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Singlet Oxygen Release and Cell Toxicity of a Chemiluminescent Squaraine Rotaxane Dye: Implications for Molecular Imaging

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The water soluble tetraguanidinium squaraine rotaxane **2** was prepared and photoconverted to its corresponding squaraine rotaxane endoperoxide (SREP), **2EP**. As expected, **2EP** undergoes a thermal cycloreversion reaction that releases $60 \pm 4\%$ singlet oxygen and produces near-infrared emission in aqueous solution. Cell toxicity assays in the dark, using human and bacterial cell cultures, showed that **2EP** (up to 20 μM) is no more toxic than its parent **2**. This suggests that SREP-derived imaging probes are not likely to exhibit a significant toxicity effect due to the slow release of stoichiometric amounts of singlet oxygen. Additional photosensitization experiments showed that tetraguanidinium squaraine rotaxane **2** is a weak photosensitizer, but nonetheless, red light irradiation of cell cultures that were pre-incubated with **2** (>3 μM) produced moderate phototoxicity. Fluorescence microscopy studies attribute the phototoxicity of **2** to its ability to penetrate into the cell cytosol. The implications of these results are discussed in the context of effective methods to activate SREP as chemiluminescent probes for in vivo optical molecular imaging.

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Introduction

Squaraine rotaxanes are interlocked molecules with a highly fluorescent squaraine dye encapsulated inside a surrounding macrocycle.[1] We are developing these compounds for potential applications in optical molecular imaging.[2] The focus here is on squaraine rotaxanes that incorporate an anthracene-containing macrocycle.[3] Recently, we discovered that irradiation of an aerated solution of organic-soluble squaraine rotaxane **1** with red light leads to a photochemical oxidation reaction that produces the squaraine rotaxane endoperoxide (SREP) **1EP** in quantitative yield (Scheme 1). Compound **1EP** is stable at temperatures below 0°C but upon warming to body temperature it undergoes an unimolecular cycloreversion reaction that releases singlet oxygen and emits near-infrared light.[4] Compounds **1** and **1EP** have almost identical fluorescent properties, which made it possible to conduct comparative studies of the signal contrast that can be obtained by whole body optical imaging. These studies included planar optical images of a mouse that contained injected target sites of **1** or **1EP**. The images were acquired as pixel intensity maps and they allowed determination of target to background ratios for both chemiluminescence and fluorescence imaging modalities. The results showed that the fluorescence intensity images were much more surface weighted. That is, target to background ratios were quite high for superficial target sites that were close to the surface of the animal, but the ratios quickly approached unity as the target depth increased much beyond 5 mm. Signal to background ratios for the chemiluminescence images decreased much more gradually with tissue depth and remained quite high at tissue depth of >2 cm. The primary reason for the higher target signal contrast with chemiluminescence imaging is the very low background due to the absence of any scattered excitation light.[5]

These results suggest that SREP, such as **1EP**, may be useful as thermally activated chemiluminescent dyes for optical imaging in living patients. However, there are several technical hurdles that must be overcome. One challenge is the need to produce imaging probe molecules or particles with very high chemiluminescence intensity. At present the chemiluminescence mechanism is not known in great detail, but there is evidence that the emitted light is due to radiative decay of the excited encapsulated squaraine dye, and that excitation of the dye is mediated by the released singlet oxygen.[4] Most importantly, chemiluminescence intensity decreases if the solvent or matrix includes agents that quench singlet oxygen. For example, experiments with **1EP** showed that addition of water to the organic solvent decreased chemiluminescence intensity. Another technical challenge is the development of methods to generate SREP-based probes in a manner that is suitable for in vivo optical imaging. There are two conceivable strategies (Scheme 2). The first is to generate the SREP outside the patient and if necessary store it at low temperature. After injection into the patient, the SREP probe warms to body temperature and becomes self-illuminating. The second strategy is to dose the patient with a probe that contains the precursor squaraine

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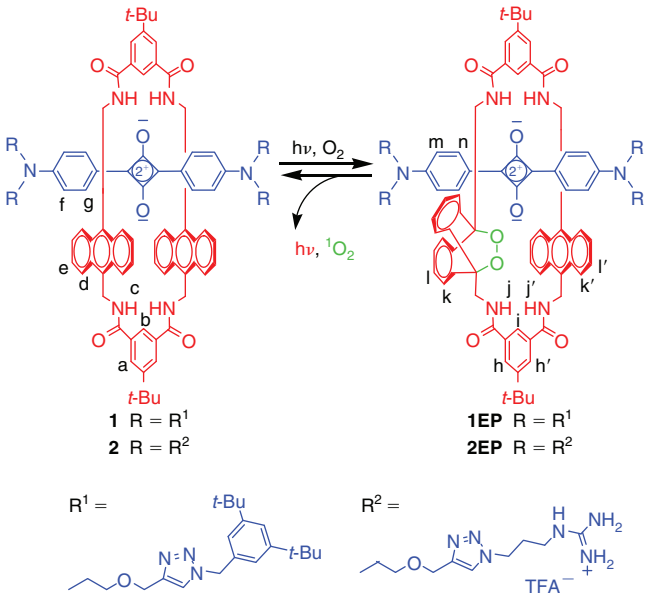
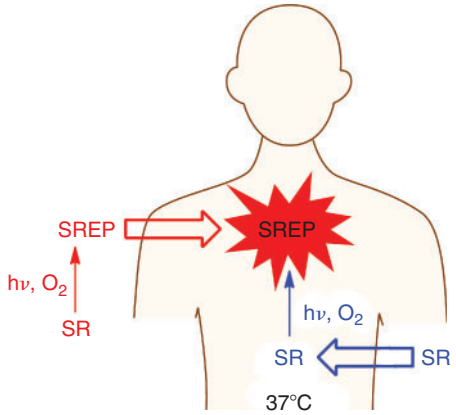


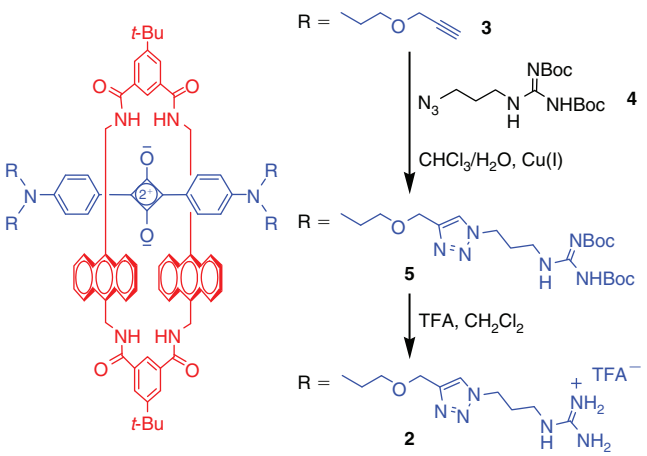
Scheme 1.



Scheme 2. Two conceivable strategies to generate squaraine rotaxane endoperoxide (SREP)-based probes for in vivo optical imaging. The first approach (shown in red) produces the SREP externally at low temperature, then injects the probe into the patient where it warms and becomes self-illuminating. The second strategy (shown in blue) is to dose the patient with a probe that contains the precursor squaraine rotaxane (SR) and then photogenerate the chemiluminescent SREP in situ.

rotaxane and then activate the probe in situ by using one or two photon irradiation to photogenerate enough localized singlet oxygen to react with the squaraine rotaxane and produce the corresponding chemiluminescent SREP. Both strategies raise the potential concern of in vivo toxicity because the probes will generate reactive singlet oxygen. In the first method, the SREP thermally releases stoichiometric amounts of singlet oxygen, and in the second approach, there is the additional problem that the administered squaraine rotaxane precursor acts as a photosensitizer and that irradiation may rapidly photogenerate a large molar excess of singlet oxygen.

As the first step in a project to assess the feasibility of these two different methods of SREP activation for in vivo chemiluminescence imaging, we have prepared a water soluble SREP and studied its photochemical and cell toxicity properties. Specifically, we report here the preparation of water soluble



Scheme 3.

squaraine rotaxane **2** and its efficient photoconversion to monoendoperoxide **2EP** (Scheme 1). We find that, as expected, **2EP** is able to undergo a thermal cycloreversion reaction that releases singlet oxygen and produces weak chemiluminescence emission in aqueous solution. Cell toxicity assays in the dark show that **2EP** is no more toxic than its parent squaraine rotaxane **2**. We also examined the ability of **2** to act as a photosensitizer and found that it is weakly effective at photogenerating singlet oxygen in homogeneous organic solution. Nonetheless, irradiation of cell cultures that have been pre-incubated with **2** produces a surprisingly potent phototoxicity effect. The implications of these results are briefly discussed in the context of our long-term aim of developing SREP as chemiluminescent probes for in vivo optical molecular imaging.

Results and Discussion

Synthesis and Studies of Chemiluminescent Cycloreversion

New squaraine rotaxane **2** was prepared by the synthetic method shown in Scheme 3. The known squaraine rotaxane tetraalkyne **3** was assembled in high yield by simply mixing the thread and macrocycle components together.[6] The protected guanidine azide **4** was attached to the four alkyne groups by conducting a copper catalyzed alkyne-azide cycloaddition reaction to give **5** in good yield,[7] and deprotection with acid gave the desired squaraine rotaxane **2**. The partial ^{1}H NMR spectra in Fig. 1 show the photoconversion of **2** into SREP **2EP**, which was achieved by simply irradiating an aerated solution with red light.[4] Upon standing in the dark, the sample of **2EP** underwent spontaneous cycloreversion to regenerate **2** in ~90% yield (Fig. 1c). Kinetic measurements in 1:9 water/acetonitrile at 37°C determined the first-order rate constant to be $0.230 \pm 0.004\,h^{-1}$, which corresponds to a half-life of ~3 h. The cycloreversion reaction released molecular oxygen and chemical trapping experiments were conducted to determine the fraction that was excited state singlet oxygen. The trapping experiments used 2,3-dimethylbut-2-ene to react with the singlet oxygen and form a stable hydroperoxide product that was easily identified and quantified by NMR spectroscopy.[8] The average of three trapping experiments in 1:9 water/acetonitrile showed that $60 \pm 4\%$ of the released oxygen was singlet oxygen. An optical imaging machine equipped with a charge-coupled device (CCD) was used to confirm that the cycloreversion reaction of **2EP** in aqueous acetonitrile was weakly chemiluminescent. In Fig. 2 is a series of false-coloured pixel intensity images of a vial, viewed

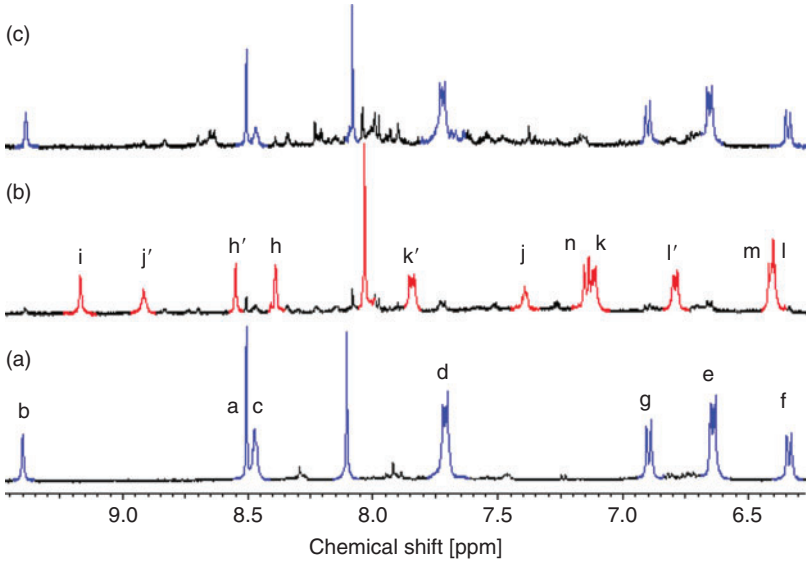


Fig. 1. Partial ^{1}H NMR spectra (CD_3OD) showing photoconversion of **2** into **2EP** and the subsequent thermal cycloreversion. (a) Starting solution of **2**. (b) Complete photo-oxidation to **2EP** after irradiation with red light for 30 min. (c) Cycloreversion of the sample back to **2** after 18 h at 37°C in the dark. See Scheme 1 for atom labelling.

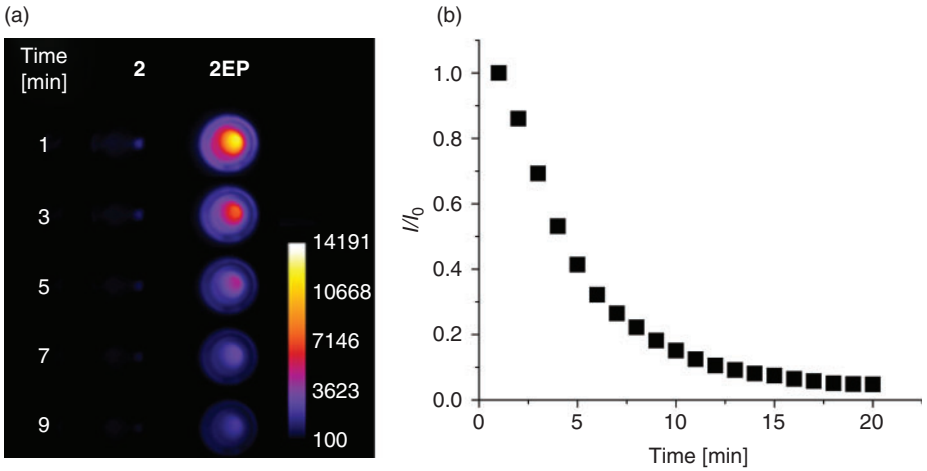


Fig. 2. (a) Pixel intensity maps of the change in chemiluminescent intensity for two vials, viewed from above, containing either **2** or **2EP** (~3 mM in 1:9 water/acetonitrile) at 37°C. (b) The change in normalized chemiluminescent intensity for **2EP** shows a decay half-life of ~4 min.

from above, as a function time. As indicated by the decay curve in Fig. 2, the chemiluminescence decay half-life is only ~4 min, which is substantially shorter than the cycloreversion half-life. The mechanistic reason for this difference in kinetic profiles is currently not clear and remains under investigation.

Photogeneration of Singlet Oxygen

To enable proper interpretation of the cell phototoxicity studies (described below), it was important to determine the ability of **2** to photogenerate singlet oxygen relative to the control photosensitizer, Rose Bengal. The comparison was made by conducting standard singlet oxygen trapping experiments using the UV-absorbing trap 1,3-diphenylisobenzofuran (DPBF).[9] A typical experiment involved red light irradiation (150 W Xenon lamp filtered to allow >520 nm) of an aerated cuvette containing **2** (or Rose Bengal as the control photosensitizer) mixed with a 15-fold molar excess of DPBF in 1:9 water/acetonitrile. The data in Fig. 3 shows consumption of the DPBF absorption band at 411 nm due to irreversible reaction with the photogenerated singlet oxygen. Inspection of this trapping data shows that Rose Bengal is about 10 times more efficient as a singlet oxygen photosensitizer than **2**.

Cell Toxicity Assays

The dark toxicity of **2** or **2EP** against cultured mammalian and bacterial cells was evaluated by conducting several complementary assays.[10] The first experiment employed the colorimetric reagent 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to compare the vitality of human A549 adenocarcinoma cells that were incubated with either **2** or **2EP** in the absence of light. Vital cells convert the MTT into a purple formazan product whose absorbance maximum is measured at 570 nm. As shown in Fig. 4a, **2EP** had no apparent effect at 20 μM, whereas the parent **2** slightly decreased cell vitality. The same trend was observed when **2** and **2EP** were evaluated using the lactate dehydrogenase (LDH) release assay. LDH is a

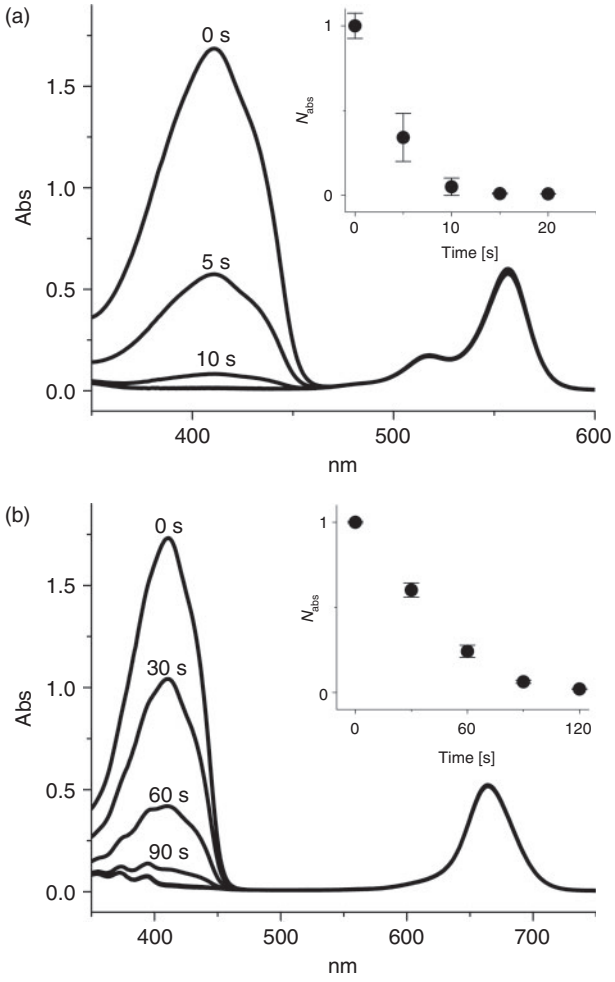


Fig. 3. Time evolution of the absorption spectra for a mixture of DPBF (75 μM) and photosensitizer (5 μM) in 1:9 water/acetonitrile, and irradiated with red light. (a) Photosensitizer is Rose Bengal. (b) Photosensitizer is **2**.

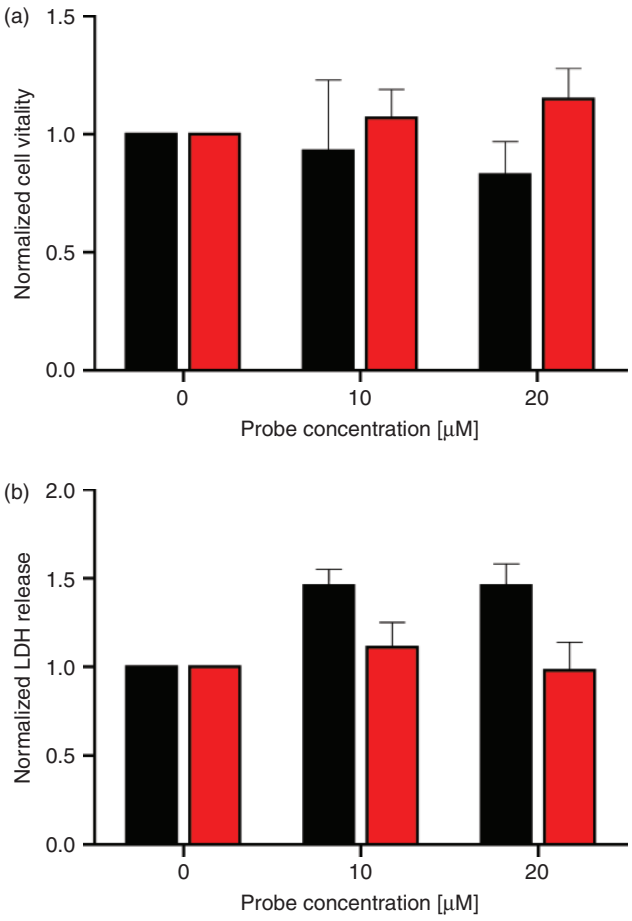


Fig. 4. (a) Cell vitality and (b) cytotoxicity assays for human A549 cells treated with **2** (black) or **2EP** (red). Cell vitality was measured using the MTT assay, and cytotoxicity was quantified by the LDH release assay. The bars indicate the normalized mean (±s.d.) compared with cells that were not treated with probe.

stable cytoplasmic enzyme present in all cells, and it is rapidly released into the cell culture medium when plasma membrane integrity is lost during cell death. As seen in Fig. 4b, squaraine rotaxane **2** induced a modest increase in cell death at 20 μM as reflected by an increase in released LDH, whereas the same dose of **2EP** produced no toxic effect. A third cell toxicity assay determined the effect of **2** and **2EP** on bacterial growth as measured by the optical density of each sample at 600 nm. The data in Fig. 5 shows both compounds had no effect on the growth of Gram-negative *Escherichia coli* UTI89, and only **2EP** had a very slight dose-dependent inhibition effect on the growth of Gram-positive *Staphylococcus aureus* NRS11. Taken together, the three cell assays indicate that the stoichiometric amount of singlet oxygen released thermally and slowly by **2EP** (up to 20 μM) induces very little, if any, cell toxicity, a conclusion that agrees with the literature on thermal oxygen release agents.[11] The data suggests that SREP, such as **2EP**, can be employed as chemiluminescence imaging probes by preparing them outside the patient and then administering them with the expectation that the stoichiometric amounts of released singlet oxygen will not cause significant cell toxicity.

A second set of cell vitality experiments attempted to mimic the hypothetical method of SREP photoactivation in situ, and thus tested the phototoxicity of squaraine rotaxane **2**. As shown in Fig. 6, red light irradiation of cell cultures that were pre-incubated with >3 μM of **2** lead to >50% loss of cell vitality. In notable contrast, a pre-incubation with >125 μM of Rose Bengal was needed to induce a similar phototoxic effect under the same cell irradiation conditions. This trend may appear to be counterintuitive since the data in Fig. 3 shows that Rose Bengal is a much more efficient photosensitizer. However, it is important to realize that the cells were washed after incubation with the photosensitizer and before the irradiation commenced. Rose Bengal is anionic and does not enter cells; whereas, cationic photosensitizers, especially those with appended guanidinium groups, have been reported to enter cells and target organelles such as the mitochondria that are sensitive to photodynamic effects.[12] The cell penetration ability of **2** was confirmed by conducting series of cell microscopy experiments. Healthy A549 cells were treated with a dose of **2** (10 μM) for 1 h and visualized by fluorescence microscopy using weak excitation power and a very short illumination time. Fig. 7 shows punctate localization of **2** within the cytosol but outside the cell nucleus. To directly image the phototoxicity of **2**, a culture of healthy cells was treated with a dose of **2** (10 μM) for 1 h, followed by irradiation with red light (>520 nm) for 30 s. Not only did the fluorescent micrograph indicate clearly that significant amounts of **2** had entered the cells, but the irradiation induced, within a few minutes, a series of signature cell morphology changes indicating cell death, including condensation of the nuclei and membrane blebbing (Fig. 8).

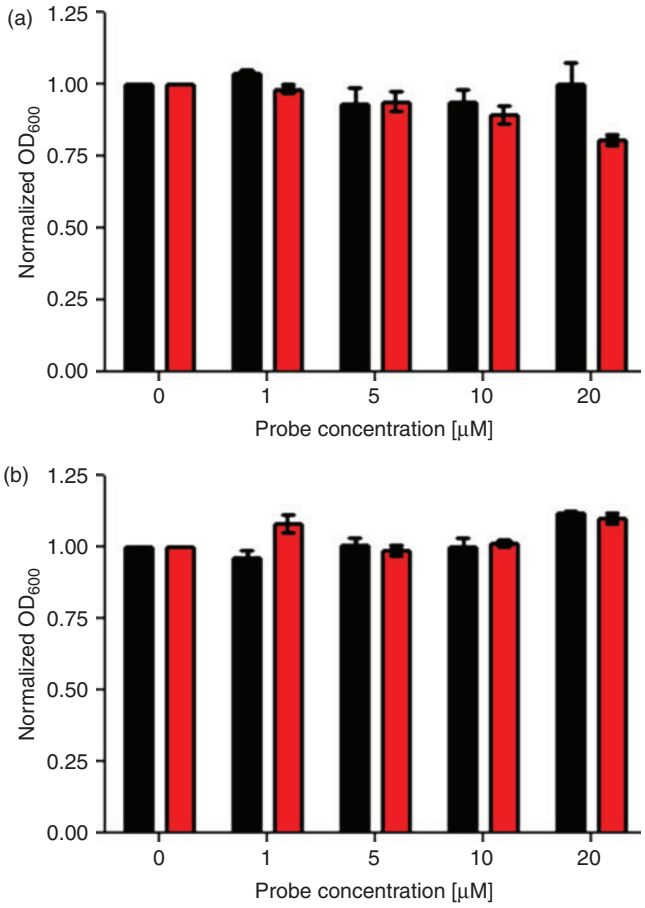


Fig. 5. Relative bacterial cell growth for (a) *S. aureus* NRS11 and (b) *E. coli* UTI89 in the presence of **2** (black) and **2EP** (red). The bars indicate the normalized mean (±s.d.) for optical density at 600 nm (normalized OD_{600}) compared with cells that were not treated with probe.

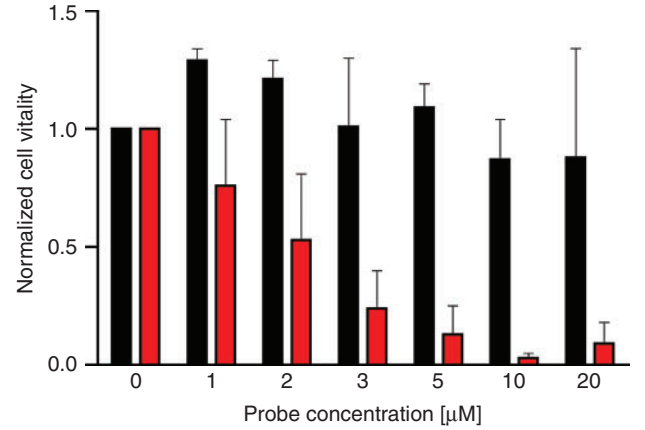


Fig. 6. Phototoxicity of probe **2** in A549 cells as measured by the MTT assay. Cells were incubated with **2** for 24 h, washed, and then either not irradiated (black bars) or irradiated with red light (red bars) for 2 h. The bars indicate normalized cell vitality (mean ± s.d.) compared with cells that were not treated with **2**.

A control sample of cells, from the same parent culture that was treated with **2** but not irradiated, did not undergo any noticeable morphological change. The fact that squaraine rotaxane **2** is reasonably phototoxic suggests that it might be problematic to attempt a hypothetic experiment where **2EP** is photogenerated

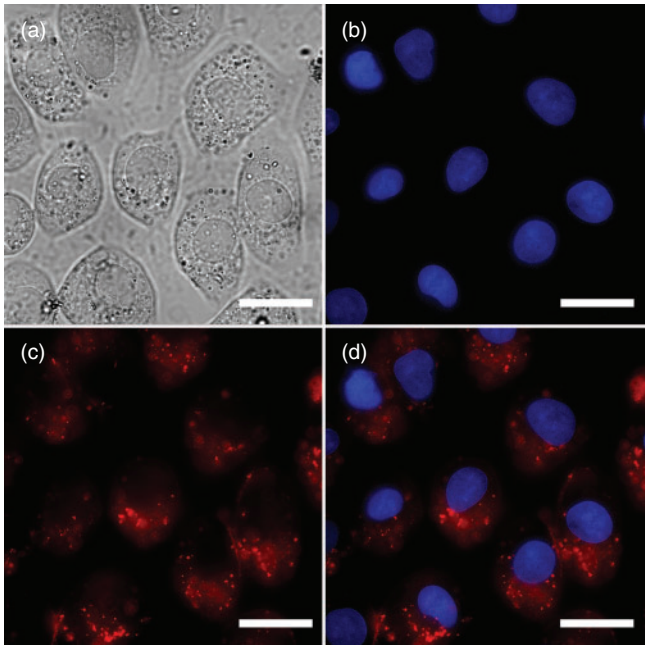


Fig. 7. Microscopic localization of squaraine rotaxane **2** in living A549 cells. Cells were treated with **2** (10 μM) for 1 h then counterstained with DAPI. The brightfield image (a) shows no morphological signs of stress or cell death. Image (d) is an overlay of cytosol staining by probe **2** (c) and nucleus staining by DAPI (b). Scale bar = 30 μm.

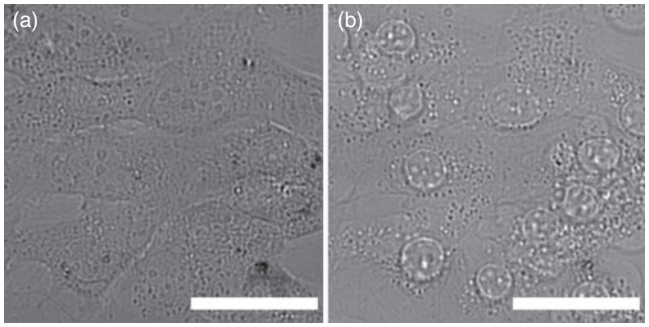


Fig. 8. Brightfield images of A549 cells treated with **2**, (a) before and (b) 6 min after exposure to red light. The cells were treated with **2** (10 μM) for 1 h then exposed to red microscope light for 30 s, which induced nuclear shrinkage and membrane blebbing. Scale bar = 30 μm.

inside a patient by a process that doses the patient with **2** followed by red light irradiation. However, it is conceivable that photoactivated in vivo imaging probes can be prepared that do not have the cell penetrating ability of squaraine rotaxane **2** and thus do not exhibit the same high degree of cell phototoxicity.

Conclusion

Extended light irradiation of a sample of water-soluble squaraine rotaxane **2** in the presence of air leads to quantitative photooxidation and formation of the corresponding endoperoxide, **2EP**. As expected, **2EP** can be stored at low temperatures (<0°C) but upon warming to 37°C it undergoes a cycloreversion reaction that releases singlet oxygen and emits near-infrared light. The chemiluminescence effect may become quite useful for a range of in vivo molecular imaging applications, but several technical challenges must be overcome. In particular,

methods must be developed to activate and deliver chemiluminescent SREP probes to the corresponding imaging target site (Scheme 2). Standard cell toxicity experiments indicate that **2EP** (up to 20 μM) does not alter cell vitality in the dark, which agrees with the known weak toxicity of stoichiometric thermal release agents for singlet oxygen. Thus, it seems that intravenous injection of SREP-derived imaging probes that are generated outside the patient will not likely cause a significant toxicity effect due to release of the relatively small amount of singlet oxygen. Nonetheless, preparing and storing fully functionalized SREP imaging probes at low temperature may be cumbersome, especially compared with the alternative method of administering the probe as the parent squaraine rotaxane and photoconverting it in situ to the SREP-probe. However, a potential drawback with this latter, in situ activation approach is phototoxicity. While squaraine rotaxane **2** can be photooxidized to produce chemiluminescent **2EP** in aqueous solution, the photoactivation process generates enough additional singlet oxygen to induce moderate cell toxicity. The phototoxicity of **2** appears to be enhanced by its ability to permeate into the cell cytosol. It may be possible to attenuate this effect by devising imaging probes containing structural variants of **2** that do not have cell penetrating ability and thus can be activated inside the patient without inducing any significant phototoxicity.

Experimental

Synthesis

*Squaraine Rotaxane Tetra(bis-(*N*-Boc)guanidine) **5***

A mixture of the known squaraine rotaxane tetraalkyne[6] (0.075 g, 0.052 mmol) and azide **4**[7] (0.11 g, 0.31 mmol) were stirred at room temperature for 24 h in a mixed solvent containing 1:1 $CHCl_3/H_2O$ (10 mL), copper sulfate (0.026 mmol), and sodium ascorbate (0.052 mmol). The copper salt was removed by stirring with Si-RAAvONa silica gel (SiliCycle, Quebec City, Canada) followed by filtration of the silica gel. The aqueous solution was extracted with excess $CHCl_3$ and the dried organic phase (Na_2SO_4) was evaporated under reduced pressure. The solid residue was purified by column chromatography (eluent $CH_3OH/CHCl_3$, 2:98) to afford **5** (0.12 g, 0.042 mmol, 80%) as a green solid. δ_H (300 MHz, $CDCl_3$) 11.43 (s, 4H, NH), 9.34 (s, 2H, ArH), 8.51 (s, 4H, ArH), 8.43 (t, *J* 5.7, 4H, *NH*CH_2), 8.23 (bs, 4H, *NH*CH_2), 8.03 (s, 4H, triazole-H), 7.70 (dd, *J* 6.6, 3.0, 8H, ArH), 6.96 (d, *J* 8.7, 4H, ArH), 6.60 (dd, *J* 6.6, 2.7, 8H, ArH), 6.13 (d, *J* 9.0, 4H, ArH), 5.20 (s, 8H, CH_2), 4.69 (s, 8H, CH_2), 4.40 (t, *J* 6.3, 8H, *CH*$_2$$CH_2$), 3.78 (m, 16H, *CH*$_2$*CH*$_2$), 3.46 (t, *J* 6.3, 8H, *CH*$_2$$CH_2$), 2.10 (m, 8H, CH_2), 1.52 (s, 18H, CH_3), 1.48 (s, 72H, CH_3). δ_C (75 MHz, $CDCl_3$) 184.1, 183.3, 172.9, 167.3, 163.6, 156.8, 153.4, 144.2, 133.5, 131.6, 131.2, 130.7, 130.6, 129.2, 128.7, 126.1, 124.1, 111.9, 105.6, 83.6, 79.6, 68.0, 64.7, 47.8, 37.6, 31.7, 30.7, 29.5, 28.5, 28.3. *m/z* (ESI-TOF) calc. for $C_{148}H_{192}N_{30}O_{26}$ $[M+H]^+$ 2806.4697, found 2806.4682.

*Squaraine Rotaxane Tetra(guanidinium) **2***

Trifluoroacetic acid (2.0 mL) was added to a solution of tetraguanidine **5** (0.12 g, 0.042 mmol) in CH_2Cl_2 (10 mL) and the solution was stirred for 24 h at room temperature. The solvent was evaporated under reduced pressure, and the residue washed with diethyl ether to remove the remaining trifluoroacetic acid. Evaporation under high vacuum afforded pure **2** as a green solid in quantitative yield. δ_H (500 MHz, CD_3OD) 9.40 (s, 2H, ArH), 8.51 (s, 4H, ArH), 8.47 (t, *J* 4.0, 4H, *NH*CH_2), 8.11 (s, 4H, triazole-H), 7.72 (dd, *J* 6.5, 3.0, 8H, ArH), 6.90 (d, *J* 9.5, 4H, ArH), 6.65 (dd, *J* 7.0, 3.0, 8H, ArH), 6.34 (d, *J* 9.5, 4H, ArH), 5.24 (d, *J* 3.5, 8H, *CH*$_2$NH), 4.72 (s, 8H, CH_2), 4.47 (t, *J* 7.0, 8H, *CH*$_2$$CH_2$), 3.83 (m, 16H, *CH*$_2$*CH*$_2$), 3.19 (t, *J* 7.0, 8H, *CH*$_2$$CH_2$), 2.14 (m, 8H, CH_2), 1.59 (s, 18H, CH_3). δ_C (60 MHz, CD_3OD) 184.8, 177.3, 167.7, 167.6, 157.6, 154.5, 153.2, 133.4, 133.3, 132.8, 130.6, 128.8, 128.1, 126.4, 123.6, 123.0, 117.1, 112.4, 68.2, 63.9, 51.0, 38.3, 35.1, 34.2, 30.6, 30.4, 29.3. *m/z* (ESI-TOF) calc. for $C_{108}H_{130}N_{30}O_{10}$ $[M+H]^+$ 2007.0576, found 2007.0506.

*Squaraine Rotaxane Endoperoxide, **2EP***

Squaraine rotaxane tetra(guanidinium) **2** was converted quantitatively to endoperoxide **2EP** by exposure to red light (150 W Xenon lamp with a longpass 520 nm filter) in either neat methanol or 1:9 water/acetonitrile. δ_H (500 MHz, CD_3CN) 9.06 (s, 2H, ArH), 8.70 (bs, 2H, *NH*CH_2), 8.47 (s, 2H, ArH), 8.32 (s, 2H, ArH), 7.89 (s, 4H, triazole-H), 7.78 (d, *J* 7.8, 4H, ArH), 7.23 (t, *J* 4.5, 2H, *NH*CH_2), 7.11 (d, *J* 5.6, 4H, ArH), 6.75 (d, *J* 7.0, 4H, ArH), 6.39 (dd, *J* 5.9, 2.9, 4H, ArH), 6.34 (d, *J* 9.4, 4H, ArH), 5.33 (d, *J* 4.4, 4H, *CH*$_2$NH), 4.64 (s, 8H, CH_2), 4.34 (t, *J* 6.8, 8H, *CH*$_2$$CH_2$), 4.13 (d, *J* 4.4, 4H, *CH*$_2$NH), 3.78 (t, *J* 4.1, 8H, *CH*$_2$$CH_2$), 3.76 (m, 8H, *CH*$_2$$CH_2$), 3.08 (t, *J* 6.8, 8H, *CH*$_2$$CH_2$), 2.03 (m, 8H, CH_2), 1.54 (s, 18H, CH_3). *m/z* (ESI-TOF) calc. for $C_{108}H_{133}N_{30}O_{12}$ $[M+H]^+$ 2042.0714, found 2042.0798.

2EP Cycloreversion and Singlet Oxygen Release Assays

The UV method to measure the rate of **2EP** cycloreversion in 1:9 water/acetonitrile, and the NMR method to quantify the released singlet oxygen using 2,3-dimethylbut-2-ene as a chemical trap are both described in ref. [4]. The chemiluminescence data in Fig. 2 was acquired using a Xenogen IVIS® Lumina imaging system (Caliper Life Sciences, Alameda, CA, USA) with a thermoelectrically cooled CCD camera. Vials containing solutions of **2** or **2EP** (~3 mM in 1:9 water/acetonitrile) were placed on a heated stage and warmed to 37°C, and each chemiluminescence image was acquired over 60 s with 8 × 8 binning, no filter, and the lens aperture fully open (Fstop = 1). Pixel intensity maps were analyzed using *ImageJ* software version 1.43r. The UV method for determining singlet oxygen photogeneration efficiency for **2** and Rose Bengal in 1:9 water/acetonitrile, using 1,3-diphenylisobenzofuran as a chemical trap, is described in ref. [9].

Cell Toxicity Assays

Mammalian Cell Vitality Assay

Human A549 (lung adenocarcinoma) cells were purchased from American Type Culture Collection, seeded into 96-microwell plates, and grown to a confluency of 85% in HF-12K media supplemented with 10% fetal bovine serum, 20000 U mL^{-1} penicillin, and 20000 μg mL^{-1} streptomycin at 37°C and 5% CO_2. The cells were treated with **2** or **2EP** (0–20 μM) and incubated for 24 h at 37°C. The Vybrant MTT Cell Proliferation Assay Kit (Invitrogen, Eugene, USA) was performed according to the manufacturer's protocol. In short, the medium was removed and replaced with 100 μL of HF-12K media containing [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT, 1.2 mM). The mixture was incubated for 5 h at 37°C. The purple formazan product was solubilized in 100 μL of SDS-HCl solution (1 g SDS in 10 mL of 10 mM HCl). The plates were incubated overnight at 37°C, and the absorbance of each microwell was measured at 570 nm using a microplate reader. The assays were performed in triplicate. To determine cell

vitality after irradiation with red light, the A549 cells at a confluency of 85% in HF-12K media, were incubated with either **2** (0–20 μM) or the positive control, Rose Bengal (0–300 μM) for 24 h at 37°C. The media was removed and 200 μL of fresh media was added to each well. The plates were irradiated with filtered light (>520 nm) from a 150 W Xenon Lamp for 2 h, and then placed in the dark for 24 h at 37°C. The above MTT assay was performed and the absorbance of each well was read at 570 nm.

Mammalian Cell Toxicity Assay

The cytotoxicity of **2** and **2EP** was evaluated by measuring the release of LDH using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, USA) according to the manufacturer's protocol. The assay monitors LDH catalyzed conversion of a tetrazolium salt (2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyl tetrazolium chloride) to a red formazan product using a microplate reader set at 492 nm. LDH release at each probe concentration was normalized to the LDH release from untreated cells. The assays were performed in triplicate.

Bacterial Growth Assay

Cultures of *S. aureus* NRS11 and *E. coli* UTI89 were grown overnight, and separate aliquots were used to seed broths in Luria-Bertani bacterial growth medium (total 3 mL solution) that also included varying amounts of **2** or **2EP** (0–20 μM). *S. aureus* was incubated at 37°C in an orbital shaker set at 180 rpm for 6 h while *E. coli* was incubated at the same settings for 4.5 h. Relative cell counts were determined by measuring the optical density of each sample at 600 nm. Assays were performed in triplicate.

Microscopy

A549 cells were treated with **2** (10 μM) for 1 h then counter-stained stained with DAPI (2 $\mu g\,mL^{-1}$) to visualize the cell nuclei. The cells were washed twice with HEPES buffer (10 mM, pH 7.2) before fluorescence microscopy using a Nikon Eclipse TE-2000 U epifluorescence microscope equipped with UV-2E/C (ex: 340/80 nm, em: 435/85 nm) and Cy5 (ex: 620/60 nm, em: 700/75 nm) filters. Images were acquired at 100 ms exposure using *Metamorph Software V6.2* (Universal) and analyzed using *ImageJ* 1.44 g. To visualize changes in cell morphology, A549 cells were seeded onto an 8-well chamber slide and incubated for 24 h at 37°C, 5% CO_2. The cells were treated with **2** (10 μM) for 1 h at 37°C, and the slide irradiated with Cy5 filtered light (ex: 620/60 nm) for 30 s, and brightfield images acquired at 5 s intervals for 15 min.

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References

[1] J. J. Gassensmith, J. M. Baumes, B. D. Smith, *Chem. Commun.* **2009**, 6329. doi:10.1039/B911064J

[2] A. G. White, N. Fu, W. M. Leevy, J.-J. Lee, M. A. Blasco, B. D. Smith, *Bioconjug. Chem.* **2010**, *21*, 1297. doi:10.1021/BC1000998

[3] J. J. Gassensmith, J. M. Baumes, A. Paek, A. Nguyen, B. D. Smith, *Org. Lett.* **2008**, *10*, 3343. doi:10.1021/OL801189A

[4] J. M. Baumes, J. J. Gassensmith, J. Giblin, J.-J. Lee, A. G. White, W. J. Culligan, W. M. Leevy, M. Kuno, B. D. Smith, *Nat. Chem.* **2010**, *2*, 1025. doi:10.1038/NCHEM.871

[5] T. Troy, D. Jekic-McCullen, L. Sambucetti, B. Rice, *Mol. Imaging* **2004**, *3*, 9. doi:10.1162/153535004773861688

[6] J.-J. Lee, A. G. White, J. M. Baumes, B. D. Smith, *Chem. Commun.* **2010**, *46*, 1068. doi:10.1039/B924350J

[7] S. Xiao, N. Fu, K. Peckham, B. D. Smith, *Org. Lett.* **2010**, *12*, 140. doi:10.1021/OL902546M

[8] M. Matsumoto, M. Yamada, N. Watanabe, *Chem. Commun.* **2004**, 483.

[9] E. Arunkumar, P. K. Sudeep, K. V. Kamat, B. Noll, B. D. Smith, *New J. Chem.* **2007**, *31*, 677. doi:10.1039/B616224J

[10] A. L. Niles, R. A. Moravec, T. L. Riss, *Expert Opin. Drug Discovery* **2008**, *3*, 655. doi:10.1517/17460441.3.6.655

[11] C. Pellieux, A. Dewilde, C. Pierlot, J.-M. Aubry, *Methods Enzymol.* **2000**, *319*, 197. doi:10.1016/S0076-6879(00)19020-2

[12] (a) E. J. Ngen, P. Rajaputra, Y. You, *Bioorg. Med. Chem.* **2009**, *17*, 6631. doi:10.1016/J.BMC.2009.07.074
(b) D. Kessel, *J. Porphyr. Phthalocyanines* **2004**, *8*, 1009. doi:10.1142/S1088424604000374