A Highly Efficient Chemiluminescence Probe for the Detection of Singlet Oxygen in Living Cells

Nir Hananya*, Ori Green†, Rachel Blau, Ronit Satchi-Fainaro,* and Doron Shabat*

Abstract: Singlet oxygen is among the reactive oxygen species (ROS) with the shortest life-times in aqueous media because of its extremely high reactivity. Therefore, designing sensors for detection of $^1O_2$ is perhaps one of the most challenging tasks in the field of molecular probes. Herein, we report a highly selective and sensitive chemiluminescence probe (SOCL-CPP) for the detection of $^1O_2$ in living cells. The probe reacts with $^1O_2$ to form a dioxetane that spontaneously decomposes under physiological conditions through a chemiexcitation pathway to emit green light with extraordinary intensity. SOCL-CPP demonstrated promising ability to detect and image intracellular $^1O_2$ produced by a photosensitizer in HeLa cells during photodynamic therapy (PDT) mode of action. Our findings make SOCL-CPP the most effective known chemiluminescence probe for the detection of $^1O_2$. We anticipate that our chemiluminescence probe for $^1O_2$ imaging would be useful in PDT-related applications and for monitoring $^1O_2$ endogenously generated by cells in response to different stimuli.

Reactive oxygen species (ROS) have an increasingly recognized role in cell signaling and stress response.1] Among all ROS, singlet oxygen ($^1O_2$) is getting substantial attention,2] especially in view of the recent progress in the field of photodynamic therapy (PDT) for cancer treatment.3] Therefore, real-time monitoring of $^1O_2$ under biologically relevant conditions is of great interest. Although the phosphorescence of $^1O_2$ at a wavelength of 1270 nm can be directly observed,4] it is very challenging to detect small amounts of $^1O_2$ by such methods because of the extremely low quantum yield under aqueous conditions.5] Alternative approaches were aimed to develop small-molecule reaction-based probes for $^1O_2$,6] among them, fluorescent turn-on probes have appeared as the most sensitive.7] Several fluorescent probes were also suitable for the imaging of $^1O_2$ in living cells.8] In general, chemiluminescence imaging might be advantageous over fluorescence imaging, since in chemiluminescence there is no need for light irradiation, and thus background signal is extremely weak.9] Explicitly, for the imaging of $^1O_2$, the advantage of chemiluminescence over fluorescence is even more substantial, since the light used for excitation of the dye could induce $^1O_2$ generation by itself.10]

Several years ago, McNeill and co-workers reported a “trap-and-trigger” chemiluminescent probe for $^1O_2$.11] They used the enol-ether precursor of Schaap’s dioxetane to trap the $^1O_2$ at the first step. In a second step, the dioxetane chemiluminescence was triggered by the addition of fluoride. In this system, chemiluminescence measurements were limited to organic solution owing to the quenching of the emitting species by water.12] We therefore speculated that a dioxetane that efficiently emits light in aqueous solution could be utilized to develop a chemiluminescent probe that is capable of real-time monitoring of $^1O_2$ production under physiological conditions.

Recently, we reported a striking substituent effect for Schaap’s chemiluminescent dioxetanes, obtained through the incorporation of an electron withdrawing group at the ortho-position of the phenol.13] These new dioxetane luminophores were able to emit light under aqueous conditions with an intensity 3000-fold greater than that of the original Schaap’s dioxetane. We sought to use the enol-ether precursor of our highly bright dioxetane luminophores as a chemiluminescent probe for the detection of $^1O_2$. Herein, we report a new highly efficient chemiluminescence probe for detection of singlet oxygen under physiological conditions.

Our strategy for real-time monitoring of $^1O_2$ by chemiluminescence is presented in Figure 1. Probe SOCL (singlet oxygen chemiluminescence) reacts with $^1O_2$ to generate a phenol-dioxetane species. This species spontaneously decomposes in water ($t_{1/2} = 10$ min) through a chemiexcitation process to produce the corresponding electronically excited benzoox ester. The decay of the latter to its ground-state is accompanied by the emission of highly bright green light. In our design, the role of the acrylic acid substituent at the ortho

---

[1] N. Hananya, O. Green, Prof. D. Shabat
[2] School of Chemistry, Faculty of Exact Sciences, Tel Aviv University
[3] Tel Aviv 69978 (Israel)
[4] E-mail: chdoron@post.tau.ac.il
[6] Department of Physiology and Pharmacology, Faculty of Medicine,
[7] Tel Aviv University
[8] Tel Aviv 69978 (Israel)
[9] E-mail: ronitsf@post.tau.ac.il

* These authors contributed equally to this work.

** Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under:
https://doi.org/10.1002/anie.201705803.
position of the phenol is to form a donor–acceptor pair that increases the emissive nature of the benzoate intermediate. The addition of the chlorine substituent at the other ortho position reduces the pKa of the phenol and thus enriches the percentage of the phenoxy ion under physiological conditions. Consequently, the chemiexcitation kinetics of the phenol-dioxetane is accelerated to enable the real-time monitoring of its formation.

**SOCL** probe was synthesized according to a previously developed method (see the Supporting Information for synthetic details). The chemiluminescence response of **SOCL** to singlet oxygen was examined by incubation of the probe with 3-(1,4-dihydro-1,4-epidioxy-4-methyl-1-naphthyl)propionic acid (EP-1). The latter is a known water-soluble compound that produces $^{1}\text{O}_2$ through a thermal decomposition.\[15\] As shown in Figure 2A, **SOCL** exhibited an expected chemiluminescent exponential decay kinetic profile in the presence of EP-1, while no chemiluminescence was obtained in the absence of EP-1 (for the chemiluminescence spectrum see the Supporting Information, Figure S1). A RP-HPLC assay showed complete conversion of **SOCL** to the dioxetane upon reaction with $^{1}\text{O}_2$. The dioxetane decomposes to its corresponding benzoate ester, in accordance with the mechanism presented in Figure 1 (data are presented in the Supporting Information, Figure S2). The **SOCL** probe was also incubated with different concentrations of EP-1 for 30 min in PBS, pH 7.4, 37°C. The obtained linear correlation between the EP-1 concentration and integrated chemiluminescence signal (Figure 2B) suggests that the $^{1}\text{O}_2$ concentration could be straightforwardly determined quantitatively. **DMAX**, a known fluorescent probe for $^{1}\text{O}_2$, is considered by Nagano and co-workers as the “best fluorescence reagent for $^{1}\text{O}_2$ detection”.\[16\] **SOCL** probe demonstrated extremely high sensitivity, with an LOD for $^{1}\text{O}_2$ below 500 nM, which is 10-fold lower than that of **DMAX** (see the Supporting Information, Figure S3, for the comparison and LOD calculations). The better sensitivity observed for **SOCL** in comparison to **DMAX** demonstrates the advantage of our chemiluminescence probe over the fluorescence-based approach. The probe’s selectivity towards $^{1}\text{O}_2$ among other ROS was also evaluated. Remarkably, no other ROS could produce light emission through the probe chemiexcitation pathway (Figure 2C).

Next, we compared the activity of our **SOCL** probe with that of methoxyvinylpyrene (MVP), a chemiluminescence probe for the detection of $^{1}\text{O}_2$.\[16\] This probe utilizes the $[2+2]$ cycloaddition of $^{1}\text{O}_2$ to an enol-ether to initiate a chemiluminescent decomposition process. **MVP** suffers from two major drawbacks. First, the reaction of **MVP** with $^{1}\text{O}_2$ results in only 10% of the chemiluminescent dioxetane and 90% of an undesired side product. Such circumstance reduces the chemiluminescence quantum yield of the probe substantially.\[17\] In addition, **MVP** has a highly hydrophobic structure that severely limits its water solubility, thus preventing the use of the probe at high concentrations under physiological conditions. These disadvantages become particularly significant when trying to detect small amounts of $^{1}\text{O}_2$, which is anyway difficult to detect in water owing to its short lifetime. Indeed, as shown in Figure 3, chemiluminescence measurements in the presence of EP-1 under physiological conditions showed significant superiority of **SOCL** over **MVP**. At low probe concentration (10 μM), **SOCL** generated about five times more light than **MVP**, whereas at high probe concentration (500 μM), the ratio between the signals produced by the probes is increased up to 100-fold. In addition, **MVP** emits blue light ($\lambda_{\text{max}} = 465$ nm) while **SOCL** emits green light ($\lambda_{\text{max}} = 515$ nm). Green light is known to penetrate living tissue deeper than blue light.\[18\] Therefore, **SOCL** has a greater potential to be further applied for in vivo $^{1}\text{O}_2$ imaging than **MVP**.

Photodynamic therapy (PDT) is gaining widespread clinical application as an anticancer treatment.\[19\] and $^{1}\text{O}_2$ is believed to be the primary cytotoxic agent that elicits cell damage upon photosensitizer irradiation.\[20\] Thus, it is essential to enable monitoring of intracellular $^{1}\text{O}_2$ produced during...
PDT. Encouraged by the high sensitivity and selectivity observed for the SOCL probe towards \( \text{O}_2 \), we sought to assess whether our probe could be used for the detection of intracellular \( \text{O}_2 \). To increase the cell permeability of the probe, we covalently attached a cell-penetrating peptide (CPP) to the acrylic acid moiety of SOCL. The synthesis of the modified probe was achieved as described in Figure 4.

\[
\text{SOCL} \rightarrow \text{SOCL-CPP}
\]

Figure 4. Synthesis of SOCL-CPP, a cell-permeable chemiluminescence singlet oxygen probe.

SOCL was reacted with N-hydroxy-succinimide (NHS) to form the corresponding NHS-ester 1. The latter was coupled with maleimide-amine 1a to generate maleimide derivative 1b. Reaction of nona-arginine–glycine–cysteine peptide (CPP, which has been shown to transport cargos into cells)[20] with the maleimide moiety of 1b afforded the SOCL-CPP probe.

In order to use SOCL-CPP as a probe for \( \text{O}_2 \) produced inside the cells, we first verified its cell permeability. SOCL-CPP also exhibits moderate fluorescent emission upon excitation at 405 nm (see the Supporting Information, Figure S4). We used this intrinsic fluorescence to image SOCL-CPP cellular internalization by fluorescence confocal microscopy. As shown in Figure 5A, upon incubation of the SOCL-CPP with human cervical adenocarcinoma HeLa cells, a clear intracellular fluorescence signal could be observed. This observation indicates the internalization capability of SOCL-CPP into the cells.

To evaluate the adaptability of our probe to detect \( \text{O}_2 \) in PDT applications, we sought to determine whether the internalization of an appropriate PDT photosensitizer could also be imaged. The photosensitizer mTHPC (Foscan) has been extensively investigated and used in clinical applications of PDT.[21] Similarly to the SOCL-CPP probe, mTHPC also has a slight intrinsic fluorescence (see the Supporting Information, Figure S5), which should be sufficient to detect its ability for cell penetration. Figure 5B clearly shows that the mTHPC fluorescence signal appears inside the HeLa cells, demonstrating its cellular internalization.

Once the cell internalization of SOCL-CPP and mTHPC was verified, we examined whether our probe is capable of detecting intracellular \( \text{O}_2 \) produced during the PDT process. HeLa cells were incubated with SOCL-CPP and mTHPC for 1 h, and washed three times to remove any remaining probe or photosensitizer from the medium. Then, the cells were irradiated with light from a white LED lamp for 60 s and their chemiluminescence signal was measured. The experiment was performed with two 96-well plates; one was kept in the dark as a negative control. As shown in Figure 6, a strong chemiluminescence signal was obtained by the cells incubated with SOCL-CPP and mTHPC following light irradiation. Conversely, no chemiluminescence signal was obtained from cells that were incubated only with SOCL-CPP (no \( \text{O}_2 \) production) or only mTHPC (no \( \text{O}_2 \) probe) following light irradiation. No chemiluminescence signal was observed from the cells that were kept in the dark.

Addition of a \( \text{O}_2 \)-specific quencher (\( \text{NaN}_3 \)[22]) results in a significant signal attenuation (see the Supporting Information, Figure S6). PDT-induced cell death by mTHPC was confirmed by standard cell-viability assay (see the Supporting Information, Figure S7). This assay also demonstrated that SOCL-CPP by itself does not induce any light toxicity. This observation is especially significant because fluorescence-based probes for \( \text{O}_2 \) are known to suffer from their intrinsic ability to act as photosensitizers.[23] These results provide strong evidence for the ability of SOCL-CPP to detect intracellular \( \text{O}_2 \) produced during the PDT mode of action. It should be noted that the dioxetane intermediate formed by the reaction of SOCL-CPP with \( \text{O}_2 \) has a half-life of several minutes. This half-life is long enough to allow chemiluminescence imaging of the PDT process, as detection can be performed shortly after light irradiation and \( \text{O}_2 \) production.

In summary, we synthesized and evaluated a new, efficient chemiluminescence probe (SOCL), for the detection and imaging of \( \text{O}_2 \). The probe reacts with \( \text{O}_2 \) to form dioxetane, which spontaneously decomposes under physiological con-
dations through a chemiexcitation pathway to emit green light with extraordinary intensity. SOCL demonstrated a promising ability to detect and image intracellular \( \text{O}_2 \) produced by a photosensitizer in HeLa cells during the PDT mode of action. In relation to aqueous solubility and light emission intensity in water, our probe exhibited significant advantages and superiority in comparison to a previously known chemiluminescence probe for \( \text{O}_2 \). These findings make SOCL and SOCL-CPP as the most effective known chemiluminescence probes for the detection of \( \text{O}_2 \). We anticipate that our chemiluminescence probe for \( \text{O}_2 \) imaging would be useful in PDT related applications, for example, for the evaluation of photosensitizers, and for the monitoring of \( \text{O}_2 \) endogenously generated by cells in response to different stimuli.

**Acknowledgements**

D.S. thanks the Israel Science Foundation (ISF), the Bina
tional Science Foundation (BSF), and the German Israeli Foundation (GIF) for financial support. This work is supported in part by a grant from the Israeli National Nanotechnology Initiative (INNI), Focal Technology Area (FTA) program: Nanomedicine for Personalized Theranostics, and by The Leona M. and Harry B. Helmsley Nanotechnology Research Fund. R.S.-F. thanks the European Research Council for the ERC Consolidator Grant Agreement n. [617445]-PolyDorm.

**Conflict of interest**

The authors declare no conflict of interest.

**Keywords:** chemiluminescence · dioxetanes · live-cell imaging · molecular probes · singlet oxygen

**How to cite:** Angew. Chem. Int. Ed. 2017, 56, 11793–11796

Angew. Chem. 2017, 129, 11955–11958

---


Manuscript received: June 7, 2017
Revised manuscript received: July 24, 2017
Accepted manuscript online: July 27, 2017
Version of record online: August 16, 2017