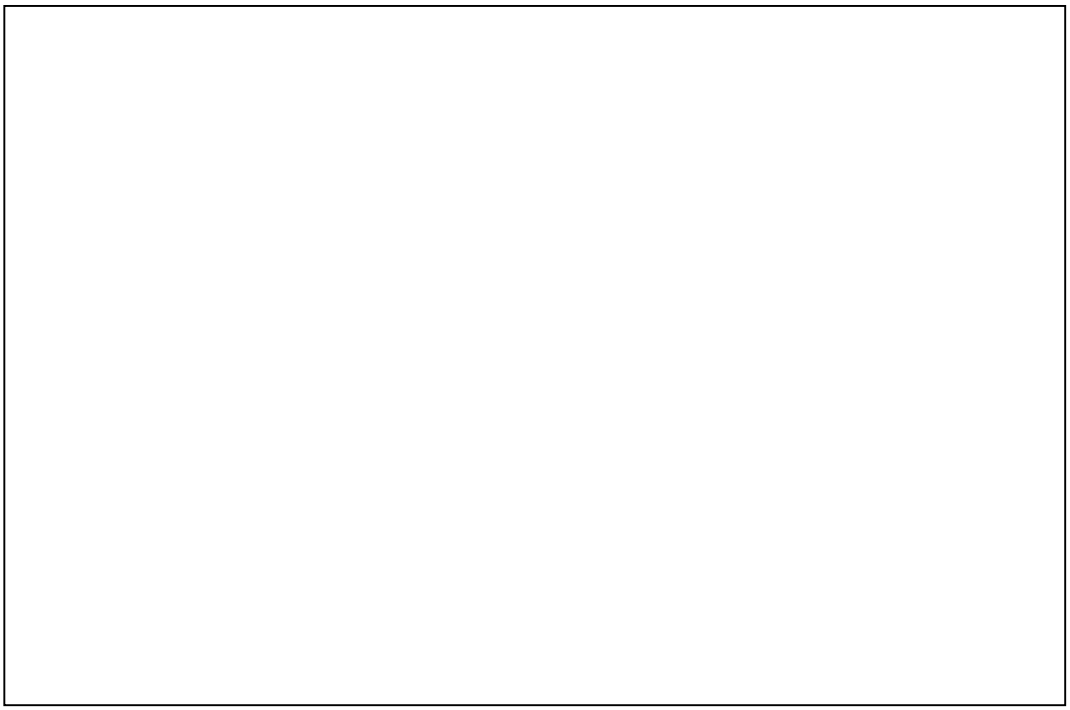

Figure 8. Relationship between ferricyanide concentration and current response to 20 mM glucose. The films were grown at 0.7 V from aqueous buffered solution containing 50 mM pyrrole and 25 units/cm^3 GOD, and a varying ferricyanide concentration.

Stability

Preliminary studies of the stability of the immobilized enzyme films were performed. We found that there was change in the response with repeated use, and that the response decreased by more than 50% from the initial value after storage for 24 h at room temperature in a glucose-free buffer solution. The stability information suggested that the decrease in the response, is partly, due to the diffusion of ferricyanide into the solution and partly due to loss of enzyme activity.

Sensitivity

The glucose sensitivity of this sensor, as optimized in this experiment, was found to be approximately 0.05 nA/mM with a linear response up to about 5 mM as shown in Figure 9.

CONCLUSIONS

Both GOD and ferri/ferrocyanide can be entrapped within polypyrrole films. Using cyclic voltammetry we can measure ferricyanide incorporation during film growth. Amperometric experiments to measure glucose suggest that the two species compete with each other for sites within the film. This is to be expected since they are both incorporated as counter ions during the deposition of the cationic polymer.

The optimum conditions for preparing the glucose sensor are the use of electrochemical polymerization of 40 mM pyrrole onto a gold microelectrode in the presence of 45 units/cm^3 of the enzyme glucose oxidase and 25 mM ferricyanide at a potential of 0.70 V *versus* Ag/AgCl. The ferricyanide entrapped within the film is able to mediate the re-oxidation of the enzyme so that glucose can be detected amperometrically using these films at 0.45 V *versus* Ag/Ag Cl. The optimized sensitivity was found to be about 0.05 nA/mM.

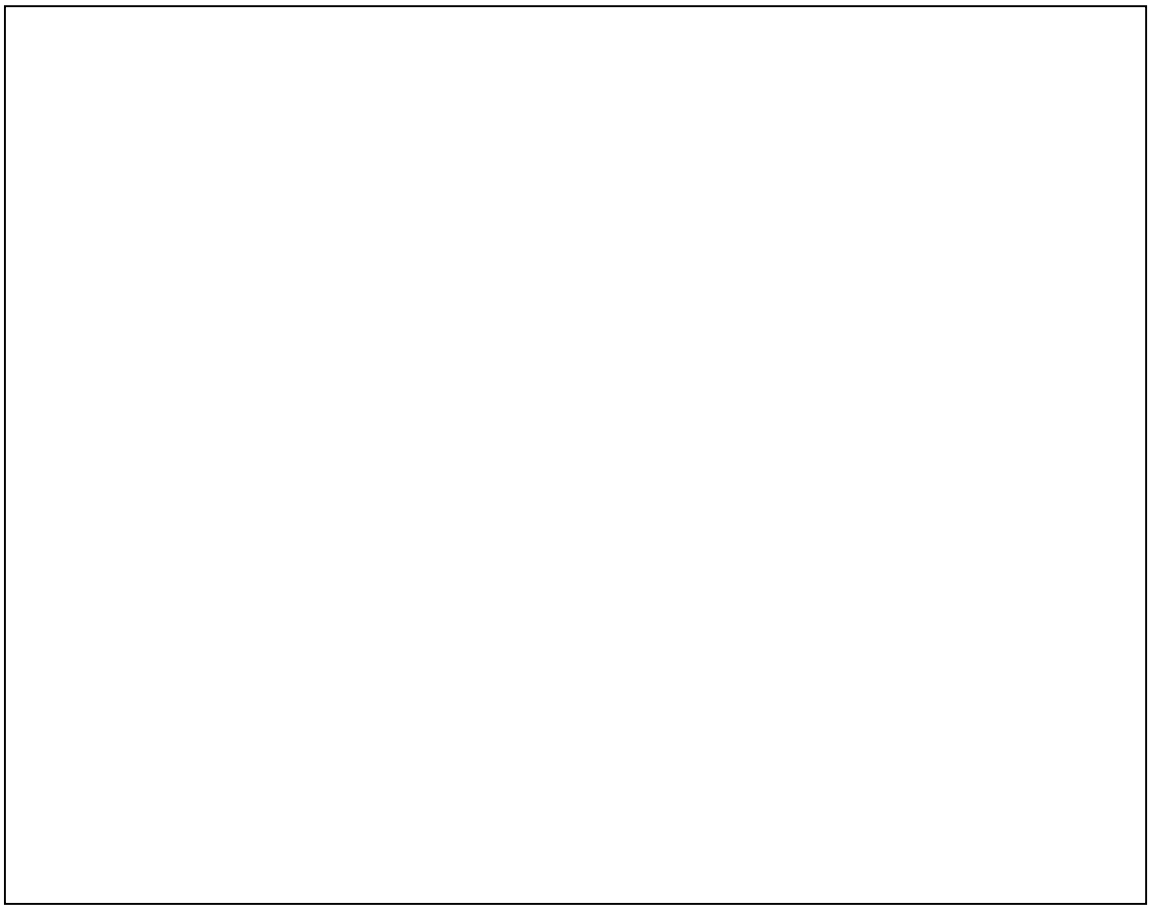

Figure 9. a) Calibration curve of the glucose sensor at 0.45 V using ferricyanide as a mediator.
b) Expanded scole showing linear behavior up to about 5 mM glucose.

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REFERANCES

1. Work, J.V.; Yacynych, A.M. *Sensors in Bioprocess control*; Work, J.V.; Yacynych, A.M., Ed., 1990 ; Vol. 6
2. Lowery, L.P.; Macateer, K.; Elatrash, S.S.; Duff, A.; O'Neill, R.D. *Anal Chem.* **1994**, 66, 1754
3. Cho, J.-H.; Shin, M-C.; Kim, H.-S. *Sensors and Actuators B* **1996**, 30, 137
4. Asavapiriyanout, S. *J.Electroanal. Chem*. **1984**, 177, 229
5. Bard, A.J.; Faulkner, L.R. *Electrochemical Methods*; John Wiley & Sons: New York, 1981
6. Adams, R. N. *Electrochemistry of Solid Electrodes*.; Marcel Dekker: New York, 1969
7. Holdcroft, S.; Funt, B.L. *J. Electroanal. Chem*. **1988**, 240, 89
8. Bartlett, P.N.; Whitaker, R.G. *J. Electroanal. Chem*. **1987,** 224, 27
9. Swoboda, B.E.P.; Massey, V. *J.Biol. Chem*. **1961**, 241, 3409
10. Diaz, A.F.; J.I.Castillo; Logan, A.; Lee, W.Y. *J.Electroanal. Chem*. **1981**, 129, 115

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