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Controlled nitric oxide photo-release from nitro ruthenium complexes: The vasodilator response produced by UV light irradiation

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Abstract

Preliminary pharmacological studies of various nitric oxide (NO) photo-releasing agents are reported based on the flash-photolysis studies of the nitro ruthenium complexes *cis*-$[Ru^{II}(NO_2)L(bpy)_2]^+$ (bpy = 2,2′-bipyridine and L = pyridine, 4-picoline and pyrazine) and $[Ru^{II}(NO_2)(bpy)(terpy)]^+$ (terpy = terpyridine) in physiological medium. The net photoreactions under these conditions are two primary photoproducts, in (**I**) there is Ru^{II}–NO_2 photoaquation, where the photoproducts are Ru^{II}–H_2O plus NO_2^- and (**II**) homolytic dissociation of NO from a coordinated nitrito to derive the Ru^{II}–OH_2 specie and NO. Based on photochemical processes, the nitro ruthenium complexes were incorporated in water in oil (W/O) microemulsion and used in the vasorelaxation induced experiment. Denuded rat aortas were contracted with KCl and nitro ruthenium complexes in microemulsion were added. Perfusion pressures were recorded while arteries were irradiated at 355 nm The time to reach maximum relaxation was longer for $[Ru^{II}(NO_2)(bpy)(terpy)]^+$ complex (ca. 50 min, $n = 6$) than for *cis*-$[Ru(NO_2)L(bpy)_2]^+$ with L = py and 4-pic complex (ca. 28 min, $n = 6$) and *cis*-$[Ru(NO_2)(bpy)_2(pz)]^{2+}$ complex (ca. 24 min, $n = 5$).

Keywords: Nitro ruthenium complexes; Nitric oxide; Nitrosyl complexes

1. Introduction

The discovery that nitric oxide (NO) plays important roles as a biological messenger in a wide range of physiological processes has stimulated great interests in the studies of its chemical and biochemical properties, especially the investigation of NO-mediated events [1]. Considering that the chemistry of NO is the primary determinant of its biological properties [2], which is limited to ca. 100 ms due to its high reactivity, the description and evaluation of its biochemical pathway in a physiological medium became complex [3]. In this context, compounds capable of releasing NO in a specific biological target could provide a useful tool to study the physiological actions of NO [4–8]. Besides this effect, the design of new NO delivery agents could also have a potential biomedical application, once it could act as a pharmaceutical agent providing NO under demand to a specific site. Therefore, it is very important to study the chemical and physico-chemical properties of such NO delivery agent in physiological medium.

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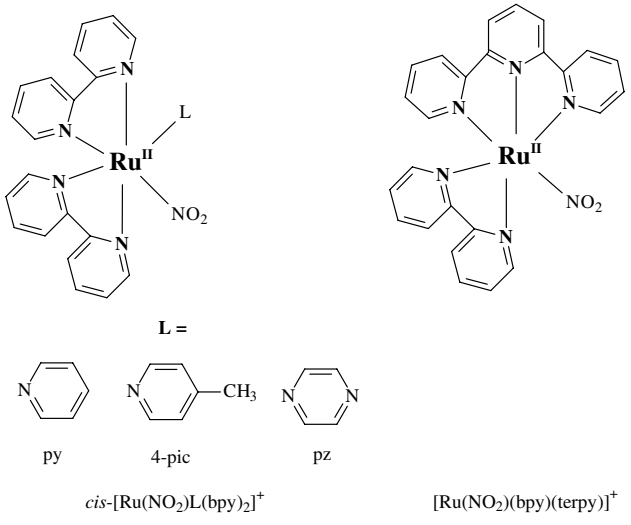
Some metallonitrosyl complexes have been used as NO delivery agents [9–13]. The stability of the complexes and the kinetics of NO release have been postulated as dependent on the co-ligands and the metal ion. Maybe the most studied system capable to release NO under stimulation is the nitrosyl ruthenium species, which is formally described as containing Ru^{II}–NO^{+} bond [2]. Nitrosyl ruthenium complexes are attractive because of their thermal stability, although under light [9–16] or electrochemical [17–20] stimulation they can be NO delivery agents. Despite numerous investigations of nitrosyl ruthenium complexes, only few examples appear as stable in a physiological pH [21], which has limited the use of those species as metal-based drugs.

We have explored the potential of nitro ruthenium complexes as an NO delivery agent in physiological conditions. Some physical chemistry properties of *cis*-$[Ru^{II}(NO_2)(bpy)_2(py)]^{+}$ have been described in dichloromethane [22]. Here, we present some photochemical properties of *cis*-$[Ru^{II}(NO_2)L(bpy)_2]^{+}$ where bpy is 2,2′-bipyridine and L = pyridine, 4-picoline and pyrazine, and $[Ru^{II}(NO_2)(bpy)(terpy)]^{+}$ with 2,2′:6′,2″-terpy is terpyridine (Scheme 1) in aqueous solution. The observation that all nitro ruthenium complexes described in this work are NO delivery agents directs us to explore its vasodilator activity in an isolated rat aorta. To avoid any side reactions in the cell using nitro ruthenium complexes, we performed the pharmacological assays with the complex inside of water in oil (W/O) microemulsion, which was used as an efficient drug delivery system. The aim of this work was to study the effect of the nitro ruthenium complex as a pro-drug that could release NO only when it was induced by light irradiation.



Scheme 1.

2. Experimental

2.1. Apparatus

The ultraviolet–visible (UV–Vis) spectra were recorded on a Hitachi U-3501 and Genesys-2 apparatus from Spectronic. The pH measurements were made using a 430 pH meter from Corning. The photochemical experiments were made using a laser flash-photolysis apparatus consisting of a Continuum Q-switched Nd-YAG laser (Continuum, Santa Clara, CA) with excitation provided by the third harmonic at $\lambda = 355$ nm. The pulse length was 8 ns, the beam diameter incident on the sample was 6 mm, and the repetition rate was 10 Hz. The pulse energy was typically 15 mJ as measured with a Field Master powermeter with an L-30V head. NO release was measured with an ISO-NOP NO meter from World Precision Instruments. High Performance Liquid Chromatography (HPLC) system consisted of a Shimadzu liquid chromatography equipped with a LC-10AD pump, SPD-10AV UV–Vis detector, and C-R6A integrator. A CLC-ODS column (5 mm; 250_4.6 mm i.d.; Shimadzu) was used in all experiments.

2.2. Chemicals and reagents

$RuCl_3 \cdot nH_2O$, 2,2′-bipyridine (bpy), 4-picoline (4-pic), pyridine (py), pyrazine (pz), and terpyridine (terpy) were purchased as high purity reagents from Aldrich Chemicals and were used as supplied. The recrystallized complex salts *cis*-$[Ru^{II}(bpy)_2(L)NO^{+}](PF_6)_3$ (L = py, 4-pic) and $[Ru^{II}(bpy)(terpy)NO^{+}](PF_6)_3$ were prepared as previously described [23,24]. The complexes were characterized by UV–Vis and infrared spectroscopy, and then compared to the published results [23,24]. UV–Vis spectra: λ (nm) ($\log\varepsilon$) $[Ru^{II}(bpy)_2(py)NO^{+}](PF_6)_3$: 334 (3.70), 300 (4.17) and FTIR ν_{NO} 1947 cm^{-1}; $[Ru^{II}(bpy)_2(4\text{-pic})NO^{+}](PF_6)_3$: 332 (3.67), 300 (4.09) and FTIR ν_{NO} 1944 cm^{-1}; $[Ru^{II}(bpy)(terpy)NO^{+}](PF_6)_3$: 365 (3.87), 328 (4.05), 307 (4.24), 286 (4.30) and FTIR ν_{NO} 1949 cm^{-1}. The *cis*-$[Ru^{II}(bpy)_2(pz)NO^{+}](PF_6)_3$ was synthesized by a similar procedure described for the synthesis of *cis*-$[Ru^{II}(bpy)_2(py)NO^{+}](PF_6)_3$, but using pyrazine as the attack ligand. Calc. for $C_{24}H_{20}N_7OP_3F_{18}Ru$: C, 30.44; H, 2.01; N, 10.53. Found: C, 30.08; H, 2.09; N, 10.23%. UV–Vis spectra: λ (nm) ($\log\varepsilon$) $[Ru^{II}(bpy)_2(pz)NO^{+}](PF_6)_3$: 432 (3.34), 338 (3.74), 300 (4.26), and FTIR ν_{NO} 1950 cm^{-1}.

The *cis*-$[Ru^{II}(NO_2)L(bpy)_2]^{+}$ with L = pyridine, 4-picoline and pyrazine, and $[Ru^{II}(NO_2)(bpy)(terpy)]^{+}$ were synthesized in situ by dissolving the nitrosyl specie in a phosphate buffer solution, pH 7.4. The ionic strength was adjusted with NaCl until 0.14 M in the photochemical experiments. All preparations and measurements were carried out under an argon atmosphere

and protected from light. Doubly distilled H_2O was used for all experiments.

2.3. HPLC mobile phase

Isocratic elution with (%) 20:80 ethanol:water containing 0.1 M of phosphate buffer solution, pH 7.4, was used at a constant flow-rate of 1.0 ml min^{-1}, at room temperature. Samples for analysis were dissolved in the mobile phase and 25 μl volumes were injected throughout the experiments and used to check the purity of the complexes. Time retention for *cis*-$[Ru^{II}(NO_2)L(bpy)_2]^+$ with L = pyridine: 6.40 min; 4-picoline: 6.58 min; pyrazine: 6.30 min and for $[Ru^{II}(NO_2)(bpy)(terpy)]^+$: 7.46 min.

2.4. NO measurements with the ISO-NOP NO-meter and the DUO-18 acquisition board

NO release was measured with an ISO-NOP NO meter from World Precision Instruments. The sensitivity of this apparatus ranges from 1 nM to 20 μM, with a 2 mm sensor, which directly detects NO concentration by an amperometric technique.

The calibration curve was constructed by dilutions of nitric oxide stock solution. Nitric oxide gas was purchased from Oxigênio do Brasil and passed through a 10 M KOH solution to remove any trace of NO_2^- specie. Nitric oxide stock solution was prepared by degassing aqueous phosphate buffer solution (pH 7.4) followed by the introduction of nitric oxide as previously described [25]. The NO concentrations were calculated according to the reported molar fraction solubility of NO (2.1 mM at 25 °C) [26], which was confirmed by titration with $KMnO_4$ as previously described [27].

The 1 cm path length quartz cell containing the sample solution was routinely thermostated at 25.0 ± 0.1 °C. The sample solution was stirred continuously during the flash-photolysis experiment and the NO measurements were made with the electrode positioned outside the light path to avoid any photoelectric interference. The output of the sensor was recorded with an IBM-PC computer linked to a DUO-18 acquisition board from WPI.

2.5. Quantum yield measurements

Light intensities were determined by the classical potassium ferrioxalate salt actinometry procedure after each photolysis experiment [28,29]. The NO quantum yield production was calculated based on NO concentrations obtained by NO meter measurement. For most runs, the initial concentration of *cis*-$[Ru^{II}(NO_2)L(bpy)_2]^+$ and $[Ru^{II}(NO_2)(bpy)(terpy)]^+$ was ca. 1.0×10^{-4} M. After the equilibration of the cell holder temperature, photolysis was initiated by irradiating the sample for a period of time ranging from 0 to 60 s with increments of 10 s. The calculated ϕ_{NO} values obtained in the range studies were plotted versus t. These plots were linear, with a negative slope, for the first 40% of the reaction. The extrapolated spectroscopic quantum yield at $t = 0$ was taken as ϕ_{NO} for the photoaquation of NO from *cis*-$[Ru^{II}(NO_2)L(bpy)_2]^+$ and $[Ru^{II}(NO_2)(bpy)(terpy)]^+$. Evaluation of ϕ_{NO} at $t = 0$ eliminates possible complications resulting from secondary photolysis of primary reaction product [30,31].

2.6. Preparation of nitro ruthenium complex formulation

Liquid water-in-oil (W/O) microemulsions were prepared according to Wu et al. [32] and Gelfuso and Lopez [33] by dissolving 64.25% (w/w) of olive oil and a 1:1 mixture of sorbitan monooleate (Span 80) and polyoxyethylene 20 sorbitan monooleate (Tween 80) (32.50% w/w) in an aqueous solution of nitro ruthenium complex. The aqueous phase consisted of complexes (7.6×10^{-4} M) dissolved in propylene glycol/isotonic 0.1 M phosphate buffer, pH 7.4 (1:1 v/v). To obtain a clear isotropic system, all the ingredients were mixed by agitating for 24 h. Microemulsions were stored at room temperature and protected from light until used in the experiments.

2.6.1. In vitro release studies of nitro ruthenium complex

The amount of nitro ruthenium complexes and their release profiles was determined in vitro using a modified Franz diffusion cell and a hydrophilic cellulose membrane. The receptor medium was phosphate buffer solution, pH 7.4, at 37.0 °C stirred at 300 rpm. The donor compartment was filled with 1.5 g of microemulsion containing *cis*-$[Ru^{II}(NO_2)L(bpy)_2]^+$ or $[Ru^{II}(NO_2)(bpy)(terpy)]^+$ complex at 7.6×10^{-4} M. The total available diffusion area of the cell was 1.3 cm^2. At regular intervals for up to 12 h, 1.5 ml of the receptor phase was removed for the determination of the total drug content by UV–Vis spectrophotometer, and replaced by an equal volume of fresh receptor solution.

2.7. Pharmacological experimental protocols

2.7.1. Vessel preparation

Male Wistar rats (400–450 g) were killed by decapitation in accordance with the Ethical Animal Committee, Ribeirão Preto Campus, University of São Paulo, Brazil. The thoracic aorta was quickly removed, dissected free, and cut into 4 mm long rings. Since the response to nitro ruthenium complexes does not require intact endothelium, in the present study we investigated the relaxation induced by this compound in endothelium-denuded arteries. The endothelium was mechanically removed and the aortic rings were placed between two stainless-steel stirrups and connected to an isometric force transducer (Letica Scientific Instruments) in order

to measure tension in the vessels. The rings were placed in a 10 ml organ chamber containing Krebs solution with the following composition ($M \times 10^3$): NaCl 130, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 14.9, glucose 5.5, and $CaCl_2$ 1.6. The solution was maintained at pH 7.4 gassed with 95% O_2 and 5% CO_2 at 37 °C. The rings were initially stretched to a basal tension of 1.5 g (previously determined by length–tension relationship experiments), before allowing them to equilibrate for 60 min in the bath, which was changed every 15–20 min. Endothelial integrity was qualitatively assessed by the degree of relaxation caused by acetylcholine (ACh, 1×10^{-6} M) in the presence of contractile tone induced by phenylephrine (1×10^{-7} M) [34]. Since our studies required endothelium-denuded aortas, the rings were discarded if there was any degree of relaxation, to avoid the possible influence of endothelial factors.

2.7.2. Light source

The radiation source for the photo-relaxation experiment was made using a UV lamp with peak intensity at 355 nm. The UV lamp was placed next to the outer wall of a jacketed glass incubation chamber. The distance from the lamp to the preparation during irradiation was about 17 cm.

2.7.3. Experimental protocols

Time-course for the relaxation induced by *cis*-$[Ru^{II}(NO_2)L(bpy)_2]^+$ (L = pyridine, 4-picoline and pirazine) and $[Ru^{II}(NO_2)(bpy)(terpy)]^+$ complexes.

The nitro ruthenium complexes (1×10^{-4} M) were added to the organ chamber when a stable contraction in response to 6×10^{-5} M KCl was achieved. Following the addition of nitro ruthenium complexes, the 355 nm light was irradiated for 50 min.

3. Results and discussion

The production of NO using a coordination compound is of great interest, as one way to mimic many NO dependent physiological processes in nature, and of developing novel NO delivery systems. One of the possibilities is to use light irradiation in the appropriate wavelength on a solution containing metalonitrosyl complexes leading to control release of NO. Unfortunately, most nitrosyl ruthenium complexes, which could act as NO delivery agent under light stimulation, have been described mainly for the photolysis in acidic medium [9,11,12]. Taking into account that a metal-based drug should be stable in physiological pH and considering that a nitro compound has been described as a NO delivery agent for some coordination compound [35–39], we decided to evaluate the possibility to have nitro ruthenium specie as a source of NO using 355 nm light irradiation at pH 7.4.

The $[Ru(NO_2)L_5]^+$, which will be used to indicate the *cis*-$[Ru^{II}(NO_2)L(bpy)_2]^+$ complex with L = pyridine, 4-picoline, pyrazine and $[Ru^{II}(NO_2)(bpy)(terpy)]^+$, was obtained based on the rate of conversion of nitrosyl to nitro specie [22]. The $[RuL_5(NO)]^{3+}$ react very rapidly in solution with hydroxide ion to give deep yellow solution. The shape of the UV–Vis spectrum changed gradually during the course of the reaction developing a new band in visible region after dissolving $[RuL_5(NO)]^{3+}$ in basic medium. This reaction is pH dependent once nitrosyl ruthenium complex is regenerated in pH 1.0. One of the spectral changes of the reaction of a nitrosyl ruthenium complex with hydroxide ion can be observed in Fig. 1.

The *cis*-$[Ru^{II}(NO_2)L(bpy)_2]^+$ (L = py, 4-pic and pz) and $[Ru^{II}(NO_2)(bpy)(terpy)]^+$ complexes were characterized by UV–Vis spectrum (Table 1).

The flash-photolyses of *cis*-$[Ru^{II}(NO_2)L(bpy)_2]^+$, where bpy is 2,2′-bipyridine and L = py, 4-pic and pz, and $[Ru^{II}(NO_2)(bpy)(terpy)]^+$ were carried out with 355 nm light irradiation in, pH 7.4, aqueous solution.

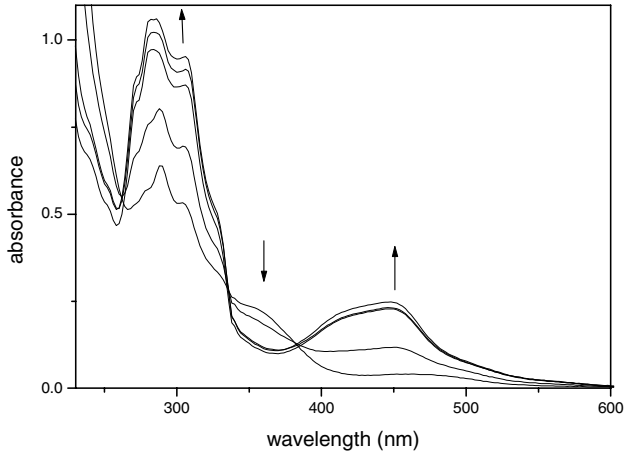


Fig. 1. Spectral profile change with pH for the conversion of $[Ru^{II}(bpy)(terpy)NO^+]^{3+}$ in $[Ru^{II}(NO_2)(bpy)(terpy)]^+$. pH is 2.48, 4.3, 5.72, 7.16 and 10.29.

Table 1
Electronic spectral data for nitro ruthenium complexes

Complex	λ (nm)/log ε[a]
cis-$[Ru^{II}(NO_2)(bpy)_2(py)]^+$	240/4.20; 286/4.35 332/3.86; 416/3.57
cis-$[Ru^{II}(NO_2)(bpy)_2(4\text{-pic})]^+$	240/4.13; 286/4.37 342/3.74; 418/3.62
cis-$[Ru^{II}(NO_2)(bpy)_2(pz)]^+$	246/4.15; 284/4.31 408/3.68
$[Ru^{II}(NO_2)(bpy)(terpy)]^+$	240/4.16; 274/4.26 282/4.34; 308/4.27 330/3.97; 420/3.69 448/3.72

[a] All data were collected in phosphate buffer solution, pH 7.4.

The electronic spectra after light irradiation of *cis*-$[Ru^{II}(NO_2)(bpy)_2(4\text{-pic})]^+$ complex could be observed in Fig. 2 as example of the photochemical experiment for a nitro ruthenium specie.

The absorption peaks of $[Ru^{II}(NO_2)L_5]^+$ observed in 425 nm region decrease in intensity, and a new peak appears at 478 nm region. The spectral changes exhibit isosbetic points (316, 372 and 460 nm) during the course of photolysis, which is consistent with the photoreaction process. The photolysis of the nitro ruthenium complexes was also carried out using a NO sensor, which is an indubitable way to prove the NO release. The observed quantum yield for the NO release from *cis*-$[Ru^{II}(NO_2)L(bpy)_2]^+$ and $[Ru^{II}(NO_2)(bpy)(terpy)]^+$ complexes during photolysis at 355 nm was obtained by in situ NO detection. The signal recorded by the NO sensor increased quickly when the photolysis was initiated, then decreased when the light was turned off owing to NO consumption by various pathways [26] (Fig. 3). The amplitude of the pulse is proportional to the concentration of free NO originated from the photolysis of *cis*-$[Ru^{II}(NO_2)L(bpy)_2]^+$ and $[Ru^{II}(NO_2)(bpy)(terpy)]^+$ complexes, which was calculated based on the calibration curve. The NO concentration decreases after each laser pulse, indicating that probably the photoproduct absorbs part of the light. The calculated quantum yields at 355 nm light irradiation are described in Table 2 for the NO formation.

The photochemical pathway was described mainly based on the observations made on UV–Vis spectrum and HPLC analysis. The resulting electronic spectrum for the photolysis of *cis*-$[Ru^{II}(NO_2)L(bpy)_2]^+$ and $[Ru^{II}(NO_2)(bpy)(terpy)]^+$ was attributed to *cis*-$[Ru^{II}(H_2O)L(bpy)_2]^{2+}$ and $[Ru^{II}(H_2O)(bpy)(terpy)]^{2+}$, respectively. The attribution of aqua ruthenium complex as a product for the photochemical process of a nitro ruthenium specie in physiological pH was made considering the pK_a of *cis*-$[Ru^{II}(H_2O)L(bpy)_2]^{2+}$ and $[Ru^{II}(H_2O)(bpy)(terpy)]^{2+}$ and by comparison to the synthesized aqua ruthenium species [40,41]. By HPLC we also could observe that the nitro ruthenium species exhibited only one peak in aqueous solution, while after light irradiation, at 355 nm, two peaks were observed for the studied complexes as described for the photolysis of *cis*-$[Ru(NO_2)(bpy)_2(4\text{-pic})]^+$ and $[Ru^{II}(NO_2)(bpy)(terpy)]^+$ as example for the photochemical studies of $[Ru^{II}(NO_2)L_5]^+$ (Fig. 4).

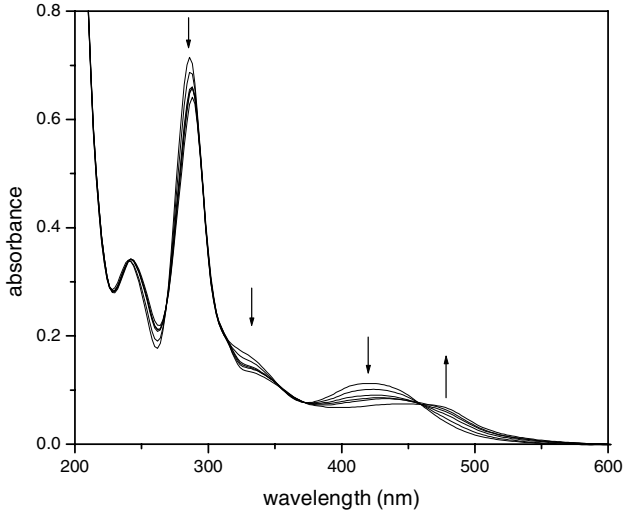


Fig. 2. Electronic spectra in UV–Vis region of *cis*-$[Ru^{II}(NO_2)(bpy)_2(4\text{-pic})]^+$ 2.0×10^{-5} M complex after flash-photolysis at 355 nm.

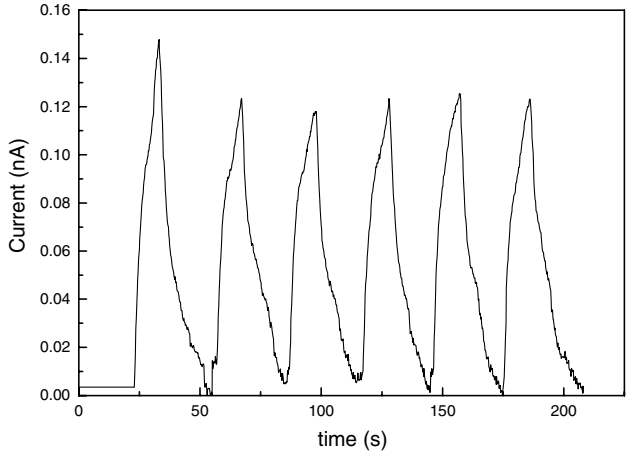


Fig. 3. Chronoamperogram of NO released by flash-photolysis of 2.0×10^{-5} M *cis*-$[Ru^{II}(NO_2)(bpy)_2(4\text{-pic})]^+$ at pH 7.4 buffer solution using 355 nm light irradiation.

Table 2
Quantum yield (ϕ_{NO}) values of nitro ruthenium complexes after flash-photolysis at 355 nm

Complexes	ϕ_{NO} mol eistein^{-1}[a]
cis-$[Ru^{II}(NO_2)(bpy)_2(py)]^+$	0.007 ± 0.001
cis-$[Ru^{II}(NO_2)(bpy)_2(4\text{-pic})]^+$	0.009 ± 0.001
cis-$[Ru^{II}(NO_2)(bpy)_2(pz)]^+$	0.037 ± 0.009
$[Ru^{II}(NO_2)(bpy)(terpy)]^+$	0.036 ± 0.009

[a] All data were collected in phosphate buffer solution, pH 7.4.

The possibility to have nitrite release after light irradiation of a nitro ruthenium complex was also checked by HPLC analysis in a similar procedure previously described [42]. This technique allows the chromatographic separation and online determination of the free nitrite by comparison to the sodium nitrite HPLC analysis, which appears at 4.30 min. We found no evidence for the presence of free nitrite originated from light irradiation at 355 nm of *cis*-$[Ru^{II}(NO_2)L(bpy)_2]^+$ and $[Ru^{II}(NO_2)(bpy)(terpy)]^+$ complexes by HPLC, although it has been related for some nitro ruthenium complexes [35–39]. Taking these results into consideration, it is possible to infer that limited amounts of NO_2^- could be produced during the photolysis of nitro ruthenium complexes, which should be converted to NO by secondary photochemical reaction. On account for UV–Vis

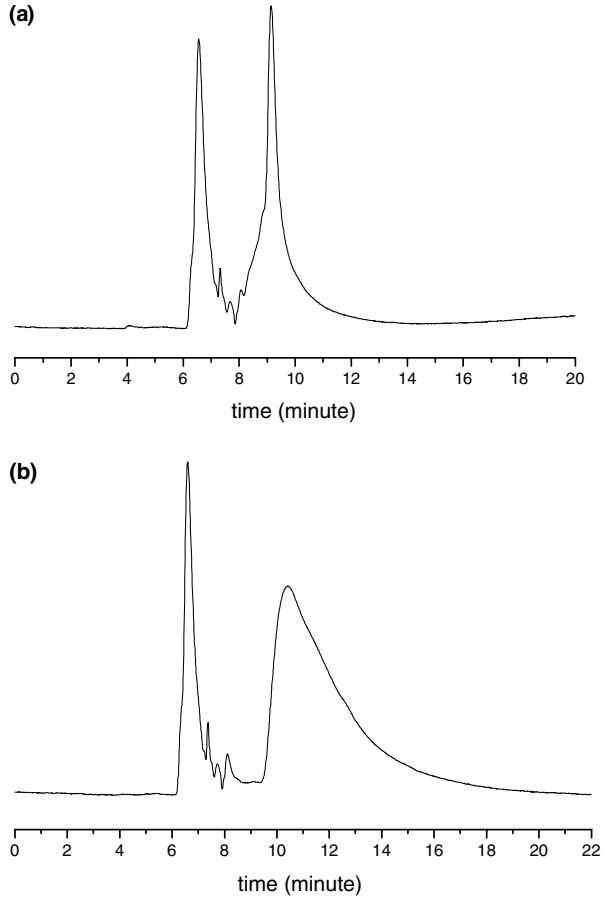


Fig. 4. Chromatograms of (a) *cis*-$[Ru^{II}(NO_2)(bpy)_2(4\text{-pic})]^+$ complex; (b) $[Ru^{II}(NO_2)(bpy)(terpy)]^+$ complex after flash-photolysis at 355 nm.

spectrum, NO measurement, HPLC analysis and by comparison to the photochemistry of sodium nitrite [43–45], we could suggest the photochemical pathway for the nitro ruthenium complexes as described in Eqs. (1)–(5).

$$[Ru(NO_2)L_5]^+ \xrightarrow[H_2O]{h\nu} [RuL_5(H_2O)]^{2+} + NO_2^- \quad (1)$$

$$NO_2^- \xrightarrow{h\nu} NO^\bullet + O^{\bullet -} \quad (2)$$

$$[Ru(NO_2)L_5]^+ \xrightarrow[H_2O]{h\nu} [Ru^{III}L_5OH]^{2+} + OH^- + NO^\bullet \quad (3)$$

$$[Ru(NO_2)L_5]^+ \xrightarrow{h\nu} \{[Ru^{III}(NO_2)L_4L]^+\}^* \quad (4)$$

$$[Ru^{III}L_5OH]^{2+} + \{[Ru^{III}(NO_2)L_4L]^+\}^* \xrightarrow{H^+} [Ru^{II}L_5(H_2O)]^{2+} + [Ru^{III}(NO_2)L_5]^{2+} \quad (5)$$

The formation of *cis*-$[Ru^{II}(OH)L_5]^+$ specie, which was observed by UV–Vis spectrum as well by HPLC (Figs. 2 and 4), could arise from the reduction of *cis*-$[Ru^{III}(OH)L_5]^+$ originated from the homolytic cleavage of Ru^{II}–ONO during photolysis. We believe that the irradiation at 355 nm of *cis*-$[Ru(NO_2)L_5]^+$ can also produce {*cis*-$[Ru^{III}(NO_2)L_4L^-]^+$}* at excited state, which can act as reducing agent, as described for similar systems [46]. The *cis*-$[Ru^{III}(NO_2)L_5]^{2+}$ complex decomposes in aqueous solution at pH 7.4 as observed by UV–Vis spectrum after controlled potential electrolysis at 0.9 V versus Ag/AgCl of an aqueous solution of *cis*-$[Ru^{II}(NO_2)L_5]^+$ complex.

The efficiency of the system was also evaluated in a microemulsion system. Recent research work has focused on the use of microemulsion in several fields, including drug delivery systems [47]. Microemulsion could be used in order to solubilize drugs [48] and improve systemic and topical drug availability [48,49]. In this work, the principal target in the use of microemulsion is to minimize any side effect that could be caused by the unreactive ruthenium compound, and also due to the interest in creating nitric oxide (NO)-releasing materials that can be used in a wide variety of biomedical applications.

Owing to the solubility of nitro ruthenium complexes in water, we assumed that the best type of microemulsion for this case is water/oil (W/O) system, where the nitro ruthenium complex is solubilized in the internal hydrophilic phase. The systematic approach used and the presentation of the various physico-chemical and biopharmaceutical aspects should facilitate the main considerations involved in designing and characterizing a specific self-dispersing NO release from a nitro ruthenium complex in a microemulsion. Among these studies, we evaluated the nitro ruthenium complex release from the microemulsion using Franz diffusion cells [50].

Two compartment of Franz diffusion cells, equipped with a synthetic membranes were employed for release studies. The synthetic thin membrane separates the donor compartment, filled with the nitro ruthenium complexes-loaded microemulsion, from the receiver one, filled with free aqueous phase. All experiments were performed at 37.0 °C and sampling was taken over 12 h, with a sample being collected in each hour. The amount of nitro ruthenium complexes released was determined spectrophotometrically, in a way that we could observe an electronic spectrum characteristic of a nitro ruthenium complex described in this work. Results showed that the systems formulated as $[Ru^{II}(NO_2)(bpy)(terpy)]^+$ and *cis*-$[Ru(NO_2)L(bpy)_2]^+$ complexes demonstrated that microemulsion systems provided a reservoir effect for nitro ruthenium complexes in ca. 1 h (Fig. 5). After this time, the nitro ruthenium specie was released from the microemulsion into the receptor solution.

The W/O microemulsion containing the nitro ruthenium complex was submitted to light irradiation at 355 nm to evaluate the NO release owing to the vasodilatation effect occasioned by free nitric oxide. In those experiments, denuded rat aortic rings were previously contracted with KCl 60 mM. The relaxation response

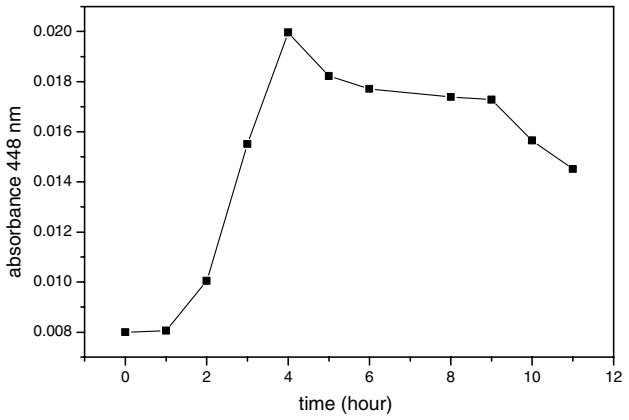


Fig. 5. Plot of absorbance at 448 nm vs. time during the liberation experiment of $[Ru^{II}(NO_2)(bpy)(terpy)]^+$ load in microemulsion to receptor solution.

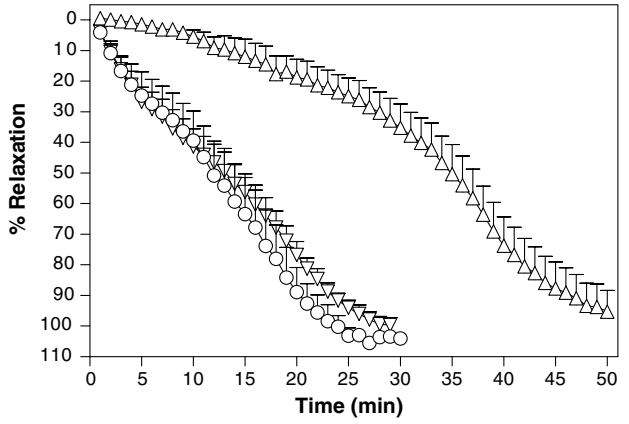


Fig. 6. Time-course for nitro ruthenium complexes-induced relaxation. Denuded thoracic aortic rings pre-contracted with 60 mM KCl and 100 μM *cis*-$[Ru^{II}(NO_2)(bpy)_2(py)]^+$ complex (○, $n = 6$), *cis*-$[Ru^{II}(NO_2)(bpy)_2(pz)]^+$ complex (▽, $n = 5$) and $[Ru^{II}(NO_2)(bpy)(terpy)]^+$ complex (△, $n = 6$) were added. Data are means ± SEM of n experiments performed on preparations obtained from different animals.

was recorded while arteries were irradiated. The rapid decrease of tension was obtained when the Krebs solution containing *cis*-$[Ru^{II}(NO_2)L(bpy)_2]^+$ complexes inside a microemulsion was irradiated at 355 nm (Fig. 6). Similar results were also observed when the $[Ru^{II}(NO_2)(bpy)(terpy)]^+$ complex in a microemulsion was used as vasodilator agent (Fig. 6). The relaxation was completely terminated when a microemulsion without the nitro ruthenium complex was submitted to the same light irradiation.

The time to reach maximum relaxation was longer for $[Ru^{II}(NO_2)(bpy)(terpy)]^+$ complex (ca. 50 min, $n = 6$) than for *cis*-$[Ru^{II}(NO_2)L(bpy)_2]^+$ with L = py and 4-pic complex (ca. 28 min, $n = 6$) and *cis*-$[Ru^{II}(NO_2)(bpy)_2(pz)]^+$ complex (ca. 24 min, $n = 5$). This difference may be an indication that nitric oxide can combine with the hydroxo-ruthenium complex inside microemulsion originating the nitro ruthenium specie. The recombination of NO to the fragment after photolysis was also observed for some *S*-nitrosothiol specie, which was accelerated due to the matrix effect [51]. In fact, we have observed that the reaction of $[RuL_5(H_2O)]^{2+}$ complex with nitric oxide when carried out in physiological solution originates $[Ru(NO_2)L_5]^+$ complex. Further investigation of the kinetic behavior of *cis*-$[Ru^{II}(H_2O)L(bpy)_2]^{2+}$ and $[Ru^{II}(H_2O)(bpy)(terpy)]^{2+}$ with nitric oxide in different pH is now in progress in our laboratory.

4. Conclusion

Considering that all of these nitro ruthenium complexes continuously release NO upon initiation of the reaction, which was observed in an aqueous physiological solution as well in a microemulsion, it is possibile for us to infer that the photo-relaxation occurs due to the NO photo-release. The report of the NO-releasing material that utilizes light as an external on/off trigger permits a precise control of the rate of NO released. This control may allow the use of this gel, topically, which can be a source of administration of NO in cancer therapy.

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