Storable near-infrared chemilumilescent probes for in vivo optical imaging

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ABSTRACT

A new class of chemiluminescent and fluorescent dyes and dye-doped nanoparticles can be stored at zero degrees and then made to emit near-infrared light by warming to body temperature (no chemical or electrical stimulus is needed). In vivo chemiluminescence imaging permits identification of target sites that are five times deeper than planar fluorescence imaging. A new imaging paradigm employs the dual modality probes first in high contrast chemiluminescence mode to locate relatively deep anatomical locations in vivo and subsequently in fluorescent mode to identify the microscopic targets within thin histopathology sections taken from the same specimen.

Keywords: chemiluminescence, optical imaging, fluorescence, near infrared, dyes, nanoparticles

1. INTRODUCTION

Radiotracers and radiopharmaceuticals are profoundly useful molecules in modern society with applications in biomedical research, nuclear medicine, and environmental science. High-energy emitters are used in medical imaging because the radiation penetrates through tissue, but nuclear medicine requires sophisticated equipment and is potentially radiotoxic. Indeed the inherent danger with radioactive molecules is what limits their wider utilization in modern society. The goal of our research is to invent relatively harmless, chemiluminescent versions of a radiotracer. That is, a suite of molecules and nanoparticles that can be stored and transported at typical kitchen freezer temperature and then made to emit visible or near-infrared light by simply warming them to room or body temperature. The subsequent chemiluminescence can then be detected with high signal sensitivity using the inexpensive technology that is already inside a common digital camera. At present, storable, thermally activated chemiluminescent imaging probes do not exist. This fact may surprise some readers because chemiluminescence is a mature field of study, and many valuable imaging and detection methods are based on stoichiometric or catalytic chemiluminescent reactions, including enzymatic chemiluminescence, electrogenerated chemiluminescence, and bioluminescence.[1] Chemiluminescence is well recognized for its inherent signal contrast advantages over fluorescence, and also the technical simplicity of collecting a chemiluminescent signal with modern detectors such as a charge-coupled device (CCD). Furthermore, the value of chemiluminescence is enhanced by the continued advances in low-light imaging technologies, including bioluminescent planar whole-body, optical tomography, and bioluminescent microscopy which are all under active academic and commercial development.[2] Deep-red and near-infrared chemiluminescent tracers that emit light in the window of 650-900 nm are particularly attractive for imaging of heterogeneous environmental and biomedical samples because there is very little undesired absorption and autofluorescence by common biomolecules, and diminished Rayleigh-Tyndall scattering by the matrix.[3,4] The most obvious long-term application is optical imaging of living patients (initially small animals for research purposes but eventually humans in clinical settings) where near-infrared chemiluminescent probes can be employed in situations that are complementary to radiopharmaceuticals. For example, radiopharmaceuticals are suitable for deep-tissue imaging but they emit ionizing radiation that has an inherent dosimetric health risk. In contrast, near-infrared chemiluminescent tracers would be restricted to shallower tissues or anatomical sites that can be reached by endoscopes. However, chemiluminescent tracers do not emit harmful radiation, so they may be suitable for

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longitudinal molecular imaging studies that require repeated dosing of the probe, or for imaging protocols that gain advantages by employing cheaper, smaller, and safer optical imaging instrumentation.

Most of the practically useful chemiluminescent systems involve oxidation reactions. Chemiluminescent oxidations are employed widely in chemical analyses and as detection systems for capillary electrophoresis and HPLC; whereas, electrogenerated and enzyme catalyzed oxidations of synthetic chemiluminescent substrates are effective in immunoassays, DNA analysis, and related diagnostics. In some optimized examples, fluorophores and enhancers are included in the analysis mixtures to enhance the chemiluminescent signal intensity and alter the wavelength. Luciferase enzymes are increasingly employed for biomedical studies and the expression of luciferase genes in cells is the basis for bioluminescence imaging.[5] The inherent advantage with bioluminescence is the amplified signal due to the enzymatic reaction, however, there are also important drawbacks. By definition the expressed luciferase requires a substrate, such as D-luciferin or colelenterazine, and external addition of these substrates obviously complicates the imaging experiment, especially for in vivo samples (an exception is the bacterial lux operon which expresses its own substrate, but it suffers from poor emission profile). Furthermore, current bioluminescent systems emit predominantly visible light which is readily scattered and does not penetrate far through heterogeneous matrices. The need for bioluminescent systems that emit red-shifted light with deeper tissue penetration is well recognized, and hybrid-BRET (bioluminescence resonance energy transfer) systems have been prepared that genetically or chemically attach luciferase enzymes to red fluorescent proteins, dyes, or quantum dots.[6] These hybrid-BRET approaches have attractive features but none of them actually produce an independent, self-illuminating nanoscale object that can emit chemiluminescence without any chemical input.

The development of storable, thermally activated chemiluminescent and fluorescent dyes would enable a range of biotechnology and nanotechnology applications that are based on multiplex detection or utilize resonance energy transfer as a signaling event. Furthermore, the ability to regenerate the chemiluminescence emission by red light irradiation would be a unique way to create chemiluminescent surface patterning effects, and also a potentially powerful method of improving detection sensitivity for gels, microarrays, and histology sections by repeated cycles of irradiation and chemiluminescence signal acquisition. Although quite a few organic molecules exhibit chemiluminescence, very few are amenable to synthetic development as storable, chemiluminescent tracers. Aromatic endoperoxides are known to undergo chemiluminescent cycloreversions and they have been studied mechanistically for several decades but never considered as imaging probes.[7] In any case, these early examples did not have properties that enabled chemiluminescence imaging. For example, the cycloreversions of anthracene endoperoxides typically require high temperatures (>60 °C) and the reactions emit UV/Vis light. The literature contains scattered reports of organic compounds that have been evaluated as sensors for singlet oxygen; that is, they react with singlet oxygen and make a product that is spontaneously chemiluminescent or becomes chemiluminescent after a second chemical reaction.

This paper describes our discovery of Squaraine Rotaxane Endoperoxides (SREPs) as a new class of fluorescent molecules that have the unique ability to become storable, thermally activated chemiluminescent tracers. The results demonstrate the potential value of these unique, self-illuminating organic compounds for programmable chemiluminescence (*i.e.*, output parameters such as emission wavelength, quantum yield, and decay rate can be controlled by molecular design). The raises the idea of a new imaging paradigm that employs the dual modality probes first in high contrast chemiluminescence mode to locate relatively deep anatomical locations in vivo and subsequently in fluorescent mode to identify the microscopic targets within thin histopathology sections taken from the same specimen.

2. RESULTS AND DISCUSSION

2.1 Photochemistry

We have developed efficient methods of making SREPs in high yield, large scale, and high purity. The prototype is **NIR-SREP** which can be stored indefinitely at -20 °C but upon warming to body temperature it undergoes a cycloreversion reaction that cleanly reverts back to the parent squaraine rotaxane (Fig. 1a).[8] The activation energy for cycloreversion is 113 kJ/mol, and the half life at 38 °C is 3.2 hours. Standard chemical trapping experiments in $CDCl_3$ using 2,3-dimethylbutene as a trap for singlet oxygen, indicated that at least 64±10 % of the released molecular oxygen was singlet oxygen. In Fig. 1b is a false-colored pixel intensity map of the emission from a solution of **NIR-SREP** in $CDCl_3$ at 38°C. The spectral plots in Fig. 1c show that the chemiluminescence and fluorescence maxima are both at 733 nm, indicating that the chemiluminescence signal is emitted from the encapsulated squaraine chromophore. The chemiluminescence decreases over time but the decay is not a simple exponential, it is a biphasic curve with an initial

rapid drop over the first few minutes followed by a slower decay with a half-life of several hours. The integrated chemiluminescence intensity is linear over a sixteen-fold concentration range, demonstrating the potential of SREPs to act as chemiluminescent tags for quantitative detection and sensing. Furthermore, the chemiluminescence can be regenerated by simply irradiating an aerated sample with red light after it had decayed (Fig. 1d), suggesting that signal regeneration may be a novel way to increase chemiluminescence detection sensitivity. The potential utility in surface based detection technologies was assessed by spotting samples of **NIR-SREP** onto reverse-phase TLC plates (glass sheets supporting a thin layer of porous silica particles with impregnated C18 hydrocarbon) and acquiring pixel intensity maps using our Xenogen IVIS\ imaging station equipped with a CCD camera. In Figs. 1e-f are chemiluminescence and fluorescence images, respectively, of a surface with a progression of spot sizes. The smallest spot is approximately 1 mm diameter, contains about 17 picomoles of **NIR-SREP**, and it is easily identified using either chemiluminescence or fluorescence imaging. This highlights the detection versatility of SREPs. Both **NIR-SREP** and the squaraine rotaxane decay product have essentially identical near-infrared fluorescence properties, thus the intrinsic bright fluorescence of a sample spot hardly changes as the chemiluminescence reaction proceeds, which means that the plate can be read by either imaging modality.

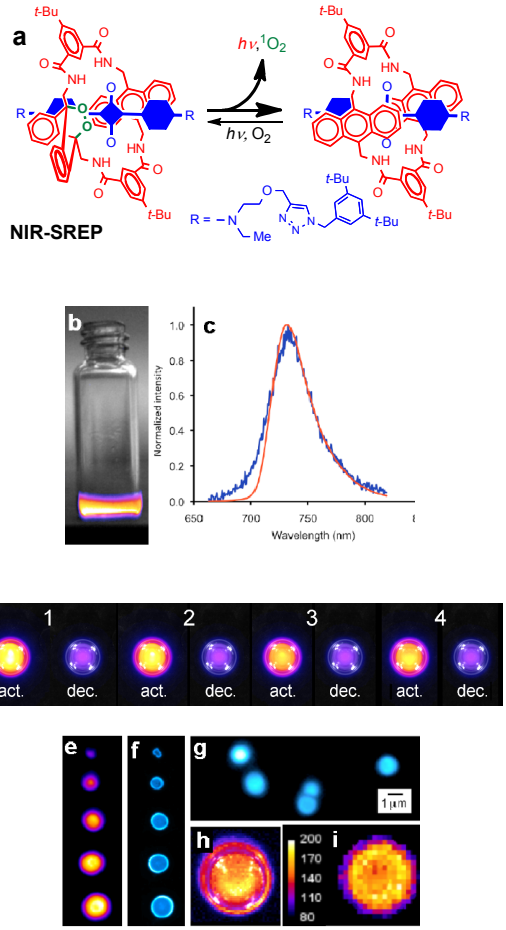

Figure 1. (a) Cycloreversion of **NIR-SREP** releases singlet oxygen and emits near-IR light. (b) False-colored pixel intensity map of the emission from a solution of **NIR-SREP** in $CDCl_3$ at 38°C. (c) Spectral plots showing that the chemiluminescence and fluorescence maxima are both at 733 nm (d) Series of four regeneration cycles of a vial containing **NIR-SREP**. After substantial signal decay (dec.), the chemiluminescence is activated (act.) by brief irradiation. (e,f) chemiluminescence and fluorescence images, respectively, of a surface with a progression of spot sizes. (g) Fluorescence micrograph of polystyrene microparticles (0.9 µm) that have been functionalized with carboxylate groups and stained with **NIR-SREP** (h) Chemiluminescence image of a vial containing an aqueous dispersion of these microparticles. (i) Chemiluminescence image of a vial containing an aqueous dispersion of silica nanoparticles (50 nm average diameter) that encapsulate **NIR-SREP** (20 µM average dye concentration).

It is technically straightforward to permanently encapsulate SREPs inside polymeric or silica microparticles and nanoparticles. The process for staining polymeric nanoparticles simply involves mixing the hydrophobic dye with commercially available particles under swelling conditions.[9] In Fig. 1g is a fluorescence micrograph of polystyrene microparticles (0.9 μm) that have been functionalized with carboxylate groups and stained with **NIR-SREP**, and in Fig. 1h is a chemiluminescence image of a vial containing an aqueous dispersion of these microparticles. A three-step process is employed to fabricate SREP-doped silica nanoparticles with controllable diameters down to 10 nm.[10] Micelle based fabrication methods are used to prepare the precursor silica nanoparticles with encapsulated squaraine rotaxane, then the nanoparticle surface is modified by straightforward silica conjugation chemistry, and finally, irradiation of the nanoparticles with red light in the presence of air converts the encapsulated squaraine rotaxane into SREP. In Fig. 1i is a chemiluminescence image of a vial containing an aqueous dispersion of silica nanoparticles (50 nm average diameter) that encapsulate **NIR-SREP** (20 μM average dye concentration).

2.2 Chemiluminescence Mechanism

Obviously, an improved understanding of the chemiluminescence mechanism will facilitate efforts to fabricate high performance chemiluminescent dyes and particles. The preliminary data strongly suggest that the chemiluminescence originates from the encapsulated squaraine chromophore whose excited state is activated by the cycloreversion process. The following experimental observations suggest that chromophore activation is mediated by the released singlet oxygen: (a) The presence of efficient singlet oxygen quenchers greatly diminishes the chemiluminescence intensity but has no effect on the fluorescence emission. (b) chemiluminescence intensity for **NIR-SREP** is five times higher when the solvent is changed from $CHCl_3$ to $CDCl_3$, which correlates with longer singlet oxygen lifetime in the deuterated solvent. (c) Thermally generated singlet oxygen in the absence of a chromophore produces a weak dimol ($2\bullet^1O_2$) emission that is observed in the DsRed channel (575-650 nm), but in the presence of a squaraine dye there is energy transfer and dye emission in the Cy5.5 channel (695-770 nm). An intriguing point with this latter observation is that the energy released by decay of excited state singlet oxygen to ground state triplet oxygen is only 94 kJ/mol and not enough to excite a squaraine chromophore to its singlet state. Previous studies of intermolecular sensitization of dye fluorescence by singlet oxygen have proposed several related mechanisms that involve dye excitation by two molecules of singlet oxygen.[11] This leads us to consider the four mechanisms in Figure 2.

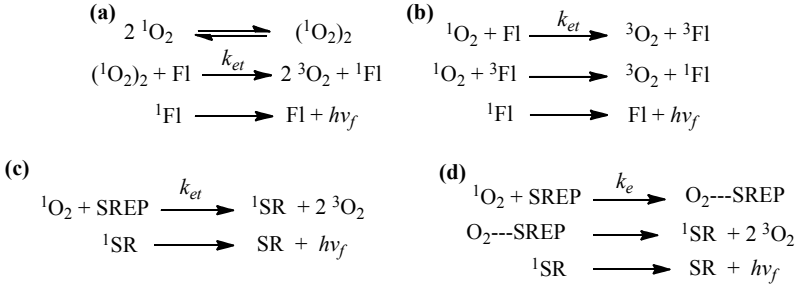

Figure 2. Four possible chemiluminescence mechanisms for SREP (squaraine rotaxane endoperoxide) cycloreversion leading to excited singlet state SR (squaraine rotaxane).

The first (Fig. 2a) envisions two singlet state oxygen molecules associating to form a dimol, which subsequently transfers energy to a fluorophore (Fl) and produces an excited singlet state (^{1}Fl). The concern with this mechanism for SREP chemiluminescence is that dimol formation is highly unfavored and likely to be present in very small amounts due to the low steady state flux of thermally generated singlet oxygen. An alternative version (Fig. 2b) requires that two singlet oxygens sequentially excite Fl to ^{1}Fl via triplet state, ^{3}Fl.[12] This is only possible if the fluorophore has a low lying ^{3}Fl state (which may be true in the case of squaraine dyes) and that the ^{3}Fl lifetime is long enough to accept energy from a second singlet oxygen. It is known that squaraine rotaxanes have relatively long triplet state lifetimes (> 200 μs); however, the likelihood that it is long enough for efficient intermolecular energy transfer with a second, thermally generated, singlet oxygen seems to be low. This leads us to consider additional mechanisms (c) and (d) in Fig. 2. These two mechanisms are more specific for SREPs. Mechanism (c) in Figure 2 envisions a singlet oxygen molecule (generated by a non-CL SREP cycloreversion reaction) exciting a second ground state SREP molecule to undergo a spin-

allowed cycloreversion that produces an excited singlet state squaraine rotaxane and two triplet oxygens. Attractive features with this mechanism are: (i) chemiluminescence requires only one bimolecular interaction of ground state SREP and singlet oxygen, (ii) the electronic energy of singlet oxygen (94 kJ/mol) nearly matches the activation energy for SREP cycloreversion (113 kcal/mol).

One general trend that we have observed with the chemiluminescence data in hand is that emission intensity appears to decay with biphasic kinetics; typically, there is an initial rapid drop from high chemiluminescence intensity over the first few minutes followed by a slower decay process with a half-life of several hours. One possible explanation is the squaraine rotaxane product of the SREP cycloreversion inhibits the chemiluminescence process by quenching the released singlet oxygen. However, evidence against this possible explanation was gained from control experiments that added squaraine rotaxane to starting samples of **NIR-SREP** and produced no change in chemiluminescence intensity or decay profile. Another possible explanation for the biphasic kinetics is that chemiluminescence is produced by a combination of two or more different processes. In the early stages, there is an efficient but rapidly decaying chemiluminescence process that is favored by a high flux of singlet oxygen, but this pathway becomes less significant as the singlet oxygen flux decreases, and in the later stages the observed chemiluminescence is due primarily to a second, slower, and less efficient mechanism. While considering these mechanisms, it is important to remember that multiple processes may contribute to the light emission and the fraction that each pathway contributes may change as the chemiluminescence proceeds and the mole fraction of reaction components changes. The mechanism may also change with the different chromophore emission energies.

2.3 Mouse Imaging

A series of systematic mouse imaging studies using nanoparticles doped with **NIR-SREP** have enabled us to assess how target signal contrast for chemiluminescence and fluorescence changes with tissue depth of the probe. Target signal contrast can be measured in several ways, but the simplest is to determine the target to background ratio (TBR) by measuring the mean pixel intensity of the target region (T) and the mean pixel intensity of a non-target region (B) that emits only background signal. We have discovered a remarkable comparative trend. Compared to fluorescence imaging, we find that chemiluminescence is much less "surface weighted" as an imaging modality. With superficial target sites (~1 mm deep), fluorescence imaging produces the highest TBR because the probe can be excited multiple times by the illumination light, which means that the target fluorescence intensity is very high. But the TBR for fluorescence imaging quickly drops close to unity with tissue depths of more than a few millimeters due to the increased contribution of the background signal. In comparison, the TBR for chemiluminescence decreases much more gradually with tissue depth, in large part because background emission from the host animal is inherently very low. This trend is illustrated by the mouse imaging experiment in Figure 3. An aliquot of carboxylate functionalized **NIR-SREP**-microparticles (50 µL) was injected subcutaneously into the dorsal side of a nude mouse rear leg. A region of interest (ROI) analysis of the dorsal images in Figure 3 indicated a TBR of 6.9 for chemiluminescence and 29 for fluorescence. The ventral images in Figure 3 required the light to penetrate a greater thickness of skin and leg tissue (~7 mm). The target signal intensities are attenuated, but the chemiluminescence TBR of 6.8 remains quite good, whereas, the fluorescence TBR of 4.4 is considerably lower. These results agree with other data indicating that planar chemiluminescence imaging can detect target sites that are five times deeper than planar fluorescence imaging.[8]

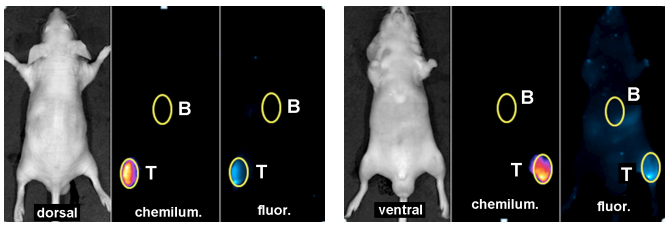

Figure 3. Planar chemiluminescence and reflected fluorescence from NIR-SREP-microparticles injected subcutaneously into the dorsal side of a nude mouse rear leg at 38 °C. (left) Dorsal bright field, chemiluminescence, and fluorescence images (chemiluminescence and fluorescence TBR = 6.9 and 29, respectively). (right) Ventral images which required light penetration through deeper tissue (chemiluminescence and fluorescence TBR = 6.8 and 4.4, respectively).

3. CONCLUSIONS

The results suggest a new paradigm for optical molecular imaging using SREP-based particles as dual modality probes. They can be used first in high contrast chemiluminescence mode to locate relatively deep anatomical locations of the probes in vivo and then employed subsequently in fluorescent mode to identify the microscopic targets within thin histopathology sections taken from the exact same specimen. This imaging paradigm should expand the scope of small animal optical imaging experiments that can be conducted using common planar imaging stations.

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REFERENCES

[1] Su, Y. S., Chen, H., Wang, Z. and Lv, Y., "Recent advances in chemiluminescence," Appl. Spectrosc. Rev. 42, 139-176 (2007).

[2] Wang, G., Cong, W., Shen, H., Qian, X., Henry, M. and Wang, Y., "Overview of bioluminescence tomography-a new molecular imaging modality," Front. Biosci. 13, 1281-1293 (2008).

[3] Bashkatov, A. N., Genina, E. A., Kochubey, V. I. and Tuchin, V. V., "Optical properties of human skin, subcutaneous and mucous tissues in the wavelength range from 400 to 2000 nm," J. Phys D: Appl. Phys. 38, 2543-2555 (2005).

[4] Rice, B. W. and Contag, C. H., "The importance of being red," Nat. Biotechnol," 7, 624-625 (2009).

[5] Xia, Z. and Rao, J., "Biosensing and imaging based on bioluminescence resonance energy transfer," Curr. Opin. Biotech. 20, 37-44 (2009).

[6] Wu, C., Mino, K., Akimoto, H. and Kawabata, M. et al., "In vivo far-red luminescence imaging of a biomarker based on BRET from cypridina bioluminescence to an organic dye," Proc. Natl. Acad. Sci. USA 106, 15599-15603 (2009).

[7] Adam, W., Kazakov, D. V. and Kazakov, V. P., "Singlet-oxygen chemiluminescence in peroxide reactions," Chem. Rev. 105, 3371-3387 (2005).

[8] Baumes, J. M., Gassensmith, J. J., Giblin, J., Lee, J. -J., White, A. G., Culligan, W. J., Leevy, W. M., Kuno, M. and Smith, B. D., "Storable, thermally activated, near-infrared chemiluminescent dyes and dye-stained microparticles for optical imaging," Nature Chem. 2, 1025-1030 (2010).

[9] Sharma, P., Brown, S., Varshney, M. and Moudgil, B. "Surfactant-mediated fabrication of optical nanoprobes," Adv. Polym. Sci. 218, 189-233 (2008).

[10] Tan, W., Wang, K., He, X., Zhao, X. J., Drake, T., Wang, L. and Bagwe, R. P., "Bionanotechnology based on silica nanoparticles," Med. Res. Rev. 24, 621-638 (2004).

[11] Fu, Y., Krasnovsky, A. A. Jr. and Foote, C. S., "Singlet oxygen dimol-sensitized luminescence from thermally generated singlet oxygen," J. Am. Chem. Soc. 115, 10282-10285 (1993).

[12] Murphy, S. T., Kondo, K. and Foote, C. S. "Singlet-oxygen-sensitized delayed fluorescence: direct detection of triplet phthalocyanine as an intermediate," J. Am. Chem. Soc. 121, 3751-3755 (1999).