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Wired Pyrroloquinoline Quinone Soluble Glucose Dehydrogenase Enzyme Electrodes Operating at Unprecedented Low Redox Potential

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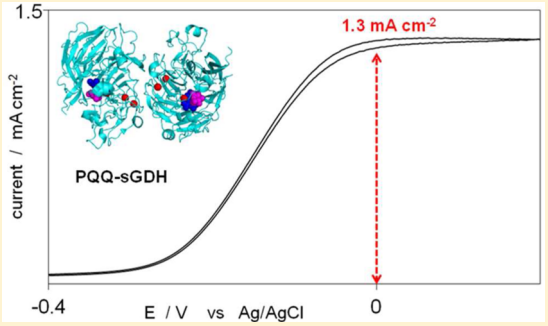
Supporting Information

ABSTRACT: We report unprecedented high current densities for the enzymatic oxidation of glucose already at 0 V versus Ag/AgCl. The modified electrodes were made by assembling pyrroloquinoline quinone (PQQ)-soluble glucose dehydrogenase (PQQ-sGDH) from *Acinetobacter calcoaceticus* with osmium-based redox polymers and a cross-linker. Both redox mediators are made of a poly(4-vinylpyridine) (PVP) polymer with Os complexes tethered to the polymer backbone via long C chains, giving the Os complexes flexibility and mobility inside the redox hydrogels. Current densities larger than 1 mA cm^{-2} were measured already below 0 V with a plateau value of 4.4 mA cm^{-2}. Similar hydrogel electrodes comprising the same redox polymers and glucose oxidase (GOx) showed less than half the current densities of the PQQ-sGDH electrodes. The current versus potential curve dependence showed a sigmoidal shape characteristic of mediated enzyme catalysis but with a current increase versus potential less sharp than expected. Surprisingly, the midwave redox potential was positively shifted with respect to the potential of the redox mediator.



Soluble pyrroloquinoline quinone (PQQ) glucose dehydrogenase (PQQ-sGDH) is a glucose oxidizing enzyme that has gained increased attention in recent years and is frequently incorporated in commercial glucose biosensors.[1−3] Although most reports on enzyme anodes oxidizing glucose use glucose oxidase (GOx), PQQ-sGDH has two undeniable advantages when compared to GOx: it is insensitive to oxygen, and it has a much higher catalytic activity.[2,3] On the other hand, PQQ-sGDH exhibits lower thermal stability than GOx and it has a higher redox potential. It appears also as an interesting candidate for enzymatic biofuel cell (BFC) applications.[4] If efficiently translated into a bioelectrochemical signal, the higher catalytic activity of PQQ-sGDH would allow for an enhancement of BFC power density, as compared to GOx.

High catalytic currents at low potentials are desired both for BFC and biosensors applications. Operation of the latter at low potential avoids oxidation of common interferences present in blood, such as ascorbic or uric acid.[3] Voltage losses within a BFC may be minimized by a clever selection of enzyme and mediator, the closer the mediator potential to the enzyme potential, the higher the BFC voltage output. Optimized BFCs generate maximum power density by producing a high current density while simultaneously operating at high voltage.[5]

Because O_2 is the natural electron acceptor of GOx,[6] several issues may arise when GOx is used at the anode in membraneless enzymatic BFCs.[3,5,7−10] First, for most BFCs designs, O_2 may be partially depleted at the anode, leaving less oxidant available for the cathodic reaction. Then, if the redox mediator is not properly chosen, O_2 reduces the efficiency of glucose oxidation, since a fraction of the electrons will not be collected by the redox mediator, but lost by direct enzyme reaction with O_2. Finally, reaction of O_2 with GOx produces toxic H_2O_2. Therefore, anodes based on PQQ-sGDH would be ideal candidates as they could overcome all those issues. In addition, their higher current densities would allow for the development of high power density BFCs. This improvement will only come to full realization if high-activity enzymatic cathodes are developed and will match the high oxidation current produced at the anode.[11]

The redox potential of PQQ-sGDH at pH 7 and in the presence of Ca^{2+} ions has been reported to be −194 mV (vs Ag/AgCl 3.5 M),[12] although there is no unanimous agreement on this value. In any case, all reported values are much more positive than that of GOx (−525 mV vs Ag/AgCl 3.5 M).[13] Despite the 300 mV difference between the two enzymes, the formal potential of PQQ-sGDH is still low enough to be a good candidate both for biosensors and BFCs applications. However, according to most literature reports on fully integrated anodes, where both the enzyme and the mediator are immobilized at the electrode surface, appreciable glucose oxidation currents are usually only observed at an overpotential of ∼350 mV from the

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enzyme redox potential, and maximum values are achieved at electrode potentials as high as 300−500 mV.[14] A wide range of redox mediators have been reported for successful wiring of PQQ-sGDH, including ferrocene derivatives,[15,16] osmium-derivatized polymers,[17−19] and phenolic compounds.[20] Given the reported redox potential for PQQ-sGDH, the challenge is to find an efficient redox mediator or molecular wire for PQQ-sGDH with much lower redox potential than previously reported, that can be coimmobilized with the enzyme. To the best of our knowledge, Tanne et al.[21] and Leech and co-workers[22,23] are the only ones to have reported catalytic responses for fully integrated PQQ-sGDH anodes at potentials close to 0 V. The first group proposed the use of an excess of free PQQ attached to multiwall carbon nanotubes to wire the enzyme and reported a catalytic current starting at −80 mV, and reaching 200 μA cm^{-2} at 100 mV; whereas the second group proposed the use of two different low-potential Os polymers, also in combination with carbon nanotubes, and reached almost 1 mA cm^{-2} at 100 mV in the presence of 60 mM glucose.

Conversely, reports on wiring of PQQ-sGDH with low-potential redox mediators have indeed been produced for soluble systems, i.e., where both the enzyme and the mediator are free to diffuse in solution. Heller and Feldman reported Os complexes as efficient redox mediators with E^0 of −105 and −125 mV, which were used in the first generations of commercially available blood glucose monitoring systems.[2,24] Okumura et al. also reported the wiring of PQQ-sGDH with different Os complexes with E^0 as low as −154.5 mV and quinone compounds with E^0 as low as −207 mV.[25]

The present work shows that high catalytic currents at unprecedented low redox potentials can be obtained for the immobilized enzyme in a redox hydrogel matrix when two different redox mediators are used. Both mediators consist of a poly(4-vinylpiridine) (PVP) polymer backbone with attached Os moieties linked to the polymer backbone by long tethers. Surprisingly, these are exactly the same polymers that have already been reported to be excellent wires for GOx, except that, because their formal redox potentials were very close to that of PQQ-sGDH, they had never been envisioned to be wired to this enzyme.

EXPERIMENTAL SECTION

All solutions were prepared in Milli-Q water. All reagents were of analytical grade. Synthesis of polymer I has previously been reported.[26] Polymer II was a generous gift from Abbott Diabetes Care.[27] Wild-type PQQ-sGDH from *Acinetobacter calcoaceticus* was produced and purified as previously described.[28,29] GOx was from Sigma and was purified as previously described.[30] Glassy carbon electrodes were carefully polished in alumina slurry of different sizes and sonicated in between. Electrodes were made hydrophilic by pretreatment in an O_2 plasma for 10 min. Polymer (10 mg/mL), enzyme (10 mg/mL), and poly(ethylene glycol 400) diglycidyl ether (PEGDGE, cross-linking agent, 1 mg/mL) were mixed together and deposited on 5 mm glassy carbon electrodes (Pine Instrument, Raleigh, NC). Electrodes were let to dry for 18 h at room temperature.

All PQQ-sGDH electrodes and GOx/polymer I electrodes were composed of a total dry loading of enzyme plus polymer plus PEGDGE of 76.5 μg cm^{-2}. GOx/polymer II electrodes were composed of a total dry loading of 200 μg cm^{-2}. The content of PEGDGE was always kept constant at 6% w/w with respect to the total loading. It should be noted that, even though the loading of the GOx/polymer II electrode is considerably higher than that of the PQQ-sGDH electrode, the plateau current versus loading increase is not linear in this loading range and increasing the total loading from 76.5 to 200 μg cm^{-2} only increased the current at glucose saturation by 25%.

In order to determine the optimum composition for the hydrogel electrodes, a series of electrodes were prepared with variable ratios of polymer and enzyme while keeping constant the total mass loading and the mass of cross-linking agent. The catalytic current was measured at 120 mM glucose for each electrode. These results are shown in Figures S1 and S2 of the Supporting Information for PQQ-sGDH/polymer I and PQQ-sGDH/polymer II, respectively. The optimum ratios, i.e., the ratio at which the catalytic current is the highest at glucose saturation, were determined to be 22/72/6% for PQQ-sGDH/polymer I/PEGDGE and 32/62/6% for PQQ-sGDH/polymer II/PEGDGE. All subsequent experiments were performed at these compositions. The optimal ratios for the GOx electrodes were determined to be 22/72/6% for GOx/polymer I/PEGDGE and 35/59/6% for GOx/polymer II/PEGDGE.

Electrochemical experiments were carried out on a CH Instruments potentiostat (model CHI 660C, Austin, TX, U.S.A.). A platinum spiral wire was used as counter electrode, while a commercial Ag/AgCl (3.5 M KCl) electrode (BASi) was used as reference electrode. All electrochemical measurements were performed in a water-jacked electrochemical cell at 37 °C, in deoxygenated buffer, rotating the electrodes at 1000 rpm. Glucose solutions were prepared 24 h before the experiments to allow for equilibration of the anomers. Experiments with PQQ-sGDH electrodes were carried out in PIPES 20 mM buffer plus 3 mM $CaCl_2$, pH = 7.2. Experiments with GOx electrodes were carried out in 20 mM sodium phosphate buffer plus 0.14 M NaCl, pH = 7.2 (PBS). Electrodes were prepared and measured at least in triplicate with a standard deviation of less than 20%.

All redox potentials reported in this manuscript are referred to the Ag/AgCl (3.5 M KCl) reference electrode. For easy of comparison, results from other authors that were originally referenced versus other electrodes have been recalculated and are expressed here versus the Ag/AgCl (3.5 M KCl) reference electrode.

Current density values quoted as milliamps per square centimeter have been normalized by the geometrical surface area of the electrode.

RESULTS AND DISCUSSION

Electrooxidation of Glucose at Low Potential. Two different polymers were used for the preparation of fully integrated electrodes comprising PQQ-sGDH. Polymer I is a PVP polymer with a [Os(*N*,*N*′-dialkylated-2,2′-biimidazole)$_3$]$^{2+/3+}$ redox center covalently attached with a redox potential of −170 mV.[26] Polymer II is also a PVP polymer, but with an Os(1,1′-dimethyl-2,2′biimidazole)$_2$-2-[6 methylpyrid-2yl]imidazole)$^{2+/3+}$ redox center covalently attached with a redox potential of −30 mV.[27,31,32] For both polymers, the redox center is tethered to the polymeric backbone through 13 atom chain spacer. The chemical structures of these polymers are shown in the Supporting Information (Figures S3 and S4). These are the same polymers that have already been proved to be excellent wires[26,31,33] for the classical and most widely employed enzyme in glucose enzyme electrodes, glucose

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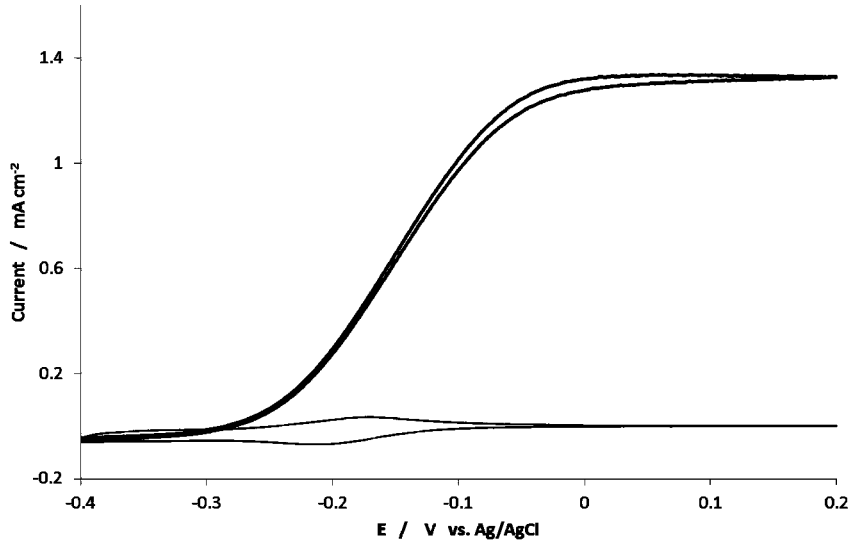

Figure 1. Cyclic voltammogram for a PQQ-sGDH/polymer I electrode in the absence (thin line) and presence (thick line) of 20 mM glucose. Scan rate: 5 mV s^{-1}, 1000 rpm, PIPES 20 mM buffer plus 3 mM $CaCl_2$, pH = 7.2, 37 °C.

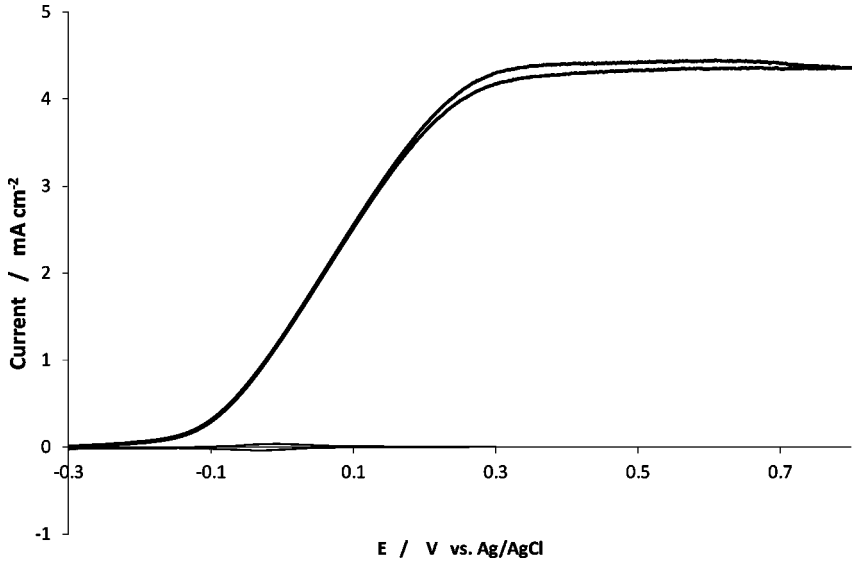

Figure 2. Cyclic voltammogram for a PQQ-sGDH/polymer II electrode in the absence (thin line) and presence (thick line) of 20 mM glucose. All conditions as in Figure 1.

oxidase from *Aspergillus niger* (GOx),[4] except that, because their formal redox potentials were very close to that of PQQ-sGDH, they had never been tested on PQQ-sGDH before.

Figures 1 and 2 show the cyclic voltammograms (CVs) both in the absence (thin line) and presence (thick line) of glucose for hydrogel electrodes made with PQQ-sGDH and polymer I (Figure 1), or PQQ-sGDH and polymer II (Figure 2), and measured at their optimum (polymer/enzyme/PEGDGE) ratio. The CVs in the absence of enzymatic substrate show the characteristic reversible wave of a diffusion confined redox species. The $E^{1/2}$ values were in agreement with previous experiments with the same redox mediators and GOx.[26,31] Upon addition of glucose, sigmoidal curves with minimal hysteresis indicating a clear enzymatic catalysis are observed for both electrodes. For the PQQ-sGDH/polymer I electrode the current starts to increase already at −320 mV and reached a plateau of maximum current density (1.33 mA cm^{-2}) at 0 V. For the PQQ-sGDH/polymer II electrode the current starts to increase at −250 mV and reaches a plateau of maximum current density (4.4 mA cm^{-2}) at ∼280 mV.

Redox Potential of PQQ-sGDH. To the best of our knowledge, this is the first time that such high current densities are reported at potentials close to 0 V for enzyme electrodes where both PQQ-sGDH and a redox mediator are jointly immobilized at the electrode surface. Surprisingly, even though the need for low-potential mediators is rather obvious, there is hardly any discussion about the generalized use of high potential mediators with PQQ-sGDH, and the absence of reports on mediators with more suitable potentials.[7,14] From our analysis of the literature, only two fully integrated PQQ-sGDH-mediated electrodes operating at relatively low potential have been reported up to date. The first one is formed by the immobilization of high amounts of PQQ, i.e., the natural redox mediator of the enzyme.[21] In this case, the PQQ acting as mediator is trapped on a carbon nanotube matrix, but probably still has some relative mobility to effectively mediate the

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electron transfer of the enzyme to the electrode. In the second one, a line of reasoning similar to ours was developed and an Os complex with appropriate low redox potential was used.[22,23]

When choosing an appropriate redox mediator, the first question that arises is which is the redox potential of PQQ-sGDH. A careful analysis of the literature reveals a rather broad window of reported redox potentials for the enzyme. This is in turn related to the fact that the electrochemistry of both free PQQ and PQQ-sGDH is, as yet, poorly understood and is still the subject of intense research.[28,34] Sato et al.[12] reported that PQQ reduction follows either two consecutive one-electron transfer steps (1E1E), or a one-step, two-electron transfer (2E) process, depending on the experimental conditions.[12] The two-step mechanism is depicted by eqs 1 and 2:

$$PQQ_{OX} + e^- \leftrightarrow PQQ_{SEM} \qquad E_1^0 \tag{1}$$

$$PQQ_{SEM} + e^- \leftrightarrow PQQ_{RED} \qquad E_2^0 \tag{2}$$

where PQQ_{OX}, PQQ_{SEM}, and PQQ_{RED}, denote the oxidized, semiquinone, and fully reduced forms of PQQ, respectively, and E_1^0 and E_2^0 are the redox potential of the first and second electron-transfer steps. The difference between E_1^0 and E_2^0 reflects the thermodynamic stability of the semiquinone intermediate.[12]

The determination of the redox potential of enzymes is not straightforward.[12,35] While several authors report the redox potential of PQQ-sGDH to be close to −115 mV,[14,16] in the above-mentioned work, E_1^0 and E_2^0 for PQQ-sGDH were determined by spectroelectrochemical techniques at pH 7 to be −217 and −172 mV.[12] We have shown earlier that the adsorbed protein on carbon cryogel showed two direct electron-transfer anodic peaks in the absence of enzymatic substrate at −159 and +145 mV, also at pH 7.[28] Ivnitski et al. also observed two redox peaks (−27 and +188 mV) under similar conditions but on an electrode modified with carbon nanotubes at pH 6.[36]

The enzyme redox potential seems to be highly dependent on pH (and not with the expected 59 mV/n pH dependence) and the presence of Ca^{2+} (or other bivalent cations). Variations were also reported depending on the presence or absence of redox mediators, on whether the reaction is occurring in solution, or on a heterogeneous phase, and in this last case, it is strongly influenced by the electrode material.[28,37−40] Very interesting examples of this complex behavior were recently reported by Lisdat et al. The DET catalytic signal of PQQ-sGDH was achieved at −68 mV, but upon addition of excess free PQQ, which putatively acts as a redox mediator, the catalytic signal is considerably enhanced, although only at the classical high redox potentials of +200 mV.[41,42] Moreover, molecules that acted as mediators at the electrode surface were completely ineffective in homogeneous phase.[43] Laurinavicius et al. suggested that once PQQ-sGDH was immobilized, PQQ dissociates from the active center of the enzyme and acts as a free mediator.[16] Differences have been reported not only in redox potentials but also in the peak separation and in the relative intensity of the peaks. The availability of Ca^{2+} ions seem to determine whether the process can be considered a 1E1E or a 2E mechanism.[12] In this scenario it is possible to envisage that, according to the electrode matrix, Ca^{2+} ions might become readily available or not depending on whether the enzyme is entrapped into a nanoporous electrode[28,36] or suspended into a hydrated hydrogel film where it finds itself in an environment which is more similar to a homogeneous solution.

Therefore, in the context of the broad window of reported redox potentials for PQQ-sGDH, expanding approximately between −200 and −100 mV, but including also some reports where a second oxidation peak is reported at potentials higher than +100 mV,[28,36,37] it is difficult to compare the mediators' potentials to that of the enzyme. If we consider this second oxidation peak, then both polymers I and II have redox potentials less positive than that of the enzyme. Disregarding this second oxidation peak, the redox potential of polymer II (−30 mV) is more positive (i.e., oxidizing) than that of the enzyme, whereas the redox potential of polymer I (−170 mV) is very close to that of the enzyme and could be considered either slightly negative or slightly positive according to which reference is considered. From our experimental results, it is clear that the enzyme is still catalyzing glucose oxidation and that the reaction is mediated by both polymers, whatever the true redox potential of the enzyme. The reactions taking place within the biofilm are

$$\text{glucose} + \text{PQQ-sGDH}_{OX} \rightarrow \text{gluconolactone} + \text{PQQ-sGDH}_{RED} \tag{3}$$

$$2\text{Os(III)} + \text{PQQ-sGDH}_{RED} \rightarrow 2\text{Os(II)} + \text{PQQ-sGDH}_{OX} \tag{4}$$

Therefore, the overall reaction is

$$\text{glucose} + 2\text{Os(III)} \rightarrow \text{gluconolactone} + 2\text{Os(II)} \tag{5}$$

The global thermodynamic potential gain (driving force) for glucose oxidation is given by the potential difference between the oxidation potential of the sugar ($E^{0\prime}_{\text{glucose/gluconolactone}}$) and that of the Os complex ($E^{0\prime}_{\text{Os(III)/Os(II)}}$): $\Delta E_5 = E^{0\prime}_{\text{Os(III)/Os(II)}} - E^{0\prime}_{\text{glucose/gluconolactone}}$. We attribute the large catalytic current values to the overall potential drop (ΔE_5). The potential difference between the enzyme ($E^{0\prime}_{\text{PQQ–SGDHOX/PQQ–SGDHRED}}$) and the Os complex is only related to the kinetics of the reaction: $\Delta E_4 = E^{0\prime}_{\text{Os(III)/Os(II)}} - E^{0\prime}_{\text{PQQ–SGDHOX/PQQ–SGDHRED}}$. Therefore, irrespective of the redox potential of the enzyme, and particularly even in the likely situation of an unfavorable potential difference between the enzyme and the mediator (ΔE_4), the global reaction is still driven forward by the overall potential gain. In other words, the thermodynamic gain from the global reaction overrules the minor loss in potential in the enzyme−mediator reaction.

This situation is analogous to that presented by Tsujimura et al. who obtained large current densities for O_2 reduction catalyzed by bilirubin oxidase using redox mediators with redox potentials up to 200 mV more positive to that of the enzyme (though still less positive than that of the O_2/H_2O couple).[44] Similarly, Gallaway and Calabrese Barton[45] also showed high catalytic currents for O_2 reduction catalyzed by another enzyme, laccase, mediated by redox mediators that have formal potentials which are very close to the enzyme's redox potential.

Therefore, in the context of the overall favorable thermodynamics, the challenge is reduced to find suitable mediators that will be able to overcome the kinetic barrier. Polymers I and II are unique in the sense that the Os complex is tethered to the polymeric backbone through long and flexible spacer arms. These tethers confer mobility to the redox center even in a cross-linked hydrogel; they allow them to sweep electrons from large-volume elements and result in high

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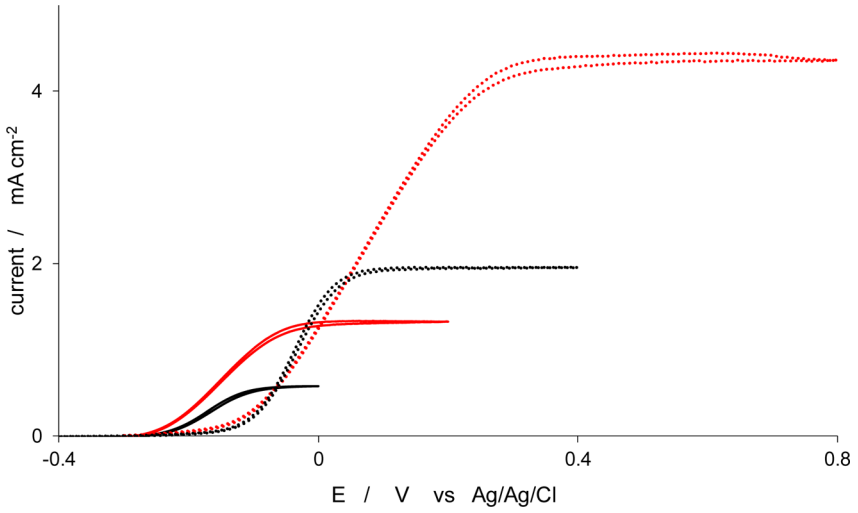

Figure 3. Cyclic voltammograms in saturated glucose solutions (50 mM) for PQQ-sGDH/polymer I electrode (red full line), PQQ-sGDH/polymer II electrode (red dashed line), GOx/polymer I electrode (black full line), and GOx/polymer II electrode (black dotted line): 5 mV s^{-1} scan rate, 1000 rpm.

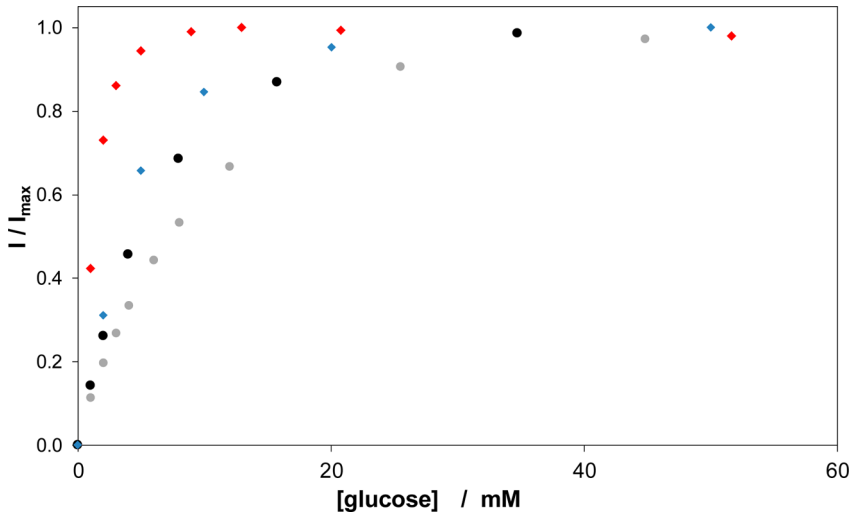

Figure 4. Normalized calibration curves: red, PQQ-sGDH/polymer I; blue, PQQ-sGDH/polymer II; black, GOx/polymer I; gray, GOx/polymer II. The normalization has been done with the maximum current density at glucose saturation reached for each electrode.

collision frequency with the enzyme.[26] Because of the high mobility of the redox centers, it is possible to efficiently reduce PQQ-sGDH in a similar way as soluble redox mediators do.[2,24,46]

Comparison of PQQ-sGDH and GOx Electrodes. It is interesting to compare the magnitude of the current densities produced by our new electrodes (PQQ-sGDH and polymers I and II) with hydrogel electrodes made with the same polymers and GOx. Because of the difference in molecular weight between GOx and PQQ-sGDH (156[6] and 100.5 kDa,[28] respectively), the question was raised as to whether we should compare electrodes with the same mass or molar loading. Because our aim was to prove that PQQ-sGDH electrodes catalyze glucose oxidation more efficiently, we decided to compare these to the optimal composition and loading for each of the GOx electrodes (see details in Experimental Section).

Figure 3 shows a set of four CVs measured at glucose saturation for electrodes modified with PQQ-sGDH/polymer I (full red line), PQQ-sGDH/polymer II (dotted red line), GOx/polymer I (full black line), and GOx/polymer II (dotted black line). The CVs for the two GOx electrodes also show the classical sigmoidal shape of mediated enzyme electrodes under saturated turnover conditions with minimal hysteresis. Comparison of the two electrodes made with polymer I reveals that the current for the PQQ-sGDH electrode is higher than the current for the corresponding GOx electrode for all potentials, and is 2.3 times higher at the plateau. For the two electrodes made with polymer II, the catalytic waves are almost superposed from the start of the scan until approximately 0 V, when the GOx electrode flattens in a plateau. The current for the PQQ-sGDH electrode continues to increase until a plateau 2.3 times higher than for the GOx electrode is reached at +280 mV.

It is also interesting to compare the ratio between the catalytic current under glucose saturation and the peak current of the mediator in the absence of glucose for each of the electrodes. This value gives a preliminary indication on the efficiency of the molecular wiring (this is only a very rough estimation; as explained later, the catalytic current is determined by several variables). For the two electrodes

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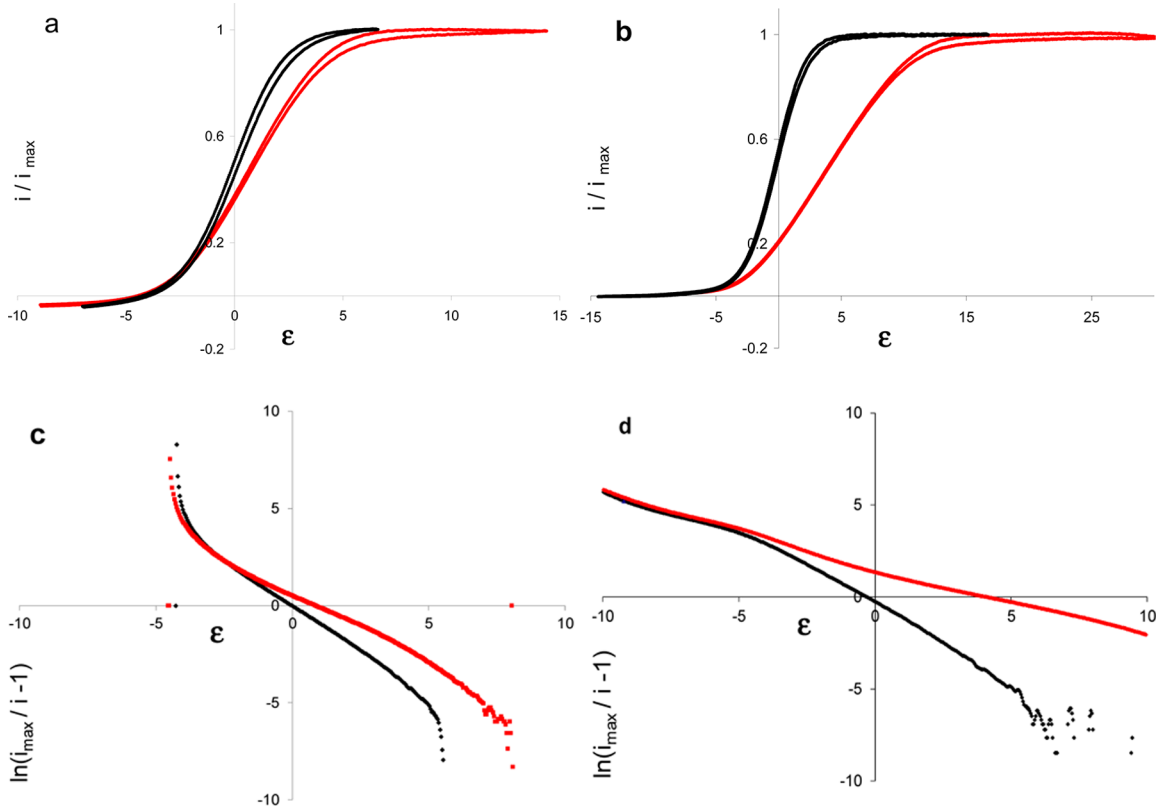

Figure 5. Normalized voltammograms for electrodes made of polymer I (a) and polymer II (b) and GOx (black line) or PQQ-sGDH (red line). (c) Corrected Tafel plots for electrodes made of polymer I (c) and polymer II (d) and GOx (black line) or PQQ-sGDH (red line).

made with polymer I these ratios are 40 and 18 for PQQ-sGDH and GOx, respectively. Because both CVs in the absence of enzymatic substrate show the same redox charge and peak current, the ratio of catalytic current/mediator current is 2.3 times higher for PQQ-sGDH than for GOx (i.e., the same ratio as the two plateau currents). It shows that the PQQ-sGDH/polymer I electrode is 2.3 times more efficient than the GOx/polymer I electrode.

Interestingly, the catalytic current/mediator peak ratios for the two electrodes made of polymer II are 118 and 18 for PQQ-sGDH and GOx, respectively. In this case, the GOx electrode was made with a higher polymer/enzyme loading, and the CV in the absence of substrate shows a peak current 2.9 times higher for the GOx electrode. Therefore, still in the frame of this rough estimation, the PQQ-sGDH is 6.6 times more efficient than the GOx electrode.

Figure 4 shows the resulting normalized calibration curves for all four enzyme electrodes presented above. The normalization has been done with respect to the maximum current at glucose saturation for each electrode. It can be seen that the two electrodes made with PQQ-sGDH (red and blue dots) show lower apparent Michaelis–Menten constant than the corresponding GOx electrodes ($K_{MS\ APP}$ = 1.3 mM for PQQ-sGDH/polymer I, 3.7 mM for PQQ-sGDH/polymer II, 5.3 mM for GOx/polymer I, and 6.8 mM for GOx/polymer II). Moreover, for each enzyme the lowest $K_{MS\ APP}$ is obtained with polymer I. Those values considerably differ from the Michaelis–Menten constants measured in homogeneous solution (K_{MS} = 43 and 25 mM for PQQ-sGDH and GOx, respectively).[6,29]

A simple analysis would attribute the higher current density of the PQQ-sGDH electrodes to the higher turnover number of this enzyme compared to GOx (k_{cat} = 5000 vs 700 s^{-1}).[6,29] However, for immobilized mediator–enzyme electrodes, the overall catalytic response is more complex and is determined by a combination of several variables: the intrinsic enzyme–substrate kinetics (k_{cat} and K_{MS}), the enzyme–mediator kinetics (k), the diffusion of both substrate and mediator (electron hopping) within the hydrogel, the hydrogel thickness, the enzyme and mediator concentrations in the hydrogel, the substrate concentration in the bulk, and the partition of substrate in between the hydrogel and the solution.[47−49] There is no unique analytical solution to fully describe the system, though several approximate analytical solutions predict the current response in different limiting cases, depending on the limiting process determining the current production. A precise estimation of each of the kinetic/diffusion variables is beyond the scope of this work. However, it is still possible to identify the limiting processes in each of our electrodes.

In particular, the current density will not be a function of the enzyme turnover rate when the overall kinetics are mainly limited by the enzyme–mediator reaction, as seems to be the case at glucose saturation. The observation that $K_{MS\ APP}$ is considerably lower than K_{MS} in solution is an indication that, at glucose saturation, the current is no longer controlled by the enzyme–substrate kinetics. We can define theoretical maximum rates both for the enzyme–substrate reaction and for the mediator–enzyme reaction. The overall rate (and hence the current) will be determined by the lower of the two theoretical maximum rates of reaction (or an interplay in the border between the two cases). These rates are a function of the glucose concentration and of distance to the electrode surface within the enzymatic biofilm. It is expected to find a certain glucose concentration where the enzyme–substrate reaction

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becomes faster, and hence nonlimiting. At this point the more sluggish mediator−enzyme reaction will take control over the overall current production, and the system becomes saturated in glucose at concentrations lower than in the homogeneous system (in presence of excess mediator). At this point, the current is no longer dependent on k_{cat}, but only on k, the enzyme−mediator bimolecular rate constant.[47−49] A further indication of this behavior is that the ratios between the PQQ-sGDH and the GOx plateau currents at glucose saturation are 2.3 both for polymer I and polymer II, while the ratio between the two values of k_{cat} is 7.1. Therefore, a higher catalytic current for the PQQ-sGDH electrodes suggest higher values of k for the PQQ-sGDH/polymer I and PQQ-sGDH/polymer II electrodes, as compared to the GOx electrodes, i.e., the redox mediators are reduced more rapidly by PQQ-sGDH than by GOx. This higher efficiency for the reoxidation of PQQ-sGDH as compared to GOx was previously estimated when we calculated the ratio of the catalytic current to the peak of CV in the absence of substrate.

Shape of the Cyclic Voltammograms. For designing biosensors and BFCs, we are interested in the steady-state amperometric response at a fixed potential for each of the electrodes. However, the potential dependence of the current (at sufficiently low scan rate) can provide extremely useful mechanistic information about the reactions within the enzymatic layer.[47] We observe that the CVs (Figure 3) for the GOx electrodes present a relatively faster and sharper current increase than the PQQ-sGDH electrodes. To analyze this feature more in detail, the relative catalytic currents are compared. The current was normalized by the maximum plateau current for each electrode, while the electrode potential was normalized as suggested elsewhere:[47,50]

$$\varepsilon = \frac{nF}{RT}(E - E^0) \tag{6}$$

where n, F, R, and T have their usual meanings, E is the applied potential, and E^0 is the redox potential of the mediator couple. Figure 5a shows the cyclic voltammograms expressed as relative current for the two enzyme electrodes made of polymer I. The onset for glucose oxidation occurs at the same potential for both electrodes, suggesting that the triggering of the bioelectrocatalytic signal is more related to the E^0 of the redox mediator than to that of the enzymes (at least for enzymes with E^0 in the potential window under study). The GOx electrode arrives at the plateau at ∼−100 mV and the PQQ-sGDH only at ∼0 V (non-normalized potentials). The half-wave potential of the GOx/polymer I electrode CV is equal to the mediator formal potential. This is not the case for the PQQ-sGDH, which displays a half-wave potential 25 mV more positive than the formal potential of the mediator. A similar behavior is observed for the electrodes made of polymer II (Figure 5b). The difference between these two electrodes is even more pronounced, with the GOx electrode reaching the plateau at ∼100 mV while the PQQ-sGDH electrode only at ∼280 mV. The half-wave potential of the GOx/polymer II CV is equal to the formal potential of the mediator, while the half-wave potential of the PQQ-sGDH electrode is 80 mV more positive.

Corrected Tafel plots are another way of analyzing the current−potential dependence.[47] A sweep in potential across the appropriate potential window is an easy way of varying the redox mediator concentration. The corrected Tafel plots for the GOx/polymer I (Figure 5c, black line) and GOx/polymer II (Figure 5d, black line) electrodes are both linear with intercept 0 and slopes of −0.96 and −0.9, respectively. The corrected Tafel plots for the two PQQ-sGDH electrodes (red lines in Figure 5, parts c and d) are linear, indicating a clear relationship in between current and potential, with intercepts and slopes considerably deviated from 0 (−0.74 and −0.39 for polymers I, and II, respectively).

This analysis faces us with an unexpected behavior which, to the best of our knowledge, has not been previously described. Both the positive shifts in the half-wave potentials for the PQQ-sGDH electrodes and the Tafel corrected plots with slopes considerably lower than unity are most intriguing. From all the different CV shapes predicted by the theoretical models,[47] none of them correspond to a steady-state CV with a half-wave potential positively shifted. The behavior in the Tafel corrected plots is not predicted either. Indeed, for limiting cases where the enzyme−mediator reaction is the rate-determining step, the half-wave potential is equal to the mediator formal potential, and the corrected Tafel slope is equal to −1, or close thereby (Nernstian behavior). On the other extreme, for the different limiting cases where the enzyme−substrate reaction is the limiting step, the CV is negatively shifted. In this context, it is important to mention that in the absence of enzymatic substrate the Os mediator couple on the different electrodes shows the expected diffusion-limited kinetics, with low peak separation and both anodic and cathodic peak currents that are linear with the square root of the scan rate (see Supporting Information Figures S5 and S6). Therefore, the anomalous behavior for the PQQ-sGDH electrodes does not seem to be explained by mediator diffusion limitations. The normalized cyclic voltammograms and the corrected Tafel plots were also analyzed for PQQ-sGDH/polymer II electrodes of different polymer/enzyme ratios (Supporting Information Figures S7 and S8). The same behavior already described in Figure 5 is observed for all electrodes. However, the effect is stronger for the polymer/enzyme ratios showing the higher catalytic responses.

We do not have at this point a strong hypothesis to explain this behavior. The CV shape could partially be explained by ohmic drop effects. PQQ-sGDH electrodes were measured in a buffer solution of lower conductivity than GOx electrodes. Moreover, while the GOx hydrogels are formed from a negatively charged enzyme and a positively charged polymer, the PQQ-sGDH electrodes are formed from both positively charged polymer and enzyme, which could account for an uncompensated resistance within the hydrogel. The different charge of the enzymes at neutral pH is explained by their different isoelectric points, 9.5 and 4.2 for PQQ-sGDH[51] and GOx,[6] respectively. In parallel to this hypothesis, we believe the CV shape could also be related to many of the open questions regarding the electrochemistry of PQQ-sGDH outlined above. Most importantly, there is not unanimous agreement on the redox potential of the enzyme. There are several reports of a second direct electron-transfer anodic peak for the immobilized enzyme in nonturnover conditions. Finally, there are also the nonexplained catalytic current increases beyond +200 mV produced by excess of free PQQ.[41,42]

■ CONCLUSIONS

We have built two new amperometric enzyme electrodes comprising a cross-linking agent, PQQ-sGDH, and two different polymers with attached Os redox centers. The first electrode (PQQ-sGDH/polymer I) showed a high current

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density of 0.65 mA cm^{-2} already at −150 mV and reached a plateau of 1.33 mA cm^{-2} at 0 V. The second electrode (PQQ-sGDH/polymer II) showed a high current of 1.26 mA cm^{-2} at 0 V and reached a plateau of 4.4 mA cm^{-2} at 280 mV. To the best of our knowledge, this is the first time that high current densities are measured at such low potentials on PQQ-sGDH amperometric enzyme electrodes where both the enzyme and the mediator are immobilized at the electrode surface.

We have compared the kinetic behavior of our new electrodes with electrodes made of GOx and the same redox polymers and found that the current is always higher for the electrodes made of PQQ-sGDH (2.3 times higher at the plateau).

Upon analyses of the current−potential dependence of the new PQQ-sGDH electrodes, we have discovered an unexpected behavior. The increase in catalytic current with electrode potential is slower than would be expected for either a system that is substrate-limited or a system that is mediator-limited (Nernstian behavior). The midwave potentials are positively shifted with respect to the mediator potential.

Most of the technological developments in the field of glucose biosensors comprising PQQ-sGDH electrodes have been carried out with coulometric sensors. The low working potential of the new electrodes (avoiding many of the interferences present in blood) makes them interesting candidates to work as amperometric biosensors as well as anodes in glucose/O_2 enzymatic biofuel cells.

■ ASSOCIATED CONTENT

Supporting Information

Plots of variation of catalytic current densities on the percent w/w enzyme in the hydrogel composition, chemical structure of polymers I and II, analysis of cyclic voltammograms in the absence of substrate, and normalized voltammograms and corrected Tafel plots for electrodes with compositions different to those shown in the main text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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