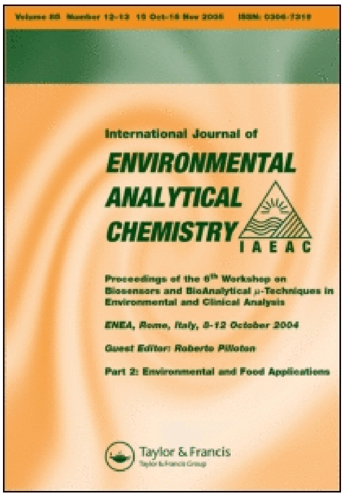

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Voltammetric analysis of glucose using poly 4-vinylpyridine modified carbon fiber electrode

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An enzyme-modified microelectrode has been constructed and evaluated. The 4-vinlypyridine was polymerized anodically at its optimum onto a carbon fiber (8 μm diameter) when its concentration was 0.003 M, pH 3.0, scan rate $50\,\mathrm{mV\,s^{-1}}$ and at a constant potential of +0.40 V *vs.* SCE. Initial investigation indicated that the type of supporting electrolyte and solvent used affected this polymerization. Glucose oxidase was then dip-coated onto the polymer modified electrode for glucose determination. The dip-coating enzyme immobilization technique has produced the best current response. The glucose analysis was only done *in vitro* for a whole blood sample. The effects of common interferents in glucose determination are also reported. The operational life of the electrode spanned over two weeks of constant use under normal laboratory condition. The *in-vivo* glucose analysis has yet to be done but preliminary work on this looked very promising. This topic will be discussed in future articles.

Keywords: Glucose; Microelecrode; Polymer

1. Introduction

Voltammetric studies using ultra microelectrodes have been the subject of growing interest in recent decades [1–3]. This is due to their unique properties that facilitate them to be used either *in vitro* or *in vivo* [4]. Overall, the rates of diffusion of reactive species to and from microelectrode surfaces are enhanced relative to the rates of unwanted (non-diffusion-controlled) side reactions and double layer charging. Hence, faster reactions than normal can be observed due to efficient supply of reactant. Additionally, ohmic distortion of responses is negligible, on account of the low currents involved [5]. Moreover, such devices are practical in a very small volume of analytes.

The use of microelectrodes in the determination of glucose has been reported [6–13]. A glucose microelectrode electrochemically deposited with tetrathiafulvalene–tetracyanoquinodimethane crystals as mediator was found suitable for glucose detection in an environment where oxygen concentrations did not frequently change [6]. Other mediator such as poly[(vinylpyridine)Os(bipyridine)$_2$Cl] in hydrogel

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formation on carbon fiber microdisk electrodes have shown 10 times better sensitivity than the conventional size electrodes prepared similarly [7]. However, there was no mention on efforts to minimize the interference.

In earlier work from this laboratory [13, 14] poly-4-vinylpyridine (P4VP) was used as a mediator for glucose monitoring, upon deposition onto a composite graphite paste electrode. However, as has been reported elsewhere [15], the paste in such electrodes is difficult to reproduce, leading to irreproducible sensors. In addition to this, the immobilized enzyme usually suffers premature denaturation around the highly lipophilic environment of the paste. In most fabrication Teflon® tube is used as an electrode body. Even though Teflon® is inert, it is reported [16] to be unsuitable for chemical detection *in vivo*. Therefore, the purpose of this study is to find an alternative material to composite graphite paste. Thus, carbon fibre was chosen as it is typically carbon with acceptable conductivity and minimal cost.

This article describes the fabrication of an immobilized enzyme P4VP-modified carbon fiber electrode as a sensor in glucose determination. Optimum conditions on preparation of P4VP from its monomer onto carbon fiber were presented. Various techniques of GOD immobilization on the P4VP-modified electrode were discussed. The most responsive electrode was used for further investigation. Finally, the results obtained were compared to a similarly fabricated conventional graphite paste electrode.

2. Experimental

2.1 *Reagents*

The 4-vinylpyridine (4VP) monomer ($\sim 96\%$) and tetramethylammonium chloride (TMAC), tetrabutylammonium perchlorate (TBAP), ascorbic acid, lactic acid and 4-acetaminophenol were from Fluka Chemika, Switzerland and were used without further purification. Acetonitrile (MeCN) from Romil Chemicals, England; Araldite® epoxy resin from Ciba Geigy, Switzerland; Triton® X-100, i.e. polyethylene glycol tert-octylphenyl ether (PEG), 10% (w/v) and glucose oxidase (GOD) from asperigillus niger (EC 1.1.3.4, Boehringer Mannheim catalogue no. 646 431, grade II lyophilized, 100,000 i.u.), from Boehringer Mannheim GmbH, Germany were used as received. β-D-glucose and mineral oil from Sigma Chemicals, USA were used as received. The stock solution of β-D-glucose was prepared in phosphate-buffered saline (PBS) and allowed to mutarotate and left overnight at 4°C before use. This was to establish equilibrium concentrations between α and β anomers. Although GOD was specific for β glucose reported concentrations were those of total glucose concentrations. The PBS solution was prepared, daily, by dissolving 2.754 g of NaCl, 2.081 g of KH_2PO_4 and 0.477 g of NaOH in 1000 mL of distilled water and then pH adjusted to 7.4 with 0.1 M NaOH. All other reagents were of the highest grade available and were used as received. Pure water was used for preparation of all aqueous solutions, and was obtained by passing distilled water through a Milli-Q Plus from Millipore Corp., USA. Mercury (extra pure, Fluka catalogue no. RdH 10008) was used without further purification. The oxygen-free nitrogen (OFN) was obtained from Nissan-IOI, Malaysia. The activities of the GOD were tested using the respective standard procedures from the manufacturers. The composite graphite powder from pencil lead 2B, Mars Lumograph 100, from Staedtler, Germany was cleaned under continuous reflux in methanol for 8 h. Whole blood samples ($n = 100$) were received from a clinical testing laboratory and were preserved in a refrigerator at 4°C. Analysis on the whole blood samples were performed within 48 h after collection.

2.2 *Instrumentation*

Most electrochemical studies and analysis were done using a Potentiostat/Galvanostat (Model 273A, Princeton Applied Research, USA). An IBM PS/2 model 80 computer with software developed in-house, was used for controlling the potentiostat and for data acquisition. A conventional three-electrode system was used with a platinum flag (1 cm^2, locally purchased) as counter electrode and a saturated calomel electrode (SCE) (Russell pH, UK) as reference. The FTIR spectrophotometer was used (model 2000 Perkin Elmer, USA). The pH measurement was established using a benchtop pH/ISE meter (model 720A Orion, MA, USA). In all experiments, the buffer was deoxygenated and experiments were performed at 25 ± 3°C. Magnification of the microelectrode was seen under a stereomicroscope (model SMZ-2T, Nikon, Japan) and photos were taken with an attached camera (model F-601, Nikon, Japan).

2.3 *Electrode preparation*

Carbon fiber (8 μm diameter, 6.4 mm long, Lot # 20590, Johnson Matthey Electronics, Ward Hill, MA, USA) was aspirated into a glass capillary (model GD-1, 1 × 90 mm, Narishige Co., Japan). The capillary was then pulled on a micropipette puller (Model PB-7 Narishige, Japan) to yield a glass tip of approximately 20 μm o.d. and later was sealed with Araldite® epoxy. The sealed glass was polished at 45°C with a micropipette grinder (EG-4, Narishige, Japan) on an extra-fine diamond abrasive plate to produce an elliptical carbon disk. Electrical contact was established by filling the capillary with a very small volume of mercury (0.5 mL) and then with carbon paste. A copper wire was then inserted in the paste. The other end of the capillary was finally sealed with epoxy. The microelectrode was cured at 70°C for two days after sitting at room temperature overnight. The protruding carbon fiber currently acted as the naked microelectrode. The latter was then washed with acetone. It was then rinsed with pure water and dried in a cold air stream prior to polymer deposition. The electrochemical cell was initially purged with oxygen-free-nitrogen (OFN) gas for 10 min. Later P4VP film was anodically electrodeposited from the 4VP solution onto the carbon fiber electrode at a potential from -0.4 to $+0.6$ V *versus* SCE and at scan rates of 50 $mV\,s^{-1}$, respectively, with supporting electrolyte either TMAC or TBAP. The pH of the solution ranges from 2.9 to 3.5 adjusted by dropwise addition of 0.1 M acetic acid or 0.1 M NaOH. Upon completion of deposition time, the microelectrode was removed carefully and washed with a mixture of acetonitrile and water. The P4VP-modified electrode was left to dry under reduced pressure for 30 min.

The GOD was immobilized onto the microelectrodes using either; (i) a dropping of GOD solution (5 μL) for 5 min or (ii) soaking for 1–3 min in 2 mL of aqueous buffer containing 200 i.u. mL^{-1} GOD. Microelectrodes were then dried for 3 h in a desiccator. Each GOD/P4VP electrode was conditioned and stored in PBS (pH 7.4) at 4°C when not in use. The composite graphite paste was a mixture of powdered graphite pencil (0.88 g) with mineral oil (0.5 mL) and PEG (0.01 mL), and the conventional P4VP modified graphite paste electrode was prepared in an earlier report [13, 14].

3. Results and discussion

3.1 *Characterization of the microelectrodes*

Microscopic examination of the microelectrode was shown in figure 1(A). The carbon fiber can be seen protruding through the Araldite epoxy and out from the glass capillary. Cyclic voltammograms (CV) of the microelectrode and the conventional carbon paste electrode were performed in 0.001 M Cr^{3+}, pH 3.0 and in 0.1 M KCl (figure 1B). The conventional carbon paste electrode produced a large background current and peaks compared with carbon fibre electrode. The well-defined sigmoidal voltammogram (typical of a microelectrode) with very much lower background current obtained indicates that the rates of diffusion of reactive species to and from microelectrode surfaces are enhanced. The E_{pc} of Cr^{3+} for both electrodes at −0.41 V *versus* SCE indicates that the Cr^{3+} is only reduced up to Cr^{2+}.

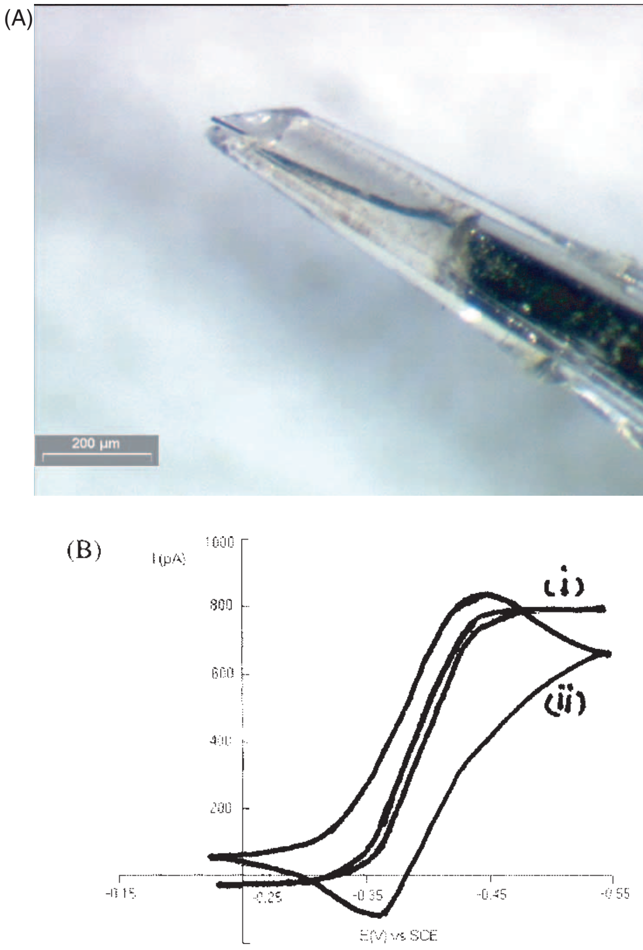

Figure 1. (A) Magnification of microelectrode seen under a stereomicroscope. (B) (i) CV for carbon fiber microelectrode. (ii) Conventional carbon paste electrode. Solution conditions: 1 mM $CrCl_3$, 0.1 M KCl, pH 3.0 and scan rate 50 mV s^{-1}.

3.2 *Choice of supporting electrolyte and organic solvent*

The use of suitable supporting electrolyte in an electrochemical cell is indispensable as this may affect the solvent medium in several ways. However, the primary function of the supporting electrolyte is to provide a conducting medium. In this report, two different supporting electrolytes, viz. TMAC and TBAP, were studied. Cyclic voltammetric studies of the system were studied at different concentrations of TMAC and TBAP. The optimum concentration for TMAC and TBAP was $\approx$0.01 M. At higher concentrations of TBAP, the background noise increased and current instability occurred. This indicates that the concentration of supporting electrolyte is critical and not necessarily always very much higher than that of the analyte. The customary practice in voltammetry is to make the supporting electrolyte concentration at least 100 times higher than the concentration of the analyte. This is particularly important when quantitative agreement is sought with the equations that are used to calculate either limiting or peak currents [17]. The CVs of both electrolytes indicate that TBAP is more suitable for electropolymerization of P4VP as it has better current stability and lower background noise. Since the polymerization is done in MeCN, TBAP is preferred due to its lower specific resistance in MeCN (37 Ω cm). Besides, TBAP is quite soluble in MeCN (70 g/100 mL), whereas TMAC has a higher association constant, 56 in MeCN.

We have conducted study on four solvents, i.e. MeCN, dimethylsulfoxide (DMSO), tetrahydrofuran (THF) and dimethylformamide (DMF). Only MeCN seems to be a useful solvent in this work. MeCN is resistant to redox process and also transparent in the region of 200 to 300 nm. It is not as strong a base as DMSO and its dielectric constant of 37 permits reasonably high conductivities. Even though DMSO has a higher dielectric constant and sufficiently resistant to redox, it is not as resistant as MeCN. FTIR analysis of P4VP shows that $\nu_{C=C}$ for the vinyl group appears at 1632 cm^{-1} and $\nu_{C\text{–}H}$ for planar deformation at 1198 and 1205 cm^{-1}. The P4VP synthesized using MeCN as solvent has all these peaks unaffected. However, when either DMF or DMSO is used, the $\nu_{C=C}$ peak disappears and of all the absorption peaks that characterize P4VP there was none to be seen.

3.3 *Electropolymerization of 4VP*

The CV of the electropolymerization of P4VP indicates that the potential range being used is the best among those investigated (figure 2). This is because the ratio of $I_{pa} : I_{pc}$ is almost unity and the scan rate, $\nu^{1/2}$, is directly proportional to the peak current I_p. The optimum condition for polymerization was observed at pH 3.0, concentration of the monomer was 0.003 M and scan rate of 50 mV s^{-1} at +0.40 V *versus* SCE. The system which was purged with OFN gas has initially produced a higher I_{pa} value. A more consistent I_{pa} was obtained after 10 min of purging. A 10% increase in peak current was, however, observed after 45 s of continuous sweeping. This is probably due to the growth of polymeric layer at the electrode surface. The cathodic peak current remains constant after 3 min of continuous sweeping indicating a steady state has been achieved. The cathodic current value seems to have dropped after 30 min of continuous scanning. Perhaps, due to the persistence dissolution or oxidation of P4VP back to its monomer formation. A similar type of electropolymerization has been reported elsewhere [13, 14]. Hence, a similar type of reaction is expected.

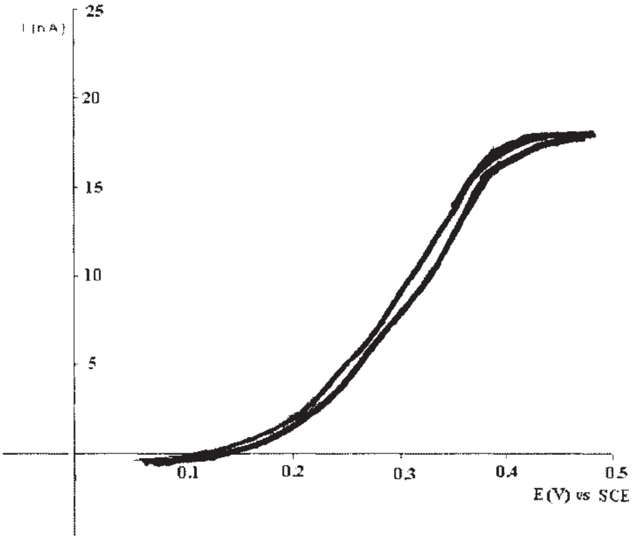

Figure 2. CV for electropolymerization of PVP at carbon fiber microelectrode. Solution conditions; 0.003 M 4VP, 0.1 M of TBAP in MeCN, pH 3.0, scan rate 50 mV s^{-1}.

Table 1. Currents recorded at the GOD/P4VP-modified carbon fiber electrode.

	P4VP microelectrodes					
	1×10^{-3} M H_2O_2		2×10^{-3} M H_2O_2		4×10^{-3} M H_2O_2	
Immobilization technique	I (pA)	j (μA cm^{-2})	I (pA)	j (μA cm^{-2})	I (pA)	j (μA cm^{-2})
Dropping (5 μL for 30 s)	2	3.98	4	7.96	7	13.93
Dip-coating	31	61.65	58	115.63	122	246.64
In situ immobilization	1	1.99	2	3.98	5	9.95
	6×10^{-3} M H_2O_2		8×10^{-3} M H_2O_2		10×10^{-3} M H_2O_2	
	I (pA)	j (μA cm^{-2})	I (pA)	j (μA cm^{-2})	I (pA)	j (μA cm^{-2})
	13	25.86	18	35.82	23	45.77
	188	373.93	250	497.25	310	616.59
	7	13.93	9	17.91	11	21.89

The current density value 794.78 nA cm^{-2} obtained, indicates that the electronic transference between the polymeric layer is feasible. The concentration of the monomer in electropolymerization is critical, as this could determine the thickness of the P4VP formed, hence the current density value and also the performance of the electrode. In this work, 0.003 M of 4VP was used to produce a suitable thin film of P4VP on the microelectrode surface. If the concentration of the monomer exceeds 0.1 M and at higher pH, precipitation may occurs whereas if the concentration were smaller than 0.002 M, very little current was detected.

3.4 *P4VP-modified microelectrode coated with glucose oxidase*

The average current recorded at the P4VP-modified carbon fiber electrodes coated with GOD with different immobilization techniques is shown in table 1.

Preliminary investigation on the response of these electrodes towards 1 and 5 mM glucose in PBS solution (pH 7.0) indicates that the dip-coated immobilization is superior than the dropping method as the current response is proportionate to the concentration of glucose. This is presumably due to the high loading of enzyme being used as well as allowable time for the adsorption of GOD to occur on the P4VP film. The microelectrode exhibits rapid and reversible electrons transfer as indicated by the small difference between the oxidation and reduction peaks, $\Delta E < 0.1$ V. Electron transfer within the film obeys a relationship similar to those for molecular diffusion in an earlier report [7]. Stacking enzyme layers onto P4VP layer by adding a higher concentration of GOD in MeCN resulted in yellowish precipitate being observed with no CV was recorded. The response time to a bolus of glucose, defined as the time for the current to rise to 90% of its maximum, was less than 2.0 s. However, a decrease of 10% in response was observed after 30 min of continuous sweeping which could be due to the dissolution of the enzyme to the bulk solution. But the response remain stable with the sweeping continued for several hours. Glucose microelectrode, fabricated with P4VP on carbon fiber, shows current densities higher than that of conventional macroelectrodes.

Since in most biochemical reaction, the activity of enzyme depends largely on the pH being used. The responses of the P4VP entrapped enzyme towards pH solution was investigated (figure 3). The result indicates that the microelectrode shows an optimum response at pH 7.4. However, very little current was detected when the pH solution was higher than 8.5 or lower than 2.5 which indicates that the enzyme is suffering from either a rapid or permanent loss of activity.

A signal is obtained through the detection of either the consumption of oxygen or the production of hydrogen peroxide as:

$$\beta\text{-D-glucose} + O_2 \xrightarrow{\text{GOD}} \text{gluconic acid} + H_2O_2 \tag{1}$$

The glucose current response was measured in a deaerated solution, at +0.18 V *versus* SCE, scan rate 50 mV s^{-1}, and at pH 7.4. When 5 mM of glucose was spiked into the

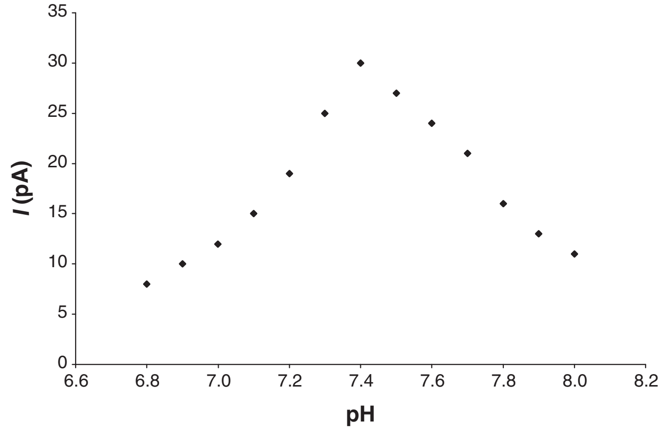

Figure 3. Variation of current response with pH for 1 mM glucose in PBS solution at +0.18 V *vs*. SCE and scan rate 50 mV s^{-1}. Only dip-coating technique immobilization was used.

solution matrix, the response increased and reached a steady state value of 152 pA after 10 s. Investigation on peak potentials confirm that these peaks were caused by electrical communication of a prosthetic group, flavin adenosine dinucleotide (FAD). Then GOD that catalyzes the overall reaction utilizes FAD and O_2 as hydrogen acceptor. It has been suggested that a glucose sensor should response linearly towards glucose from 1 mM to at least 15 mM of glucose concentration [18]. In this work, fabricated microelectrode responses linearly towards glucose at a concentration between 1 and 16 mM (figure 4A and B). This is a typical linear range obtained as reported elsewhere [6]. A drop in current response was observed when the glucose concentration exceeded 17 mM. A possible explanation for this is either the enzyme layer is saturated with substrate glucose or the membrane restricted the influx of glucose to the enzyme [6, 7]. There is also a suggestion that this was caused by oxidation of reduced GOD in the electrode [6].

The concentration dependence of the amperometric sensor was also tested in deaerated and aerated solutions over the range of 1–20 mM glucose solution. The current increased linearly with concentration. A drop in current response was observed

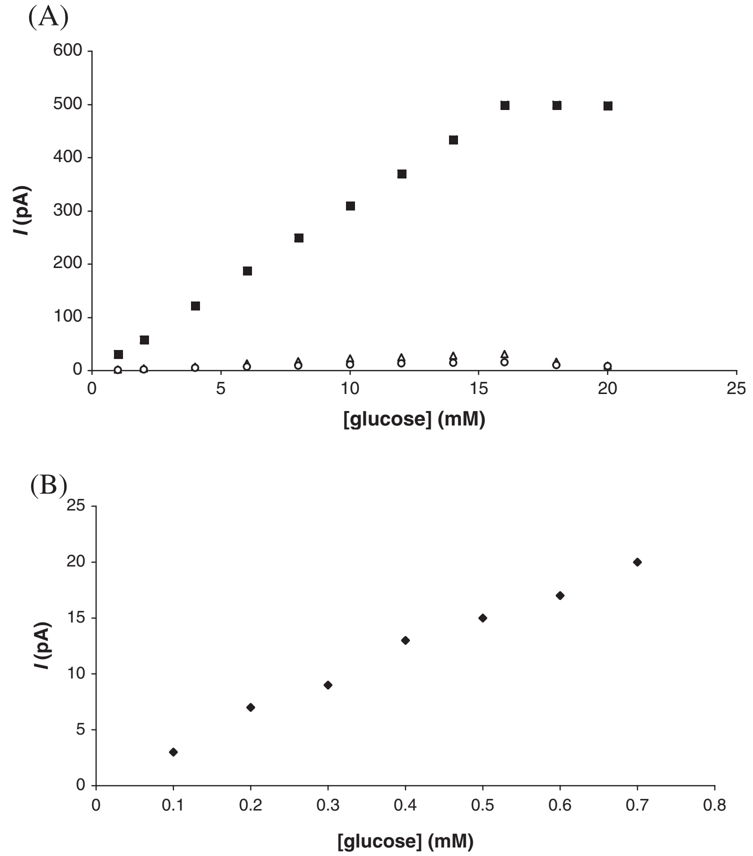

Figure 4. (A) The current response of GOD/P4VP-modified carbon fibre electrode at different glucose concentrations. Dip coating (■), *in situ* immobilization (○) and drop wise (Δ). (B) The current response of GOD/P4VP-modified carbon fibre electrode at different glucose concentration by a dip-coating immobilization technique.

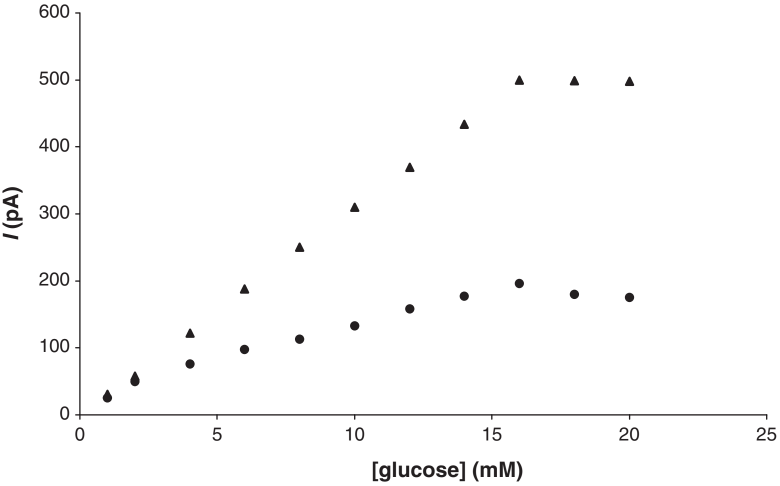

Figure 5. Measurement of steady states current against glucose concentration under aerated (●) and deaerated (▲) (N_2 purged).

when the concentration exceeded 17 mM (figure 5). At moderate glucose concentration, the electrode current decreased substantially in the presence of air. This is consistent with the results previously reported [19]. The current responses to 5 mM glucose fluctuated by less than 1% with flow rates from 0.25 to 1.00 mL min^{-1}. The latter will be discussed further on the interference by oxygen.

3.5 *Kinetics of GOD/P4VP-modified carbon fiber electrode*

The apparent Michaelis-Menten constant, K_m, gives the indication of the affinity of the enzyme for the substrate [20]. However, K_m is not an intrinsic property of the enzyme but rather of the system as a whole. Kinetic measurements of glucose was made according to the Eadie–Hofstee equation

$$J_{ss} = j_{max} - K_m(j_{ss}/C) \tag{2}$$

where j_{ss} is the steady-state catalytic current density, j_{max} is the maximum current density under saturating substrate conditions, K_m is the apparent Michaelis–Menten constant, and C is the concentration of glucose in solution.

The apparent Michaelis–Menten constant, K_m, of the immobilized enzyme was determined from equation (1) [6, 20–22].

The kinetic behaviour of these electrodes was evaluated from the amperometric response at E_{app} *versus* SCE = +0.18 V to various concentrations of glucose ranging from 1 to 40 mM. A glucose response data plotted according to equation (2), should give a straight line with a slope equal to the negative of the apparent Michaelis–Menten constant, K_m, and intercepts equal to current density at its maximum j_{max}. The K_m value and j_{max} were 7.1 mM and 118.62 μA cm^{-2} respectively. The j_{max} was calculated on the basis of the protruding carbon fiber. This value compared favourably with other previous results in which j_{max} was in the range of 100–800 μA cm^{-2} [23].

3.6 *Interference studies*

Equation (1) shows that oxygen is essential in the enzymatic reaction of a glucose sensor. However, oxygen is accused of competing with the counter electrode as the electron sink, which then further reduced the current response [6]. The effect of O_2 on the present microelectrode is somewhat comparable to previous reports [6, 18, 22]. This is indicated by the linearity range of glucose concentration being extended to 17 mM. A reduction in amperometric response was, however, observed during aeration of glucose solution ranging from 1 to 20 mM (figure 5). Measurements of interference by oxygen were made on the basis of comparative study, from negligible oxygen level to air-saturated conditions.

Other interferents studied were those normally found in biological fluid i.e. 4-acetaminophenol, ascorbic and lactic acids. The responses of the GOD/P4VP microelectrode towards each of these interferents, in the absence of glucose, were tested and shown in figure 6. The most severe interference was observed from 4-acetaminophenol. This is presumably because of the relatively small molecular size of 4-acetaminophenol. Since it is uncharged, utilization of perm-selective membranes was not effective in reducing its interference [24]. The P4VP-modified micro-electrode, however, has not responded to the interference as well as the P4VP-modified composite paste electrode. Efforts to further attenuate the interference signal has been reported [14]. This is done by engineering the specific enzymatic reaction within the GOD/P4VP layer. A layer of glutaraldehyde cross-linked with ascorbate oxidase was placed over the cross-linked glucose oxidase layer to reduced interference caused by ascorbic acid [6, 14].

Investigation on whole blood samples at pH 5, after spiking with 5 mM glucose solution showed that 88.5 ± 1% ($n = 10$) was recovered. The decrease in the recovery of glucose in whole blood is probably caused by other possible interferents commonly

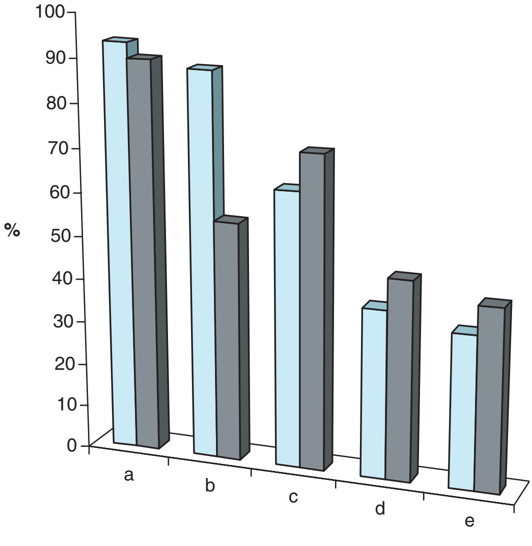

Figure 6. Recovery measurement of glucose sensor toward: (a) 5 mM glucose, (b) whole blood sample, (c) 4-acetaminophenol, (d) ascorbic acid, and (e) lactic acid. Comparisons were made between P4VP-modified carbon fiber electrode (light shade) and P4VP-modified carbon paste electrode (dark shade).

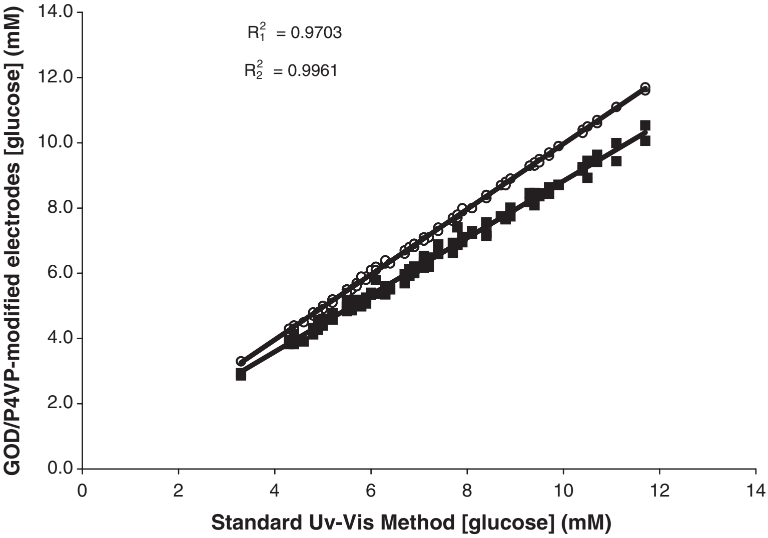

Figure 7. Correlation plot between carbon fiber electrode (○) (correlation coefficient R_2) and GOD/P4VP-modified carbon paste electrode (■) (correlation coefficient R_1) with standard UV method.

found in human blood. They are alkali metal ions, organic acids and possibly phosphates. Since the recovery of the 5 mM glucose solution in PBS (pH 7.4) is 93.5 ± 1% ($n = 10$) the assumption that phosphate ions might have interfered could be possible. The amperometric responses of the GOD/P4VP-modified carbon fiber electrode and GD/P4VP-modified composite paste electrode towards 100 whole blood samples was performed and compared with the results obtained using the standard UV method [25]. There is a direct correlation between both electrodes and the corresponding standard methods, with the microelectrode ($R_2^2 = 0.9961$) relatively superior to the composite carbon paste electrode (figure 7).

3.7 *Lifespan of the P4VP/GOD carbon fiber microelectrode*

The current response of the electrode, kept in a buffer solution at 4°C, towards glucose remained fairly constant for 5 days and then a decrease of 9% in current response was observed (figure 8). A possible explanation is that the enzyme could have dissolved while being kept in the buffer solution at 4°C, and the loss of activity resulted in a decrease of current signals. The stability in storage was not less than 14 days. A comparative study on the storage of P4VP/GOD carbon fiber microelectrode in buffer solution and being kept dry at 4°C has been carried out and it was found that the difference in response measured was less than 1%. However, the current response of the GOD/P4VP carbon fiber microelectrode that was kept dry towards glucose remained fairly constant for 7 days and then a decrease of 5% in current response was observed, slightly better than the one being stored in buffer solution. Further investigation on its reproducibility indicated that the relative error of the response was less than 1% with measurements being carried out ($n = 10$) at the 4th, 8th and 12th day from its fabrication and performed in 5 mM glucose.

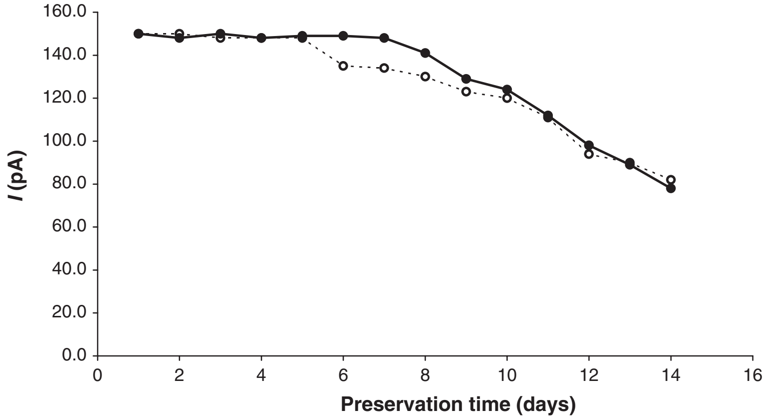

Figure 8. Response of the GOD/P4VP-modified microelectrode to 5 mM glucose measured at intervals. Microelectrodes were kept dry (●) at 4°C and microelectrodes were stored in phosphate buffer solution (○) at 4°C.

4. Conclusion

Unlike other microelectrode fabrication used for glucose determination, the present electrode offers a cheaper alternative. Besides cheap materials being used, the fabrication is simple, fast and easy. The results obtained in this work compares favourably, if not better, with other previous reports. At present, the electrode still suffers serious interferences from 4-acetaminophenol and ascorbic acid. Thus, various techniques to minimize the effect of these interferences is still in an investigative stage. An effort to use the microelectrode for *in vivo* glucose analysis is in progress and this will be discussed further in the next article.

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