Electrical Communication between Electrodes and Enzymes Mediated by Redox Hydrogels

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Different redox polymers based on poly(allylamine) with covalently attached ferrocene and pyridine groups that coordinate iron and ruthenium complexes were prepared, and hydrogels were obtained by cross-linking them with epichlorohydrin. Charge propagation from the underlying electrode, through the redox polymer and electrical communication with the enzyme FADH₂ of glucose oxidase, was studied by cyclic voltammetry and electrochemical impedance spectroscopy. The effects of electrolyte composition, concentration of enzyme and substrate, and electrode potential are reported. The role of different redox mediators covalently attached to the polymer backbone is discussed in terms of driving force and electrostatic barriers.

Amperometric biosensors have been developed by using different strategies. Soluble redox mediators were inadequate for long-term stability devices. An alternative approach involved the use of high molecular weight mediators. In this way, polymeric mediators can effect electron transfer between an enzyme and an electrode with the subsequent decrease of mediator loss.

An interesting attempt was the use of conductive polymers such as polypyrrole functionalized with ferrocene moieties. The monomer, ferrocene-modified pyrrole, was electropolymerized onto the electrode, and the enzyme was entrapped within the resulting film. It was possible to measure the catalytic current due to glucose oxidation as well as to demonstrate the regeneration of reduced ferrocene attached to the polymer by the entrapped enzyme in an excess of glucose. However, this design leads to unstable electrodes.

Another approach for increasing mediators’ molecular weights was the covalent attachment of redox species to enzymes with successful results. Nevertheless, this case implied the entrapment of the modified enzyme by using semipermeable membranes.

More recently, experiments employing redox polymers have proved to be very promising. These polymers represent a “bridge” between the electrode and the enzyme’s redox site. Three essential effects are involved when measuring the resulting signal: (i) electrical connection between the redox polymer and the electrode, (ii) charge propagation through the polymer, and (iii) connection between redox sites in the polymer and the enzymatic active site.

The initial results were obtained with modified electrodes using quinone polymers. Polysiloxanes and other polyquinones also proved to be effective for redox mediation. However, in these cases redox polymers were soluble only in organic media, which required introduction of the polymer and the enzyme in two separate steps. Skotheim et al. reported that polysiloxanes with covalently bound ferrocenes with highly flexible chains were effective to prove mediation with GOx. The authors showed that the catalytic response depended on the average spacing between redox sites and the length of the alkyl side chains containing the active redox group.

Heller introduced a different fruitful strategy by working with soluble redox hydrogels. The enzyme was initially immobilized in a two-dimensional structure through electrostatic complexation on the electrode. Increased currents could be estimated to be obtained in three-dimensional structures. A strong interaction between enzyme and redox polymer was achieved by electrostatic association of both components, and 3D biosensors involved covalent cross-linking with bifunctional reagents. This allowed large current densities to be obtained in the presence of freely diffusing substrate under anaerobic conditions.
Most of the work was carried out with glucose oxidase (GOx) and poly(vinyl(pyridine)osmium,15,16 as well as with other enzymes,17,18 and more recently with osmium poly(vinylimidazole).19,20

A different alternative was proposed by Oyama et al. for the entrapment of a redox enzyme in a thermoshrinkable redox polymer.21 In this case, a cold aqueous solution of polymer and enzyme is placed on the electrode, and the resulting mixture becomes insoluble for temperatures higher than the phase separation temperature (∼25 °C).

Willner et al. achieved electrical communication for various layers of GOx when developing organized networks on a self-assembled monolayer of functionalized thiol on gold electrodes.22 Enzyme electrodes in a self-assembled monolayer configuration have also proved to be effective for NAD(P)⁺-dependent enzymes.23

The aim of the present work is to report the application of a polyacationic hydrogel24 derivatized with different redox couples in amperometric biosensors. The behavior of such electrodes is analyzed in terms of the mechanisms of charge propagation and enzyme catalysis.

EXPERIMENTAL SECTION

Materials. Ferrocene- and pyridinecarboxaldehydes (Aldrich), high molecular weight poly(allylamine) hydrochloride (Aldrich), and sodium borohydride (Riedel-de-Haën) were used as received. Glucose oxidase (GOx, EC 1.1.3.4 type VII-S from Aspergillus niger, 186 kDa) with activity 277 IU mgs⁻¹ (Sigma Chemical Co.) was used.

[Ru(NH₃)₅Cl]Cl₂ was prepared from RuCl₃ according to the procedure described in ref 25. [Fe(CN)₆]³⁻Nₐ₉ was prepared according to ref 26.

Glucose and 2-deoxyglucose solutions were prepared from a stock solution equilibrated in the anomers, and kinetics herein refer to the total glucose concentration.

Doubly distilled water was further purified from a Milli-Q reagent water system (Millipore).

Synthesis of Ferrocene-Allylamine Polymer. Ferrocene-carboxaldehyde was dissolved in methanol and added dropwise within an hour to a methanolic solution of poly(allylamine). Poly-(allylamine) was previously dissolved in a slight excess of triethylamine. The mixture was stirred for another hour at room temperature, and sodium borohydride was carefully added in portions at 0 °C. Stirring continued for 1 h; finally, methanol was removed in vacuum, and the residue was extracted with diethyl ether several times. The aqueous solution was further purified by membrane dialysis (Sigma) against water, and water was further removed by freeze drying.

XPS studies of the ferrocene–allylamine polymer revealed a N/F ratio of 10.5 as compared to ferrocene nitrile, used as reference, which yielded 0.93 under similar conditions. Atomic absorption analysis of iron in the polymer confirmed the ratio 1:10 of ferrocene modified to unmodified monomeric units.

Synthesis of Pyridine-Allylamine Polymer. Pyridinecarboxaldehyde was dissolved in methanol and then poured into a methanolic solution of poly(allylamine). The reaction was allowed to proceed at room temperature, and after an hour, reduction by sodium borohydride was achieved under conditions similar to those used for the ferrocene polymer. After removal of methanol, the solid residue was further washed with diethyl ether. Finally, the aqueous solution of the modified polymer was purified by membrane dialysis, and water was removed by freeze drying. H-NMR from the pyridine-modified polymer shows, after integration of the respective signals, 50% of covalent attached pyridine comparing the aromatic protons to the aliphatic signals.

Preparation of Hydrogel-Modified Electrodes. Glassy carbon electrodes were chemically modified with the soluble ferrocene-poly(allylamine) polymer or poly(allylamine)–pyridine polymer as follows: 5 mL of a 1–4% solution of the polymer was mixed with an equal amount of 0.4–2%GOx solution in 10 mM Tris buffer (pH 9). The cross-linking agent, chlorohydrin (1 mL), was finally added, and the mixture was left for 48 h at room temperature.

Admittance analysis of EQCM data at 10 MHz has shown that the sol–gel process by which the hydrogel is formed takes more than 15 h for the viscous resistance to increase to half its final value.

Coordination of Metal Centers to Polymer Pyridines. In both cases, the electrodes were initially modified with 5 mL aliquots of 3–10%pyridine-poly(allylamine) polymer mixed with an equal aliquot of 0.5%GOx solution in 20 mM Tris buffer (pH 9) and finally cross-linked with 1 mL of epichlorhydrin. The mixture was left to react for 48 h at room temperature.

For the ruthenium complex, the modified electrodes were immersed in a 10 mM [Ru(NH₃)₅Cl][CF₃COO]₂ solution of pH 4.9 in contact with amalgamated Zn and were kept under argon for 90 min.

For coordination of the pentacyanoiron complex, the modified electrodes were immersed in a 10 mM solution of [Fe(CN)₅NH₃]⁻Nₐ₉ for 10 min and then thoroughly washed. The coordinated ammonium in the iron complex is relatively labile and undergoes aquation to yield the aquopentacyanoferate(II) anion, which generates the pentacyanoferate(II) pyridine complex in the presence of modified poly(allylamine) by ligand exchange.

Electrodes with 25 and 125 mmol dm⁻³ GOx are reported below, with the total enzyme concentration referred to the total volume of precursor solution. The thickness of the hydrogel membrane is affected by swelling changes upon oxidation–reduction; however, RED experiments allowed us to estimate a thickness of ∼10 µm. Furthermore, using Heller's estimation of hydrogel thickness based on a density of 1 g cm⁻³ and the total mass of material deposited onto the electrode, a dry thickness close to 5 µm could be evaluated.

Oxygen removal from the working solutions was achieved by purging with nitrogen. Electrochemical experiments were described elsewhere;24 electrodes of 0.2 cm² were employed. All

potentials in this paper are quoted with respect to the saturated calomel electrode (SCE).

RESULTS

Poly(allylamine) (PAA) was modified according to two different procedures: ferrocene was covalently attached to the polymer, purified, and further cross-linked on the electrodes, while ruthenium and iron pentacyano complexes were introduced directly to polymer-modified electrodes. In this latter case, pyridine was previously attached to poly(allylamine), and this polymer was cross-linked on the electrode surfaces (see Scheme 1).

Figures 1–3 show the cyclic voltammograms of electrodes modified with hydrogels of ferrocene, \([\text{Ru(NH}_3\text{)}_5\text{py}]^{2+/3+}\), and \([\text{Fe(CN)}_5\text{py}]^{2-/3-}\), respectively, with similar redox charge and enzyme load (see Table 1). Figures 1 and 2 also depict the catalytic waves for the oxidation of glucose catalyzed by GOx immobilized in the gels. The sigmoidal oxidation waves centered at the respective \(E_{1/2}\) (0.350 V for ferrocene and 0.033 V for ruthenium complex) prove effective mediation by the redox centers attached to the polymer backbones. \(^{24}\)

It should be noted that, for iron pentacyano complex attached to the PAA polymer, there is no enzyme catalysis, in spite of the oxidation–reduction potential of this redox couple in the polymer (0.25 V). However, addition of ferrocene ammonium monosulfonate as soluble mediator to the solution in contact with the iron pentacyano polymer proved that the enzyme was active toward glucose oxidation.

Table 1. Data Obtained with Cyclic Voltammetry

<table>
<thead>
<tr>
<th>redox couple</th>
<th>(E_{1/2}) (mV)</th>
<th>(E_p - E_{1/2}) (mV)</th>
<th>(Q^a) (µC)</th>
<th>(\Gamma^b) (nmol cm(^{-2}))</th>
<th>(cD_{1/2} \times 10^{10^c}) (mol cm(^{-2}) s(^{-1/2}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>([\text{Fe(CN)}_5\text{NH}_3\text{Na}_3]^-)</td>
<td>350</td>
<td>59(67)</td>
<td>13–62</td>
<td>0.7–3.3</td>
<td>3–7</td>
</tr>
<tr>
<td>([\text{Ru(NH}_3\text{)}_5\text{Cl}]^2^-)</td>
<td>33</td>
<td>57</td>
<td>90–105</td>
<td>4.5–5.5</td>
<td>5</td>
</tr>
<tr>
<td>([\text{Fe(CN)}_5\text{py}]^{2/-3})</td>
<td>214</td>
<td>71</td>
<td>30–96</td>
<td>1.5–5.0</td>
<td>1.3–2</td>
</tr>
</tbody>
</table>

\(^a\) Integration at low sweep rate. \(^b\) Randles–Sevcik equation. \(^c\) Values in parentheses after prolonged oxidation–reduction cycles.
Note in Figures 1–3 that current never reaches zero at the extremes of the potential scans; therefore, the redox polymers are never fully oxidized nor fully reduced at any measured sweep rate. When propagation of charge in the polymer has a diffusion-

type behavior, the extent of polymer oxidation or reduction will depend on sweep rate.

Cyclic voltammetry in background electrolyte shows linear $i$ vs $v^{1/2}$ plots for all three hydrogels, which indicates propagation of charge in the volume of the polymer network by a diffusion-like process ($D_e$, electron diffusion coefficient), such as electron hopping between neighboring redox sites or counterion motion.

Figure 4 shows cyclic voltammograms of a glassy carbon electrode modified with poly(allylamine), GOx, and epichlorohydrin under analogous conditions but containing no fixed redox groups, in contact with 1 mM ferrocene ammonium monosulfonate solution. Typical curves of soluble redox species are apparent, with a diffusion coefficient of $8 \times 10^{-7}$ cm$^2$ s$^{-1}$ calculated from linear $i$ vs $v^{1/2}$ plots. This value is 20% the value of the diffusion coefficient in aqueous solution. Similar results have been obtained for other hydrogels, such as BSA cross-linked with GOx and glutaraldehyde, with a value of $2.4 \times 10^{-6}$ cm$^2$ s$^{-1}$ for ferrocene monosulfonate.

Diffusion of counterions inside the film to compensate charge when the hydrogel is oxidized or reduced has been ruled out as the rate-determining step of charge propagation, since redox anions in the external electrolyte can easily diffuse through the hydrogel and their electrochemistry can be recorded.

Table 1 shows values of $cD_e^{1/2}$ ($c$ is the volume concentration of redox sites) in the range of $10^{-10}$ mol cm$^{-2}$ s$^{-1/2}$, calculated with the Randles–Sevcik equation for semiinfinite diffusion in a reversible system. Electron transfer under diffusion control suggests for these polymers charge propagation beyond the first layer, which for ferrocene and ruthenium polymers implies a three-dimensional wired enzyme.

For a reversible redox reaction, $E_{f} - E_{1/2}$ should be 56.5/ n mV at 25 °C, and $E_{1/2}$ can be calculated as $E_{f}^{1/2} + 28/n$ mV at 25 °C. In the present case, the ruthenium complex follows a Nernstian behavior, and the other couples were considered almost reversible for $E_{1/2}$ calculation. Table 1 summarizes the data obtained from cyclic voltammetry in 0.1 M KNO$_3$ solution.

The values of the formal reversible potentials of the redox systems covalently attached to the hydrogels can be compared

to the respective formal potentials of the soluble redox couples: 

\[ \text{FCH}_2\text{NH}_2, \ 0.36 \text{ V}^{29} \ \text{[Ru(NH}_3)_5\text{Py}]^{3+/2+}, \ 0.05 \text{ V}^{30} \ \text{and} \ \text{[Fe(CN)}_5\text{Py}]^{3+/2+}, \ 0.25 \text{ V}^{31} \]

Polymer microenvironment effects are likely to modify the free energy of the redox sites in these hydrogels.

The formal potentials of redox couples in the ferrocene-bound polymer shift with the logarithm of anion activity in the range 0.1—1 M, with a slope lower than the value predicted by a Nernst equation for anion permselectivity, RT / F. Exchange of water upon redox switching the PAA-Fc polymer has also been detected by EQCM.\(^{(32)}\) According to Oyama et al.\(^{(33)}\) and Hillman et al.,\(^{(34)}\) the above evidences suggest that the redox change in the PAA hydrogels can be described by

\[
\text{Fc}_t + t_s \text{C}^+_t + t_s \text{A}^-_s + t_s \text{H}_2\text{O}_t + e^- \rightleftharpoons \\
\text{Fc}^+_t + \text{A}^-_t + t_c \text{C}^+_s + \text{H}_2\text{O}_t + e^- \quad (1)
\]

where subindexes f and s indicate species in the film and in solution, respectively, A\(^-\) is the anion, C\(^+\) the cation, and Fc\(^+\) Fc\(^+\) the ferrocene redox couple in the polymer, and t\(_c\) and t\(_s\) are the transport numbers of cations and anions in the hydrogel.

The features of cyclic voltammetry of multilayer-modified electrodes are determined by the charge transfer process at the electrode—polymer interface,\(^{(36)}\) while the subsequent layers propagate charge by a diffusion-like process.\(^{(36)}\)

For the modified electrodes, \(\Delta E_{p}\) exceeds the expected value for a reversible redox couple which is likely to arise from the presence of (i) a bulky molecule such as GOx\(^{(37)}\) and (ii) a highly charged protein. In the absence of GOx, PAA-Fc cross-linked with epichlorohydrin exhibits a symmetrical surface wave with 30 mV peak separation at low sweep rate and linear \(i_p\) vs \(v\).

The rate of charge transfer at the glassy carbon electrode—polymer interface was studied by electrochemical impedance spectroscopy (EIS). Figures 5—7 depict typical Nyquist plots for the three redox polymers in supporting electrolyte and in the presence of 0.1 M glucose in solution at the formal redox potential. In the absence of glucose, the impedance data for the three hydrogels studied can be analyzed by a simple Randles circuit,\(^{(38)}\) where Warburg impedance is apparent at low frequencies and confirms the semiinfinite diffusion behavior of charge propagation where Warburg impedance is apparent at low frequencies and confirms the semiinfinite diffusion behavior of charge propagation in the polymer, i.e., \(\Delta E_{p} \propto L\), where \(D_e\) is the diffusion coefficient for charge propagation and \(L\) the thickness of the hydrogel. This has been confirmed by chronoamperometric transients which show a Cottrell behavior for ferrocene-modified polymer in 0.1 M KNO\(_3\). It should be noted that the low-frequency pure capacitive region characteristic of polymer film electrodes\(^{(39)}\) is never reached in the present polymers in the frequency range investigated (±0.01 Hz). For a Randles circuit, the low-frequency impedance is given by

\[
Z = R_\Omega + R_{ct} + \sigma \omega^{-1/2} - j(2\pi C + \sigma \omega^{-1/2}) \quad (2)
\]

with \(j = -1/2\) and \(\omega = 2\pi f\), with \(f\) the ac perturbation frequency, and \(\sigma = RT/2F^2D_e^{1/2}\).

From a plot of Re(Z) versus \(\omega^{-1/2}\), \(\sigma\) could be evaluated, and the quantity \(CD_e^{1/2}\) was calculated.

Table 2 summarizes the data from EIS in background electrolyte. The uncompensated resistance, \(R_\Omega\), takes account of the

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(35) Laviron, E. J. Electroanal. Chem. 1979, 100, 263.
resistance of the hydrogel layer and a negligible contribution of the external solution resistance. The charge transfer resistance, $R_{ct}$, and the exchange current density, $j_0 = RT/FR_{ct}$, account for the rate of charge transfer between the glassy carbon electrode and the first layer of redox polymer and depends on the surface redox concentration, which is determined by the redox concentration of the polymer and the distribution of polymer redox sites on the surface.

For ferrocene hydrogel, prolonged oxidation—reduction cycles of the electrode lead to a decrease of redox charge which results in larger $E_p - E_{p/2}$ and smaller $j_0$, as shown in Tables 1 and 2 in parentheses. This is thought to arise from the unstable ferricenium species, which can undergo nucleophilic attack and decompose.

**Enzyme Catalysis.** The catalytic waves for the anaerobic oxidation of $\alpha$-D-glucose substrate, $S$, in Figures 1 and 2 can be described by the enzymatic catalytic cycle. Redox mediation by cosubstrate attached to the polymer backbone, $O$, and by the enzyme glucose oxidase, $\text{GOx}$, immobilized in the hydrogel can be represented by eqs 3–6:

\[
\text{GOx(FADH}_2) + 2O \rightarrow \text{GOx(FAD)} + 2R
\]  

The concentrations of oxidized and reduced redox sites in the polymer, soluble substrate, and the different forms of enzyme entrapped in the gel layer are described by $C_D$, $C_R$, $C_O$, $C_E$, $C_{ER}$, and $C_{ET}$, respectively; $C_T = C_D + C_R$, and $C_{ET}$ is the total enzyme concentration. Electrode reaction 3 takes place at the electrode—gel interface with fast kinetics, and a first-order reaction layer at the electrode surface is established rather than reaction throughout the film, since $L \gg (D_O)^{1/2}$.

For charge propagation in the hydrogel under semiinfinite diffusion conditions and reaction 4 under steady state, the catalytic wave can be described by case II of Pratt and Bartlett kinetic analysis:

\[
I_{\text{cat,II}} = \frac{2FA\sqrt{2kD_OC_{ET}C_T}\exp[\beta(F/RT)(E - E^{*})]}{1 + \exp[\beta(F/RT)(E - E^{*})]}
\]  

where $I_{\text{cat,II}} = 2FA(2kD_OC_{ET})^{1/2}C_T$, where $E^*$ is the formal redox potential of mediator couple in the polymer, $\beta$ takes into account the interactions between neighboring redox centers, and $k$ is the bimolecular enzyme oxidation coefficient. The potential dependence of the catalytic current results from the potential dependence of the surface concentration of $O$, which follows a modified Nernstian behavior.

\[
R \rightleftharpoons O + e \tag{3}
\]

\[
E_D + S \rightleftharpoons E_R - S \rightarrow E_R + P \tag{4}
\]

with $K_S = k_d/k_{-1}$ and $k_{-d} \ll k_{-1}$.

Since $\text{FADH}_2/\text{FAD}$ is a two-electron redox center and $O/R$ a one-electron mediator, the oxidation of $\text{GOx}$ involves two one-electron steps according to the global eq 5.

\[
\text{GOx(FADH}_2) + 2O \rightarrow \text{GOx(FAD)} + 2R \tag{5}
\]
Results for catalytic activity of all hydrogels studied are summarized in Table 3.

Note that, as expected from eq 6, the catalytic current increases with the square root of the total enzyme load, \( C_{ET}^{1/2} \), as was previously shown for two GOx concentrations in the redox hydrogel with similar redox concentration.\(^{24}\)

The dependence of the catalytic current with substrate concentration can be described for these films by case VII of Pratt and Bartlett.\(^{41}\)

\[
I_{cat,VII} = 2FA \sqrt{\frac{2C_T D e k_{cat} C_{ET}}{1 + K_S/C_S^{1/2}}}
\]

where the maximum catalytic current for excess substrate is \( I_{cat,max} = 2FA(2C_T D e k_{cat} C_{ET})^{1/2} \).

Figure 8 depicts the catalytic currents for the anaerobic oxidation of glucose and 2-deoxyglucose as a function of substrate concentration for \( E \gg E^o \). In this case, the rate-determining step is the enzymatic reaction (eq 4) with fast reoxidation of (FADH\(_2\))-GOx by the redox mediator. Best fits of eq 7 with data in Figure 8 yield \( I_{cat,max} = 59.5 \pm 3.8 \mu A \text{ cm}^{-2} \) and \( K_S = 10.9 \pm 2.9 \text{ mM} \) for glucose and \( I_{cat,max} = 16.3 \pm 1.2 \mu A \) and \( K_S = 15.2 \pm 4.5 \text{ mM} \) for deoxyglucose. The difference between both substrates results in a ratio of \( k_{cat,max}^{glucose/k_{cat,max}^{deoxy}} = 3.6 \), in good agreement with \( (k_{cat,glucose/k_{cat,deoxy}})^{1/2} = 4.2^{24,43} \).

Nyquist impedance plots in Figures 5 and 6 show that, in excess glucose, a second semicircle is apparent at low frequency instead of the linear Warburg impedance observed in glucose-free solutions. However, for iron pentacyano complex attached instead of the linear Warburg impedance observed in glucose-excess glucose, a second semicircle is apparent at low frequency, as expected from the lack of catalysis found by voltammetry (Figure 3), and only Warburg impedance characteristic of semi-infinite diffusion of electrical charge in the redox hydrogel was observed.

In excess glucose, the reaction scheme depicted in eqs 3–5 can be approximated by an EC’ mechanism,\(^{44,45}\) with fast regeneration of the reduced enzyme and the reoxidation of FADH\(_2\)-GOx by ferricenium or \([Ru(NH_3)_6^{3+}\)]\(^{3+}\) the rate-limiting step. If \( K_S/C_S + 1 \ll K_S/C_O \), the EC’ mechanism is given by eq 3, followed by

\[
O + Z \xrightarrow{k_f} R + P
\]

with \( k_f = 2C_{ET} \) and \( c_Z = C_{ET} \).

The differential equation describing the concentration of the oxidized redox mediator in the hydrogel layer of thickness \( L \gg (D_e t)^{1/2} \) is\(^{44,45}\)

\[
\frac{\partial C_O}{\partial t} = D_e \frac{\partial^2 C}{\partial x^2} - k_c C_O
\]

with the following initial and boundary conditions:

\((42)\) Gibson, O. H.; Swoboda, B. E.; P.; Massey, V. J. Biol. Chem. 1964, 239, 3927.


expected to be positioned across the hydrogel film. This finally results in the oxidation of substrate glucose. The rate of reoxidation, thus, fixes an upper limit for the propagation length of charge by diffusion in the hydrogel with the kinetic length for $C_S \gg K_S$: $\mu = (D_e/2kC_E)^{1/2}$.27

Higher rates of enzyme catalysis, however, were observed for ferrocene hydrogel than for the ruthenium polymer due to the higher redox potential driving force. Iron pentacyanopyridine hydrogel, with a higher redox potential than ruthenium, failed to show catalysis due to the strong local repulsive barrier for the formation of $\text{FADH}_2$–redox site encounter pair.48 $\text{GOx}$ is a highly negatively charged glycoprotein,49 and the redox moiety attached to the polymer backbone must penetrate the funnel leading to the $\text{FADH}_2$ redox site buried in the protein structure.50 The electrostatic work to bring the redox mediator at the reaction site can be described by the model of Wherland and Gray,48 with $R_E$ and $R_O$ the radii of reactants (large GOx and oxidant moiety respectively), $r$ their sum, $\kappa = 0.329\mu^{1/2}$ A$^{-1}$ (with $\mu$ the ionic strength) for water at 25 °C, $\epsilon$ the dielectric constant of water (78.3 at 25 °C), $z_O$ and $z_E$ the charge of mediator and enzyme, respectively, and $e$ the unit electrical charge:

$$W = \left[ \frac{e^{-\kappa R_E}}{1 + \kappa R_E} + \frac{e^{-\kappa R_O}}{1 + \kappa R_O} \right] z_O z_E e^2 \epsilon / 2 \epsilon r^2 \quad (13)$$

Equation 13 assumes the protein to be a sphere with a totally symmetric charge distribution, and the electrostatic barrier corresponds to the approximation of the oxidant molecule to the GOx protein, which would result in a very low concentration of reactants at the reaction site, i.e., a fraction $e^{-W/kT}$.

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