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Synthesis, properties, and biosensor applications of cycloruthenated 2-phenylimidazoles

Valentin S. Soukharev [a], Alexander D. Ryabov [b,*], Elisabeth Csöregi [c]

[a] Division of Chemistry, G.V. Plekhanov Russian Economic Academy, Stremyanny per. 28, 113054 Moscow, Russia
[b] Department of Chemistry, M.V. Lomonosov Moscow State University, 119899 Moscow, Russia
[c] Lund University, Department of Biotechnology, P.O. Box 124, 221 00 Lund, Sweden

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Abstract

The cycloruthenation of 2-phenylimidazole (phim) by $[Ru(\eta^6\text{-}C_6H_6)(\mu\text{-}Cl)Cl]_2$ in acetonitrile in the presence of NaOH has been carried out. The unstable intermediate $[Ru(phim)(MeCN)_4]PF_6$ formed has been converted into the complexes $[Ru(phim)(4,4'\text{-}Me_2bpy)(MeCN)_2]PF_6$ (**2**) and $[Ru(phim)(LL)_2]PF_6$ (**3**, LL = phen (**a**), bpy, 4,4′-Me_2bpy), which were characterized by the mass-spectrometry, ^{1}H-NMR spectroscopy, UV–vis spectrophotometry, and cyclic voltammetry. The $Ru^{II/III}$ redox potentials of complexes **3** equal 130–250 mV (vs. Ag–AgCl) at pH 7 (0.01 M phosphate). Such potential range is favorable for fast exchange of electrons with the active sites of redox enzymes. In fact, the second-order rate constant for the oxidation of reduced glucose oxidase (GO) from *Aspergillus niger* by the electrochemically generated Ru^{III} derivative of complex **3a** equals (8.1×10^6 M^{-1} s^{-1}). The second-order rate constant for the oxidation of **3a** by the Compound II of horseradish peroxidase is 9.3×10^7 M^{-1} s^{-1}. Complexes **3** were used as mediators for the fabrication of enzyme electrodes by simple co-adsorbing with GO or horseradish peroxidase on graphite electrodes. These electrodes were tested in flow-injection systems and showed linear responses in the range of D-glucose and H_2O_2 concentrations 0.1–30 mM and 1–200 μM, respectively. The new mediators reported herein seem promising for the construction of amperometric biosensors based on GO, horseradish peroxidase, and similar enzymes.

Keywords: Cyclometalation; Ruthenium complexes; Electron transfer; Mediators; Glucose oxidase; Horseradish peroxidase; Kinetics; Electrochemistry

1. Introduction

Cyclometalated complexes of platinum metals have been an active area of research during the last four decades [1,2]. For many years, these complexes were primarily used in organic synthesis [3–8], however, they have recently found their way into promising new applications [9], such as in bioorganometallic chemistry [10,11]. We have recently demonstrated that cationic cyclometalated ruthenium(II) derivatives of 2-phenylpyridine with diimine ligands such as bpy or phen, viz. $[Ru(phpy)(LL)_2]PF_6$, are highly efficient in the mediated electron transfer with redox enzymes [12]. In particular, the rate constants of ca. 10^7 M^{-1} s^{-1} were obtained for the oxidation of reduced active site of glucose oxidase (GO) by the Ru^{III} species generated electrochemically. The second-order rate constants for the oxidation of Ru^{II} into Ru^{III} by the HRP Compound II are ca. 10^8 M^{-1} s^{-1} [12]. The redox potentials of the 2-phenylpyridine derivatives in water were found to be in the range 265–340 mV vs SCE, a range that is somewhat higher than ideal for use in modern intelligent analytical bioelectronic devices. In an attempt to decrease this potential range, we looked at a smaller and more electron-rich 2-phenylimidazole ligand as an alternative to 2-phenylpyridine [13]. Smaller ligand size and increased electron-richness are factors that are favorable for improving mediator performance. Therefore, in this paper we report the first cycloruthenation of 2-pheny-

Abbreviations: bby, 2,2′-bipyridine; phen, 1,10-phenanthroline; GO, glucose oxidase; HRP, horseradish peroxidase.

* Corresponding author. Address: Department of Chemistry, Carnegie Mellon University, 4400 Fifth Avenue, Pittsburgh, PA 15213, USA. Fax: +1-412-268-1061.
E-mail address: ryabov@andrew.cmu.edu (A.D. Ryabov).

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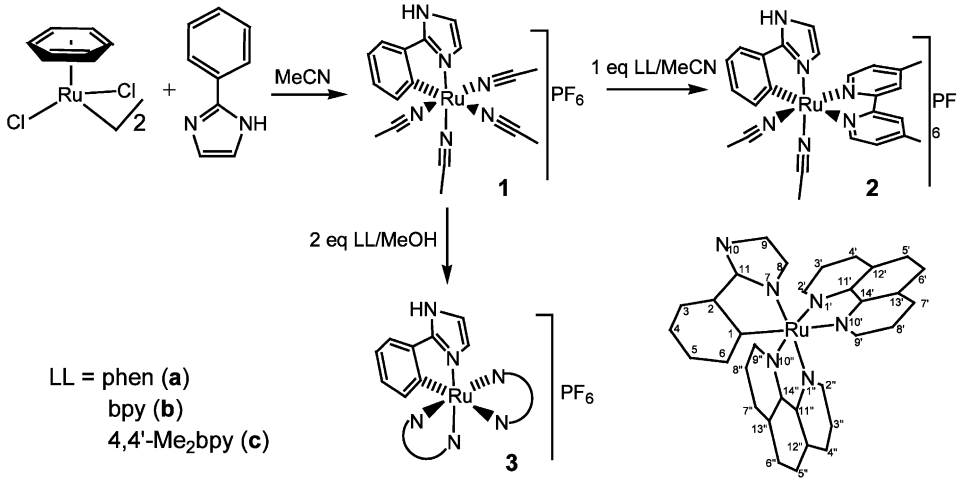
limidazole. Cyclometalation of 2-phenylimidazole derivatives by Pd^{II} has been known for more than two decades [14–16], but these were the *N*-alkylated imidazoles. Later, their cycloauration was reported, but the *N*-proteo derivative was not metalated [17]. Although cyclometalation of 2-phenylimidazoline by Pd^{II} and Pt^{II} was eventually achieved [18], similar reactions with 2-phenylimidazole remain problematic and therefore indirect synthetic routes to the corresponding Pd^{II} complexes were developed [19]. We have found that cycloruthenation of 2-phenylimidazole is feasible and the complexes $[Ru(phim)(LL)(MeCN)_2]PF_6$ (**2**) and $[Ru(phim)(LL)_2]PF_6$ (**3**) are isolated and characterized (Scheme 1). In addition, we have shown that their redox potentials are in fact lower than of the corresponding 2-phenylpyridine complexes, but they display the same order of reactivity in the GO- and HRP-catalyzed processes mentioned above. Complexes **3** were also tested as components of D-glucose and hydrogen peroxide biosensors. They were co-adsorbed on a graphite electrode with both GO and HRP. The performance of the sensors in the flow-injection system was found to be significantly higher than of traditional mediators such as formylferrocene or sodium 2,6-dichloroindophenol.

2. Results and discussion

2.1. Synthesis of new cycloruthenated compounds

The synthetic procedures used in this work, which are essentially that previously described for 2-phenylpyridine and *N*,*N*-dimethylbenzylamine [12], are summarized in Scheme 1. Direct activation of the *ortho* C–H bond of 2-phenylimidazole by the dimer $[Ru(\eta^6\text{-}C_6H_6)(\mu\text{-}Cl)Cl]_2$ in acetonitrile in the presence of NaOH [20] resulted in the formation of the easily oxidizable cyclometalated intermediate **1**. The empirical formula of **1** was determined by mass-spectral analysis and is consistent with the structure given in Scheme 1. The absence of η^6-coordinated benzene in **1** was confirmed by ^{1}H-NMR spectroscopy, but no attempts were made at further characterization due to instability of this yellow material, which becomes very soon greenish even under argon atmosphere. Instead, complex **1** was further allowed to react either with a stoichiometric amount of 4,4′-Me_2bpy in acetonitrile to afford complex **2c** in 44% yield, or with phen, bpy, or 4,4′-Me_2bpy in refluxing methanol to form **3a**–**c** in 70–80% yield. These transformations indicate that a variety of cycloruthenated 2-phenylimidazole derivatives can indeed be prepared by our methodology as shown in Scheme 1.

The new complexes **2** and **3** were characterized by mass-spectrometry, ^{1}H-NMR spectroscopy, UV–vis spectrophotometry, and cyclic voltammetry (Table 1). In general, their properties match those of 2-phenylimidazole and *N*,*N*-dimethylbenzylamine derivatives obtained previously as shown in Scheme 1[12], or by the use of *cis*-$[RuCl_2(LL)_2]$ a metalating agents [21–24]. In particular, there are 22, 22, and 18 resonances from the aromatic protons in the spectra of complexes **3a**–**c**, respectively. For each of these complexes, the two imidazole ring protons are singlets which are strongly separated. The upfield resonance from H8 is seen at ca. δ 6.0 due to shielding by the ring current from adjacent aromatic rings of the diimine ligands [25]. Its counterpart, H9, is shifted by almost 1 ppm downfield. The protons from the ruthenated aromatic ring appear at higher field, as in all previous cases reported. Interestingly, the resonances from H6 and H5 are broadened, the degree of broadening increases in the series **3a** < **3b** < **3c**. None of the other protons is broadened except H10, which is seen at the lowest field. As expected, there



Scheme 1.

Table 1
Yields and spectral properties of new cycloruthenated complexes **2c** and **3a**–**c**

Complex (yield, %)	Mw (M^+)	MS: m/z (M^+)	^{1}H-NMR (δ, CD_2Cl_2)[a]	UV–vis in MeOH: λ, nm (ε, M^{-1} cm^{-1})
2c(44)	510.58	510(28%), 469(35%, −MeCN), 427(100%, −2MeCN)	2.25s, 2,28s, 2.38s, and 2.65s (CH_3 of MeCN and 4,4′-Me_2bpy), 5.85s (H8), 6.74s (H9), 6.79d (H3′), 7.01t (H4), 7.20t (H5), 7.45d (H3), 7.54d (H8′), 7.85s and 8.07s (H12′ and H13″), 7.90d (H2′), 8.09d (H6), 9.13d (H9′), 9.65b(H10)	236 (38 950), 295 (52 450), 348 (9230), 472 (6360), 566 (3620)
3a(83)	604.7	605	6.04s (H8); 6.23d (H6); 6.70t (H4); 6.80t (H5); 6.94s (H9); 7.48d (H3); 7.39m, 7.59m (2H), 7.65m (H3′, H8′, H3″, H8″); 8.00–8.24m(H5′, H6′, H5″, H6″); 8.26d, 8.35d, 8.43d, 8.67d (H2′, H9′ H2″, H9″); 9.93b (H10)	222 (57 800), 263 (62 650), 490 (12 500), 543 (12 490)
3b(79)	556.6.6	557	6.01m (H6, H8); 6.55t (H5); 6.83s (H8); 6.85t (H4); 7.13t, 7.21m (2H), 7.34t (H3′, H8′, H3″, H8″); 7.54d (H3); 7.65t, 7.72m (3H) (H4′, H7′, H4″, H8″); 7.89m (3H), 8.14m (H4′, H13′, H4″, H13″); 8.15d, 8.22d, 8.25d, 8.31d (H2′, H9′, H2″, H9″); 9.95b (H10)	247 (39 750), 294 (49 650), 370 (12 800), 563 (10 700)
3c(68)	612.7	613	2.53s (6H), 2.60s (3H), 2.78s (3H) (CH_3); 5.72b (H6); 5.89s (H8); 6.35b (H5); 6.89d (H9); 7.04d, 7.07d, 7.20d, 7.22d (H3′, H8′, H3″, H8″); 7.27b (H4), 7.64b (H3); 7.94s, 8.02s, 8.07s, 8.17s H2′, H9′ H2″, H9″); 9.91b (H10)	251 (35 950), 292 (47 750), 373 (11 700), 567 (8630)

[a] For the numbering scheme, see Scheme 1.

are 4 methyl resonances from 4,4′-Me_2bpy in the spectrum of **3c**.

There are four methyl singlets observed in the ^{1}H-NMR spectrum of **2c**. They are attributed to the two diastereotopic acetonitrile ligands and the two CH_3 groups of 4,4′-Me_2bpy. The down-field resonances are well resolved and could be unambiguously assigned (Table 1) on the assumption that the MeCN ligands are mutually *cis* and the σ-bound carbon is *trans* to the nitrogen of 4,4′-Me_2bpy. In fact, all four crystallographically characterized compounds of this type possess such a geometry [12,26,27]. This assumption allows us to rationalize that the down-field shifted resonances from H9′ and H6, at δ 9.13 and 8.09, respectively, are due to the deshielding effect of the adjacent acetonitrile ligands.

As expected, **3a**–**c** are strongly absorbing species in the visible region (Table 1) and their redox potentials are in the range 130–250 mV (Table 2) in accord with the electronic properties of the diimine ligands. Thus, the Lever concept for predicting redox potentials of transition metal complexes seems to work in this case as well [28]. In total, the new Ru^{II} complexes are oxidized at a 100 mV lower potential than the corresponding 2-phenylpyridine complexes.

Table 2
Redox potentials and rate constants for electron transfer between GO/HRP and Ru^{III}/Ru^{II}, respectively

Complex	E'°, mV	GO: $10^{-6} \times k_3$, M^{-1} s^{-1}	HRP: $10^{-7} \times k_3$, M^{-1} s^{-1}
2c	280	1.2±0.2	–
3a	250	8.1±0.3	9.3±0.9
3b	205	4.8±0.5	2.3±0.4
3c	130	5.2±0.9	6.5±0.3

2.2. Kinetic evaluation of new cycloruthenated compounds as mediators of GO and HRP

The new compounds were tested in enzymatic reactions with GO and HRP. The second-order rate constants for the oxidation of reduced GO by electrochemically generated Ru^{III} species were obtained from the studies of complexes **2** and **3** by cyclic voltammetry in the presence of D-glucose and GO as it was described in our previous work [12]. Fig. 1 shows cyclic voltammograms of **3c** and D-glucose in the absence and in the presence of 10^{-6} M GO. The current increases substantially in the latter case indicative of a fast interaction

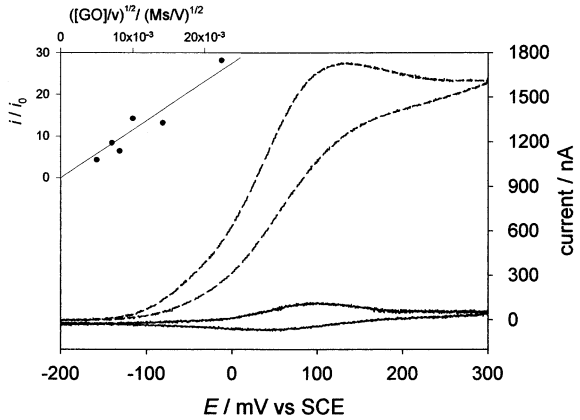


Fig. 1. Cyclic voltammograms of **3c** (1×10^{-4} M) without (solid line) and with (dashed line) 1×10^{-6} M GO and 0.05 M D-glucose; pH 7 (0.01 M phosphate), scan rate 5 mV s^{-1}, 25° C. Inset: plot for evaluating the rate constant k.

between the reduced enzyme and Ru^{III} as shown in Scheme 2 (Eqs. 1, 2 and 3). The inset illustrates the plot for calculating the second-order rate constant k according to the accepted routine [29] on the standard assumption that the rate-limiting step is a transfer of the first electron from reduced flavin adenin dinucleotide ($FADH_2$). The rate constants obtained equal $(4–8) \times 10^6\ M^{-1}\ s^{-1}$ (Table 2).

$$\text{D-glucose} + \text{GO(ox)} \rightarrow \text{D-}\delta\text{-gluconolactone} + \text{GO(red)} \quad (1)$$

$$\text{GO(red)} + 2Ru^{III} \rightarrow \text{GO(ox)} + 2Ru^{II} \quad (k) \quad (2)$$

$$2Ru^{II} - 2e \rightarrow 2Ru^{III} \quad \text{(electrode)} \quad (3)$$

The steady-state rate constants k_3 for oxidation of Ru^{II} into Ru^{III} by the Compound II of HRP, generated from the native enzyme in the presence of H_2O_2, were determined spectrophotometrically as described previously [12]. Fig. 2 demonstrates the dependence of the steady-state rate of HRP-catalyzed oxidation of **3c** as a function of [**3c**]. The data obtained were fitted to Eq. (4),

$$\text{rate} = \frac{2k_1k_3[Ru^{II}][H_2O_2][HPR]}{k_1[H_2O_2] + k_3[Ru^{II}]} \quad (4)$$

which is true for catalysis by HRP when the reactivity of Compound I is much higher than that of Compound II, a relation that holds for organometallic substrates of HRP [30], Scheme 3 (Eqs. 4, 5 and 6).

$$\text{HRP} + H_2O_2 \rightarrow \text{HRP-I} \quad k_1 \quad (5)$$

$$\text{HRP-I} + Ru^{II} \rightarrow \text{HRP-II} + Ru^{III} \quad k_2 \quad (6)$$

$$\text{HRP-II} + Ru^{II} \rightarrow \text{HRP} + Ru^{III} \quad k_3 \quad (7)$$

The rate constants obtained are listed in Table 2. The data indicate clearly that the complexes are very reactive toward HRP. The rate constants are close to those previously reported for complexes based on 2-phenylpyridine [12]. Remarkably, their redox potentials are lower, making them more useful as mediators of the redox enzymes.

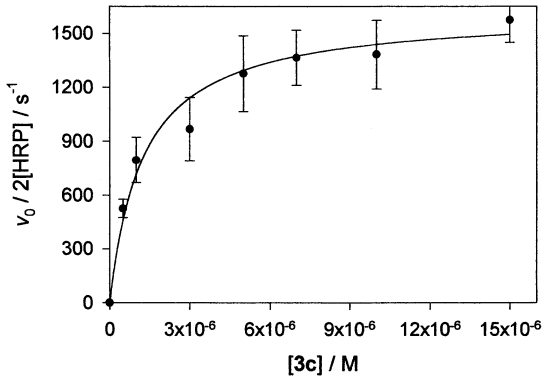


Fig. 2. Initial rate of HRP-catalyzed oxidation of **3c** by H_2O_2 against the concentration of **3c**: 25°C, $[H_2O_2]$ 2.0×10^{-4} M, [HRP] 5×10^{-11} M, 0.01 M phosphate, pH 7.

2.3. Enzyme electrodes based on cycloruthenated complexes

The novel mediators **3** were evaluated as constituents of amperometric biosensors for D-glucose and H_2O_2 in combination with GO and HRP, respectively. The electrodes were made using a simple adsorptive technique involving successive deposition of mediator and enzyme from their solutions. The adsorbed complexes **3** exhibited ca. 100 mV higher redox potentials than in solution. Such a behavior was also observed for the osmium and ruthenium complexes $[M(R\text{-}bpy)_3]^{2+}$ [31], indicative of a hydrophobic environment of the adsorbed complexes and suggesting a common adsorption mechanism. An increase in redox potential does not compromise the mediating properties of the cycloruthenated complexes. Fig. 3A shows cyclic voltammograms of the **3c**/GO electrode in the absence and in the presence of D-glucose. The voltammogram in the presence of D-glucose indicates a catalytic process as shown in Scheme 2. Fig. 3B illustrates a substantial increase in the cathodic current for the **3c**/HRP electrode on addition of H_2O_2 to the solution. The electrocatalytic process in this case is accounted for in terms of Scheme 3 plus an electrode reaction $Ru^{III} + e \rightarrow Ru^{II}$ involving the reduction of the enzymatically generated Ru^{III} species at the electrode.

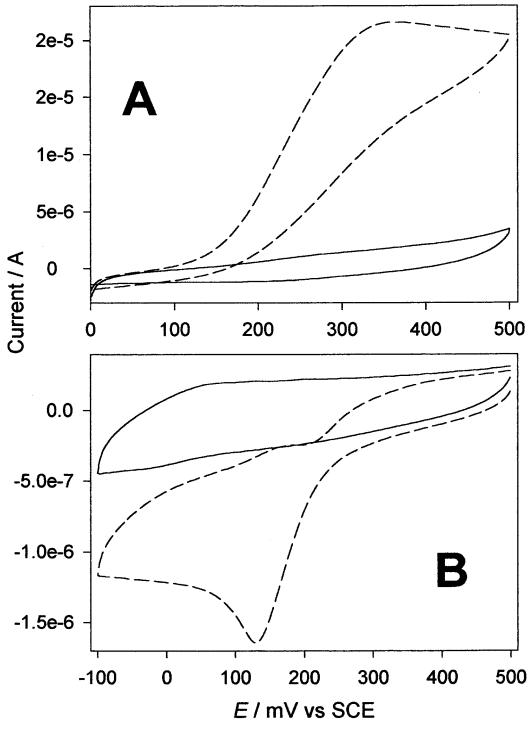


Fig. 3. (A) Cyclic voltammogram of the **3c**/GO electrode in solution of the supporting electrolyte (solid line), and after addition of 50 mM D-glucose (dashed line). (B) Cyclic voltammogram of the **3c**/HRP electrode (solid line), and after addition of 2 mM H_2O_2 (dashed line): 0.1 M phosphate, pH 7; scan rate, 2 mV s^{-1}.

The electrodes thus made were tested in the flow-injection system. The applied potentials were 250–350 and −50 mV vs. Ag–AgCl for the glucose and H_2O_2 sensor, respectively. Calibration curves are presented in Fig. 4. Linear responses were observed in the concentration ranges of 0.1–30 mM for D-glucose and 1–200 μM for H_2O_2. The operational stability of the electrodes was determined by injecting 10 mM of glucose for the GO electrode and 500 μM of H_2O_2 for the HRP electrode. Reproducibility of the GO and HRP electrodes was 85–95% for the duration of 2 and 1 h, respectively. This is a good stability for so easily made electrodes [32]. The performance of the cycloruthenated complexes was compared with conventional mediators, formylferrocene and sodium 2,6-dichloroindophenol. All electrodes were prepared and tested similarly. The data obtained is summarized in Table 3. As seen, the sensitivity of the **3**-based electrodes is 2–10 times higher than that of electrodes with the conventional mediators due to much higher rates of steps 2 and 7 in GO and HRP biosensors, respectively.

In conclusion, cycloruthenation of 2-phenylimidazole by $[Ru(\eta^6\text{-}C_6H_6)(\mu\text{-}Cl)Cl]_2$ is the first easy entry to the cyclometalated non-*N*-alkylated imidazole derivatives. This is important for the creation of Ru-based mediators of electron transport between redox enzymes and electrodes, since utilization of 2-phenylimidazole rather than its *N*-alkylated derivatives does not increase the size and hydrophobicity (hence, provides better aqueous solubility) of the complexes. Both factors are important when these complexes are used as mediators. The approach described herein allows for the synthesis of a family of versatile mediators having high second-order rate constants for the oxidation of GO or the reduction of horseradish peroxidase. The graphite electrodes with co-adsorbed the ruthenium complexes and enzymes allow for easy determination of D-glucose and hydrogen peroxide concentrations.

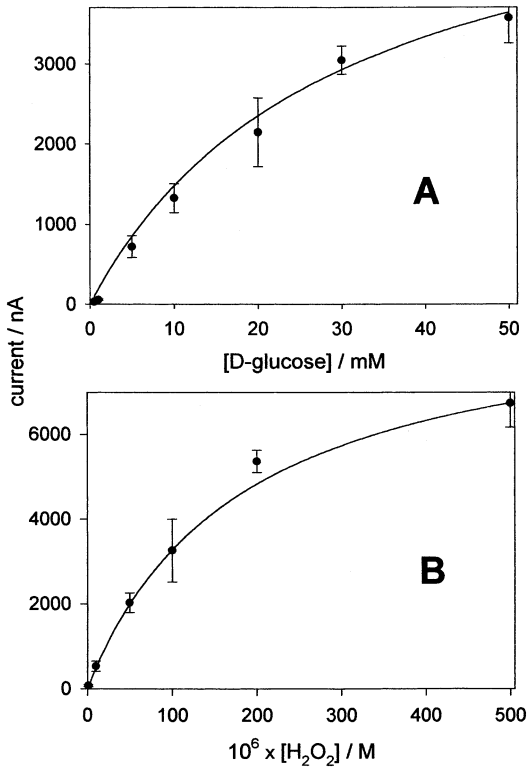


Fig. 4. Calibration curves for the **3c**/GO (A) and **3c**/HRP electrode (B) in the flow-injection system: 0.1 M phosphate, pH 7, flow rate 0.5 ml min^{-1}.

3. Experimental

3.1. Materials and methods

GO from *Aspergillus niger* was purchased from Serva. Horseradish peroxidase ($R/Z = 3$), $RuCl_3 \cdot nH_2O$ and 1,3-cyclohexadiene were obtained from Aldrich. All spectral grade solvents were received from Acros. Other analytical grade chemicals were obtained elsewhere. The dimer $[Ru(\eta^6\text{-}C_6H_6)(\mu\text{-}Cl)Cl]_2$ was prepared following the literature procedure [33]. Mass-spectra were obtained using a Jeol SX-102 instrument with 3-nitrobenzyl alcohol as the matrix. ^{1}H-NMR spectra were recorded on a Bruker DRX 500 MHz spectrometer.

3.2. Synthesis of ruthenacyclic complexes

3.2.1. Complex **1** (adapted from Ref. [20])

A suspension of $[Ru(\eta^6\text{-}C_6H_6)(\mu\text{-}Cl)Cl]_2$ (0.2 g, 0.4 mmol), 2-phenylimidazole (0.115 g, 0.8 mmol), NaOH (0.03 g, 0.8 mmol) and KPF_6 (0.292 g, 1.6 mmol) in 15 ml of acetonitrile was stirred at 50 °C for 24 h under argon. The resulting dark yellow suspension was filtered over Al_2O_3 using a mixture of CH_2Cl_2:MeCN (10:1) as the eluent. The first yellow band was collected, concentrated in vacuum, and the yellow material was precipitated by addition of hexane.

3.2.2. Complexes ***3a–c***

A solution of **1** (0.1 g, 18 mmol) and two equivalents of LL in 20 ml of MeOH was refluxed for 3 h under argon. The solvent was evaporated, the residue redissolved in CH_2Cl_2 and chromatographed on the Al_2O_3 column using CH_2Cl_2:MeCN (10:1) as eluent. The first dark-reddish band was collected, concentrated, and the dark solid was precipitated by the addition of hexane. Satisfactory C, H, N analyses were obtained for **3a–c**. Spectral properties of the new complexes are summarized in Table 1.

3.2.3. Complex ***2c***

Complex **1** (0.115 g, 0.2 mmol) was stirred with 4,4′-Me_2bpy (30 mg, 0.16 mmol) in 15 ml of acetonitrile for

Table 3
The performance of enzyme sensors in a flow-injection system

Mediator	Working potential, mV vs. Ag–AgCl	Sensitivity, mA M^{-1} cm^{-2}	
		Mediator/GOx electrode	Mediator/HRP electrode
3a	350	2.5	450
3b	300	2.0	510
3c	250	1.9	580
Formylferrocene	300	0.3	230
Sodium 2,6-dichloroindophenol	350	0.5	150

24 h under argon. The solvent was evaporated, the residue was redissolved in CH_2Cl_2 and chromatographed on the Al_2O_3 column (eluent CH_2Cl_2). The first dark reddish band was separated, the volume reduced to 3 ml, complex **2c** was precipitated by addition of hexane, and after filtration 58 mg (0.088 mmol, 44%) of **2c** was collected which showed satisfactory C, H, N analysis.

3.3. Kinetic and electrochemical measurements

Spectrophotometric and kinetic measurements were carried out on a Shimadzu UV-160A spectrophotometer equipped with a CPS-240A cell positioner/temperature controller. Cyclic voltammograms were obtained on a PC-interfaced IPC BAS 50W Electrochemical Analyzer (Bioanalytical System Inc., USA). A three-electrode scheme was used with the working glassy carbon electrode, Ag–AgCl reference electrode, and auxiliary Pt electrode. Flow-injection measurements were done in a single channel system, which contained a manual sample injection valve (Valco Instruments Co Inc, USA) equipped with a 100 μl injection loop. A peristaltic pump (Alitea AB, Sweden) pumped the carrier solution at a flow rate of 0.5 ml min^{-1} to a flow-through wall-jet electrochemical cell through Teflon tubes (diameter 0.5 mm). A potentiostat (Zäta-Elektronik, Sweden) was used to maintain the working potential. The enzyme-modified graphite, Ag–AgCl electrode and a platinum wire were the working, reference, and counter electrodes, respectively. The resulting current was monitored with a single channel strip-chart recorder (Model BD 111, Kipp&Zonen, The Netherlands). The kinetic measurements and experiments in the flow-injection system were done in 0.01 and 0.1 M phosphate buffer, respectively, pH 7.

The rate constants k for step 2 were calculated from the electrochemical data [29] by plotting the ratio between the catalytic limiting current (i) and the diffusion-controlled peak current (i_0) against $([GO]/v)^{1/2}$ (an example is shown as inset in Fig. 1). The rate constants were calculated from the slope of the linear plots.

3.4. Electrode fabrication

Enzyme-modified graphite electrodes were prepared as follows. Rods of spectroscopic-grade graphite were cut, polished with a fine Emery paper (Tufback, Durite P1200, USA), rinsed with water, and air-dried at room temperature. Next, 6 μl of a mediator solution in MeOH (2 mg ml^{-1}) were syringed on the electrode surface, and, after drying, 6 μl of the mixture consisting of enzyme (5 mg ml^{-1} for GO and 2 mg ml^{-1} for HRP) and polyethyleneglycol diglycidyl ether (0.5 mg ml^{-1}) were added on the electrode surface. The coated electrodes were allowed to cure for 24 h at room temperature. All the electrodes were thoroughly rinsed with phosphate buffer before use.

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