Complexation of ruthenium with glucose oxidase modified by 4-pyridineacetic and 4-imidazoleacetic acids

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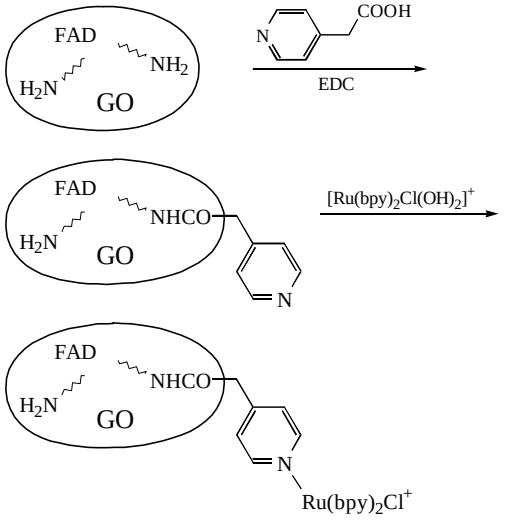
Glucose oxidase (GO) conjugated with 4-pyridineacetic and 4-imidazoleacetic acids reacted with *cis*-[Ru(bpy)$_2$Cl$_2$] to afford the Ru-containing catalytically active species Ru–X–GO (X = pyridine or imidazole) capable of the intramolecular electron transfer from reduced FAD to an electrochemically generated Ru^{III} centre.

Our current interests are aimed at understanding the intimate mechanistic features of the interaction between redox enzymes and transition metal species.[1–6] Recently,[7] it has been demonstrated that the coordination of bis(2,2′-bipyridine) or bis(1,10-phenanthroline) ruthenium(II/III) complexes with glucose oxidase (GO) from *Aspergillus niger* presumably *via* histidine residues affords the biocatalyst (Ru–GO) capable of an efficient Ru-mediated electron exchange between the active site of the modified enzyme and an electrode. Thus, the intrinsic natural donor ligands of the enzyme served as traps for the ruthenium centres. Searching for more efficient electron transfer relays and with the goal to compare the electrocatalytic properties of different preparations of modified GO, we have prepared and investigated the properties of the enzyme first covalently conjugated with 4-pyridineacetic or 4-imidazoleacetic acid *via* surface amino groups of the protein using a water-soluble carbodiimide followed by the complexation of *cis*-[Ru(bpy)$_2$Cl$_2$] to the introduced pyridine or imidazolyl moieties (Scheme 1).

Glucose oxidase (Serva, 220U) was modified by 4-pyridineacetic acid (PAA) (Aldrich) as described elsewhere.[8] PAA (4.3 mg), NaHEPES (20 mg), and urea (48 mg) were dissolved in water (400 μl). The solution was adjusted to pH 7.0. Next, GO (10 mg) was introduced, and 5 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, Sigma) was added. The solution was readjusted to pH 7.0 and allowed to stand overnight (16 h) in an ice bath. The chemically modified enzyme was separated by gel filtration chromatography on a Sephadex G-50 (fine) column equilibrated with a 0.1 M phosphate buffer (pH 7.0). The same buffer was used as an eluent. The first eluted yellow fraction contained the modified enzyme (py–GO), which was then concentrated to 600 μl by ultrafiltration using a 100000 cut-off membrane (Amicon). The modification of GO



Scheme 1

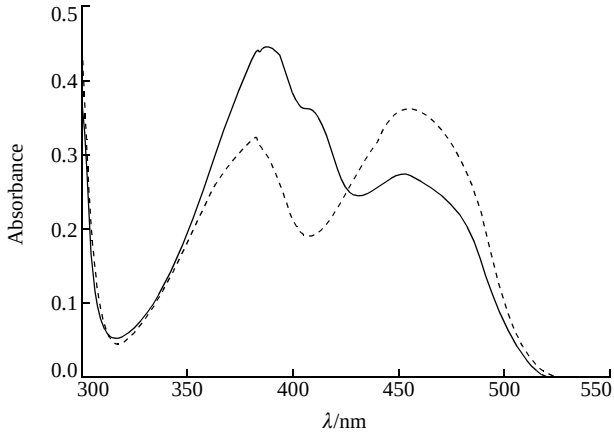


Figure 1 UV–VIS spectra of native GO (solid line) and GO covalently conjugated with 4-pyridineacetic acid (py–GO) (broken line) recorded at pH 7.0 (0.1 M phosphate) and 22±2 °C. Concentrations of the enzyme preparations were about 4 mg ml^{-1}.

by 4-imidazoleacetic acid (IAA) (ACROS) to afford im–GO was carried out similarly using 3.9 mg (2.45×10^{-5} mol) of IAA, 10 mg (6.25×10^{-8} mol) of GO, 5 mg (2.6×10^{-5} mol) of EDC, 48 mg (8×10^{-4} mol) of urea and 20 mg (7.68×10^{-5} mol) of NaHEPES in 400 ml of H_2O.

To prepare the ruthenium-modified enzyme (Ru–py–GO), the complex *cis*-[Ru(bpy)$_2$Cl$_2$] (1 mg) was dissolved in 200 μl of a phosphate buffer (pH 7.0), and 180 μl of this solution was added to 600 μl of the yellow solution of py–GO. Urea necessary to yield a 2 M solution was added, and the resulting solution was kept at room temperature for 4 h. Ru–py–GO was then separated on a Sephadex G-50 (fine) column. Two fractions were collected. A comparison of the UV–VIS spectra recorded at pH 7.0 (0.1 M phosphate) with that obtained for *cis*-[Ru(bpy)$_2$Cl$_2$] showed that the second fraction contained the unbound ruthenium complex. The enzyme-containing first fraction was concentrated to 600 μl by ultrafiltration to yield a 12.3 mg ml^{-1} solution of Ru–py–GO. The protein concentration was determined by the Lowry procedure[9] using native GO as a standard. The enzymatic activities of native GO and Ru–py–GO solutions were determined colorimetrically by measuring the rate of bleaching of 2,6-dichlorophenol–indophenol.[10] The activity of Ru–py–GO was 70% with respect to the native enzyme. Ru–im–GO was prepared similarly; its catalytic activity toward 2,6-dichlorophenol–indophenol was 65%.

The UV–VIS spectra of GO and py–GO clearly show that the enzyme surface is modified with PAA (Figure 1). The spectra of im–GO and Ru–im–GO presented in Figure 2 (curves *1* and *2*) clearly demonstrate that the coordination of ruthenium species with the modified enzyme provides noticeable spectral changes. The addition of an excess of D-glucose to Ru–py–GO and Ru–im–GO induces the expected spectral changes in both cases

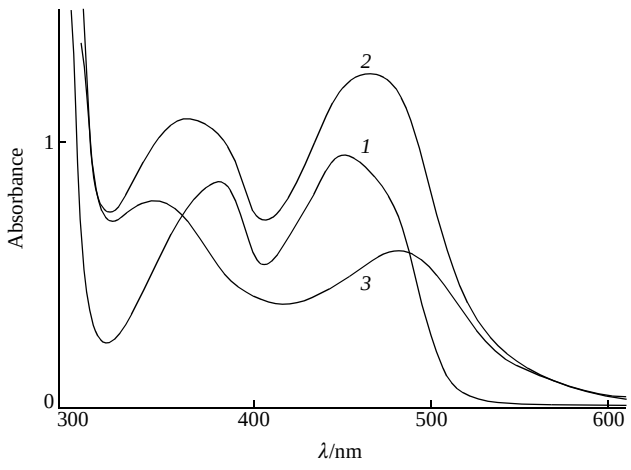


Figure 2 UV–VIS spectra of (*1*) im–GO, (*2*) Ru–im–GO and (*3*) the product obtained after addition of D-glucose (0.02 M) to the Ru–im–GO at 22±2 °C. Concentrations of the enzyme preparations were 10 mg ml^{-1}; pH (*1*) 5.5 and (*2*,*3*) 7.0.

associated with the reduction of flavine adenine dinucleotide (FAD) into $FADH_2$ and with partial conversion of Ru^{III} into Ru^{II} (Figure 2, curve *3*). As can be seen, the intensity of the broad band at 465 nm, where the contribution from FAD is dominant, drops due to reduction of the latter into $FADH_2$. Thus, there is a maximum shift since a new band at 482 nm corresponds predominantly to Ru^{II} species. The reduction of Ru^{III}, which is most likely generated during the gel filtration, occurs due to the glucose oxidase–catalysed oxidation of β-D-glucose into δ-D-gluconolactone, which is accompanied by the reduction of the oxidised FAD into $FADH_2$ (equation 1)[11] followed by the intramolecular reoxidation of the reduced enzyme into its catalytically active form by Ru^{III} (equation 2):[7]

$$Ru^{III}\text{–im–GO(ox)} + \beta\text{-D-glucose} \longrightarrow Ru^{III}\text{–im–GO(red)} + \delta\text{-D-gluconolactone}; \quad (1)$$

$$Ru^{III}\text{–im–GO(red)} \xrightarrow{k} Ru^{II}\text{–im–GO(ox)}. \quad (2)$$

Assuming that Ru^{II} is the only species absorbing at 482 nm, the amount of the 'catalytically active' ruthenium, *viz*. involved in reaction (2), can be estimated from the spectral changes in Figure 2 (curves *2* and *3*) using the molar absorption coefficient obtained for *cis*-$[Ru(bpy)_2Cl_2]$ in the presence of 4-imidazoleacetic acid (ε = 5660 dm^3 mol^{-1} cm^{-1} at 475 nm). The calculations performed according to the procedure in ref. 7 taking into account the independently determined catalytic activity of the enzyme samples suggest that the Ru–im–GO preparation contains 1.8 active ruthenium centres per protein molecule. The same approach applied to Ru–py–GO gives practically the same value (1.5).

The electrochemical properties of Ru–py–GO and Ru–im–GO were investigated by cyclic voltammetry both in the presence and in the absence of D-glucose. The representative data are shown in Figures 3 and 4. As can be seen in Figures 3 (curve *1*) and 4 (curve *1*), the cyclic voltammograms of Ru–py–GO and Ru–im–GO are characterised by weak signals from coordinated ruthenium around 300 mV *versus* Ag/AgCl (288 and 305 mV, respectively). The addition of D-glucose to both preparations leads to a current increase indicative of the coupling between the electrochemically generated Ru^{III} species and $FADH_2$ of reduced glucose oxidase. Therefore, in addition to reactions (1) and (2), the third step should be added to accomplish the electrocatalytic cycle which accounts for the origin of catalytic currents in Figures 3 (curve *2*) and 4 (curve *2*).

$$Ru^{II}\text{–im–GO(ox)} - 1e \longrightarrow Ru^{III}\text{–im–GO(ox)}. \quad (3)$$

As in the previous work,[7] the rate constants for the intramolecular electron transfer according to reaction (2) were estimated using computer simulation of the data displayed in Figures 3 and 4 as described elsewhere.[12] The model applied is given below:

$$FADH_2\text{–}M^{II} \rightleftharpoons FADH_2\text{–}M^{III} + e\ (E_1^0\ \text{electrode});$$
$$FADH_2\text{–}M^{III} \longrightarrow FADH\text{–}M^{II} + H^+\ (k_1);$$
$$FADH\text{–}M^{II} \rightleftharpoons FADH\text{–}M^{III} + e\ (E_2^0\ \text{electrode});$$
$$FADH\text{–}M^{III} \longrightarrow FAD\text{–}M^{II} + H^+\ (k_2);$$
$$FAD\text{–}M^{II} \longrightarrow FADH_2\text{–}M^{II}\ (k_3).$$

The following assumptions have been made: $k_1 = k_2$ and $E_1^0 = E_2^0$; k_3 = 800 s^{-1} (saturating glucose concentration).[13] The rate constants thus obtained are equal to 0.55 and 2 s^{-1} for Ru–py–GO and Ru–im–GO, respectively, at 22±2 °C and pH 7. These should be compared with the rate constant of 12 s^{-1} previously obtained[7] for native glucose oxidase modified by *cis*-$[Ru(bpy)_2Cl_2]$. The comparison suggests that the coordinative loading of Ru complexes onto the enzyme surface without pretreatment with artificial donor centres is more advantageous for the preparation of bioelectrocatalysts capable of the intramolecular electron transfer from the reduced cofactor ($FADH_2$) at the metal centre. Note that the rate constants obtained in this work are very close to those reported for GO randomly modified with ferrocenecarboxylic acid residues.[14] The highest rate constant of 3.6 s^{-1} was calculated for the enzyme with 13 ferrocene units.[14]

In conclusion, the procedure for loading electroactive ruthenium species onto glucose oxidase involving covalent attachment of

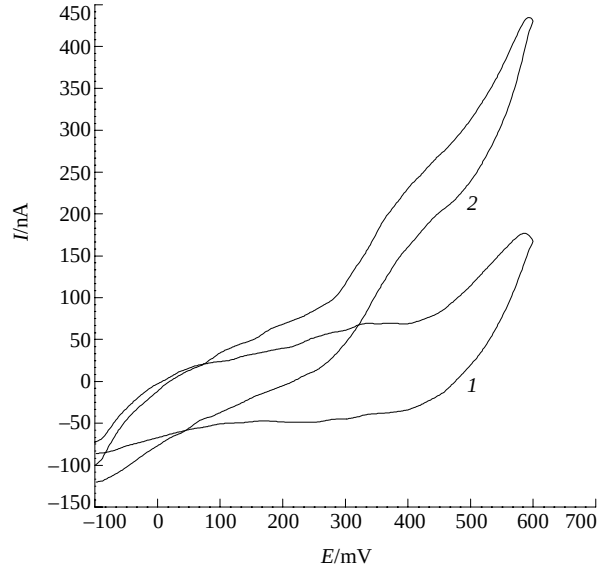


Figure 3 Cyclic voltammograms of Ru–py–GO (*1*) in the absence and (*2*) in the presence of D-glucose (0.033 M); [Ru–py–GO], 10 mg ml^{-1}, pH 7, 0.01 M phosphate, scan rate 2 mV s^{-1}, 22±2 °C.

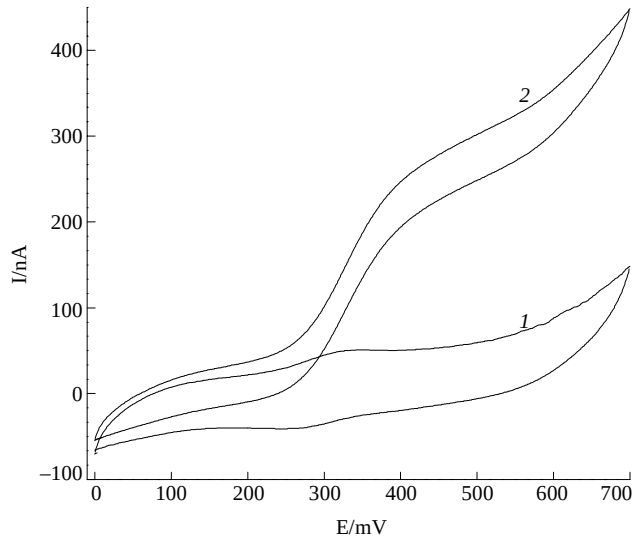


Figure 4 Cyclic voltammograms of Ru–im–GO (*1*) in the absence and (*2*) in the presence of D-glucose (0.033 M); [Ru–im–GO], 10 mg ml^{-1}, pH 7, 0.01 M phosphate, scan rate 2 mV s^{-1}, 22±2 °C.

4-pyridineacetic and 4-imidazoleacetic acids followed by the complexation with *cis*-[Ru(bpy)$_2$Cl$_2$] affords biocatalysts the efficacy of which in the intramolecular electron transfer reaches the same level as in the case of GO covalently modified with ferrocenecarboxylic acid, but somewhat lower than in the case of native GO coordinatively enriched with *cis*-[Ru(bpy)$_2$Cl$_2$].

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