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Photochemical and pharmacological aspects of nitric oxide release from some nitrosyl ruthenium complexes entrapped in sol–gel and silicone matrices

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Abstract

The entrapped $[Ru(terpy)(L)NO](PF_6)_3$, where terpy = 2,2′:6′,2″-terpyridine and L = 2,2′-bipyridine (bpy) and 3,4-diiminebenzoic acid (NH · NHq) complexes into sol–gel processed polysiloxane and silicone matrices, shows NO release characteristics when submitted to light irradiation at 355 and 532 nm, as judged by NO measurement using a NO-sensor electrode. The pharmacological properties of doped matrix showed vasodilator characteristics by visible light irradiation, which is of great interest because the target delivery system can avoid the occurrence of side effects possibly by the aquo ruthenium specie. All matrices obtained showed to be amorphous materials. The scanning electron micrographs of the matrices showed irregularly shaped particles, with broad size of 1000 μm for both matrices and homogeneous distribution.

Keywords: Immobilized ruthenium complexes; Nitric oxide; Photo-release

1. Introduction

Nitric oxide (NO) is an endogenously produced free radical in mammals that is responsible for mediating a wide variety of important physiological processes including neurotransmission, platelet and tumor cell adhesion, immune response, and vasodilation [1–7]. The several biological functions of nitric oxide make NO-releasing agents potential therapeutic candidates for a range of disease states, which are dependent on its site and source of production [8,9]. A possible strategy would be to employ nitrosyl ruthenium compounds that display relatively low thermal reactivity but are photochemically active to release NO when subjected to luminous stimulus [10–18]. Unfortunately, many of these species present NO releasing ability only at low pH, which make them unsuitable for clinical therapy. The use of solid matrices with nitrosyl ruthenium species capable of providing local and controlled NO delivery could overcome this kind of problem and yield an important investigative tool to assess the effects of NO on cells and tissues [19–21].

With the aim of stabilizing nitrosyl ruthenium compounds that could act as NO-releasing agents in a control-

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lable manner, as well as limiting NO exposure to selected sites within the body, we have entrapped [Ru(terpy)(L)-NO](PF$_6$)$_3$ complexes (where terpy = 2,2′:6′,2″-terpyridine and L = 2,2′-bipyridine (bpy) and 3,4-diiminebenzoic acid (NH · NHq) into sol–gel processed polysiloxane and silicone matrices. Recently some physico-chemical properties of nitrosyl complexes entrapped in a silicate sol–gel matrix have been described [19,20]. Although the authors have suggested its use in therapeutic applications due to the controlled NO release, there is no experimental indication that kind of system will work in biological experiments.

In this study, NO measurements were carried out and we have found that [Ru(terpy)(L)NO](PF$_6$)$_3$ complexes can also promote NO-release from the sol–gel and silicone membranes by ultraviolet and visible light irradiation. Some pharmacological properties of the entrapped [Ru(terpy)(NH · NHq)NO](PF$_6$)$_3$ complexes have also been described based on the photolysis at 532 nm showing the versatility of these materials for therapeutic applications.

2. Experimental

2.1. Chemicals and reagents

$RuCl_3 \cdot nH_2O$, terpyridine (terpy) and bipyridine (bpy) were purchased as high purity reagents from Aldrich Chemicals and were used as supplied. Doubly distilled water was used for all experiments. All preparations and measurements were carried out under an argon atmosphere and protected from light.

2.2. Synthesis of ruthenium complexes

The recrystallized complex salt [RuCl(bpy)(terpy)]Cl, [Ru(bpy)(terpy)(NO)](PF$_6$)$_3$, [RuCl(NH · NHq)(terpy)]Cl and [Ru(NH · NHq)(terpy)NO](PF$_6$)$_3$ were prepared as previously published [22–24].

2.3. Preparation of the membranes

The hybrid composite was synthesized by preparing a mixture of 24 mL tetraethoxysilane (Aldrich, Milwaukee, WI, USA) and 19 mL of bidistilled water. The reagents were carefully added and three drops of concentrated HCl were added in order to catalyze the reaction. The nitrosyl ruthenium complexes (*ca*. 0.001 g) were added with constant stirring into 4.5 mL of hydrolyzed. The solution was stirred for 5 min at 60 °C and aliquots (2.0 mL) of the mixture were then transferred to a Petri dish (58 mm diameter) to complete the reaction. The molds remained opened for 3 days until they no longer exhibited weight loss due to evaporation of residual water and solvent.

The silicone resin was prepared by mixing polydimethylsiloxane (PDMS Dow Corning) (2.000 g) with tetraethoxysilane (TEOS, Fluka) (0.500 g) in 2.5 cm^3 of isopropylic alcohol. To this mixture we add the nitrosyl ruthenium compound (0.001 g), previously dissolved in the 0.5 cm^3 of acetonitrile, and five drops of di-*n*-butyltin–dilaurate complex, 5% in hexane (Gelest) as catalyst. The final mixture was stirred for 5 min. The wet gels were air-dried at room temperature for 24 h.

2.4. Apparatus

Ultraviolet-visible (UV–Vis) spectra were recorded on a Hitachi U-3501 and a Hewlett Packard HP8452A diode array spectrophotometer. Infrared (IR) spectra were recorded on either a Protegé 460 series or a MB Bomem 102 FTIR spectrometer. Scanning electron microscopy was performed using a LEO 440 microscope equipped with an Oxford EDS detector. The pH measurements were made using a 430 pH meter from Corning. NO release was measured with an ISO-NOP NO meter from Word Precision Instruments. Continuous photolysis with monochromatic irradiations at 355 and 436 nm was carried out using a 150 W Xenon lamp in a model 6253, Oriel Universal Arc Lamp Source. The irradiation wavelength was selected with an Oriel interference filter, with 10 nm band path, for photolysis at the appropriate wavelengths. The progress of the photoreactions was monitored spectrophotometrically.

2.5. Nitric oxide release measurement

The experiment was performed using a photo-reactor (0.18 m^3) containing 250 W fluorescent lamps (Philips), which were used as a visible light source. The 450–800 nm wavelength range was achieved using a filter. A total of 0.200 g of membrane containing incorporated nitrosyl ruthenium complexes were added to a quartz UV–Vis reservoir containing 10 mL of 0.01 M phosphate buffer solution pH 7.4. This reservoir was connected by a polyethylene tube (20 cm; 1 mm diameter) to another chamber containing 10 mL of phosphate buffer solution where the NO-sensor (amino-700 from Innovative Instruments, Inc) was adapted. Argon flux was bubbling in both reservoirs during the experiment.

The reservoir containing the membrane was submitted to light irradiation and the NO was detected by measuring the current.

2.6. Vessel preparation

Male *Wistar* rats (180–200 g) were killed by decapitation and the thoracic aorta was quickly removed and dissected free and cut into rings 4 mm long. The endothelium was mechanically removed by gently rolling the lumen of the vessel on a thin wire. The aortic rings were placed between two stainless-steel stirrups, and connected to an isometric force transducer (50630-45, Harward Bioscience, South Natick, MA, USA) and the other was connected to a fixed support in the chamber in order to record the tension on a Harward Bioscience Oscillograph polygraph. The rings were placed in a 10 mL organ chamber containing Krebs

solution with the following composition (mmol/L): NaCl 130, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 14.9, Glucose 5.5, $CaCl_2$ 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, before allowing to equilibrate for 60 min in the bath fluid that was changed every 15–20 min. Endothelial integrity was qualitatively assessed by the degree of relaxation caused by acetylcholine (ACh,1 μmol/L) in the presence of contractile tone induced by phenylephrine (0.1 μmol/L). Because our studies require endothelium-denuded aortas, the rings were discharged if there was any degree of relaxation to ACh, in order to avoid possible influence of endothelial factors. The tissues were washed, pre-contracted with the EC_{50} of phenylephrine (0.1 μmol/L). On top of the contraction, the chamber was loaded with $0.134\ g \pm 0.004$ $(n = 7)$ of the membrane containing entrapped $[Ru(NH \cdot NHq)(terpy)NO]^{3+}$, which was added in a dialysis bag to avoid any adverse physiological effect due the organic reagents. Similar experiment was run with the control (membrane without ruthenium complex -0.129 ± 0.004 $(n = 7)$). After addition of the membrane, the light was activated and time-course for relaxation induction was evaluated. Relaxation was calculated as a percentage of the maximal contraction induced by phenylephrine.

Light irradiation for the pharmacological assays was performed using a photo-reactor. The reactor consists of a 0.5 m^3 box inside which aluminum was placed to increase light reflection. Three 250 W fluorescent lamps (Philips) were used as a visible light source with 450–800 nm wavelength range achieved with a filter (7.05 cm^2 area) positioned in front of each channel on the chamber.

2.7. Statistical analysis

Relaxant responses to the NO donor were measured from the plateau of the phenylephrine contraction and were expressed as percent reversal of the phenylephrine pre-contraction. Data are expressed as mean ± SEM. In each set of experiments,*n* indicates the number of rats studied. We considered the maximal relaxing effect for compounds when the concentration used reached the baseline. Statistical significance was tested by one-way ANOVA (Bonferroni's Multiple Comparison Test) and values of $p > 0.05$ were considered to be significant.

3. Results and discussion

The nitrosyl ruthenium complexes, $[Ru(terpy)(L)NO]^{3+}$ (L = bpy; NH · NHq), used in the present study have been proposed as a NO donor agents in aqueous acidic solution by external stimulation [24].

These species react very rapidly in aqueous medium with hydroxide ion to give deep yellow solutions (Eq. (1)).

$$[RuL_5(NO)]^{3+} + 2OH^- \overset{K_{eq}}{\rightleftharpoons} [RuL_5(NO_2)]^{+} + H_2O \qquad (1)$$

Spectral changes accompanying this reaction for $[Ru(bpy)(terpy)NO]^{3+}$ are shown in Fig. 1. The product was characterized as $[Ru(NO_2)(L)(terpy)]^{+}$ (nitro form) based mainly on the similarity of the analogous ruthenium complexes [24]. The spectral profiles show that, above pH 3.00 for $[Ru(bpy)(terpy)NO]^{3+}$ and pH 6.00 for $[Ru(NH \cdot NHq)terpy)NO]^{3+}$, the nitro form exists in significant amounts at equilibrium. Low pH is somehow crucial for both nitrosyl ruthenium species generate NO release by light irradiation. In this regard, novel strategies to preserve the properties of $[Ru(terpy)(L)NO]^{3+}$ should be developed for biomedical applications. We found that silicone and sol–gel matrices could allow stabilizing the nitrosyl species as well be a convenient target delivery system for NO release.

The physico-chemical properties of $[Ru(terpy)(L)NO]^{3+}$ seems to be unaffected by the entrapping process with sol–gel or silicone membranes as judged by the UV–Vis and FTIR analysis. The solid sate absorbance spectra for sol–gel and silicone samples doped with nitrosyl ruthenium complexes show some similarities to those obtained in aqueous solution. Fig. 2 shows the electronic spectrum of $[Ru(bpy)(terpy)NO]^{3+}$, as an example of a [Ru(ter-

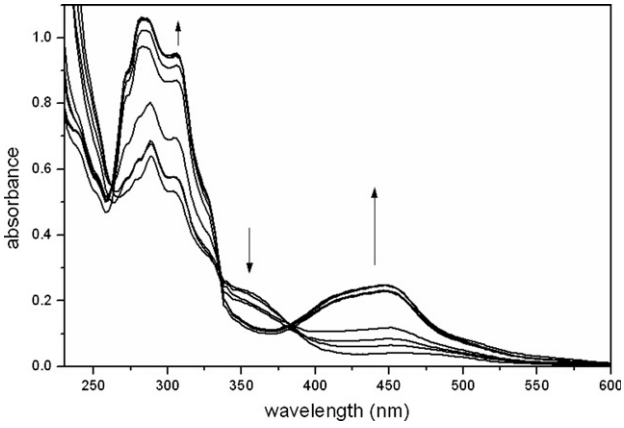

Fig. 1. Spectral profile change with pH for the conversion of $[Ru(NH \cdot NHq)(terpy)NO]^{3+}$ in $[Ru(NH \cdot NHq)(terpy)(NO_2)]^{+}$. Concentration of the complex = 2.6×10^{-5} M. pH 2.02, 2.40, 3.20, 3.58, 6.30, 7.40.

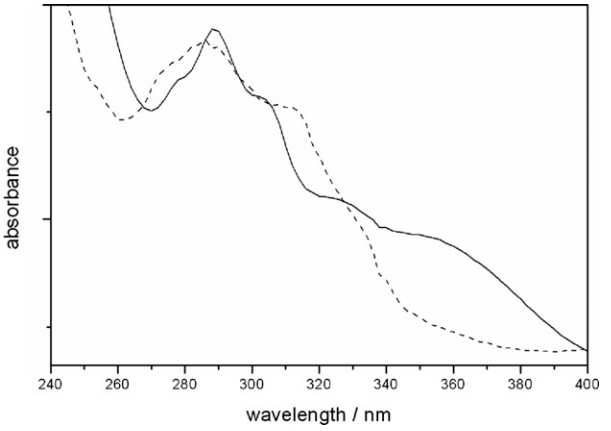

Fig. 2. Electronic spectrum of $[Ru(bpy)(terpy)NO]^{3+}$ in solution pH 2.03 (solid line) and in silicone matrix (dashed line).

py)LNO]$^{3+}$ species in solid state (dashed line) and in aqueous solution (solid line) performed at room temperature. A series of three bands were observed in both spectra in the region of 250–300 nm attributed to intra-ligand transition. However, the electronic spectrum in solution shows a shoulder at 360 nm, attributed to a metal ligand charge transfer band [24], which was not observed in the solid state, probably due the medium effect.

Scanning electron microscopy (SEM) of the studied membranes in this study was applied to determine the distribution pattern and size of particles in the solid matrices. All matrices obtained were found to be amorphous materials (Figure S1: Supporting Material). The SEM micrographs revealed irregularly shaped particles with broad size of 1000 μm for both membranes as well as a homogenous distribution. The samples of [Ru(bpy)(terpy)-NO](PF$_6$)$_3$ complex doped in sol–gel and silicone matrices formed three-dimensional networks while the samples of [Ru(NH · NHq)(terpy)NO](PF$_6$)$_3$ complex showed edges and conchoidal fractures, as well as globular particle shapes with low-dimensional structures.

The entrapped [Ru(terpy)LNO]$^{3+}$ complex on the matrices was submitted to light irradiation at 355 nm, while the [Ru(NH · NHq)(terpy)NO]$^{3+}$ complex was also irradiated at 532 nm. NO monitoring of these photochemical processes in aqueous solution, as observed for [Ru(NH · NHq)-(terpy)NO]$^{3+}$ as an example of nitrosyl ruthenium complex entrapped in silicone matrix, showed NO release (Fig. 3) although in the absence of light no current increase was recorded. The amount of available NO *per* volume of the matrices can be controlled by the amount of [Ru(bpy)(terpy)LNO]$^{3+}$. It implies that tissue exposure to such materials could be convenient because it provides different dosages of NO. After exhaustive photolysis, the UV–Vis spectrum of the entrapped [Ru(terpy)LNO]$^{3+}$ showed [Ru(terpy)-L(H$_2$O)]$^{2+}$ characteristics (Figure S2: Supporting Material).

The stability of the solid matrices to entrap the nitrosyl compounds was tested by rinsing the samples in 0.1 mol L^{-1} HCl and also in phosphate buffer solution at pH 7.40 for 30 min. The electronic spectra of these dispel aliquots showed insignificant absorption, which was considered as a qualitative indicator of the efficiency of the entrapment of ruthenium nitrosyl complexes in sol–gel and silicone matrices.

Due the spectroscopic and photochemical characteristics of [Ru(NH · NHq)(terpy)NO]$^{3+}$ complex in aqueous solution [24] as well encapsulated in a solid matrix, some pharmacological assays was performed. In a vasodilator test, denuded rat aortic rings were previously contracted with phenylephrine and vasorelaxation was verified in the presence of doped matrix. In this experiment, we used the sol–gel matrix (*ca.* 0.200 g), which was kept in a cellulose dialysis tubing to avoid any other side reaction. The addition of the membrane without entrapped compound in the absence of light did not produce vasodilation response. However, as shown in Fig. 4, in the presence of visible light irradiation and after depletion of endogenous photoactivable NO stores, NO release caused relaxation of denuded pre-contracted aorta with maximum within about 100 s. In pre-contracted aortic rings, pre-incubation with oxyhaemoglobin 10 μmol/L for 30 min, a known NO scavenger [25], completely abolished the relaxation induced by the matrix indicating that NO is released in the extracellular medium.

Vasodilation using sol–gel or silicone as target delivery system shows similar vasorelaxation effects observed for [Ru(NH · NHq)(terpy)NO]$^{3+}$ in physiological solution [7]. The entrapped ruthenium complexes induced E_{max}: 29.9 ± 3.6% for sol–gel matrix. It seems to be less effective when compared to the [Ru(NH · NHq)(terpy)NO]$^{3+}$ in physiological solution, which is possibly explained by the fact that activation of guanylyl-cyclase is nitric oxide concentration dependent. In addition, the membrane releases NO extracellularly and some NO amount can be lost outside the cells. In this way, less NO could be available inside the cell to promote the guanylyl-cyclase activation and

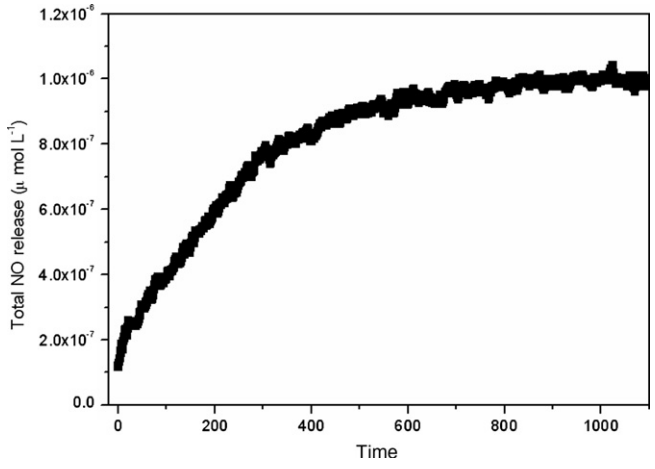

Fig. 3. Time course of NO release from [Ru(NH · NHq)(terpy)NO]$^{3+}$ complex in silicone matrix under 532 nm light irradiation. Mass membrane = 0.200 g.

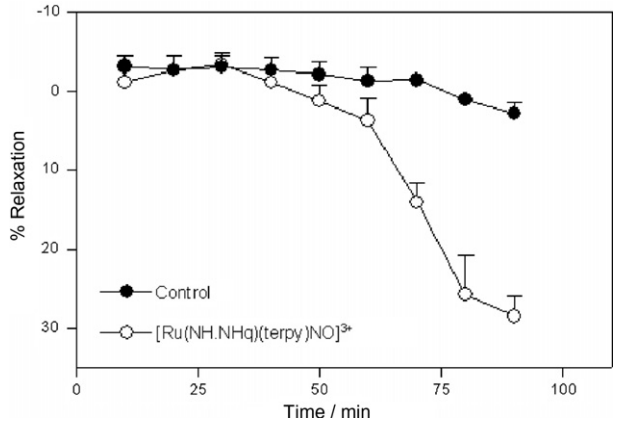

Fig. 4. Time-course for [Ru(NH · NHq)(terpy)NO]$^{3+}$-induced relaxation. Denuded aortic rings were pre-contracted with 100 nmol L^{-1} phenylephrine and [Ru(NH · NHq)(terpy)NO]$^{3+}$ doped in sol–gel matrix (○, $n = 7$) and control (sol–gel matrix) (●, $n = 7$) were added. Data are means ± SEM of n experiments performed on preparations obtained from different animals.

aorta relaxation. Apparently, the observed vasodilation using the target delivery system suggests that local delivery of NO may help increasing the local potency.

4. Conclusions

In the present study, we showed for the first time that the NO released from entrapped $[Ru(NH \cdot NHq)(terpy)\text{-}NO]^{3+}$ by light irradiation induces rat aorta relaxation, as demonstrated by its vasodilatory effect. This NO-release ability might represent an effective mechanism for some therapeutic applications because NO has been shown to prevent biofilm formation. Studies are being conducted at our laboratory to quantify NO optimal amount required to prevent, for example, bacterial adhesion, which may be useful in the development of patch-based topical therapies.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.poly.2007.03.042.

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