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ORIGINAL PAPER

Stable, colourless and water-soluble electron-transfer mediators used in enzyme electrochemistry

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Abstract Electron-transfer mediators are needed to transport charge between electrodes and enzymes, as enzymes hardly react on conventional electrode surfaces. Several complex ions were tested for their stability, absorbance and ability to work as electron-transfer mediators. A set of five stable mediators covering the potential range from 0 mV to +800 mV vs. SHE was established: [Fe-DCTA]$^{0/1+}$ (DCTA is 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid), [Co-terpyridine$_2$]$^{2+/3+}$, [Fe(CN)$_6$]$^{4-/3-}$, [W(CN)$_8$]$^{4-/3-}$ and [Mo(CN)$_8$]$^{4-/3-}$. These mediators are water-soluble, pH-independent and able to transfer one electron at a time. This set offers promising mediator candidates whenever indirect electrochemistry is needed, as is not restricted to any particular enzyme. It is especially useful for redox titrations and other enzyme research, where colourlessness is required. As all charge is consumed by the desired redox reactions, and not by degradation reactions, even amperometric and coulometric titrations are possible.

Keywords Colourless · Complex ion · Redox enzyme · Indirect electrochemistry · Mediator · Redox titration

1 Introduction

The electrochemically active component of most redox enzymes is encapsulated deep inside the enzyme [1, 2]. Due to this spatial isolation, enzymes hardly react on conventional electrode surfaces. The use of indirect electrochemistry helps to overcome this problem: electron-transfer mediators transport the charge between the electrode and the enzyme (Fig. 1). In most cases the mediator is a soluble molecule that diffuses freely in the electrolyte, though in sensors and biofuel cells the trend is to bind the mediator to the electrode or to the enzyme [3]. Mediators can be roughly divided into two categories: organic molecules with conjugated double bonds, and complex ions with central metal atoms.

In addition to the ability to transport electrons, mediators should preferably fulfil certain criteria: (i) Stability: If the mediator decomposes or takes part in side reactions, a true equilibrium between the enzyme and the electrode will never be achieved. Furthermore, in amperometric applications it is not possible to distinguish whether the current is consumed by the enzyme or by side reactions. (ii) Colourlessness: In enzyme research, changes in light absorption spectrum of the enzyme molecule are often measured to gain information about the reactions. In these applications it is advantageous to use colourless mediators. (iii) Solubility: The concentration of the mediator should be high enough to enable fast equilibration. (iv) pH-independence: If the equilibrium potential and the reactions of the mediator are pH-independent, its application range is wider. (v) Number of electrons: The mediator molecule should preferably carry as many electrons as the enzyme exchanges at a time, in order to reach fast equilibration and to prevent side reactions.

The charge or the shape [4] of the mediator molecule should not prevent the mediator from entering or leaving the active site of the enzyme. Further a mediator should only exchange electrons with the active site, but not interact with enzyme in any other way. These highly enzyme-specific aspects are not covered by this study.

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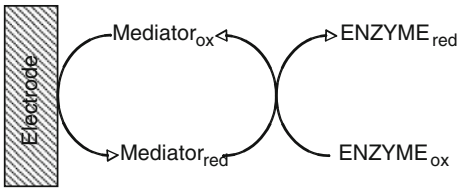

Fig. 1 Working principle of a soluble mediator. The mediator transports electrons from the electrode to the enzyme. The opposite direction is equally possible

An electron-transfer mediator brings electrodes and enzymes into equilibrium, but only near its own equilibrium potential. Therefore, the choice of a suitable mediator depends in particular on the applied potential. Lists of commonly used mediators and their equilibrium potentials have been published [2, 5–8].

Mediator stability is often ignored in biochemical research [9], probably due to lack of published stability data. For example, ferrocenes, one of the most popular groups of mediators, are unstable in their oxidised form [5, 6].

In the present study, the aim was to find highly stable mediators covering the potential range of 0–800 mV vs. SHE. Furthermore, these mediators should be colourless, water-soluble, pH-independent and able to carry one electron at a time. A literature survey [1, 6, 10] and preliminary experiments showed that most organic mediators are unstable and strongly coloured. In addition, they usually transport two electrons at a time. Therefore, in this study the selection was restricted to complex ion mediators.

2 Experimental details

2.1 Chemicals

Various complex ions were selected for screening. Some of them, such as ruthenium complexes and ferrocenes, were collected from the biochemical literature. Others, especially complexes with hexadentate ligands, were new as mediators. All chemicals were of analytical grade and purchased from Merck (Germany), if not stated otherwise. The group of ferrocene derivatives was purchased from Aldrich (Germany). The enzyme cytochrome *c* oxidase was prepared in-house from *Paracoccus denitrificans*.

The mediators were tested in buffer solution. 100 mM potassium phosphate buffer, pH 8, was prepared by dissolving K_2HPO_4 and KH_2PO_4 (both analytical grade, Merck, Germany) in deionized Milli-Q® water. This buffer was chosen on the basis of its compatibility with many enzymes, electrochemical stability and reasonable conductivity. To test the influence of pH on mediators, phosphate buffer pH 6.0 (prepared similarly) and borate buffer pH 9.5 (100 mM boric acid + KOH + 100 mM K_2SO_4) were used. The organic buffers, Tris, CHES, HEPES and Bicine were found to decompose upon oxidation at potentials positive to +100 mV, and could therefore not be used.

2.2 Screening of mediators

The complex ions were screened using a three-dimensional electrode described in [11]. The working electrode consisted of gold-plated graphite grains with a diameter of 1 mm and a total geometric surface area of 150 cm^2. The total volume of the electrode was 10 cm^3, of which 5 cm^3 was void volume between the grains. Deaerated 500 μM mediator solution was pumped in to fill this void volume, after which the pump was stopped. Trace amounts of oxygen were removed by reducing the solution for 5 min at −300 mV (all potentials vs. SHE). The electrode potential was then swept three times with a rate of 1 mV s^{-1} from −300 to +500 mV and back. Due to the large electrode surface area, all the electrochemical reactions–including side reactions—that can take place in this potential range, could be seen as current peaks. Clear, constant current peaks indicated suitability as mediator. Additional peaks indicated side reactions, decreasing peak height degradation of the complex ion. The use of this flow-by cell was practical, as after each experiment, the mediator solution could easily be pumped out and replaced with the next mediator solution to be screened. However, the large surface area restricted the maximum positive potential to +500 mV. At stronger oxidation, current due to surface oxidation started to overshadow the mediator reactions.

For all electrochemical experiments, a Princeton Applied Research potentiostat 263A (USA) was used. The experiments were carried out at room temperature, 20–25 °C.

2.3 Detailed electrochemical testing of mediators

Promising complex ions from the screening tests were further tested using an Optically Transparent Thin Layer Electrode (OTTLE) [12]. In addition, some complex ions that, according to the literature, could be active in the range +500 to +800 mV were included in these tests. The OTTLE cell (Fig. 2) consisted of a gold minigrid working electrode (6 μm thread, 300 lines per inch, 70% transmittance, Buckbee-Mears Europe GmbH, Germany), a platinum wire counter electrode and a Ag/AgCl reference electrode. The gold grid was placed between two PTFE gaskets and two quartz windows to construct a working electrode compartment with a thickness of 0.2 mm, an area of 0.8 cm^2 and a volume of 16 μL. The counter and reference electrodes were connected to this compartment by capillaries.

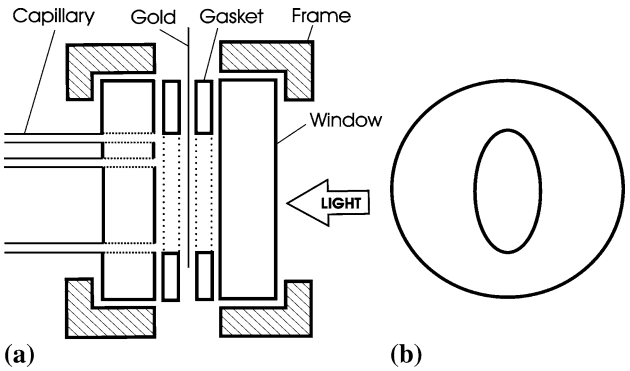

Fig. 2 (**a**) Cross-section of the OTTLE cell. (**b**) The 0.1-mm thick gasket with an elliptical aperture for the light. This aperture forms the working electrode compartment

An OTTLE cell is advantageous in bioelectrochemical research as it makes it possible to measure the absorbance spectrum of the electrolyte solution during electrochemical experiments. In addition, the maximum mass transport distance of only 0.2 mm helps to achieve equilibrium in the entire solution on a reasonable time scale.

The cell, including capillaries, was filled with a 200 μM mediator solution and placed in a vacuum-tight box made of acrylic glass. The air inside the box was exchanged with argon and the dissolved oxygen in the electrolyte was removed by reducing the solution at −300 mV. This deoxygenation cycle was repeated 2–5 times until the reduction current dropped below 20 nA. The electrode potential was then swept three times at a rate of 0.1 mV s^{-1} from −300 to +500 mV and back. For mediators of higher equilibrium potential, the potential range was correspondingly broader. Using the current–potential curves, the exact equilibrium potential and the degradation rate of the mediator were determined.

2.4 Testing of mediators together with a redox enzyme

Cytochrome *c* oxidase from *Paracoccus denitrificans* was used to test the mediators in practice in redox titrations, the method being adapted from [13, 14]. This is the final enzyme of the mitochondrial electron transport chain; it catalyses the reduction of oxygen to water. The gold minigrid of the OTTLE cell was pretreated for 12 h in a 4 mM cysteamine solution to avoid adsorption of the enzyme on gold. The cell was then filled with buffer solution, including about 150 μM of the enzyme and 2–3 mediators of 50–200 μM each. 0.02% of dodecyl maltoside was added to the buffer as detergent to keep the enzyme soluble.

After deoxygenation, the electrode potential was swept stepwise from 0 to +500 mV and back, the step size being 20 mV/20 min. After each step, the absorbance spectrum of the electrolyte was measured in the range of 400–800 nm. Using the spectrum, especially the absorbance at 445 nm, the progress of the redox reactions of the well-known enzyme could be followed.

2.5 Measurement of absorbance spectra

The spectra of the mediators, without enzyme, in the wavelength range 400–800 nm were measured in a standard 10 mm quartz cuvette, using a Cary 1c UV-visible spectrophotometer (Varian, Australia). The mediator concentration was 500–1000 μM.

To measure the enzyme spectra, the OTTLE cell and vacuum-tight box were fitted to a double wavelength spectrophotometer (designed in-house). The optical path length of 0.2 mm was optimized for cytochrome *c* oxidase; mediators influenced the absorbance less than 1%.

3 Results and discussion

3.1 Set of five mediators

Screening and testing of complex ions resulted in a set of five stable mediators, covering the range from 0 to +800 mV. The set consists of $[Fe\text{-}DCTA]^{0/1+}$ (DCTA is 1,2-diamino-cyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid), $[Co\text{-}terpyridine_2]^{2+/3+}$, $[Fe(CN)_6]^{4-/3-}$, $[W(CN)_8]^{4-/3-}$ and $[Mo(CN)_8]^{4-/3-}$. The preparation of these mediators is presented in Table 1.

The equilibrium potentials of the five mediators are shown in Table 2. At the equilibrium potential, the red/ox ratio of a mediator is 50:50, which means that the "potential buffering" capacity or mediation capacity is 50% of its total concentration. According to the Nernst equation, 60 mV apart from the equilibrium potential this capacity is 10%, and 120 mV apart 1%. The values are true for one-electron mediators. For two-electron mediators, the corresponding capacities would be 50%, 1% and 0.01%, respectively. It can be seen that the potentials are distributed evenly enough to give a reasonable mediation capacity throughout the range 0–800 mV.

The equilibrium potentials of the five mediators are practically pH-independent (Table 2). If hydrogen atoms were to take part in the redox reactions of a mediator, as is the case for quinone mediators, a pH-dependence of the equilibrium potential would be expected. Its magnitude is typically 60 mV/pH unit (for $1H^+/1e^-$ mediators). The apparent pH-dependence, 3–5 mV/unit, of the cyano complexes is not a true pH-dependence. Due to their very negative charge (−4/−3), the cyano complexes interact with other ions of the solution, such as potassium [18]. As the ionic strengths of the three buffer solutions are not equal, the equilibrium potentials show a slight variation.

Table 1 Preparation of mediators

$[Fe\text{-}DCTA]^{0/1+}$	Mixing $FeSO_4$ and DCTA-Na_2 solutions, 1:1 (Merck)
$[Co\text{-}terpyridine_2]^{2+/3+}$	Dissolving 2,2′:6′2″-terpyridine in $CoSO_4$ solution, 2:1 (Fluka) [15]
$[Fe(CN)_6]^{4-/3-}$	Dissolving $K_3[Fe,(CN)_6]$ (Merck)
$[W(CN)_8]^{4-/3-}$	Dissolving $K_4[W(CN)_8]$ (University of the Free State, RSA) [16]
$[Mo(CN)_8]^{4-/3-}$	Dissolving $K_4[Mo(CN)_8]$ (University of the Free State, RSA) [17]

Table 2 Equilibrium potentials of the five mediators

Mediator	Equilibrium potential vs. SHE (mV)		
	pH 6.0	pH 8.0	pH 9.5
$[Fe\text{-}DCTA]^{0/1+}$	+96	+97	+93
$[Co\text{-}terpyridine_2]^{2+/3+}$	+266	+266	+265
$[Fe(CN)_6]^{4-/3-}$	+434	+441	+450
$[W(CN)_8]^{4-/3-}$	+525	+530	+541
$[Mo(CN)_8]^{4-/3-}$	+790	+797	+799

3.2 Stability, colourlessness and water-solubility

When a mediator is repeatedly reduced and oxidised, side reactions may occur. The degradation rates of the five mediators were measured separately at pH 6.0, 8.0 and 9.5. As pH did not influence the results, only the values for pH 8.0 are listed in Table 3.

The central metal atom is mainly responsible for accepting and donating the electron; the ligands are less influenced by the redox reactions. However, the ligands may break off during a redox cycle as the stability constants of metal complexes vary, depending on the metal oxidation state. Polydentate ligands or "chelators" are known to have the highest stability constants [19]. With some simplification it can be stated that a ligand with many coordination bonds to the central metal atom does not break off as easily as a ligand with only one bond.

Table 3 Degradation rates of the mediators at pH 8.0

Mediator	Cycling range (mV)	Duration of cycle (h)	Loss per cycle (%)
$[Fe\text{-}DCTA]^{0/1+}$	−300 to 500	4.4	<1
$[Co\text{-}terpyridine_2]^{2+/3+}$	−300 to 500	4.4	<1
$[Fe(CN)_6]^{4-/3-}$	−300 to 500	4.4	<1
$[W(CN)_8]^{4-/3-}$	−300 to 650	5.3	<1
$[Mo(CN)_8]^{4-/3-}$	−300 to 800	6.1	<10[a]
For comparison:			
DCIP[b]	−300 to 500	4.4	4
1,2NQ4S[b]	−300 to 500	4.4	13

[a] At the peak potential of +790–800 mV, the surface oxidation current was too strong to allow exact measurement of degradation rate

[b] DCIP (2.6−dichlorophenolindophenol) and 1,2NQ4S (1,2-naphtoquinone-4-sulfonic acid) are organic 2-electron mediators having equilibrium potentials +135 mV and +170 mV

Correspondingly, mediators with polydentate ligands, such as DCTA or terpyridine, have a high probability of being stable. Of all the monodentate ligands tested, the cyano groups were the only stable ones.

Complex ions of transition metals are coloured to some degree due to the splitting of metal d-orbitals. However, the absorbances of the five mediators are very low (Table 4). Only the reduced $[Co\text{-}terpyridine_2]^{2+}$ has an extinction coefficient higher than 1 mM^{-1} cm^{-1}. The red colour probably originates from a combination of the cobalt ion and the conjugated double bonds of the terpyridine groups. Even this extinction coefficient is lower than that of most organic mediators.

As can be expected of complex ions, all five mediators are readily water-soluble, at least up to a concentration of 5 mM. No primary solvents, such as dimethyl sulfoxide, are needed, in contrast to ferrocene and its derivatives.

3.3 Alternative mediators

In addition to the set of five mediators, a few complex ions were found that fulfil most of the requirements of a good mediator (Table 5). Although they all have some drawbacks, they can be useful when the "set of five" is not adequate, e.g. for controlling unwanted mediator–enzyme interactions. In some cases, a mediator (e.g. cyanide complexes [6]) can interact with an enzyme in a way that alters the equilibrium potential of the enzyme. Therefore, it is generally recommended that different mediators should be used and the results compared [2].

Of these alternative mediators, ferrocenes are most frequently applied in enzyme electrochemistry. Their particular drawback is the hydrolysis of the oxidised form [5]. As can be seen in Table 5 (ferrocene acetic acid, −300 to 450 mV), a restriction of potential to less positive values can substantially enhance their stability. Due to their aromatic sandwich structure, ferrocenes are not readily soluble. Therefore, ferrocene acetic acid and ferrocene methanol were dissolved in DMSO (dimethyl sulfoxide). The DMSO solution was then added to the buffer to achieve a mediator concentration of 200 μM. The final DMSO concentration was 0.1%.

Iron complexes with hexadentate ligands, such as EDTA, DCTA, HEDTA (*N*-(2-hydroxyethyl)ethylenediamine-*N*,*N*′,*N*′-triacetic acid) and EDDS (ethylenediamine-*N*,*N*′-disuccinic acid) were found to be extremely stable.

Table 4 Absorption maxima and extinction coefficients of the mediators in the range 400–800 nm

Mediator	Reduced form		Oxidized form	
	max./nm	ext./mM^{-1} cm^{-1}	max./nm	ext./mM^{-1} cm^{-1}
$[Fe\text{-}DCTA]^{0/1+}$	None	<0.05	400	0.28
$[Co\text{-}terpyridine_2]^{2+/3+}$	445 (505)	1.37 (1.22)	435	0.28
$[Fe(CN)_6]^{4-/3-}$	None	<0.05	420	0.95
$[W(CN)_8]^{4-/3-}$	400 (430)	0.10 (0.09)	None	<0.05
$[Mo(CN)_8]^{4-/3-}$	None	<0.05	Not measured	
For comparison:				
Cytochrome *c* oxidase	440	200	420	150
DCIP	463	0.30	605	20
1,2NQ4S	400	0.41	400	1.8

Table 5 Alternative mediators: potentials, degradation rates and restrictions at pH 8.0

Mediator	Potential (mV)	Cycling range (mV)	Loss per cycle (%)	Restrictions
$[Ru(NH_3)_6]^{2+/3+}$	+3	−300 to 500	6	a
$[Fe\text{-}EDTA]^{0/1+}$	+65	−300 to 500	<1	b
$[Co\text{-}bipyridine_3]^{2+/3+}$	+323	−300 to 500	3	c
$[Co\text{-}phenantroline_3]^{2+/3+}$	+370	−300 to 500	<1	c
Ferrocene acetic $acid^{-1/0}$	+368	−300 to 500	7	a,d
(ferrocene acetic $acid^{-1/0}$	+368	−300 to 450	3	a,d)
Ferrocene $methanol^{0/1+}$	+430	−300 to 500	10	a,d
Ferrocene carboxylic $acid^{-1/0}$	+530	−300 to 650	25	a,d

[a] Low stability

[b] Slow reaction rate at electrode surface

[c] Tendency to exchange ligands with other complex ions

[d] Low solubility

However, they suffered from slow kinetics on the gold electrode. The oxidation and reduction peaks of Fe-HEDTA and Fe-EDDS showed such a hysteresis that the equilibrium potentials could not be measured. Even $[Fe\text{-}DCTA]^{0/1+}$, the fastest of these complexes, reacted slower than other tested mediators. Hexadentate ligands probably cover the central atom too efficiently to allow adequate contact to the electrode.

The exact degradation rates are not universal, but restricted to the test applied here. They merely serve for comparison between different mediators. If the cycling range is broadened to very positive values, most mediators become unstable due to strong oxidation. Even $[Fe(CN)_6]^{4-/3-}$ was degraded by 5–10% per cycle in a cycling range of −300 to +800 mV. If the cycling speed is increased, the loss per cycle decreases, as the time that a mediator spends at each potential gets shorter.

3.4 Mixing of mediators

Mixing of different mediators should ideally broaden the active potential range as in Fig. 3a, if the mediators do not interact with each other. However, some interactions are common.

The ligands are not covalently bound to the central metal atom, but dissociate and associate in a dynamic manner. In a mixture of different complex ions, a ligand can associate with the neighbouring central metal atom and constitute a new complex ion. The result can be loss of mediation capacity (Fig. 3b) if the new complex is not active in the applied potential range. Therefore, it is favourable to use a mixture of complex ions that have common central atoms (such as Co) or common ligands (such as CN) to avoid mediator loss. However, redistribution of ligands is still possible. At best it can constitute a continuum of useful mediators, as in Fig. 4.

3.5 Mediators together with an enzyme

The following complex ions were tested for their ability to equilibrate cytochrome *c* oxidase with the gold electrode in the OTTLE cell: $[Fe\text{-}DCTA]^{0/1+}$, $[Co\text{-}terpyridine_2]^{2+/3+}$, ferrocene $acetic\ acid^{-1/0}$, $[Fe(CN)_6]^{4-/3-}$ and $[W(CN)_8]^{4-/3-}$. Each complex ion worked well as mediator.

A redox titration of the enzyme is shown in Fig. 5. Here $[Fe\text{-}DCTA]^{0/1+}$, $[Co\text{-}terpyridine_2]^{2+/3+}$ and $[Fe(CN)_6]^{4-/3-}$ were used as a set of mediators to control the potential of the enzyme in the range 0 to +500 mV vs. SHE. As can be seen, the positive and negative sweeps coincide, which means that the enzyme was close to equilibrium. The two-wave behaviour is similar to that found in the literature [13].

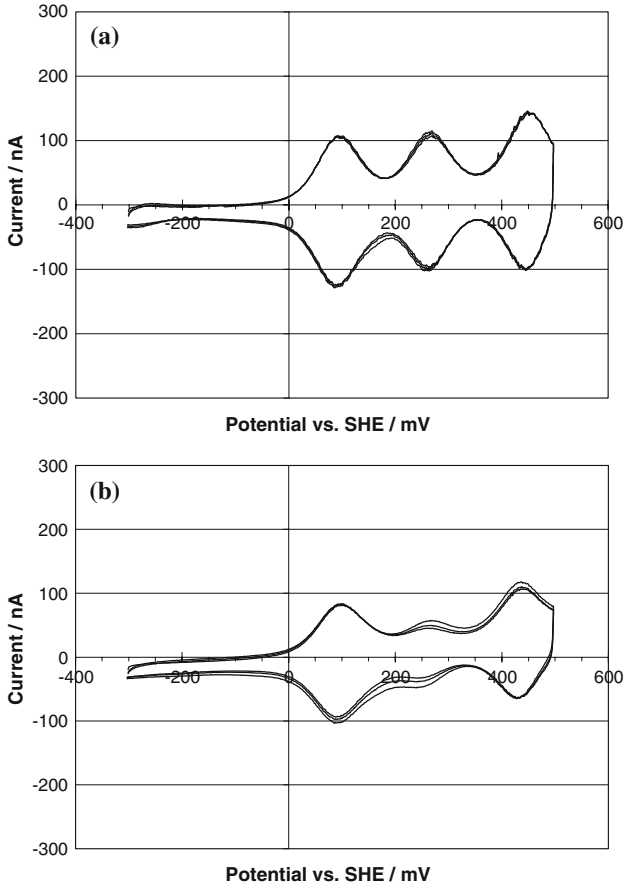

Fig. 3 (**a**) Combination of $[Fe\text{-}DCTA]^{0/1+}$, $[Co\text{-}terpyridine_2]^{2+/3+}$ and $[Fe(CN)_6]^{4-/3-}$ at pH 9.5. Concentration of each mediator 100 μM. No interaction between the mediators detected. (**b**) Same as (**a**), but pH 6.0. $[Co\text{-}terpyridine_2]^{2+/3+}$ is degraded, possibly by binding of terpyridine by iron

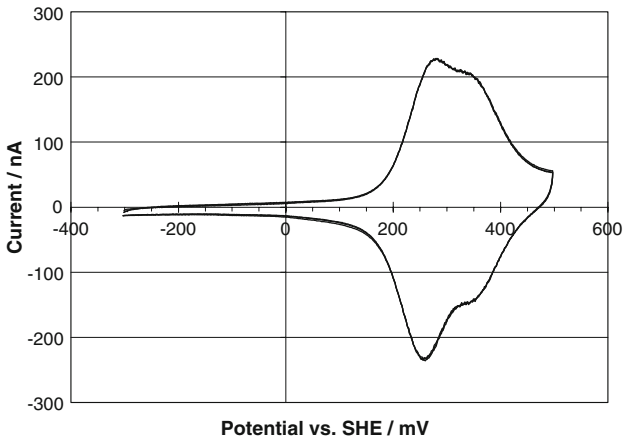

Fig. 4 Combination of $[Co\text{-}terpyridine_2]^{2+/3+}$, $[Co\text{-}bipyridine_3]^{2+/3+}$ and $[Co\text{-}phenantroline_3]^{2+/3+}$. Concentration of each mediator 100 μM. Ligands are exchanged between cobalt ions to constitute new mixed complex ions. They are stable as the three cycles shown here coincide perfectly

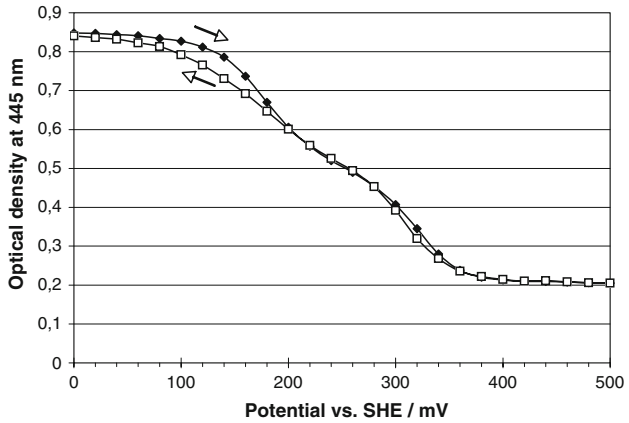

Fig. 5 Redox titration of cytochrome *c* oxidase. Potential was changed stepwise (20 mV/20 min) from 0 to +500 mV and back. The slight hysteresis at +120 mV is caused by the slow electrochemical kinetics of $[Fe\text{-}DCTA]^{0/1+}$. Degradation of the enzyme during the 17 h long experiment was compensated by normalizing the return sweep with the factor 1.05

Compatibility of a mediator with cytochrome *c* oxidase does not necessarily imply that it can mediate electrons to other enzymes. However, all tested complex ions that fulfilled the requirements of stability, solubility and one-electron transport, worked well with this enzyme. This indicates that the above-mentioned general requirements may be more important for a mediator than any enzyme-specific steric compatibility.

4 Conclusions

There are very few mediators that are both stable and colourless. Of the widely applied mediators, ferrocenes are unstable and most organic complexes of ruthenium and osmium are coloured. Thus there is a need for new mediators. The design of a complex ion mediator involves the choice of a central metal atom and suitable ligands. The properties of the complex ion can be partly predicted from its components, but ultimately each mediator must be tested separately.

The stability of the complex ions was difficult to predict. Polydentate ligands were more stable than monodentatate ligands, as expected. Of all the monodentate ligands tested, only the cyano groups were found to be stable. The colour was influenced by both the metal ion and the ligands. Large systems of conjugated double bonds in the ligands increased the absorbance considerably. The solubility was strongly influenced by ligands. Complex ions are generally water-soluble, but bulky organic ligands may limit the solubility. For example, ruthenocene and tris(4,7-diphenyl-1,10-phenantroline)ruthenium could not be tested, as their

solubility was less than 100 μM. For inorganic and non-aromatic organic ligands, solubility caused no problems.

As the redox reaction of a complex ion is merely a change of the metal oxidation state, hydrogen ions did not participate in the reactions, and the potentials were generally pH-independent. Likewise, the choice of metal ion decided if the mediator tended to transport one or two electrons. $Fe^{2+/3+}$ and $Co^{2+/3+}$are examples of one-electron mediators, as the oxidation states +II and +III of these metals show high stability.

The established set of five mediators, $[Fe\text{-}DCTA]^{0/1+}$, $[Co\text{-}terpyridine_2]^{2+/3+}$, $[Fe(CN)_6]^{4-/3-}$, $[W(CN)_8]^{4-/3-}$ and $[Mo(CN)_8]^{4-/3-}$, fulfils the requirements of stability, colourlessness, water-solubility and pH-independence. All five mediators transport one electron at a time. Together, the mediators cover the potential range of 0 to +800 mV.

The set offers promising mediator candidates whenever indirect electrochemistry is needed, as it is not restricted to any particular enzyme. For applications like bioelectrochemical fuel cells or enzymatic production of fine chemicals, the stability of these mediators is of importance. Stability combined with colourlessness is especially useful for redox titrations and other enzyme research, where measurement of enzyme spectra is required. As no charge is consumed by mediator degradation, even amperometric and coulometric enzyme titrations using these mediators are possible.

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