# EVALUATION OF RUTHENIUM(II) COMPLEXES AS ELECTRON TRANSFER MEDIATORS ACCESSIBLE FOR AMPEROMETRIC GLUCOSE SENSORS AND BIOFUEL CELLS

Yasuo NAKABAYASHI*, Toshiya ADACHI, Hideto KITAMURA, Yuri HIROSAKI and Osamu YAMAUCHI

## Abstract

The mediation of dipolar ruthenium(II) ammine complexes containing pyridinium ions $[Ru(L^+)(NH_3)_5]^{3+}$ ($L^+$: pyridinium ions) in glucose oxidation was investigated using a voltammetric method. These ruthenium(II) complexes had appropriate redox potentials of 0.10–0.18 V vs. Ag/AgCl and high $k_s$ values of $5.7 \times 10^6$–$1.7 \times 10^7 M^{-1}s^{-1}$, which are the second-order rate constants for electron transfer from glucose oxidase in reduced form to $[Ru(L^+)(NH_3)_5]^{1+}$. In particular, the $k_s$ values for $[Ru(L^+)(NH_3)_5]^{3+}$ were greater than those of osmium(II)-polypyridine complexes which possesses similar redox potentials and which are most commonly used. All the dipolar ruthenium(II) complexes used in this study were therefore found to be useful as the electron transfer mediators of amperometric glucose sensors and biofuel cells.

## 1. Introduction

Creating an electrical contact between redox enzymes and electrodes is the most fundamental principle for developing amperometric biosensors and biofuel cells[1,2]. Since the 1970s, there have been numerous papers describing electrocatalysis by enzymes attached to electrodes, in which authors have focused either on fundamental studies of enzyme electron transfer and catalytic mechanism or on applications to biosensors. It is only comparatively recently that papers have appeared dealing specifically with enzymes as fuel cell catalysts. Enzyme-based fuel cells have remained a popular focus for research due to the high turnover rates associated with enzymes that lead to a high biocatalysis rate. Because any one development depends on others, it is instructive to view how the use of enzymes as fuel cell catalysts relates chronologically to breakthroughs in technology or understanding. Most of the fuel cells reported use glucose as the fuel and dioxygen as the oxidizer.

The enzyme glucose oxidase (GOx, EC 1.1.3.4) specifically converts glucose into gluconolactone, which subsequently hydrolyzes spontaneously to gluconic acid with concomitant reduction of dioxygen to hydrogen peroxide. This oxidation reaction is accompanied by reduction of the cofactor flavin adenine dinucleotide (FAD). The flavin cofactor FAD is responsible for the redox properties of the enzyme, and available evidence suggests that it is firmly bound, but not covalently linked, to the polypeptide protein of

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the enzyme. This enzyme GOx, a structurally rigid glycoprotein, is most widely used as a biosensor in an increasing number of clinical, environmental, agricultural, and biotechnological applications. Thus GOx accepts electrons from glucose through the flavin moiety and transfers them to dioxygen but not to simple electrodes. The reason that reduced flavin adenine dinucleotide (FADH₂), known as an active site, is not oxidized electrochemically through the electrode reaction is as follows:

$$FADH_2 \longrightarrow FAD + 2H^+ + 2e^- \tag{1}$$

The FAD/FADH₂ redox centers of the homodimer are located at the depth of GOx from *Aspergillus niger* active site pocket of *ca.* 10 Å[3]. So the idea came to use a non-physiological redox-active molecule as an electron acceptor for GOx in reduced form, which is usually called a mediator. Numerous artificial electron acceptors are used as electron transfer mediators for coupling the glucose/GOx system to an electrode surface. Transition metal complexes, such as iron[4-6] and osmium[7-17] compounds, are most commonly used. In addition, there are papers on using ruthenium complexes[7,8,14,18-22]. In a recent work, Dempsey et al have evaluated novel mediators for a glucose biosensor based on ruthenium and osmium picolinate complexes[23]. Ruthenium complexes are of greater advantage than related osmium complexes, owing to the lower cost and their higher reactivity toward GOx[14]. Taking the example of glucose, one can describe the operation of a ruthenium complex as an electron transfer mediator as follows:

$$\beta\text{-D-glucose} + \text{GOx}_{ox}(\text{FAD}) \longrightarrow \text{glucono-}\delta\text{-lactone} + \text{GOx}_{red}(\text{FADH}_2) \tag{2}$$

$$\text{GOx}_{red}(\text{FADH}_2) + 2\ \text{Ru(III)} \longrightarrow \text{GOx}_{ox}(\text{FAD}) + 2\ \text{Ru(II)} \tag{3}$$

$$2\ \text{Ru(II)} \longrightarrow 2\ \text{Ru(III)} + 2\ e^- \tag{4}$$
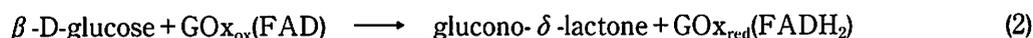
where GOx_ox(FAD) and GOx_red(FADH₂) are oxidized and reduced forms of GOx, respectively. A ruthenium complex as an electron transfer mediator is required to have (1) an appropriate redox potential of ruthenium(III/II) couple, ideally *ca.* 0.0 V (vs. Ag/AgCl), that in turn enables the electrode to be polarized at a potential that does not give rise to interfering electrochemical reaction, (2) chemical stability in both oxidized and reduced forms, and (3) a high value of $k_s$, which is the second-order rate constant for electron transfer from GOx_red(FADH₂), as shown as Eq. 3, in order to minimize competition with dioxygen. Consequently, the choice of the electron transfer mediator is certainly important for achieving high sensitivity and selectivity in the amperometric glucose sensor and for creating power in the biofuel cell.

The reactivity between a metal complex and GOx_red(FADH₂) can be discussed through the use of the Marcus relationship[24,25] for the electron transfer reaction:

$$k_s = (k_{11}\ k_{22}\ f\ K_s)^{1/2} \tag{5}$$

where $k_s$ is the second-order rate constant for the electron transfer cross-reaction between the mediator and GOx (Eq. 3), $k_{11}$ and $k_{22}$ are the self-exchange rate constants for the mediator and GOx, and $K_s$ is the equilibrium constant of Eq. 3. Frequently, $f$ is close to unity. Differential excitation flash photolysis work has shown that the $k_{11}$ values of $[M(PP)_3]^{3+/2+}$ complexes (M=Fe, Ru, PP=polypyridine)[26] are in the range of $10^8$–$10^9 M^{-1}s^{-1}$, and $10^3$–$10^5$ times those which have

been obtained for $[Ru(NH_3)_6]^{3+}$ ($2.5 \times 10^5 M^{-1} s^{-1}$) and $[Ru(NH_3)_5(py)]^{2+}$ (py=pyridine, $1.7 \times 10^4 M^{-1}$ $s^{-1}$)[25]. However, $[Ru(PP)_3]^{3+/2+}$ (PP=2,2'-bipyridine, 1,10-phenanthroline) complexes cannot act as electron transfer mediators for glucose oxidation since the redox potentials of Ru(III/II) couples are too high (> 1.0 V vs. Ag/AgCl). On the other hand, the $[Ru(NH_3)_5(py)]^{2+}$ complex possesses a small $k_s$ value ($1.0 \times 10^5 M^{-1} s^{-1}$), although the redox potential of Ru(III/II) couple is satisfactorily low ($E_{1/2}$=0.09 V vs. Ag/AgCl)[27].

In this paper, we describe the results of studies on the interactions between GOx and dipolar ruthenium(II) complexes $[Ru(L^+)(NH_3)_5]^{3+}$, as shown in Fig. 1, which are chemically stable in both oxidized and reduced forms because of the electron-withdrawing effect
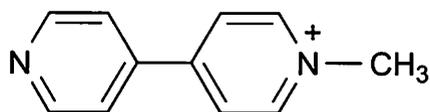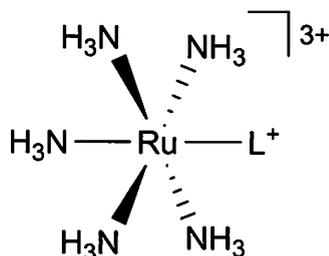


N-methyl-4,4'-bipyridinium (meq$^+$)
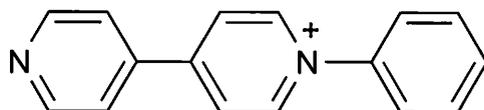
N-phenyl-4,4'-bipyridinium (phq$^+$)
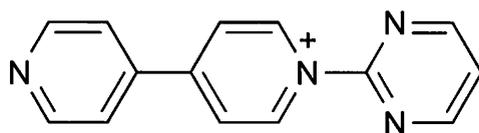
N-(2-pyrimidyl)-4,4'-bipyridinium (pymq$^+$)
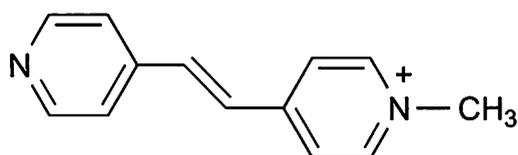
N-methyl-4-[trans-2-(4-pyridyl)ethenyl]pyridinium (mebpe$^+$)

Fig. 1   Structures of ruthenium(II) complex and ligands used in this study.

of the pyridinium ions $L^+$. The factors to be satisfied by electron transfer mediators for amperometric glucose sensors and biofuel cells are discussed, and the evaluation of $[Ru(L^+)(NH_3)_5]^{3+}$ as electron transfer mediators is made on the basis of the redox potentials of ruthenium(III/II) couples and the second-order rate constants for electron transfer between $GOx_{red}$ (FADH$_2$) and the ruthenium(III) complex (Eq. 3).

## 2. Experimental

### 2.1 Materials

The complex $[RuCl(NH_3)_5]Cl_2$, ammonium hexafluorophosphate, and D-glucose were supplied by Wako Pure Chemicals. GOx from *Aspergillus niger* (EC 1.1.3.4, 25 units per mg of solid, type II, molecular weight $1.86 \times 10^5$)[28] was purchased from Sigma. All other chemicals were of reagent grade and were used as received. A stock aqueous solution of glucose (1 M (M=mol dm$^{-3}$)) was left for at least 24 h at room temperature to allow for equilibration of the anomers. All aqueous solutions were prepared with Millipore "Milli Q" grade water.

### 2.2 Physical measurements

Cyclic voltammograms were obtained by using an Als/chi electrochemical analyzer model 600A with a 20-mL one-compartment three-electrode electrochemical cell. A three-electrode system was used with a 3-mm diameter glassy carbon working electrode (BAS 11-2012), an Ag/AgCl reference electrode (BAS 11-2020), and a platinum wire counter electrode. Cyclic voltammetric measurements were carried out in a deaerated 0.1 M phosphate buffer (pH 7.0) at 25.0 $\pm$ 0.1 °C. Absorption spectra were recorded on a Shimadzu UV-3101PC (quartz cell length was 10 mm). $^1$H NMR spectra were obtained using a JEOL JNM-EX 270 MHz spectrometer using D$_2$O as the solvent and sodium 3-(trimethylsilyl)propionate (TSP) as the internal reference.

### 2.3 Syntheses

The ligands *N*-methyl-4,4'-bipyridinium iodide ([meq$^+$]I)[29], *N*-phenyl-4,4'-bipyridinium chloride ([phq$^+$]Cl)[30], *N*-(2-pyrimidyl)-4,4'-bipyridinium chloride ([pymq$^+$]Cl)[31], and *N*-methyl-4-[*trans*-2-(4-pyridyl)ethenyl]pyridinium iodide ([mebpe$^+$]I)[31] were prepared according to published procedures. The complexes $[Ru(L^+)(NH_3)_5]Cl_3$ (L$^+$=meq$^+$, phq$^+$, pymq$^+$, mebpe$^+$) were synthesized from $[RuCl(NH_3)_5]Cl_2$ *via* slight modifications of published methods[30,32]. Absorption spectral data of $[Ru(L^+)(NH_3)_5]Cl_3$ in CH$_3$CN were as follows: $[Ru(meq^+)(NH_3)_5]Cl_3$ : $\lambda_{max}$ / nm ($\varepsilon$ / M$^{-1}$ cm$^{-1}$) 587 (1.53 × 10$^4$), 267 (1.65 × 10$^4$) (lit. : 590 (1.58 × 10$^4$), 268 (1.63 × 10$^4$))[30]. $[Ru(phq^+)(NH_3)_5]Cl_3$ : $\lambda_{max}$ / nm ($\varepsilon$ / M$^{-1}$cm$^{-1}$) 625 (2.04 × 10$^4$, 280 (1.93 × 10$^4$) (lit. : 628 (1.93 × 10$^4$), 280 (1.85 × 10$^4$))[30]. $[Ru(pymq^+)(NH_3)_5]Cl_3$ : $\lambda_{max}$ / nm ($\varepsilon$ / M$^{-1}$cm$^{-1}$) 677 (1.76 × 10$^4$), 284 (2.06 × 10$^4$) (lit. : 673 (1.80 × 10$^4$), 285 (2.15 × 10$^4$))[31]. $[Ru(mebpe^+)(NH_3)_5]Cl_3$ : $\lambda_{max}$ / nm ($\varepsilon$ / M$^{-1}$cm$^{-1}$) 595 (1.62 × 10$^4$), 312 (2.40 × 10$^4$) (lit. : 595 (1.61 × 10$^4$), 312 (2.38 × 10$^4$))[31].

### 2.4 Kinetic measurements

The second-order rate constant for a given electron transfer mediator was determined by cyclic voltammetry according to the experimental method described by Cass et al[33] and used frequently in the literature. The magnitude of the catalytic current at a glassy carbon

electrode was measured in samples with increasing concentrations of $GOx_{ox}$ (2 – 12 $\mu$M) containing a 0.1 M glucose and 0.1 mM ruthenium(II) complex at scan rates of 1 – 20 mV s$^{-1}$. The change in the catalytic current with the addition of $GOx_{ox}$ was compared with the diffusion current obtained in the absence of $GOx_{ox}$ at various scan rates, and thereby related to the rate constant using the scheme of Nicholson and Shain[34].

## 3. Results and discussion

### 3.1 Characterization of $[Ru(L^+)(NH_3)_5]^{3+}$

Absorption spectral and electrochemical data of $[Ru(L^+)(NH_3)_5]^{3+}$ in a 0.1 M phosphate buffer (pH 7.0) are shown in Table 1. All the ruthenium(II) complexes show intense, broad $d_\pi(Ru^{II}) \rightarrow \pi^*(L^+)$ metal-to-ligand charge-transfer (MLCT) bands in the region 580–677 nm, the energies of which are related to the electron-donor ability of the ruthenium center and to the electron-acceptor ability of $L^{+[35,36]}$. The MLCT band maximum of the pymq$^+$ complex is red-shifted by ca. 100 nm with respect to the meq$^+$ complex. Given an almost constant HOMO energy, this shows that pymq$^+$ has the lowest LUMO energy of the 4,4'-bipyridinium ligands used in this study, in keeping with the highly electron-deficient nature of the 2-pyrimidyl group. Accordingly, the acceptor strength of $L^+$ hence increases in the order meq$^+$ < phq$^+$ < pymq$^+$. The MLCT energy of mebpe$^+$ complex is very similar to that of the meq$^+$ complex. As shown in Table 1, all the ruthenium(II) complexes exhibit reversible ruthenium(III/II) waves and possess the redox potentials of ruthenium(III/II) couples in the region 0.10–0.18 V vs. Ag/AgCl. In the 4,4'-bipyridinium ligands, the redox potentials increase in the order meq$^+$ < phq$^+$ < pymq$^+$. This order is in conformity with the acceptor strength of $L^+$; that is, the stronger the electron-acceptor ability of $L^+$, the more positive the redox potential of the ruthenium(III/II) couple. However, the variations in the redox potentials are only small, showing that the HOMO energy is relatively insensitive to changes in the substituent at nitrogen. The corresponding red (bathochromic) shifts in the MLCT bands are mainly the result of stabilization of the ligand-based LUMOs. The redox potential of the mebpe$^+$ complex is shifted by ca. –50 mV with respect to that of the meq$^+$ complex, due to the mildly electron-donating character of the ethylene unit.

Table 1. Absorption spectral data of $[Ru(L^+)(NH_3)_5]Cl_3$ and redox potentials of Ru(III/II) couples in 0.1 M phosphate buffer (pH 7.0)

| $L^+$ | $\lambda_{max}$ / nm ($\varepsilon$ / $M^{-1}$ cm$^{-1}$) | Assignment | $E_{1/2}$[a] / V vs. Ag/AgCl ($\Delta E_p$ / mV) |
|---|---|---|---|
| meq$^+$ | 580 (11300) | $d_\pi \longrightarrow \pi^*$(meq$^+$) | 0.152 (60) |
| phq$^+$ | 617 (20400) | $d_\pi \longrightarrow \pi^*$(phq$^+$) | 0.165 (60) |
| pymq$^+$ | 677 (14800) | $d_\pi \longrightarrow \pi^*$(pymq$^+$) | 0.180 (63) |
| mebpe$^+$ | 588 (10100) | $d_\pi \longrightarrow \pi^*$(mebpe$^+$) | 0.101 (60) |

a) Scan rate, 50 mV s$^{-1}$.

## 3.2 Mediation of ruthenium complexes in glucose oxidation

The function of the ruthenium complexes as electron transfer mediators can be described according to the reaction mechanisms shown in Eqs. 2-4. Two properties for an electron transfer mediator are essential to fabricate the amperometric glucose sensors. Firstly, the electron transfer mediator should possess an appropriate redox potential for the electrode reaction given by Eq. 4. Amperometric glucose sensors are necessary to detect glucose at near 0.0 V vs. Ag/AgCl, where the risk of interfering reactions is minimized, and also where the background current and noise levels are lowest. Secondly, in order to enhance the
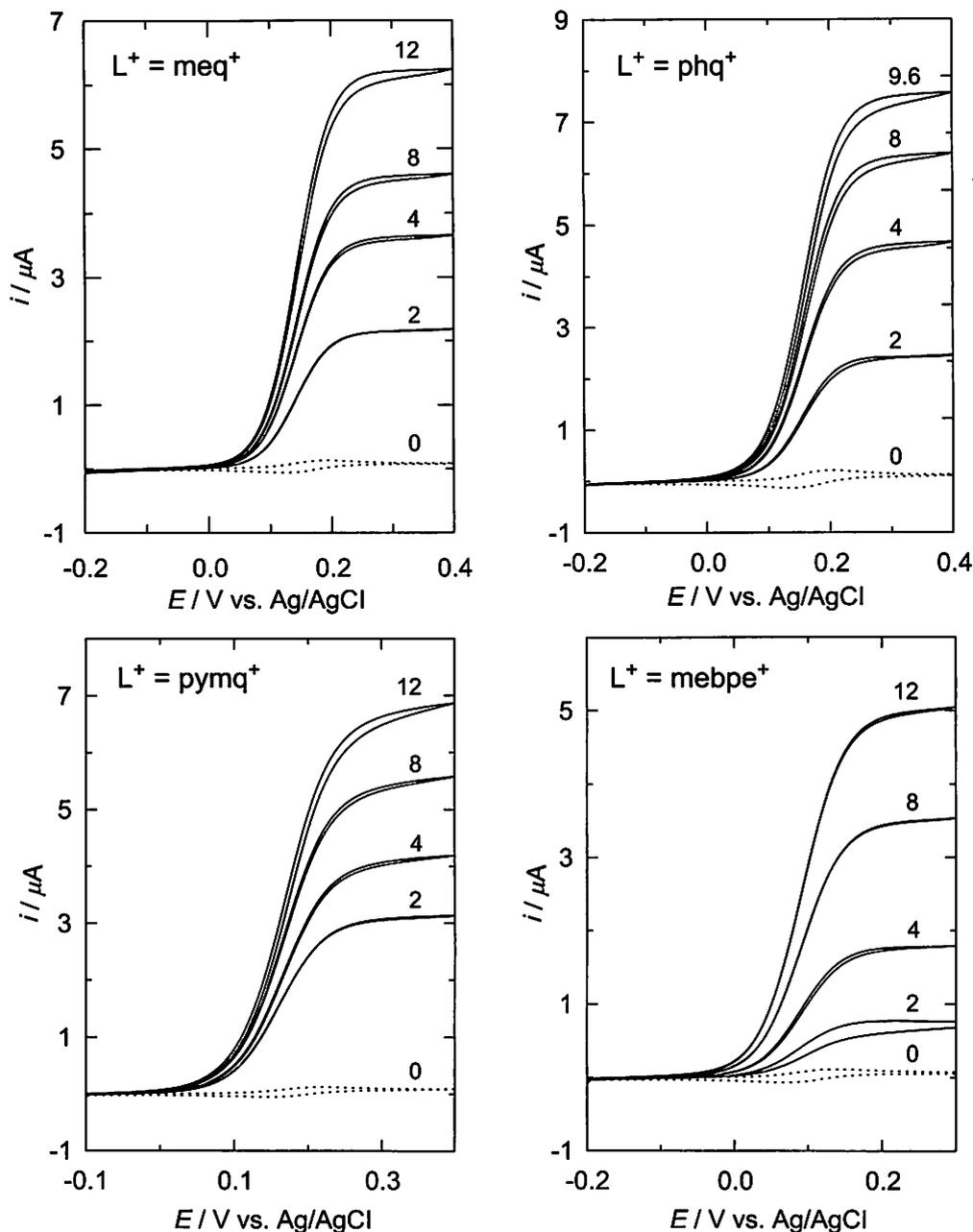


Fig. 2  Cyclic voltammograms of 0.1 mM $[Ru(L^+)(NH_3)_5]^{3+}$ in 0.1 M phosphate buffer (pH 7.0) containing 0.1 M glucose in the absence (dotted lines) and presence (solid lines, μM) of $GOx_{ox}$. Scan rate, 2 mV s$^{-1}$.

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sensitivity and minimize competition with dissolved dioxygen, an electron transfer mediator should possess a high value of second-order rate constant $k_s$ (Eq. 3).

The features of the cyclic voltammograms for all the ruthenium(II) complexes were attributed to the ruthenium(III/II) couples and to reversible electron transfer, with the anodic and cathodic peak heights being proportional to the square root of the scan rate. Table 1 summarizes the half-wave potentials $(E_{1/2})$ of the ruthenium(III/II) couples, corresponding to the redox potentials and the peak separations $(\Delta E_p)$ between the two peak potentials. The $\Delta E_p$ values of $60-63$ mV indicate that the electron transfers of all the ruthenium(III/II) couples are electrochemically reversible; that is, the rates of heterogeneous electron transfer are fast. As the redox potentials of all the ruthenium(III/II) complexes were more positive than the redox potential of the FAD/FADH$_2$ couple ($-0.41$ V vs. Ag/AgCl at pH 7.0)[37], the mediation reaction (as shown in Eq. 3) is considered thermodynamically possible. As previously described, amperometric glucose sensors preferably should detect glucose at near 0.0 V. Although [Ru(mebpe$^+$)(NH$_3$)$_5$]$^{3+}$ may be considered the best electron transfer mediator of all the ruthenium(II) complexes, as evaluated solely by the redox potentials of the ruthenium(III/II) complexes, the second-order rate constant $k_s$ for the electron transfer reaction (Eq. 3) must also be considered for the evaluation of electron transfer mediators.

The second-order rate constant for the reaction, as shown in Eq. 3, can be estimated using the electrochemical method[33]. When both glucose and GOx$_{ox}$ were added together to a ruthenium(II) complex solution, so-called catalytic current waves were observed for all the ruthenium(II) complexes (Fig. 2). The magnitude of the catalytic current at the glassy carbon electrode was measured for sample solutions containing increasing amounts of GOx$_{ox}$, with the bulk concentrations of ruthenium(II) complex and glucose kept constant. The change in
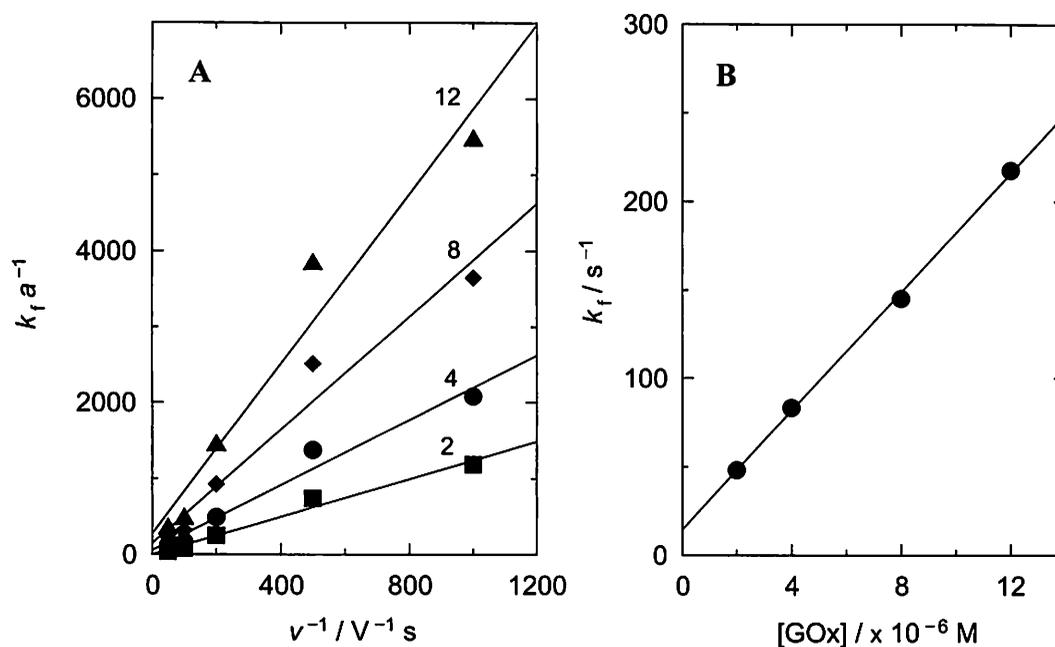


Fig. 3A Plots of the kinetic parameter $k_f\, a^{-1}$ as a function of $v^{-1}$ for various GOx$_{ox}$ concentrations ($\mu$M) for [Ru(pymq$^+$)(NH$_3$)$_5$]$^{3+}$.

    3B. Plot of the pseudo-first-order rate constant $k_f$ as a function of GOx$_{ox}$ concentrations for [Ru(pymq$^+$)(NH$_3$)$_5$]$^{3+}$.

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Table 2. Redox potentials of Ru(III/II) couples and rate constants of glucose oxidation at pH 7.0 and 25℃

| Complex | $E_{1/2}$ / V vs. Ag/AgCl ($\Delta E_p$ / mV) | $k_s$ / M$^{-1}$ s$^{-1}$ |
|---|---|---|
| [Ru(meq$^+$)(NH$_3$)$_5$]$^{3+}$ | 0.152 (60) | $9.1 \times 10^6$ |
| [Ru(phq$^+$)(NH$_3$)$_5$]$^{3+}$ | 0.165 (60) | $5.7 \times 10^6$ |
| [Ru(pymq$^+$)(NH$_3$)$_5$]$^{3+}$ | 0.180 (63) | $1.7 \times 10^7$ |
| [Ru(mebpe$^+$)(NH$_3$)$_5$]$^{3+}$ | 0.101 (60) | $8.0 \times 10^6$ |
| [Os(Him)$_2$(dmbpy)$_2$]$^{2+}$ a) | 0.154 (60) | $2.3 \times 10^6$ |
| [OsCl(Him)(bpy)$_2$]$^+$ a) | 0.107 (59) | $1.2 \times 10^5$ |

a) Him: imidazole, dmbpy: 4,4'-dimethyl-2,2'-bipyridine,
bpy: 2,2'-bipyridine [17].

the catalytic current $i_k$ with additions of GOx$_{ox}$ was compared with the diffusion current $i_d$ obtained in the absence of GOx$_{ox}$. Using a working curve (see Fig. 14 of reference 34) of $i_k$ $i_d^{-1}$ vs. kinetic parameter, $(k_f$ $a^{-1})^{1/2}$, we replotted the data as $k_f$ $a^{-1}$, where $k_f$ is the pseudo-first-order rate constant for mediation of glucose oxidation and $a = F v R^{-1} T^{-1}$ ($F$: Faraday constant, $v$: scan rate, $R$: gas constant, $T$: absolute temperature), against $v^{-1}$ to obtain straight lines under first-order conditions, as shown in Fig. 3A for [Ru(pymq$^+$)(NH$_3$)$_5$]$^{3+}$ at various GOx$_{ox}$ concentrations. From the slope of each line, the corresponding scan rate independent $k_f$ value was obtained. The second-order rate constant $k_s$ in Eq. 3 was next determined from the slope of the plot of the $k$ f value against the concentration of GOx$_{ox}$ (Fig. 3B). Table 2 summarizes the $k_s$ values determined for all the ruthenium(II) complexes and two osmium(II)-polypyridine complexes possessing similar redox potentials [16,17]. The $k_s$ values of $5.7 \times 10^6 - 1.7 \times 10^7$ M$^{-1}$s$^{-1}$ for [Ru(L$^+$)(NH$_3$)$_5$]$^{3+}$ are greater than those of the osmium(II) complexes. In particular, the rate constant for [Ru(pymq$^+$)(NH$_3$)$_5$]$^{3+}$ ($1.7 \times 10^7$ M$^{-1}$s$^{-1}$) is very close to the upper limit for the best organic[38] and inorganic[21,39] mediators of GOx.

Ferrocene and its derivatives have been extensively characterized and would appear to have satisfied most of the criteria mentioned previously. However, the oxidized forms (the ferricinium cations) undergo slow hydrolysis in aqueous solution[40], and ferrocene and its derivatives are not easily soluble in aqueous solution, which might cause problems in the fabrication of glucose sensors. On the other hand, both the ruthenium(II) and ruthenium(III) complexes used in this study were stable and easily soluble in aqueous solution. In addition, the $k_s$ value of $2.6 \times 10^4$ M$^{-1}$s$^{-1}$ for ferrocene[33] is significantly smaller than that of a dipolar ruthenium(II) complex. These facts suggest that the electrostatic ruthenium(III/II) complex-GOx interaction and/or "antenna effect" of pyridinium ions introduced into GOx may contribute to greater $k_s$ values.

For a glucose sensor to be of practical use, it is proposed that the enzyme GOx and an electron transfer mediator [Ru(L$^+$)(NH$_3$)$_5$]$^{3+}$ complex can be immobilized in electropolymerized films[41] or onto the surface of an electrode by polyion complex formation[42,43]. In particular, the surface functionalization of an electrically conductive polymer film (for example, polypyrrole film) with a pyridinium ion may be effective for the covalent immobilization of GOx[44]; that is,

$[Ru(L^+)(NH_3)_5]^{3+}$ complexes lead to the electrical wiring of GOx.

## 3.3 Interactions of GOx with $[Ru(L^+)(NH_3)_5]^{3+}$

The $k_s$ values for the dipolar ruthenium(II) complexes bearing a net charge of 3+ were greater than the values for the osmium(II) complexes bearing net charges of 1+ and 2+. This points to the electrostatic ruthenium(III/II) complex-GOx interaction as one of the most important factors, where the isoelectric point for *Aspergillus niger* $GOx_{ox}$ has been reported to range from pH 3.9 to 4.3[45]. Thus $GOx_{ox}$ is negatively charged at pH 7.0. For comparison of the cyclic voltammograms of 0.2 mM $[Ru(L^+)(NH_3)_5]^{3+}$ in a 0.1 M phosphate buffer (pH 7.0) in the absence (a) and presence (b) of 0.1 mM $GOx_{ox}$, all the ruthenium(II) complexes in the presence of $GOx_{ox}$ display a decrease in magnitude of the peak currents and the same redox potentials, as shown in Fig. 4A for $[Ru(pymq^+)(NH_3)_5]^{3+}$. The decrease in the magnitudes of the peak currents suggests that effective diffusion coefficients of the ruthenium(III/II) complexes may decrease by means of forming adducts with $GOx_{ox}$, as illustrated in Scheme 1. The change in binding constant at 25 °C can be determined according to Eq. 6:

$$[Ru(L^+)(NH_3)_5]^{3+} + GOx_{ox} \; \overset{K_{Ru(II)}}{\rightleftharpoons} \; [Ru(L^+)(NH_3)_5]^{3+} \cdot GOx_{ox}$$

$$+ e^- \big\Vert - e^- \qquad\qquad\qquad + e^- \big\Vert - e^-$$

$$[Ru(L^+)(NH_3)_5]^{4+} + GOx_{ox} \; \overset{K_{Ru(III)}}{\rightleftharpoons} \; [Ru(L^+)(NH_3)_5]^{4+} \cdot GOx_{ox}$$
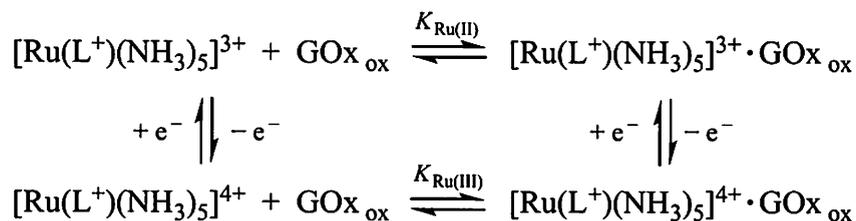
Scheme 1.  Adducts formation between $[Ru(L^+)(NH_3)_5]^{+/3+}$ and $GOx_{ox}$.

$$E_b' - E_f' = 0.059 \log (K_{Ru(II)} / K_{Ru(III)}) \tag{6}$$

where $E_b'$ and $E_f'$ are the thermodynamic redox potentials for the bound and free complexes, respectively, and $K_{Ru(II)} / K_{Ru(III)}$ is the ratio of binding constants for the $[Ru(L^+)(NH_3)_5]^{3+}$ and $[Ru(L^+)(NH_3)_5]^{4+}$ complexes. For no shift of the redox potential, we calculate $K_{Ru(II)} / K_{Ru(III)} = 1$; i.e., the strength for electrostatic binding of $[Ru(L^+)(NH_3)_5]^{3+}$ is the same as that of $[Ru(L^+)(NH_3)_5]^{4+}$, although the charge on both forms is different. The cyclic voltammetric behavior of $[Os(dmbpy)_3]^{2+}$ (dmbpy: 4,4'-dimethyl-2,2'-bipyridine) in the presence of $GOx_{ox}$ was the same as that of $[Ru(L^+)(NH_3)_5]^{3+}$, suggesting that association of $[Os(dmbpy)_3]^{2+}$ with $GOx_{ox}$ takes place. However, no difference in absorption spectral behavior was observed either in the absence or presence of $GOx_{ox}$. We recently reported that there is only an electrostatic effect between $[Os(dmbpy)_3]^{2+}$ and $GOx_{ox}$[17]. Fig. 4B shows variation in the intense $d_\pi(Ru^{II}) \rightarrow \pi^*(pymq^+)$ MLCT band of $[Ru(pymq^+)(NH_3)_5]^{3+}$ with increasing amount of $GOx_{ox}$, for example, 13 nm red shift and 11% hyperchromicity (Table 3). All the MLCT bands of $[Ru(L^+)(NH_3)_5]^{3+}$ exhibit hyperchromism and red (bathochromic) shifts (usually attributed to a change of solvent polarity) in the presence of $GOx_{ox}$. Absorption spectral and $^1H$ NMR data of $[Ru(pymq^+)(NH_3)_5]^{3+}$ $- FAD, - FMN$ (flavin mononucleotide), and $- GOx$ systems are shown in Table 3. All proton signals of $^1H$ NMR spectra for the $[Ru(pymq^+)(NH_3)_5]^{3+} - FAD$ and $- FMN$ systems showed substantial upfield shifts arising from face-to-face stacking between $pymq^+$ and the flavin rings of FAD and FMN. These facts suggest that the pyridinium ions coordinated monodentately to ruthenium may be introduced into $GOx_{ox}$ through the active site pocket of *ca.* 10 Å and act
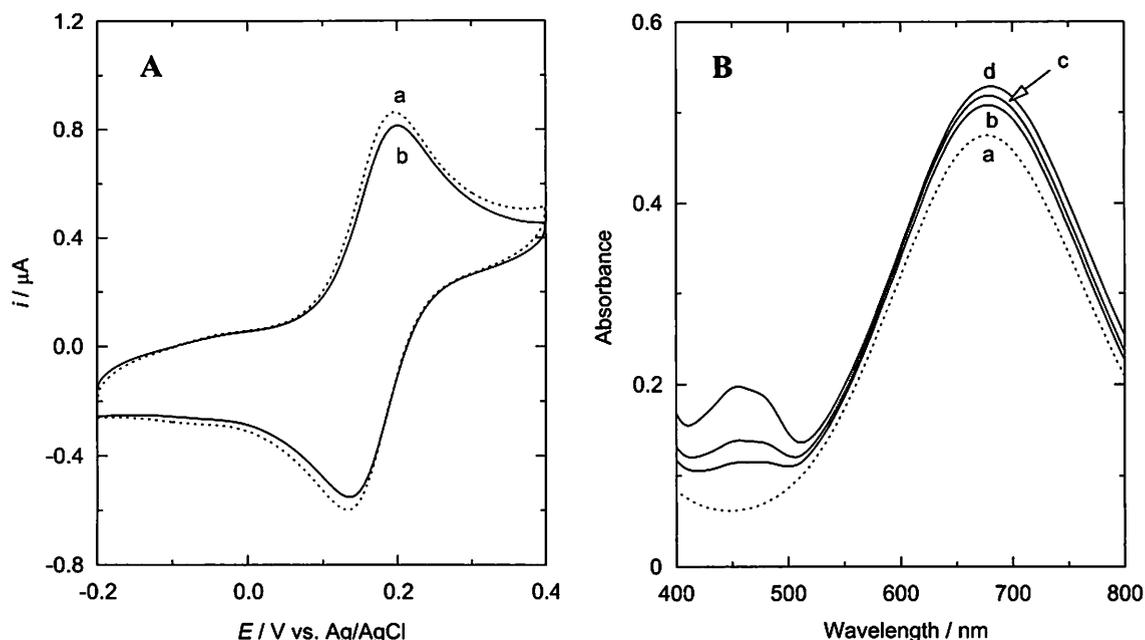
Fig. 4A  Cyclic voltammograms of 0.2 mM [Ru(pymq$^+$)(NH$_3$)$_5$]$^{3+}$ in 0.1 M phosphate buffer (pH 7.0) in the absence (a) and presence (b) of 0.1 mM GOx$_{ox}$. Scan rate, 50 mV s$^{-1}$.

   4B  Absorption spectra of 50 μM [Ru(pymq$^+$)(NH$_3$)$_5$]$^{3+}$ in 0.1 M phosphate buffer (pH 7.0) in the absence (a) and presence (b: 20; c: 30; d: 50 μM) of GOx$_{ox}$.

Table 3.  UV-vis (in 0.1 M phosphate buffer, pH 7.0) and $^1$H NMR (in D$_2$O) data of [Ru(pymq$^+$)(NH$_3$)$_5$]$^{3+}$ –FAD, –FMN, and –GOx systems

| Composition | $\lambda_{max}$[a) / nm | $\Delta\delta$ / ppm |
| --- | --- | --- | --- | --- | --- | --- | --- |
| | | H$_6$[b) | H$_9$[b) | H$_a$[c) | H$_b$[c) | H$_c$[c) | H$_d$[c) |
| [Ru(pymq$^+$)(NH$_3$)$_5$]$^{3+}$ only | 677 | | | | | | |
| [Ru(pymq$^+$)(NH$_3$)$_5$]$^{3+}$ + FAD | 682 | 0.121 | 0.004 | 0.028 | 0.129 | 0.009 | 0.127 |
| [Ru(pymq$^+$)(NH$_3$)$_5$]$^{3+}$ + FMN[d) | 681 | 0.013 | 0.060 | 0.004 | 0.007 | −0.009 | 0.069 |
| [Ru(pymq$^+$)(NH$_3$)$_5$]$^{3+}$ + GOx | 690 | | | | | | |

a) [Ru] = 50 $\mu$M, [FAD] = [FMN] = [GOx] = 100 $\mu$M (in 0.1 M phosphate buffer, pH 7.0)
b) $\Delta\delta = \delta_{flavin} - \delta_{flavin \cdot Ru}$   c) $\Delta\delta = \delta_{Ru} - \delta_{flavin \cdot Ru}$   d) Flavin mononucleotide



FAD or FMN                                        pymq$^+$
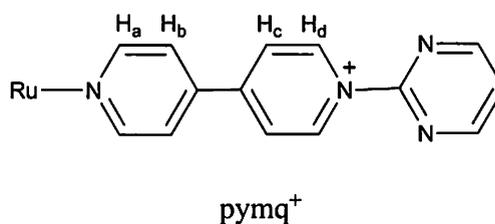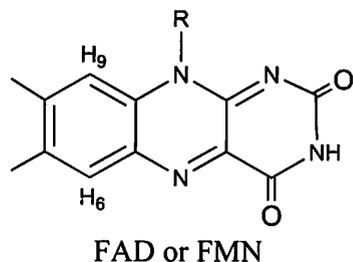
as an antenna that find FAD of the enzyme. This suggests the importance of the antenna strategy in the electron transfer mediator design; that is, an electron transfer mediator should contain a linear wire made of pyridinium ions.

## 4. Conclusions

In this paper, the mediation of dipolar ruthenium(II) ammine complexes containing pyridinium ions $[Ru(L^+)(NH_3)_5]^{3+}$ ($L^+$: pyridinium ion) in glucose oxidation was investigated using a voltammetric method. These ruthenium(II) complexes had appropriate redox potentials of 0.10–0.18 V vs. Ag/AgCl, which, in turn, enable the electrode to be polarized at a potential that does not give rise to interfering electrochemical reactions, and high values of $k_s$ of $5.7 \times 10^6$–$1.7 \times 10^7 M^{-1}s^{-1}$, which are the second-order rate constants for electron transfers from $GOx_{red}$ to $[Ru(L^+)(NH_3)_5]^{4+}$, to minimize competition with dissolved dioxygen. The $k_s$ values of $[Ru(L^+)(NH_3)_5]^{3+}$ were greater than those of osmium(II) complexes possessing similar redox potentials, which the ones most commonly used. This suggests that the electrostatic ruthenium(III/II) complex-GOx interaction and/or "antenna effect" of pyridinium ions introduced into GOx may contribute to greater $k_s$ values. Consequently, these dipolar ruthenium(II) complexes will be useful as electron transfer mediators for amperometric glucose sensors and biofuel cells. Our next step will be to design the amperometric glucose sensors and the anodes of biofuel cells using immobilized GOx and $[Ru(L^+)(NH_3)_5]^{3+}$ on the glassy carbon electrode surface.

## Acknowledgement

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