DOI: 10.1002/elan.201500479

Evaluation of the Oxo-bridged Dinuclear Ruthenium Ammine Complex as Redox Mediator in an Electrochemical Biosensor

Paulo A. Raymundo-Pereira,[a, b] Ana C. V. Mascarenhas,[a] and Marcos F. S. Teixeira*[a]

Abstract: The mediation of electron-transfer by oxo-bridged dinuclear ruthenium ammine $[(bpy)_2(NH_3)Ru^{III}(\mu\text{-}O)Ru^{III}(NH_3)(bpy)_2]^{4+}$ for the oxidation of glucose was investigated by cyclic voltammetry. These ruthenium (III) complexes exhibit appropriate redox potentials of 0.131–0.09 V *vs.* SCE to act as electron-transfer mediators. The plot of anodic current *vs.* the glucose concentration was linear in the concentration range between 2.52×10^{-5} and 1.00×10^{-4} mol L^{-1}. Moreover, the apparent Michaelis-Menten kinetic (K_M^{app}) and the catalytic (K_{cat}) constants were 8.757×10^{-6} mol L^{-1} and 1,956 s^{-1}, respectively, demonstrating the efficiency of the ruthenium dinuclear oxo-complex $[(bpy)_2(NH_3)Ru^{III}(\mu\text{-}O)Ru^{III}(NH_3)(bpy)_2]^{4+}$ as mediator of redox electron-transfer.

Keywords: Oxo-bridged dinuclear ruthenium ammine complex · Redox Mediator · Electron Transfer · Electrochemical Biosensor · Glucose Oxidase (*Aspergillus niger*)

1 Introduction

The redox properties of ruthenium polynuclear oxo-complex have been studied with special attention to the stability of the oxidation states of the metallic cation, and many studies are based on qualitative molecular orbital interaction schemes $d\pi_{(metal)}-p\pi_{(ligand)}$ [1–4]. These polynuclear oxo complexes exhibit electron transfer processes with multiple steps that result in a high stability in different oxidation states and the ability to exchange charges between metallic cations. Notably, a wide range of dinuclear ruthenium complexes have been chemically and/or electrochemically studied [5,6]. The complexes $[(bpy)_2(H_2O)Ru^{III}(\mu\text{-}O)Ru^{III}(H_2O)(bpy)_2](ClO_4)_4$ (where bpy = 2,2'-bypyridyne) and $[(NH_3)_5Ru^{III}-O-Ru^{IV}(NH_3)_4-O\text{-}Ru^{III}(NH_3)_5]^{6+}$ are examples that have been intensively studied by Meyer's [7,8] and Kaneko's groups [9,10], respectively. These complexes were investigated for their ability to catalyse water oxidation in an artificial photosynthetic system [11] and electrocatalytic processes [12]. In addition, ruthenium dinuclear oxo-complexes can be used as artificial electron-transfer mediators in biosensors [13]. However, the ruthenium dinuclear oxo-complex $[(bpy)_2(NH_3)Ru^{III}(\mu\text{-}O)Ru^{III}(NH_3)(bpy)_2](ClO_4)_4$ has not been studied to date as a redox mediator in the development of glucose biosensors.

The use of artificial redox mediators allows the flow of electrons from the redox enzyme to the surface of the electrode center, decreasing redox potential and ideally preventing interference by electro-oxidizable species such as ascorbic acid, therefore, increasing the selectivity and sensitivity of the (bio)sensor [14]. The determination of the optimal redox mediator for the production of a biosensor is extremely important for its efficiency. A mediator should be selected that possesses a lower redox potential than the other electrochemically active interfering compounds present in the sample. Furthermore, a high electrochemical constant is very desirable, which is important to ensure that the response of the biosensor is not limited by the kinetics of electrodes or by oxygen interference [15]. An ideal mediator is also characterized by reversible kinetics, a high chemical stability in both reduced and oxidized forms and unreactivity with oxygen [13]. To date, several transition-metal complexes such as iron [16–18], osmium [19,20] have been investigated as redox mediators and are the most used, but ruthenium complexes can also be used, due to their higher reactivity with glucose oxidase (GOx) [13,21].

This study describes the electrochemical performance of the oxo-bridged dinuclear ruthenium ammine $[(bpy)_2(NH_3)Ru^{III}(\mu\text{-}O)Ru^{III}(NH_3)(bpy)_2]^{4+}$ incorporated into a Nafion film coating a glassy carbon electrode that acts as an electron-transfer mediator for the development of glucose biosensors. The electrochemical performance of the modified Ru–O–Ru/Nafion®/GC electrode was performed by cyclic voltammetry. The electrocatalytic activity of the glucose biosensor GOx/oxo-bridged dinuclear ruthenium ammine/Nafion®/GC was also studied.

[a] P. A. Raymundo-Pereira, A. C. V. Mascarenhas, M. F. S. Teixeira
Faculty of Science and Technology, São Paulo State University (UNESP), Rua Roberto Simonsen 305, CEP 19.060–900, Presidente Prudente, SP, Brazil
Telephone: +55 18 3229 5874; Fax: +55 18 3221 5682
*e-mail: funcao@fct.unesp.br

[b] P. A. Raymundo-Pereira
São Carlos Institute of Chemistry, University of São Paulo, CP 380, CEP 13566–590, São Carlos, São Paulo, Brazil

2 Experimental Section

2.1 Materials

All the reagents used were of analytical grade and were used without further purification. Silver nitrate, sodium phosphate, *cis*-$(bpy)_2Ru^{II}Cl_2$, and ammonium sulfate were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sodium perchlorate was obtained from Merck (Darmstadt, Germany). A 5 % (w/v) Nafion® alcoholic solution was obtained from Sigma–Aldrich (St. Louis, MO, USA). High-purity deionised water (resistance, 18 MΩ cm) was obtained from the Millipore Milli-Q system. Acetate-buffer solution (pH 4.75) was prepared from anhydrous sodium acetate and acetic acid from Sigma–Aldrich (St. Louis, MO, USA) and was used as a supporting electrolyte. All experiments were performed at room temperature, which was approximately 25 °C.

Glucose oxidase (GOx) from *Aspergillus niger* (1.0 mg/mL) was purchased from Sigma–Aldrich. Phosphate-buffer solution (pH 7.0) was prepared from Na_2HPO_4 and NaH_2PO_4 from Sigma–Aldrich (St. Louis, MO, USA). The initial amount of enzyme contained in the vessel was diluted with phosphate-buffer solution (PBS), aliquoted into Eppendorf® microtubes and frozen until use. The glucose solution 0.001 mol L^{-1} was prepared in acetate-buffer solution and was used as stock solution.

2.2 Apparatus

Electrochemical measurements were carried out with a μ-Autolab type III (Eco Chimie, Netherlands) potentiostat/galvanostat coupled to a computer and controlled by GPES 4.9 software. All measurements were carried out in a 50-mL thermostated glass cell at 25 °C, with a three-electrode configuration: a glassy carbon electrode (diameter 3.0 mm) coated with polymeric film as the working electrode, a saturated calomel electrode (SCE) as a reference and platinum wire as an auxiliary electrode. During the measurements, the solution within the cell was neither stirred nor aerated. The ultraviolet-visible (UV–vis) absorption spectra were recorded on a Shimadzu UV-1650PC spectrometer with a system interface for a computer.

2.3 Ruthenium Oxo-complex Synthesis

The *bis*(oxo-bridged) dinuclear ruthenium-bipyridyl-ammine complex ($[(bpy)_2(NH_3)Ru^{III}$-O-$Ru^{III}(NH_3)$-$(bpy)_2](ClO_4)_4$) was synthesized as proposed by Meyer et al. [6,12], based on pretreatment of the $[(bpy)_2(H_2O)Ru^{III}$-O-$Ru^{III}(H_2O)(bpy)_2]^{4+}$ complex. Initially, the ruthenium complex was prepared from its monomeric *cis*-$(bpy)_2Ru^{II}Cl_2$ precursor following well-established synthetic procedures and was used without further purification. Specifically, 1.0 g of $Ru(bpy)_2Cl_2{\cdot}2H_2O$ was dissolved in 12.5 mL water and was heated for 1 h at 100 °C in a water bath, following which 0.65 g of $AgNO_3$ was added and the mixture was heated for a further 2.5 h in the water bath. The AgCl that formed was filtered off using fine-porosity filter paper and the filtrate was diluted with 30 mL water. Ten milliliter of saturated $NaClO_4$ was subsequently added and precipitation was induced by overnight storage in a refrigerator. The complex obtained was dried *in vacuo* at room temperature for 72 h. An aqueous solution of 0.1 M Na_3PO_4 (20 mL) containing 0.47 mmol of $[(bpy)_2(H_2O)Ru^{III}$-O-$Ru^{III}(H_2O)(bpy)_2]^{4+}$ and 7.57 mmol of $(NH_4)_2SO_4$ was heated in a steam bath for 1 h. The solution was cooled to room temperature and a saturated aqueous solution of $NaClO_4$ was added. The solution was kept at room temperature overnight, and the precipitate was collected on a frit.

2.4 Biosensor Preparation

Prior to modification, the surface of the GC electrode was polished with a 0.3 μm alumina slurry and was then rinsed thoroughly with double-distilled water, sonicated for 5 min in ethanol and 5 min in water, and was air-dried. A polymeric coating layer was prepared only on the disk electrode by casting Nafion® solution (ion exchange capacity: 0.8 meq g^{-1}) using a micropipette (5 μL), which was then air-dried for 24 h at room temperature. The coated electrode was then immersed into 1.0×10^{-3} mol L^{-1} oxo-bridged dinuclear ruthenium ammine complex in aqueous solution for 24 h. The polymeric membrane on the surface electrode acquired a green-blue color. Subsequently, a 25-μL aliquot of the GOx solution was placed onto the polymeric membrane as a droplet and the solvent was then evaporated at room temperature.

3 Results and Discussion

3.1 Characterization UV-Vis of the Oxo-bridged Dinuclear Ruthenium Ammine

The absorption spectrum in the UV-Vis region for the oxo-bridged dinuclear ruthenium ammine $[(bpy)_2(NH_3)Ru^{III}(\mu\text{-}O)Ru^{III}(NH_3)(bpy)_2]^{4+}$ in acetonitrile (Figure 1) shows two intense absorption bands of high energy centered at 245 and 286 nm in the UV region, which are typical for bipyridine systems and ligand transitions $bpy(\pi)\rightarrow bpy(\pi^*)$. Two bands of mean energy absorption at 360 and 420 nm were observed, which probably induced charge-transfer transitions of the metal-ligand $d\pi(Ru)\rightarrow\pi^*(bpy)$, and a low energy absorption centered at 646 nm due to transitions between molecular orbitals composed of orbital dπ of ruthenium and pπ of the oxo-bridge, typical of oxo-ligands linked to ruthenium dimers Ru^{III}–O_{oxo}–Ru^{III} [22]. These results agree with data in the literature for the ruthenium dinuclear polypyridyl oxo-complex [3,15,22] and thus confirm the presence of this oxo-complex. Moreover, the electron donor ability of the metallic center (ruthenium) combined with the electron acceptor nature of the bipyridine, produces

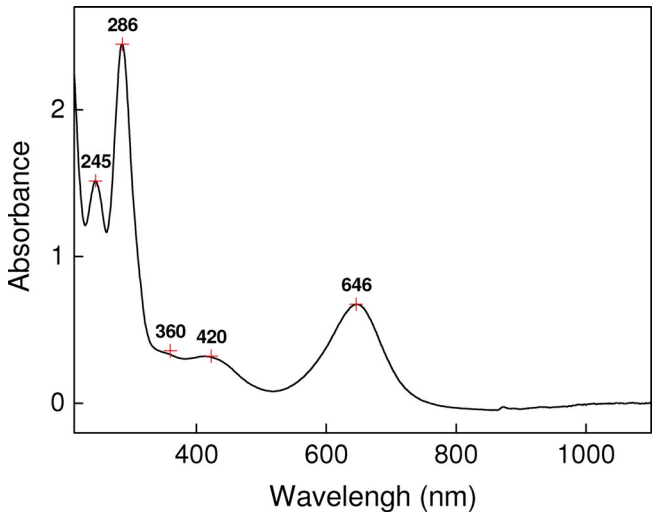
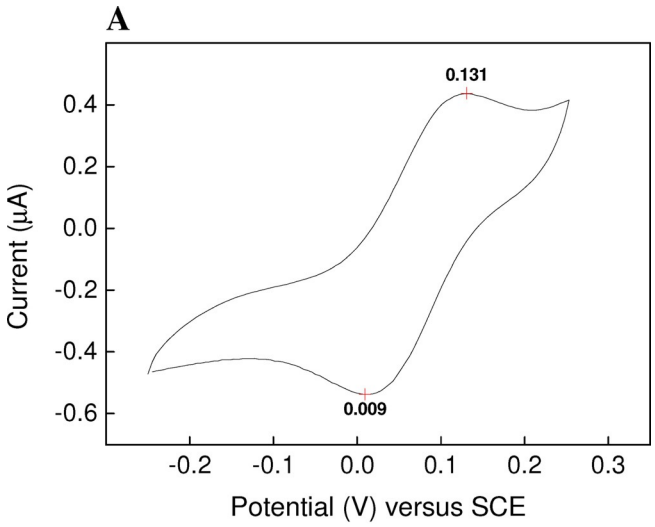


Fig. 1. Absorption spectrum of the ruthenium dinuclear polypyridyl oxo-complex (1.0×10^{-4} mol L^{-1}) in acetonitrile.



an effective interaction of molecular orbitals and an extensive electronic delocalization [15], which confers excellent properties on this oxo-complex, to be used as an electron-transfer mediator in electrochemical biosensors.

3.2 Electrochemical Properties of the Immobilized Ruthenium Dinuclear Oxo-complex

In reports by Teixeira and co-workers [6,23,24], metal complexes that are confined to electrode surfaces are active catalysts for a variety of electrochemical reactions and the surfaces of the electrodes that are modified by these complexes can be obtained simply by coating cation exchange polymer film. The electrochemical performance of the modified electrode with the oxo-bridged dinuclear ruthenium ammine complex incorporated into Nafion® polymeric film was analysed in 0.1 mol L^{-1} acetate buffer by cyclic voltammetry, to investigate the electrochemical properties of the oxo-complex on the conductive substrate. Figure 2(A) shows a typical cyclic voltammogram with two peaks: an anodic peak at +0.131 V (E_{PA}) and a cathodic peak at −0.009 V (E_{PC}), which remains stable after the second cycle. The anodic and cathodic peak potentials in Figure 2(A) can be attributed to the redox process of the ruthenium dinuclear oxo-complex immobilized onto the Nafion® polymer film. Equation 1 shows the processes involved in redox-cyclic voltammograms:

$$\left[(bpy)_2(NH_3)Ru^{III}(\mu-O)Ru^{III}(NH_3)(bpy)_2\right]^{4+} \rightleftarrows \left[(bpy)_2(NH_3)Ru^{III}(\mu-O)Ru^{IV}(NH_3)(bpy)_2\right]^{5+} + e^- \quad (1)$$

The voltammetric profile of modified electrodes with ruthenium dinuclear polypyridyl oxo-complex $[(bpy)_2(NH_3)Ru^{III}(\mu\text{-}O)Ru^{III}(NH_3)(bpy)_2]^{4+}$ immobilized onto the Nafion® polymeric membrane agree with the results obtained by [23] for studies on modified carbon

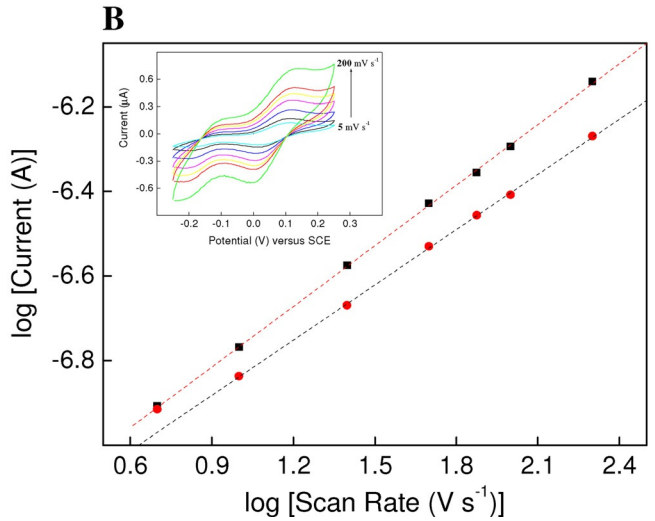


Fig. 2. (A) Cyclic voltammograms obtained for electrode coated with Nafion film containing oxo-bridged dinuclear ruthenium(III) $[(bpy)_2(NH_3)Ru^{III}(\mu\text{-}O)Ru^{III}(NH_3)(bpy)_2]^{4+}$ in 0.1 mol L^{-1} acetate-buffer solution. Scan rate 25 mV s^{-1}. (B) Representative logarithm current *vs.* logarithm scan rate. The insert show cyclic voltammograms obtained at different scan rates for modified electrode.

paste electrodes with Ru-red immobilized in zeolite (Na-Y). These processes can be attributed to a quasi-reversible system by a single electron transfer between the redox couple $Ru^{III}(\mu O)Ru^{III}/Ru^{III}(\mu O)Ru^{IV}$, a $\Delta E_p \approx 122$ mV confirms a quasi-reversible electrochemical system. The surface concentration of electroactive species (Γ/mol cm^{-2}) was estimated from the background-corrected electric charge (Q), under the anodic peaks in accordance with the theoretical relationship [6] as follows: $\Gamma = Q/nFA$, where Q (C) is the background-corrected electric charge, calculated by integrating the anodic peak of the cyclic voltammogram ($v = 5$ mV s^{-1}) in the aqueous solu-

tion of acetate-buffer; n is the number of electrons transferred; F is the Faraday constant (96,485.34 C mol^{-1}); and A is the electrode geometric area. Under the conditions described above, Q was approximately 2.022×10^{-6} C, and the estimated surface concentration was 2.965×10^{-10} mol cm^{-2}. To determine the influence of oxygen on the electrochemical behavior of the complex, some experiments were performed in deaerated solutions, and the profile showed no marked alteration in redox potential or current with the dissolved O_2.

The effect of the potential scan rates (5–200 mV s^{-1}) on the voltammetric response for an electrode coated with Nafion film containing dinuclear of ruthenium(III)-bipyridyl-ammine complex in 0.1 mol L^{-1} acetate-buffer solution was investigated, to elucidate the electron-transfer mechanism. The recorded cyclic voltammograms revealed that the anodic peak current increased and the peak potential shifted as the scan-rate increased. The anodic and cathodic peak currents varied linearly with the square-root of the scan rates. This linearity indicates that the redox process follows a diffusion-controlled mechanism [25]. This behavior suggests a mobility of the counterions of the supporting electrolyte, which are necessary for charge transport or to keep the electroneutrality at the electrode surface during the redox process [23,24]. This behavior is common for this type of polymer film as such as reported by Martin *et al.* [26] and Bertoncello *et al.* [25]. To confirm the diffusion-controlled mechanism, a plot of log [peak current] *versus* log [scan rate] was performed. Figure 2(B) shows the dependence of the logarithm of the anodic and cathodic peak current on the logarithm of the scan rates for the glassy carbon electrode coated with ruthenium dinuclear oxo-complex in medium of 0.1 mol L^{-1} acetate buffer. The slopes obtained for the anodic and cathodic curves were 0.48 ($R=0.9999$) and 0.44 ($R=0.9976$), respectively. The obtained values are close to the theoretical value of 0.5 for a diffusion-controlled mechanism [27]. These features are very convenient diagnostic tools to test for the diffusion-controlled mechanism of a redox process, which is consistent with our results.

The degree of kinetic reversibility exhibited by the redox process on the surface depends on the scan rate of potentials. It is expected that a redox process exhibits a reversible behavior when the potential scan rate is small and an irreversible behavior when the potential scan rate is high [27].

3.3 Application of the Electrode Coated with Nafion Film Containing Dimeric Oxo-bridged Ruthenium Complex as a Mediator of Electron-Transfer in Glucose Determination

The direct reduction or oxidation of glucose at a non-modified electrode is not suitable for analytical application, due to the slow electrode kinetics and high overpotentials that are required for the redox reactions of glucose on many electrodes. For this reason, redox mediators have been widely used to decrease the overpotential and increase the kinetics of electron transfer. To verify mediation of electron-transfer from the glassy carbon electrode coated with Nafion® film containing oxo-bridged $[(bpy)_2(NH_3)Ru^{III}(\mu\text{-}O)Ru^{III}(NH_3)(bpy)_2]^{4+}$, the glucose oxidase (GOx) enzyme from *Aspergillus niger* was immobilized onto the electrode surface and cyclic voltammograms were obtained in the absence and presence of 2.5×10^{-5} mol L^{-1} glucose in 0.1 mol L^{-1} acetate buffer solution, as shown in Figure 3(A). With the addition of glucose in solution, the anodic and cathodic peak current of the modified electrode increased significantly ($\Delta I=0.061$ μA). Increasing the anodic and cathodic peak current clearly showed the mediation of electron-transfer to

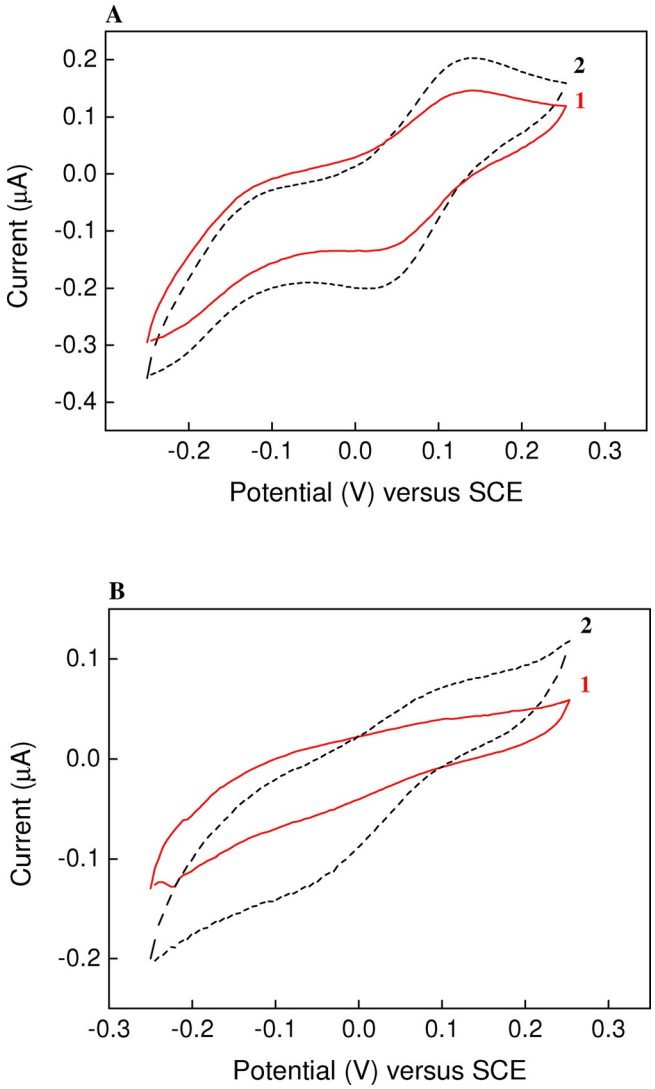


Fig. 3. (A) Cyclic voltammograms obtained for GOx/oxo-bridged dinuclear ruthenium amine/Nafion/GC electrode in 0.1 mol L^{-1} acetate-buffer solution in the absence (curve 1) or the presence of 2.5×10^{-5} mol L^{-1} glucose (curve 2). Scan rate 25 mV s^{-1}. (B) Cyclic voltammograms obtained for GOx/Nafion/GC electrode without the incorporation of the mediator in 0.1 mol L^{-1} acetate-buffer solution in the absence (curve 1) or the presence of 2.5×10^{-5} mol L^{-1} glucose (curve 2).

glucose by the central metallic cations of the complex immobilized in the polymeric film. Nakabayashi and coworkers [13, 21] described the operation mechanism of the biosensor as a reaction between the analyte and biosensor that is similar to an electrochemical-chemical mechanism in three steps (two chemical and one electrochemical). The initial step is the chemical or enzymatic step (biocatalysis), where the glucose oxidase enzyme specifically converts glucose to gluconolactone, which spontaneously hydrolyzes to gluconic acid, with concomitant reduction of O_2 to hydrogen peroxide. This oxidation reaction is accompanied by the reduction of the flavin adenine dinucleotide (FAD) cofactor. Flavin adenine dinucleotide is responsible for the redox properties of the enzyme, and available evidence suggests that it is firmly attached, but is not covalently bonded to the enzyme protein polypeptide. Thus, GOx accepts electrons from glucose via the flavin moiety and transfers them to dioxygen, but this does not occur for non-modified electrodes. This is the reason why reduced flavin adenine dinucleotide ($FADH_2$), known as the active site, is not electrochemically oxidized by the electrode reaction:

$$FADH_2 \rightarrow FAD + 2H^+ + 2e^- \quad (2)$$

Thus, the idea developed to use a non-physiological redox molecule as an electron acceptor from the reduced form of GOx, the mediator. Many artificial electron receptors are used as electron-transfer mediators for coupling the glucose/GOx system to an electrode surface [28]. Taking glucose as an example, we can describe the function of a ruthenium complex as a mediator of electron transfer as follows:

$$\beta\text{-D-glucose} + GOx_{ox}\ (FAD) \rightarrow \text{glucono-}\delta\text{-lactona} + GOx_{red}\ (FADH_2) \quad (3)$$

$$GOx_{red}\ (FADH_2) + 2\ Ru(IV)_{(electrode)} \rightarrow GOx_{ox}\ (FAD) + 2\ Ru(III)_{(electrode)} + 2\ H^+ \quad (4)$$

$$2\ Ru(III) \rightarrow 2\ Ru(IV) + 2e^- \quad (5)$$

where GOx_{ox}(FAD) and GOx_{red}($FADH_2$) are the oxidized and reduced forms of GOx, respectively. The role of ruthenium complexes as electron-transfer mediators can be described according to the reaction mechanisms shown in Eqs. (3), (4) and (5). Two important electron-transfer mediator properties are particularly necessary to develop voltammetric glucose biosensors. Firstly, an electron-transfer mediator should have an appropriate redox potential for the reaction at the electrode, given by Eq. (5). Electrochemical glucose biosensors are required to detect glucose at a potential near 0.0 V, where the risk of interference between reactions is minimized and when the background current and noise levels are lower. Secondly, to increase the sensitivity and to minimize competition with dissolved O_2, an electron-transfer mediator must have a high value of the second-order constant k_s in Eq. (4). Consequently, the choice of the mediator of electron-transfer is certainly important to achieve a high sensitivity and selectivity of glucose biosensors. Ruthenium complexes offer great advantages compared to complexes of osmium for example, due to their lower cost and higher reactivity with GOx [29]. Moreover, the low redox potential of the mediator minimizes the oxidation of endogenous species, such as ascorbic acid and uric acid [30]. The redox potentials recorded by ruthenium dinuclear polypyridyl oxo-complex $[(bpy)_2(NH_3)Ru^{III}(\mu\text{-}O)Ru^{III}(NH_3)(bpy)_2]^{4+}$ were more positive than those of $FAD/FADH_2$ (−0.41 V *vs.* SCE at pH = 7.0) present in the enzyme active center [13, 21], the mediation reaction is considered thermodynamically possible (GOx_{red} ($FADH_2$) + 2Ru(IV) → GOx_{ox} (FAD) + 2Ru(III) + $2H^+$). The oxo-bridged dinuclear ruthenium amine complex can be considered to be a good electron-transfer mediator at the observed redox potential (0.13 V *vs.* SCE).

In the Figure 3(B) were conducted a control experiment by using Nafion modified glassy carbon electrode without the incorporation of the mediator, demonstrating the mediator effect. The Figure 3(B) shows the voltammetric response for a GOx/Nafion-modified carbon electrode without the incorporation of the redox mediator in the presence of glucose. The cathodic current (curve 2 in figure) in presence of glucose is resulting from its catalytic oxidation by enzyme in the electrode surface. The GOx/Nafion film itself shows that in the absence of the ruthenium dinuclear polypyridyl oxo-complex is difficult to measure the electrogerate current of the peroxide due to absence of the well-defined peaks. There are only an increase of the diffusion current in approximately 0.2 V vs. SCE. Apparently, the current is due the reduction of hydrogen peroxide generated by enzymatic catalysis. Comparatively, the sensitivity obtained for the electrode containing the oxo-bridged dinuclear ruthenium amine complex was better and it is evident the electronic mediation with glucose oxidase.

3.4 Glucose Voltammetric Determination Using GOx/Oxo-bridged Dinuclear Ruthenium Amine/GC Electrode

Successive cyclic voltammograms were performed, to obtain the analytical curve, at a range of potentials between −0.25 V and +0.25 V in a 0.1 mol L^{-1} acetate buffer, at a scan rate of 25 mV s^{-1} for successive additions of 50 µL from a glucose standard solution at 0.001 mol L^{-1}. Figure 4(A) shows the voltammograms obtained for different concentrations of glucose. The values of the anodic peak current varied linearly, according to variation in the glucose concentration in solution. The calibration curve (see Figure 4(B)) was linear in the glucose concentration range between 2.52×10^{-5} and 1.00×10^{-4} mol L^{-1} and can be represented by the equation (ΔI (µA) = $3.77\text{x}10^{-2} + 8.30\times10^{-4}$ [Glucose] (µmol L^{-1}) with a linear correlation coefficient $R = 0.9976$ and the limit-order detection (LOD) was 9.63 µmol L^{-1}. The standard

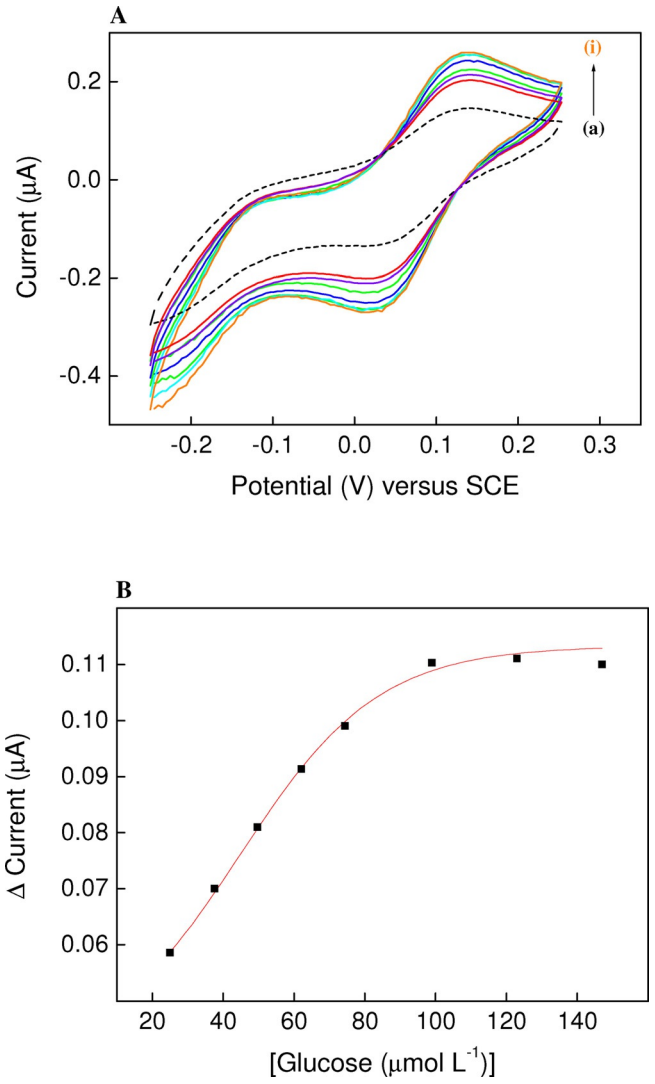


Fig. 4. (A) Cyclic voltammograms obtained for GOx/oxo-bridged dinuclear ruthenium amine/Nafion/GC electrode in 0.1 mol L^{-1} acetate-buffer solution in the absence (a) or the presence of glucose: 2.50×10^{-5} (b); 3.76×10^{-5} (c); 4.97×10^{-5} (d); 6.20×10^{-5} (e); 7.44×10^{-5} (f); 9.90×10^{-5} (g); 1.23×10^{-4} (h); and 1.47×10^{-4} mol L^{-1} (i). Scan rate 25 mV s^{-1}. (B) Analytical curve obtained by the peak current *vs.* [glucose] in solution. The data were abstracted from Fig. A.

deviation for six successive determinations of glucose was 4.4%. Furthermore, the repeatability inter-day (five different electrodes) was also evaluated for a 5.0×10^{-5} mol L^{-1} glucose solution in 0.1 mol L^{-1} acetate-buffer solution. RSD of 3.78% was obtained, indicating a high repeatability of the electrode fabrication. The performance of the developed biosensor was compared with that of other glucose biosensors. Table 1 shows the analytical parameters (operating potential and sensitivity) of electroanalytical methods using different mediators that have been reported for the glucose biosensors. We can conclude that the mediator showed good results, confirming the efficiency of this voltammetric biosensor for the detection of glucose [13,14,31–35].

The K_M^{app} provides important information about the relationship and affinity between the enzyme and substrate. An estimated value of the rate of the enzyme-substrate intermediate dissociation without conversion of substrate to product is provided by the apparent Michaelis–Menten kinetic constant (K_M^{app}) [36]. Thus, the probability of product formation per binding is inversely proportional to K_M^{app}. The value of K_M^{app} can be obtained by linear regression analysis using the Lineweaver–Burk equation [37]: $I_{ss}^{-1}=I_{max}^{-1}+K_M^{app}\,I_{max}^{-1}\,[glucose]^{-1}$, where I_{ss} is the steady-state current measured for the enzymatic product and I_{max} is the maximum current under conditions of substrate (glucose) saturation. The equation of the Lineweaver–Burk plots for the GOx/$[(bpy)_2(NH_3)Ru^{III}(\mu\text{-}O)Ru^{III}(NH_3)(bpy)_2]$/ Nafion®/GC biosensor was $I_{ss}^{-1}=3.643x10^6+34.38\,[glucose]^{-1}$ (correlation coefficient of 0.995). Thus, the K_M^{app} can be simply obtained by multiplying the slope ($1/I_{ss}$ vs 1/[glucose]) by I_{max}. The I_{max} and K_M^{app} source values obtained in this study were 2.547×10^{-7} A and 8.757×10^{-6} mol L^{-1}, respectively. Table 2 lists the K_M^{app} source values reported in the literature for different glucose biosensors using glucose as a substrate.

The K_M^{app} value obtained in this study indicates a high affinity of the substrate (glucose) for the active site of GOx immobilized within the biosensor. This suggests that the enzyme immobilized onto the transducer $[(bpy)_2(NH_3)Ru^{III}(\mu\text{-}O)Ru^{III}(NH_3)(bpy)_2]$/Nafion/GC, has a high catalytic activity and a high affinity for glucose, due to an increase in the electron transfer rate. Further-

Table 1. Comparison of the analytical parameters obtained for the determination of glucose using GC/oxo-bridged dinuclear ruthenium amine/GOx with those from the literature.

Mediator	Redox Potential (vs Ag/AgCl)	Sensitivity	Ref.
$[Ru(NH_3)_5(pyridinium)]^{3+}$	0.10–0.18 V	–	[13]
$Ru_3(\mu_3\text{-}O)(AcO)_6(Py)_3(ClO_4)$	−0.190 and −0.106 V	15.4 mA mol L^{-1}	[14]
CFe*-RP/GOx/Ts	0 V	5.4 mA mol L^{-1}	[31]
$[Ru(CO)Cl(PPh_3)_2TSC^{N-S}]$	−0.9 V	2.1 mA mol L^{-1}	[32]
Ru-RP	−0.15 V	24.3 mA mol L^{-1}	[33]
$[Ru(NH_3)_6]^{3+}$	0 V	–	[34]
$[Ru(trpy)(phen)(OH_2)]^{2+}$	0.52 V	–	[35]
trans-$[Ru(2,2-bpy)_2(OH_2)(OH)]^{2+}$	0.50 V	0.4 mA mol L^{-1}	[35]
$[Ru(4,4-bpy)(NH_3)_5]^{2+}$	0.24 V	7.2 mA mol L^{-1}	[35]
$[(bpy)_2(NH_3)Ru^{III}(\mu\text{-}O)Ru^{III}(NH_3)(bpy)_2]^{4+}$	0.13 V (vs. SCE)	0.86 mA mol L^{-1}	This Study

Table 2. Comparison of the K_M^{app} values for different glucose biosensors.

Biosensor	K_M^{app} (M)	Ref.
GOx/ZnO/GOx/CNTs/GC	2.5×10^{-3}	[38]
ZnO/(PSS/PDDA)/GOx	3.1×10^{-3}	[39]
GOx/AuNPs/PtNPs/CNTs/Au	11×10^{-3}	[40]
GOx/SnS_2/Nafion/GC	7.6×10^{-3}	[41]
Au/MPS/TH/(SCGNPs/TH)/GOx/HPR	1.2×10^{-3}	[42]
GOx/graphene-CTS/GC	4.4×10^{-3}	[43]
(GOx/AuNPs/CNTs)/GC	15×10^{-3}	[44]
GOx oxo-bridged dinuclear ruthenium amine /Nafion/GC	8.76×10^{-6}	This study

more, the important contribution of the electron-transfer mediator ruthenium dinuclear oxo-complex $[(bpy)_2(NH_3)Ru^{III}(\mu\text{-}O)Ru^{III}(NH_3)\ (bpy)_2]$ is clearly demonstrated, because the K_M^{app} values for the biosensor GOx/$[(bpy)_2(NH_3)Ru^{III}(\mu\text{-}O)Ru^{III}(NH_3)(bpy)_2]$/Nafion/GC were three orders of magnitude lower than those previously reported in the literature (see Table 2).

In addition to K_M^{app} and I_{max}, an another important constant is the catalytic rate constant k_{cat}, defined as the maximum amount of substrate that can be converted to product by one mol of enzyme per unit time. In agreement to Lineweaver–Burk [37], in steady state conditions, k_{cat} can be calculated by the equation $I_{max}=k_{cat}$ [E], where I_{max} is the maximum current under conditions of substrate (glucose) saturation obtained when the enzyme concentration ([E]) exists completely as the enzyme–substrate complex. The I_{max} and k_{cat} values obtained in this study were 2.547×10^{-7} A and 1,956 s^{-1}, respectively. Bright and Gibson [38] and Gibson et al. [39] report k_{cat} values for the transformation of the glucose–enzyme$_{ox}$ complex to the lactone–enzyme$_{red}$ complex of 1,000 and 1,150 s^{-1}, respectively. The k_{cat} value obtained for our biosensor GOx/oxo-bridged dinuclear ruthenium amine/Nafion/GC was two-fold higher than those in the literature, indicating the efficiency of ruthenium dinuclear oxo-complex as an electron transfer mediator.

4 Conclusions

Cyclic voltammetry at the modified electrode in acetate buffer showed a single-electron reduction/oxidation of $Ru^{III}ORu^{III}/Ru^{III}ORu^{IV}$. The modified electrode exhibited electron-transfer mediator properties towards glucose oxidation in acetate buffer. The kinetic studies were performed to evaluate the performance of the Nafion film containing the oxo-bridged dinuclear ruthenium amine complex as an electron mediator. Furthermore, we observed an increase in catalytic current and a decrease in overpotential, compared to the unmodified electrode.

Acknowledgments

The authors are grateful to FAPESP (2005/01296-4) and CNPq for research support. The scholarships granted by the Foundation for Research Support of the State of São Paulo, FAPESP (2008/07298-7 and 2012/17689-9), and the National Council for Scientific and Technological Development, CNPq (146712/2010-2) to Paulo A. Raymundo-Pereira and Ana C. V. Mascarenhas, respectively, are gratefully acknowledged. The authors thank SJT.

References

[1] A. Bahreman, J. A. Cuello-Garibo, S. Bonnet, Yellow-light sensitization of a ligand photosubstitution reaction in a ruthenium polypyridyl complex covalently bound to a rhodamine dye, *Dalton T*, **2014**, *43*, 4494–505.
[2] F. F. Li, M. Feterl, J. M. Warner, A. I. Day, F. R. Keene, J. G. Collins, Protein binding by dinuclear polypyridyl ruthenium(II) complexes and the effect of cucurbit[10]uril encapsulation, *Dalton T*, **2013**, *42*, 8868–77.
[3] F. Liu, J. J. Concepcion, J. W. Jurss, T. Cardolaccia, J. L. Templeton, T. J. Meyer, Mechanisms of water oxidation from the blue dimer to photosystem II, *Inorg. Chem.*, **2008**, *47*, 1727–52.
[4] T. R. Weaver, T. J. Meyer, S. A. Adeyemi, G. M. Brown, R. P. Eckberg, W. E. Hatfield, et al., Chemically Significant Interactions between Ruthenium Ions in Oxo-bridged Complexes of Ruthenium(Iii), *J. Am. Chem. Soc.*, **1975**, *97*, 3039–48.
[5] C. A. Bignozzi, S. Roffia, F. Scandola, Intervalence Transfer in Cyano-Bridged Binuclear and Trinuclear Ruthenium Complexes, *J. Am. Chem. Soc.*, **1985**, *107*, 1644–51.
[6] M. F. S. Teixeira, F. H. Cincotto, P. A. Raymundo-Pereira, Electrochemical investigation of the dimeric oxo-bridged ruthenium complex in aqueous solution and its incorporation within a cation-exchange polymeric film on the electrode surface for electrocatalytic activity of hydrogen peroxide oxidation, *Electrochim. Acta*, **2011**, *56*, 6804–11.
[7] W. Kutner, J. A. Gilbert, A. Tomaszewski, T. J. Meyer, R. W. Murray, Stability and Electrocatalytic Activity of the Oxo-bridged Dimer $[(bpy)_2(H_2O)RuORu(OH_2)(bpy)_2]^{4+}$ in Basic Solutions, *J. Electroanal. Chem.*, **1986**, *205*, 185–207.
[8] J. R. Schoonover, J. F. Ni, L. Roecker, P. S. Whiter, T. J. Meyer, Structural and resonance Raman studies of an oxygen-evolving catalyst. Crystal structure of $[(bpy)_2(H_2O)Ru^{III}ORu^{IV}(OH)(bpy)_2](ClO_4)_4$, *Inorg. Chem.*, **1996**, *35*, 5885–92.
[9] T. Abe, T. Goto, K. Ohzeki, M. Kaneko, Electrocatalysis for proton reduction by a coated Nafion (R) membrane dispersing ruthenium moieties, *Electrochim. Acta*, **2000**, *45*, 4009–14.
[10] M. Yagi, K. Kinoshita, M. Kaneko, Charge transfer distance between trinuclear ruthenium complexes incorporated in a polymer membrane studied by potential-step chronoamperospectrometry, *Electrochim. Acta*, **1999**, *44*, 2245–50.
[11] K. Nagoshi, S. Yamashita, M. Yagi, M. Kaneko, Catalytic activity of $[(bpy)_2(H_2O)Ru\text{-}O\text{-}Ru(H_2O)(bpy)_2]^{4+}$ for four-electron water oxidation, *J. Mol. Catal. a-Chem.*, **1999**, *144*, 71–6.
[12] J. A. Gilbert, D. S. Eggleston, W. R. Murphy, D. A. Geselowitz, S. W. Gersten, D. J. Hodgson, et al., Structure and Redox Properties of the Water-Oxidation Catalyst

[(bpy)$_2$(H$_2$O)Ru-O-Ru(H$_2$O)(bpy)$_2$]$^{4+}$, *J. Am. Chem. Soc.*, **1985**, 107, 3855–64.
[13] Y. Nakabayashi, Y. Hirosaki, O. Yamauchi, Dipolar ruthenium(II) ammine complexes as electron transfer mediators of amperometric glucose sensors, *Bioelectrochemistry*, **2006**, *69*, 216–22.
[14] K. Yamamoto, H. S. Zeng, Y. Shen, M. M. Ahmed, T. Kato, Evaluation of an amperometric glucose biosensor based on a ruthenium complex mediator of low redox potential, *Talanta*, **2005**, *66*, 1175–80.
[15] H. Jude, F. N. Rein, P. S. White, D. M. Dattelbaum, R. C. Rocha, Synthesis, structure, and electronic properties of a dimer of Ru(bpy)(2) doubly bridged by methoxide and pyrazolate, *Inorg. Chem.*, **2008**, *47*, 7695–702.
[16] A. E. G. Cass, G. Davis, G. D. Francis, H. A. O. Hill, W. J. Aston, I. J. Higgins, et al., Ferrocene-Mediated Enzyme Electrode for Amperometric Determination of Glucose, *Anal. Chem.*, **1984**, *56*, 67–71.
[17] N. J. Forrow, G. S. Sanghera, S. J. Walters, The influence of structure in the reaction of electrochemically generated ferrocenium derivatives with reduced glucose oxidase, *J. Chem. Soc. Dalton*, **2002**, 3187–94.
[18] J. Tatur, W. R. Hagen, H. A. Heering, Voltammetry of Pyrococcus furiosus ferritin: dependence of iron release rate on mediator potential, *Dalton T*, **2009**, *15*, 2837–42.
[19] G. Kenausis, Q. Chen, A. Heller, Electrochemical glucose and lactate sensors based on ″wired″ thermostable soybean peroxidase operating continuously and stably at 37 degrees C, *Anal. Chem.*, **1997**, *69*, 1054–60.
[20] V. Shklover, S. M. Zakeeruddin, R. Nesper, D. Fraser, M. Gratzel, Tris(4,4′-dimethoxy-2,2′-bipyridine)osmium(II), amperometric properties and crystal structure, *Inorg. Chim. Acta.*, **1998**, *274*, 64–72.
[21] Y. Nakabayashi, Y. Hirosaki, O. Yamauchi, Dipolar ruthenium-ammine complexes with 4,4′-bipyridinium ions accessible for both amperometric and colorimetric glucose sensors, *Inorg. Chem. Commun.*, **2006**, *9*, 935–8.
[22] H. Y. Ye, J. L. Chen, Z. N. Chen, Oxo-linked dirutheniu-m(III) complex with 2,2′-bipyridine in ortho-metalation, *Inorg. Chem. Commun.*, **2007**, *10*, 1023–5.
[23] M. F. S. Teixeira, M. E. Bergamini, C. M. P. Marques, N. Bocchi, Voltammetric determination of L-dopa using an electrode modified with trinuclear ruthenium ammine complex (Ru-red) supported on Y-type zeolite, *Talanta*, **2004**, *63*, 1083–8.
[24] M. F. S. Teixeira, L. H. Marcolino-Junior, O. Fatibello, E. R. Dockal, E. T. G. Cavalheiro, Voltammetric determination of dipyrone using a N, N′-ethylenebis(salicylideneaminato)oxovanadium(IV) modified carbon-paste electrode, J. Brazil. *Chem. Soc.*, **2004**, *15*, 803–8.
[25] P. Bertoncello, P. Ugo, Preparation and voltammetric characterization of electrodes coated with Langmuir-Schaefer ultrathin films of Nafion (R), *J. Brazil. Chem. Soc.*, **2003**, *14*, 517–22.
[26] C. S. Martin, M. F. S. Teixeira, A novel Mn-containing conducting metallopolymer obtained by electropolymerization in aqueous solution of a tetranuclear oxo-bridged manganese complex, *Dalton T*, **2011**, *40*, 7133–6.
[27] E. Laviron, General Expression of the Linear Potential Sweep Voltammogram in the Case of Diffusionless Electrochemical Systems, *J. Electroanal. Chem.*, **1979**, *101*, 19–28.
[28] B. C. Janegitz, R. Pauliukaite, M. E. Ghica, C. M. A. Brett, O. Fatibello, Direct electron transfer of glucose oxidase at glassy carbon electrode modified with functionalized carbon nanotubes within a dihexadecylphosphate film, *Sensor Actuat B-Chem.*, **2011**, *158*, 411–7.
[29] A. D. Ryabov, Y. N. Firsova, A. Y. Ershov, I. A. Dementiev, Spectrophotometric kinetic study and analytical implications of the glucose oxidase-catalyzed reduction of [M-III(LL)(2)Cl-2](+) complexes by D-glucose (M=Os and Ru, LL=2,2 ′-bipyridine and 1,10-phenanthroline type ligands), *J. Biol. Inorg. Chem.*, **1999**, *4*, 175–82.
[30] J. Wang, Electrochemical glucose biosensors, *Chem. Rev.*, **2008**, *108*, 814–25.
[31] G. Cui, J. H. Yoo, B. W. Woo, S. S. Kim, G. S. Cha, H. Nam, Disposable amperometric glucose sensor electrode with enzyme-immobilized nitrocellulose strip, *Talanta*, **2001**, *54*, 1105–11.
[32] H. M. Deng, A. K. L. Teo, Z. Q. Gao, An interference-free glucose biosensor based on a novel low potential redox polymer mediator, *Sensor Actuat B-Chem.*, ***2014***, *191*, 522–8.
[33] E. Kosela, H. Elzanowska, W. Kutner, Charge mediation by ruthenium poly(pyridine) complexes in ′second-generation′ glucose biosensors based on carboxymethylated beta-cyclodextrin polymer membranes, *Anal. Bioanal. Chem.*, **2002**, *373*, 724–34.
[34] H. Yildirim, E. Guler, M. Yavuz, N. Ozturk, P. K. Yaman, E. Subasi, et al., Ruthenium (II) complexes of thiosemicarbazone: Synthesis, biosensor applications and evaluation as antimicrobial agents, *Mat. Sci. Eng. C-Mater.*, **2014**, *44*, 1–8.
[35] J. M. Zen, A. S. Kumar, C. R. Chung, A glucose biosensor employing a stable artificial peroxidase based on ruthenium purple anchored cinder, *Anal. Chem.*, **2003**, *75*, 2703–9.
[36] F. C. Vicentini, B. C. Janegitz, C. M. A. Brett, O. Fatibello-Filho, Tyrosinase biosensor based on a glassy carbon electrode modified with multi-walled carbon nanotubes and 1-butyl-3-methylimidazolium chloride within a dihexadecylphosphate film, *Sensor Actuat B-Chem.*, **2013**, *188*, 1101–8.
[37] H. Lineweaver, D. Burk, The determination of enzyme dissociation constants, *J. Am. Chem. Soc.*, **1934**, *56*, 658–66.
[38] H. J. Bright, Q. H. Gibson, Oxidation of 1-Deuterated Glucose by Glucose Oxidase, *J. Biol. Chem.*, **1967**, *242*, 994–1003.
[39] Q. H. Gibson, V. Massey, B. E. P. Swoboda, Kinetics+Mechanism of Action of Glucose Oxidase, *J. Biol. Chem.*, **1964**, *239*, 3927–34.

Received: July 14, 2015
Accepted: August 14, 2015
Published online: September 14, 2015

