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Nitric oxide photorelease from hydrogels and from skin containing a nitro-ruthenium complex

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ABSTRACT

Nitric oxide (NO) is a gaseous molecule that has specific functions dictated by its localization and its kinetics of release. As NO-donors have a range of potential uses in the skin, much attention has been paid to the development of topical NO delivery systems. The aim of this work was to study the release rate and the skin penetration of the NO-donor *cis*-$[Ru(NO_2)(bpy)_2(4\text{-pic})]^+$ from different gel formulations and their potential as topical NO delivery systems under light stimuli. Among the formulations developed, the anionic gel retarded the nitro-ruthenium complex diffusion and also obstructed NO release after light irradiation. On the other hand, NO release before light irradiation was observed when the complex was dispersed in the cationic chitosan gel, possibly due to oxi-redox reactions between the amino groups of the polymer and the drug molecule. Finally, the non-ionic gel released the NO after light irradiation to the same extent as a drug aqueous solution at the same pH. The drug dispersed in this gel also penetrated into the stratum corneum skin layer, and the nitro-ruthenium complex present in the skin was able to release the NO after light stimuli, suggesting the potential use of this formulation as a topical NO delivery system.

1. Introduction

Nitric oxide (NO) is an endogenous molecule that plays paradoxical roles in the regulation of physiological functions where its absence or its excess can cure or cause pathological diseases (Smith and Simmons, 1998; Shishido et al., 2003). Therefore, the local controlled release of NO is essential in order to avoid side effects related to its concentration. The topical application of NO-donors is a promising strategy to transport NO to a target area (Smith and Simmons, 1998; Hardwick et al., 2001; Paoloni et al., 2003; Seabra et al., 2004). Furthermore, skin itself, i.e., the site of NO-donor application, can be the NO target area since it is known that NO has an antimicrobial action (Ormerod et al., 1999; Weller et al., 2001; Ghaffari et al., 2005, 2006), participates in wound healing (Bohl-Masters et al., 2002; Witte et al., 2002) and also has an antitumor action depending on its concentration (Wink et al., 1998; Trikha et al., 2001).

There are a great number of agents that release NO in living systems; however, most of them involve the formation of NO by either simple dissociation or by a complex series of reactions (Butler and Glidewell, 1987; Sauaia et al., 2003), making difficult to control the NO release and concentration in specific sites. Many NO-donors are also thermally unstable (Shishido et al., 2003), which makes the control of NO release difficult for a long period. Different approaches have been used to control the NO release in a specific site. For example, the encapsulation of less stable donors as diazeniumdiolates in drug delivery systems as nanoparticles (Smith and Simmons, 1998; Hetrick et al., 2008) or sol–gel matrix (Nablo and Schoenfisch, 2003), the encapsulation of gaseous NO in liposomes (Huang et al., 2009) or the synthesis of stable NO-donors (Chen et al., 2006).

In view of the high instability of NO and its permeability across cell membranes from extracellular to intracellular domains, the NO photolabilisation from metal compounds is a strategy of great value to generate NO rapidly at known concentrations within biological preparations at specific locations (Macyk et al., 2005). Some nitrosyl ruthenium species, such as those containing polypyridyl ligands (Sauaia and Silva, 2003), have received special attention as NO-donors due to their thermal stability and potential to release NO under light or under electrochemical stimulation (Sauaia et al., 2003). The use of light to promote nitric oxide (NO) formation is a

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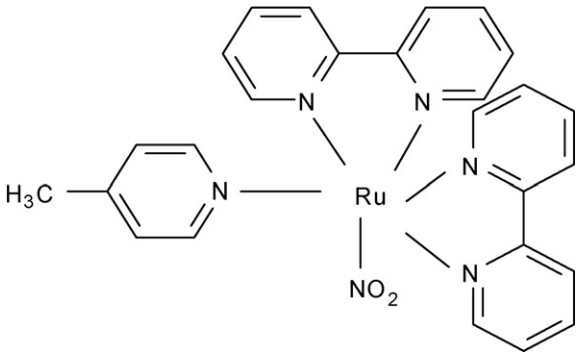


Fig. 1. Structure of *cis*-[Ru(NO$_2$)(bpy)$_2$(4-pic)]$^+$.

fascinating approach. Light irradiation can be used without causing damage or side reaction. The photo-reactivity is modulated by the wavelength excitation and intensity of the light source. It permits to control the amount of the NO released and therefore is a perfect tool to be used in clinical medicine (Maranho et al., 2009). A particular complex, *cis*-[Ru(NO)(bpy)$_2$(4-pic)]$^{3+}$ (Fig. 1), where bpy is 4,4′-bipyridine and 4-pic is 4-picoline, has been explored in an attempt to assess its therapeutic application due to NO release by light stimulation (Sauaia et al., 2003). In physiological pH this nitrosyl ruthenium complex is converted in a nitro-ruthenium specie, *cis*-[Ru(NO$_2$)(bpy)$_2$(4-pic)]$^+$, by hydroxide electrophilic attack (Sauaia and Silva, 2003). By employing this nitro-ruthenium complex, many important results were observed in vascular relaxation (De Lima et al., 2005). For instance, it was verified that the complex is able to relax vascular aortic rings pre-contracted with norepinephrine (NOR), phenylephrine (Phe) or prostaglandin F2α (PGF2α) by light irradiation in an extension similar to that obtained when the well-characterized relation drug, the sodium nitroprusside (SNP), was used (De Lima et al., 2006).

However, despite the many advantages that topical application of NO-donors can bring, topical administration of *cis*-[Ru(NO$_2$)(bpy)$_2$(4-pic)]$^+$ has not been studied yet. It may be due to the lack of studies of this complex behavior in a formulation different from an aqueous solution. Or better, to topically administer this complex easily, semi-solid formulations that do not spread away from the site of administration and, more importantly, that do not interfere in NO release from the NO–ruthenium complex are required instead of a simple solution. These formulations have yet to be studied. Furthermore, the nitro-ruthenium complex must at least penetrate the outermost layer of the skin, the stratum corneum, and, then, release the NO after stimulus, once the NO is a diffusible gas, presenting half-life of low seconds (Ignarro et al., 1987; Vallance and Collier, 1994) and it could easily dissipate to the environment.

Therefore, the aim of this work was to develop a simple formulation containing *cis*-[Ru(NO$_2$)(bpy)$_2$(4-pic)]$^+$ for NO topical release under light stimuli. The *in vitro* skin penetration of the nitro-ruthenium complex from these formulations as well as the NO release after light irradiation of this tissue was also evaluated.

2. Materials and methods

2.1. Chemicals

Cis-[Ru(NO$_2$)(bpy)$_2$(4-pic)]$^+$ and *cis*-[Ru(bpy)$_2$(4-pic)(H$_2$O)]$^{2+}$ were synthesized as described by Sauaia and Silva (2003). RuCl$_3$·3H$_2$O, 2,2′-bipyridine and 4-picoline were purchased as high purity reagents from Aldrich Chemicals. Hydroxyethyl cellulose (HEC) was purchased from Galena (Campinas, SP, Brazil), polyacrylate (Carbopol® 940) was from Via Farma (São Paulo, SP, Brazil), and Chitosan (MW ~500,000–5,000,000 g mol^{-1}, greater than 80% deacetylation—Hydagen® CMFP) was a generous gift from Cognis Brazil (São Paulo, SP, Brazil). Methanol was obtained from J.T. Baker (Phillipsburg, NJ, USA) and trifluoroacetic acid from Tedia (Fairfield, OH, USA). Distilled water (Mili-Q®, Millipore Simplicity 185, Bedford, MA, USA) was used to prepare all solutions of the experiments. All other reagents were BDH or HPLC reagents.

2.2. Skin tissue

The membrane used for the *in vitro* experiments was dermatomed (700 μm) from pig ear skin. It was obtained immediately after the slaughter of the animal (Frigorífico Pontal Ltd., Pontal, Brazil) and stored at −20 °C for a maximum of 30 days before use.

2.3. Analytical chemistry

Cis-[Ru(NO$_2$)(bpy)$_2$(4-pic)]$^+$ was quantified by a Shimadzu Instrument HPLC System (LC 10-AD, Kyoto, Japan) equipped with two LC 10-AT VP solvent pump units, a SLC-10A system controller, a CTO-10AS VP column oven and a SPD-10A VP UV–vis detector operating at 290 nm. Injections of 20 μL of the samples were performed automatically with an autoinjector model SIL 10AD. The peak area ratios for calibration curves and quantification were obtained using Class VP chromatography data system software. Separation was carried out at 37 °C on a Shim-pack CLC-ODS C$_{18}$ column (250 mm × 4.6 mm i.d., 5 μm particle size, Shimadzu, Kyoto, Japan). A Shim-pack CLC-ODS C$_{18}$ (10 mm × 4 mm i.d., 5 μm particle size, Shimadzu, Kyoto, Japan) was used as guard column. The isocratic reversed mobile phase for the analysis of the ruthenium complex consisted of methanol:phosphate buffer 0.01 mol L^{-1} pH 7.0 (60:40, v/v) plus 0.84% of trifluoroacetic acid (HTFA), at a flow rate of 1.0 mL min^{-1}. After addition of HTFA, the buffer solution was adjusted with 5 mol L^{-1} sodium hydroxide aqueous solution until pH 7.0. At these conditions, the *cis*-[Ru(NO$_2$)(bpy)$_2$(4-pic)]$^+$ elution retention time was 6.1 min. A linear calibration curve ($y = 53003x + 8975.1$, $r = 0.999$) was obtained over the working concentration range of 0.05–25 μg mL^{-1}. The aqua-ruthenium complex (*cis*-[Ru(bpy)$_2$(4-pic)(H$_2$O)]$^{2+}$) was also eluted at these conditions showing a retention time of 8.3 min and a linear calibration curve ($y = 59260x + 34214$, $r = 0.999$) over the concentration range of 0.25–25 μg mL^{-1}. The method was validated showing an intra- and inter-day precision and accuracy of no less than 96% and 92%, respectively. It was also selective during all the analyses performed in this work. The limits of quantification and detection of the method were 0.05 μg mL^{-1} and 0.01 μg mL^{-1}, respectively, for the nitro-ruthenium complex and 0.25 μg mL^{-1} and 0.02 μg mL^{-1}, respectively, for the aqua-ruthenium complex.

2.4. Preparation of the formulations containing *cis*-[Ru(NO$_2$)(bpy)$_2$(4-pic)]$^+$

The composition of the formulations containing the nitro-ruthenium complex is shown in Table 1. In order to prepare a non-ionic, anionic and cationic hydrogel, three different gel-forming polymers, HEC, polyacrylate and chitosan, respectively, were dispersed in appropriate amounts of an aqueous solution saturated with the *cis*-[Ru(NO$_2$)(bpy)$_2$(4-pic)]$^+$ (Table 1). A chemically crosslinked chitosan gel was also obtained by mixing glutaraldehyde (GLU) aqueous solution at 0.03% to chitosan hydrogel. A drug-saturated phosphate buffer solution at pH 5.0 was used as a control. The pH of all formulations was adjusted to 5.0 when necessary.

Table 1
Composition of hydrogels saturated with *cis*-[Ru(NO_2)(bpy)$_2$(4-pic)]$^+$.

Formulation code	Components (%, w/w)						
	HEC	Polyacrylate[a]	Chitosan	Propylene glycol	Acetic acid (0.5%)	GLU	Phosphate buffer, pH 5.0
A	2	–	–	5	–	–	93
B	–	0.8	–	5	–	–	94.2
C	–	–	2	5	93	–	–
D	–	–	2	5	92.9	0.03	–

GLU, glutaraldeyde.
[a] NaOH was added until pH 5.0.

2.5. In vitro release studies of nitro-ruthenium complex from the formulations

The *in vitro* release behavior of *cis*-[Ru(NO_2)(bpy)$_2$(4-pic)]$^+$ from the hydrophilic gels was assessed using a 23 μm cellulose membrane (MW 12,000–14,000; Fisher Scientific, USA) mounted between the donor and the receptor compartment of a modified Franz diffusion cell (1.15 cm^2 of effective area of diffusion and with a receptor volume of 5.5 mL). Formulations (1 g) were placed over the membrane in the donor compartment. The receptor fluid was perfused continuously at 1 mL h^{-1} using a peristaltic pump (Pump Pro MPL580, Watson-Marlow Bredel Pumps, UK). Samples were collected automatically (Fraction collector PTFCII-Pharmatest, Germany) every hour up to 12 h and analyzed by HPLC to determine the amount of the drug that was released from the formulation as a function of time. The sink conditions were maintained throughout the study.

The diffusion coefficient (D) of the drug in the formulations was calculated according to Eq. (I):

$$Q = 2C_0\sqrt{\frac{Dt}{\pi}} \quad \text{(I)}$$

where Q is the amount of drug released per unit area; C_0 is the initial concentration of the drug in the formulation; and t is the time elapsed since the release experiment started (Merclin et al., 2004).

2.6. NO release profile from the nitro-ruthenium complex-loaded formulations

The potential of the phosphate buffer solution at pH 5 (control) and of the formulations containing the *cis*-[Ru(NO_2)(bpy)$_2$(4-pic)]$^+$ (A, B, C) as NO delivery systems under light stimuli was evaluated using a specific NO selective electrode (ISO-NOP NO meter, World Precision Instruments, Sarasota, FL, USA). The sensitivity of this apparatus ranges from 1 nmol L^{-1} to 20 μmol L^{-1}, with a 2 mm sensor, which directly detects NO concentration by an amperometric technique. Quartz cuvettes containing the *cis*-[Ru(NO_2)(bpy)$_2$(4-pic)]$^+$ formulations were irradiated with UV–vis light from a 125 W mercury-arc lamp (Philips, São Paulo, Brazil) without a glass bulb. To verify the NO release profile from the control solution, the NO sensor was immersed vertically directly inside the quartz cuvette containing this solution. The NO release from the hydrogel formulations was determined according to the system illustrated in Fig. 2: the quartz cuvettes containing the hydrogels were connected by a silicone tube to a glass bottle containing phosphate buffer at pH 7.4. The NO sensor was immersed in this solution for the measurement of NO release, which was carried via vacuum from the cuvettes containing the formulations to the phosphate buffer solution. To avoid any release before the light irradiation, formulations were protected from light using aluminum foil. The system was previously deaerated with argon. The output of the sensor was recorded with an IBM-PC computer linked to a DUO-18 acquisition board from WPI. A NO release profile was constructed by plotting current *versus* time. The same light intensities were used in all experiments.

2.7. Indirect quantification of NO release from *cis*-[Ru(NO_2)(bpy)$_2$(4-pic)]$^+$ formulations

The amount of NO released over time (1 h) by UV–vis light irradiation from the control solution and from the HEC (A) and chitosan gel (C) was determined indirectly by the amount of the aqueous complex *cis*-[Ru(bpy)$_2$(4-pic)(H_2O)]$^{2+}$ formed after NO release. This amount is equimolar to NO release (Scheme 1) (De Lima et al., 2005).

Weighed samples of the formulations containing the nitro-ruthenium complex were placed in the quartz cuvettes and

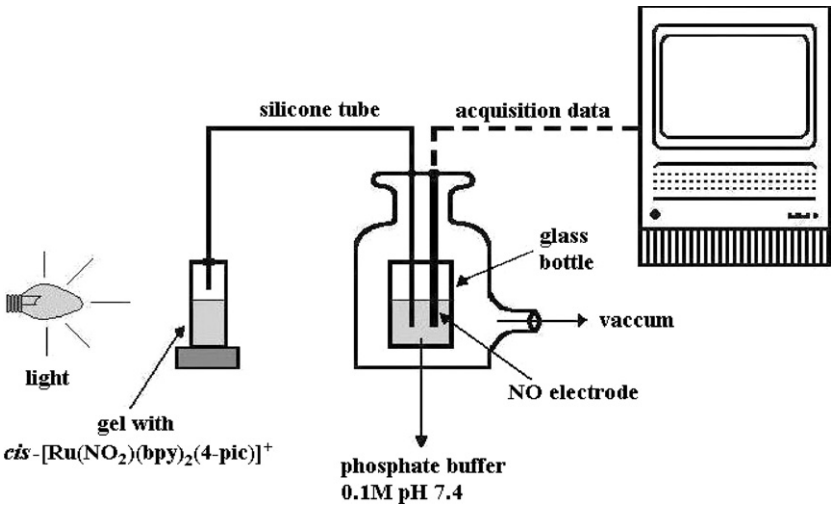
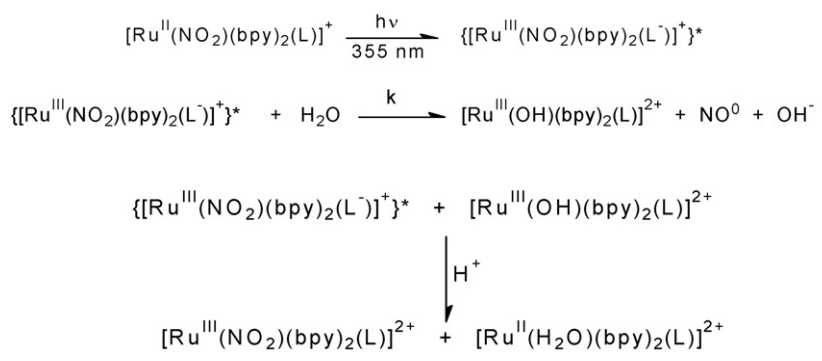


Fig. 2. Schematic illustration of the system used to determine the NO released from the hydrogels containing the *cis*-[Ru(NO_2)(bpy)$_2$(4-pic)]$^+$ during 1 h of light irradiation.



Scheme 1. Photochemical pathway for the nitro-ruthenium complexes (De Lima et al., 2005).

irradiated for 1 h using a 125 W mercury-lamp light. The amount of the ruthenium aqueous complex formed was then determined by HPLC before and after the irradiation. The samples were protected from light before and after light irradiation experiments.

2.8. In vitro permeation study

In vitro skin penetration experiments were carried out for 24 h using static Franz diffusion cells and pig ear skin as a membrane. The skin was mounted in the cells with the dermal side facing downward into the receptor medium, using 5.5 mL of phosphate buffer at pH 7.4. To achieve a higher reproducibility, the skin samples were allowed to prehydrate with receptor fluid for 1 h before the formulation was applied. The donor compartment was then filled with 1.0 g of the HEC gel containing the nitro-ruthenium complex. The total available diffusion area of the cell was 1.15 cm^2. The system was maintained at 37 °C, and the receptor medium was stirred at 500 rpm. At the end of the experiment, the amount of the nitro-ruthenium complex that permeated across the skin was analyzed by HPLC as described above. *In vitro* permeation studies were performed in replicates of four. To avoid any release before the light irradiation, formulation and the diffusion cells were protected from light using aluminum foil.

2.9. Skin uptake

After 24 h of the permeation study, the skin was removed from the diffusion cell, washed with distilled water to remove the excess of formulation and pinned to a piece of Parafilm™ with the stratum corneum (SC) face up. The part of the skin, which had been exposed to the formulation, was then tape-stripped 15 times using Scotch Book Tape n 845 (3M, St. Paul, MN). The tape strips were subsequently immersed in 5 mL of methanol in a vial submitted to vortex stirring (Phoenix, model AP56, Araraquara, SP, Brazil) for 1 min to extract the permeant. Aliquots of the extract were then analyzed by HPLC. Removal of the SC was almost complete after 15 tape-strippings as indicated by the glistening of the exposed (viable epidermal) surface (Lopez et al., 2003).

The remaining skin was cut into small pieces, homogenized by a tissue homogenizer (T25 Ultraturrax, IKA-Labortechnik, Staufen, Germany) for 1 min with 5 mL of methanol and bath-sonicated for 30 min. An aliquot of the filtered homogenate was then analyzed by HPLC to determine the quantity of the NO–ruthenium complex and its aqua-complex *cis*-$[Ru(bpy)_2(4\text{-pic})(H_2O)]^{2+}$ in the epidermis and dermis ("viable skin").

The extraction procedure of the *cis*-$[Ru(NO_2)(bpy)_2(4\text{-pic})]^+$ and the *cis*-$[Ru(bpy)_2(4\text{-pic})(H_2O)]^{2+}$ from the skin with methanol was validated. The recovery percentages obtained were not less than 94% and 95% for the SC and "viable epidermis", respectively, when the NO–ruthenium complex was analyzed, and not less than 71% and 66% for the SC and "viable epidermis", respectively, when the aqua-ruthenium complex was analyzed.

2.10. NO photorelease from the skin containing *cis*-$[Ru(NO_2)(bpy)_2(4\text{-pic})]^+$

In order to evaluate the NO photorelease from skin containing *cis*-$[Ru(NO_2)(bpy)_2(4\text{-pic})]^+$, a new series of permeation studies was performed as described elsewhere. After 24 h of *cis*-$[Ru(NO_2)(bpy)_2(4\text{-pic})]^+$ skin permeation from the HEC gel, the donor compartment formulation was removed, and the skin, still fixed in the diffusion cell, was washed with distilled water three times to remove excess of the formulation. The skin was then irradiated using a 125 W mercury-lamp light for 10 min. After the membrane was finally removed from the diffusion cell, the permeant was extracted according to the "*skin uptake*" procedure already described. The amount of the nitro-ruthenium and the aqua-ruthenium complexes present in the skin homogenate was then analyzed by HPLC.

To confirm NO photorelease from the skin, the NO release profile from the skin containing the nitro-complex was also verified using the NO selective sensor. In this way, after the permeation experiments, the skin was removed from the diffusion cells, washed with distilled water to remove the excess of the formulation and placed in a quartz cuvette containing a pH 7.4 phosphate buffer solution. Then, it was irradiated for 10 min with the 125 W mercury-lamp light. NO measurements were made with the NO sensor immersed vertically inside the quartz cuvette containing the skin.

2.11. Statistics

All results were expressed as the mean ± standard deviation. Statistical comparisons were performed using one-way ANOVA with Tukey's multiple comparison by GraphPad Prism® software for the release studies of nitro-ruthenium complex from the formulations. Student's *t*-tests were used to compare molar concentrations of *cis*-$[Ru(NO_2)(bpy)_2(4\text{-pic})]^+$ ($[Ru^{II}\text{-}NO_2]$) and *cis*-$[Ru(bpy)_2(4\text{-pic})(H_2O)]^{2+}$ ($[Ru^{II}\text{-}H_2O]$) before and after light irradiation. In all analyses, p values <0.05 were considered to be statistically significant.

3. Results and discussion

The *cis*-$[Ru(NO_2)(bpy)_2(4\text{-pic})]^+$ was incorporated in three hydrogels currently used for topical administration of drugs. Hydrogels were selected for these studies instead of ointments, also commonly used in topical administration, or other more elaborated delivery system because it is the first time that skin permeation of this NO-donor is studied. Therefore, formulations that interfere as little as possible in the characteristics of the complex, as its sta-

bility, were chosen for this study. The polymers chosen to form the gels have different ionic characteristics (non-ionic, cationic and anionic hydrogels) for the evaluation of interactions of the positively charged NO-donor complex with the vehicles and its possible influence in the NO release. The influence of the formulation in nitro-ruthenium complex release was first evaluated as described below.

3.1. *In vitro release studies of cis-[Ru(NO$_2$)(bpy)$_2$(4-pic)]$^+$ from the formulations*

Since it is likely that the polymeric formulations will not penetrate the skin but the skin penetration of the nitro-ruthenium complex is required to target the site of NO release into skin, the complex must be released from formulations to enter at least the outermost skin layer. In this way, the rate of complex release can influence the extent of NO absorption (Lu and Jun, 1998; Davis and Hadgraft, 1993). The rate of the release depends, in turn, on the thermodynamic activity of the drug in the vehicle as well as on its diffusivity (Lu and Jun, 1998). In an attempt to maximize the nitro-ruthenium complex release from the formulations studied, the solutions used to disperse the former-hydrogel polymers were saturated with *cis*-[Ru(NO$_2$)(bpy)$_2$(4-pic)]$^+$. In this way, the initial concentration of the complex in each formulation was slightly different (157 μg/g, 130 μg/g and 112 μg/g for formulations A, B and C, respectively).

The nitro-ruthenium complex release profile from the formulations studied exhibited a linear relationship ($r > 0.99$) when the amount of drug released was plotted against the time square root, indicating that the Higuchi model could describe the release. Therefore, the diffusion coefficient (D) of the *cis*-[Ru(NO$_2$)(bpy)$_2$(4-pic)]$^+$ was calculated according to Higuchi's equation (Eq. (I)) (Higuchi, 1962) (Fig. 3).

As can be seen in Fig. 3, the D of the nitro-ruthenium complex from the non-ionic and the cationic gels was not statistically different from that of the phosphate buffer solution ($p > 0.05$). Only the polyacrylate gel retarded the drug D when compared to the control (phosphate buffer solution at the same pH of the gels) ($p < 0.05$). As the *cis*-[Ru(NO$_2$)(bpy)$_2$(4-pic)]$^+$ is positively charged in the pH of the formulation, an electrostatic interaction between the drug and the negatives charges of the polymer is feasible. Merclin et al. (2004) observed the same behavior for the positively charged 5-aminolevulinic acid dispersed in a polyacrylate gel. This possible

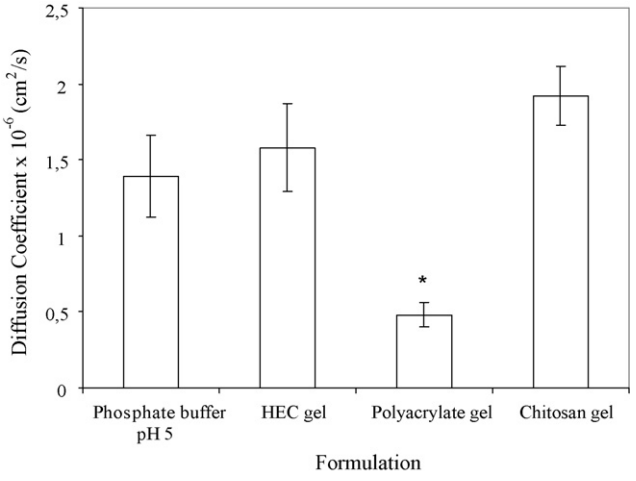


Fig. 3. Diffusion coefficient of *cis*-[Ru(NO$_2$)(bpy)$_2$(4-pic)]$^+$ after 12 h from different formulations. Data shown are the mean ± S.D. of five replicates. $^*p \leq 0.05$, representing statistically lower release rates from the control and from the other hydrophilic gels.

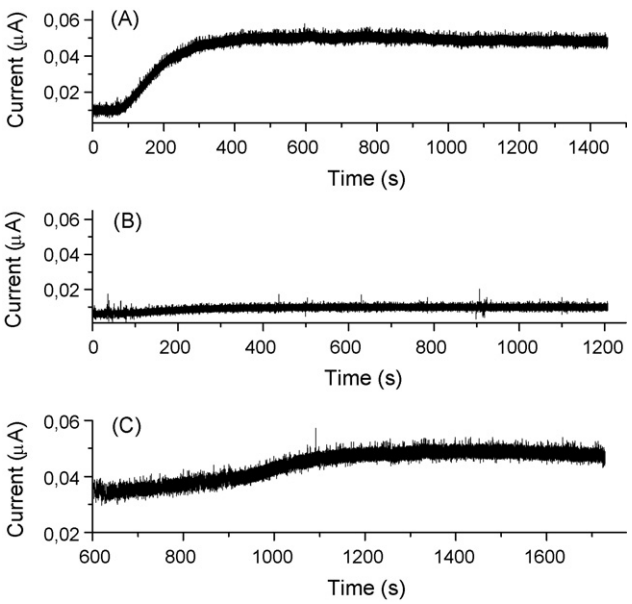


Fig. 4. Chronoamperograms of NO released during UV–vis light irradiation of the formulations containing the nitro-ruthenium complex: (A) phosphate buffer solution (pH 5) irradiation; (B) HEC hydrogel irradiation; (C) chitosan hydrogel irradiation.

interaction between drug and polymer, besides affect drug release from formulation, can also affect the NO release from the complex. Therefore, the NO release from nitro-ruthenium complex-loaded formulations was evaluated as described below.

3.2. *NO release from nitro-ruthenium complex-loaded formulations*

The photolysis of *cis*-[Ru(NO$_2$)(bpy)$_2$(4-pic)]$^+$ dispersed in the formulations was carried out using a NO sensor, which is an indubitable way to prove NO release. Fig. 4 shows the chronoamperograms of NO released from the control solution (phosphate buffer, pH 5) and from the HEC and chitosan gel formulations.

As can be seen in the Fig. 4 the presence of a current is a direct proof of NO release from the complex dispersed in the HEC and chitosan gel formulations when they were irradiated as described before. Formulations in the absence of the nitro-ruthenium complex were also irradiated and no current was observed, as expected.

Nitric oxide is released from nitrosyl ruthenium complex with conventional light sources, depending on exclusively of the wavelength of absorption. Typically, absorption of light by a molecule involves electron excitation, which may initiate photochemical reaction of the energized ruthenium species causing NO release. The nitric oxide released from *cis*-[Ru(NO$_2$)(bpy)$_2$(4-pic)]$^+$ after UV–vis light irradiation could be explained by reduction of coordinated nitrite by excited *cis*-[Ru(NO$_2$)(bpy)$_2$(4-pic)]$^+$ in a similar mechanism observed for *cis*-[Ru(NO)(bpy)$_2$(4-pic)]$^+$ after UV–vis irradiation (Sauaia et al., 2003). Despite of the important biological consequences of UV radiation to the skin and eyes, as carcinogenesis, aging and wrinkling of the skin (Matsumura and Ananthaswamy, 2004), UV radiation has been used as a therapeutic agent for various skin diseases, as vitiligo, psoriasis, parapsoriasis, cutaneous T-cell lymphomas and chronic eczemas (Rosenbaum et al., 1985; Krutmann, 2000; El-Mofty et al., 2006; Parsad et al., 2006; Stein et al., 2008). A specific portion of UV spectrum, the UVA-1 (340–400 nm) region can penetrate deeper into skin, in comparison to UVA-2 (320–340 nm) and UVB (280–320 nm) regions and thus therapeutic doses are able to reach the dermis (Weichenthal

and Schwarz, 2005). Additionally, UVA-1 region is considered to be relatively safer (Seité et al., 2000).

The chronoamperogram of NO released from the polyacrylate gel is not illustrated in Fig. 4 because the NO was not released from this gel in detectable amounts, that is, no current was observed as in the control formulations (without nitro-ruthenium complex). According to Suzuki et al. (2005), the NO-releasing activity of nitrobenzene compounds was closely related to the conformation of the nitro group, the absorption wavelength, and the length of the conjugated π-electron system. The attraction forces between the positively charged nitro-ruthenium complex and the negative polyacrylate chain indicated by the release studies (Fig. 3) could have modified the original conformation of the nitro-ruthenium complex in this study. In this way, the electron-transfer reaction that occurs during the light irradiation of the molecule could be changed, hindering NO_2 reduction to NO and its subsequent release from the polyacrylate gel. Therefore, we decided not to continue to study this gel for the moment.

Also related to Fig. 4, it is important to mention that despite the fact that the NO release profile is the same for all formulations presented in this figure the intensity of the current generated by NO release is different. However, it is not possible to affirm only using these results that, for instance, the amount of NO released from the HEC gel formulation is smaller than that released from the phosphate buffer solution due to the smaller current intensity presented by the gel NO release profile. The amperometric technique used to verify the NO presence was only qualitative since the NO sensor cannot be directly immersed in the semi-solid formulations. As shown in Fig. 1, the NO released from the irradiated formulation was carried via vacuum until a phosphate buffer solution and the NO sensor was put in contact with this solution. This long route may hinder the proper quantification of the NO. Furthermore, the formulation was not stirred in this determination and this could also have affected the nitro-complex irradiation and the NO release from the bulk formulation due to the formation of boundary layers. In this way, to quantify, and not only qualify, the NO release from the formulations after light irradiation an HPLC analysis was performed. By this method NO release from the formulations was determined indirectly by the quantification of the formation of the aqua-ruthenium complex *cis*-$[Ru(bpy)_2(4\text{-pic})(H_2O)]^{2+}$ that is equimolar to NO release (De Lima et al., 2005). Table 2 shows the *cis*-$[Ru(NO_2)(bpy)_2(4\text{-pic})]^{+}$ and the *cis*-$[Ru(bpy)_2(4\text{-pic})(H_2O)]^{2+}$ molar concentrations in the formulations before and after 1 h of light irradiation.

According to Table 2, the HEC gel formulation released approximately 36% of NO, an amount not statistically different ($p > 0.05$) from that released from the phosphate buffer solution in the same period of light irradiation. The chitosan gel also released NO after irradiation; however, an important amount of the aqua-complex was observed in the gel before light irradiation as well. Once the *cis*-$[Ru(NO_2)(bpy)_2(4\text{-pic})]^{+}$ releases the NO under light and electrochemical stimulation (Sauaia et al., 2003), it seems that the chitosan gel formulation itself can reduce the nitro-ruthenium complex molecule, releasing the NO. Chitosan is a cationic linear $\beta \rightarrow$ 1,4-linked polysaccharide of 2-amino-deoxyglucose and 2-amino-2-N-acetylamino-D-glucose (Berger et al., 2004). It is likely that it is the acetamido and/or the amino groups of its backbone that reduce the drug molecule, promoting NO release.

In order to confirm this hypothesis, a chemical crosslinked chitosan gel was obtained by mixing glutaraldehyde (GLU) aqueous solution to chitosan hydrogel. In this gel, the amino groups of the chitosan molecules are covalently crosslinked with glutaraldehyde (Berger et al., 2004). Therefore, they are not available to reduce the nitro-ruthenium complex dispersed in the gel formulation. Actually, the aqua-complex was not present in detectable amounts in the crosslinked chitosan gel before light irradiation (Fig. 5), corroborating the hypothesis that the amino groups of the chitosan molecules are mainly responsible for the electrochemical reduction of the nitro-ruthenium complex into the uncrosslinked gel before light irradiation.

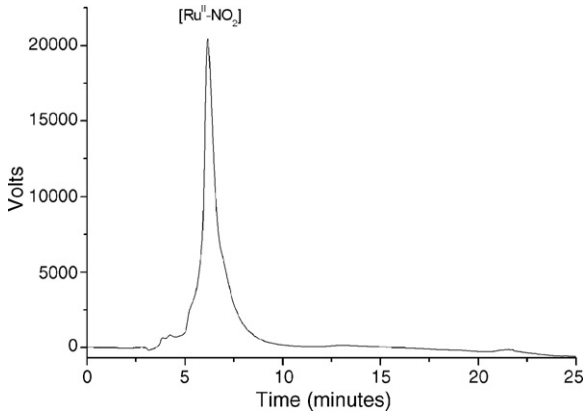


Fig. 5. Chromatogram of a solution extracted from the chitosan gel crosslinked with 0.03% of glutaraldehyde containing the *cis*-$[Ru(NO_2)(bpy)_2(4\text{-pic})]^{+}$ before light irradiation.

3.3. In vitro permeation study

Once one of the aims of this work was to obtain a semi-solid formulation that did not interfere in NO release, the HEC gel was selected to administer the nitro-ruthenium complex in the skin. The *in vitro* passive skin penetration experiments of the *cis*-$[Ru(NO_2)(bpy)_2(4\text{-pic})]^{+}$ dispersed in the HEC gel showed that the nitro-ruthenium complex did not cross through the skin in detectable amounts after 24 h. This was also detected in the viable epidermis but not in a quantifiable amount. Nevertheless, 2.90 $\mu g\,cm^{-2}$ ($\pm 0.20\ \mu g\,cm^{-2}$) of the *cis*-$[Ru(NO_2)(bpy)_2(4\text{-pic})]^{+}$ was quantified in the stratum corneum (SC).

To evaluate the NO photorelease from the skin, after 24 h of the administration of the nitro-ruthenium complex-loaded HEC gel, this formulation was removed from the surface of the skin and this tissue was irradiated for 10 min. Fig. 6A shows the chromatograms obtained from HPLC analysis of the skin before and after light irradiation. It is clear that, despite the fact that the aqua-ruthenium complex could not be detected, the nitro-ruthenium complex peak disappeared after irradiation, suggesting NO release. To determine the NO release a NO sensor was immersed vertically inside the quartz cuvette containing the skin. Therefore, Fig. 6B shows the chronoamperograms of NO released from the skin during light irradiation, confirming the NO photorelease from the skin containing the nitro-ruthenium complex.

Based on the quantitative results of NO released from the developed formulations demonstrated in Table 2, the amount of complex which is present in SC should be able to release around 3 nmol cm^{-2} of NO in a few minutes. The amount of NO delivered is expressed as total nanomoles of NO instead of a concentration (*e.g.*, mM) once NO is readily converted to reactive nitrogen and oxygen species. Therefore, the exact molar concentrations of NO and its byproducts in solution are not known (Hetrick et al., 2008).

Bactericidal action against pathogens common in skin infections as *P. aeruginosa* and *S. aureus* was achieved by Nablo and Schoenfisch (2003) in cell culture after NO release from a sol–gel surface at a flux less than 1 pmol s^{-1} cm^{-2}. Ghaffari et al. (2005) also observed a bactericidal action after the release of 200 ppm of NO without compromising the viability and function of culture skin cells. Similar results were described by Hetrick et al. (2008) that developed NO-releasing nanoparticles. Finally, Janczyk et al. (2004)

Table 2
Molar concentration of *cis*-$[Ru(NO_2)(bpy)_2(4\text{-pic})]^+$ ($[Ru^{II}\text{-}NO_2]$) and *cis*-$[Ru(bpy)_2(4\text{-pic})(H_2O)]^{2+}$ ($[Ru^{II}\text{-}H_2O]$) present in the formulations before and after 1 h of light irradiation.

Formulation code	Before light irradiation		After light irradiation	
	$[Ru^{II}\text{-}NO_2]$ (nmol)	$[Ru^{II}\text{-}H_2O]$ (nmol)	$[Ru^{II}\text{-}NO_2]$ (nmol)	$[Ru^{II}\text{-}H_2O]$ (nmol)
A	730 (±0)	–	520 (±01)	260 (±10)
B	800 (±50)	–	450 (±70)	290 (±20)
C	460 (±30)	200 (±10)	370 (±60)*	330 (±0)*

Data shown are the mean ± S.D. of five replicates.
* $p \leq 0.05$, representing statistically different amounts from the control and from the other hydrophilic gel. No statistical significant differences were found between formulation A and B employing *t*-test.

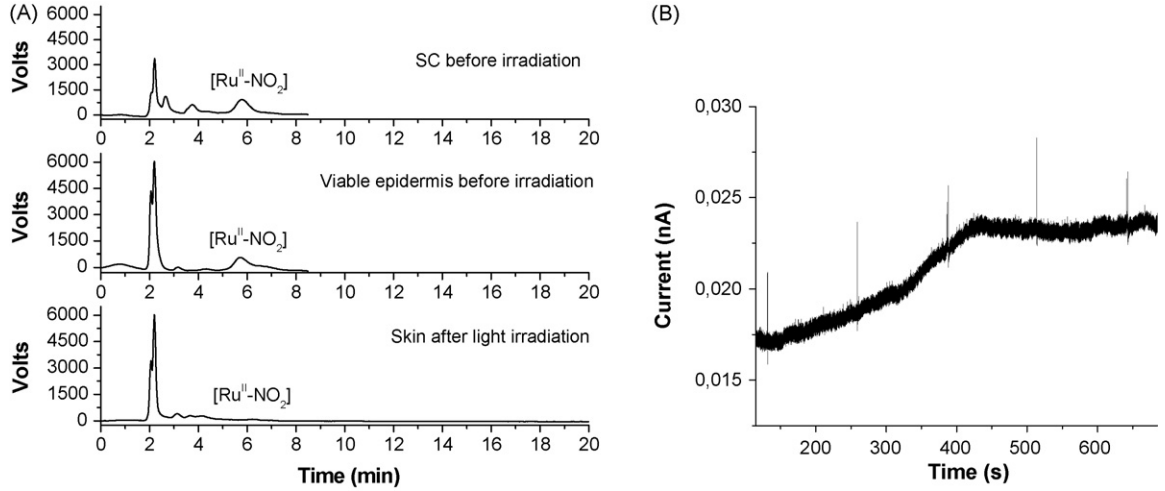


Fig. 6. NO photorelease from the skin containing the *cis*-$[Ru(NO_2)(bpy)_2(4\text{-pic})]^+$ after 10 min of light irradiation. (A) Chromatograms of skin samples containing the nitro-ruthenium complex before and after the light irradiation. (B) Chronoamperogram of NO released during UV–vis light irradiation of the skin containing the nitro-ruthenium complex.

showed NO-dependent phototoxicity of Roussin's black salt (RBS) after UVA irradiation, employing melanoma cells (human SK-MEL 188 and mouse S91). The maximum concentration of NO released was 37 nmol L^{-1}, which was sufficient to cause serious melanoma cells damage. It is important to emphasize that all these studies were conducted in cell culture, where NO released was in direct contact with the cells.

Since NO, as a gas, spreads out easily through different membranes (Wang et al., 2002), its photorelease from the nitro-ruthenium complex present on the formulation developed in our work would be enough to allow NO penetration into the skin. However, considering that the half-life of NO is only a few seconds (Ignarro et al., 1987; Vallance and Collier, 1994), the presence of the NO-donor in the SC layer is an interesting find as well, since it could target the NO diffusion to the viable layers of the skin.

4. Conclusions

In summary, it was verified that the nature of the polymer used to disperse the NO-donor *cis*-$[Ru(NO_2)(bpy)_2(4\text{-pic})]^+$ can influence not only the diffusion of the nitro-ruthenium complex but also NO release. NO can be released from the nitro-ruthenium complex dispersed in the HEC gel by light stimulus in the same magnitude as that from a drug-loaded phosphate buffer solution. Moreover, the nitro-ruthenium complex can penetrate the stratum corneum and, by light stimuli, release NO into the skin.

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