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A Mechanistically-Distinct Approach to Fluorescence Visualization of Singlet Oxygen.

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We describe fluorescence detection of $^1O_2$ by a new strategy. Oxidation of a non-fluorescent sulfoxide by $^1O_2$ occurs via intramolecular oxygen atom transfer in a reactive persulfoxide intermediate. The resulting sulfone shows significantly enhanced (> 50-fold) emission. This approach complements known methods, and is being extended to biological $^1O_2$ imaging.

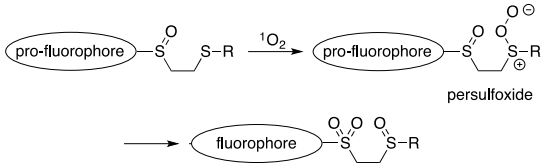
Reactive oxygen species (ROS) play manifold important roles in cell biology.[1] They are linked to aging and numerous disease states due to their ability to damage proteins and DNA. They are also important in oxidative stress-induced cell apoptosis. While background ROS damage is primarily the result of "leakage" from oxidative metabolism in mitochondria, it is now clear that there are controlled mechanisms for the release of both low and high concentrations of ROS in intracellular media. At lower concentrations, ROS play a role – incompletely understood – in cell signaling pathways. $^1O_2$ is an outlier, as it is not generated in the mitochondria, but rather in systems where a photosensitizer is present. This is important for the detailed study of photodynamic therapy (PDT),[2] where it the primary ROS responsible for inducing the apoptosis of cancer cells.

The study of ROS biology is complicated by the numerous chemical species involved, including $^1O_2$. Critical in deciphering their biological roles has been the development of ROS-selective fluorescent probes, such that the presence and localization of ROS can be imaged, and even quantified, in cells.[1-4] We describe here a new approach to fluorescence-based detection of $^1O_2$.

We have previously devoted much effort to designing or discovering new mechanisms by which sulfoxide metal ion-binding or oxidation can be converted to a turn-on fluorescence signal.[5,6] While there are already a number of useful probes for ROS imaging,[3,6] new signaling mechanisms are still needed. Each mechanism brings with it characteristic advantages and limitations; the more signaling mechanisms available, the more complementary approaches to fluorescent probes can be developed and explored.

In the present case, our approach to $^1O_2$ detection was inspired by our observation that the oxidation of non-fluorescent sulfoxides can produce highly-fluorescent sulfones.[5] $^1O_2$ displays unique sulfur oxidation chemistry, differentiating it from other ROS.[7,8] The reaction of $^1O_2$ with sulfides is known to form reactive persulfoxide intermediates, which undergo two primary reactions: direct decomposition to release $^3O_2$, and oxygen atom transfer to other sulfides.[8] While oxidation of sulfoxides by $^1O_2$ has no intramolecular precedent, it has been shown in select studies to be a viable intermolecular reaction.[9] We reasoned that the high-energy persulfoxide intermediate should be capable of intramolecular oxygen atom transfer to a proximal sulfoxide,[9,10] which in turn could produce a fluorescence response (Scheme 1). Here we describe validation of this mechanistically and conceptually distinct approach to $^1O_2$ detection.

Elaborating on the role of $^1O_2$ in biological systems, $^1O_2$ is unusual in that it not only damages proteins and DNA, but also oxidizes lipids to lipid peroxides via ene reactions,[1-4] a membrane-disrupting process not prevalent for other ROS. It is the most important oxidant in PDT.[2] Infusing cancer cells with an appropriate sensitizer, followed by irradiation with light, turns endogenous $^3O_2$ to $^1O_2$. This leads to such egregious oxidative damage to the tumor cell that it undergoes apoptosis. $^1O_2$ is also generated by other, non-sensitized, cellular processes, such as neutrophilic oxidative burst.[11]



Scheme 1. Detection of $^1O_2$ via persulfoxide formation.

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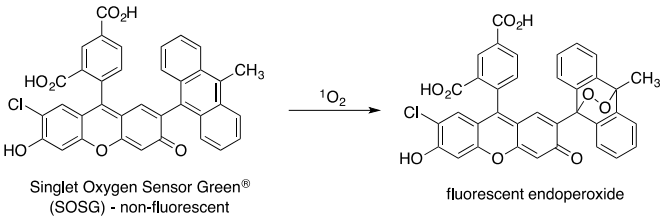


Scheme 2. Reaction of SOSG with singlet oxygen.

As promising as PDT is for the treatment of light-accessible tumors, improvement of sensitizers requires further study of the related sub-cellular processes. Among the most relevant are sensitizer localization, $^1O_2$ quantification, and kinetic analysis.

This makes fluorescence detection of $^1O_2$ an important problem.

$^1O_2$ can be imaged, and even quantified, by its near-IR phosphorescence (1274 nm). However, this requires specialized instrumentation and is limited by the low phosphorescence quantum yield.[4] More commonly employed are $^1O_2$-responsive small-molecule probes.[2-4] An archetypal example is the widely-used probe, Singlet Oxygen Sensor Green (SOSG; Scheme 2).[12,13] SOSG exploits the Diels-Alder reaction of $^1O_2$ and an anthracene-bearing xanthene derivative. Prior to the reaction, SOSG exhibits weak green fluorescence. Following formation of the endoperoxide, the emission increases substantially in intensity. SOSG is in widespread use, and can be effective for $^1O_2$ quantification. However, its use can be limited by membrane-permeability, unpredictable cellular localization, endoperoxide photobleaching, the ability of the endoperoxide itself to sensitize $^1O_2$ formation, as well as other factors. Thus, while SOSG is a powerful tool, there are clear potential advantages to mechanistically orthogonal alternatives.

The chemistry of $^1O_2$ reactions with sulfides is surprisingly diverse.[7,8] Most important for this study is the generation of a reactive persulfoxide intermediate (Scheme 1). The anticipated fluorescence response is based on oxidization of a non-fluorescent sulfoxide to a fluorescent sulfone. As we have shown, the photophysics of fluorescence quenching in such sulfoxides is complex, but tractable. The "dark" excited state processes originate with relaxation of the initially-formed excited state to an intramolecular charge-transfer (ICT) state.[5] This ICT state then partitions between a small amount of radiative decay, modest deactivation via sulfoxide pyramidal inversion, and dominant relaxation to a non-radiative twisted intramolecular charge transfer (TICT) state. Oxidation of the sulfoxide blocks ICT and the subsequent non-radiative processes, leading to fluorescence enhancement.

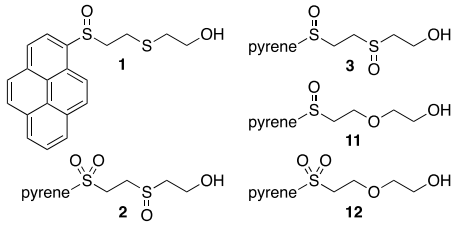
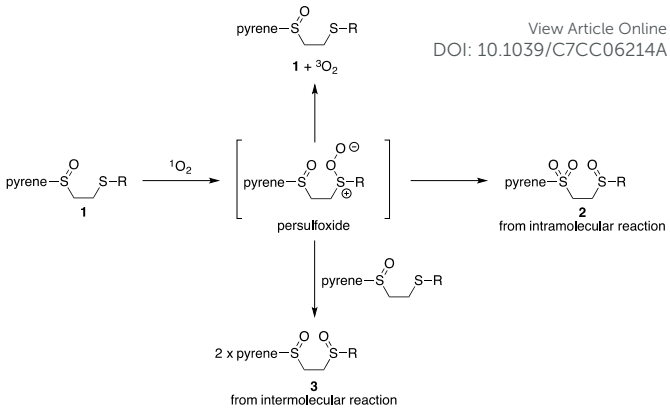


Chart 1. Singlet oxygen probe and control molecules. See Supporting Information for synthetic details.



Scheme 3. Expected partitioning of the persulfoxide intermediate.

We chose pyrene as our initial reporting fluorophore, based on our understanding its photophysics, and the relative ease of synthesizing pyrene derivatives. A set of probes and control molecules were designed and prepared (Chart 1), based on the expected partitioning of the persulfoxide intermediate (Scheme 3). The set comprises the initial probe (**1**), the anticipated products of intramolecular (**2**) and intermolecular oxygen atom transfer (**3**), and control compounds **11** and **12**. The quantum yields of **1**–**3** confirm the initial hypothesis that intramolecular oxygen atom transfer to form **2** would lead to enhanced fluorescence: ϕ (**1**) = 0.01; ϕ (**2**) = 0.55; ϕ (**3**) = 0.01.[14]

While the hydroxyethyl group of **1** improves its solubility in polar solvents, **1** is not entirely water soluble. We thus examined photooxidation of **1** in THF/CH_3OH and DMF/H_2O mixtures, using tetraphenylporphyrin (TPP) as the sensitizer.

There is a pronounced solvent dependence in the partitioning between the intramolecular oxidation product (**2**) and the intermolecular oxidation product (**3**). In pure THF, formation of **3** predominates, while in THF/CH_3OH mixtures increased formation of **2** is observed as the proportion of CH_3OH increases (Table 1).[14-17] Similar results are observed in DMF/H_2O.[14] As expected for unimolecular formation of **2** and bimolecular formation of **3**, the product distribution is concentration-dependent (Table 2). Further, **3** is inert to photooxidation, and **12** is not formed during attempted oxidation of **11**. These observations exclude direct oxidation of the sulfoxide moiety of **1** by $^1O_2$, and indicate that the formation of **2** from **1** occurs solely through an intramolecular process.

Table 1.[a-d]

CH_3OH:THF	**2** (%)	**3** (%)
10:90	6	94
30:70	14	86
50:50	33	67
70:30	33	67
90:10	52	48

[a] All reactions at 10^{-4} M. [b] The quantum yield of **2** does not vary appreciably as a function of solvent composition (ϕ = 0.55±0.01). [c] Typical reaction times 4-8 hours. [d] For details of all parameters, including qualitative kinetic analysis, see Supporting Information.

Table 2.[a,b]

Concentration	**2** (%)	**3** (%)
10^{-4} M	17	83
10^{-5} M	27	73
10^{-6} M	33	67
10^{-7} M	48	52

[a] Reactions in 70:30 CH_3OH:THF, to ensure complete sensitizer dissolution. [b]See Supporting Information for details.

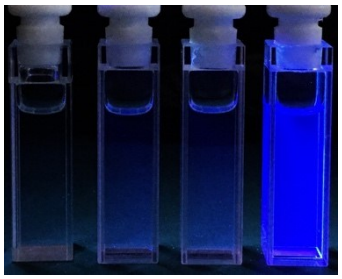


Figure 1. Emission from blank, **1**, **3**, and **2** (10^{-4} M; 70:30 CH_3OH:THF).

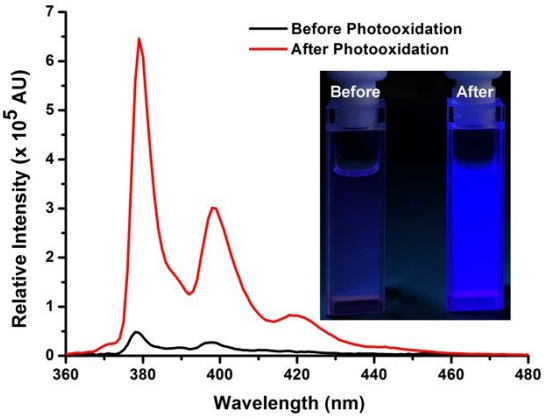
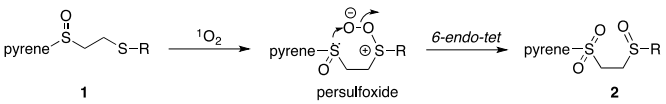


Figure 2. Photooxidation of **1** (10^{-6} M; 70:30 CH_3OH:THF).

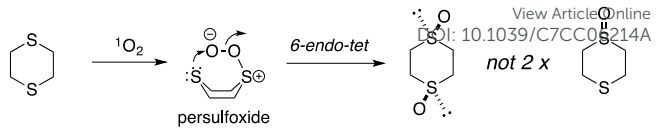
These trends are affirming, in that the ultimate objective is the development of probes that function in intracellular (aqueous) media at low concentration. The utility of this approach to $^1O_2$ detection can readily be seen by visual inspection of samples of **1**–**3**, and of **1** before and after photooxidation (Figures 1, 2).[14,17]

A clear molecular design issue is the suppression of the formation of **3**. In this respect, we note that the intramolecular oxygen atom transfer (**1** → **2**) is a *6-endo-tet* reaction, in Baldwin's terms,[18] and it is thus a generally disfavored reaction (Scheme 4).

Two approaches to suppressing formation of **3** are apparent. First, the distance between the S atoms could be increased, leading to a more favoured transition state.[18] However, this approach could lead to both chemical and entropic problems. Alternatively, some form of cyclic constraint could be put in place to enforce intramolecular oxidation, overriding unfavorable orbital overlap considerations. An important precedent for this is the



Scheme 4. *6-endo-tet* O-atom transfer.



Scheme 5. Photooxidation of dithiane.

intramolecular reaction to be favoured, despite the disfavoured reaction of 1,4-dithiane with $^1O_2$, which leads to formation of the corresponding *cis*-bis-sulfoxide to the exclusion of intermolecular disproportionation,[19] despite bis-sulfoxide formation being a *6-endo-tet* reaction that proceeds through a chair conformation (Scheme 5).[20] This example is not directly structurally related to our next generation of molecules, but illustrates the general advantage of cyclic constraint.

The above results and discussion define a conceptually and mechanistically distinct approach to $^1O_2$ detection. Further development will require controlling inter- vs. intramolecular oxygen atom transfer from the key persulfoxide intermediate. In addition, analogous water-soluble probes with longer-wavelength excitation and emission are essential. With the body of evidence provided, and the underpinning mechanistic understanding, these are attainable goals. Additional studies in these respects are ongoing.

Still, it should be noted that these efforts will surely not provide "the" definitive approach to $^1O_2$ imaging. It is highly unlikely that there will ever be a universal single-molecule probe for fluorescence visualization of any specific molecule or ion of biological significance. Rather, a panoply of complementary probes will be required, each with its own optimal use. Thus, each distinctly new approach leads to new possibilities.

Conflicts of interest

There are no conflicts to declare.

Notes and references

§ The authors thank Prof. Ed Clennan (University of Wyoming) for insightful discussions.

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9 Diphenylsulfoxide (Ph_2SO) does not react directly with $^1O_2$. However, in the presence of $^1O_2$ and diethylsulfide (Et_2S), it is readily oxidized to diphenylsulfone, providing clear evidence that the Et_2S-derived persulfoxide can oxidize sulfoxides. See refs 7, 8.

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13 SOSG is commercially available from ThermoFisher Scientific.

14 See Supporting Information.

15 The addition of even small amounts of CH_3OH is known to stabilize persulfoxides. (See ref. 2, and Supporting Information.) Still, it is not immediately obvious why CH_3OH so strongly influences the ratio of **2**:**3**. See Supporting Information, Section 6, for discussion.

16 The solubility of **1** and the sensitizer (tetraphenylporphyrin; TPP) preclude the use of entirely hydroxylic solvent systems.

17 Regardless of how much **3** is formed, it remains non–emissive, and thus invisible to fluorescence visualization.

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19 C.S Foote, J.W. Peters., *J. Am. Chem. Soc.*, 1971, **93**, 3796–3796; C. S. Foote, J. W. Peters. Int. Congr. Pure Appl. Chem., 1971, **4**, 129–154. It must be noted that the bis-sulfoxide forms only in dry, aprotic solvents (benzene, acetonitrile). In protic solvents, no bis-sulfoxide is formed, only the monosulfoxide arising from intermolecular reaction. In contrast, observe that increased % CH_3OH increases the proportion of intramolecular reaction of **1**. We are unable to explain the discrepancy at this time.

20 While the boat conformation of 1,4-dithiane is higher in energy than the chair form, the energetics differ surprisingly little from those of cyclohexane. Thus, rapid chair-to-chair inversion at RT, via the requisite boat conformers, is expected. The conformational energy profile of the *cis*-bis-sulfoxide is not unambiguously resolved. See: F. Lautenschlaeger, G. F. Wright, *Can. J. Chem.* 1963, **41**, 1972-1983; A. Watanu, Sulfur Reports, 1981, **1**, 147-207; F. Freeman, E. Derek, *J. Comp. Chem.* 2002, **24**, 909-919; J. S. Cruz-Sánchez, E. Juariti, *Tetrahedron Lett.* 2002, **43**, 9369-9372. Overall, we anticipate the ready availability of appropriate conformations in seeming sterically congested, constrained systems, e.g., derivatives of the cis or trans isomers of 1,2-cyclohexanedithiol.