Chapter V. Rational design of mediators for optimising electron transfer rates between redox enzymes and electrodes

Abstract

Glucose oxidase (GOx) was used as a model enzyme to study the electron transfer between enzymes and redox mediators. Since this enzyme can be used in many biosensor configurations where electrochemical signal amplification is required such as in DNA sensors, high electron transfer rate constants, which result in high currents and low limits of detection, are desired.

Osmium complexes of the type [Os(2,2'-bipyridyl)$_2$LL'] and [Os(4,4'-dimethyl-2,2'-bipyridyl)$_2$LL'] were rationally designed and synthesised to study the effect of charge, potential, ionic strength and pH on the electron transfer rate constant. The effect of the mediator global charge from 0 to +5 on the electron transfer rate constant was studied and experimental results showed an exponential dependence of the constant in the mediator global charge, from 0.66 x 10^5 to 6.67 x $10^5 M^{-1}s^{-1}$.

Electron transfer rate constants achieved with osmium mediators having different redox potentials, from 175 to 650mV (*vs.* Ag/AgCl), also showed an exponential dependence on the donor-acceptor potential difference from 0.68 x 10^5 to 1.81 x $10^6 M^{-1}s^{-1}$, in accordance to Marcus' theory. A theoretical maximum electron transfer rate constant k_{ET} = 1.87 x $10^6 M^{-1}s^{-1}$ for a mediator having E_{redox} = 848mV (*vs.* Ag/AgCl) was obtained. Moreover, with the experimental results it was possible to obtain the Marcus reorganisation energy λ = 0.87eV and the distance decays constants β = 2.62 and 0.57Å^{-1} for Os(bpy)$_2$Cl(pyNH$_3^+$) and Os(bpy)$_3$, respectively.

The study of the ionic strength effect demonstrated the influence of the glucose oxidase and osmium mediators charge screening on the kinetics of electron transfer, being these constants higher when using lower ionic strength conditions. Plots of the logarithm of the electron transfer rate constant *vs.* the ionic strength function for Os(bpy)$_2$Cl(pyNH$_3^+$) (E_{redox} = 272mV (*vs.* AgAgCl)) and Os(bpy)$_3$ (E_{redox} = 647mV (*vs.* AgAgCl)) allowed to obtain its infinite ionic strength rate constant k_0 = 3.9 x $10^3 M^{-1}s^{-1}$ and 5.1 x $10^5 M^{-1}s^{-1}$, the radius of the enzyme R_{enz} = 2.5 and 23.6Å and the charge of the enzyme Z_{enz} = -5.3 and -31.9, respectively.

The effect of pH of the media on the kinetics was also studied, the electron transfer rate constant at pH 4.0 decreasing 96% compared to the value at pH 8.0. Studies with negatively and positively charged osmium complexes suggested that a local His residue, which could be on the enzyme surface or close to the active site, may be playing a decisive role in the mechanism of electron transfer between redox mediators and glucose oxidase.

Keywords: homogeneous electron transfer rate constant, redox enzyme electrochemistry, osmium redox mediator, rational mediator design, electrochemical amplification schemes.

Introduction

Most of electrochemical biosensors use enzymes as biorecognition elements or as labels that produce an electrochemical signal proportional to the biorecognition event. In electrochemical DNA sensors, where the biorecognition element is the DNA probe, the enzymatic label can be included in the system by direct attachment of the enzyme to the complementary strand (target) or by incubation with enzyme-antibody conjugates after the hybridisation, which recognise the antigen-modified complementary strands. Additionally, enzymes can be used as part of a cascade that amplifies the signal from the label. These enzymes have to be able to transfer electrons with the electrode surface in order to generate detectable currents. Glucose oxidase is a ubiquitous enzyme that can be used in such schemes of amplification (DellaCiana *et al.*, 1995; Male *et al.*, 1998; and Gooding *et al.*, 2000).

Due to the fact that most of redox enzymes do not exchange directly electrons with electrodes, a wide range of chemical compounds have been described are used as mediators, which allow enzymes to transfer electrons to electrodes (Fultz and Durst, 1982 and Johnson *et al.*, 1983). The requirements for the mediators are: suitable solubility in aqueous or organic solvents, well-known and reversible electrochemical behaviour, chemical stability in both oxidised and reduced forms, appropriate redox potential allowing to work at potentials lower than the oxidation potentials of interfering substances, absence of direct reaction with the enzyme substrates or other substances, and insensitivity to pH and ionic strength effects of the media. Ferrocene derivatives have been extensively used as electron donors/acceptors for redox enzymes, due to their well-known, reversible and pH-independent redox behaviour. Apart from these characteristics, it is possible to introduce active groups on either or both of the cyclopentadienyl rings while retaining the redox properties. These redox couples have been shown to have high electron transfer rate constants for their reaction with oxidoreductases (Cass *et al.*, 1984, 1985; Green and Hill, 1986; Jönsson and Gorton, 1989; and Liaudet *et al.*, 1990). Zakeeruddin *et al.* (1992) and Fraser *et al.* (1993) synthesised Rh and Os complexes, and demonstrated that they can act as electron transfer acceptors for these enzymes. And not only did they demonstrate their electrocatalytic capacity, but also studied the mediator global charge optimisation for glucose oxidase mediation, observed the role of redox potential, pH and ionic strength in mediation, and incorporated the synthesised mediators in enzyme electrodes in order to compare the current intensities. Apart from metal complexes, some other molecules have been used as electron acceptors/donors. Kulys *et al.* (1994) studied the kinetics between glucose oxidase and phenothiazines, phenoxazines and Wurster's salts, and their dependence on the redox potential. Scheller *et al.* (1989) used benzoquinone to study the effect of the capacity of transferring electrons and protons on the electron transfer rate constant, since the relation between single- and double-electron transfer is of great interest in the chemistry of flavin oxidases. The same objective was pursued by Kulys and

Čénas (1983 and 1988), who used a series of quinonic acceptors and studied the effect of the working pH and of the redox potential and global charge of the mediator. Coury *et al.* (1990, 1991 and 1993) applied cyclic voltammetry to sulphite oxidase reactions and studied the kinetics of reactions between sulphite oxidase and several kinds of electron transfer mediators, like Rh and Co complexes, cytochrome *c* and different dyes. Apart from studying the influence of the mediator redox potential, the global charge and the ionic strength on the electrocatalytic voltammetry, they investigated the effect of sulphite stabilisers in solution and they used chronoamperometry for quantification of sulphite concentration and determination of both sensitivity and dynamic range. With these studies, they elucidated the mechanism of the catalytic cycle of the enzyme and they determined the apparent self-exchange rate constants of the enzyme heme site, which contributed to rationally design new biosensors.

In electrochemical sensors, one of the most important requirements for a mediator is to present a high electron transfer rate constant (k_{ET}) with the enzyme in order to obtain high currents. One of the main problems in DNA electrochemical sensors is the limit of detection of the system. On one hand, the amount of hybridised DNA that has to be detected is usually in the femtomolar or attomolar range. On the other hand, the sensitivity of the system has a crucial importance, for example, in the detection of 1 mismatch in a complementary sequence. The need for a low limit of detection and a high sensitivity is solved when high currents are obtained, and this requirement explains the increasing interest in electrochemical signal amplification methods. In these schemes, the optimisation of the homogeneous electron transfer rate constant between the label (enzyme) and the transducer (electrode) by the choice of the appropriate mediator, is the first step to consider to obtain high current densities, which may lower the limit of detection several orders of magnitude. That is why the study of electron transfer kinetics is important for the optimisation of amperometric mediated biosensors (Frew and Hill, 1988; Pishko *et al.*, 1990a, b; Katakis and Heller, 1994; Csöregi *et al.*, 1994, 1995; Ye *et al.*, 1994; Taylor *et al.*, 1995; Narváez *et al.*, 1996, 1997; Ruzgas *et al.*, 1996; Kenausis *et al.*, 1996; Hedenmo *et al.*, 1996, 1997; and Popescu *et al.*, 1999). Apart from the direct improvement of the limit of detection and the sensitivity, other advantages from the fast electron exchange between enzyme and mediator and the consequent high currents are, as it is demonstrated elsewhere, (Katakis and Heller, 1992 and Katakis, 1994) the lower competition from O_2 (for oxidase-based electrodes) and from electrooxidisable interfering substances, such as ascorbate (vitamin C), urate and acetaminophen (Johnson *et al.*, 1983). Moreover, high current densities make possible the miniaturisation of the sensors, a necessary condition for DNA arrays. The miniaturisation of the hybridisation detection device, together with the miniaturisation of the sample volume, pre-treatment and amplification systems, allow the integration of the processes in microfluidic devices.

Accessibility is an important factor for electron transfer. It includes steric effects, orientation and distance dependence. For an electron transfer reaction to occur, the reactants must approach each

other to facilitate electron coupling. Apart from accessibility, other factors that influence the electron transfer are the redox potential and the electrostatic interactions, and the pH and the ionic strength of the media. Marcus' semiclassical theory (1965) describes the biological electron transfers and postulates an expression that includes the parameters that influence the electron transfer rate constant:

$$k_{ET} = 10^{13} \exp(-\beta (r - r_0)) \exp - \left(\frac{(\Delta G^0 + \lambda)^2}{4RT\lambda} \right) \qquad \text{(Eq. V.1)}$$

where β is the distance decay constant in $Å^{-1}$, r is the distance between donor and acceptor in Å, r_0 is the value of r at which the frequency of motion of the nuclei equals $10^{13}s^{-1}$ in Å, ΔG^0 is the free energy of the reaction, and λ is the Marcus reorganisation energy, both energies in eV. This equation describes a decay of the electron transfer constant with distance. However, some deviations from this dependence have been observed. It has been demonstrated experimentally that the deviations could be due to the existence of electronic-coupling "pathways", through polypeptide sigma bonds and hydrogen bonds present inside the protein, where an electron tunnelling effect is produced. The existence of these "pathways" in proteins like cytochrome *c* and myoglobin was experimentally verified using semi-synthesised proteins with incorporated redox complexes (Mayo *et al.*, 1986; and Wuttke *et al.*, 1992; and Casimiro *et al.*, 1993). Moreover, Beratan and Onuchic (1987, 1989, 1991a and 1992) described a theoretical model for the electron transfer rate constant that included these electronic "pathways" and their model was successfully applied to the study of the electron transfer between proteins and metal complexes (Cowan *et al.*, 1988; Beratan *et al.*, 1990, 1991b; Jacobs *et al.*, 1991; Onuchic *et al.*, 1992a, b; and Ullmann and Kostić, 1995).

The thermodynamic driving force depends on the redox potential of the two partners of the reaction, which has an influence on the kinetics of electron transfer. It has been mentioned above than one of the requirements for a mediator in an electrochemical biosensor is to have a low redox potential in order to work at potentials that decrease the oxidation currents from interfering compounds. But on the contrary, a high thermodynamic driving force, which results in a high electron transfer rate, is desired. Consequently, it is necessary to compromise between low interfering currents and high rates. The electrostatic interactions also intervene on the electron transfer, favouring or disfavouring the approach and orientation of the reactants. The pH and the ionic strength of the media can change the role or the importance of these interactions, increasing or decreasing the electron transfer rate constants.

This work examines the effect of the global charge and the redox potential of several osmium mediators, the ionic strength and the pH of the media on the electron transfer rate constant between glucose oxidase and these mediators. Rational design of the mediator by the choice of

their ligands allowed the synthesis of two series of osmium complexes. The first series differs only on the mediator global charge, from 0 to +5, as the mediators have similar sizes and redox potentials. A second series includes mediators with the same size and global charge, differing only on the redox potential, varying from 175 to 650mV *vs.* Ag/AgCl. These studies demonstrate how this data can be used for the elaboration of enzymatic mechanisms and for rationally designing amplification schemes for affinity sensors.

Materials and methods

Materials. The enzyme glucose oxidase (GOx) from *Aspergillus Niger* (EC 1.1.3.4.) was obtained from Sigma (G-7141). Enzymatic activity was monitored periodically by spectrophotometric assay at pH 7.0. Enzyme solutions were made by dissolving weighed amount of enzyme in standard buffer solution of 0.05M tris[hydroxymethyl]amino methane hydrochloride (Sigma, T-3253) containing 0.2M NaCl (Panreac, 131659) and titrating to pH 7.0 using NaOH (Panreac, 131687). Enzyme concentrations were calculated by spectrophotometry. Glucose solutions were made by preparing a 0.25M solution of anhydrous D-(+)-glucose (Panreac, 131341) in the standard buffer. This solution was always left to mutarotate overnight.

K_2OsCl_6 was obtained from Alfa (Stk. 12177). 2,2'-Bipyridyl, pyridine-3,5-dicarboxylic acid and isonicotinic acid were purchased from Fluka (14454, 82794, and 58930). 4,4'-Dimethyl-2,2'-bipyridyl, 2,2'-bipyridine-4,4'-dicarboxylic acid, pyridine, 3-pyridinepropanol, 4-(aminomethyl)pyridine and 1-(3-aminopropyl)imidazole were obtained from Aldrich (24,573-9; 28,281-2; 36,057-0; P7,120-7; A6,560-3; and 27,226-4). Sodium dithionite was obtained from Panreac (211685). Mediator solutions were made dissolving weighed amount of mediator in Milli-Q water.

Instrumentation. Spectrophotometry was conducted with a Hewlett Packard HP-8452A photodiode-array spectrometer interfaced to a PC. Cyclic voltammetry experiments were performed using an AUTOLAB PGSTAT10 potentiostat connected with a single-compartment and three-electrode electrochemical cell with a glassy carbon working electrode, Ag/AgCl reference electrode and Pt counter electrode. Before each experiment the surface of the working electrode was polished with alumina of 5, 1 and 0.3μm (Buehler, 40-6351-006, 40-6361-006 and 40-6363-006), rinsed with water and sonicated in Milli-Q water. Working electrode areas (3.08 ± 0.02 and $4.46 \pm 0.10 mm^2$) were determined by chronoamperometry experiments from background-subtracted Cottrell plots.

Synthesis of mediators. Figure V.1 shows the chemical structure of the osmium mediators, which were characterised electrochemically.

$Os(bpy)_2Cl_2$[1], $Os(dmebpy)_2Cl_2$[2] and $Os(dcarbpy)_2Cl_2$. These complexes were synthesised by refluxing K_2OsCl_6 with 2,2'-bipyridyl (bpy), 4,4'-dimethyl-2,2'-bipyridyl (dmebpy) or 2,2'-bipyridine-

4,4'-dicarboxylic acid (dcarbpy) (complex : ligand, 1 : 2) for 1h in DMF. Then, the precipitated KCl was filtered and the product was reduced by sodium dithionite.

$[Os(bpy)_2ClL]Cl$ and $[Os(dmebpy)_2ClL]Cl$[3] (L = pyridine, pyridine-3,5-dicarboxylic acid, isonicotinic acid, 3-pyridinepropanol, 4-(aminomethyl)pyridine or 1-(3-aminopropyl)imidazole). They were prepared by refluxing [1] or [2] with the corresponding ligand (complex:ligand, 1:1) for 30min in ethylene glycol. To introduce an imidazole derivative, L = 1-(3-aminopropyl)imidazole (complex : ligand, 1 : 5), the mixture was heated at 120˚C for 1h. The product was isolated as PF_6^- salt and ion-exchanged with Cl^- for use.

$[Os(dmebpy)_2LL']Cl_2$ (L = pyridine or 4-(aminomethyl)pyridine and L' = 1-(3-aminopropyl) imidazole). [3] was refluxed the ligand L' (complex : ligand, 1 : 6) for 2h in ethylene glycol. The product was isolated as PF_6^- salt and ion-exchanged with Cl^-.

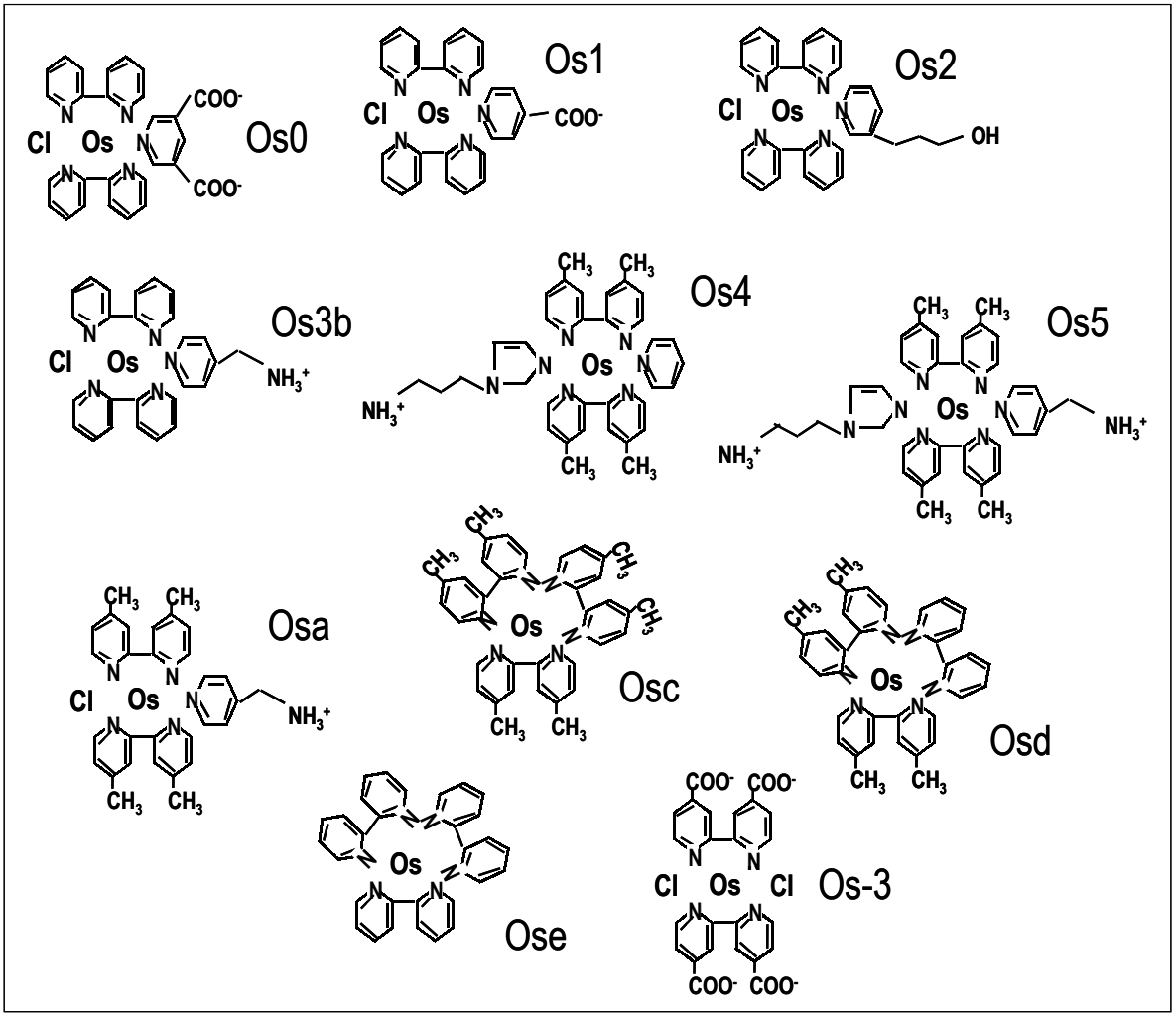


Figure V.1. Chemical structure of the osmium mediators. Os0 = $Os(bpy)_2Cl(py2COO^-)$; Os1 = $Os(bpy)_2Cl(pyCOO^-)$; Os2 = $Os(bpy)_2Cl(pyOH)$; Os3b = $Os(bpy)_2Cl(pyNH_3^+)$; Os4 = $Os(dmebpy)_2(py)(imNH_3^+)$; Os5 = $Os(dmebpy)_2(pyNH_3^+)(imNH_3^+)$; Osa = $Os(dmebpy)_2Cl(pyNH_3^+)$; Osc = $Os(dmebpy)_3$; Osd = $Os(dmebpy)_2(bpy)$; Ose = $Os(bpy)_3$; Os-3 = $Os(dcarbpy)_2Cl_2$.

$[Os(bpy)_2(L\text{-}L)]Cl_2$ and $[Os(dmebpy)_2(L\text{-}L)]Cl_2$ (L-L = 2,2'-bipyridyl, 4,4'-dimethyl-2,2'-bipyridyl or 1,10-phenanthroline-5,6-dione). Synthesis were performed by refluxing [1] or [2] with the corresponding ligand (complex : ligand, 1 : 3) for 3h in ethylene glycol. The product was isolated as PF_6^- salt and ion-exchanged with Cl^-.

Determination of electron transfer rate constants. Electron transfer rate constants were derived from steady-state cyclic voltammetry, which was conducted in a 400µL standard 3-electrode cell with 0.05M tris-HCl buffer solution, 0.2M NaCl and pH 7.0 unless otherwise mentioned. The buffer was Ar-saturated, containing 0.25M glucose, at 25˚C. Initially, cyclic voltammograms of the corresponding mediator were obtained (from 0.05 to 1mM final concentration). Upon addition of increasing concentrations of glucose oxidase (from 0.015 to 22.371µM final concentration), electrocatalytic voltammograms were recorded. For each mediator concentration, the GOx concentration range was adjusted in order to obtain an electrocatalytic plateau for each enzyme addition and to accurately determine the rate constant. Care was taken to avoid bubbling of Ar through solutions when cyclic voltammograms were recorded. Experiments were performed at scan rates of 2, 10 and 20mV s^{-1} in unstirred solutions. Measurements for rate constant determination were taken at 2mV s^{-1}.

Results and discussion

Mediators characterisation. Mediator redox potentials obtained from cyclic voltammetry are summarised at Table V.1. Mediator global charges in their oxidised form are also shown.

Table V.1. Global charges and mediator redox potentials (E_{redox} in mV *vs.* Ag/AgCl) for osmium complexes obtained by cyclic voltammetry.

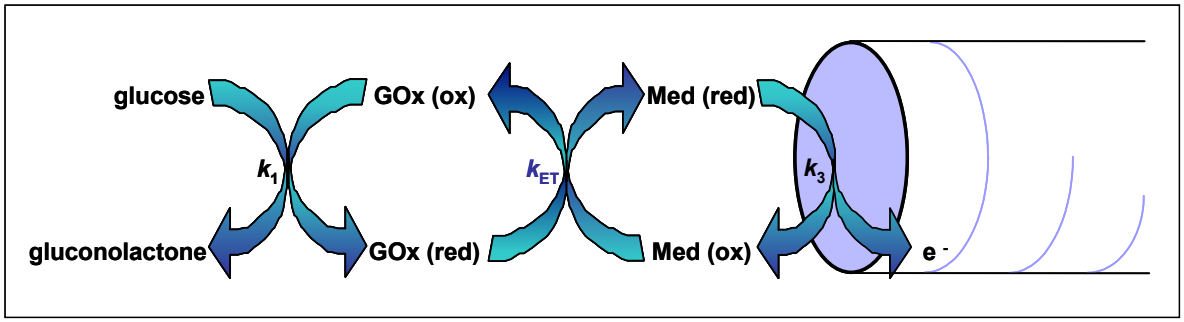
	Os0	*Os1*	*Os2*	*Os3b*	*Os4*	*Os5*	*Osa*	*Osc*	*Osd*	*Ose*	*Os-3*
Z_{med}	0	+1	+2	+3	+4	+5	+3	+3	+3	+3	-3
E_{redox}	286	283	280	272	280	280	178	463	570	647	120

* Os0 = $Os(bpy)_2Cl(py2COO^-)$; Os1 = $Os(bpy)_2Cl(pyCOO^-)$; Os2 = $Os(bpy)_2Cl(pyOH)$; Os3b = $Os(bpy)_2Cl(pyNH_3^+)$; Os4 = $Os(dmebpy)_2(py)(imNH_3^+)$; Os5 = $Os(dmebpy)_2(pyNH_3^+)(imNH_3^+)$; Osa = $Os(dmebpy)_2Cl(pyNH_3^+)$; Osc = $Os(dmebpy)_3$; Osd = $Os(dmebpy)_2(bpy)$; Ose = $Os(bpy)_3$; Os-3 = $Os(dcarbpy)_2Cl_2$.

Mediators Os0, Os1, Os2, Os3, Os4 and Os 5 belong to the same series. They have similar size (approximately 12Å in diameter) and similar redox potential, about 280mV *vs.* Ag/AgCl, but they differ on the global charge, from 0 to + 5. This series was useful for the evaluation of the effect of the global charge on the kinetics of electron transfer between mediator and enzyme. These mediators were also used in the study of the effect of the ionic strength of the media. Additional results were obtained with the $Os(dcarbpy)_2Cl_2$ mediator (Os-3), which has negative global charge (-3) and more negative redox potential (120mV *vs.* Ag/AgCl). Mediators Osa, Osb (Os3), Osc, Osd and Ose belong to the other series. They have similar size and the same global charge (+3), and

differ on their redox potential, varying from 175 to 650mV *vs.* Ag/AgCl. With this series it was possible to study the effect of the mediator redox potential and the pH of the media on the electron transfer rate constant.

Determination of the electron transfer rate constants. The principle of the enzymatic catalysis is shown at Scheme V.1. GOx catalyses the oxidation of ß-D-glucose to glucono-D-lactone (FAD and $FADH_2$ are the oxidised and reduced form of the flavin adenine dinucleotide group of GOX). After this reaction, the reduced GOx is regenerated (oxidised) by the redox mediator. Then, the reduced mediator transfers its electrons to the electrode, which is held at an oxidising potential, and it is regenerated (oxidised). Since the glucose/gluconolactone and $FADH_2$/FAD couples are $2e^- + 2H^+$ systems, 2 equivalents of osmium complex are involved in the mediation.



Scheme V.1. Electrocatalytic reaction mechanism between GOx and a redox mediator.

The homogeneous electron transfer rate constant corresponding to the reaction between a mediator and an enzyme in solution can be determined by cyclic voltammetry according to Nicholson and Shain theory (1964). In order to apply this theory to the determination of the electron transfer rate constant between GOx and an osmium complex, two criteria have to be fulfilled: the electrode reaction of the mediator must be fast compared to the rate between the mediator and the enzyme, and saturated glucose concentration is required to ensure a pool of reduced enzyme. If these hypotheses are fulfilled, the limiting step of the overall mechanism will be the reaction between the reduced glucose oxidase and the oxidised mediator, which will facilitate the calculation of the electron transfer rate constant between these two partners.

Figure V.2 illustrates the systematic increase of electrocatalytic currents for the Ose mediator as the concentration of GOx increases. In the absence of GOx, the only species in solution are glucose and mediator. In this case, cyclic voltammetry shows a reversible wave with an oxidation and a reduction peak, corresponding to the oxidation and reduction of the mediator, since glucose is not electroactive in this redox potential window. The anodic peak current is proportional to the square root of the scan rate, indicating a diffusional system. Addition of GOx increases the oxidation peak intensity and decreases the reduction peak intensity. When the reaction between the reduced enzyme and the oxidised mediator is so fast than the diffusion of the reduced mediator from the

bulk to the electrode is limiting, the oxidation catalytic intensity reaches a plateau, the reduction disappears completely and the cyclic voltammogram acquires a sigmoidal shape. Since the diffusion is limiting, the plateau intensity does not depend on the scan rate (at time scales where the assumptions are fulfilled). Experiments must be performed under anaerobic conditions to avoid the competitive reaction of O_2 for the reduced GOx.

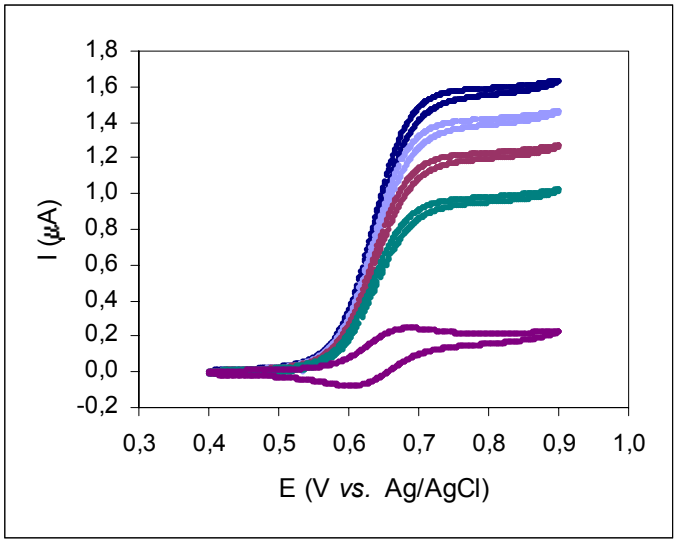


Figure V.2. Cyclic voltammetry for the Ose mediator at 2mV s^{-1} in 400µL of a 0.05M tris-HCl buffer solution, 0.2M NaCl, pH 7.0, Ar-saturated, containing 0.25M glucose, at 25°C. Initially, the cyclic voltammogram of 0.25mM Ose was obtained (no GOx). Upon addition of increasing concentrations of GOx (0.15, 0.22, 0.29 and 0.36µM final concentrations), electrocatalytic voltammograms were recorded.

The theoretical treatment of Nicholson and Shain (1964) for the reactions of the type EC_{cat}:

$$Z + O \rightarrow R$$

$$R \leftrightarrow O + ne^-$$

where O and R are the respective oxidised and reduced forms of the mediator and Z is the reduced enzyme, provides an equation for the steady-state current, i_{SS}, which is an expression derived from the Savéant and Vianello equation (1965):

$$i_{SS} = nFAC_{med}^*(D_{med}k_{ET}C_{enz}^*)^{1/2} \qquad \text{(Eq. V.2)}$$

where i_{SS} is the steady-state current, A is the electrode area, C_{med}^* is the mediator concentration, D_{med} is the mediator diffusion coefficient, k_{ET} is the electron transfer rate constant, and C_{enz}^* is the enzyme concentration. This equation predicts a scan rate-independent steady-state intensity and a dependence of this intensity on the square root of the concentration of the enzyme. Plots of i_{SS} *vs.* $C_{enz}^{*1/2}$ for constant C_{med}^* are linear and according to this equation will provide the k_{ET} values.

The linear dependence predicted by Equation V.2 is confirmed by experimental data (*results not shown*). Slight negative deviations from linearity at high GOx concentrations suggest that at fast

enzyme turnover, the electrocatalytic reaction sequence is not limited by the reaction between mediator and enzyme. Possible reasons could be accumulation of systematic errors, loss of mediator, adsorption of mediator on the walls of the electrochemical cell, or poisoning and blocking of the electrode surface, due to an accumulation of GOx. Another possibility, perhaps more probable, could be that a fast consumption of oxidised mediator breaks down the assumptions. In order to determine the electron transfer rate constants, values that disturb the linearity were neglected, assuming linear regressions with $r^2 = 0.99$ as acceptable results. Such plots were used to calculate the rate constants in what follows.

Effect of the mediator global charge on the electron transfer rate constant. As previously mentioned, the effect of the mediator global charge on the rate constant of electron transfer was studied with mediators Os0, Os1, Os2, Os3, Os4 and Os5, which have similar size (approximately 12Å in diameter) and redox potential (about 280mV *vs.* Ag/AgCl), and differ on the global charge, from 0 to +5. Results for electron transfer rate constants are shown in Figure V.3. Experimental data shows an exponential increase on the electron transfer rate constant from 0.66×10^5 to $6.67 \times 10^5 M^{-1}s^{-1}$ that can be explained as the result of the electrostatic attraction, since GOx is negatively charged at pH 7.0 (pI = 4.2).

Apart from the global charge, the local charges can also have an important role in the kinetics of electron transfer. In fact, observations of electron transfer mechanisms show that the global charges dominate the electrostatic interactions at large distances, but the local charges become more important as the distance decreases. Moreover, theory postulates that a precursor complex between the two reactant species might be formed, and that the electron transfer rate constant may increase proportionally to that complexation constant. GOx contains many groups, such as amine, imidazole or sulfhydryl groups, which can interact with oxidised mediators and form this precursor complex. Although both cationic and anionic groups are located on the GOx surface, as mentioned above at pH 7.0 the enzyme has an essentially negative electrostatic surface potential, because of excess of glutamate and aspartate over lysine and arginine. This fact favours the idea of the electrostatic complexation between mediators with positively charged groups and anionic sites of the enzyme. Experimental results also support this hypothesis, since the observed trend for the electron transfer rate constant between GOx and Os mediators is Os5 (with two amine groups) > Os 4 (with one amine group and global charge +4) > Os 3 (with one amine group and global charge +3) > Os 2 (with one hydroxyl group) > Os 1 (with one carboxylic group) > Os 0 (with two carboxylic groups). In fact, it has been reported that the formation of this complex seems to be more favourable with mediators having amino or hydroxyl groups than with mediators having carboxylic groups (Bourdillon *et al.*, 1993; and Fraser *et al.*, 1993).

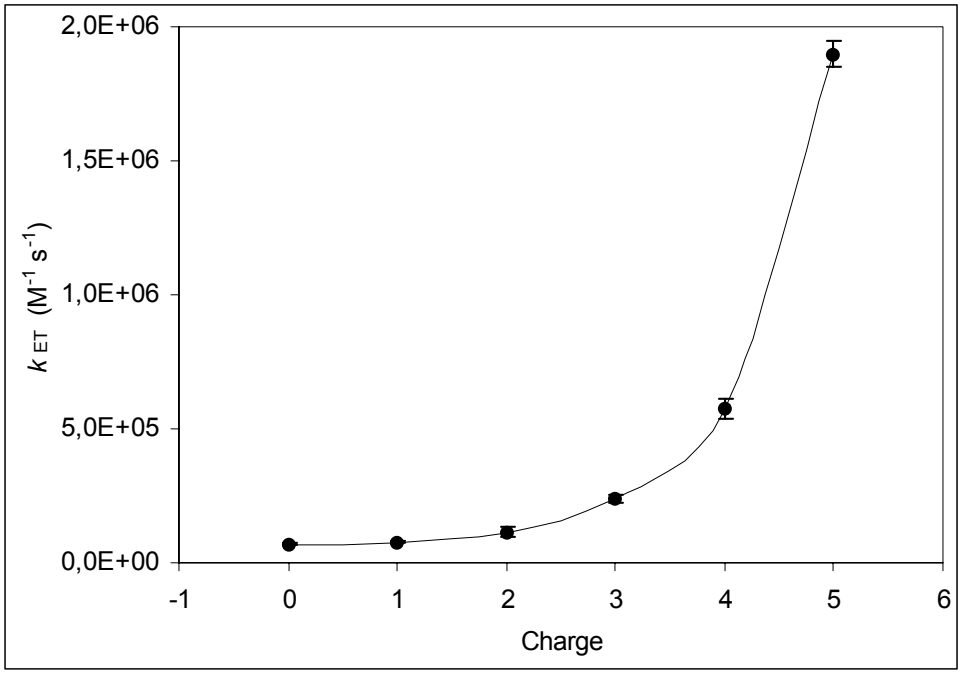


Figure V.3. Variation of the electron transfer rate constant (k_{ET}) with the mediator global charge. Electron transfer rate constants were derived from steady-state cyclic voltammetry at 2mV s^{-1} in 400μL of a 0.05M tris-HCl buffer solution, 0.2M NaCl, pH 7.0, Ar-saturated, containing 0.25M glucose, at 25°C. Initially, cyclic voltammograms of the corresponding mediator were obtained. Upon addition of increasing concentrations of GOx, electrocatalytic voltammograms were recorded. Plots of i_{SS} *vs.* $C_{enz}{}^{*1/2}$ for constant $C_{med}{}^{*}$ provided the k_{ET} values according to Equation V.2.

Although it has been observed that the funnel where the active centre of GOx is located is quite inaccessible, the effect of the residues of the active centre on the electron transfer must be considered. Takabe *et al.* (1986) (although not with GOx but with cytochrome) studied the influence of the positive local charges. Their work showed that residues next to prosthetic group play a more important role on the electrostatic interactions than other residues and global charge. Consequently, if the electronic contacts between mediators and enzymes mentioned above could occur close to the active site, the kinetics would be even more favoured.

Another possible explanation for the mechanism of mediation takes into account the conducting "pathways" in the enzyme. In general terms, the GOx shell is insulating in order to inhibit the indiscriminate electron transfer with other biomolecules present in physiological conditions. However, the shell can be crossed by electronic-coupling "pathways", through which the electron transfer is possible. According to Wuttke *et al.* (1992) and Casimiro *et al.* (1993), the effective electron tunnelling distance can be reduced not only through physical contact between the redox partners, but also through these electron conducting "pathways", whose termini are on the enzyme surface. In this way, the observed trend could be due to the preferential approach, or even the complexation, of the mediator to negatively charged groups of residues located at the enzyme surface, which are the terminal extremes of electron conducting "pathways" that cross the insulating enzyme. This explanation is highly likely because the channel to the active centre of glucose

oxidase is not very accessible to the solvent and probably even less to the mediator. In fact, from the tertiary structure of glucose oxidase, it is known that the FAD centre is located at 13Å from the surface, at the bottom of a funnel that has a cross section of 10 x 10Å at the top (Hecht *et al.*, 1993). Consequently, access to the flavin site seems rather difficult for the osmium mediators, as their diameter is approximately 12Å. It can be therefore speculated that a surface local charge close to such a conducting "pathway" explains the behaviour observed in Figure V.3.

Effect of the pH on the electron transfer rate constant. Several authors have studied the reaction mechanism between oxidoreductases and the natural acceptor O_2, and have predicted the formation of a complex between the active centre and the dioxygen (Bright and Appleby, 1969; Yamasaki and Yamano, 1973; Choong and Massey, 1980; Massey and Hemmerich, 1980; Voet and Andersen, 1984; Ghisla and Massey, 1986; Schreuder *et al.*, 1990; Hecht *et al.*, 1993; and Meyer *et al.*, 1998). All the models converge in the importance of a His in the electron transfer reaction between GOx and O_2. But unlike O_2, the entrance of the osmium complex through the funnel towards the active centre seems unlikely, which rules out the hypothesis of a precursor complex with FAD. Nevertheless, Bourdillon *et al.* (1993) suggested the formation of a precursor complex between a ferrocene mediator and the GOx enzyme site, the energetics or kinetics of which may influence the kinetics of electron transfer, and proposed a His residue or a thiol group that are in the close vicinity of the flavin as responsible for the enzyme binding site to the ferrocene mediator. To obtain more information about the electron transfer mechanism between GOx and osmium complexes, the effect of pH on the rate constant was studied.

GOx shows a broad activity plateau between pH 4.0 and pH 7.0, presenting its maximum activity at pH 5.5 when the acceptor is dioxygen. By contrast and like results reported by other authors (Nakamura *et al.*, 1976; and Zakeeruddin *et al.*, 1992), kinetic experiments with synthetic mediators show an increase of the overall rate constant k_{ET} with pH, presenting the highest electron transfer rate constants at pH 8.0, and a sharp drop as the medium becomes acidic to give only 4% of the response at pH 4.0. This distinct dependence indicates that the factors that influence the electron transfer with redox mediators are different to those that influence the reaction with dioxygen. In Figure V.4, normalised results of the variation of the electron transfer rate constant with the pH for the Osa and Ose mediators are plotted and for qualitative comparison, the pH activity profile when O_2 is the acceptor. The electron transfer rate constants with Ose were at least 25-fold higher than those with Osa (18.10 x 10^5 $M^{-1}s^{-1}$ for Ose and 0.74 x 10^5 $M^{-1}s^{-1}$ for Osa, at pH 7.0), due to the higher difference in redox potential, as expected from Equation V.2. However, the observed pH trend was the same for both mediators. The reaction between glucose oxidase and mediator is a multistep reaction, where two electrons and two protons are exchanged between the reduced form of the enzyme ($FADH_2$) and two molecules of oxidised mediator and the buffer. The observed trend could be explained in different terms. Regarding the redox potential, when pH increases, the redox potential value of the osmium mediator does not change, but the redox potential of the $FADH_2$

becomes more negative (Stankovich *et al.*, 1978). This implies that at higher pH, the thermodynamic driving force is larger, which favours the electron transfer kinetics. Additionally, at pH above the pI, glucose oxidase has a global negative charge, which favours the attraction with the positively charged mediators. Finally, the sharp jump around pH 7.0 could be related to the pK_a for the deprotonation of the neutral to the anionic semiquinone (pK_a = 7.3), the FAD$^{\bullet}$ being more reactive towards the osmium mediator than the FADH and, consequently, accelerating the electron transfer. This is so because above pH 7.3, it is not necessary to transfer a proton from the reduced form of FAD, a proton that in any case the mediator is not able to abstract. The $Os(dmebpy)_2(phen)$ mediator was used to test the effect that a proton-abstracting mediator of similar characteristics would have on electron transfer. No improvement of the electron transfer rate constant was observed. Therefore, it seems certain that direct or close contact between FAD and mediator can be ruled out. So, one possible explanation for the observed pH behaviour is the effect of the driving force on the electron transfer, although the abrupt change, even if it coincides with the semiquinone pK_a, it cannot be explained.

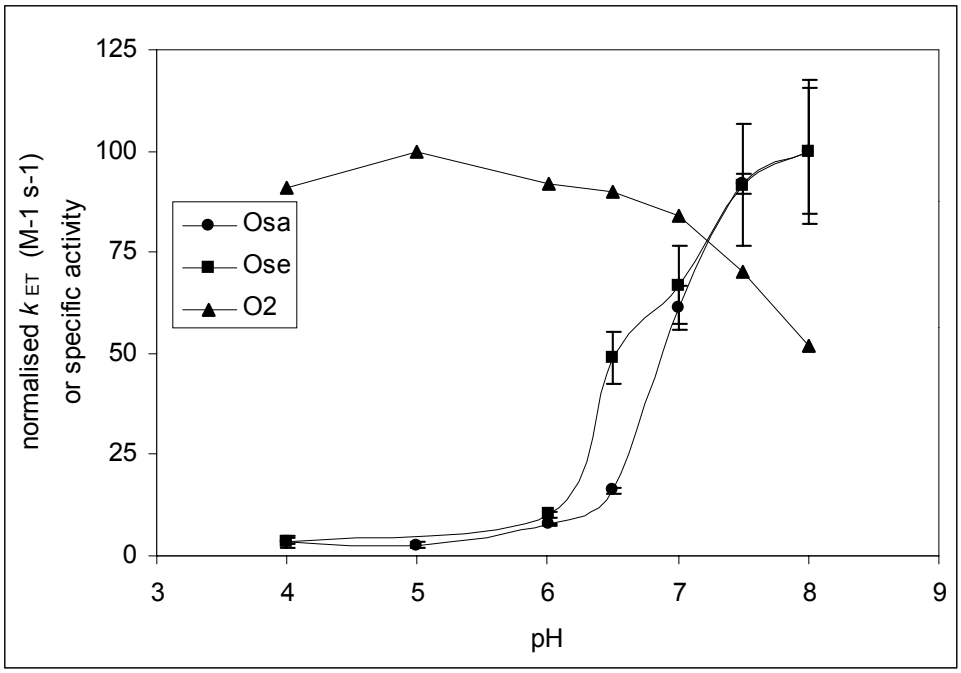


Figure V.4. Variation of the normalised electron transfer rate constant (k_{ET}) and specific activity with the pH for the Osa and Ose mediators and O_2. Electron transfer rate constants were derived from steady-state cyclic voltammetry at 2mV s^{-1} in 400µL of a 0.05M tris-HCl buffer solution, 0.2M NaCl, at different pH values, Ar-saturated, containing 0.25M glucose, at 25˚C. Initially, cyclic voltammograms of the corresponding mediator were obtained. Upon addition of increasing concentrations of GOx, electrocatalytic voltammograms were recorded. Plots of i_{SS} *vs.* $C_{enz}{}^{*1/2}$ for constant $C_{med}{}^{*}$ provided the k_{ET} values according to Equation V.2. For comparison, the pH activity profile when O_2 is the acceptor is given (pH activity profile from Toyobo Co., Ltd.).

However, it is also necessary to remember the importance of the local charges of the enzyme. As mentioned above, His may intervene on the kinetics of electron transfer. There are several His on the GOx surface and at the active site participating in the catalytic mechanism, which have an imidazole group. At pH higher than ~6.8, this group is deprotonated and may favour the approach

or interaction with positive mediators. At pH lower than ~6.8, the imidazole is protonated and the electrostatic repulsion with positive mediators may contribute to decrease the electron transfer rate constant. It is very unlikely that the pK_a of the active site buried histidine is 6.8. To clarify this point, additional experiments were performed with the $Os(dcarbpy)_2Cl_2$ (Os-3) mediator. This osmium mediator is negatively charged due to the four carboxylic groups, which are deprotonated at pH 7.0, conferring a negative total charge to the complex (-3). As expected, at pH 7.0 it was not possible to observe electrocatalysis, because both GOx and the Os-3 mediator are negatively charged and mediation does not occur due to the electrostatic repulsion between them. The same kinetic experiments were performed at pH 5.0, and it was possible to detect electrocatalytic currents. Moreover, the electron transfer constant obtained in this experiment was 8.54 x $10^5 M^{-1}s^{-1}$, higher than expected taking into account that the Os-3 mediator has a redox potential of 120mV *vs.* Ag/AgCl (lower than any mediator used in this work). As at this pH, GOx is still overall negative, the observed mediation cannot be explained in terms of global electrostatic interaction. However, this high electron transfer rate constant could be explained if considering the approach or complexation of Os-3 to a local residue of GOx. This local residue could be a His of the GOx surface, which moreover could be an ending of a conducting "pathway", or of the GOx active centre but, as mentioned, the pK_a of the active site His is unlikely to be unchanged. Unlike when positively charged mediators are used, at pH lower than ~6.8, the protonated His could favour the approach (by local electrostatic interactions) or the complex formation (between the carboxylic groups and active local residues) between mediator and enzyme. Although these experiments do not demonstrate the precursor or complex formation, they do suggest the importance of the His residues in the electron transfer kinetics and most likely they prove the terminus of the electron conducting "pathways" discussed above.

Another factor that may have an important role on the electron transfer are steric effects. It has been reported that acidic pH can provoke a change in the tertiary structure of the GOx, which would facilitate the accessibility to the active centre and the penetration of the redox mediator, increasing the kinetic rate constant (Kulys and Cénas, 1983). At pH 7.0, the distance from the active site to the surface is 13Å, whilst at pH 5.0 the distance is 8Å. The groups responsible for such changes in the conformation of the enzyme could be carboxylic residues or groups of the ionic bridges that bind the two protein subunits (Akulova *et al.*, 1978). However, the general results do not follow this trend because at low pH the electron transfer rate constants are lower than at high pH. Although the effect of structure change would have been present in these experiments, the effects of the thermodynamic driving force or most likely the electrostatic interaction with a surface residue with pK_a close to 7.0 have a more important contribution to the overall rate constant.

Effect of the mediator redox potential on the electron transfer rate constant. The effect of the mediator redox potential on the electron transfer rate constant was examined using the mediators Osa, Osb, Osc, Osd and Ose, with the same global charge (+3) and approximately the same size

(around 12Å in diameter), and with redox potential varying from 175 to 650mV *vs.* Ag/AgCl. Results are plotted in Figure V.5. Experimental data showed an exponential increase on the electron transfer rate constant from 0.68 x 10^5 to 1.81 x $10^6 M^{-1}s^{-1}$.

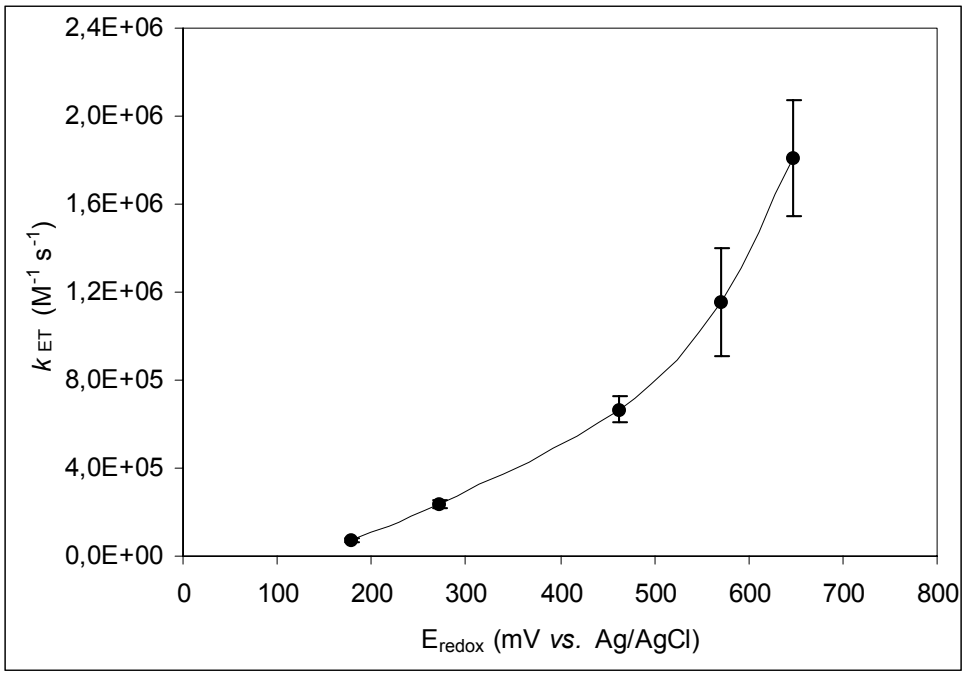


Figure V.5. Variation of the electron transfer rate constant (k_{ET}) with the mediator redox potential (E_{redox} *vs.* Ag/AgCl). Electron transfer rate constants were derived from steady-state cyclic voltammetry at 2mV s^{-1} in 400µL of a 0.05M tris-HCl buffer solution, 0.2M NaCl, at pH 7.0, Ar-saturated, containing 0.25M glucose, at 25°C. Initially, cyclic voltammograms of the corresponding mediator were obtained. Upon addition of increasing concentrations of GOx, electrocatalytic voltammograms were recorded. Plots of i_{SS} *vs.* $C_{enz}{}^{*1/2}$ for constant $C_{med}{}^*$ provided the k_{ET} values according to Equation V.2.

In Figure V.6, the logarithm of the obtained rate constants *vs.* the difference in redox potentials between mediators and the active centre of GOx is plotted. At high mediator redox potentials, the logarithm of the constant changes slowly. Nevertheless, no “inverted region” was observed, since the maximum mediator redox potential was only 650mV *vs.* Ag/AgCl. In general, the data follow the trend expected from the Marcus theory of electron transfer, which postulates that electron transfer rate constants increase with increasing thermodynamic driving force (due to the higher difference between the reversible potentials of FAD/$FADH_2$ and the electron acceptor couple). The standard redox potential of the FAD/$FADH_2$ couple in a pH 7.0 solution can be assumed to be -245mV *vs.* Ag/AgCl (Stankovich *et al.*, 1978). Consequently, the shift of the mediator redox potential in the positive direction results in an increase in the thermodynamic driving force and larger electron transfer rate constants.

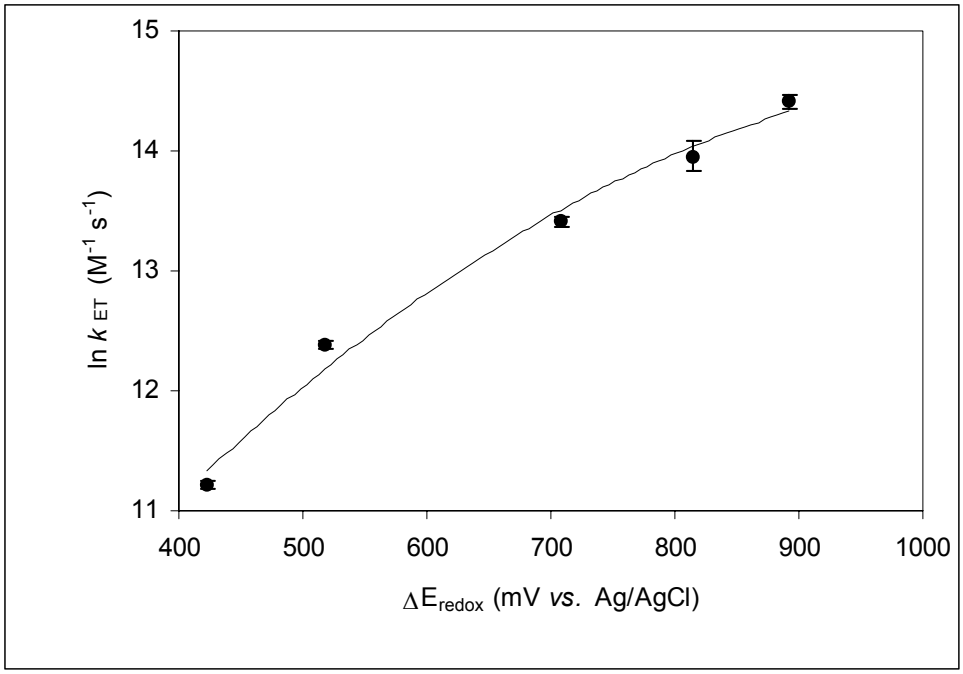


Figure V.6. Variation of the logarithm of the electron transfer rate constant (k_{ET}) with the difference in redox potentials between mediator and GOx active centre (ΔE_{redox} *vs.* Ag/AgCl).

The results obtained in Figure V.6 give an experimental equation, $y = -7\ 10^{-6}\ x^2 + 0.0153\ x + 6{,}083$. With this experimental equation and Equation V.1, it is possible to obtain $\lambda = 0.87$eV. Although the reorganisation energy varies from system to system, this value can be assumed to be the same for all the mediators used in this section because they are similar in structural terms.

With the experimental equation and Equation V.1, and assuming r_0 = 3Å (the distance at Van der Waals contact) and r = 8.9 and 30Å (distance between redox partners, obtained in next section with the effect of the ionic strength on the electron transfer between GOx and the mediators Osb and Ose, respectively), it is possible to obtain $\beta = 2.62$ and $0.57 Å^{-1}$, respectively. The exponential distance decay constant, β, describes the effectiveness of the medium (separating donor and acceptor) in coupling the fixed redox sites. $\beta = 2.62 Å^{-1}$ indicates a very weak coupling and $\beta = 0.57 Å^{-1}$ a very strong coupling, which is translated in higher rates for Ose than for Osb (*see next section*). Both values are very extreme, since in proteins it is reasonable to expect distance decay constants in the vicinity of $1 Å^{-1}$. These extreme values indicate that the electron transfer distances obtained (and used to calculate the distance decay constants) may be perturbed by experimental errors. The difference in β for the two mediators, although is due to the different assumed distances between redox partners, may also reflect a different electron transfer "pathway", i.e. a different intervening polypeptide structure. However, since the most important difference between these two mediators is their redox potential (272mV for Osb and 647mV for Ose), another possibility would be that β depends on this parameter. Apart from the reorganisation energy and the distance decay constant, the experimental equation provides a hypothetical maximum electron transfer rate constant, $k_{ET} = 1.87 \times 10^6 M^{-1} s^{-1}$, for a mediator with E_{redox} = 848mV (*vs.* Ag/AgCl).

Although all the mediators have the same global charge, one could think about the nature of these local charges. Whereas Osa and Osb have amino groups, which could be favouring the docking of the mediatior, Osc, Osd and Ose present higher electron transfer rate constant without having amino groups. The observed trend however suggests that the redox potential plays a more important role than local charges.

Effect of the ionic strength on the electron transfer rate constant. An experimental approach to estimate the magnitude of the electrostatic component of donor-acceptor interaction is the determination of the electron transfer constant as a function of the ionic strength. High ionic strength conditions disfavour the association of oppositely charged species and favour the association of like-charged molecules. This effect was observed using the mediators of both series in order to see the effect of the charge and the redox potential independently. In the ionic strength range from 0.2 to 1.0M, the enzyme glucose oxidase does not change its activity when assayed with glucose, O_2 and the *o*-dianisidine dye. This observation implies that the differences observed on the electron transfer rate constant are only due to the effect of the ionic strength on the reaction rate constant of glucose oxidase with mediator.

In Figure V.7, results for the normalised electron transfer rate constants for mediators Os0, Os2 and Os3, with similar redox potential (280mV (*vs.* Ag/AgCl)) but with different global charge, are shown.

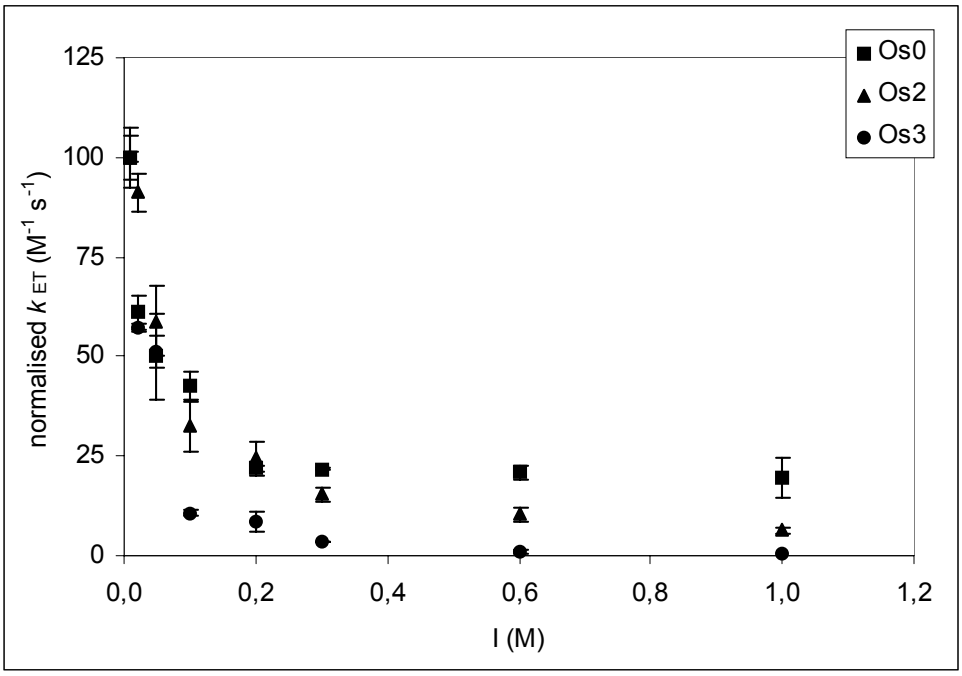


Figure V.7. Variation of the normalised electron transfer rate constant (k_{ET}) with the ionic strength (I) for the Os0, Os2 and Os3 mediators. Electron transfer rate constants were derived from steady-state cyclic voltammetry at 2mV s^{-1} in 400µL of a 0.05M tris-HCl buffer solution, different NaCl concentrations, at pH 7.0, Ar-saturated, containing 0.25M glucose, at 25°C. Initially, cyclic voltammograms of the corresponding mediator were obtained. Upon addition of increasing concentrations of GOx, electrocatalytic voltammograms were recorded. Plots of i_{SS} *vs.* $C_{enz}{}^{*1/2}$ for constant $C_{med}{}^{*}$ provided the k_{ET} values according to Equation V.2.

It can be clearly seen that the electron transfer rate constant increases as the ionic strength decreases, and this effect is much more drastic with the mediator of charge +3 (Os3) than with the mediator of charge +2 (Os2), and more important with the mediator of charge +2 (Os2) than with the neutral mediator (Os 0) at ionic strengths ≥ 0.1M (at lower ionic strengths, the error associated to the measurement does not allow one to appreciate significant differences). Comparing the results at 0.01 and 0.1M, whereas the constant for Os3 decreases 89%, for Os2 it decreases 67% and for Os3 it decreases 58%. High ionic strength produces an environment that screens glucose oxidase and mediator global charges, decreasing the electrostatic forces and slowing the kinetics of electron transfer. As expected, this masking effect is much more evident when using more positively charged mediators because the electrostatic interactions represent a higher contribution to electron transfer. In fact, there should not exist such effect for the Os0 mediator, since the global charge is 0. However, there is a trend, and the explanation would be the effect of the local charges, in this case the carboxylic groups of this mediator and the still positive charge of the central ion.

In Figure V.8, results for the electron transfer rate constant with mediators Osa, Osb and Ose, having different redox potentials but the same global charge (+3) are shown.

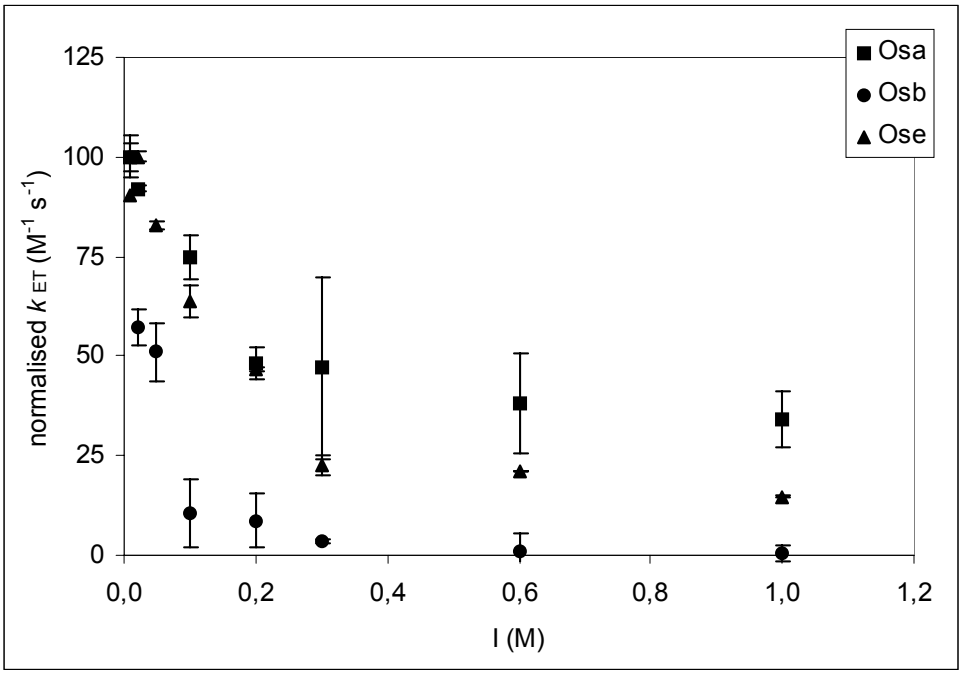


Figure V.8. Variation of the normalised electron transfer rate constant (k_{ET}) with the ionic strength (I) for the Osa, Osb and Ose mediators. Electron transfer rate constants were derived from steady-state cyclic voltammetry at 2mV s^{-1} in 400μL of a 0.05M tris-HCl buffer solution, different NaCl concentrations, at pH 7.0, Ar-saturated, containing 0.25M glucose, at 25°C. Initially, cyclic voltammograms of the corresponding mediator were obtained. Upon addition of increasing concentrations of GOx, electrocatalytic voltammograms were recorded. Plots of i_{SS} *vs.* $C_{enz}{}^{*1/2}$ for constant $C_{med}{}^{*}$ provided the k_{ET} values according to Equation V.2.

In these results, there is no direct correlation between mediator redox potential and effect of the ionic strength, as at ionic strengths ≥ 0.1M the influence of the ionic strength follows the trend Osb >

Ose ≥ Osa (as before, at lower ionic strengths, the error associated to the measurement does not allow one to appreciate the differences). Comparing the results at 0.01 and 0.1M, whereas the constant for Osa and Ose decreases 25 and 33%, respectively, for Osb it decreases 89%. The differences between Osb and Ose cannot be explained in terms of mediator global charge, but they can be explained in terms of side ligand groups in the mediators. Whereas Ose has no side groups, Osb has one amine group that could be more susceptible to ionic strength changes. This local charge phenomena may explain that the effect of ionic strength on the kinetics of Osb is much more drastic than the effect on Ose. The problem appears with Osa, which has also one amine group but it is the less influenced than Osb. The dimethyl groups of Osa make the mediator more hydrophobic, which might be decreasing the effect of screening on the rate constant or the orientation of the mediator during electron transfer.

Figures V.9 and V.10 show the variation of the logarithm of the electron transfer rate constant with the ionic strength function, f(I), defined by Wherland and Gray (1976), for Osb and Ose, respectively:

$$\ln k_{ET} - \ln k_0 = f(I) = -3.576 \left[\frac{e^{-KR_{enz}}}{1+KR_{med}} + \frac{e^{-KR_{med}}}{1+KR_{enz}} \right] \left[\frac{Z_{enz} Z_{med}}{R_{enz} + R_{med}} \right] \qquad \text{(Eq. V.3)}$$

where k_{ET} is the electron transfer rate constant, k_0 is the infinite ionic strength electron transfer rate constant, K = 0.329 $(I)^{½}$ $Å^{-1}$, R_{med} and R_{enz} are the radii of the mediator (theoretical value of 6.4 Å), and the enzyme, respectively, and Z_{med} and Z_{enz} are the charges of the mediator and the enzyme, respectively. Figure V.9 shows a linear plot for the variation of the logarithm of the electron transfer rate constant with the ionic strength function for Osb. From this regression line it is possible to obtain the infinite ionic strength rate constant when all the electrostatic contributions are screened out (f(I) = 0): k_0 = 3.9 x $10^3 M^{-1}s^{-1}$. The Figure V.9 inset shows the squared correlation coefficients for a series of plots at different R_{enz}. The statistically best value, which is obtained for the maximum r^2, gives R_{enz} = 2.5Å. This value is very low compared to the hydrodynamic enzymatic radius of 43Å (Schreuder *et al.*, 1990) or its structural determination (60 x 52 x 77Å) (Savéant and Vianello, 1965). The discrepancy may be due to the scatter of the data and the accumulated errors. It may however reflect the specific docking of the mediator to an electron conducting "pathway" as explained before. In any case, assuming the best-fit enzyme radius, it is possible to obtain Z_{enz} = -5.3. This value is lower than the global enzyme charge, -80 (Voet *et al.*, 1981), because it considers the charge given by the residues inside a sphere of radius 2.5Å, again possibly reflecting the very specific docking of this mediator. Still, this value is negative, which correlates with the expected anionic active centre of GOx. Assuming the theoretical mediator radius and the experimental enzyme radius, the distance between these two redox partners is $r = R_{enz} + R_{med}$ = 2.5 + 6.4 = 8.9Å, which reflects the distance at which the electron travels from one partner to the other. Although the best fit has provided

enzyme experimental radius R_{enz} = 2.5Å, as mentioned above experimental errors could change this value. For example, with R_{enz} = 3, 5, 7, 9 and 11Å, the R^2 value is still 0.99, and provides Z_{enz} = -5.8, -8.1, -11.2, -15.4 and -20.6, respectively. As expected, the negative charge increases as the radius increases, as more residues are being considered. This uncertainty in the distance value could explain the high value obtained for β in the previous section. Simply using R_{enz} = 11Å, β = 1.07$Å^{-1}$ would be obtained.

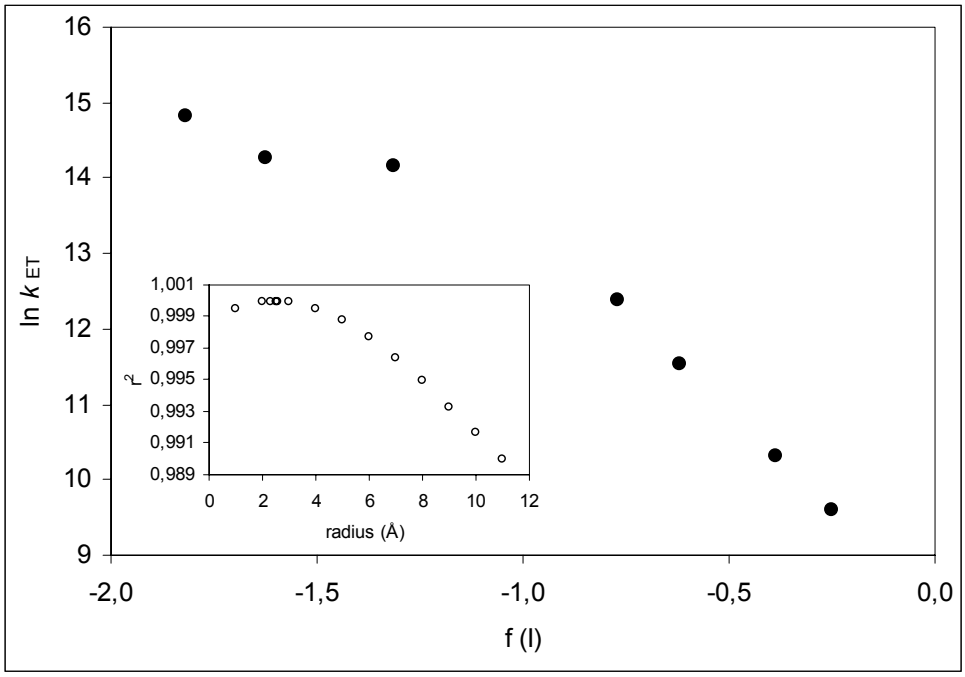


Figure V.9. Variation of the logarithm of the electron transfer rate constant (k_{ET}) with the ionic strength function (f(I)) for the Osb mediator. Inset: variation of the squared correlation coefficients for the ionic strength function with the radius of the enzyme.

On the other hand, whereas Osb produces a linear plot, Ose does not (Figure V.10). In this case, the logarithm of the electron transfer rate constant deviates from the linearity at low ionic strengths. In fact, since this mediator is the most oxidizing, the electron transfer rate constant achieves the internal electron transfer rate constant value at 0.05M, and consequently, lower ionic strength values do not increase the electron transfer rate constant in a significant way. Calculating the regression line for the four points corresponding to the highest ionic strengths, the infinite ionic strength rate constant k_0 = 5.1 x $10^5 M^{-1}s^{-1}$ has been obtained for Ose. This value is higher than the corresponding value for Osb (k_0 = 3.9 x $10^3 M^{-1}s^{-1}$) because this mediator possesses a higher internal electron transfer, due to its more positive redox potential. Moreover, from Figure V.10 inset, it is possible to obtain R_{enz} = 23.6Å and Z_{enz} = -31.9. In this case, r^2 shows less fitting to the regression line. As mentioned above, again experimental errors can be assumed or a less specific docking of the mediator might explain the results. In the same way, considering for example R_{enz} = 19, 21, 26, 28 and 30Å, where the r^2 is still 0.89, Z_{enz} = -20.6, -25.2, -38.8, -45.0 and -51.5, are obtained respectively. The statistically optimum distance between these two redox partners is *r* =

R_{enz} + R_{med} = 23.6 + 6.4 = 30Å. This distance is higher than the distance obtained for Osb (8.9Å), indicating that Ose allows the electron transfer to occur at higher distances with the same efficiency. As with Osb, simply using R_{enz} = 19Å, β = 0.90Å^{-1} would be obtained.

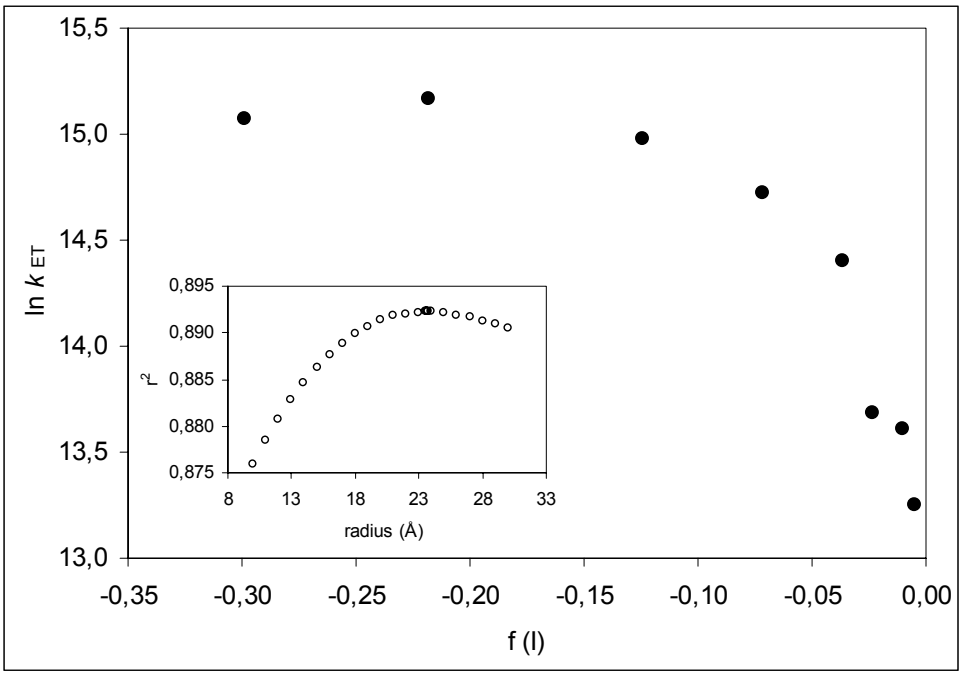


Figure V.10. Variation of the logarithm of the electron transfer rate constant (k_{ET}) with the ionic strength function (f(I)) for the Ose mediator. Inset: variation of the squared correlation coefficients for the ionic strength function with the radius of the enzyme.

Implications of findings to the design of affinity sensors. It was shown that mediators with more positive global charge result in higher electron transfer rate constants, which could lower the limit of detection of affinity sensors several orders of magnitude. When incorporating these mediators to the final configuration, an amperometric DNA sensor with GOx as label or as part of the amplifying cascade, more factors have to be considered. In principle, since hybridisation is usually carried out at pH 7.0-8.0, GOx is negatively charged and does not interact with the DNA (negatively charged due to the phosphate groups). However, the interaction between the positive charged mediators and DNA is possible. In this case, the way to incorporate the mediator in the system is crucial. For example, in a reagentless DNA sensor where the redox mediator is already attached to the electrode surface, its charge may increase the non-specific adsorption of the target DNA. If instead of having the mediator immobilised, it is incorporated to the system at the same time as the hybridisation step, the electrostatic interaction of the mediator with the probe may also increase the non-specific adsorption of the target DNA and/or decrease the hybridisation yield. In these two examples, it will be necessary to compromise between the kinetics of the mediator and its charge, trying to achieve an electron transfer rate constant as high as possible but without having non-specific electrostatic interactions. If, on the other hand, the mediator is incorporated after the

hybridisation step, there will be no interferences. However, the system will not be reagentless and will not allow real-time measurements.

Regarding the mediator redox potential, once again it is necessary to compromise. Although the higher the mediator redox potential, the higher the electron transfer rate constant and the lower the limit of detection, high mediator redox potential implies amperometric steady-state measurements at high fixed potential, which increases the interference currents from other oxidisable compounds that may be present in the real sample, such as ascorbic acid. However, since direct DNA analysis is rarely performed, samples can be cleaned from interfering substances.

Finally, using low ionic strength in the DNA “hybridisation + real-time detection” step, not only favours the kinetics of electron transfer between the enzymatic label and the redox mediator, but also increases the hybridisation stringency conditions because the electrostatic repulsion between the probe and the target is higher. Consequently, low ionic strengths decrease the limit of detection and increase the selectivity of the DNA sensor. But it is still necessary to have a certain amount of salt in the “hybridisation + real-time detection” solution to allow the base pairing to occur and to carry out electrochemical measurements.

Conclusions

Electron transfer between GOx and osmium mediators has been studied as a means to better understand the mechanisms of the redox reactions and to further use the redox system in electrochemical signal amplification schemes. Electron transfer rate constants between GOx and mediators showed an increasing trend with the positive mediator global charge. The trend can be explained in terms of global (at large distances) and local (at small distances) electrostatic interactions, or existence of redox conducting “pathways” inside the enzyme that accelerate the electron transfer kinetics. Whereas mediators contribute to the local interactions through the active groups of their ligands, the enzyme contributes through its residues, which appear to be on the surface rather than in the active site. The increase of electron transfer rate constants at high pH can be due to different effects: the higher thermodynamic driving force, the positive global charge of the enzyme and/or a more reactive deprotonated $FAD^{\bullet}$ (compared to the $FADH_2$). Nevertheless, the assumption of a His residue that contributes to the proton transfer is also possible. As when a negatively charged mediator was used, the trend was the opposite, His could be in this case favouring electrostatic attraction or complexation between GOx and the mediator. The effect of the redox potential of the mediator on the electron transfer rate constant showed a dependence according to Marcus’ theory of electron transfer, where the electron transfer rate constants increase with increasing thermodynamic driving force. This trend allows to calculate the theoretical maximum electron transfer rate constant, the reorganisation energy for a series of osmium complexes and the distance decay constants for particular osmium complexes. The study of the effect of the ionic

strength demonstrated that high ionic strength screens the charges of redox enzymes and mediators, decreasing the electron transfer rate constants. Calculations of the ionic strength function defined by Wherland and Gray allowed to obtain infinite ionic strength constants for particular mediators, the radius of the enzyme and its charge. Moreover, the non-dependence of the kinetics on the ionic strength at I < 0.05M, shown that the internal kinetic limit has been achieved, demonstrating the fast intrinsic electron transfer kinetics of the rationally designed osmium mediators.

Summarising, complexes more positively charged and complexes with higher redox potential exhibit larger electron transfer rate constants in their reaction with glucose oxidase, and even larger at low ionic strength and high pH conditions. Rationally designing the mediators for a detection system, it is possible to amplify, in a simple way, the electrochemical response from DNA amperometric biosensors with oxidoreductase labels. This rational design is an easy, promising and applicable tool to obtain high current densities from the hybridisation in the DNA arrays, where miniaturisation is pursued.

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Abbreviations

bpy: 2,2'-bipyridyl
dcarbpy: 2,2'-bipyridine-4,4'-dicarboxylic acid
dmebpy: 4,4'-dimethyl-2,2'-bipyridyl
DMF: dimethylformamide
enz: enzyme
FAD: oxidised flavin adenine dinucleotide
$FADH_2$: reduced flavin adenine dinucleotide
GOx: glucose oxidase
His: histidine
$imNH_3^+$: 1-(3-aminopropyl)imidazole
L: ligand
med: mediator
O: oxidised mediator
phen: 1,10-phenanthroline-5,6-dione
pI: isoelectric point
py: pyridine
$pyCOO^-$: isonicotinic acid
$py2COO^-$: pyridine-3,5-dicarboxylic acid
$pyNH_3^+$: 4-(aminomethyl)pyridine
pyOH: 3-pyridinepropanol
R: reduced mediator
Z: reduced enzyme