A photosensitive {Ru–NO}^{6} nitrosyl bearing dansyl chromophore: novel NO donor with a fluorometric on/off switch†

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The synthesis, fluorescence properties and NO photolability of [(Me_2bpb)Ru(NO)(Ds-im)]BF_4, a {Ru–NO}^{6} nitrosyl–fluorophore conjugate, have been investigated and its potential as a trackable NO donor has been evaluated.

Effective tumor imaging and treatment could be achieved *via* combination of photodynamic therapy (PDT)[1] and fluorescence-guided resection (FGR)[2] with suitably designed photochemotherapeutics. Recent *in vitro* studies have shown that inducible nitric oxide synthase (iNOS)[3] can produce elevated levels of NO[4] that limit tumor metastasis and induce apoptosis in cancer cells.[5,6] A photosensitive NO-releasing drug (NO donor) with a fluorescent tag could therefore act as a ''trackable'' PDT agent. Although many NO donors have been synthesized, including organic nitrates (R–ONO_2, such as nitroglycerin), *S*-nitrosothiols (R–SNO), and diazenium-diolates (NONOates),[7,8] there are few reports of NO donors that contain light-harvesting or light-emitting chromophores for such use.[9–12] In limited cases, NO donors have been derivatized with fluorescent tags. For example, a piperazine-based NONOate was derivatized with a dansyl group to generate the fluorescent NO donor GLO/NO.[9] However, this molecule spontaneously releases NO in buffered solution ($t_{\frac{1}{2}}$ = 5.6 min)—an undesirable property for targeted NO delivery in cancer treatment. In other work, the N-terminus of *S*-nitrosoglutathione (GSNO) has been derivatized with a dansyl group.[10] GSNO also releases NO spontaneously in solution (over several hours), although its NO release is somewhat more selective as it can be accelerated by exposure to light. However, in both cases (GLO/NO and dansyl-GSNO), the conjugated fluorophore does not report the status of the drug, *i.e.* whether the NO is released or not released. Lippard and co-workers have done much research in the area of fluorescence-based ''NO sensors'' (NO generated from endogenous sources),[13] but fluorescence has not yet been utilized as a convenient means for monitoring NO delivery from an exogenous NO source, such as a photosensitive NO pro-drug.

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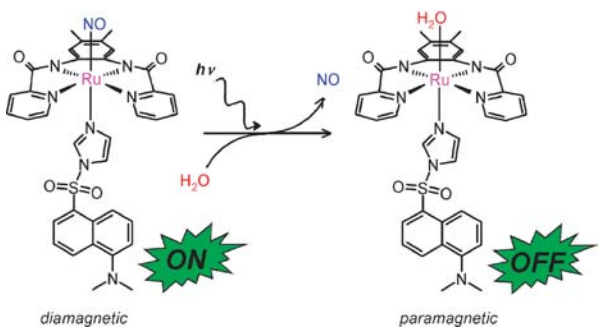
† Electronic supplementary information (ESI) available: Synthesis, experimental details, spectral properties, ^{1}H-NMR spectra of **1** and **2**, structure of **2**, X-band EPR spectrum of a photolyzed solution of **1**, and crystallographic details of **2**. CCDC reference number 683303. For ESI and crystallographic data in CIF format see DOI: 10.1039/b805332d

Among metal nitrosyls, Roussin's red salt esters (RSEs) of the formula [$Fe_2(\mu$-$SR)_2(NO)_4$] release NO under exposure to light.[11] However, the by-products of their photodecomposition follow poorly defined pathways, and as such cannot be designed to reliably bind and/or quench a fluorescent tag in aqueous solutions. In addition, iron nitrosyls in general are unstable in water, and undergo spontaneous NO release or NO → NO_2 conversion even under dark conditions.[14] Ruthenium nitrosyls on the other hand, exhibit much greater stability in aqueous solution and undergo clean NO photorelease without further decomposition or dissociation of other bound ligands.[15] When derived from ligands containing negatively-charged groups such as carboxamido-N or phenolato-O donors, such nitrosyls afford paramagnetic Ru(III) species as photoproducts[16] which could quench the emission signal of a bound fluorophore.[17]

In the present work, we report a {Ru–NO}^{6} nitrosyl [(Me_2bpb)Ru(NO)(Ds-im)]BF_4 (**1**) with a conjugated dansyl group which allows its detection in solution *via* the fluorescence of the dansyl tag in the NO-bound form; upon photorelease of NO, the Ru(III) photoproduct causes complete quenching of the fluorophore tag thus allowing a highly sensitive fluorometric method to follow NO delivery (Scheme 1). The ruthenium nitrosyl has been synthesized from the designed dicarboxamido ligand H_2Me_2bpb (*N*,*N*-bis(pyridinecarboxamido)-1,2-dimethylbenzenediamine; where H = dissociable carboxamide protons).[16b] This ligand, in the deprotonated form, employs two carboxamido-N donors (in addition to two pyridine N) to bind the metal center and exhibits preference for the +3 oxidation state of Ru. As shown below, the planar tetradentate ligand frame chelates the metal center in the equatorial plane, while leaving the axial



Scheme 1 Schematic of paramagnetic fluorescence quenching observed with [(Me_2bpb)Ru(NO)(Ds-im)]BF_4 (**1**, shown on left) upon photorelease of NO and generation of the Ru(III) photoproduct (shown on right).

positions open for binding of one NO molecule, and one other exogenous ligand.[12]

Previously, we have reported the structures and properties of {Ru–NO}^{6} nitrosyls derived from the Me_2bpb^{2-} ligand with various sixth donors (such as Cl^-, OH^-, py).[12,16b] These nitrosyls rapidly release NO when exposed to low-intensity (mW) UV light in the range of 350–450 nm. In the present work, the fluorescent ligand dansyl-imidazole (Ds-im)[13b] has been employed as the sixth donor to isolate the nitrosyl–fluorophore conjugate [$(Me_2bpb)Ru(NO)(Ds\text{-}im)]BF_4$ (**1**). Reaction of the parent nitrosyl [$(Me_2bpb)Ru(NO)(Cl)$] with 1 equivalent of $AgBF_4$ in refluxing MeCN afforded the solvato species [$(Me_2bpb)Ru(NO)(MeCN)]BF_4$ in solution. Further heating with 3 equivalents of Ds-im for several hours generated a greenish-orange solution. The solvent was then removed and the residue was redissolved in CH_2Cl_2. Addition of Et_2O to this solution and storage at −20 °C for several days afforded the target nitrosyl [$(Me_2bpb)Ru(NO)(Ds\text{-}im)]BF_4$ (**1**) as a light green solid. Coordination of the Ds-im fluorophore to ruthenium is confirmed by the ^{1}H-NMR spectrum of **1** (see ESI†) and mass spectral data. Additionally, the IR spectrum of **1** exhibits characteristic ν_{SO} stretches (1184 and 1170 cm^{-1}), as expected for the aryl-sulfonamide moiety. The ν_{NO} and ν_{CO} stretches, at 1868 cm^{-1} and 1639 cm^{-1}, respectively, are typical for {Ru–NO}^{6} nitrosyls derived from this ligand.[12,16b]

The nitrosyl–fluorophore conjugate **1** is soluble and stable in a variety of solvents including MeCN, MeOH and H_2O. In aqueous solutions, the NO moiety of **1** is stable over a range of biologically relevant pH values (pH 5–8), and the bound fluorophore does not dissociate under any tested conditions. The electronic absorption spectrum of **1** (Fig. 1, inset) exhibits a prominent $d\pi(Ru) \rightarrow \pi^*(NO)$ transition near 400 nm, typical for nitrosyls of this type.[18] In aqueous phosphate buffer (pH 7.4), the fluorescence spectrum of **1** (Fig. 1) exhibits an emission peak at 505 nm (λ_{ex} = 380 nm), similar to other diamagnetic metal complexes that contain the bound Ds-im moiety.[13a] The related nitrosyl in which imidazole (but no attached dansyl group) is the sixth ligand, namely [$(Me_2bpb)Ru(NO)(im)]BF_4$ (**2**, see ESI†), does not exhibit any fluorescence (Fig. 1).

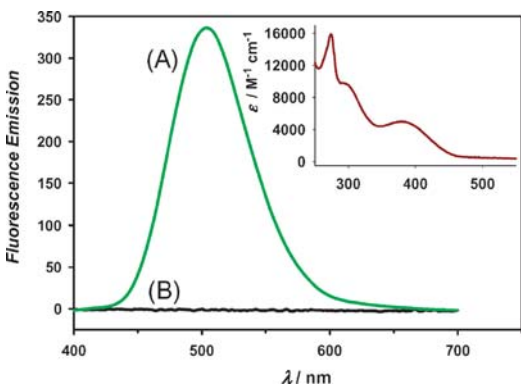


Fig. 1 Fluorescence emission spectrum (λ_{ex} = 380 nm) of the nitrosyl–fluorophore conjugate [$(Me_2bpb)Ru(NO)(Ds\text{-}im)]BF_4$ (**1**, trace A), and the corresponding nitrosyl without dansyl group, namely [$(Me_2bpb)Ru(NO)(im)]BF_4$ (**2**, trace B) in aqueous phosphate buffer (pH 7.4). Inset: electronic absorption spectrum of **1**.

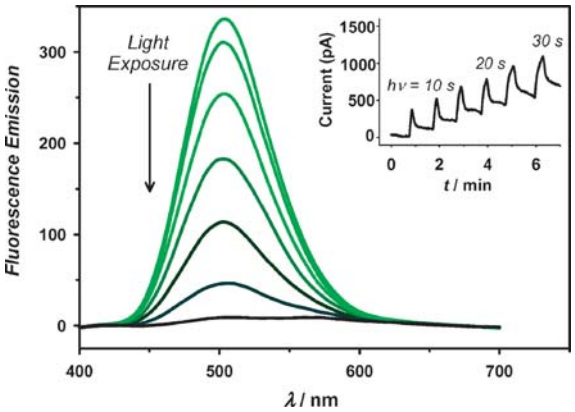


Fig. 2 Fluorescence quenching observed upon photolysis of **1** ($\lambda_{irr} \geq$ 400 nm, 0.5 W) in aqueous phosphate buffer (pH 7.4). Inset: NO amperogram recorded with an inNO nitric oxide monitoring system fitted with the ami-NO-2000 electrode during this experiment.

Much like other {Ru–NO}^{6} nitrosyls of the type [$(Me_2bpb)Ru(NO)(X)]BF_4$ (X = py, Cl^-, OH^-), both **1** and **2** rapidly release NO upon exposure to UV light. However, unlike others, photorelease of NO from **1** can be conveniently monitored by changes in its fluorescence spectrum. As shown in Fig. 2, the fluorescence intensity of **1** is systematically quenched over the course of several minutes of illumination ($\lambda_{irr} \geq$ 400 nm, 0.5 W). Photorelease of NO from **1** during such illumination is readily detected by an NO-sensitive electrode (see amperogram, Fig. 2, inset). We ascribe the quenching of fluorescence of **1** as due to formation of the paramagnetic Ru(III) photoproduct [$(Me_2bpb)Ru(H_2O)(Ds\text{-}im)]^+$ in solution. This paramagnetic species exhibits a strong EPR signal with g = 2.21 and 1.86 (see ESI†), typical of a low-spin d^5 system. Quantum yield measurements indicate that **1** photoreleases NO with moderate efficiency (ϕ_{400} = 0.08) in aqueous solution.

That the NO delivery by **1** can be faithfully followed by monitoring the quenching of its fluorescence has been demonstrated in the present work. Photorelease of NO from

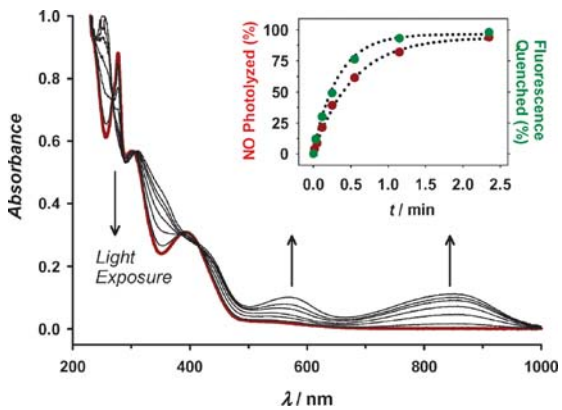


Fig. 3 Changes in the electronic absorption spectrum of **1** (in MeCN) upon exposure to a filtered halogen light source ($\lambda_{irr} \geq$ 400 nm, 0.5 W). Absorptions in the low energy region arise from the Ru(III) photoproduct. Inset: correlation between percent absorption increase at 600 nm and percent fluorescence quenched during the same photolysis experiment.

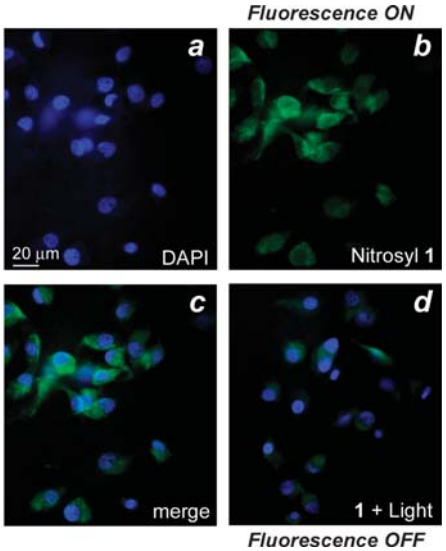


Fig. 4 Fluorescence microscopy images using the fluorophore–nitrosyl conjugate **1** in MDA-MB-231 breast cancer cells (fixed with 4% paraformaldehyde). (a) Cells stained with nuclear stain DAPI; (b) same slide viewed in green fluorescence mode to monitor accumulation of **1**; (c) merged a + b image; (d) cells exposed to light with quenched fluorescence following NO photodelivery. Light-exposed slides (d) were illuminated with a filtered halogen light source ($\lambda_{irr} \geq 400$ nm, 0.5 W) for 1 min.

$\{Ru–NO\}^6$ nitrosyls of this type is routinely monitored by changes in their electronic absorption spectra.[16] Formation of the Ru(III) photoproduct in solution is evidenced by increases in absorption around 600 and 900 nm. As shown in Fig. 3, such changes in the electronic absorption spectrum of **1** are observed in MeCN upon exposure of the solution to UV light. It is important to note that during such illumination, the increase in absorbance at 600 nm is strongly correlated with the quenching of the fluorescence of **1** (Fig. 3, inset). Clearly, NO release from **1** can be determined by the extent of the fluorescence quenching. In this regard, **1** can be used as a fluorometric NO donor.

In order to evaluate the potential of **1** as a "trackable" NO donor in a cellular environment, we have employed **1** to deliver NO to human breast cancer cells.[19] Treatment of a preparation of MDA-MB-231 cells with a 200 μM solution of **1** in phosphate buffer (pH 7.4) affords a bright green cytoplasmic staining pattern (Fig. 4) that neatly overlays the nuclear staining by DAPI (shown in blue). Such a concentration of **1** is not toxic to the cells; the cells remain viable in the growth medium for hours if kept away from light. Brief exposure (1 min) of the cell preparation to light ($\lambda_{irr} \geq 400$ nm, 0.5 W) quenches this green fluorescence, indicating photorelease of NO within the cell preparation (Fig. 4). The "turning off" of the NO drug fluorescence within the cell is caused by the paramagnetic photoproduct. It is therefore evident that **1** can not only be tracked within the target cell, but also the NO delivery by this nitrosyl can be easily seen by the loss of its green fluorescence. Experiments to determine the downstream signaling effects of NO in such cells are in progress in this laboratory. The results will be reported in due course.

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