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Rapid generation of HNO induced by visible light†

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We present a new method for controlled generation of HNO, based on the combination of a pH photoactuator induced by visible light with an HNO donor activated by pH increase. This method avoids the use of UV light, and in the future could be extended by using an IR photoactuator.

HNO (azanone, commonly referred as nitroxyl) is a highly reactive compound that has gained considerable attention due to its biological significance.[1] It is thought to be an intermediate in biochemical pathways and it has various pharmacological effects, including cardioprotective action.[2] Moreover, it has been recently shown that it can be produced in biological systems by nitric oxide reduction in the presence of hydrogen sulfide.[3] Natural phenols such as the amino acid tyrosine and vitamins C and E can also produce HNO from NO.[4] It shows high reactivity towards biological targets (specially thiols and heme-proteins) and with itself ($2HNO \rightarrow H_2O + N_2O$; $k_{dim} = 8 \times 10^6\ M^{-1}\ s^{-1}$).[5] For this reason, when required, HNO must be generated *in situ* from appropriate donors, and the synthesis of these compounds is an important research area in this field. Donor molecules that release HNO under physiological conditions, especially those that allow precisely controllable release, are desirable for studying the activity of HNO at the cellular level.

Several HNO donor compounds have been developed since the first one reported in 1896, $Na_2N_2O_3$, Angeli's salt, which is still widely used as an HNO source for biological experiments.[1,6] Another family of compounds routinely used for the generation of HNO is related to Piloty's acid (PA) and its derivatives, which release HNO *via* heterolysis once the sulfohydroxamic moiety is deprotonated.[1,7] Piloty's acid (without the nitro substituent) has been previously used as an inhibitor of yeast aldehyde dehydrogenase and as a relaxator of precontracted rabbit aortic rings.[8] The pH at which the HNO donation becomes spontaneous will depend on the derivative's pK_a.

A weak point of these spontaneous donors is that they do not permit site-specific or temporally controlled release, which is required for investigating the role of HNO as a signalling molecule. The controlled release of many small bioactive molecules is achieved with chemical donors that are activated by light irradiation.[9] Many light induced HNO donors were prepared and studied in the last decade.[10] The main drawback of these photoactive donors is the need of ultraviolet light (instead of visible or IR light sources), which is harmful for biological systems, shows more scattering (Tyndall effect) and less transmittance on biological tissues, and also presents the need of using expensive optical materials. On the other side, the synthesis of these photoactive systems is quite demanding, especially compared with those of Angeli's salt or Piloty's acid derivatives.

One alternative would be PA activation by a pH increase induced by a photoactive actuator that releases a basic ligand upon visible light excitation. One family of these compounds consists of ruthenium(II)–polypyridyl (Ru–bpy) complexes including an organic molecule which does not have chemical activity while it is coordinated to the metal center but recovers its activity when it is released, after irradiation at the MLCT band of the Ru–bpy complex (generally between 400 and 500 nm, depending on the coordinated ligands).

In the last few years this family of caged compounds, based on ruthenium–polypyridyl complexes, has been shown to have several applications such as in drug delivery or other biological processes activated by light,[11–18] advanced microscopy techniques,[19] pH manipulation,[20] molecular machines[21,22] and other applications. The photoinduced release of a basic ligand gives rise to an increase of the pH of the solution in the irradiated zone in the order of tens of nanoseconds.[20]

Herein we combined two well-studied systems (the ruthenium complexes as photoactive actuators and the Piloty's derivatives as HNO donors) to develop a new fast HNO generation method that allows precisely controllable release without the use of a UV light source. As the rate of HNO generation depends on

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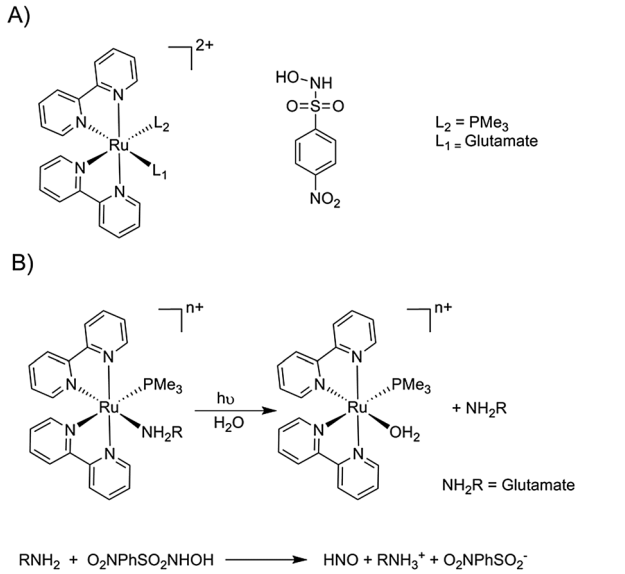

Fig. 1 Structures of Ru–bpy complexes and PA derivate used in this work (A). HNO photo-induced reaction mechanism (B).

the pH and the derivative's pK_a (very slow at pH values lower than the acid's pK_a and fast at higher pH values), it is easy to control the production of HNO according to the irradiation conditions, the actuator concentration, and the PA derivative's pK_a. The HNO released was quantified and the kinetics parameters were determined with the use of a well-known probe for HNO, a water-soluble manganese(III) porphyrinate.[23]

Fig. 1 shows the structures of the Piloty's acid derivative (HNO donor) and the Ru–bpy complexes (pH actuator) along with the reactions related to the photochemical HNO generation. Upon irradiation at the MLCT band, the complex $[Ru(bpy)_2(PMe_3)(Glut)]^{2+}$ releases the base (Glut = Glutamate) and the PA is deprotonated, generating HNO. The photodelivery quantum yield of this actuator is 0.13,[24] and the photoinduced pH increase takes place within tens of nanoseconds.[20] However, the rate of HNO generation is determined by the rate of the PA decomposition. This rate is highly pH dependent. The pH at which the HNO donation takes place depends on the derivative's pK_a. *N*-Hydroxy-4-nitro benzene sulfonamide[25] (4-nitro Piloty's acid) was used as the HNO donor.

The pH increase depends on the actuator's concentration. Complete photolysis of the actuator (in water, pH = 7) generates a free base concentration equal to the actuator initial concentration. For example, concentrations of the actuator of 1, 10 and 100 mM increase the pH up to 10.2, 10.7 and 11.2, respectively, after the base (pK_a 9.47) is released in the irradiated region. Although the acuo-complex (Ru–H_2O) is formed, after Ru–Glut is irradiated, which is in equilibrium with its conjugated base (hydroxo-complex, Ru–OH, pK_a = 10.7, see the ESI†) in this case, as the pK_a of glutamate is more than one unit lower than the ruthenium acuo-complex pK_a, the acid–base equilibrium of the complex can be considered negligible.

The MLCT band of the complex is strongly dependent on the coordinated ligands.[26] Thus, the maxima for the Ru–H_2O complex differ from the one for the Ru–OH derivative, which makes UV-vis spectrometry a good tool to estimate the pH increase in very small volume samples. This pH change in the sample was calculated by irradiating solutions, with different concentrations of Ru–Glut, using a 405 nm laser diode. Ru–H_2O UV-vis spectra obtained by irradiation of the sample in the cell were compared with Ru–H_2O UV-vis spectra at different pH values obtained with buffer solutions. For initial concentrations of Ru–Glut of 3 and 10 mM at pH = 7, the final pH values obtained were 10.4 and 10.7, respectively (see the ESI†).

To measure the HNO production in our system, we used the water-soluble manganese(III) porphyrinate (MnTSPP) (λ_{max} = 465 nm; ε_{max} = 87 500 M^{-1} cm^{-1}). Mn(III) porphyrinates are known to react quickly with HNO to give the Mn(II) nitrosyl derivative, which presents a considerable shift of *ca.* 40 nm in the Soret band.[23] Even though this reaction competes with HNO dimerization, as the rate of the reaction between HNO and the Mn(III) porphyrin is higher than that of dimerization (see the ESI†), almost all HNO is trapped by the MnTSPP. Due to the high absorbance of the HNO probe and the pH actuator under the experimental conditions, it is necessary to use a small optical cell path (approximately 0.1 mm). To achieve this, a cell was fabricated as described in the ESI.† The nitrosyl derivative is oxygen sensitive, and it slowly goes back to the Mn(III) complex under air.[23,27] However, we did not observe this in our system, probably due to the slow diffusion of oxygen into the flat solution inside the thin cell.

Upon irradiation of a sample containing the HNO donor, the pH actuator and the HNO probe, the band of the Mn(III) porphyrin at 465 nm decreases while a new band at 425 nm, attributed to the nitrosyl derivative, appears (Fig. 2). This result clearly demonstrates HNO generation in our system.

When a sample containing only the PA and the manganese porphyrinate is irradiated, no changes in the UV-vis spectra are observed. The changes observed in the spectra when a solution containing the probe and the actuator is irradiated are only due

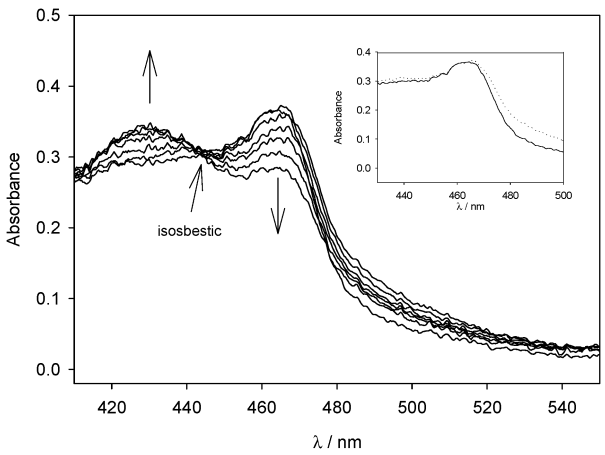

Fig. 2 Selected spectra of photo-induced HNO generation in phosphate buffer 2.5 mM, pH = 7.4 and Ru–Glut 3 mM (irradiation time = 16 s, isosbestic point at 442 nm). Inset: Initial spectra (line) and first spectra after complete photolysis of the actuator (dotted).

to the actuator spectral changes and happened only during the time of irradiation; after that, no changes were observed.

The rate constant obtained for a sample of actuator (3×10^{-3} M), PA (1.5×10^{-4} M) and MnTSPP (5×10^{-3} M) was $k = (1.03 \pm 0.05) \times 10^{-2}$ s^{-1}. In order to calculate the amount of HNO generated in the sample (10 μl), the UV-vis spectra were simulated to fit with the experimental final spectra, showing a decrease of approximately 20% on the Mn(III) porphyrin at $\lambda = 465$ nm (which corresponds to the probe that has not reacted with HNO), indicating an accumulated concentration of HNO of $\sim 1 \times 10^{-4}$ M (ESI†). An initial spectra were also simulated and compared with the experimental spectra, showing an excellent agreement (ESI†). For higher concentrations of the actuator, the pH increase reaches the pK_a of Ru–H_2O, and the observed rate constant is four times higher (see the ESI†). However, a higher pH is more harmful for biological systems.

Although under physiological conditions the pH is buffered, the actuator must increase the pH of the solution in order to deprotonate and decompose the HNO donor. In order to find the optimal conditions of actuator's concentration to achieve the necessary pH jump, 3 mM and 15 mM solutions of the actuator were irradiated using the 405 nm laser diode, and the final pH of the solution was calculated by using the UV-vis spectra of the actuator during its photolysis in buffer solution (pH = 7.4, 2.5 mM NaH_2PO_4/Na_2HPO_4 buffer), obtaining a final pH value of 9.7 and 10.7, respectively.

Then, HNO production was induced by irradiation using the 405 nm laser diode in the same buffer solution. Selected spectra of the HNO photo-induced generation reaction, for 10 μl of the sample of the actuator (3×10^{-3} M), PA (2×10^{-4} M) and MnTSPP (2×10^{-4} M), are shown in Fig. 2. UV-vis spectra were acquired until the reaction was completed. Under these experimental conditions, during irradiation of the sample, almost all changes in the UV-vis spectra are due to the photolysis of the actuator (Fig. 2, inset). After photolysis is completed, the increase of the Mn porphyrin Soret band at 425 nm and the decrease of the band at 465 nm indicate that HNO is being generated even in a buffer solution (isosbestic point at 442 nm). The rate constant was calculated, and the amount of produced HNO was estimated. Under these conditions $k = (0.65 \pm 0.20) \times 10^{-2}$ s^{-1} and the yield of generated HNO was similar to the one calculated for distilled water (Table 1).

The same procedure was applied for higher actuator concentrations. Selected spectra acquired upon HNO generation in buffer solutions are shown in the ESI.† The calculated rate constant was also higher due to the higher pH reached (Table 1).

Fig. 3 shows the rate of production of the porphyrin–Mn–NO product (which represents the rate of HNO production) of light-induced HNO generation under different experimental conditions. A summary of the rate constants and amounts of HNO generated for each sample is shown in Table 1. These values were compared with HNO generation induced by a macroscopic pH change in HNO donor solutions, showing an excellent agreement (see the ESI†).

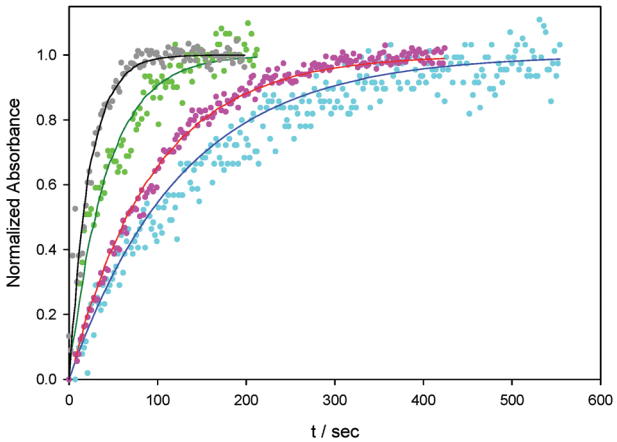

Fig. 3 Reaction rate of photo-induced HNO generation under different experimental conditions. Experimental (dotted) and simulated (line) values for EXP 1 (blue), EXP 2 (green), EXP 3 (pink) and EXP 4 (gray). Experimental conditions are shown in the ESI.†

During the time needed to photolyze the actuator and produce the pH jump, the rate of HNO production induced by light (v_I) is higher than the rate of dimerization (v_D) (Table 1). This indicates that this is a fast method for HNO generation induced by visible light. This rate constant is strongly pH dependent, which allows controlling it by changing the concentration of the actuator, the light power, and the PA derivatives and their concentration.

By increasing the actuator's concentration, it is possible to obtain a change in pH on more concentrated buffered solutions. This could be useful to generate HNO in extracellular media but could not be used inside a cell. Anyway, this problem may be solved by using another PA derivative which has a lower activation pH, modifying the substituents on the aromatic ring of the compound,[28] so that a lower pH jump could produce HNO, as it has been shown before.

Although the MLCT band for this Ru–bpy complex is in the blue region of visible spectra ($\lambda_{max} = 446$ nm), it is possible to tune the position of this band and make it to occur at higher wavelengths. But the photorelease quantum yield decreases when the energy of the MLCT band decreases, so the photoactivity ($\varepsilon^*\phi$) does not increase.[26] However, some strategies have been studied to avoid this problem, allowing photorelease

Table 1 Summary of experimental conditions, rate constants and HNO generation in the photo-induced processes ($n = 2$)

Medium	[Ru–Glut] (mM)	$k \times 10^2$ (M^{-1} s^{-1})	τ (s)	[HNO] $\times 10^4$ (M)	[AP] $\times 10^4$ (M)	$v_I \times 10^6$ (M s^{-1})	v_D/v_I
Water	3	1.03 ± 0.05	67 ± 3	1.2	1.5	1.5 ± 0.1	0.002
Buffer[a]	3	0.65 ± 0.20	107 ± 33	1.7	2.0	1.3 ± 0.4	0.013
Buffer[a]	6	2.90 ± 0.83	24 ± 7	0.9	1.0	2.9 ± 0.8	0.048

[a] $H_2PO_4^-/HPO_4^{2-}$ buffer solution (pH = 7.4, 2.5 mM).

of the coordinated ligand with longer wavelength light sources increasing the photoactivity,[29,30] even with IR light, using two-photon microscopy techniques[17,31] or using nanoparticle upconversion.[32]

We have described a new method of HNO controlled generation, by using a pH actuator that increases the pH, in the irradiated region, using a visible light source, which activates the HNO donor.

The use of visible light, instead of UV light, decreases the Tyndall effect, increases light transmittance, and is less harmful for biological tissues. HNO generation can be activated using a visible light laser diode of a few mW or less (in a small irradiated region), in water and also in buffered solutions. This method presents the possibility of being expanded to HNO generation induced with a light source of visible long-wavelength or even IR. Generated HNO was quantified, the rate constant of HNO generation was measured and compared with the activation with a macroscopic change of pH, and similar values were obtained, indicating that this is a fast method for light induced HNO generation. The rate constant is strongly pH dependent, which allows controlling it by changing the concentration of the actuator, the light power, and the PA derivatives, providing a lot of versatility to the present method.

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