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PAMAM dendrimers functionalized with ruthenium nitrosyl as nitric oxide carriers

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The functionalization of three generations of polyamidoamine (PAMAM G0, G2 and G3) dendrimers with the NO-donor trans-(Ru(NO)(NH\textsubscript{3})\textsubscript{4})(ina)]\textsubscript{2}(BF\textsubscript{4})\textsubscript{2} (ina = isonicotinic acid) is reported. PAMAMs were modified through a peptide-type bond between the carboxyl group of the ina ligand and the dendrimer superficial amines. Compounds were characterized by FT-IR, UV–Vis, CV, DPV, \textsuperscript{1}H NMR, ICP-OES, and the structure of the complex trans-(Ru(NO)(NH\textsubscript{3})\textsubscript{4})(ina)]\textsubscript{2}(BF\textsubscript{4})(SiF\textsubscript{6})\textsubscript{2} was determined by single crystal X-Ray analysis. The experimental data indicated the immobilization of 4, \~{~}8 and \~{~}27 nitrosyl complexes on the G0, G2 and G3 dendrimer’s surface, respectively, which corresponds to \~{~}1.0–1.43 \textmu mol NO per mg of dendrimer. FT-IR, UV–Vis and electrochemical assays suggest that the functionalization of PAMAM did not alter the coordination sphere of the ruthenium nitrosyl complex neither the formal reduction potential of Ru\textsuperscript{III}NO\textsuperscript{2+}/Ru\textsuperscript{II}NO\textsuperscript{0} couple regarding to the complex not attached to PAMAM. The NO release in these compounds, through light irradiation (\textlambda = 355 nm) and one-electron reduction (E\textsubscript{u}'), was investigated.

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1. Introduction

The free radical nitric oxide (NO\textsuperscript{-}) is an important signaling molecule\textsuperscript{[1,2]}, endogenously produced in the human body, which is associated with several biological functions [3]. The wide range of physiological and pathophysiological functions in which NO is involved, demands methods for storage and control the NO delivery at specific sites. Different compounds have been developed as effective NO donors, such as nitrosothiols, diazeniumdiolates, organic nitrates and nitrites, and also metal nitrosyl complexes\textsuperscript{[4,5]}. These compounds are helpful to better understand the biological functions of NO and some of them exhibited potential therapeutic activities\textsuperscript{[6–11]}.

Among the different NO donors, ruthenium nitrosyl complexes of the type trans-[Ru(NO)(NH\textsubscript{3})\textsubscript{4}(L)]\textsuperscript{1+}\textsuperscript{[12]} are particularly attractive due to their water solubility, low toxicity to the host cells [7], stability in aqueous solution in the presence of oxygen [13] and robustness regarding substitution reactions [14]. The NO release in these compounds can be triggered either by one-electron reduction [12] or by light irradiation [15].

Similar to other NO donors, ruthenium nitrosyl complexes have been attached to different platforms aiming diverse purposes [16,17], such as the production of materials with a high NO payload [18], the controlled NO bioavailability at specific sites [19] and the improvement of NO donors stability [20]. Silica [21,22], xerogels [23–25], liposomes [26,27], nanoparticles [28] and dendrimers [18,29,30], are common examples of matrices that have been used as NO-releasing vehicles [17,31]. Dendrimers are attractive molecules for NO transport, once they can provide a scaffold for storing large amounts of NO on a single framework [18]. They also have a well defined branching structure with a multivalent surface, which can be useful for simultaneous interactions to multiple receptors [32,33]. These molecules have been extensively studied as drug delivery systems and imaging contrast [34–36]. Among the variety of dendrimers, Polyamidoamine (PAMAM) is a noteworthy class due to its water solubility and amine or carboxyl surface, which allows tailoring through different kinds of reactions with molecules or ions of interest [37–39].

Some dendrimers have already been synthesized aiming nitric oxide transport and release [18,19,29,30,20]. For example Stasko and Schoenfisch [18] synthesized polypropyleneimine (PPI) dendrimers (G3 and G5) functionalized with N-diazeniumdiolates which were able to store \~{~}5.6 \textmu mol NO per mg of compound. Following a similar approach, Lu et al. [20] synthesized a series of amine PPI dendrimers (G2–G5) functionalized with different surface groups,
in which the NO storage capacity was in the range of 0.9–3.8 μmol NO per mg of compound [20]. Stasko and collaborators [29] also synthesized a G4 PAMAM functionalized with S-nitrosothiols which were able to store ~2 μmol NO per mg of compound. Benini and collaborators [30] functionalized PAMAM dendrimers (G0, G2 and G3) with the [Ru(eda)[NO]]– complex, which were capable of storing around 1.4–1.8 μmol NO per mg of compound.

In this context, this work reports the synthesis and characterization of the complex trans-[Ru(NO)(NH3)4(ina)]4+ (ina = isonicotinic acid) attached to PAMAM dendrimers of generations 0, 2 and 3. Some reactivity aspects of these compounds regarding NO release are also discussed. The ina ligand was chosen because it provides a carboxyl group for the attachment to PAMAM's superficial amines. Furthermore, after the amide bond formation, the structure of the complex trans-[Ru(NO)(NH3)4(ina)]4+ attached to the dendrimer becomes similar to the structure of trans-[Ru(NO)(NH3)4(isn)]4+ (isn = isonicotinamidic), which exhibited the best results among the complexes of type trans-[Ru(NO)(NH3)4(L)]n+ in tests against Chaga's disease [7,8].

2. Experimental section

2.1. Chemicals and reagents

The analytical grade reagents (Aldrich or Merck) and solvents (Malinckrodt, Baker, Merck or Panreac) were used as purchased, except PAMAM dendrimers (20 wt.% in methanol), which were dried under vacuum to remove methanol before using. Ruthenium trichloride (RuCl3.xH2O) was the starting reagent for the synthesis of all the complexes described herein. The synthesis and manipulations were performed under an argon atmosphere using standard techniques [40]. Eu2+ solution was prepared by adding Eu2O3 (99.99%) in a deaerated acid solution (0.10 mol L−1 trifluoroacetic acid) containing Zn(Hg). The reduction of Eu2+ to Eu3+ was completed after 40 min, and then the solution was used immediately. Deionized water (Millipore) was used throughout this work.

2.2. Instruments

Electronic spectra were recorded in a Hitachi U-3501 or Agilent 8453 UV–Vis spectrophotometer model using a 1.00 cm quartz cell. The solid-state infrared measurements were recorded in a Bomem MB 102 FTIR spectrophotometer using KBr pellets. 128 scans and resolution of 4 cm−1 in the 4000–400 cm−1 range. The FTIR measurements for PAMAM (oil) were performed in a silicon window using the same conditions described before for the assays using KBr pellets. The elemental analyses were performed on a Perkin–Elmer CHN 2400. Ruthenium analysis was carried out using a Perkin Elmer Optima 3000 DV Induced Coupled- Plasma Optical Emission Spectrometer (ICP-OES). Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) experiments were performed with a PAR model 264A Potentiostat. The three-electrode system, saturated calomel, glassy carbon and platinum wire, was used as reference, work and auxiliary electrodes respectively. The potential values were converted and reported as normal hydrogen electrode (NHE). 1H NMR spectra were recorded in a Bruker DRX 400 spectrometer using a trifluoroacetic acid-d solution (CF3COOD, 1 mol L−1) in D2O. 3-(Trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt was used as internal reference. Electron paramagnetic resonance (EPR) spectra were obtained in a Bruker EMX Plus X-band spectrometer at room or liquid nitrogen temperature. Samples were irradiated with a Nd:YAG LASER (Continuum, model Surelite-II) operating in the third harmonic (λ = 355 nm). A power meter (Coherent, Lasermate-P) measured the average energy per pulse.

2.3. Measurements

All experiments were carried out at 25 ± 1 °C in a phosphate buffer pH 7.4 (0.10 mol L−1), μ = 0.20 mol L−1, or in aqueous solution pH 2.0, μ = 0.10 mol L−1 (CF3COOH/CF3COONa). All manipulations were performed in the absence of oxygen. The inert gas (argon with high purity 99.998%) was deoxygenated by passing through a Cr(II) solution prior to use [40]. The nitrogen gas (99.999% of purity) was used without further purification. The complexes were stored under vacuum and protected from light and moisture. For ruthenium analysis, a calibration curve was prepared using a commercial standard ruthenium solution (1000 mg/L of Ru in HNO3 2% water solution). The samples were prepared dissolving the ruthenium complexes in a trifluoroacetic acid solution (1.0 × 10−3 mol L−1) and aliquots were taken for the analysis (ICP-OES). NMR, EPR and UV–Vis spectra of the solutions containing air-sensitive complexes were obtained under argon atmosphere. Solutions were transferred through Teflon tubing to specific cell or tube using the inert gas pressure.

2.4. Synthesis of trans-[Ru(NO)(NH3)4(ina)](BF4)3

The complexes trans-[Ru(NH3)4(ina)](SO4)Cl [41] and trans-[Ru(NO)(NH3)4(ina)](BF4)3 were synthesized and characterized as described in the literature [13,14]. Theoretical elemental analysis for trans-[Ru(NH3)4(ina)](SO4)Cl·2H2O: C, 15.67; H, 4.60; N, 15.23. Experimental elemental analysis: C, 15.50; H, 4.68; N, 14.92. Yield: 65–70%. Theoretical elemental analysis for trans-[Ru(NO)(NH3)4(ina)](BF4)3·2H2O: C, 11.65; H, 3.42; N, 13.58. Experimental elemental analysis: C, 11.51; H, 3.52; N, 13.43. Yield: 53–60%.

2.5. X-ray data collection and structure determination

Crystals of trans-[Ru(NO)(NH3)4(ina)](BF4)2SiF6·H2O were obtained from an aqueous solution (4.0 mol L−1 of HBF4) and trans-[Ru(NO)(NH3)4(ina)]1+ maintained at ~10 °C for one week. The presence of SiF62− as counterion in the crystals was originated from the reagent HBF4 that contains SiF62− as impurity (~0.2%), as previously reported [13]. The presence of SiF62− as counterion in the crystals was also identified by FT-IR measurements through the bands at 740 and 480 cm−1 [42], which are absent in the amorphous solid trans-[Ru(NO)(NH3)4(ina)](BF4)2·2H2O. The band at 1080 cm−1, assigned to BF3 anion [42], was also observed in the FT-IR for the complex trans-[Ru(NO)(NH3)4(ina)](BF4)2SiF6·H2O. It is important to emphasize that the amorphous solid of trans-[Ru(NO)(NH3)4(ina)]1+ has three BF3 as counterions, as confirmed by the FT-IR measurements and elemental analysis. Theoretical elemental analysis for trans-[Ru(NO)(NH3)4(ina)](BF4)2SiF6·H2O: C, 12.66; H, 3.36; N, 14.76. Experimental elemental analysis: C, 12.41; H, 3.53; N, 14.56.

2.5.1. Crystal data

A yellow crystal of dimensions 0.163 × 0.036 × 0.026 mm3 was selected and mounted on an Enraf–Nonius Kappa-CCD diffractometer with graphite monochromated Mo Kα (λ = 0.71073 Å) radiation. Data were collected at room temperature up to 52° in 2θ and final unit cell parameters were based on all reflections.

2.5.2. Data collection and processing

Data collections were made using the collex program [43]; integration and scaling of the reflections were performed with the HKL Denzo-Scalepack system of programs [44]. Absorption corrections were carried out using the Gaussian method [45]. The structure was solved by direct methods with SHELXS-97 [46]. The model was refined by full-matrix least squares on F2 by means of SHELXL-97 [47]. All hydrogen atoms were stereochemically positioned and
refined with the ridging model except for the ones of the water molecule which were found from a difference map and refined with the O–H distance restrained to 0.82(2) Å. Fig. 1 was prepared using ORTEP-3 for Windows [48]. Listing of atomic coordinates and equivalent isotropic displacement parameters, full intramolecular bond distances and angles, hydrogen coordinates, and anisotropic thermal parameters are available from the authors and were deposited at the Cambridge Crystallographic Data Centre, reference numbers CCDC 929821. These data are also shown in Supplementary material (Tables 1S–3S).

2.6. Functionalization of PAMAM dendrimers with trans-[Ru(NO)(NH3)4(ina)](BF4)3

Functionalization of PAMAM (G0, G2 and G3) with trans-[Ru(NO)(NH3)4(ina)](BF4)3 was performed via an amide bond formation using Yamaguchi reagent (2,4,6-trichlorobenzoyl chloride) [49–51]. Similar procedures were applied for the three PAMAM generations, as described below.

Under absence of air and moisture, a solution containing trans-[Ru(NO)(NH3)4(ina)](BF4)3 (75 mg, 0.121 mmol) in dry DMF (4 mL) was treated with triethylamine (Et3N) (0.133 mmol, 20 µL) and 2,4,6-trichlorobenzyl chloride (0.133 mmol, 22 µL). The resulting suspension was stirred at room temperature (25 °C) for ~24 h and then, 4-Dimethylaminopyridine (DMAP, 0.242 mmol) and PA-MAM (0.080 mmol of superficial NH2) dissolved in dry and degassed DMF (~5 mL) were added in sequence. The mixture was stirred at room temperature (25 °C) during 96 h for G0, 120 h for G2 and 168 h for G3. The resulting suspension was filtered and the solid was washed with DMF and dried under vacuum. The obtained products are de-

ethenol and dried under vacuum. The obtained products are described throughout this text for simplicity as Gx/RuNO (where x = 0, 2 or 3, and represents PAMAM generations). Ruthenium nitrosyl attached to PAMAM were hygroscopic, and this property increased with the increasing of the dendrimers generation. Yield = 40–45%.

2.7. Nitric oxide release

Nitric oxide release from ruthenium nitrosyl complexes (attached or not to PAMAM) was performed in solution through light irradiation and by one-electron reduction [12–15].

In the photochemical experiments, samples were irradiated (λirr = 355 nm) with a Nd:YAG Laser, 10 ns pulse width, attenuated to approximately 2 mJ/pulse, in a 1.00 cm path length quartz cells capped with a rubber septum and under constant argon flow. The progress of the photoreaction, in aqueous solutions pH 2.0 (µ = 0.10 mol L−1 CF3COOH/CF3COONa; CRu = ~9.0 × 10−5 mol L−1), was monitored spectrophotometrically (UV–Vis). Also, RuIII, one of the photoproducts (Eq. (1)) [12,15], was detected through EPR spectroscopy after the light irradiations (tirr = 30 min) of aqueous solution of ruthenium nitrosyl complex (Cru ~ 2.0 × 10−3 mol L−1, pH 2.0, µ = 0.10 mol L−1 CF3COOH/CF3COONa).

trans-[RuII(NO)(NH3)4(L)]3+ × bv H2O trans-[RuIII(NH3)4(H2O)(L)]3+ + NO

(1)

The detection of the nitric oxide released from ruthenium nitrosyl complexes after light irradiation was performed through EPR spectroscopy, using the NO spin trap 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) [52,53]. Thus, a phosphate buffer solution (pH 7.4, 0.10 mol L−1, µ = 0.20 mol L−1) containing ruthenium complexes (CRu = 7.5 × 10−4 mol L−1) and PTIO (CPTIO = 7.5 × 10−6 mol L−1) was irradiated for 5 min. A solution containing only PTIO was irradiated for 10 min, in the same conditions described above and was used as control.

Fig. 1. ORTEP-3 structure representation for trans-[Ru(NO)(NH3)4(ina)](BF4)3(SiF6)2H2O with displacement ellipsoids displayed at 30% probability.
For the NO release from chemical reduction, trans-[Ru(NO)(NH₃)₄(ina)][BF₄]₂·H₂O (attached or not to PAMAM) was solubilized in deaerated aqueous solution pH 2.0, μ = 0.10 mol L⁻¹ (CF₃COOH/CF₃COONa) and then an excess of Eu²⁺ (4 equivalents) was added [13,14]. Differential pulse voltammetry (DPV) and electronic spectroscopy were used to help the detection and characterization of the produced species.

3. Results and discussion

3.1. Synthesis

The crystallographic data for trans-[Ru(NO)(NH₃)₄(ina)][BF₄]₂·H₂O are listed in the Table 1, and the selected bond lengths and angles are shown in Table 2. Four NH₃ ligands occupy the equatorial positions and, ina and NO⁺ ligands are in trans position in the axial direction (Fig. 1). The mean of the Ru–NH₃ distance is 2.113 ± 0.009 Å and the Ru–NO distance is 1.741(4) Å. These values are similar to those found for other nitrosylamminerruthenium complexes such as [Ru(NH₃)₅(NO)][Cl] [54] and trans-[Ru(NO)(NH₃)₄(L)]|X|ₙ (where L = N-heterocyclic and X = PF₆⁻, Cl⁻ or BF₄⁻) [12,13]. The Ru–N–O bond angle was 176.8(4)°, which is compatible with a nitrosyl coordinated structure [12] for the NO ligand. The ina ligand has two values for the C–O distances in the carboxyl group, 1.323(6) and 1.196(6) Å, which suggests that one of the oxygen atoms in this product is protonated, and therefore the ligand is the isonicotinic acid and not the corresponding anion.

PAMAM dendrimers of generations 0, 2 and 3 have, respectively, 4, 16 and 32 superficial primary amines. Ruthenium complexes were immobilized onto PAMAM via the attachment of dendrimer terminal amines to the carboxyl group of the complex trans-[Ru(NO)(NH₃)₄(ina)][BF₄]₂ through an amide bond formation (Fig. 2) [49–51]. Size exclusion chromatography (Sephadex G-25) was used to isolate the desired product (Gx/RuNO) and the second, to the excess of trans-[Ru(NO)(NH₃)₄(ina)][BF₄]₂·H₂O.

Two other amidation protocols employing mixed anhydride (treatment with ethyl chloroformate and triethylamine) [50], and another using condensation reagents such as DCC/DMAP (Steglich conditions) failed to give pure products in reasonable yields (less than 10%).

Another synthetic route was tried out without success through the attachment of the isonicotinic acid to dendrimers [49–51] (Fig. 1S, Supplementary Material). Then, this product was submitted to the reaction with trans-[Ru(NH₃)₅(SO₂Cl)₂(Cl)]Cl, following the sulfite/sulfate route [12–14]. The problem with this route was the oxidation step of the metal center (Ru²⁺ to Ru³⁺) and of the ligand (SO₂ to SO₄²⁻) using hydrogen peroxide [12–14,55]. This oxidation step needs to be carried out before the nitric oxide coordination to ruthenium, but it unfortunately compromised the dendrimer structure.

3.2. Spectroscopy

The nitrosyl complexes (attached or not to PAMAM) are EPR silent, indicating that no Ru²⁺ is formed on the anchoring of the complex to dendrimers.

As expected, the 1H NMR spectra of Gx/RuNO (x = 0, 2 and 3) showed similarities to the 1H NMR spectra of the synthesis precursors: Fig. 3 for G0/RuNO, and Figs. 2S and 3S (Supplementary material) for G2/RuNO and G3/RuNO, respectively. As can be noticed comparing the 1H NMR spectra of PAMAM G0 and of G0/RuNO in Fig. 3, the signals with chemical shift (δ) between 2.7 and 4.0 ppm are related to the dendrimer’s moiety of the G0/RuNO product. This is an indication of the carboxyl attachment (of the ina ligand) to the terminal PAMAM amines. The only significant differences between the 1H NMR spectra of PAMAM G0, G2 and G3 and Gx/RuNO (x = 0, 2 and 3) is the presence of two doublets, centered at δ = 8.36 and 8.75 ppm for the Gx/RuNO species. These signals are also presented in the 1H NMR spectrum of trans-[Ru(NO)(NH₃)₄(ina)][BF₄]₂·H₂O, with δ = 8.36 and 8.75 ppm (Fig. 45, Supplementary material), and their presence is consistent with the coordination of the N-heterocyclic ligand to the metal center through the pyridine-type nitrogen [13,14].

An attempt to estimate the number of PAMAM’s superficial amines functionalized with the ruthenium complex was carried out through the integration of the hydrogen signals in the 1H NMR spectra (Fig. 2 and Figs. 2S and 3S; Tables 4S and 5S, Supplementary material). The integrations were performed using the area of the characteristic aromatic hydrogens peaks of the ina ligand (which corresponds to four H for each ina ligand, Fig. 2) and the area of the aliphatic hydrogens of the PAMAM dendrimers (Fig. 2). These results are summarized in Table 3.

An alternative method to estimate the number of ruthenium nitrosyl attached to PAMAM was performed by analyzing the ruthenium content through ICP-OES (Table 3). The ratio between the theoretical (Gx fully functionalized) and the experimental concentration of ruthenium for a known mass of Gx/RuNO was used as an indication of the degree of the trans-[Ru(NO)(NH₃)₄(ina)][BF₄]₂·H₂O incorporation on the dendrimer.

According to the 1H NMR and ICP-OES data, PAMAM G0 was fully functionalized with trans-[Ru(NO)(NH₃)₄(ina)][BF₄]₂·H₂O, and G2 and G3 were functionalized with ~8 and ~27 ruthenium nitrosyls, respectively. Thus, according to these results, the compounds G0/RuNO, G2/RuNO and G3/RuNO described in this work were able to storage ~1.43, ~1.03 and ~1.28 μmol NO per mg of compound, respectively. Analogous results were obtained by Benini and collaborators [30], in which PAMAM G0, G2 and G3 were

**Table 1**

Crystallographic data for trans-[Ru(NO)(NH₃)₄(ina)][BF₄]₂·H₂O.

<table>
<thead>
<tr>
<th>Formula</th>
<th>C₁₁₉₁₂B₁₄F₁₄N₁₉O₁₉Ru₁</th>
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<tbody>
<tr>
<td>Space group</td>
<td>P2₁/n</td>
</tr>
<tr>
<td>α (Å)</td>
<td>7.9618(2)</td>
</tr>
<tr>
<td>β (Å)</td>
<td>24.3982(8)</td>
</tr>
<tr>
<td>γ (Å)</td>
<td>9.9705(3)</td>
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<tr>
<td>α (°)</td>
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</tr>
<tr>
<td>β (°)</td>
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</tr>
<tr>
<td>γ (°)</td>
<td>90</td>
</tr>
<tr>
<td>V (Å³)</td>
<td>1914.06(10)</td>
</tr>
<tr>
<td>Z</td>
<td>4</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
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</tr>
<tr>
<td>μ (Mg m⁻¹)</td>
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</tr>
<tr>
<td>R₁ [F &gt; 2σ(F)]</td>
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</tr>
<tr>
<td>wR₁</td>
<td>0.0626</td>
</tr>
<tr>
<td>wR₁ (all data)</td>
<td>0.1246</td>
</tr>
</tbody>
</table>

**Table 2**

Selected bond lengths and bond angles for trans-[Ru(NO)(NH₃)₄(ina)][BF₄]₂·H₂O.

<table>
<thead>
<tr>
<th>Selected bond lengths (Å)</th>
<th>Selected bond angles (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ru–N</td>
<td>1.741(4)</td>
</tr>
<tr>
<td>Ru–O</td>
<td>1.132(5)</td>
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<tr>
<td>Ru–N(1)</td>
<td>2.124(3)</td>
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<tr>
<td>Ru–N(2)</td>
<td>2.100(4)</td>
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<td>Ru–N(3)</td>
<td>2.116(4)</td>
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<td>Ru–N(4)</td>
<td>2.119(4)</td>
</tr>
<tr>
<td>Ru–N(5)</td>
<td>2.118(4)</td>
</tr>
<tr>
<td>O(11)–C(1)</td>
<td>1.323(6)</td>
</tr>
<tr>
<td>O(12)–C(1)</td>
<td>1.196(6)</td>
</tr>
<tr>
<td>O(12)–C(1)–O(11)</td>
<td>124.0(4)</td>
</tr>
<tr>
<td>O(12)–C(1)–O(11)</td>
<td>124.0(4)</td>
</tr>
</tbody>
</table>
functionalized, respectively, with ~4, ~12 and ~29 [Ru<sup>III</sup>(edta)(H<sub>2</sub>O)]<sup>2+</sup> complexes.

The electronic spectrum of trans-[Ru(NO)(NH<sub>3</sub>)<sub>4</sub>(ina)]<sup>2+</sup> showed intense bands at 232 nm (ε = 11.2 ± 0.3 × 10<sup>3</sup> M<sup>-1</sup> cm<sup>-1</sup>) and at 275 nm (ε = 3.9 ± 0.2 × 10<sup>3</sup> M<sup>-1</sup> cm<sup>-1</sup>), assigned as internal ligand transitions (IL) of ina; one band at 335 nm (ε = 221.9 ± 7.3 M<sup>-1</sup> cm<sup>-1</sup>), assigned to Ru d–d transitions, with contributions from a MLCT transition (dnRu(II) → π*(NO)), and a weaker band at 466 nm (ε = 23.9 ± 6.9 M<sup>-1</sup> cm<sup>-1</sup>) assigned to dnRu(II) → π(NO) and π(ina) → π*(NO) transitions. These above assignments were tentatively carried out by analogy to previously reported data for other ruthenium tetraamine nitrosyls [12–14]. The UV–Vis spectra of Gx/RuNO (x = 0, 2 and 3) exhibited the absorption maxima governed by the ruthenium nitrosyl moiety (Fig. 5S, Supplementary material). These functionalized dendrimers (Gx/RuNO) showed bands at 232, 275, 335 and 466 nm, and the assignments for these bands were similar to the ones described above for trans-[Ru(NO)(NH<sub>3</sub>)<sub>4</sub>(ina)]<sup>2+</sup>. PAMAM G0, G2 and G3 have a band at λ<sub>max</sub> = 278 nm (ε = 130–150 M<sup>-1</sup> cm<sup>-1</sup>). This low intensity band, in comparison to that observed for trans-[Ru(NO)(NH<sub>3</sub>)<sub>4</sub>(ina)]<sup>2+</sup> in the same region (275 nm, ε = 3.9 ± 0.2 × 10<sup>3</sup> M<sup>-1</sup> cm<sup>-1</sup>), confirms the small contribution of the dendrimer moiety for the absorption maxima in the UV–Vis spectra of Gx/RuNO products. Similar results were found by Stasko and collaborators [29], which functionalized two PAMAM G4 dendrimers with S-nitrosothiol (N-acetyl-D,L-penicillamine or N-acetyl-L-cysteine), and also by Benini et al. [30].

According to the discussed above, using the ε values for λ = 232 and 275 nm for trans-[Ru(NO)(NH<sub>3</sub>)<sub>4</sub>(ina)][BF<sub>4</sub>]<sub>3</sub>, would be possible to estimate the number of the PAMAM amines functionalized with the ruthenium nitrosyl complex [30,56]. Thus, assuming that the ε values for the G0 functionalized with four trans-[Ru(NO)(NH<sub>3</sub>)<sub>4</sub>(ina)][BF<sub>4</sub>]<sub>3</sub>, would be in principle approximately four times of that observed for the free complex [30], the number of functionalized amines was estimated and these results are shown in Table 6S (Supplementary material). Using the same approach was possible to calculate ~8 and ~27 trans-[Ru(NO)(NH<sub>3</sub>)<sub>4</sub>(ina)][BF<sub>4</sub>]<sub>3</sub> attached to PAMAM G2 and G3, respectively. These results are consistent with the ones calculated from NMR and ICP-OES data (Table 3). Nevertheless, the approach using only UV–Vis is not conclusive and must be performed together with other techniques (as 1H NMR and ruthenium analysis) in order to obtain a more reliable value.

The formal reduction potentials of the Ru<sup>III</sup>NO<sup>2+</sup>/Ru<sup>II</sup>NO<sup>0</sup> couple (E<sub>NO<sup>2+</sup>-NO</sub>) for trans-[Ru(NO)(NH<sub>3</sub>)<sub>4</sub>(ina)]<sup>2+</sup> and for Gx/RuNO (x = 0, 2 and 3) in solution, were similar, ~60 mV versus NHE (Table 4). Analogous results were obtained for other ruthenium nitrosyl immobilized on PAMAM dendrimers [30] and in other matrices [17]. It is interesting to recall that these potentials are in the accessible range for biological reducing agents [7,57], which potentially enables the title compounds as candidates for therapy use.

The ν(NO<sup>-</sup>) frequencies for the complex trans-[Ru(NO)(NH<sub>3</sub>)<sub>4</sub>(ina)][BF<sub>4</sub>]<sub>3</sub> (attached or not to PAMAM) are also presented in Table 4. All the ruthenium compounds described in this work showed a medium to strong intensity band in the 1930–1933 cm<sup>-1</sup> range ascribed to ν(NO<sup>-</sup>) (Figs. 6S–8S, Supplementary material), which is compatible with a nitratosium (NO<sup>-</sup>) character of the NO ligand (as previously discussed in the crystallographic results in the Section 3.1) [12]. Comparing the FT-IR spectra of the products (Gx/RuNO) with the starting compounds is also possible to notice the appearance of a medium intensity band in the region of 1640–1680 cm<sup>-1</sup> for Gx/RuNO (x = 0, 2 and 3), assigned to the amide I (νC=O) [30,42,58], which is present on PAMAM (G0, G2 and G3) but is absent in the complex trans-[Ru(NO)(NH<sub>3</sub>)<sub>4</sub>(ina)][BF<sub>4</sub>]<sub>3</sub> (Figs. 6S–8S, Supplementary material). This is an indicative of the amide bond formed between the ina ligand and the dendrimer. Other differences observed in the FT-IR spectra of Gx/
RuNO in relation to that of trans-[Ru(NO)(NH₃)₄(ina)](BF₄)₃ are the appearance of strong broad bands around 3070–3080 cm⁻¹ (ν(NH)) and 2940–2960 cm⁻¹  (ν(NH) and ν(CH)) [42] for Gₓ/RuNO, which are present in PAMAM (G0, G2 and G3) dendrimers [58] in the same region (Figs. 6S–8S, Supplementary material) and are absent in the FT-IR spectrum of trans-[Ru(NO)(NH₃)₄(ina)](BF₄)₃.

Thus, the electrochemical (DPV and CV) and spectroscopic (UV–Vis and FT-IR) results suggest that the functionalization of dendrimers with trans-[Ru(NO)(NH₃)₄(ina)](BF₄)₃ did not significantly alter the spectroscopic and electrochemical characteristics of the ruthenium complex.

3.3. Photochemical NO release

NO release from ruthenium tetraamine nitrosyl (Eq. (1)) can be achieved by irradiation of these complexes in solution with light.
in the range of 300–370 nm [15]. Thus, solutions containing trans-
[Ru(NO)(NH₃)₄(ina)]³⁺ (attached or not to PAMAM G2) were irradiated
at 355 nm in phosphate buffer (pH 7.4) in the presence of
PTIO. This last compound is a well known nitric oxide spin trap
[52,53]. PTIO reacts with NO yielding PTI, as illustrated in Fig. 4.
Both PTIO and PTI are paramagnetic species, and therefore can be
detected and distinguished through EPR spectroscopy [52,53].
Thus, performing the photochemical reaction for trans-
[Ru(NO)(NH₃)₄(ina)]³⁺ and for G2/RuNO in the presence of PTIO,
was possible to detect the NO release from these compounds
due to the observation of the signals related to the PTI species
(Fig. 5) [52,53]. The values obtained for the hyperfine splitting con-
fant (Fig. 5) were: \( a_N^1 = 0.82 \) mT for PTIO; \( a_N^1 = 0.98 \) mT and \( a_N^2 = 0.44 \) mT for PTI, which are consistent with the ones reported
previously [52].

This photochemical reaction was also performed for trans-
[Ru(NO)(NH₃)₄(ina)]³⁺ and for G3/RuNO, in the absence of PTIO,
in aqueous solution (pH 2.0, \( \mu = 0.10 \) mol L⁻¹ CF₃COOH/CF₃-
COONa), and it was monitored through UV–Vis spectroscopy
(Fig. 9S, Supplementary material). It was possible to observe an
increase at 275 nm and at 300–350 nm. Also an isosbestic point
(Fig. 9S, Supplementary material). It was possible to observe an
development of the signals related to the PTI species
(Fig. 5) [52,53]. The values obtained for the hyperfine splitting con-
fant (Fig. 5) were: \( a_N^1 = 0.82 \) mT for PTIO; \( a_N^1 = 0.98 \) mT and \( a_N^2 = 0.44 \) mT for PTI, which are consistent with the ones reported
previously [52].

This photochemical reaction was also performed for trans-
[Ru(NO)(NH₃)₄(ina)]³⁺ and for G3/RuNO, in the absence of PTIO,
in aqueous solution (pH 2.0, \( \mu = 0.10 \) mol L⁻¹ CF₃COOH/CF₃-
COONa), and it was monitored through UV–Vis spectroscopy
(Fig. 9S, Supplementary material). It was possible to observe an
absorbance decrease at \(-230 \) nm concomitant with an absorbance
increase at 275 nm and at 300–350 nm. Also an isosbestic point
was observed at 249 nm. This photochemical behavior for the
nitrosyl complex (attached or not to PAMAM) was similar to that
previously described for other ruthenium tetraammine nitrosyls
in solution [15] or immobilized in different matrices [17,22].
The formation of the trans-[Ru⁶⁺(NH₃)₄(H₂O)₄(ina)]³⁺ photoprod-
t (solution [15] or immobilized in different matrices [17,22].
The formation of the trans-[Ru⁶⁺(NH₃)₄(H₂O)₄(ina)]³⁺ photoprod-
uct [15]) (pH 2.0, \( \mu = 0.10 \) mol L⁻¹ CF₃COOH/CF₃-
COONa) was confirmed through EPR spectroscopy (Fig. 6) [12,14,15].

The g-factor for trans-[Ru⁶⁺(NH₃)₄(H₂O)₄(ina)]³⁺ (not attached to
PAMAM) is \( g = 2.543 \), and for trans-[Ru⁶⁺(NH₃)₄(H₂O)₄(ina)]³⁺
attached to PAMAM G3 are \( g_L = 2.481 \) and \( g_0 = 2.675 \). The other g-
factor for trans-[Ru⁶⁺(NH₃)₄(H₂O)₄(ina)]³⁺ (not attached to
PAMAM) was not observed and this difficulty was previously reported
and thoroughly discussed elsewhere [55,59]. Also, when comparing
the EPR spectra for trans-[Ru⁶⁺(NH₃)₄(H₂O)₄(ina)]³⁺ attached or not
to PAMAM (Fig. 6), a shoulder could be observed in the G3/RuNO
spectrum. This result was tentatively attributed to matrix effects [17].

Furthermore, as far as the compounds described in this work
are concerned, the photochemical NO release activation pathway
requires light excitation in a non favorable region of the spectra
(\( \lambda < 400 \) nm). Thus some coordination sphere tailoring is necessary

![Fig. 4. PTIO conversion into PTI through the reaction with NO [53, 54].](image)

### Table 4

<table>
<thead>
<tr>
<th>Compound</th>
<th>( E_{\text{red}}(^{\text{NO}}/^{\text{NO}^-})^a ) (mV vs. NHE)</th>
<th>( \nu(^{\text{NO}})^{-1}b )</th>
</tr>
</thead>
<tbody>
<tr>
<td>trans- [Ru(NO)(NH₃)₄(ina)]¹⁺</td>
<td>59</td>
<td>1933</td>
</tr>
<tr>
<td>G0/RuNO</td>
<td>57</td>
<td>1931</td>
</tr>
<tr>
<td>G2/RuNO</td>
<td>58</td>
<td>1939</td>
</tr>
<tr>
<td>G3/RuNO</td>
<td>59</td>
<td>1931</td>
</tr>
</tbody>
</table>

| \( a \) Potential of the cathodic peak obtained by differential pulse voltammetry, pH 2.0, \( \mu = 0.10 \) M (CF₃COOH/CF₃-
COONa), \( T = 25 \pm 1 \) °C; scan rate = 5 mV/s, pulse height = 50 mV.
| \( b \) KBr pellet, resolution of 4 cm⁻¹.

\[ \text{Fig. 5. } \text{EPR detection of NO release from the ruthenium nitrosyl upon photochemical reaction. EPR spectra of irradiated solutions containing: (A) only PTIO (control), irradiated for 10 min; (B) PTIO + trans-[Ru(NO)(NH₃)₄(ina)]³⁺ and (C) PTIO + G2/RuNO, both irradiated for 5 min. (phosphate buffer solutions pH 7.4; \( C_{\text{NO}^-} = 7.5 \times 10^{-4} \) mol L⁻¹; \( C_{\text{PTIO/PTI}} = 7.5 \times 10^{-4} \) mol L⁻¹; \( \lambda_{\text{irr}} = 355 \) nm, \( \sim 2 \) mJ/pulse; \( T = 25 \pm 1 \) °C).} \]

3.4. NO release from chemical reduction

It is well established that the NO dissociation from trans-
[Ru(NO)(NH₃)₄(L)]³⁺ complexes can be triggered by the reaction with Eu³⁺ ions [12–14], as shown in Eqs. (2) and (3).

\[
\text{trans-[Ru}^\text{II}(\text{NO})^\text{−}]_4(\text{L})^3^+ + \text{Eu}^3^+ \rightarrow \text{trans-[Ru}^\text{II}(\text{NO})^\text{−}]_4(\text{L})^2^+ + \text{Eu}^2^+ \tag{2}
\]

\[
\text{trans-[Ru}^\text{II}(\text{NO})^\text{−}]_4(\text{L})^2^+ + \text{H}_2\text{O} \rightarrow \text{trans-[Ru}^\text{II}(\text{NO})^\text{−}]_4(\text{H}_2\text{O})(\text{L})^2^+ + \text{NO} \tag{3}
\]

Thus, the NO dissociation from ruthenium nitrosyl attached to
PAMAM was similarly investigated through the reaction with
Eu²⁺ ions. As observed in Fig. 7, the DPV of a solution containing
G3/RuNO showed a cathodic peak (E_{\text{cp1}}) at \( \sim 0.060 \) V versus NHE,
ascribed to the RuNO²⁻/RuNO³⁻ process. After the reaction of G3/
RuNO with Eu²⁺, the process described above disappeared, whereas
a new one was observed with E_{\text{ap1}} \sim 0.295 V versus NHE, which
was assigned to the [RuH₂O]²⁺/[RuH₂O]³⁻ couple in trans-
[Ru(NO)(NH₃)₄(H₂O)(O)]²⁺ (Fig. 7). Also, an electrochemical anodic
wave with E_{\text{ap2}} \sim 0.753 V versus NHE can be observed. This last
electrochemical process is due to the oxidation of free NO in solution
[14,60], confirming that the NO was released from G3/RuNO after the reaction with Eu²⁺.

The reaction between Gx/RuNO and Eu²⁺ was also investigated spectrophotometrically, as shown in the Fig. 8 for G3/RuNO. After the addition of Eu²⁺ to the solution containing G3/RuNO, a new band at 492 nm appeared, consistent with the trans-[Ru(NO)(NH₃)₄(H₂O)(ina)]²⁺ formation, and which was assigned to a metal to
ligand charge transfer (MLCT) dπRu(II) \( \rightarrow \pi^*(\text{ina}) [12–14].

Therefore, as suggested by DPV and electronic spectroscopy
data, the ions trans-[Ru(NO)(NH₃)₄(ina)]³⁺ attached to PAMAM,
after reduction, exhibited nitric oxide dissociation and the respective
formation of the aqua species trans-[Ru(NH₃)₄(H₂O)(O)]²⁺.
Toledo et al. [57] found a correlation between the rate constant for NO dissociation ($k_{\text{NO}}/C_0$), represented in Eq.(3), and the sum of the electrochemical parameters ($P_{\text{EL}}$), introduced by Lever [61], as shown in Eq.(4): $\frac{k_{\text{NO}}/C_0}{0.81 \times \sum E_i} + 0.48 \text{ (with } R = 0.997) \quad (4)$

Using this correlation (with $\sum E_i = 0.57$) [57,61], it was possible to estimate the rate constant for the NO dissociation from trans-[Ru(NO)(NH$_3$)$_4$(ina)]$^{2+}$ as $k_{\text{NO}} \sim 0.0183 \text{ s}^{-1}$, with $t_{1/2} \sim 38 \text{ s}$. Since no drastic changes were observed between the ruthenium nitrosyl properties in solution or attached to dendrimers [30] it is likely that the $k_{\text{NO}}$ for Gx/RuNO would be around $0.02 \text{ s}^{-1}$ [30].

The NO release mechanism for the NO-releasing dendrimers described in the literature [18–20,29,30,62] is diversified. Dendrimers functionalized with N-diazeneimdiolates [18–20] release NO spontaneously in physiological conditions with a $t_{1/2}$ for NO release varying from 1.4–293 min, depending on the dendrimers surface modifications [18,20,62]. Dendrimers functionalized with S-nitrosothiols [29] release NO by two main pathways: photo-initiated decomposition, with $t_{1/2}$ in the range of 34–200 min, and transition metal-mediated catalytic decomposition (based on Cu$^+/Cu^{2+}$ redox couple) with $t_{1/2}$ in the range of 1.5–106 min [29]. PAMAM functionalized with [Ru(edta)(NO)]$^{-} \quad$ [30] release NO in an analogous way to that observed for the compounds described in this work (by chemical reduction) with $t_{1/2}$ in the range of 4–8 min.

Since the NO liberation from ruthenium nitrosyl compounds can be triggered on a controlled way by photochemical and thermal activation (reduction), these compounds offer the possibility of designing and exploring them as new platforms for NO transport.

Therefore, when dealing with hypoxic conditions, as in the infections like Chaga’s Disease and Leishmaniosis, or even in solid tumors, the environment might provide the necessary reducers for the Ru$^{5+}$/Ru$^{3+}$ reduction in the nitrosyl compounds. Thus, Gx/RuNO could be good candidates to be used in these therapies.
Indeed, some preliminary experiments (in vitro) have been carried out with the compounds Gx/RuNO against Trypanosoma cruzi (Y strain). Results collected to date indicate that the trypanocidal activity of G0/RuNO and G2/RuNO (C60 = 200 × 10⁻⁶ mol L⁻¹) were approximately 82% and 88%, respectively. These compounds exhibited a trypanocidal activity slightly higher than that observed for the complex trans-[Ru(NO)(NH₃)₄(CN)₂]BF₄ (72% of trypanocidal activity for C60 = 200 × 10⁻⁶ mol L⁻¹). The in vivo experiments, in which these differences might be more evident, are in course and these results will be reported later on.

4. Conclusion

The functionalization of PAMAM dendrimers with trans-[Ru(NO)(NH₃)₄(CN)₂]⁺ complexes was performed in one-step synthesis. As far as the data collected are concerned, no significant changes in the chemical and photochemical properties of the ruthenium nitrosyl complex occurs with its attachment to the dendrimers. The Gx/RuNO compounds are robust and able to store a high payload of nitric oxide, which can be released on a controlled way when triggered by light irradiation or by chemical reduction.

Acknowledgments

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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.jica.2013.07.009.

References