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Lifetime and Diffusion of Singlet Oxygen in a Cell

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In time- and spatially resolved experiments, singlet molecular oxygen, $O_2(a^1\Delta_g)$, was created in a single nerve cell upon irradiation of a sensitizer incorporated in the cell nucleus using a focused laser beam. The singlet oxygen thus produced was detected by its infrared phosphorescence. Data obtained indicate that, contrary to common perception, this reactive species can be quite long-lived in a cell and, as such, can diffuse over appreciable distances including across the cell membrane into the extra-cellular environment. These results provide a new perspective for mechanistic studies of photoinduced cell death and intracellular signaling.

Introduction

The lowest excited electronic state of molecular oxygen, singlet oxygen, $O_2(a^1\Delta_g)$, is a reactive species that is significant in a range of chemical and biological processes.[1] Of particular interest are the roles played by singlet oxygen in mechanisms by which cellular function is altered in both plant and animal systems. It is acknowledged that the presence of singlet oxygen can result in cell death and that it initiates "controlled" cell death or apoptosis.[2] These processes form the basis for photodynamic therapy, PDT, a medical treatment used to destroy undesired tissue, including cancerous tumors.[3]

A common method by which singlet oxygen is generated in or near cells is to irradiate the system with UV or visible light. In this case, light absorbed either by a molecule inherent to the system, or by a molecule specifically added to the system, will generate an excited electronic state which, in turn, can transfer its energy of excitation to ground-state oxygen, $O_2(X^3\Sigma_g^-)$, producing singlet oxygen. This approach of photosensitization is employed in PDT, and it is also significant for a plethora of systems exposed to sunlight.

The specific roles played by singlet oxygen in photoinduced cell death are generally not fully understood. This is particularly true for processes that depend on the spatial localization of singlet oxygen in the cell.[4] Attempts to better understand these processes are often limited by the fact that photosensitizers which produce singlet oxygen may not be unequivocally localized in one cellular domain. This limitation becomes significant when light is not delivered to a specific sub-cellular domain but, rather, bathes the entire cell and its surroundings. Thus, it would be of great benefit to selectively produce singlet oxygen with unequivocal sub-cellular spatial resolution and then, even better, to detect the singlet oxygen thus produced in a direct optical experiment. Depending on how the latter is performed, one could then obtain unique information about the extent to which this reactive species diffuses in the cell within its lifetime.

Direct optical detection of singlet oxygen is, in general, not a trivial exercise because the pertinent and readily accessible spectroscopic transitions are all very improbable.[5,6] With this caveat in mind, detection of the weak $O_2(a^1\Delta_g) \rightarrow O_2(X^3\Sigma_g^-)$ phosphorescent transition at 1270 nm has nevertheless provided an unparalleled amount of useful information from a range of chemically pertinent systems.[6] The efficiency of a $\rightarrow$ X phosphorescence, however, is particularly small in aqueous systems of biological significance. On this basis, the prognosis of using direct optical techniques to obtain information about singlet oxygen in biological systems has been characterized as dismal, at best, especially with respect to time-resolved experiments at the single cell level.[7] Indeed, direct optical measurements of singlet oxygen in biological systems have heretofore only been possible using bulk suspensions where the signal observed derives from a large ensemble of cells.[8,9] We recently demonstrated, however, that singlet oxygen can, in fact, be directly monitored via the a $\rightarrow$ X transition from a single cell.[10] On this basis, we set out to further develop techniques by which singlet oxygen could be produced and optically detected with sub-cellular resolution.

Results and Discussion

For the present experiments, the intent was to produce singlet oxygen by irradiation of a sensitizer deposited in the cell. To this end, 5,10,15,20-tetrakis(*N*-methyl-4-pyridyl)-21*H*, 23*H*-porphine (TMPyP) was incorporated into neurons from the hippocampus of Wistar rats using a procedure described elsewhere.[10] TMPyP is an efficient singlet oxygen photosensitizer,[11] and it has been studied in the context of PDT.[12] Upon incorporation into a cell, it has been reported to localize in the nucleus.[13] Images of our cells based on the 670 nm fluorescence of TMPyP confirm that this molecule indeed principally localizes in the nucleus (Figure 1). However, as clearly seen in Figure 1 a non-negligible amount of TMPyP remains in the cytoplasm.

Neurons on a polylysine-coated cover slip in an isotonic solution were placed on the stage of an inverted microscope. For reasons outlined below, experiments were first performed

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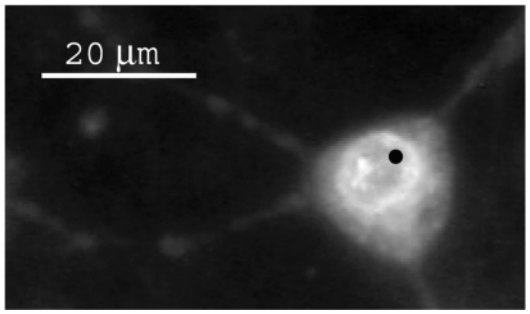


Figure 1. Fluorescence image of a neuron that had been incubated with TMPyP. The superimposed spot in the nucleus approximates the cross-sectional area at the waist of our focused laser beam. The latter was selectively positioned in the cell by translating the sample on the microscope stage.

with cells in which the intracellular H_2O had been exchanged with D_2O.[10] A given cell was irradiated using a femtosecond pulsed laser that was focused into the sample through the microscope objective.[11,14,15] The irradiation wavelength was 420 nm, which coincides with the Soret absorption band of TMPyP. On the basis of the optics used, the cross-sectional diameter at the waist of the focused beam was calculated to be approximately 1.4 μm.[16] Along the axis of propagation, the beam diverges such that at a distance of 10 μm from the waist its cross-sectional diameter is $\sim$6 μm. Most importantly, the respective areas transected by the laser beam are smaller than the corresponding area of the cell nucleus (Figure 1).

Upon selective irradiation into the nucleus of a cell containing TMPyP, we observed a 1270 nm emission signal from the cell in a single photon counting experiment.[15] Given the optics used,[15] it is important to note that we are collecting emission from the entire cell and its immediate surroundings. Upon irradiation into the cytoplasm, the intensity of the observed signal decreased. The signal intensity dropped even further upon irradiation into the TMPyP-free domain immediately outside the cell (Figure 2).[17]

Our data indicate that there is a discrete position-dependent near-IR emission signal superimposed on a weak background signal. We assign this position-dependent signal to singlet oxygen phosphorescence based on the following experiments. (a) The intensity of the signal from a cell saturated with oxygen was greater than that from a cell saturated with nitrogen (Figure 2). (b) Singlet oxygen has a discrete a $\rightarrow$ X phosphorescence band centered at $\sim$1270 nm. Although this band exhibits a slight solvent dependence,[18,19] emission is never observed at 1200 nm. Upon using a filter with a band-pass centered at 1200 nm, we were unable to observe the position-dependent signal that was recorded when using a filter centered at 1270 nm. (c) Sodium azide is an acknowledged quencher of $O_2(a^1\Delta_g)$ that can readily penetrate cell membranes.[20] Our position-dependent signal disappeared upon the addition of an aliquot of sodium azide to the solution surrounding the cells (final [NaN_3] of $\sim$10 mM) (Figure 2). (d) The medium in which $O_2(a^1\Delta_g)$ is dissolved can significantly influence the a $\rightarrow$ X phosphorescence experiment.[5,6] In particular, deuteration of the surrounding solvent can result in an appreciable increase in the $O_2(a^1\Delta_g)$ lifetime, τ_Δ, which, in turn, results in an increase in the quantum efficiency of a $\rightarrow$ X phosphorescence. The intensity of the position-dependent signal observed from a cell containing D_2O was greater than that observed from a cell containing H_2O (Figure 2), providing final unequivocal evidence that our signal indeed comes from singlet oxygen.

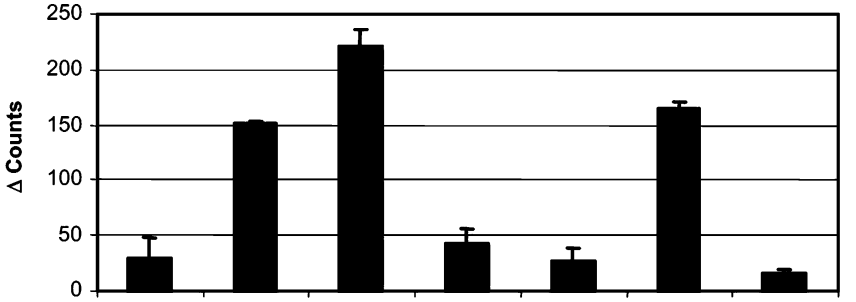
On the basis solely of a solvent effect, the singlet oxygen phosphorescence intensity in D_2O should be $\sim$19 times greater than that in H_2O.[10] This isotope effect will be mitigated by any quenchers that reduce τ_Δ (e.g., proteins). The isotope effect we observe upon irradiation of TMPyP in the nucleus of cells that had been incubated with D_2O and H_2O, respectively, is 15 ± 5 (Figure 2). Thus, for these particular experiments, the data indicate that singlet oxygen deactivation is dominated by interactions with the solvent in the cell and not by interaction with cellular components. In support of these data, we were able to record a time-resolved a $\rightarrow$ X phosphorescence signal upon irradiation of TMPyP in the nucleus of a single cell incubated with D_2O (Figure 3).[15] The lifetime obtained, $\tau_\Delta = 45 \pm 18$ μs, is consistent with that expected for a moderate amount of quenching (from bulk solution phase experiments, τ_Δ in quencher-free $D_2O = 68.1 \pm 2.5$ μs[21]).

It has been suggested that, once formed in a cell, singlet oxygen will not diffuse over a great distance simply because the presence of quenchers inherent to the cell will reduce τ_Δ significantly.[22] For our particular system, in which singlet oxygen was created in the nucleus, the data clearly indicate that quenchers inherent to the cell do not dominate $O_2(a^1\Delta_g)$ deactivation. This observation is consistent with reports that DNA is not a particularly good quencher of singlet oxygen. If we assume that the quenching rate constant of $\sim 5 \times 10^5$ M^{-1} s^{-1} obtained from a bulk solution phase experiment using dissolved DNA[23] is applicable to our study, and if we assume an effective DNA concentration of 40 mM in the nucleus,[24] then one should obtain a τ_Δ value of $\sim$30 μs in a D_2O-incubated cell. This expected value of τ_Δ is indeed consistent with our experimental value of 45 ± 18 μs.

With such a large value of τ_Δ, the $O_2(a^1\Delta_g)$ formed in our system should diffuse over appreciable distances. Indeed, it is entirely possible that, as a consequence of diffusion across the cell membrane, some fraction of the optical signal we detect originates from an extracellular population of $O_2(a^1\Delta_g)$. When envisioning such diffusion into the extracellular medium, it is useful to consider the cell, sitting on the polylysine base, as a "fried egg" with the nucleus as the yolk. As such, "vertical" diffusion (e.g., along the propagation axis of the irradiating laser beam) could allow for facile transport of singlet oxygen across the cell membrane.

To test how far $O_2(a^1\Delta_g)$ can diffuse after it has been selectively created in the nucleus of the cell, we performed experiments in which the cell was exposed to bovine serum albumin (BSA). This protein is an efficient $O_2(a^1\Delta_g)$ quencher ($k_q = 5 \times 10^8$ M^{-1} s^{-1}),[25] but it is sufficiently large that it will not penetrate the cell membrane (MW $\sim$65 kDa). Thus, in our study, it is a quencher specific for extracellular singlet oxygen. Recall that upon addition of NaN_3, which is small enough to readily penetrate the cell membrane, the singlet oxygen signal is completely quenched (Figure 2).

In D_2O-based experiments, when the medium surrounding the cell contains 0.77 mM BSA, we observe a decrease in our signal intensity of $\sim$30% (Figure 2). This same concentration of BSA in a bulk solution-phase experiment causes the intensity of a singlet oxygen signal to decrease by 96%. Moreover, when the BSA concentration surrounding the cell is increased to 1.3 mM, we still observe this same 30% decrease in signal intensity relative to the BSA-free system. These data clearly indicate that, once formed in the cell nucleus, $\sim$70% of our signal derives from singlet oxygen within the cell. Nevertheless, a significant fraction of the singlet oxygen produced is able to diffuse into the extracellular medium. As such, our solvent isotope effect of 15 ± 5 and τ_Δ value of 45 ± 18 μs reflect a weighted mean of this singlet oxygen distribution. On this basis, we estimate



Location	Ext	Cyt	Nuc	Nuc	Nuc	Nuc	Nuc
Atmosphere	O_2	O_2	O_2	N_2	O_2	O_2	O_2
Solvent	D_2O	D_2O	D_2O	D_2O	D_2O	D_2O	H_2O
Quencher	None	None	None	None	NaN_3	BSA	None
n	3	5	15	5	9	8	7

Figure 2. Histogram that shows the intensity of the 1270 nm emission signal observed upon irradiation of a single cell under a range of conditions. The variable *n* is the number of independent data sets[15] recorded from different cells on different days. Error bars reflect the standard error of the mean (Ext = extracellular; Cyt = cytoplasm; Nuc = nucleus).

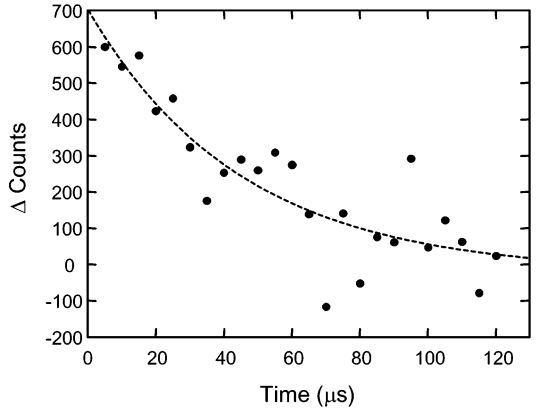


Figure 3. Time-resolved singlet oxygen phosphorescence signal recorded upon selective irradiation of TMPyP in the nucleus of a neuron incubated with D_2O. The dashed line is a single-exponential fit to the data and yields $\tau_\Delta = 45 \pm 18$ μs.

that τ_Δ in the D_2O-incubated cell is 35 μs, which is consistent with our estimate based on DNA quenching (vide supra).

Our data consistently imply that, in the nuclei of our cells incubated with H_2O, τ_Δ is ~3 μs (τ_Δ in quencher-free H_2O ~3.5 μs[20,26,27]). Under this latter condition, let us consider how far singlet oxygen could diffuse over a period *t* of twice its lifetime (i.e., 6 μs). Using a diffusion coefficient, *D*, for oxygen in water of 2×10^{-5} cm^2 s^{-1},[28] the distance traveled by singlet oxygen in 6 μs is ~268 nm (root-mean-square radial displacement = $(6tD)^{1/2}$). This distance is significantly larger than what is currently believed.[22]

Our data are unique in that we have spatially resolved the direct optical characterization of singlet oxygen in the cell. In itself, this provides a credible explanation for the difference between our results and the results of experiments in which cell suspensions were used.[8,9] Specifically, in these latter studies, the data obtained reflect the behavior of singlet oxygen averaged over the entire cell and its surroundings.

Conclusions

On the basis of unique experiments performed on a single cell, we have demonstrated that singlet oxygen can be quite long-lived and, as such, diffuse over appreciable distances in the cell. On one hand, our results will be pertinent when considering mechanisms for intracellular signaling, for example. Perhaps most importantly, however, we have demonstrated that one can optically create and detect, with sub-cellular resolution, the main cytotoxic agent responsible for oxygen-dependent photoinduced cell death.

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(15) The 840 nm output of an amplified Ti:sapphire laser was frequency-doubled to yield 420 nm. A pulse repetition rate of 1 kHz was used with powers at the sample of 0.6 μW. The microscope objective had a magnification of 60× and a numerical aperture of 0.9. For sample irradiation, however, in which the laser beam did not fill the back aperture of the objective, the effective numerical aperture, NA_{eff}, was 0.4. Luminescence from the sample was collected by the microscope objective and transmitted through a 1270 nm interference filter onto a near-IR photomultiplier tube (Hamamatsu model R5509−42, −80°C, rise time of 3 ns). For each laser pulse, data were recorded over two separate 50 μs sampling periods; the first began 1 μs after the laser pulse (signal channel) and the second began 500 μs after the laser pulse (background channel), and the difference between the two was obtained. For irradiation at a specific point in a given cell, the integration period for one acquisition was 10 s and was repeated 10 times

to enhance the signal-to-noise ratio (i.e., at 1 kHz, each data set derives from 10^5 laser pulses). In the time-resolved experiment, the sampling period was reduced to 5 μs and counts were accumulated as the signal sampling window was successively delayed relative to the laser pulse.

(16) From the Rayleigh equation using $\lambda = 420$ nm, diameter $\sim 2 \times 0.61\lambda/NA_{eff} = 1.4$ μm.

(17) An important aspect of our pulsed-laser-based single photon counting experiments relative to steady state experiments is that we are able to temporally discriminate against comparatively intense and short-lived background luminescence from the sensitizer and optics that would otherwise interfere with the singlet oxygen signal.

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