A singlet oxygen monitor as an in vivo photodynamic therapy dosimeter


*aPhysical Sciences Inc., 20 New England Business Center, Andover, MA, USA 01810-1077;
bDartmouth-Hitchcock Medical Center, One Medical Center Drive, Lebanon, NH 03756;
cMassachusetts General Hospital, Wellman Laboratories of Photomedicine, 40 Blossom Street, Boston, MA, USA 02114-2605

ABSTRACT

In this paper we describe the development and testing of instruments to measure singlet molecular oxygen produced by the photodynamic process. Singlet oxygen is an active species in photodynamic therapy, and we are developing two instruments for PDT researchers with the goal of a real-time dosimeter for singlet oxygen. We discuss both an ultra-sensitive point sensor, and an imaging system that provides simultaneous 2D maps of the photosensitizer fluorescence and the singlet oxygen emission. Results of in vitro tests to characterize the sensors and preliminary in vivo results are presented.

Keywords: Photodynamic therapy, singlet oxygen, dosimetry

1. INTRODUCTION

Photodynamic therapy (PDT) is a relatively new, rapidly developing, and promising modality for cancer treatment.1-4 PDT uses certain compounds known as photosensitizers (PSs) that are preferentially retained in malignant tumors. With visible light, the photosensitizers initiate a reaction that selectively kills the malignant cells to which they are attached. PDT is being used in clinical trials for bladder, brain, skin and other cancers. PDT is also being applied to important areas outside of cancer treatment including age related macular degeneration and actinic keratosis, a pre-cancerous skin condition. There is considerable evidence that singlet O2 (O2(a1Δ) produced by energy transfer from optically excited photosensitizers is the active species in cancer cell or endothelial cell necrosis. Despite the general acceptance of this role of singlet O2 in PDT, there have been limited demonstrations of its importance in vivo.5-7 If singlet O2 is indeed the critical species that determines PDT efficacy, a device that is conducive to real-time measurement of singlet O2 in vivo could provide the critical parameter in PDT dosimetry and the potential of individualized therapeutic design.

Background of Photo-Induced Processes

The earliest reports of photo-induced processes date back to 1900.6 Three essential ingredients are required: 1) a light source, 2) oxygen, and 3) a photosensitizer (PS) molecule capable of absorbing the wavelength of the light source. The first definitive studies were those of Kautsky et al. in 1931.9 The first evidence that singlet O2 was the species responsible for killing tumors was reported in 1976 by Weishaupt et al.1 In a very clever set of experiments, they used filtered light at 630 nm to irradiate tumor samples in aqueous solutions and a selective quencher of singlet oxygen, they showed that only those cells exposed to HPD, oxygen, and light were killed.

The emergence of PDT as a FDA approved treatment modality has produced tremendous activity in clinical applications. Numerous new photosensitizers have been developed. Photofrin II was the first widely used photosensitizer. It has strong photodynamic effect, and its major absorption band for photoactivation is at about 630 nm. From a fundamental perspective, 630 nm light does not penetrate tissue as deeply as longer wavelengths. The cw dye lasers used in PDT are expensive and relatively difficult to operate and are rapidly being replaced by high power diode lasers that operate in the

* lee@psicorp.com; phone 1 978 689-0003; fax 1 978 689-3232; psicorp.com
630 to 690 nm. Our singlet oxygen monitor takes full advantage of these miniature diode laser sources. There has also been considerable activity to develop photosensitizers with longer wavelength absorption bands to treat tumors in greater depth. They also may be excellent receptors for diode laser excitation.

There has been great interest in developing a sensor for singlet O₂ that could be used as a real-time dosimeter during PDT treatments. Correlations of the singlet O₂ produced with treatment efficacy could be one important use of such a sensor. Some researchers have attempted to develop dosimeters based on the fluorescence intensity of the PS in the tumor, but photobleaching of the PS precludes this as an accurate method. Pogue et al. used small electrodes to measure total oxygen content in tumors during PDT in animal studies have demonstrated deoxygenation during treatments. Other researchers have shown that oxygenation of tissue can enhance PDT efficiency. Since singlet O₂ appears to be the active species, a dosimeter for singlet O₂ produced during PDT would be a valuable tool for improving treatment outcomes. Our device measures directly the singlet O₂ dose which is proportional to the product of the PS concentration, the ground state oxygen concentration and the light intensity within the tissue being irradiated. Thus, we can define the dose as \( \int [PS(t)] \times [O₂(t)] \Phi(t) \, dt \) where \([PS(t)]\) is the photosensitizer concentration, \([O₂(t)]\) is the ground state oxygen concentration and \(\Phi(t)\) is the light irradiance. In short, our diode laser-based, singlet oxygen monitor provides the integrated product of the factors that produce the singlet O₂.

The dye sensitizer absorbs the excitation light and is excited to a singlet state as indicated in Fig. 1. This singlet state radiates strongly to the ground state of the dye and has characteristic emissions in the visible portion of the spectrum, e.g. peaks near 630 and 703 nm in Photofrin II. This emission can be used to locate tumor tissue and there are several groups investigating this effect as a diagnostic tool. A rapid intramolecular transfer process populates a metastable triplet state in the dye indicated by \(T_1\) in Fig. 1. Collisions between this metastable dye molecule and ground state oxygen (present in the tumor) populate the singlet O₂ via an energy transfer process. The excited metastable, singlet O₂ destroys the cancer cells via two distinct mechanisms: a) direct disruption of cell function by rupturing of the cell wall or destruction of the cell mitochondria, and b) constriction of the vascular network in the tumor that provides nutrients to the tumor.

2. DESCRIPTIONS OF SINGLET OXYGEN MONITORS

2.1 Ultra-sensitive Point Sensor

Our point (non-imaging) singlet O₂ monitor uses the weak but unique spectral signature of the O₂(a₁Δ → X₃Σ) transition shown above in Fig. 1. The radiative emission from the singlet O₂ is extremely weak (radiative rate of ~ 0.2 s⁻¹ in aqueous media such as tissue) and occurs at a wavelength \(\lambda \approx 1.27 \mu m\), a particularly challenging region of the spectrum for sensitive detection. Indeed, the weak optical emission of singlet O₂ is one of the major difficulties in monitoring singlet O₂ produced by irradiated photosensitizers. While optical filtering provides some measure of sensitivity, temporal discrimination is also required and is at the heart of our method. Referring to Fig. 1, the prompt dye fluorescence from the \(1S_0\) state has a lifetime on the order of 10 ns since it is from a radiatively allowed transition. It decays much more rapidly than the emissions from the singlet O₂ (lifetime of 4 μs in aqueous media and as short as 0.1 μs in biological

![Fig. 1. Mechanism for production of singlet molecular oxygen in the photodynamic therapy.](image-url)
PS excitation with a pulsed diode laser is a key element in our sensor. Until recently, the most sensitive optical sensors for singlet O\textsubscript{2} emission were solid state, liquid nitrogen cooled germanium (Ge) photodiode detectors. While these devices can provide high sensitivity ($D^* \sim 10^{15}$ cm$^2$ Hz$^{1/2}$/W), they operate at what is known as the "gain/bandwidth limit" and the highest sensitivity Ge devices have a temporal response time of 1 ms. This is inadequate for isolating the singlet O\textsubscript{2} emission from prompt dye emission that may leak through to the detector. The detector simply cannot discriminate between laser on and laser off conditions with adequate temporal resolution.

We use a novel near-IR photomultiplier tube (PMT) with a time response < 5 ns and with low enough dark current that we used photon counting methods to optimize the sensitivity. We summarize our strategy in Fig. 2. Our detection method is similar to that described by Wilson et al. who also use the near-IR PMT approach. However, we use a fiber-coupled diode laser as our excitation source whereas Wilson uses a Q-switched, frequency doubled, Nd:YAG laser and has reported impressive results.

In Fig. 3, we illustrate typical signals recorded with the near-IR PMT. Figures 3(a) and (b) show data for the PS C\textsubscript{6}-e6 in acetone (Fig. 3a) and water (Fig. 3b) for a 5 µs diode laser excitation pulse width. The temporal evolution of the production of singlet O\textsubscript{2} (via transfer from the PS triplet state) and its subsequent quenching (by the solvent molecules) are evident in these data. During the square wave diode laser pulse, the singlet O\textsubscript{2} signal grows in both the acetone and water solvents. For the acetone solution (Fig. 3a), the quenching is relatively weak and the singlet O\textsubscript{2} emission by the end of the diode laser pulse is several times stronger than the near-IR fluorescence from the PS. In contrast, for the more severe quenching aqueous environment, the singlet O\textsubscript{2} emission is much weaker. Note also the dramatic reduction in $\tau_\Lambda$ due to water quenching when compared to acetone, a relatively weak quencher of singlet O\textsubscript{2}.

The temporal evolution shown in Fig. 3(b) is typical of the singlet O\textsubscript{2} signatures that we observe even in vivo. Most of the bright dye fluorescence promptly terminates at the end of the diode laser pulse, and we sum the intensity (photoelectron counts) after the diode laser is shut off to obtain our singlet O\textsubscript{2} signal. However, in tissue, the singlet O\textsubscript{2} becomes so highly quenched that some weak emitters can cause spectral interferences, even when observing during the time that the diode laser is off. The relatively slow emission (phosphorescence) from triplet state of the PS is a potential interference for in vivo studies. The triplet state lifetime is typically on the order of microseconds and the emission (albeit weak) can occur subsequent to the diode laser pulse. This requires additional optical filtering to isolate the singlet O\textsubscript{2} spectral feature from the broadband emission from the PS dye. We used a series of three narrow band interference filters with center wavelengths of 1.22, 1.27, and 1.32 µm to spectrally discriminate between the PS and singlet O\textsubscript{2} emission for the in vivo studies. With this approach, we have succeeded in detecting singlet O\textsubscript{2} production from two photosensitizers in tumors implanted in rats and from healthy human skin containing topical ALA photosensitizer. A photograph of our prototype point sensor is shown in Fig. 4.
2.2 2D Imaging Sensor

We have also developed and are testing a system with the capability of simultaneously recording 2D images of both the PS fluorescence and singlet O₂ emission. Here we use near-IR and visible wavelength sensitive cameras to obtain spatially resolved images of the singlet O₂ emission and PS fluorescence, respectively. The near-IR camera uses a high
quantum efficiency photocathode and an electron bombardment intensifier to provide near single-photo electron detection in the 1 to 1.5 µm spectral region. Similar to the non-imaging sensor described above (point sensor), we use fiber coupled diode lasers each having an appropriate excitation wavelength for particular photosensitizers, e.g., 635 nm for protoporphyrin IX (Pp IX) and 660 nm for chlorin e6 (Cl-e6).

The near-IR camera cannot be gated, and we could not use the same detection strategy employed for the PMT-based sensor. Thus, to differentiate the singlet O₂ emission at 1.27 mm from the bright PS fluorescence and any background signal (spectral discrimination), we used a set of filters (1.22, 1.27, and 1.32 µm) to detect emissions of singlet O₂ and background signals. The images at 1.22 and 1.32 µm contain only PS fluorescence and the image at 1.27 µm contains contributions from both the singlet O₂ and PS. Therefore, the singlet O₂ signal is calculated by subtracting the average of the signals at 1.22 and 1.32 µm from the 1.27 µm signal. Fig. 5(a) shows the schematic of an early version of the imaging sensor; excitation diode laser, near-IR camera, and visible camera. We have recently completed an integrated system that combines the visible and near IR cameras on a single platform so that both cameras share a common optical path and view the same field as indicated in Fig. 5 (b) and (c).

3. RESULTS

3.1 Ultra-sensitive Point Sensor

Upon completion of the prototype device, we undertook a series of studies using several photosensitizers in solution and with some tumor cells suspensions. Photosensitizers included: Cl-e6, BPD, ALA, and Photofrin II all in aqueous solutions. Fig. 6 shows a comparison of the time resolves signals we observed with Cl-e6 in an aqueous solution with the
solution oxygenated (Fig. 6a) and deoxygenated (Fig. 6b). For the deoxygenated case, only the PS fluorescence remains and reproduces the temporal shape of the diode laser pulse. These and similar data demonstrated that the system was capable of sensitive detection of singlet O₂ in highly quenching aqueous solutions.

Fig. 6. Comparison of near-IR emission observed in Cl-e6 in water using a 2 s diode laser pulse: oxygenated sample and same sample deoxygenated.

We completed an animal study using Copenhagen rats and a R3327-MatLyLu prostate cancer cell line that somewhat follows the human disease pattern and metastasizes into lymph nodes and lungs. Since the R3327-MatLyLu cells are syngeneic, there is no need for immune suppression in the rats, which enhances the model's clinical relevance. Tumors were induced by subcutaneous injection of a suspension of 10⁵ R3327-MatLyLu cells into the flanks of two-months-old male Copenhagen rats of 150 to 200 g weight. Implant sizes were repeatedly assessed by caliper measurement. Tumor volumes (V) were calculated using the equation \( V = \text{length} \times \text{width} \times \text{height} \times 0.5236 \). Tumors were observed while growing to treatment dimensions of about 0.3 cm³, which requires approximately 8 to 10 days.

Once the tumors had reached treatment size, the animals received an injection of either intravenous Cl-e6, or intraperitoneal ALA. After a waiting period of 3 hours that allows for accumulation of the PS in the tumor, light exposure was done in the anesthetized animals through the shaved skin. Irradiations of the tumors were performed using a continuous wave diode laser source (HPD, Inc.) with a wavelength that matched the absorption profile of the PS.

Before the therapeutic light exposure, the singlet O₂ probe was set up to allow rapid contact with the tumor surface for measurements. The singlet O₂ emission was recorded immediately before starting the therapeutic light exposure, at several separate times during the irradiation, and at the end of the light exposure. Each measurement consisted of approximately 30,000 pulses with the pulsed diode laser source and the signal photoelectrons were summed. The measurement required interrupting the therapeutic light exposure, but only for approximately 10 to 30 seconds.

Fig. 7 shows results for three rats where the Cl-e6 was used as the PS. Each had a tumor implanted. One had no PS, one was infused to a PS concentration of 0.5 mg/kg, and the third was infused to a PS concentration of 1 mg/kg. The singlet O₂ was measured at two locations on the tumor in each rat. Fig. 7a shows the temporal evolution of the singlet oxygen emission (in photoelectron counts) for three rats, each with a different concentration of PS. Figure 7b shows the two site average, temporally integrated photoelectron counts at 1.27 µm (the singlet O₂ wavelength) as a function of total PDT fluence. These data clearly show the reduction of the singlet O₂ signal as the total light dose was increased. The causes of this diminution are likely a combination of tumor hypoxia and PS photobleaching. The data demonstrate that the sensor is capable of monitoring singlet O₂ in vivo during PDT treatments of tumors.
3.2 2D Imaging Sensor

To characterize the imaging system, we completed a series of *in vitro* studies that used two photosensitizers, Cl-e6 and Pp IX in several solvents including protein-laden aqueous solutions that are severe quenchers of singlet O₂. Fig. 8 shows the images of both the singlet O₂ emission and Cl-e6 fluorescence with *in vitro* samples as a function of the PS (Cl-e6) concentration. The intensities of singlet O₂ and PS fluorescence were integrated and plotted in Fig. 8(b). There is a good correlation between singlet O₂ produced by light treatment and PS fluorescence.

We also obtained spatially resolved images from a tumor model on mice using the high resolution, near-IR camera. Fig. 9 shows images of singlet O₂ and PS fluorescence during a PDT treatment for a control mouse and a tumor-loaded mouse. The control mouse had no tumor, so the injected PS dye was spread through the entire body. The weak singlet O₂ emission and PS fluorescence were observed due to PS dye spread and defined by where PDT excitation light was illuminated. Comparing to the control case, tumor-loaded mice showed much higher intensities of singlet O₂ and PS fluorescence from the tumor area due to the accumulation of PS in the tumor. We believe these are the first reported spatial images of singlet O₂ emission from live animals.

In this paper we have described two sensors for singlet O₂ detection with point and spatially imaging capabilities. These systems rely upon ultra-sensitive detection of 1.27 μm radiation from the (a →X) molecular transition in oxygen. We have developed portable prototype platforms that are undergoing animal and preclinical studies to determine ultimate sensitivity for *in vivo* detection of singlet O₂. These results will be used to develop potential applications for these systems in PDT research and eventually as tools in a clinical setting.
Fig. 8. Images of singlet O2 emission and PS fluorescence simultaneously recorded in the configuration shown in Fig. 5(a): (a) various concentrations of Cl-e6 in PBS, 25 second accumulation; (b) Plot of singlet O2 emission and PS fluorescence as a function of Cl-e6 concentration.

Fig. 9. Images of control (no tumor) and tumor-loaded mouse. The both mice were injected with 3 mg/kg Cl-e6 dye and incubated for 2 hours.

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