Naga Adithya Chandra Pandurangi

Direct electrical contacting of pyrroloquinoline quinone dependent glucose dehydrogenase (PQQ-GDH) based on polyaniline and carbon nano onion-modified gold electrodes

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supervised by Dr. Ioanis Katakis

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Naga Adithya Chandra Pandurangi
Master Program in Nanoscience, Materials and Processes, 2019-2020
e-mail: adithyapandurangi@gmail.com

Supervisor: Ioanis Katakis
Department of Chemical Engineering, Universitat Rovira i Virgili,
Campus Sescelades, Av. Paisos Catalans 26, 43007, Tarragona, Spain.

Abstract

The interaction of enzymes with electrodes is critical for the integration of bioelectronic systems for applications in biosensors and bionic devices for energy harvesting or biocomputing. To create such devices and systems that are robust, direct electron transfer (DET) between enzyme and electrode is the deciding factor for sensitivity and reliability. This work focuses on fabricating such a reliable bioelectronic interface based on pyrroloquinoline quinone-dependent glucose dehydrogenase [PQQ-GDH, EC 1.1.5.2]. In order to achieve DET, the electrode surface was modified by carbon nano onions (CNO) and polyaniline copolymer on gold electrodes that facilitated direct electron transfer in the presence of glucose. The CNO were adsorbed on to the surface and polymer film was formed by co-electropolymerization of 2-amino benzoic acid (ABA) and 3-aminophenyl boronic acid (APBA) monomers. The resulting electrodes show behaviour that can be interpreted as direct electron transfer. At the enzyme saturation, the current density of 120 µA/cm^2 was achieved. This indicates that the CNO modified electrodes are capable of glucose electro-oxidation and polymer acts as conductive film improving the electron transfer. Preliminary attempts to optimise the electrode modification conditions and the effect of operating variables are reported.

Key words: pyrroloquinoline quinone, PQQ, glucose dehydrogenase, PQQ-GDH, direct electron transfer, carbon nano onions, polyaniline, electropolymerisation.

1. Introduction

Pyrroloquinoline quinone (PQQ) dependent glucose dehydrogenase (GDH, EC 1.1.5.2) is a redox enzyme, dependent on that can convert glucose to gluconolactone. It is widely used in glucose sensors [1], biofuel cells [2] , and also in logic gates in bio-electronic systems [3,4]. Katz et al. showed mediated electron transfer (MET) from PQQ-GDH for the first time[5]. MET can be achieved by many compounds such as ferrocene derivatives, 2,6-dichlorophenolindophenol (DCIP) and meldola blue (MB) [6] etc. (referred to as mediators), whereby these species shuttle electrons from the enzyme redox cofactor (PQQ) to the electrode. However, MET is subject to electrode crosstalk when electrodes need to be placed close to each other and to lower electron

transfer efficiency (due to diffusion). In making the electronic and biological systems integration possible, the efficient contact of PQQ-GDH to the electrode by direct electron transfer (DET) plays a crucial role. In contrast to MET, DET from enzyme to electrode, is necessary for cost efficiency, increased rate of electron transfer, simple construction and usage of devices[7]. The DET between enzyme and electrodes is a basis of the third-generation biosensors[8]. PQQ-GDH enzyme-based electrodes, capable of DET are widely used in biofuel cells [9,10] and in biosensors [11–13]. However, the electrical communication of the enzyme to the electrode depends on accessing the active site of the enzyme and transferring electrons to the electrode. This greatly depends on the orientation of the immobilized enzyme. Although PQQ-GDH presents a relatively accessible cofactor, such oriented immobilisation can be substantially guided by molecular simulation so electron conducting pathways can come in contact with the electrode surface[14].

Thus, the most important element for achieving DET is the binding mechanism of the enzyme to the electrode. This can be done by two strategies, first is the molecular approach by using nano catalysts[15] (the connectors) like carbon nanotubes (CNT) [16,17] or gold nanoparticles (AuNP) linked to the amine moieties of the enzyme or carboxylic ends of PQQ by linker (by covalent coupling or affinity binding like EDC-NHS coupling or thiol-Au affinity). Second is the non-specific entrapment of the enzyme on to the electrode which is bulk approach using different hydrogel configurations[18,19].

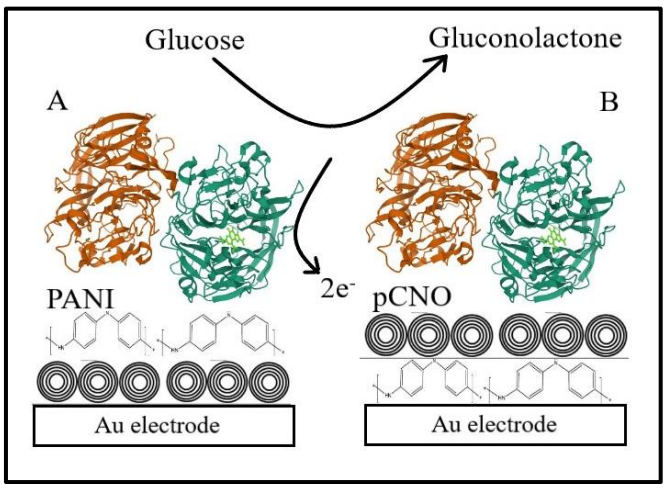
By molecular approach, Hiraoki et al. reported that the His-tag affinity binding is efficient in conducting electrons uninhibitedly as the catalytic site is oriented towards the substrate with increased current density values[20]. 1,4-benzenedithiol was used as a linker between gold electrode and AuNP covalently bound to the enzyme using dative bonding of thiol to gold[21].

In bulk approach, conductive polymers, especially polyaniline and its derivatives, have been shown to provide an interface for transferring electrons. There are several ways to synthesize polymer on electrode surface. One of the most efficient techniques is electropolymerisation. Electropolymerisation can be performed by various methods like cyclic voltammetry, differential pulse voltammetry, potentiostatic pulse method etc., on the electrode surface. Electropolymerized surfaces can also serve as an immobilisation matrix[17]. It has the additional advantage to provide patterning of closely spaced electrodes with high resolution, a useful characteristic for bioelectronic devices. The electrical contacting of PQQ-GDH through conducting polymers has been also shown using electrospinning. The electro-spun conductive polyaniline-based derivatives on Indium-Tin Oxide (ITO) electrode for DET by Gladisch et al. provided the 3D nano-porous structure that served as an electrically active matrix for proton and electron transfer[19].

Carbon nano onions (CNO) are members of the fullerene family of materials, 8-15 nm in size, and were discovered at the same time as carbon nanotubes (CNT)[22]. However, in comparison with CNT, the interaction of CNO with enzymes has not been studied well. CNOs can be functionalised with different groups such as hydroxy (-OH) and carboxylic (-COOH) that can be used for enzyme immobilisation. The CNO with no functionalisation are referred to as pristine carbon nano onions (pCNO). In this work the interaction of pCNO with immobilised PQQ-GDH enzyme in electropolymerized conducting polymer matrices on gold electrodes was studied.

The electrode-linker-connector-enzyme contacting system supported by the conductive polymer matrix for receiving electrons can provide a promising and generic approach for the efficient electrical contacting of the PQQ-GDH active site to the electrode with DET. In order to have a high rate of electron transfer, it is considered according to the dimensions of the enzyme (85 Å x 60 Å x 61 Å) that the peak current density should be at least 1 mA /cm^2 (calculated for a closely packed monolayer of enzyme molecules). The peak current density of 1.2 mA /cm^2 has been achieved by V. Scherbahn et al. using bucky paper electrodes modified with poly(3-aminobenzoic acid-co-2-methoxy aniline-5-sulfonic acid)- PABMSA [23] .

The objective of the current work is to achieve direct electrochemical contact using inclusive approach (of both molecular and bulk approach), between pyrroloquinoline quinone-dependent glucose dehydrogenase (PQQ-GDH, EC 1.1.5.2), polyaniline and pristine carbon nano onions (pCNO)-modified gold electrodes. For this reason, first the interaction of (PQQ)-GDH enzyme with polymer and pCNO individually was studied by either forming a layer of electropolymerized polyaniline derivates, or by adsorbing pCNO on bare gold electrode surface to achieve DET. The second approach was to combine the two elements by electropolymerizing the aniline derivatives on CNO modified electrode surfaces to examine if this combination would further enhance the DET[15] (see scheme 1A). The third approach was the chemisorption of CNO on polymer film-modified gold electrodes (see scheme 1B). The polymer film is synthesized by electropolymerisation of aniline derivatives by cyclic voltammetry. The resulting electrodes were used for immobilizing PQQ-GDH enzyme and investigated using electrochemical techniques for obtaining information on their properties and behaviour in the presence of the enzymatic substrate (glucose).



Scheme 1: Enzyme contacting scheme (A) PQQ-GDH immobilised on PANI-pCNO-Au electrode, (B) PQQ-GDH immobilised on pCNO- PANI-Au electrode.

2. Experimental

2.1 Materials

Aniline derivatives : anthranilic acid (2-amino benzoic acid, ABA), 3- amino phenyl boronic acid (APBA) as well as N-hydroxysuccinimide (NHS), 3-(N-morpholino) propane sulfonic acid (MOPS), D-glucose, $CaCl_2$, Hydrogen peroxide (H_2O_2), ferrocene methanol and Potassium Ferricyanide($K_3Fe[CN]_6$) were purchased from Sigma- Aldrich Chemie GmbH (Germany). Potassium Chloride and Sodium Fluoride were from Scharlau S.L. (Spain). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide (EDC), 1,4-Piperazinediethanesulfonic acid (PIPES), phosphate buffer saline (PBS) tablets and Triton X-100 were acquired from ACROS Organics (Thermo Fisher Scientific, USA). Sulphuric acid (H_2SO_4) was from J.T. Baker. was from ACROS Organics (Thermo Fisher Scientific, USA).

1-Pyrene butanoic acid succinimidyl ester (PBSE) was from ANASPEC, Canada. CNO was gained as a gift from Gardona et al. [24], synthesized by annealing nano diamonds method as specified by Kuznetsov et al.[25]. PQQ-dependent glucose dehydrogenase (PQQ-GDH; E.C. 1.1.5.2) from a microorganism not specified by the company was purchased from Sorachim SA, Switzerland.

All aqueous solutions are prepared in ultrapure water (18.2 ± 0.2 MΩ·cm) from Simplicity UV water purification system. All the solutions were at pH 7.0 whenever the enzyme was used, except for the solution of electropolymerisation.

2.2 Electrode Preparation

Au electrodes (1 mm diameter, geometrical area - 0.033 cm^2 teflon coated) were used in all cases. Electrodes were firstly rinsed in deionized water, isopropanol and polished with alumina powder (0.3 µm) on abrasive paper. The electrodes were cleaned with piranha solution (H_2SO_4:H_2O_2- 2:1) for 20 minutes. As piranha solution is highly corrosive and dangerous, care should be taken by avoiding any contact and cleaning should always be performed under fume hood. Followed by sonication of electrodes for 5 minutes in ultra-pure water. For 2 minutes, electrodes were exposed to piranha solution prior to the modification and finally rinsed with ultra-pure water. At all times, electrodes were stored in closed containers with inert atmosphere (N2 or Ar gas) except after enzyme immobilization.

For CNO modification, the pristine carbon nano onions (pCNO) solution was sonicated for 20 min and then added on the electrode surface (two steps of 2 µL each/electrode of 1.7 mg/mL CNO solution in DMF). After this, the electrodes were placed under vacuum (-20 cm Hg) at 80 ºC until the pCNO solution dried completely (10 minutes approximately). Polymer film was synthesized onto the pCNO-modified as well as bare gold electrode, electropolymerisation by cyclic voltammetry was performed in a solution of 1 M H_2SO_4 and a total concentration of 0.1 M (0.05 M ABA+0.05 M APBA) aniline

derivatives. The electrodes were rinsed with deionized water after synthesizing polymer.

No further treatment is required for adsorptive enzyme binding on to electrode. covalent binding of the (PQQ)-GDH enzyme, (i) to the polymer film, the carboxylic groups of 2-antraanilic acid are activated by 25 mM NHS and 100 mM EDC for 20 min and (ii) to the pCNO modified surface, the electrodes were incubated with 10 mM PBSE ethanolic solution , agitated for 1 hour , rinsed with pure ethanol and dried with nitrogen.

Modification nomenclature can be interpreted by layer name. Pristine carbon nano onion layer represented by pCNO and copolymer film by poly[ABA/APBA]. For instance, in PQQ-GDH-poly[ABA/APBA]-Au, the PQQ-GDH represents the uppermost layer followed by second layer of copolymer on the gold electrode.

2.3 Enzyme immobilization

Prior to electrode functionalisation with PQQ-GDH, the activity of the enzyme was measured. The procedure has been adapted from Toyobo's protocol[26]. After this, a PQQ-GDH solution (2.4 mg/mL) in MOPS buffer (50 mM, pH 7.0) containing 1 mM $CaCl_2$ (1 mM) was deposited on the electrodes at room temperature for 1 hour. The enzyme-modified electrodes were stored (4ºC) in MOPS-buffer.

2.4 Electroactive surface area (EASA)

A three-electrode cell was used for electrochemical characterization. Using $[Fe(CN)6]^{3-}$ as an electrochemical probe, the apparent electroactive area was studied after each surface modification step. The Randles-Sevcik equation was used to calculate the area (1),

$$i_p = 268600 n^{\frac{3}{2}} D^{\frac{1}{2}} C \nu^{\frac{1}{2}} A \qquad (1)$$

where n is the number of electrons transferred (n = 1, in this case), D is the diffusion coefficient for the electroactive species (7.20 x 10^{-6} cm^2 s^{-1})[27], C is the electroactive species concentration (1 x 10^{-6} mol cm^{-3}), ν is the scan rate (V s^{-1}), and A is the electroactive surface area of the electrode (cm^2). The slope of the peak current (i_p) vs. $\nu^{1/2}$ plot was used to obtain the electroactive surface area over the range of scan rates.

2.5 Electrochemical Measurements

All voltammetric measurements of the modified electrodes were performed at 50 mV/s scan rate using a potentiostat/galvanostat PGSTAT 12 Autolab controlled with 2.1.3 software. Prior to each electrode surface modification step, electrodes were analyzed in potassium ferricyanide (III) (1 mM) prepared in 0.1 M KCl solution at different scan rates(ν). An in-house made three electrode electrochemical cell containing 10.0 mL of the electrolyte solution, an external Ag/AgCl as reference electrode and a Pt wire as counter electrode was used for all measurements. For each experiment minimum 3 electrodes have been maintained. All the experiments have been conducted at room temperature without control of the atmosphere

since the enzyme is not sensitive to oxygen concentration.

2.6 Electron pathway

The electron transfer pathways from the active site of the GDH enzyme i.e., PQQ to the surface of the enzyme and their electron coupling value in their binding states have been studied using HARLEM software. The PQQ-GDH structure (1C9U) was downloaded for this study from RCSB protein data bank[28].

3. Results and Discussion

The aim of the current work is to design an enzyme electrode which can obtain DET between PQQ-GDH and pCNO and polyaniline modified electrodes. The pCNO adsorbed, lead to increase in active surface area and act as an ideal base for enzymatic interaction with the electrode. For the improvement of electrochemical communication by increased current density and for enhanced stability a new supportive interface is developed and the interaction with enzyme is studied. A polyaniline film is prepared for this purpose on both bare and pCNO modified electrodes. The electrode behavior in the presence of glucose is investigated.

3.1 Electroactive surface area (EASA)

The results demonstrate that the electrodes modified with different layers resulted to different apparent electroactive surface areas (EASA) on gold electrodes. Large current response was observed in pCNO+ poly[ABA/APBA] modified electrodes suggesting that this modification provides large apparent electrochemical area in comparison to other approaches. Calculated EASA of all the electrodes is shown in table 1 in which bare Au electrode EASA is around 0.015 cm^2. Possible reason for the smaller EASA of poly[ABA/APBA] modified electrode compared to pCNO modified electrode is the presence of moderately electron withdrawing negatively charged carboxylic acid (-COOH) functional groups in ABA. This functional group may be responsible for smaller current by shielding the diffusion of negatively charged ferricyanide molecules.

Table 1:EASA after each surface modification step and % decrease of EASA by enzyme immobilisation.

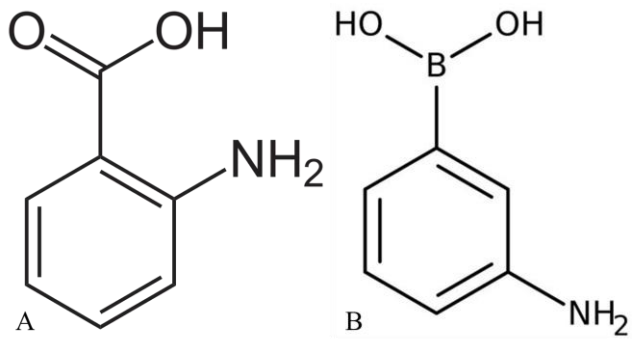
Surface modification	EASA (± 0.0005, cm^2)	% Decrease
Bare Au electrode	0.0150	
poly[ABA/APBA]-Au	0.0028	62.5
PQQ-GDH-poly[ABA/APBA]-Au	0.0017	
poly[ABA/APBA]-pCNO-Au	0.5548	3.6
PQQ-GDH-poly[ABA/APBA]-pCNO-Au	0.0201	
pCNO-poly[ABA/APBA]-Au	0.0411	46
PQQ-GDH-pCNO-poly[ABA/APBA]-Au	0.0113	
pCNO-Au	0.0277	35
PQQ-GDH-pCNO-Au	0.0097	

The poly[ABA/APBA] layer on Au electrode has shown increased current response as illustrated in cyclic voltammogram in Figure 1. Possible reason behind current response is that the layer with nano-size pCNO and polymer chains is able to increase electroactive area leading to more porous surface. After immobilizing the (PQQ)-GDH on surfaces a decrease in apparent EASA of

30-60% was observed which may be due to insulating barrier formed by the enzyme. The actual electroactive surface area that was used for calculating the current density values was the difference between EASA (before PQQ-GDH) and EASA (after PQQ-GDH).

3.2 Electropolymerization

The co-polymer formation collectively brings different functional groups into the polymer. Electropolymerization is done by using 2-ABA and 3-APBA monomers (0.05 M each) which have -COOH and B-$(OH)_2$ at ortho and meta positions respectively with amine of the benzene ring (see scheme 2). The solution preparation and electropolymerisation process has been adopted from Schubart et.al.[17] and parameters are optimized for high conductivity of the polymer.



Scheme 2: Chemical structure of aniline derivatives (A) 2-aminobenzoic acid (ABA) and (B) 3-aminophenylboronic acid (APBA).

The electropolymerization of poly[ABA/APBA] was performed by cyclic voltammetry for up to 30 cycles, after which no significant improvement in peak currents was observed. In Figure 1, the cyclic voltammograms for cycles 5, 10, 20, 25 and 30 are given. The results show that with increasing number of cycles, the current response was also increasing, especially for lower potential peak. The peaks present in figure 1 demonstrate various oxidation states of polyaniline. The lower potential redox peak indicates the presence of mixed state (emeraldine) after being converted from polyaniline reduced state (leucoemeradine) and higher potential peak indicates the fully oxidized form (peringraniline) after conversion from redox emeraldine[29]. The peak height shown in figure 1 represents the quantity of oxidized polymer suggesting that a conductive layer is being formed by the polymer film. Therefore, most polymer is present in green emeraldine form at higher potentials, which is also highest conductive form of polyaniline[29]. The reason for addition of NaF is because APBA readily oxidizes in the presence of fluoride (F^-) ions and aids in the polymer formation [30].

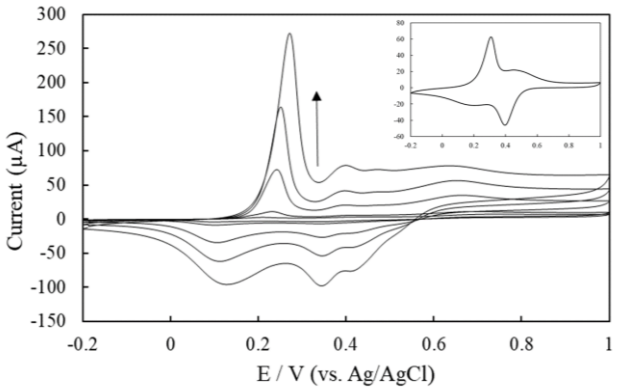


Figure 1:Cyclic voltammograms of poly[ABA/APBA] on Au electrode (0.1 M) in the presence of 0.15 M sodium fluoride (NaF) and 1 M H_2SO_4 during electropolymerization: cycles-5,10,20,25,30, Inset: Cyclic voltammogram of poly[ABA/APBA] after electropolymerisation in monomer free 1M H_2SO_4 solution.

The -COOH functional groups in ABA are used to link the enzyme to poly[ABA/APBA] by covalent coupling using EDC-NHS. The adsorptive binding technique also been performed for verifying the enzymatic interaction in the absence of covalent or affinity binding. For linking CNO to the PQQ-GDH enzyme, PBSE was used as it has been shown to work for carbon nanostructures, especially multiwall carbon nano tubes (MWCNT) by Slaughter et.al. [23].

3.3 Electrochemical Characterization

3.3.1 DET studies

The enzyme electrodes were studied by cyclic voltammetry in absence and presence of glucose initially in phosphate buffer (PBS). The PQQ-GDH-poly[ABA/APBA]-Au electrodes were initially studied, showing absence of catalytic activity of PQQ (Figure 2(i)). The decrease in the current values with increase in the glucose levels might indicate the interaction of $B(OH)_2$ functional group in the 3-APBA of copolymer with glucose as functional group has tendency to cross link and form diol bridge in area where the enzyme is not adsorbed. It is evident that the polymer layer is unable to interact with the enzyme during the catalytic reaction.

To study the effects of ionic strength, the same electrodes were studied in MOPS solution. In Figure 2(ii), it can be seen that there is an increase in current with increase in glucose level which may represent increase capacitance and not catalytic activity of PQQ. The reason for absence of catalytic activity may be due to enzyme inactivation due to the covalent coupling method.

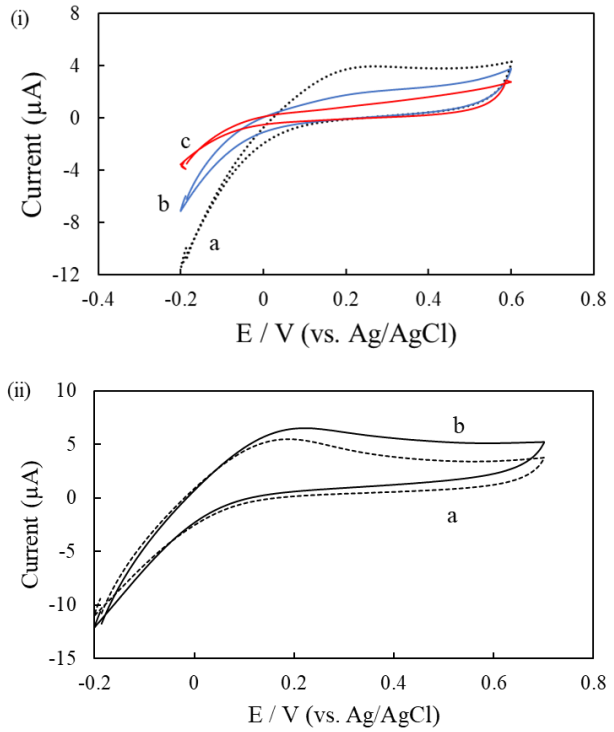


Figure 2: PQQ-GDH-poly[ABA/APBA]-Au electrode in, (i) 0.1 M PBS pH 7.0 with (a) 0 mM glucose (dotted), (b) 20 mM glucose (blue), (c) 50 mM glucose (red) (ii) 20mM MOPS pH 7.0 +1mM$CaCl_2$ with (a) 0mM glucose (dotted), (b) 20mM glucose (black); sweep rate is 50 mV/s.

To examine if this were the case, PQQ-GDH was immobilised by adsorptive binding on top of poly[ABA/APBA]-pCNO-Au electrode. The electrochemical response of the electrodes in the presence of glucose was studied and cyclic voltammograms are shown in Figure 3. However, they also showed similar response, i.e. absence in catalytic activity of PQQ even with increase in glucose levels.

In the above cases, the loss of enzyme interaction with polymer might be due to various reasons. For covalent coupling, the carboxylic functional groups of copolymer where not able to connect to amine ends of electron pathways of the

enzyme. For the adsorptive binding, the absence of response might be due to enzyme wash-off.

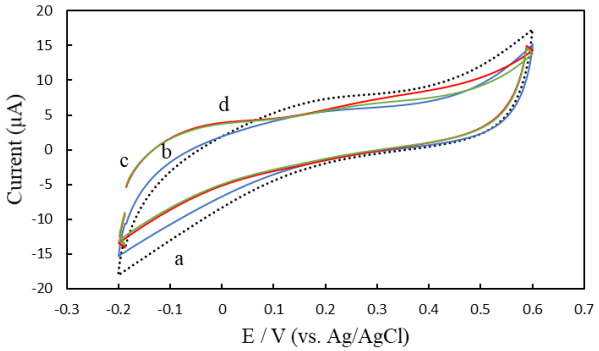


Figure 3: PQQ-GDH- poly[ABA/APBA]-pCNO-Au electrode in 0.1M PBS pH 7.0 with (a) 0 mM glucose (dotted line), (b) 20 mM glucose (blue), (c) 50 mM glucose (green) (b) 100 mM glucose (red); sweep rate is 50 mV/s.

On the other hand, the electrodes modified with pCNO show well defined catalytic activity in the presence of glucose. Figure 4(i) shows the behaviour of PQQ-GDH-pCNO-Au electrode in 0.1M PBS pH 7.0 in absence of glucose. The peaks 1 and 2 represent the catalytic activity of PQQ as proposed by Sato et al.[31] , re-proposed by Flexer et al.[18] The peak observed appear to be redox reactions of PQQ which are released due to some denatured enzymes and can be depicted by equations 2 and 3[18].

$$PQQ_{ox} + e^- + H^+ \leftrightarrow PQQ_{semiquinone} \quad (2)$$

$$PQQ_{semiquinone} + e^- + H^+ \leftrightarrow PQQ_{red} \quad (3)$$

The redox reaction of PQQ highly depends on the solution composition. The equations (2) and (3) represent the two consecutive one electron (1E1E) mechanism. This is highly probable in the presence of Ca^{2+} in the solution as it stabilizes the PQQ_{semi} thus converting to two step reaction[31]. In the case of PBS solution, the reaction might be a 2-electron process. In either case, there will be redox peak observed in absence of substrate related to the catalytic activity of PQQ. It should be noted that the electrochemistry of PQQ has not been fully researched yet. Also, the PQQ redox behaviour is dependent highly upon the electrode material. The reason for selecting gold electrode in this study was the reversible response of PQQ stated by Sato et al. [31].

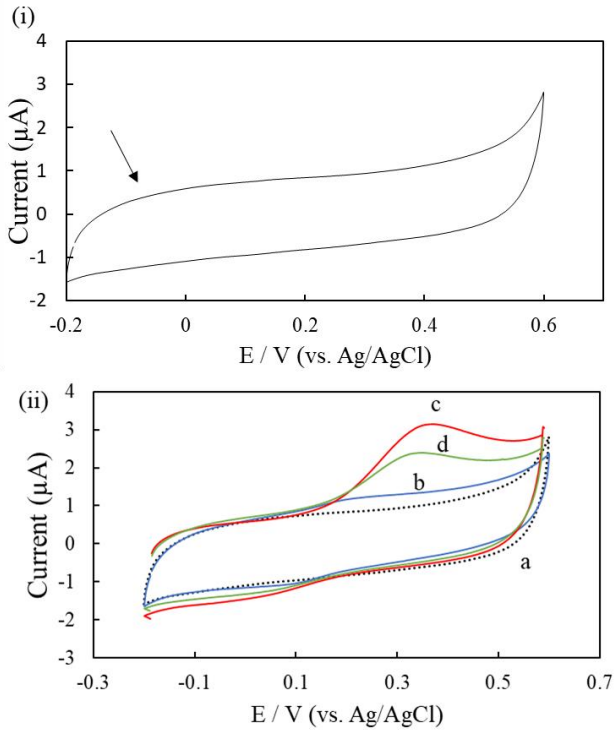


Figure 4: (i)PQQ-GDH-pCNO-Au electrode in 0.1M PBS pH 7.0 in absence of glucose, (ii)PQQ-GDH-pCNO-Au electrode in 0.1M PBS pH 7.0 with (a) 0 mM glucose (dotted), (b) 20 mM glucose (blue), (c) 50 mM glucose (red) (b) 100 mM glucose (green); sweep rate is 50 mV/s.

For these electrodes, the presence of glucose at 20, 50, 100 mM concentrations lead to increase in the oxidation peak observed (presumably of PQQ) that is dependent on glucose concentration. The results are shown in Figure 4(ii). While the oxidation peak is increasing substantially with glucose

concentration, there is no significant change in the reduction peak intensity, a fact that supports that the observed behaviour is due to direct electron transfer from the reduced PQQ.

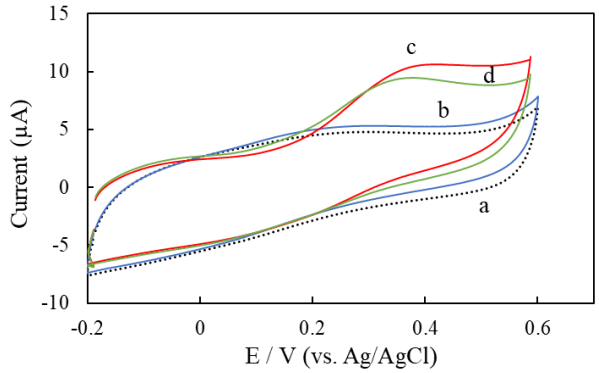


Figure 5: PQQ-GDH-pCNO-poly[ABA/APBA]-Au electrode in 0.1M PBS pH 7.0 with (a) 0 mM glucose (dotted), (b) 20 mM glucose, (c) 50mM glucose (b) 100 mM glucose; sweep rate is 50 mV/s.

The PQQ-GDH-pCNO-poly-[ABA/APBA]-Au electrodes showed similar behaviour in the absence of glucose but much more enhanced oxidation current compared to the previous electrode (without the poly[ABA/APBA]) in the presence of glucose. The results are shown in Figure 5. This indicates that the supporting polymer film enhances DET from the enzyme to the electrode.

It is worth noting that the oxidation peaks of the polymer differ from the redox peaks of PQQ. The co-polymer poly[ABA/APBA] oxidation peaks appear between 0.2 and 0.4 V as can be observed from Figure 1, whereas the redox peaks of PQQ are observed from figure 4(i) between -0.2 and 0.2 V and 0.4 to 0.6 V which we assume to be the oxidation peak corresponding to the direct electron transfer from the reduced PQQ.

To confirm the activity of PQQ both bare and electropolymerized electrodes -modified by pCNO (i.e., PQQ-GDH- pCNO-Au and PQQ-GDH-pCNO-poly[ABA/APBA])are also tested in MOPS buffer. The electrodes showed catalytic activity in the absence of substrate, however they did not show efficient direct electron transfer after 20 mM glucose concentration, when compared to activity in PBS buffer. The reason behind the less activity in MOPS buffer might be the less proton mobilization and accumulation, hindering the catalytic reaction. Thus, this proves that the active centre of PQQ-GDH enzyme can be accessed with pCNO with PBSE linker in the absence of substrate.

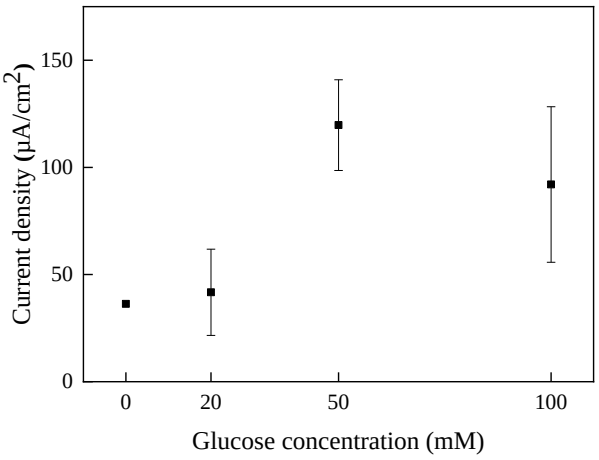


Figure 6: Current density values of PQQ-GDH-pCNO-poly[ABA/APBA]-Au electrode with respect to glucose concentration (mM).

Further, direct electron transfer of PQQ-GDH was studied in the presence of increased substrate concentrations with multiple PQQ-GDH-pCNO-poly[ABA/APBA]-Au electrodes. Figure 6 shows the increase in current density values with increase glucose levels for PQQ-GDH-pCNO-poly[ABA/APBA]-Au electrode.

This indicates that PQQ-GDH enzyme is able interact with the CNO, subsequently the CNO can transfer electrons to the polymer matrix on Au electrode. This also signifies the efficient proton (H^+) mobilisation from the enzyme to the solution from the redox reaction of PQQ. The maximum current density achieved is approximately 120 $\mu A/cm^2$ at 50 mM glucose concentration.

3.3.2 MET studies

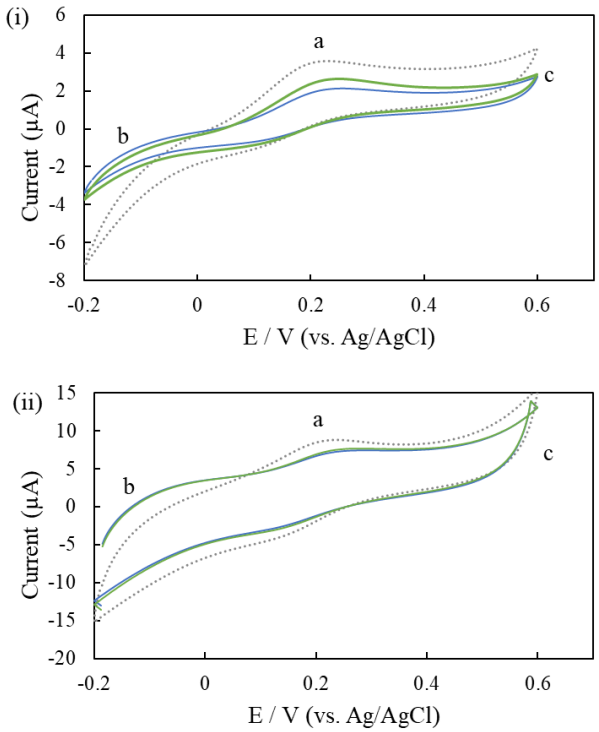


Figure 7: (i) PQQ-GDH- poly[ABA/APBA]-Au electrode in 0.1M PBS pH 7.0 + 1 mM 1-ferrocene methanol with (a) 0 mM glucose (dotted), (b) 20 mM glucose (blue), (c) 50 mM glucose (green), (ii) PQQ-GDH-pCNO- poly[ABA/APBA]-Au electrode in 0.1M PBS pH 7.0 + 1 mM 1-ferrocene methanol with (a) 0 mM glucose (dotted line), (b) 20 mM glucose (blue), (c) 50 mM glucose(green) ; sweep rate is 50 mV/s.

The electrodes have been also tested in the presence of 1-ferrocenemethanol as diffusional electron mediator redox species in 0.1 M PBS solution pH 7.0. Figure 7 shows the response of electrodes on which have been electropolymerized by poly[ABA/APBA] on bare and pCNO modified electrodes with immobilized enzyme. No significant variation can be observed with increase in glucose levels even in the presence of mediator. This signifies that the polymer alone cannot interact with the enzyme due to its hydrophobic characteristics.

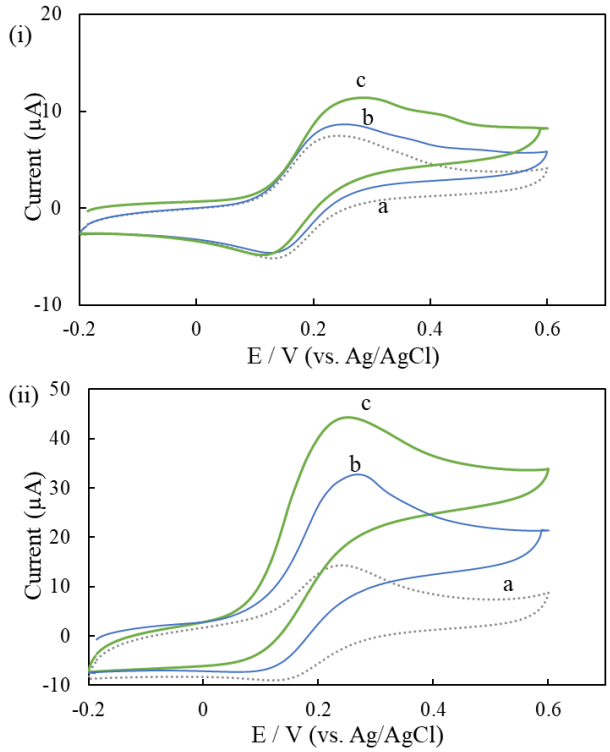


Figure 8: (i) PQQ-GDH-pCNO- Au electrode in 0.1M PBS pH 7.0 + 1 mM 1-ferrocene methanol with (a) 0 mM glucose (dotted), (b) 20 mM glucose (blue), (c) 50 mM glucose (green), (ii) PQQ-GDH-pCNO- poly[ABA/APBA]-Au electrode in 0.1M PBS pH 7.0 + 1 mM 1-ferrocene methanol with (a) 0 mM glucose (dotted line), (b) 20 mM glucose (blue), (c) 50 mM glucose (green); sweep rate is 50 mV/s.

On the contrary, the electrodes modified with pCNO showed electron transfer in the presence of mediator. The results are shown in Figure 8. The pCNO-Au electrodes showed poor current response in comparison with the pCNO-poly[ABA/APBA]-Au electrodes. This again confirms that this system is more efficient for electron transfer with or without the aid of mediator. Another aspect is that this value is close

to the maximum achievable theoretical value. This observation makes clear that further improvement of the DET mechanism is required.

3.4 Electron Pathways

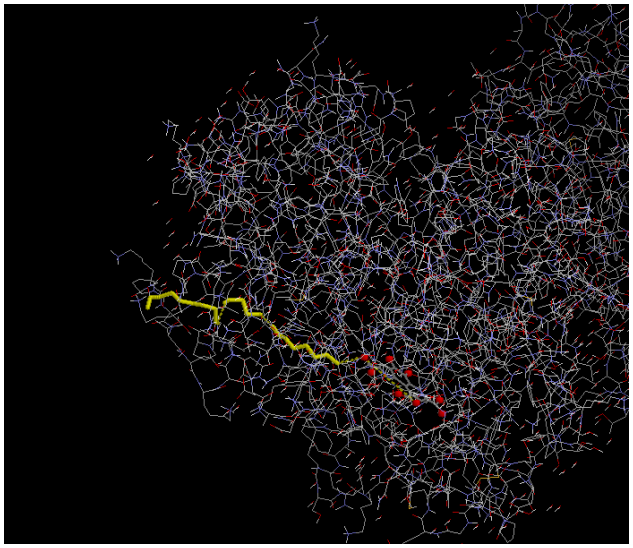


Figure 9: Best electron pathway between end of PQQ ($1C9U$PQQ1004:C.O2B) and surface atom of enzyme subunit-glutamate ($1C9U$GLU68:B.OE2) simulated using HARLEM software.

Previously, the electron pathways have been studied from both ends of PQQ consisting of 8 (total - 30) atoms to several atoms on the surface of the enzyme. The best electron pathway has been found between -COOH functional end of PQQ and the glutamate subunit of the enzyme and the dimensionless electronic coupling value (H_{AB})[14] between them is about 1.005857×10^{07}. The result is shown in Figure 9. This electron transfer pathway and preferential affinity towards glutamate may be considered for the oriented immobilisation, if the active site of the enzyme is not accessible.

4. Conclusions and future work

The poly[ABA/APBA]-Au and poly[ABA/APBA]-pCNO-Au electrodes have not been able to effect direct electron transfer from PQQ in absence and presence of glucose probably due to loss of interaction between enzyme and polymer. The electrodes (pCNO-Au and pCNO-poly[ABA/APBA]-Au) are able to directly interact with the PQQ-GDH accessing the active site of enzyme. Maximum current density value up to 140 µA/cm^2 at 50 mM glucose concentration by direct electron transfer was observed for pCNO-poly[ABA/APBA]-Au.

In future, optimization of polymer/CNO architecture for efficient enzymatic interaction should be studied by varying monomer concentrations for electro-polymerization and/or CNO concentrations. A composite matrix of CNO and conductive polymer can be fabricated by *in situ* polymerization technique. The effects of PQQ as mediator in solution can be studied. Finally, the created successful system can be applied for micrometric patterned electrodes and can be investigated for compatibility and end usage in biosensors and bionic systems.

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