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Reaction of ruthenium nitrosyl complexes with superoxide

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ABSTRACT

The reaction between the ruthenium nitrosyl complexes *trans*-$[Ru^{II}(NO)L_1L_2]^{n+}$, where $L_1 = (NH_3)_4$ and L_2 = imN, nic, 4-pic and $P(OEt)_3$ or L_1/L_2 = Hedta, with superoxide ($O_2^{\cdot -}$) have been probed in aqueous medium at (25 ± 0.1) °C by UV–Vis, DPV and EPR spectroscopies. The reaction involves one electron transfer from $O_2^{\cdot -}$ to *trans*-$[Ru^{II}(NO)L_1L_2]^{n+}$ yielding *trans*-$[Ru^{II}(H_2O)L_1L_2]^{n+}$ and nitric oxide (NO) as main reaction products. Using cytochrome *c* as a probe for superoxide and a competitive kinetic approach, the apparent bimolecular rate constant for the reaction between *trans*-$[Ru^{II}(NO)L_1L_2]^{n+}$ and $O_2^{\cdot -}$ have been determined to be in the range of $(6.3 \pm 0.5) \times 10^3$ ($L_1 = (NH_3)_4$; L_2 = 4-pic) to $(5.8 \pm 0.2) \times 10^6$ ($L_1 = (NH_3)_4$; $L_2 = P(OEt)_3$) $M^{-1}\,s^{-1}$. For $L_1 = (NH_3)_4$ and $L_2 = P(OEt)_3$ the peroxynitrite formation as a by-product of $O_2^{\cdot -}$ and NO reaction was detected using tyrosine as a probe monitoring the formation of 3-nitro-tyrosine by HPLC-DAD. These nitrosyl compounds can be activated by superoxide, thus holding radical scavenger potential *in vivo*, besides being useful to modulate the local NO levels.

1. Introduction

Superoxide ($O_2^{\cdot -}$) is widely produced by mammalian cells, mostly by the mitochondrial respiration process [1–3]. It is estimated that 1–3% of all oxygen uptake by mammalian respiration is converted into superoxide [1]. Although, under conditions of oxidative stress, the consumption of molecular oxygen and consequently the production of $O_2^{\cdot -}$ increases dramatically [1,4]. As a consequence of its continuous production and reactivity, superoxide is a key molecule in human physiology [1,5].

Nitric oxide (NO) is a widely studied molecule due to its role in human physiological pathways [6–12]. Nitric oxide reacts with superoxide at rate constant close to the diffusion limit in water, generating peroxynitrite ($ONOO^-$) [13–15]. This is a high reactive molecule and may be responsible for cells damages and oxidative stress [16–21]. From this perspective the control of the local NO concentration becomes very important.

Metal based nitric oxide carriers, chemically or photochemically activated, have been deserving attention as much as NO itself, since the controlled delivering or uptake of NO is required [22–26]. Ruthenium nitrosyl complexes, *trans*-$[Ru^{II}(NO)L_1L_2]^{n+}$, where $L_1 = (NH_3)_4$ and L_2 = N-heterocyclic or phosphane ligands [23]; L_1/L_2 = Hedta are able to dissociate NO or HNO after reduction of coordinated NO^+ by reductants such as ascorbic acid and thiols (e.g. GSH) [23]. As consequence of the NO releasing ability, these compounds were tested *in vitro* and *in vivo* as candidate drugs for vasodilatator and as pro-drugs against tripanosomatides (*Trypanosoma cruzi* and *Leishmania major*) and cancer [27]. Furthermore, in a reductive environment, as found under hypoxia conditions, where the iNOS is inhibited [28], these compounds could act as an alternative and controlled NO source.

With an aim to better understand the chemical reactivity of these ruthenium nitrosyl complexes when administrated in biological medium, this paper describes the reactivity of the *trans*-$[Ru^{II}(NO)L_1L_2]^{n+}$ towards superoxide in aqueous medium.

2. Materials and methods

2.1. Reagents and solutions

All reagents were purchased from Sigma-Aldrich, Fluka or Merck and used as received. A 5.0×10^{-2} M Phosphate buffer pH = (7.2 ± 0.1) μ = 0.1 M was prepared using high purity water (18.2 M Ω cm) from a Milli-Q purification system (Millipore, Bedford, MA). Horse-heart ferricytochrome *c* was purified using a Sephadex G-25 column (0.5 × 15 cm) [29]. The concentration of the purified ferricytochrome *c* (Cyt *c*) solution was calculated by UV–Vis spectroscopy [30].

2.2. Instrumentation

UV–Vis spectra were recorded on a Hitachi U-3501 spectrophotometer using 1.00 cm quartz cells. Samples were irradiated with a Nd:YAG *LASER* (Continuum, model Surelite-II) operating in the third harmonic (355 nm) pumping an optical parametric oscillator (OPO) system (Continuum, model 10-05-00 SSP-4) adjusted to 440 nm using a wavelength meter (Coherent, model Wavemate).

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A power meter (Coherent, Lasermate-P) measured the average energy per pulse. The selective detection of nitric oxide was carried out chronoamperometrically using a NO selective electrode (Innovative Instruments Inc., model inNO-T). The electrode was polarized in water for 12 h before the measurements. Electron paramagnetic resonance (EPR) experiments were carried out on a Bruker ESP300E spectrometer at X-Band frequency. Measurements were carried out at 100 K using a variable temperature system (Euroterm, model B-VT 2000). For spectra calibration, a sealed capillary containing a small amount of solid DPPH$^{\cdot}$ (g = 2.0036) was introduced into the samples tubes and the spectra were recorded. HPLC analyses were carried out in a LC-10AD-VP system coupled to a SPD-M10AVD UV–Vis diode array detector (Shimadzu). Differential pulse voltammetry (DPV) measurements were performed on an EG&G PAR model 264A, using a glassy carbon electrode as a working electrode, a saturated calomel electrode (SCE) as reference and a platinum plate as auxiliary electrode. Solutions of CF_3COOH/CF_3COONa, pH = (5.5 ± 0.1) μ = 0.1 M were used as the background electrolyte. Experimental conditions for DPV were scan rate of 20 mV/s and pulse height of 100 mV. Both *trans*-$[Ru^{II}(NO)(NH_3)_4(P(OEt)_3)]^{3+}$ and complex *trans*-$[Ru^{II}(H_2O)(NH_3)_4(P(OEt)_3)]^{2+}$ are robust species in aqueous acidic solutions and therefore the *trans*-$[Ru^{II}(NO)(NH_3)_4(P(OEt)_3)](PF_6)_3$ salt was used as internal standard when quantification was necessary. The measured potentials were converted and reported as normal hydrogen electrode (NHE). ^{31}P NMR spectra were obtained in a Bruker AVANCE (400 MHz) spectrometer, using a 5 mm probe. The counter-ion hexafluorophosphate was used as internal reference (PF_6^-; δ = −144 ppm).

2.3. Synthesis of ruthenium amines nitrosyl complexes

Ruthenium nitrosyl complexes *trans*-$[Ru^{II}(NO)L_1L_2](X)_n$ (L_1 = $(NH_3)_4$ and L_2 = 4-picoline (4-pic), nicotinamide (nic), imidazole (imN), and triethylphosphite ($P(OEt)_3$); and L_1/L_2 = Hedta, X = hexafluorophosphate (PF_6^-) or tetrafluoroborate (BF_4^-), and their synthetic precursors were prepared and characterized as described in the literature [31–37]. When necessary, the solutions were degassed using purified and dried argon and manipulations were carried out using standard inert atmosphere techniques [38].

2.4. Generation and detection of superoxide

Superoxide was generated photochemically or enzymatically (xanthine/xanthine oxidase) as previously described in the literature [29,39]. For the photochemical generation, air saturated solutions containing 1.0×10^{-3} M flavin mononucleotide (FMN), 1.0×10^{-2} M of diethylenetriaminepentaacetic acid (DTPA) were irradiated for 4 min using a Nd:YAG *LASER* with a repetition-rate of 1 pulse per second (8 ns duration) with average energy of 2.5 ± 0.9 mJ per pulse. To probe the superoxide generation, the reduction of a ferricytochrome *c* solution was monitored using UV–Vis spectroscopy (Fig. S1) [30]. For the enzymatic assay [29], 1.0×10^{-3} M xanthine solutions in phosphate buffer were used. Xanthine oxidase solutions (0.016 U/mL) were freshly prepared in the same buffer. After mixing the solutions, the reaction proceeded for a period of 20 min. Superoxide generation was confirmed through the detection of the ferricytochrome *c* reduction by UV–Vis [29,30].

2.5. Competitive kinetic

A competitive kinetic experimental method [40–42] was used to estimate the bimolecular apparent rate constants for the reaction between superoxide and the ruthenium nitrosyl complexes. A solution at concentration of 2.0×10^{-5} M of ferricytochrome *c* was used as a competitor ($k_{Cyt\ c} = 1.4 \times 10^6\ M^{-1}\ s^{-1}$ for superoxide) [43] and the reaction course was monitored by UV–Vis spectroscopy. Concentrations of ruthenium nitrosyl complexes ranged over the range of 1.0×10^{-2} to 1.0×10^{-5} M depending on the nature of the ligand L. Control experiments without addition of the ruthenium complexes were loaded between each sample. By probing the absorbance at 550 nm for ferricytochrome *c* in the control experiments and in the samples containing the ruthenium nitrosyl complexes, the bimolecular apparent rate constant (k_{app}) for the reaction were calculated by plotting $(S/1 - S)\ k_{Cyt\ c}$ [Cyt *c*] versus [*trans*-$[Ru^{II}(NO)L_1L_2]^{n+}$] where S is the percentage of inhibition of the ferricytochrome *c* reduction by the ruthenium nitrosyl complexes as follows:

$$\left[\frac{S}{1-S}\right] k_{Cyt\ c}|\text{Cyt } c| = k_{app}\left[\textit{trans}\text{-}[Ru^{II}(NO)L_1L_2]^{n+}\right]$$

2.6. Peroxynitrite detection

Peroxynitrite detection during the reaction of superoxide with the ruthenium nitrosyl complexes was carried out using tyrosine as a probe and analyzing its nitration product, 3-nitro-tyrosine [44–46] by HPLC-DAD detection. Solutions of ruthenium nitrosyl complexes were photolyzed in the presence of L-tyrosine (2.7×10^{-3} M) in the same photolysis conditions described before. Chromatography analyzes were performed using a 5.0×10^{-2} M phosphate buffer (KH_2PO_4; pH = 3.0 ± 0.1) and methanol (95:5 v/v). A Waters Spherisorb S5 ODS (250 × 2.0 mm i.d. and 5 μm particle size), chromatographic column was used at the flow rate of 0.3 mL/min and at a temperature of 22 °C. Under these experimental conditions, the detection limit for 3-nitro-tyrosine was determined as 2.0×10^{-7} M, being comparable with the one reported in the literature [46]. Photolyzed solution (20 μL) was injected and the 3-nitro-tyrosine formation was followed at 365 nm. 3-Nitro-tyrosine is not the only product of the reaction between tyrosine and peroxynitrite and therefore the result was used only for semi quantitative purposes. Nevertheless an estimative of the 3-nitro-tyrosine amount formed was performed by the standard addition method.

3. Results and discussion

The reaction of superoxide with the ruthenium nitrosyl complexes was studied by chronoamperometric measurements, DPV and UV–Vis, ^{31}P NMR, and EPR spectroscopies. Samples were EPR silent, suggesting that the metal center was not oxidized to Ru(III) in the course of the reaction.

After the photochemical generation of superoxide in presence of *trans*-$[Ru^{II}(NO)(NH_3)_4(P(OEt)_3)]^{3+}$ the complex *trans*-$[Ru^{II}(H_2O)(NH_3)_4(P(OEt)_3)]^{2+}$ was observed in solution by DPV (Fig. 1, peak a, $E^{0'}$ = 0.70 V versus NHE) [47] and ^{31}P NMR (δ = 148 ppm, data not shown) measurements [46]. After 4 min of photolysis, approximately 30% of the initial amount of *trans*-$[Ru^{II}(NO)(NH_3)_4(P(OEt)_3)]^{3+}$ (Fig. 1, peak b, $E^{0'}$ = 0.12 V versus NHE) was converted into the aqua complex. Nitric oxide evolution was not detected chronoamperometrically (Fig. S2) using a selective NO electrode. Using DTPA/FMN/light as superoxide source and cytochrome *c* reduction as a probe [43], the concentration of superoxide in solution was calculated as being approximately 9.6×10^{-6} M (Fig. S1). As shown by the DPV response, the concentration of *trans*-$[Ru^{II}(H_2O)(NH_3)_4(P(OEt)_3)]^{3+}$ formed was equal to 9×10^{-6} M. Thus, the reaction of superoxide with the *trans*-$[Ru^{II}(NO)(NH_3)_4(P(OEt)_3)]^{3+}$ yielded 92% of the predicted amount of the aqua complex for a stoichiometric reaction. The phosphite complex was chosen to be studied in more details because its aqua species

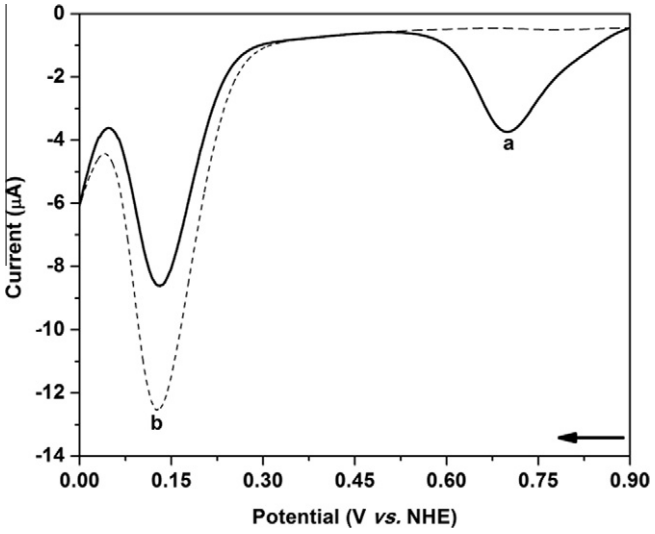

Fig. 1. Differential pulse voltammogram of the photolyzed solution containing *trans*-$[Ru^{II}(NO)(NH_3)_4(P(OEt)_3)]^{3+}$ DTPA and FMN. Dotted line: before irradiation; solid line: after 4 min of photolysis, peak a and b: aqua and nitrosyl complexes respectively. $C_{Ru} = 3.0 \times 10^{-5}$ M; pH = (5.5 ± 0.1), μ = 0.1 M CF_3COOH/CF_3COONa; scan rate: 20 mV/s; pulse height: 100 mV; T = (25 ± 0.1) °C.

is more stable in acidic solution regarding to *trans*-$[Ru^{II}(H_2O)L_1L_2]^{n+}$ where L_2 = N-heterocycles [23].

The kinetic measurements were carried out by monitoring absorbance changes at 550 nm and the kinetics calculations were performed as described in Section 2.5. The ruthenium nitrosyl complexes spectra were discussed previously [48] and they photochemical behavior as well [49]. According to these data these compounds are not photoactive in the wavelength utilized (λ_{irrad} = 440 nm) precluding eventual side reactions. Fig. 2 shows the spectra of the reduction of cytochrome *c* by superoxide and its inhibition when the solution was photolyzed in the presence of ruthenium nitrosyl complexes.

The bimolecular apparent rate constant (k_{app}) was calculated using the Fig. 2 data, the control experiment (Fig. S1) and the equation described in the Section 2.5. The values of k_{app} obtained for the

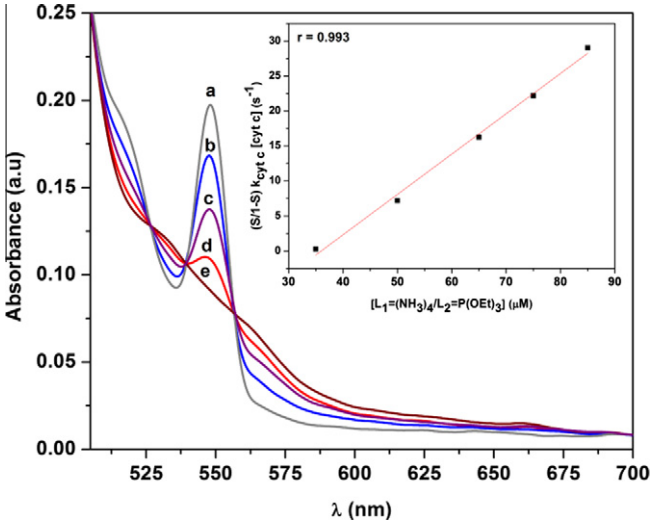

Fig. 2. UV–Vis spectra of the reaction between *trans*-$[Ru^{II}(NO)(NH_3)_4((POEt)_3)](PF_6)_3$ with superoxide using cytochrome *c* as probe. a: $C_{Ru} = 3.5 \times 10^{-5}$ M; b: $C_{Ru} = 5.0 \times 10^{-5}$ M; c: $C_{Ru} = 6.5 \times 10^{-5}$ M; d: $C_{Ru} = 7.5 \times 10^{-5}$ M; e: $C_{Ru} = 8.5 \times 10^{-5}$ M; pH = (5.5 ± 0.1) μ = 0.1 M; T = (25 ± 0.1) °C. $C_{Cyt\ c} = 2.0 \times 10^{-5}$ M.

ruthenium nitrosyl complexes are given in Table 1. The plots for k_{app} calculation for the other ruthenium nitrosyls herein studied are in the Supplementary Material section (Fig. S3).

The data of Table 1 show that the k_{app} values were influenced by the nature of the ligands L_1 and L_2. A clear trend was not observed between the nitrosonium reduction potential of the $[RuNO]^{3+/2+}$ couple or the $\nu(NO^+)$ (Table S1) in all of the title compounds and their respective values of k_{app}.

Since superoxide and nitric oxide are present in solution, the possibility of peroxynitrite formation was also investigated, using tyrosine nitration as probe [46]. Under the experimental conditions used, the formation of $ONOO^-$ was observed only for the complex *trans*-$[Ru^{II}(NO)(NH_3)_4(P(OEt)_3)]^{3+}$. The utilization of tyrosine for peroxynitrite identification is extensive discussed in the literature [44–46,50]. Since the reaction medium is not anaerobic, the dissolved CO_2 in the solution can participate of the reaction. As discussed by Lancaster [45], only one third of the peroxynitrite produced can nitrosilate the tyrosine, through the generation of $NO_2^{\cdot}$ and $CO_3^{\cdot -}$. Also, as proposed by Houk and co-workers, there is more than one possible pathway for tyrosine nitration [50]. A simplified scheme of these reactions is presented in the Fig. S4. Thus, in the present experimental approach, tyrosine was used only for identification and to estimate the amount of peroxynitrite formed during the reaction. An estimative of 3-nitro-tyrosine amount formed, carried out by HPLC-DAD, suggest that when the concentration of *trans*-$[Ru^{II}(NO)(NH_3)_4(P(OEt)_3)]^{3+}$ was 1.25×10^{-4} M, the concentration of 3-nitro-tyrosine in solution was 1.7×10^{-5} M.

The superoxide may act as an oxidant or reductant, depending on the reaction conditions. The reported pK_a value for the perhydroxyl radical ($HO_2^{\cdot}$) is 4.7 [51]. Thus, in most of the cases, especially in biochemical processes, the predominant form of superoxide is the deprotonated form, $O_2^{\cdot -}$. Since the redox potential for superoxide is reported to be −0.16 V [52], the reduction of the coordinated NO^+ is thermodynamically feasible (Table S1).

Molecular orbitals analysis of the ruthenium nitrosyl complexes indicates that the LUMO orbitals have a predominant NO^+ ligand character (for example, L = py – LUMO: 28% Ru, 67% NO^+, 3% py; LUMO + 1: 28% Ru, 70% NO^+) [48]. Thus, it is reasonable to suppose that the reduction of these complexes by superoxide occurs preferentially on the nitrosonium ligand. On this perspective, and taking in account the experimental data presented herein, the reaction between superoxide and the ruthenium nitrosyl complexes would be coherent with the sequence of reactions:

$$trans\text{-}[Ru^{II}(NO)L_1L_2]^{n+} + O_2^{\cdot -} \xrightarrow{k_{app}} trans\text{-}[Ru^{II}(NO)L_1L_2]^{(n-1)+} + O_2 \quad (1)$$

$$trans\text{-}[Ru^{II}(NO)L_1L_2]^{(n-1)+} + H_2O \xrightarrow{k_{-NO}} trans\text{-}[Ru^{II}(H_2O)L_1L_2]^{(n-1)+} + NO \quad (2)$$

The ruthenium nitrosyl complexes can undergo to a second chemical or electrochemical reduction at the coordinated nitric oxide, yielding HNO [53]. However, the second reduction reaction

Table 1
Apparent bimolecular rate constants (k_{app}) for superoxide reaction with ruthenium nitrosyl complexes in aqueous medium at (25 ± 0.1) °C.[a]

L_1/L_2	k_{app} ($M^{-1}\ s^{-1}$)
$(NH_3)_4/P(OEt)_3$[b]	$(5.8 \pm 0.2) \times 10^6$
$(NH_3)_4$/imN	$(1.3 \pm 0.1) \times 10^5$
$(NH_3)_4$/nic	$(7.2 \pm 0.5) \times 10^4$
$(NH_3)_4$/4-pic	$(6.3 \pm 0.5) \times 10^3$
Hedta	$(3.2 \pm 0.3) \times 10^4$

[a] pH = (7.2 ± 0.1).
[b] pH = (5.5 ± 0.1).

is not possible on thermodynamic grounds with superoxide, since the $E^{0\prime}_{(NO^0/HNO)}$ value for the ruthenium tetraamine complexes is more negative than −0.50 V [53]. Furthermore, the back reaction of the Eq. (2), is not favorable since $K_{eq} \approx 30\ M^{-1}$ [23].

The apparent bimolecular rate constant (k_{app}), Table 1, are in the range of $(6.3 \pm 0.5) \times 10^3$ to $(5.8 \pm 0.2) \times 10^6\ M^{-1}\ s^{-1}$. These values are in agreement with those reported for other d^6 low spin metal complexes and metalloproteins, such as cytochrome *c* [54]. Regarding the reaction of superoxide with other nitric oxide donors, Aleryani and co-workers [55] reported that the values for the specific rate constants for *S*-nitrosocysteine and *S*-nitrosoglutathione were $(7.69 \pm 0.64) \times 10^4$ and $(1.28 \pm 0.05) \times 10^4\ M^{-1}\ s^{-1}$, respectively. These values are in a good agreement with the ones measured for the ruthenium nitrosyl complexes described herein.

Superoxide can also act as a nucleophilic agent. Ruthenium nitrosyl complexes are susceptible to nucleophilic attack on the coordinated NO^+ by OH^- or thiols (RS^-) to yield the species, *trans*-$[Ru^{II}(NO_2)(NH_3)_4L]^{(n-1)+}$ and *trans*-$[Ru^{II}(N(O)SR)(NH_3)_4L]^{n+}$ respectively, as described in the literature [56,57]. As mentioned before, the NO liberation was observed by chronoamperometric measurements using a selective NO electrode suggesting that the nucleophilic attack of superoxide on *trans*-$[Ru^{II}(NO)L_1L_2]^{n+}$ through the formation of *trans*-$[Ru^{II}(NOOO)L_1L_2]^{(n-1)+}$ is unlikely to occur, at least to any appreciable extent. If the formation of an intermediate species with superoxide and coordinated NO^+ has occurred, the products would probably be peroxynitrite and a Ru(III). In our experiments, no paramagnetic species was detected by EPR during the experiments, hence suggesting that the nucleophilic attack at the coordinate NO^+ did not occur.

According to Weinstock [58], the reaction of transition metal complexes with superoxide proceeds by an outer-sphere electron transfer mechanism. It is interesting to recall at this point that the k_{app} values obtained for the reaction of superoxide with the ruthenium nitrosyl complexes, and the values calculated and reported in the literature for the complexes $[Ru^{III}(edta)(H_2O)]^-$ and *trans*-$[Ru^{III}(NH_3)_6]^{3+}$ [58] are in the same order of magnitude. Ruthenium nitrosyl complexes are robust concerning substitution reactions (the same is not true for photochemical reactions) in aqueous medium for at least one week [23]. As substitution reactions do not take place for these complexes on the time scale of the reactions here described, it is feasible to suppose that the reduction of the NO^+ by superoxide follows an outer-sphere electron transfer mechanism.

Another important reaction involving free nitric oxide and superoxide is the peroxynitrite formation. Under our experimental conditions, peroxynitrite could be formed after the reactions of Eqs. (1) and (2), by the interaction of nitric oxide with superoxide (Eq. (3)).

$$NO + O_2^{\cdot -} \rightarrow ONOO^- \quad (3)$$

The formation of peroxynitrite was only observed when *trans*-$[Ru^{II}(NO)(NH_3)_4(P(OEt)_3)]^{3+}$ reacts with superoxide. The detection of 3-nitro-tyrosine by HPLC-DAD confirmed the formation of peroxynitrite during the reaction. For all other ruthenium nitrosyl complexes studied, peroxynitrite was not detected. The literature reports that the presence of superoxide dismutase (SOD) or $[Fe^{III}(edta)]^-$ increases the yield of the tyrosine nitration [59]. However, in our experiments, even SOD or $[Fe^{III}(edta)]^-$ were not used since they can react with superoxide ($k = 2 \times 10^9$ [60] and $k = 1.9 \times 10^6\ M^{-1}\ s^{-1}$ [61], respectively) diminishing the yield of the ruthenium nitrosyl complexes reduction.

The failure of $ONOO^-$ detection for the nitrosyl complexes (except for $L_2 = P(OEt)_3$) can be tentatively explained by taking in account their respective specific rate constants for NO liberation (Eq. (2)). Since the measured superoxide concentration is almost the same in all the experiments and the reaction between the nitrosyl complexes and superoxide is fast, the formation of $ONOO^-$ (Eq. (3)) would be dependent of the NO concentration generated from in solution.

Among the compounds studied herein, the complex *trans*-$[Ru^{II}(NO)(NH_3)_4(P(OEt)_3)]^{3+}$ exhibited the highest values of k_{app} and k_{-NO}, which would generate the major NO concentration in solution, and thus turning more feasible the formation of $ONOO^-$ in an appreciable extension. The high limit for the NO concentration in our experiments would be equal to the one of the produced superoxide ($\approx 1.0 \times 10^{-5}$ M). Despite the high value for reaction between superoxide and NO ($k \approx 10^9\ M^{-1}\ s^{-1}$) [14,15] we must to have in mind that the reaction between NO and $O_2^{\cdot -}$ is first order in both reactants and the half life for superoxide ion in solution small due to its dismutation [62].

For the other complexes the values of k_{app} and k_{-NO} are much lower than those for the $L_2 = P(OEt)_3$ and therefore the available concentration of free NO in solution is lower. These observations are in agreement with the experimental data, since no 3-nitrotyrosine formation was detected when $L_1 = (NH_3)_4$; L_2 = imNand L_1/L_2 = Hedta ($k_{-NO} = 0.16$ and $7.3 \times 10^{-3}\ s^{-1}$; $k_{app} = (1.3 \pm 0.1) \times 10^5$ and $(3.2 \pm 0.3) \times 10^4\ M^{-1}\ s^{-1}$, respectively). However, under situations where superoxide production is continuous and the concentration of this molecule is relatively high, as found for example inside mitochondria [2,3] the formation of peroxynitrite would be possible. It is interesting to recall that the concentration of superoxide in tissues may range from *ca.* 1.0×10^{-9} to *ca* 1.0×10^{-6} M in basal or in inflammatory conditions, respectively [1,10].

Two other possible biological reductants for the ruthenium nitrosyls are cysteine (Cys) ($pK_{aCys} = 8.3$, $E^{0\prime}_{Cysteine/Cystine} = -0.245$ V, pH = 7.0) [63] and NADPH ($E^0_{NADP/NADPH} = -0.324$ V, pH = 7.0) [64], which are found in the millimolar concentration range [65]. For cysteine, more than 95% is in the protonated form under physiological conditions, with the concentration of the active reductant, Cys^-, *ca.* 1.0×10^{-5} M. The specific rate constant for the reaction between Cys^- and NADPH with ruthenium nitrosyl complexes are 1.6×10^5 and $2.0 \times 10^1\ M^{-1}\ s^{-1}$, respectively [56,66]. Comparing the rate constants and the concentrations of the species NADPH, Cys^- and superoxide, under inflammatory conditions or circumstances where the concentration of superoxide is in the micromolar scale, it is reasonable to infer that superoxide along with Cys^- would be candidates to act as important reductants for ruthenium nitrosyl complexes in biological systems.

After the reduction of nitrosonium ligand by $O_2^{\cdot -}$ and consequently NO liberation the aqua species *trans*-$[Ru^{II}(H_2O)L_1L_2]^{(n-1)+}$ is formed. NO itself is a multi-target biologically active species, but the aqua species of transition metal complexes is also active as discussed in the literature [67–69]. The aquation step of the transition metal complexes, as the platinum and the ruthenium species, is a key step for their biological properties [67–69], since the aquo species can bind on DNA [68–70] and other proteins like albumin [69]. Also *trans*-$[Ru(H_2O)(NH_3)_5]^{n+}$, where Ru is in the oxidation state II or III, *trans*-$[Ru^{II}(H_2O)(NH_3)_5(pz)]^{2+}$, and $[Ru^{II}(H_2O)(edta)]$ showed anti-cancer activity [69]. Thus, the complexes *trans*-$[Ru^{II}(NO)L_1L_2]^{n+}$ would exhibit two possible different pathways of action *in vivo*, as consequence of the reaction with $O_2^{\cdot -}$ yielding NO and *trans*-$[Ru^{II}(H_2O)L_1L_2]^{(n-1)+}$ both biologically active.

4. Conclusions

Ruthenium nitrosyls complexes react with superoxide by reduction of the coordinated NO^+, yielding NO and the corresponding aqua complex as products. The apparent bimolecular rate constants found for this reaction are within the same range reported for other metal complexes described in the literature. Experimental evidence

for the peroxynitrite formation was observed only for the complex *trans*-$[Ru^{II}(NO)(NH_3)_4(P(OEt)_3)]^{3+}$, which suggest that the specific rate constant for NO release is a determinant step for $ONOO^-$ formation. As showed by the experimental data, it is likely that superoxide would be one of the possible candidates as a chemical reductant in biological media for the *trans*-$[Ru^{II}(NO)L_1L_2]^{n+}$ species. Also, both reaction products, NO and *trans*-$[Ru^{II}(H_2O)L_1L_2]^{(n-1)+}$, could be biologically active species.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.poly.2012.11.021.

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