

Amperometric and Photometric Responses of *in Situ* Coupled Glucose Oxidase-Poly (Propylene Imine) Dendrimer Based Glucose Biosensor

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In this work, poly (propylene imine) dendrimer (PPI) was coupled to glucose oxidase enzyme (GOx) *in situ* and used as a bioreceptor in a glucose biosensor. The coupling was accomplished by the use of 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC)/N-hydroxysuccinimide (NHS) linker. Ultraviolet visible spectroscopy, Fourier transform infra red spectroscopy and scanning electron microscopy were used to confirm the coupling reaction. A biosensor was then prepared by drop coating the GOx-PPI onto a glassy carbon electrode and then used for the amperometric detection of glucose. The bio-recognition of glucose by the GOx-PPI was observed with a detection limit of 0.1 mM. Photometric responses showed good enzyme stability and activity with a Michaelis-Menten constant of 0.127 mM.

Keywords: glucose oxidase, poly (propylene imine) dendrimer, biosensor, glucose amperometric, photometric

1. INTRODUCTION

The quantitative monitoring of the blood glucose level is of great significance in biomedical applications. A metabolic disorder resulting from insulin deficiency and hyperglycemia is shown by blood glucose concentrations higher than the normal range of about 3.9 mM -6.2 mM (empty stomach) and 3.9 mM - 7.8 mM (2 h after food). Rapid and early detection of glucose levels in the blood can greatly reduce the risk of diabetes mellitus, induced heart disease, blindness and kidney failure. Diabetes mellitus is one of the leading causes of the death in the world [1-3]. Due to the global prevalence of diabetic patients, which represent near about 6.4% of the world's population, there is a

need to develop cost-effective, simple, accurate, portable and rapid sensors that are a socially acceptable [4]. Electrochemical glucose biosensors are based on glucose oxidase (GOx) enzyme catalytic oxidation of glucose to gluconolactone. The quantification of glucose can be achieved by electrochemical detection of the enzymatic release of H₂O₂. Most often GOx is used as a catalyst to catalyse the oxidation of glucose by the O₂ to produce gluconic acid and hydrogen peroxide (H₂O₂) [5, 6].



In direct electrochemical detection, it is difficult to exchange the electrons between the solid surface of the electrodes and the enzyme. This is because of the inaccessibility of its redox centre at the electrode interface and loss of some degree of bioactivity of the enzyme due to the conformational changes caused by adsorption on the electrode surface. These challenges present the need for electrode surface modification as a way of creating a biocompatible environment for the enzyme [7]. Polymers are suitable candidates for electrode modification of biosensor platforms. Nowadays, polymers are being used in the field of biosensors due to their high surface-to-volume ratio, surface area, electrical conductivity and considerable mechanical strength.

Dendritic macromolecules are synthetic three-dimensional hyperbranched, globular shaped polymers that emanate from a central core, having a defined number of generations and functional end groups [8, 9]. Dendrimer have been used in catalysis [10, 11] and very recently as biosensors [12, 13]. Poly (propylene imine) (PPI) is one of the notable and fascinating dendrimers that have been found to be compatible with bioreceptors such as enzyme and DNA [14, 15]. PPI can also be used as hydrogen donors, because of the high density of -NH₂ groups [16]. Due to the presence of an easily accessible multiple terminal or end functional groups, PPI is ideal for the construction of star shaped polymer-conjugation and can be applied in biosensors as a platform for the fabrication of DNA-, immuno-, and enzyme sensors [17, 18]. The interaction between PPI and enzyme can be used to aid enzyme immobilisation owing to the amine functional group of the dendrimer. Furthermore, the PPI may form a conjugate that can enhance GOx activity and probably prolong the shelf life by inhibiting degradation.

In this study, GOx was conjugated to PPI and used as a bioreceptor in the development of a glucose biosensor. Carboxylic acid groups of GOx were converted to reactive NHS-ester using EDC and NHS, and thereafter the PPI G2 was covalently coupled by the reaction with NHS-activated carboxylic group of GOx. The stability of the conjugate was also investigated.

2. EXPERIMENTAL

2.1 Reagents and Materials

All reagents were of analytical grade and were used without further purification. Glucose oxidase (GOx) from *Aspergillus niger* (Aldrich, EC 1.1.3.4 Type X), H₂O₂ (30 wt % in H₂O), *o*-

dianisidine dye (Aldrich), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), KH_2PO_4 , K_2HPO_4 , KCl, $\text{K}_3[\text{Fe}(\text{CN})_6]$, $\text{K}_4[\text{Fe}(\text{CN})_6]$ were purchased from Sigma-Aldrich. Generation 2 Poly(propylene imine) (PPI) dendrimer was purchased from SyMO-Chem, Eindhoven, Netherlands. 5 mg ml^{-1} GOx in phosphate buffer solution (PBS 0.1 M, pH 7) was stored at 4°C . Ultra pure Mili Q water (from Milipore) was used throughout the experiments. 0.05 M phosphate buffer solution (PBS) at pH 5.0 was used as the electrolyte. All solutions were purged with high-purity argon for at least 15 min to remove oxygen and a blanket of argon was then kept over the solutions during all the measurements.

2.2 Instrumentation

All electrochemical experiments were performed using an IVIUM CompactStat electrochemical potentiostat/galvanostat workstation (Ivium Technologies B.V., Netherland). A conventional three electrode system consisting of a working 3 mm diameter glassy carbon electrode, a Ag/AgCl reference electrode (3 M NaCl type) and an auxiliary platinum wire. Amperometric studies were carried out under stirred conditions and all experiments were done at room temperature. The surface morphology of *in situ* coupled GOx and PPI dendrimer were studied by scanning electron microscope TESCAN (Vega 3 XMU) and the samples were fixed on the holder by using carbon coating.

The UV-Vis spectroscopy experiments were performed with a GBC UV/Vis 920 spectrophotometer (GBC Scientific Instruments, Australia). FTIR spectra were obtained from a PerkinElmer spectrum 100 spectrophotometer. The IR spectra were recorded on an IR universal ATR sampling system accessory transmission mode, with an accumulation of 16 scans and a resolution of 4 cm^{-1} in the range of 4000 to 550 cm^{-1} .

2.3 *In situ* coupling of Glucose Oxidase and Poly (propylene imine)

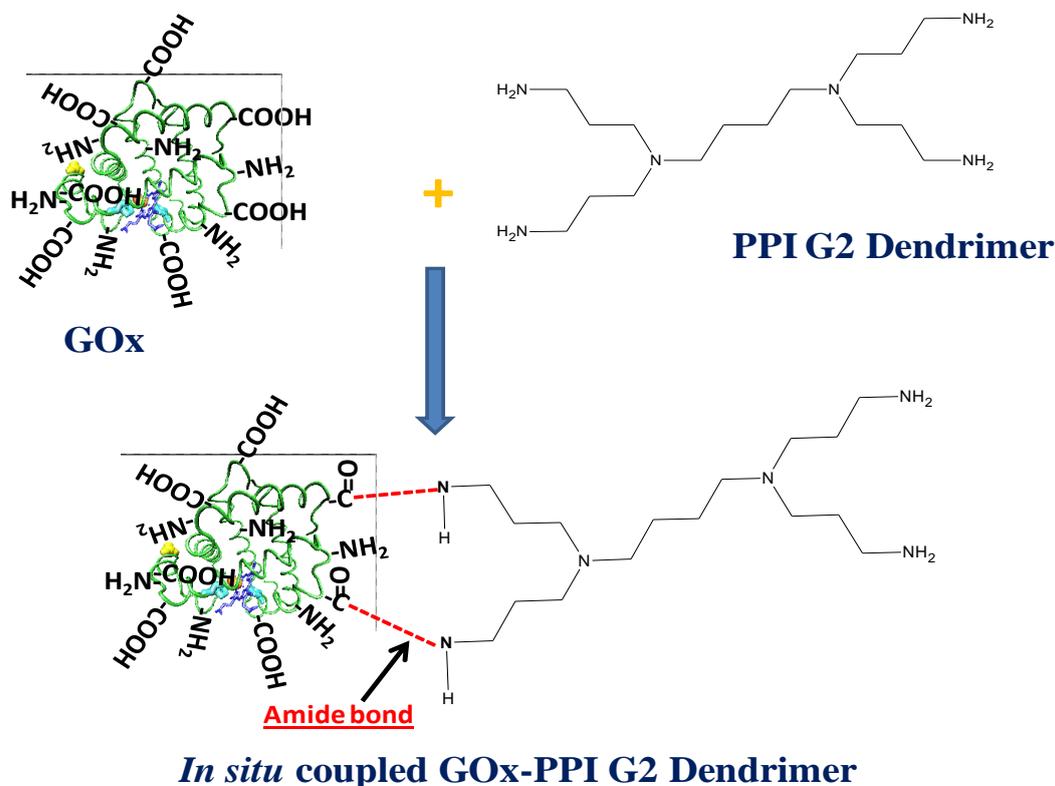
Covalent *in situ* coupling between GOx and the generation 2 Poly(propylene imine) dendrimer (PPI) was performed using EDC and NHS. Variation of crosslinking conditions was accomplished by altering the reaction time, reaction volume, pH and the molar ratio of GOx, PPI and EDC/NHS.

2.4 Determination of Glucose Oxidase Activity

The pristine enzyme and coupled PPI -enzyme activities were determined using spectrophotometric and amperometric methods. In the spectrophotometric method, the activities were determined according to the sigma methods using a Spectronic 20D Milton Roy model spectrophotometer [19]. The free enzyme assays were performed by using two processes; first one was an unmodified working glassy carbon electrode and second one was PPI-GOx modified.

2.5 GOx –PPI coupling

GOx was conjugated onto PPI dendrimer by an EDC/NHS activation procedure [20]. For enzyme activation, 5 mg ml⁻¹ of GOx was mixed in a phosphate buffer solution (1M, pH 7.5) which containing 0.02M EDC and 0.02 M NHS solution for 3 h with gentle stirring at room temperature. For the immobilisation of GOx, the PPI dendrimer (10 mM) was mixed in GOx activated phosphate buffer solution for 4 h at room temperature with gentle stirring. The *in situ* coupled GOx with EDC/NHS-crosslinked PPI G2 was stored in a refrigerator under dry conditions at 4 °C.



Scheme 1. Preparation scheme for *in situ* coupled PPI-GOx dendrimer based Glucose Biosensor

2.6 Preparation of the Glucose bioactive electrode

Prior to enzyme immobilisation, the glassy carbon electrode was mechanically polished with 1.0, 0.3 and 0.05 μm $\alpha\text{-Al}_2\text{O}_3$ powder and sonicated successively in ethanol and water. The polished GCE was then electrochemically cleaned by cyclic voltammetry between -0.6 V and +0.6 V at 50 mV s⁻¹ in 0.05M PBS (pH 7.0) until stable cyclic voltammograms were obtained.

The glucose bioactive electrode was prepared by drop coating 20 μL solutions of the GOx-PPI couple onto the GCE surface and left to dry at room temperature. This electrode will be referred to as GOx-PPI/GCE. Finally, the GOx-PPI/GCE bio-electrodes were washed with phosphate buffer (50 mM, pH 7.0, 0.9% NaCl) to remove any unbound GOx-PPI at the surface of the GCE.

2.7 Photometric analysis

The bioelectrode was dipped in reaction mixture containing 3 mL PBS solution, 25 μL of HRP (4.1 mM in PBS), 25 μL of *o*-dianisidine dye (1% in deionized water) and 100 μL of glucose solution (5.52 mM). The mixture of reaction mixture was kept for approximate 3 min and the absorbance was taken.

3. RESULT AND DISCUSSION

3.1 Characterisation of GOx-PPI

The coupling between GOx and PPI was confirmed by UV and FTIR spectroscopy. The UV spectra of GOx, PPI dendrimer and GOx-PPI couple are given in Fig. 1. The pristine GOx absorbed at 278 nm and 221 nm. The 278 nm band of the GOx shifted to 260 nm in the GOx-PPI owing to the chemical interaction between the dendrimer and the GOx. The dendrimer showed no absorbance in the 206 nm to 280 nm range.

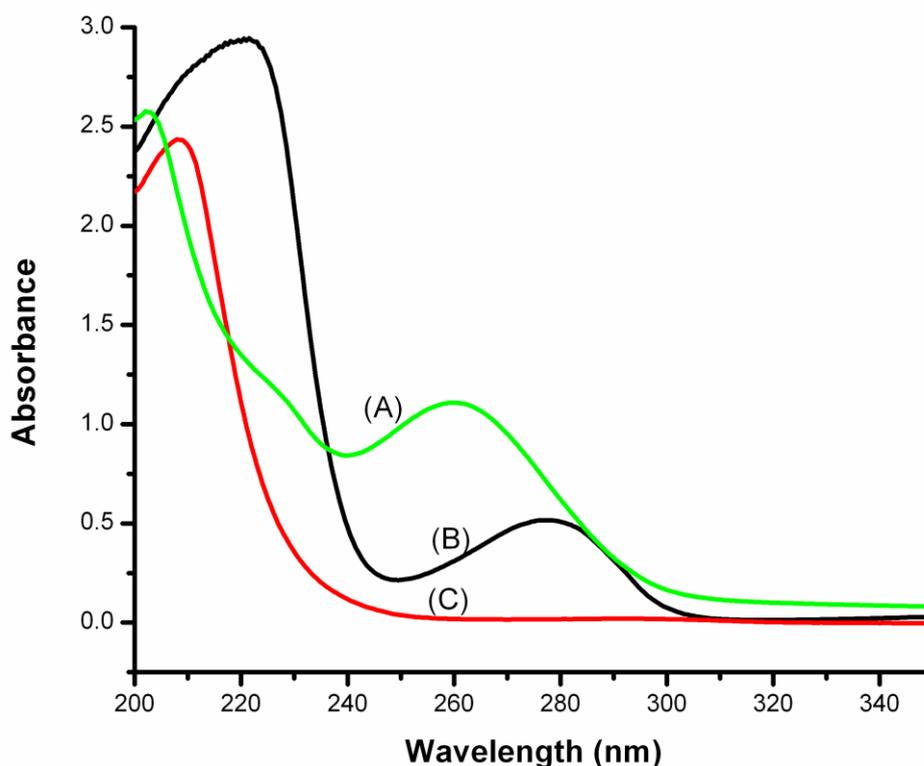


Figure 1. UV spectra of (A) PPI-GOx dendrimer, (B) GOx and (C) PPI dendrimer.

The FTIR spectra of PPI, GOx-PPI and GOx are shown in Fig. 2. Two N-H stretch bands for NH_2 group at 3460 cm^{-1} and 1629 cm^{-1} can be seen for PPI in Fig. 2 (spectrum A). In Fig. 2 (spectrum

C), the several peak positions of 1640, 1530, 1076 cm^{-1} were attributed to the presence of $-\text{COOH}$ groups and 3280 cm^{-1} was attributed to N-H stretching of NH_2 groups. The presence of 1637 cm^{-1} peak corresponding to amide bands as seen in Fig. 2 (spectrum B) confirms the attachment of GOx to PPI via carbodiimide chemistry [21-24]. These amide band is absent in Fig. 2 (spectra A & C). Furthermore, the broadening of the absorption band within 2880 cm^{-1} - 3718 cm^{-1} has been attributed to C-H and N-H stretching vibrations peculiar to amide interaction [24].

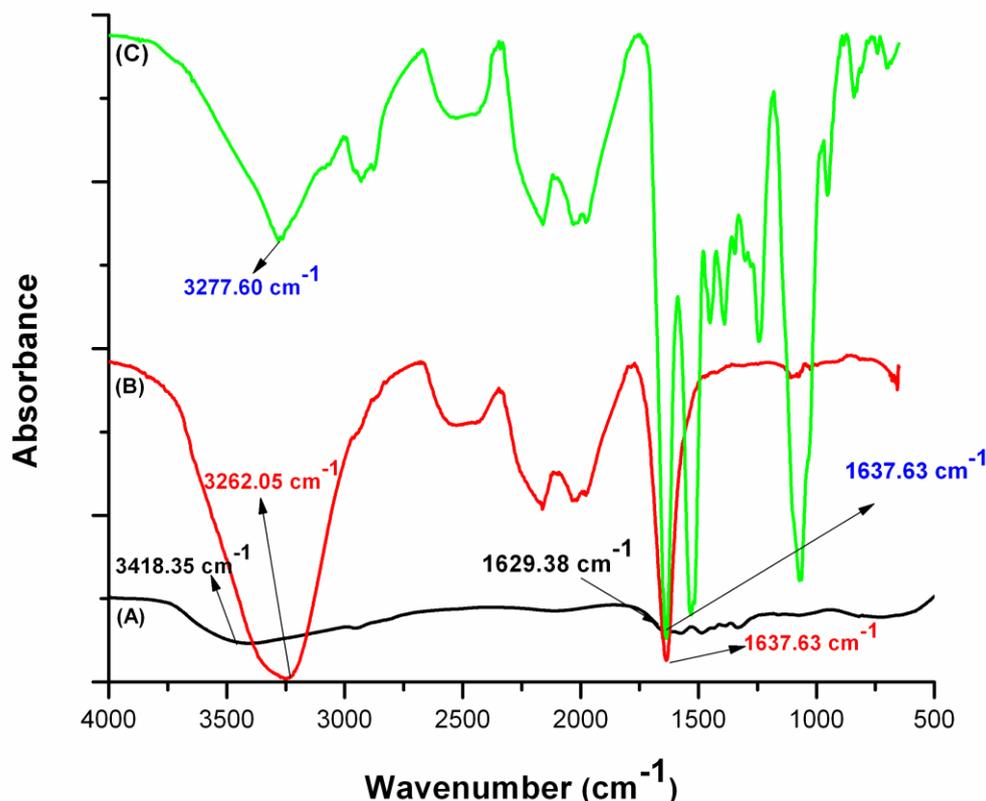


Figure 2. FTIR spectra in KBr monitored in the region between 4000 cm^{-1} and 500 cm^{-1} ; (a) PPI (b) GOx-PPI dendrimer and (c) GOx.

The SEM images presented in Figure 3 further corroborate the coupling of GOx to PPI. Fig. 3B shows homogeneous nano-globular deposition of PPI dendrimer in contrast to the bare electrode (Fig. 3A). The GOx-PPI couple (Fig. 3D), shows a combination of the morphologies of both PPI (Fig. 3B) and GOx (Fig. 3C). The poorly distributed droplets of enzyme are now seen to be covered with globular dendrimer with a better distribution on the electrode surface thus suggesting the coupling reaction between GOx and PPI. This improved distribution can be attributed to the interaction of PPI with the carbon substrate [25, 26].

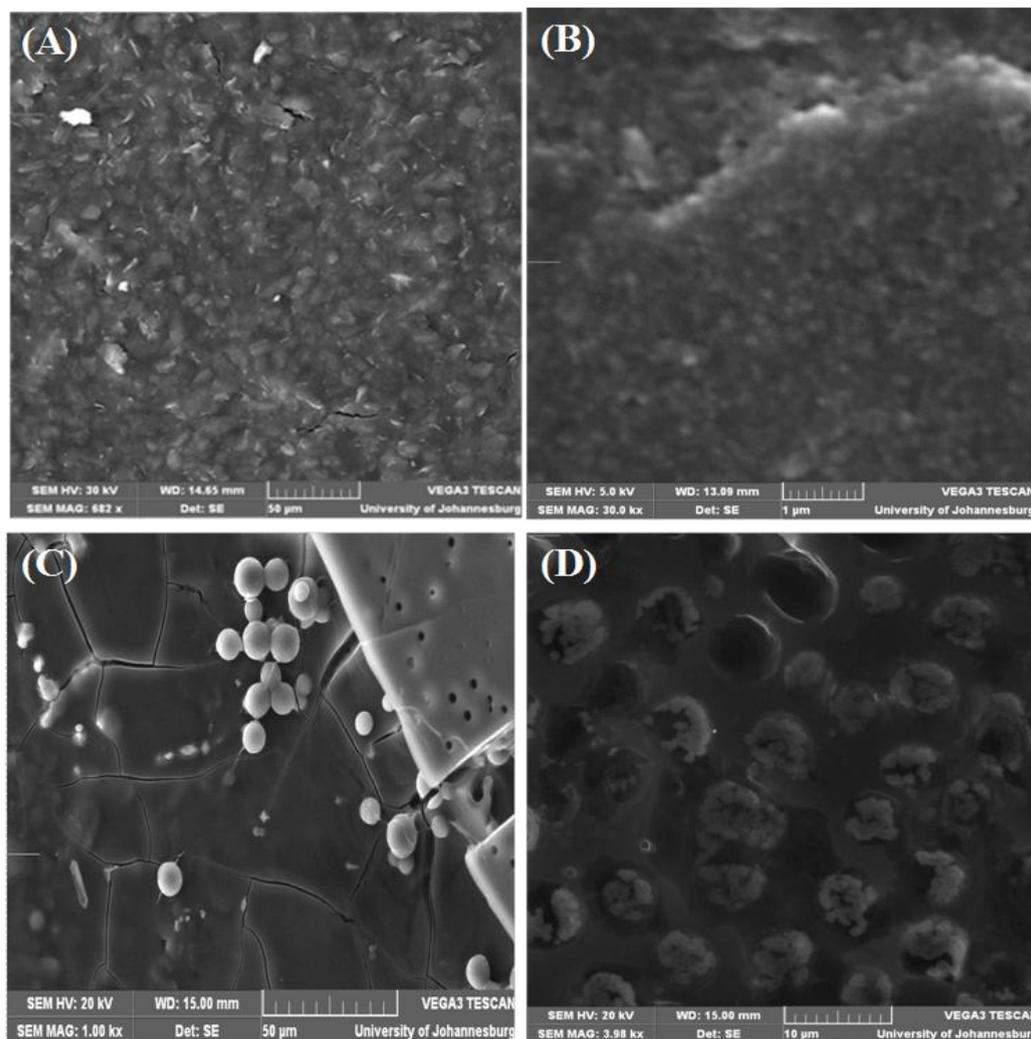


Figure 3. SEM image of (A) bare SPCE, (B) PPI dendrimer modified SPCE, (C) GOx modified SPCE (D) GOx-PPI dendrimer modified SPCE.

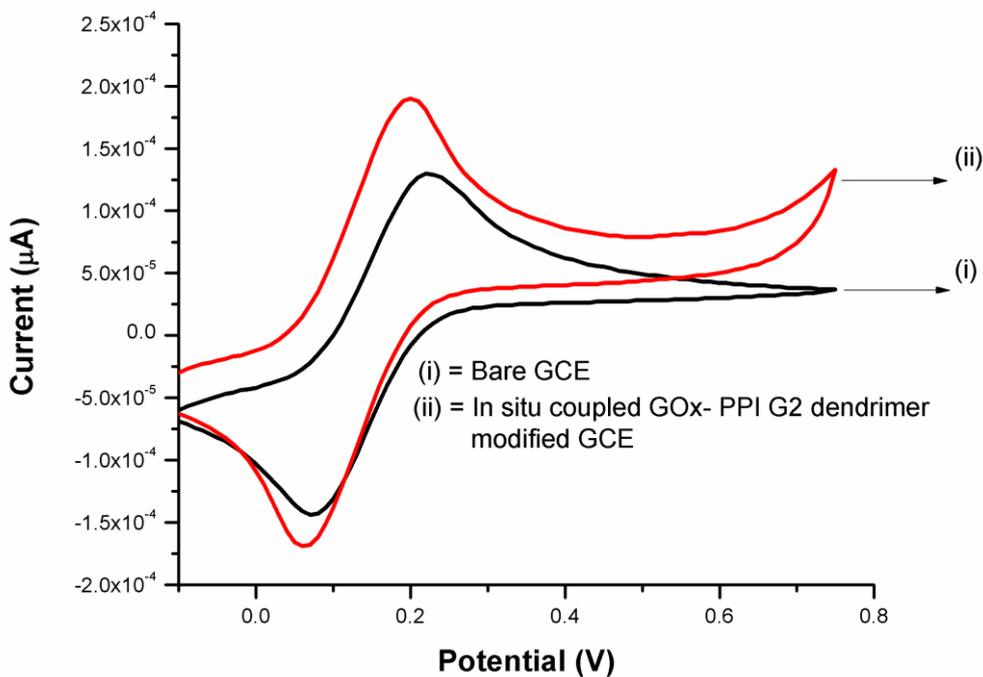
3.2 Electrochemical Studies of GOx-PPI

Fig. 4A shows the effect of the immobilised GOx-PPI in the presence of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ ions. An increase in current was observed for the GOx-PPI modified electrode over the bare GCE owing to the nano-dimensional nature of PPI which increases the surface area of the electrode [26].

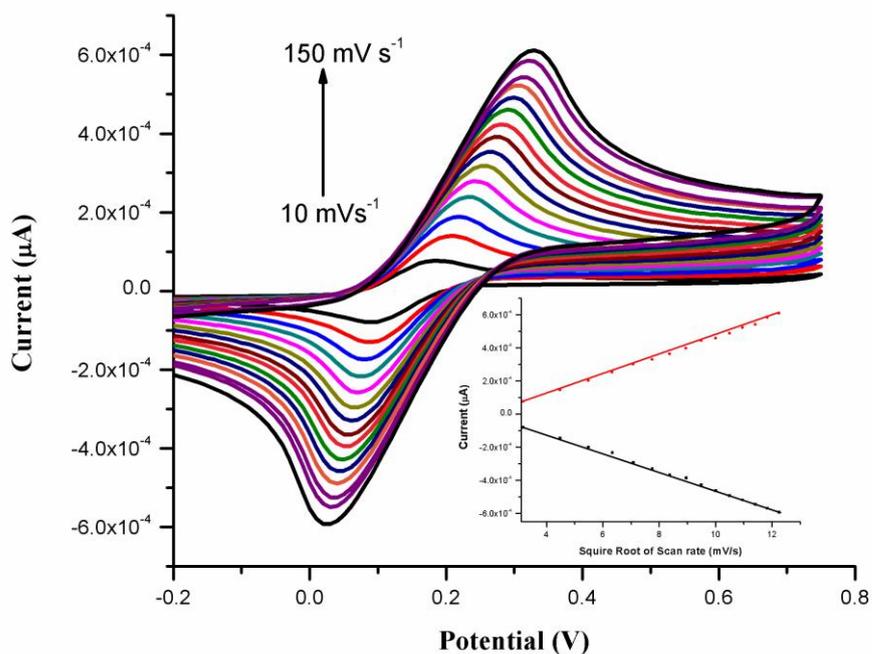
In the scan rate study (Fig. 4B), the peak current varies linearly with increased current suggesting that the kinetics of FC is diffusion controlled on the GOx-PPI modified electrode (GOx-PPI/GCE) as seen in Fig. 4B (inset) where a correlation coefficient 0.9937 was calculated. This diffusion controlled interfacial kinetics suggests that the GOx-PPI/GCE can be used for quantitative analytical purposes. Furthermore, the stability of the GOx-PPI film is depicted by shift in peak potential as scan rate increases. A large shift or irregular peak potential is expected when the film on the electrode is unstable or leaching into the solution, but this is not the case in Fig. 4B.

The redox specie used to monitor glucose enzymatic oxidation (metabolism) is H_2O_2 . Oxidation of H_2O_2 can occur in a potential as high as +800 mV [27]. The catalytic oxidation of glucose

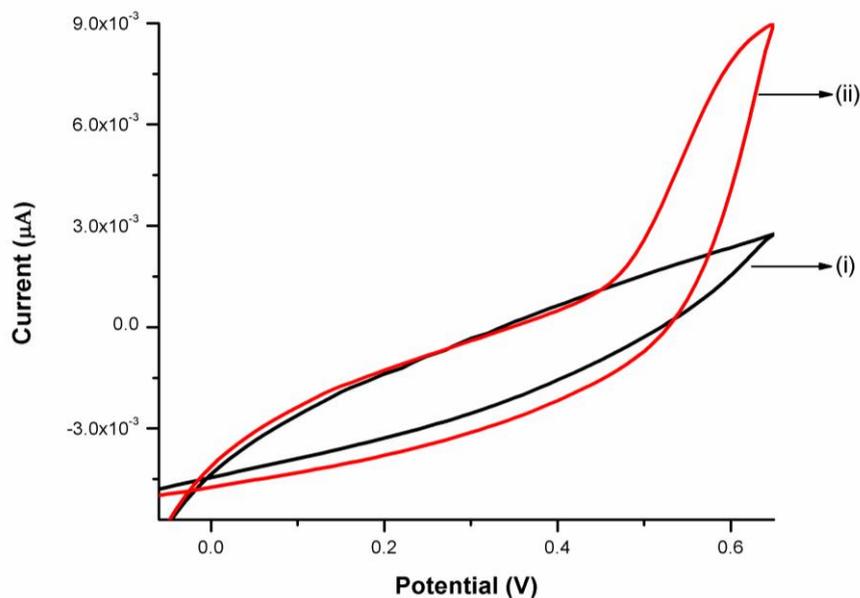
on the GOx-PPI modified GCE occurred between 450 mV and 600 mV as seen in Fig. 4C. This lowered oxidation potential reduces the risk of interferences. This oxidation is an evidence of successful immobilisation of the GOx-PPI on GCE. It also shows that the enzyme maintains its activity after coupling it to PPI.



A)



B)



C)

Figure 4. A) Cyclic voltammetry studied of (i) bare GCE, (ii) PPI modified GCE; (iii) GOx-PPI modified GCE $[\text{Fe}(\text{CN})_6]^{3-/4-}$. B) Cyclic voltammograms of GOx-PPI/GCE bioelectrode on increasing scan rate from 10 mV s^{-1} to 150 mV s^{-1} . Inset is the corresponding plots of peak current versus the square root of scan rate. C) Cyclic voltammetry responses of a PPI-GOx dendrimer in a PBS solution (pH 7.5) in the absence (i) and presence (ii) of 30 mM glucose.

3.3 Chronopotentiometric determination of glucose

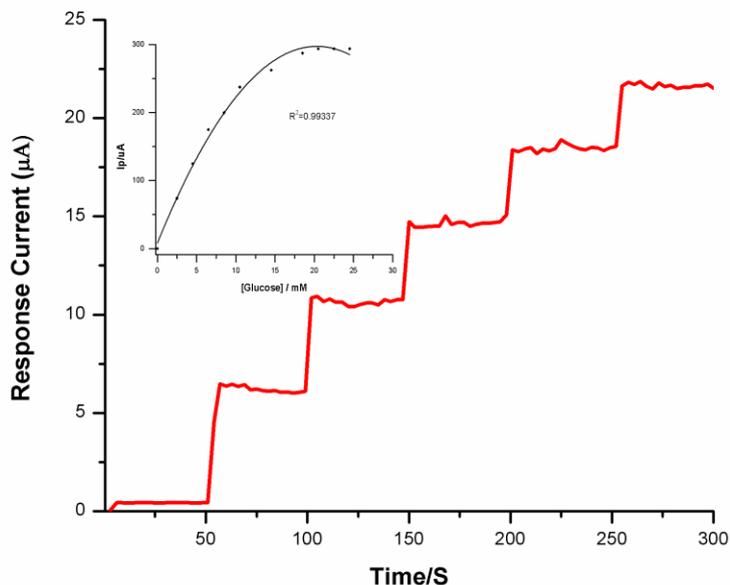


Figure 5. Typical amperogram recorded at *in situ* coupled GOx-PPI G2/GCE on successive addition of 2 mM glucose to a 0.1 M PBS pH 7.5 solutions. Potential poised at +400 mV (vs. Ag/AgCl); solution stirred at 250 rpm. Inset is the corresponding plot between current Vs concentration of glucose.

Chronoamperometric experiments for the detection of glucose using the GOx-PPI modified GCE were investigated at +400 mV and +600 mV. The amperometric responses at these two potentials were similar and thus +400 mV was chosen as the optimum oxidation potential (Fig. 5). A response time of ca 5 sec was needed to obtain the glucose signal. A calibration plot of the biosensor response to glucose (Fig. 5 inset) gave a correlation of 0.99337 and a linear range of 0.4 mM to 14 mM. A detection limit of 0.1 mM was calculated.

3.4 Photometric response studies

Photometric studies were carried out to determine the GOx activity, stability and shelf life of GOx-PPI/GCE bioelectrode. Fig. 6 shows the variation of the difference between the initial and final absorbance value obtained at 500 nm after 3 min of incubation of the biosensor in glucose. It can be seen that the value of absorbance increases linearly with glucose concentration in the range of 0.552 mM to 22.1 mM. This experiment was carried out in triplicates with good reproducibility within 2% error.

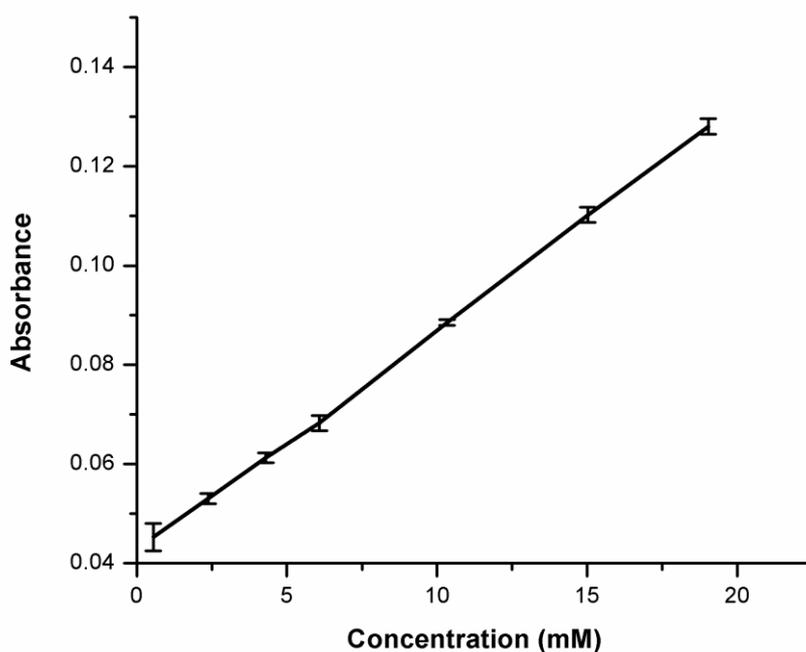


Figure 6. Photometric response of *in situ* coupled GOx-PPI dendrimer modified SPCE as a function of glucose concentration (0.552 mM to 22.1 mM) in phosphate buffer (50 mM, pH 7.5) containing 0.9% NaCl.

The value of Michaelis-Menten constant (K_m) determined using the Lineweaver-Burke plot (i.e. a plot of the inverse of absorbance versus the inverse of glucose concentration) was 0.127 mM (2.29 mg dL⁻¹). The value of K_m , is a measure of enzyme activity. The low value obtained compares well with that obtained from literature [28, 29]. This rate constant shows that the enzyme maintained its activity in the conjugated form.

3.5 Enzyme activity studies

The noticeable enzyme activity ($U\text{cm}^{-2}$) is defined as one unit of enzyme activity that results in the conversion of $1\mu\text{mol}$ of glucose into glucolactones per minute. It can be calculated by using the equation [30],

$$\alpha_{app}^{enz}(U\text{cm}^{-2}) = \frac{AV}{\varepsilon_{st}} \quad (2)$$

Where A is the difference in absorbance before and after incubation, V is the total volume (3.015 cm^3), ε is the millimolar extinction coefficient (7.5 for o-dianisidine at 500 nm), t is the reaction time (3 min), and s is the surface area (cm^2) of the GCE. The apparent enzyme activity was found to be ~ 0.032 ($U\text{ cm}^{-2}$) indicating that 0.032 units of enzyme per cm^2 actively participate in the enzymatic reaction.

The shelf life of PPI-GOx biosensor was measured as a function of absorbance with respect to time with regular interval of 1 week. It was observed that GOx-PPI biosensor retained about 80% of glucose oxidase activity even after 9 weeks, when stored at 4°C .

4. CONCLUSION

The successful coupling of poly (propylene imine) dendrimer (PPI) to glucose oxidase and its use as a biosensor for glucose is reported for the first time. The *in situ* coupling integrates the multifunctionality and nano-scopic properties of PPI into the enzyme moiety thereby enhancing immobilization, while retaining the enzyme activity on the electrode surface. The proposed biosensor displayed interesting amperometric response characteristics towards the efficient detection of glucose. These analytical parameters include low over potentials (+400 mV versus Ag/AgCl), very fast response time (~ 5 s), linear concentration range extending upto 14 mM. This glucose biosensor was highly sensitive; to the extent that it was able to chronoamperometrically detect the target glucose concentration as low as 0.4 mM ($R^2 = 0.9933$) in phosphate buffer with detection limit of 0.1 mM. Photometric results showed the good enzyme stability and activity with linear response in the range of 0.552 mM to 22.1 mM. The activity of the enzyme, as deduced from relatively low Michaelis-Menten constant (0.127 mM) indicates high enzyme affinity towards glucose, was found to be better than previous reports. In addition, the GOx-PPI G2 dendrimer glucose biosensor exhibits both good reproducibility and long term stability (9 weeks). Owing to the similarities in the functional groups of enzyme, there is a possibility of extending this simple carbodiimide coupling reaction to other types of enzymes. The result of which can lead to improved enzyme activity, ease of enzyme handling at room temperature and elongation of enzyme shelf life.

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