

Photochemical and Electrochemical Study of the Release of Nitric Oxide from $[\text{Ru}(\text{bpy})_2\text{L}(\text{NO})](\text{PF}_6)_n$ Complexes (L = Imidazole, 1-Methylimidazole, Sulfite and Thiourea), Toward the Development of Therapeutic Photodynamic Agents

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The development of NO photoreleaser compounds has important potential applications on medicine, particularly on preventing topic infections and controlling cancers. Due to these expectations, the photochemical release of nitric oxide from complexes of $[\text{Ru}(\text{bpy})_2\text{LX}]^{n+}$, where L = imidazole, 1-methylimidazole, sulphite and thiourea and X = NO^+ and NO_2^- was investigated employing spectroscopic and electrochemical techniques. The release of NO was confirmed by chronoamperometry using a NO selective electrode, while the other product, mainly $[\text{Ru}^{\text{II}}\text{H}_2\text{O}]$, was detected by UV-Visible spectroscopy and electrochemical techniques for all complexes except for thiourea. The amount of NO released by these complexes upon irradiation was determined using a new developed method using square wave voltammetry.

Keywords: ruthenium nitrosyl complexes, photochemical, photodynamic therapy, metallodrug

Introduction

During the last decades, a large number of studies concerned with NO activity in biological systems have been published revealing novel physiological roles and promising therapeutical applications.¹⁻¹⁰ The NO molecule is the smallest well-known biological messenger that does not depend on specific transporters.¹¹ This diatomic molecule acts as a neurotransmitter,^{6,7} immune regulator,¹² besides being involved in the mechanisms of cellular death by apoptosis and necrosis.¹³⁻¹⁸ Insufficient NO limits NO-mediated signal transduction of normal or protective physiological processes. Dysfunction of the normally protective endothelium is found in several cardiovascular diseases, including hypertension, coronary heart disease, and arterial thrombotic disorders and other diseases such as diabetes.¹⁹⁻²¹

The molecule of NO reacts with several transition metals producing stable compounds that can be used

as source of controlled nitric oxide donors.²² There are different mechanisms of NO release from metal complexes, among them it can be mentioned the chemical or electrochemical reduction and light induction.^{10,23-26} The literature describes spectroscopic and photochemical studies of a series of nitrosyl ruthenium complexes,^{25,27,28} in particular, the *trans*- $[\text{RuCl}([\text{15}]\text{aneN}_4)\text{NO}]^{2+}$ species²⁸ when irradiated with laser of 355 nm in physiologic medium produces NO and the *trans*- $[\text{RuCl}([\text{15}]\text{aneN}_4)\text{H}_2\text{O}]^+$ species. The quantum yield found for the NO release was high (0.61 mol einstein⁻¹) when compared with similar complexes.²⁷ Once nitric oxide is a quite reactive species in biological fluids its therapeutical use requires a controlled and site-specific deliver system to achieve optimum action. Light has become one key element on delivering these features, which has been used in cancer treatment (e.g., photodynamic therapy, PDT) for many decades using reactive oxygen generators^{29,30} During the last years several therapeutic applications of NO donors have emerged including topic treatment, cloth for preservation of donor organs or to improve wound healing, bandages for sexual

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dysfunction, therapeutic socks for diabetics who show lower NO production among others.³¹⁻³³ Thus, it is essential an overall investigation of the routes of NO release for its practical use and evaluate if sun light could be used.

A series of ruthenium nitrosyl complexes have been prepared by our group showing promising pharmacological properties such as antiparasitic,³⁴ brain ischemia/reperfusion protection,³⁵ inflammatory pain inhibition,³⁶ and vasodilator.³⁷ At least in some of these studies, there are evidences supporting they may work by increasing NO levels, which activates sGC (soluble guanylate cyclase) pathway signaling,³⁵ or by inhibiting enzymatic activity through nitrosation reaction.³⁸ Despite these interesting biological data, it is well known that nitric oxide lacks selectivity and shows relative high reactivity in biological fluids. So, there is an increasing interest on developing strategies for selective delivery and release of this key molecule, which could maximize its effect and minimize undesired reactions. Certainly, the use of light is among the most prominent strategy, which has been similarly used in cancer for many decades (photodynamic therapy). As a first step toward the usage of this methodology is to evaluate the photochemical behavior of the therapeutical compounds under controlled photolysis.

In a previous paper³⁹ we showed that the $[\text{Ru}(\text{bpy})_2\text{L}(\text{NO})](\text{PF}_6)_n$ ($n = 1$ for $\text{L} =$ sulfite and $n = 3$ for $\text{L} =$ imidazole, respectively) complexes release NO after NO^+/NO reduction mediated by thiols. Also, this complex type showed biological activities such as fungicidal,³⁴ anti-inflammatory,³⁶ neuroprotector³⁵ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) inhibitor for Chagas' disease.³⁸ Aiming to evaluate NO release through another mechanistic route, it was carried out photochemical studies of $[\text{Ru}(\text{bpy})_2\text{L}(\text{NO})]^{n+}$ complexes, where L is imidazole (imN), 1-methylimidazole (mimN), sulfite (SO_3) and thiourea (tu), and their nitrite species as well. The product of these reactions was followed by UV-Vis spectroscopy and square wave voltammetry technique supporting the analytical use of this later technique for metallonitrosyl investigations in the field.

Experimental

Chemicals and reagents

Ultrapure water prepared in a Milli-Q system (Direct-Q 3 from Millipore) was employed throughout this work. $\text{RuCl}_3 \cdot x\text{H}_2\text{O}$, imidazole, 1-methylimidazole, sulfite and thiourea were obtained from Aldrich and used without further purification. *Cis*- $[\text{Ru}(\text{bpy})_2\text{L}(\text{NO})](\text{PF}_6)_n$ complexes ($\text{L} =$ imidazole, 1-methylimidazole, sulfite and

thiourea) complexes were prepared according to procedures described in the literature.²³

Apparatus

Samples prepared in buffered solution were irradiated aiming to photorelease nitric oxide, and their quantum yields were calculated based on the amount of photolysed species formed. In these quantitative experiments, it was employed a homemade photochemical reactor UNT 001 containing 355 nm lamps (Figure available in the Supplementary Information), temperature controlled at 25 °C, where the samples were irradiated and the products of these photolysis monitored by square wave voltammetry, following the decrease of the wave at -0.10 to 0.25 V (*vs.* Ag/AgCl) due to $[\text{RuNO}]^{6/7}$ redox couple. Qualitative experiments to observe the NO release were also carried out using a NO meter in NO-T model CC (Innovative Instruments). Quantum yield measurements were performed using a Hummingbird diode laser (Quantum Tech) in 377 nm and 419 nm, and product of formation was calculated using a UV-Visible-NIR (near infrared spectroscopy) Hitachi model U-3501 spectrophotometer, using 1.0 cm path length quartz cells until no spectroscopic changes were observed. Electron paramagnetic resonance (EPR) spectra were obtained in a Bruker EMX Plus X-band spectrometer at room or liquid nitrogen temperature.

Electrochemical experiments for determination of NO release

Measurement of the nitric oxide released was accomplished using an electrochemical potentiostat Epsilon (Bioanalytical System Inc.) employing a conventional three-electrode cell consisting of platinum, glassy carbon, and Ag/AgCl as auxiliary, working and reference electrodes, respectively. These measurements were carried out at 25 °C in a 0.1 mol L^{-1} aqueous solution of sodium trifluoroacetate (NaTFA), pH 2.0, for $\text{L} =$ imidazole, 1-methylimidazole, pH 4.2, for $\text{L} =$ thiourea and pH 7.4, for $\text{L} =$ sulfite, as supporting electrolyte using square wave voltammetry. The difference in the pH was necessary due to the nitrosyl/nitrite interconversion reaction, which occurs above this pH for each specie (equation 1). According to the equation 1, the pK_a are 5.5; 6.4 and 10.3 for $\text{L} =$ imidazole, thiourea and sulfite, respectively.

Analytical routine was followed for the development of a quantitative method for measuring the release of nitric oxide. First of all, it was established the parameters that showed the best results for current *vs.* concentration relationship by varying frequency (10-90 Hz), width

(10-70 mV) and increment (1-10 mV) of the wave, where only one parameter was changed each time during optimization (analytical curves are available in the Supplementary Information).

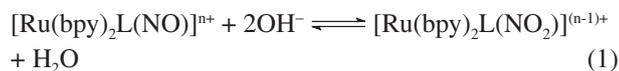
Analytical standard curve was prepared using nitric oxide metal complexes by taking the current value at the peak for the NO⁺⁰ redox process for each concentration, which resulted in a linear relationship.

A solution of each nitrosyl compound was irradiated and the amount of NO released was monitored with successive electrochemical measurements of the current at the peak of the potential of the coordinated NO⁺⁰ redox couple. Once, these voltammograms were analyzed and peak currents extracted at each time point, the NO concentration were calculated by interpolation of the standard curve, prepared previously. Since the stoichiometric relationship between NO released and bound to the complex is the same, then the difference in NO concentration before and after the irradiation is equal to the concentration of NO released in the process.

Results and Discussion

The absorption spectra of the *cis*-[Ru(bpy)₂L(NO)]ⁿ⁺ nitrosyl complexes, where L = imidazole, 1-methylimidazole, sulfite and thiourea, showed bands from 310-326 nm attributed to (bpy)π*←dπ(Ru) MLCT transition. The (NO⁺)π*←dπ(Ru) transitions were not detected in the spectra probably because it is overlapped by the (bpy)π*←dπ(Ru) transitions. These bands are expected to shift to higher energy due to the stabilization of the Ru^{II} dπ orbitals as consequence of the (bpy)π*←dπ(Ru) backbonding interaction.⁴⁰⁻⁴²

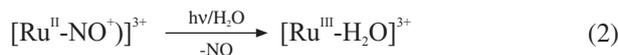
The wavelength used in the irradiation of the complexes was chosen according to their absorption bands. In aqueous solution ruthenium nitrosyl complexes exhibit the equilibrium displayed in the equation 1.^{23,43,44}



where for the complexes investigated here the pK_a values for this equilibrium are 10.3, 5.5, and 6.4 for L = SO₃²⁻, imidazole and thiourea, respectively.²³ So, for these studies it was chosen a pH where only one species is predominant, such as in the RuNO⁺ form.

Photochemical release of nitric oxide has been widely investigated during the last years as a selective strategy to deliver this pharmacological relevant molecule; however; still several drawbacks prevent its suitable use. There are several studies concerning photochemical reactivity

of ruthenium nitrosyl complexes when irradiated in the ultraviolet and visible range. Interestingly, many of these reports has suggested that NO release is a consequence of the photoelectronic energy transfer from the metal center to the nitrosyl species forming the oxidized metal center and reduced NO as final products as illustrated in equation 2.^{25,26,45-48}



However, Ru^{III}-H₂O species is not always observed as a photoproduct, where in some cases it has been detected Ru^{II}-H₂O species instead. Rose and Mascharak⁴⁵ had proposed that the photochemical behavior of the {RuNO}⁶ regarding to the NO release could be classified in two types of mechanisms: the first one is observed the formation of the NO and [Ru^{III}-solvent], while the second one is observed the formation of NO and [Ru^{II}-solvent] as final products. The first type is noticed for the Ru-nitrosyl complexes having as auxiliary ligands ammine, carboxamide, thiolate, heme and phenolate, whereas the second type for polypyridines and Schiff base as auxiliary ligands. For the second type of mechanism, despite it is not very clear, the formation of Ru^{II} species seems to proceed from a spontaneous reduction of the transient Ru^{III} species caused by incident light.^{45,46} In our case, the irradiations conducted at 377 nm (for nitrosyl complexes) and at 477 nm (for nitrite complexes) for the solutions of the complexes with L = SO₃²⁻, imidazole and 1-methylimidazole showed profiles compatible to Ru^{II}-H₂O species as supported by UV-Vis, EPR and ¹H NMR (nuclear magnetic resonance) spectra (data available in the Supplementary Information). These results suggested that these complexes follow the second type of mechanism proposed by Rose and Mascharak.⁴⁵ To confirm this, these complexes were fully reduced by Zn/Hg amalgam and the spectrum recorded (Figure 1) was the same obtained after photolysis, as described above. Also, the ¹H NMR spectrum of the product of irradiation at 350 nm for the sulfite complex, showed a profile compatible with the [Ru(bpy)₂(SO₃)(H₂O)]²⁺ complex ion. Additionally, the EPR spectrum was collected for a solution containing the [Ru(bpy)₂(SO₃)NO]⁺ after 60 minutes of laser irradiation at 350 nm and it did not show any significant signal of paramagnetic Ru^{III}, which is compatible with a EPR silent species such as Ru^{II}-H₂O as proposed here (the spectrum is available in the Supplementary Information). The ruthenium complexes containing L = imidazole and 1-methylimidazole showed similar behavior, where their photoproducts were also EPR-silent consistent with Ru^{II}-H₂O. However, for the complex containing thiourea as auxiliary ligand, the electronic spectrum profile suggested

the formation of another species instead the $\text{Ru}^{\text{II}}\text{-H}_2\text{O}$, which was not fully characterized and is still under investigation. Nevertheless, this photoproduct was also EPR-silent supporting Ru^{II} is maintained as final species.

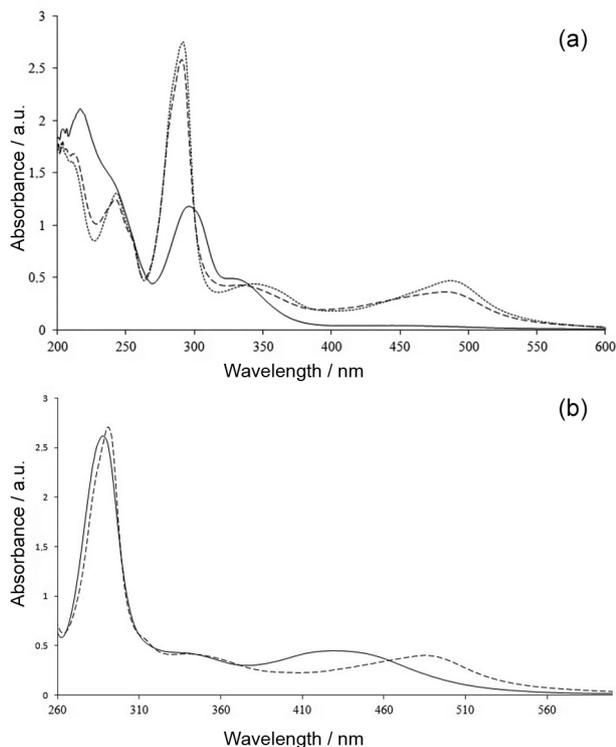


Figure 1. UV-VIS spectra of $[\text{Ru}(\text{bpy})_2\text{imN}(\text{X})]^{n+}$ complex ion upon irradiation at 377 nm for 30 min, for $\text{X} = \text{NO}^+$ and 447 nm for $\text{X} = \text{NO}_2^-$ and reduction by Zn/Hg amalgam. (a) $\text{X} = \text{NO}^+$: solid line: before irradiation; dashed line: after irradiation; dotted line: reduced by Zn/Hg amalgam; (b) $\text{X} = \text{NO}_2^-$: solid line: before irradiation, dashed line: after irradiation.

Figure 2 shows spectral changes for the $\text{cis-}[\text{Ru}(\text{bpy})_2(\text{imN})(\text{X})]^{n+}$ complexes, where $\text{X} = \text{NO}_2^-$ and NO^+ , before and after irradiation for 30 min at 447 nm and 377 nm, respectively. After irradiation, both solutions exhibited a decreasing in the absorbance of the bands at 433 nm for NO_2^- followed by the appearance of a new band at 488 nm for both complexes in an agreement with the formation of the same specie.

Silva and co-workers^{46,47} and Rose and Mascharak⁴⁵ reported photochemical studies of a similar ruthenium nitrosyl complexes. They proposed upon irradiation an electron transfer from the metal center to the nitrosyl ligand occurs generating an intermediate species with a consequent NO release and formation of the $[\text{Ru}^{\text{III}}\text{-OH}_2]$ which is further reduced forming as final product $[\text{Ru}^{\text{II}}\text{-OH}_2]$ moiety, besides the nature of the reducing agent being unknown, with the suggestion that the NO also can act in this process. This observation is the same as detected in the compounds presented here (equations 3-5).

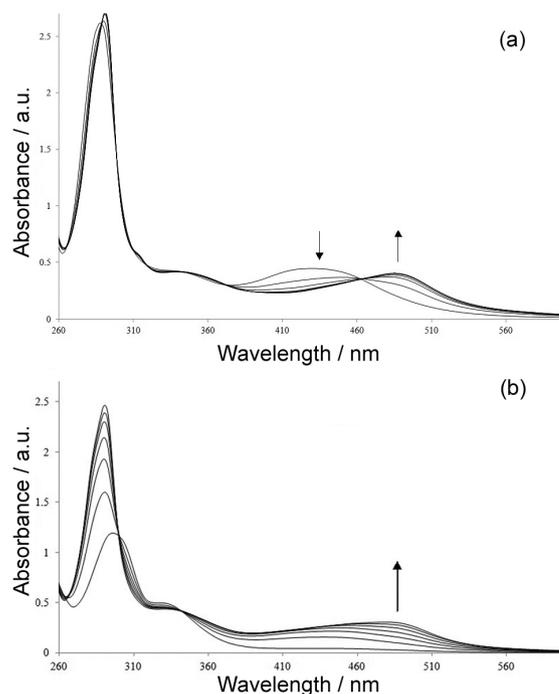
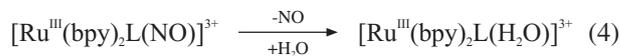


Figure 2. Spectral changes during photolysis of: (a) $\text{cis-}[\text{Ru}(\text{bpy})_2(\text{imN})(\text{NO}_2)]^+$, $[\text{Ru}] = 6.0 \times 10^{-5} \text{ mol L}^{-1}$, in buffer solution, pH 7.4, $\mu = 0.1 \text{ mol L}^{-1}$, $\lambda_{\text{irr}} = 447 \text{ nm}$ and (b) $\text{cis-}[\text{Ru}(\text{bpy})_2(\text{imN})(\text{NO})]^{3+}$, $[\text{Ru}] = 6.0 \times 10^{-5} \text{ mol L}^{-1}$, in buffer solution, pH 2.1, $\mu = 0.1 \text{ mol L}^{-1}$, $\lambda_{\text{irr}} = 377 \text{ nm}$.



Since free NO was verified by the NO meter (Figure 3) and the final product obtained from the irradiation showed similar electronic spectra to $[\text{Ru}^{\text{II}}\text{-OH}_2]$ species, a mechanism of mono-electronic reduction is likely for all the nitrosyl species, except the $[\text{Ru}(\text{bpy})_2(\text{tu})\text{NO}]^{3+}$, which

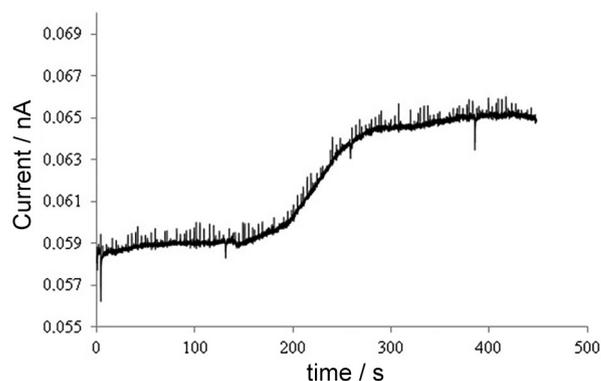


Figure 3. Chronoamperogram of a buffered aqueous solution (pH 7.40) containing the complex ion $[\text{Ru}(\text{bpy})_2(\text{SO}_3)\text{NO}]^+$ after irradiation at 377 nm.

Table 1. Quantum yield for the photolysis of $\text{cis}-[\text{Ru}(\text{bpy})_2\text{L}(\text{X})]^{n+}$ in buffer solutions

L/X	λ_{irr} / nm	$\lambda_{\text{monitored}}$ / nm	(ϕ) Formation of $\text{Ru}^{\text{II}}\text{-OH}_2$	Reference
Sulfite/ NO^{a}	377	488	0.00182	This work
imN/ NO^{b}	377	488	0.00315	This work
imN/ NO_2^{a}	447	488	0.00252	This work
1-meimN/ NO^{b}	377	488	0.00689	This work
1-meimN/ NO_2^{a}	447	488	0.00336	This work
Pyridine/ NO_2	355	416	0.00700	46
4-Pycoline/ NO_2	355	418	0.00900	46
Pyrazine/ NO_2	355	408	0.03700	46

^apH: 7.4; ^bpH: 2.0.

is observed the formation of a different final species, as mentioned before, not identified yet.

Quantitative data is presented in Table 1 which summarizes the quantum yield for the formation of the $[\text{Ru}^{\text{II}}\text{-OH}_2]$ species after irradiation of the nitrosyl complexes type $[\text{Ru}(\text{bpy})_2\text{LNO}]^{n+}$ presented in this work. This measurement assumes that the formation of the $[\text{Ru}^{\text{II}}\text{-OH}_2]$ and NO release are equimolar. It is important to remark that for all of the complexes, quantum yield measurement was based only on the formation of the aquo species as detected by electronic absorption, and free NO was detected qualitatively by NO meter (Figure 3).

As the pH for these experiments was kept in 2.0-7.4 range, for the $[\text{Ru}(\text{bpy})_2(\text{SO}_3)(\text{NO}_2)]^-$ it was not possible to proceed this measurement, since in this pH range this compound is presented in nitrosyl form instead of nitrite form.²³

The data collected in this work can be compared to similar compounds prepared elsewhere, $\text{cis}-[\text{Ru}(\text{bpy})_2(\text{L})(\text{NO}_2)]^+$, where L = pyridine,⁴⁶ 4-pycoline⁴⁶ and pyrazine⁴⁶ (Table 1). Despite the usage of a much longer wavelength, the results presented here were still considerably attractive granting further attempt to modulate compounds which can act as NO donors under irradiation.⁴⁶ Here, it was observed the formation of the aqua complex for all the species upon irradiation in agreement to their electrochemical and spectroscopic profiles. However, the best nitric oxide photo releaser compound was found to be the complex $[\text{Ru}(\text{bpy})_2(1\text{-meimN})\text{NO}](\text{PF}_6)_3$ which was directly followed by the $[\text{Ru}(\text{bpy})_2(1\text{-meimN})\text{NO}_2]\text{PF}_6$. The other compounds also present significant photochemical release of nitric oxide in the same way.

In order to quantify the amount of NO released, for the nitrosyl complexes, the photochemical release of NO was also monitored by square wave voltammetry following the decrease of the peak current centered at -0.10 to 0.25 mV range attributed to the coordinated $\text{NO}^{+/0}$ redox process

(Figure 4). By observing the linear correlation between the peak current and the complex concentration ($R = 0.999$, Figures available in the Supplementary Information) it was possible to calculate the amount of NO released after irradiation (see Experimental section). Table 2 summarizes the results of the NO release after 10 min of irradiation, where it can be observed the co-spectator ligand 1-methylimidazole (1-meimN) promotes a better NO releasing if compared to the worse process for sulfite complex, being the trend of the percentage on NO released the same as the quantum yield.

These results are in good agreement with the quantum yield data, where the highest ϕ for the formation of the $\text{Ru}^{\text{II}}\text{-OH}_2$ species was observed for the $[\text{Ru}(\text{bpy})_2(1\text{-meimN})\text{NO}]^{3+}$ complex, which also presents the highest percentage of NO release quantified by square wave voltammetry (87.1%). Interestingly, this new approach to quantify indirectly

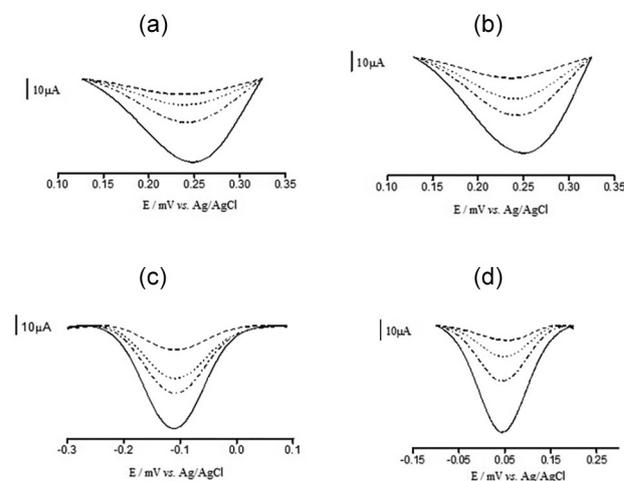


Figure 4. Photolysis of the $\text{cis}-[\text{Ru}(\text{bpy})_2\text{L}(\text{NO})]^{n+}$ complexes (0.1 mol L^{-1}) during 20 min of irradiation at 355 nm, monitored by square wave voltammetry, using glassy carbon electrode in buffer solutions, where L: 1-methylimidazole (pH = 2.03) in (a); imidazole (pH = 2.03) in (b); sulfite (pH = 7.4) in (c) and thiourea (pH = 4.16) in (d). Time of irradiation: 0 min (solid line), 5 min (dotted-dash line), 10 min (dotted line) and 20 min (dashed line).

Table 2. Results of the calculation of the liberation photochemical of nitric oxide for the *cis*-[Ru(bpy)₂L(NO)]ⁿ⁺ complexes, using the technique of square-wave voltammetry

L	[Ru-NO] / (mol L ⁻¹) t = 0	[NO] / (mol L ⁻¹) t = 10 min	NO / %	(φ) Formation of Ru ^{II} -OH ₂
imN ^a	1.995 × 10 ⁻⁴	1.321 × 10 ⁻⁴	66.3	0.00315
1-meimN ^a	1.723 × 10 ⁻⁴	1.501 × 10 ⁻⁴	87.1	0.00689
Thiourea ^b	2.451 × 10 ⁻⁴	1.820 × 10 ⁻⁴	74.3	–
Sulfite ^c	2.369 × 10 ⁻⁴	1.159 × 10 ⁻⁴	49.0	0.00182

^apH: 2.0; ^bpH: 4.2; ^cpH: 7.4.

NO release and has showed promising use for further investigation of metallonitrosyl complexes.

Conclusions

Square-wave voltammetry has been shown indeed a reliable analytical tool for quantitative measurements of the amount of nitric oxide released, particularly important when working with slow-releasing compound. We should mention there is still a limited number of reliable methods for nitric oxide quantification, and even less for slow NO-releasing compounds.^{49,50}

Here, we have shown that the photolysis of *cis*-[Ru(bpy)₂L(X)]ⁿ⁺ complexes, where L is imidazole, 1-methylimidazole, sulfite and thiourea, produces indeed NO when irradiated with light from 350-380 nm. A limitation faced by most of these studies relies on the very short wavelength required for NO release, usually in the ultraviolet range, which is not a practical option for its therapeutic use. Interestingly, the nitrite-based metal complexes were also capable of releasing nitric oxide with a reasonable quantum yield, but remarkably using visible light at 477 nm (blue light).

In summary, the presented data of quantitative release of NO expressed by quantum yield measurements along with qualitative detection of NO using selective electrode of NO showed 1-methylimidazole ligand promotes the highest amount of nitric oxide released, upon irradiation, when compared to other ones.

Methyl group on imidazole has caused a better NO release, which supports a stronger sigma donation, by this ligand, to ruthenium compared with others presented here. The difference on pK_a for NO₂⁻/NO⁺ equilibrium can be an interesting hint for their potential biological usage where very low pK_a compounds could be converted to mainly NO⁺ species in stomach and higher ones (e.g., sulfite) could be still kept as NO⁺ in blood. However, if nitrite species can indeed release NO using even longer wavelength, it is suggestive that this equilibrium could be actually used to produce several inorganic NO donors.

Supplementary Information

Supplementary data (analytical curves for the square wave voltammetry determination, NMR and EPR spectra) are available free of charge at <http://jbcbs.s bq.org.br> as PDF file.

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