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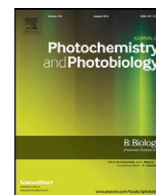

Direct measurement of singlet oxygen by using a photomultiplier tube-based detection system

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ABSTRACT

The effective dosimetry for photodynamic therapy (PDT) can be specified by direct measurement of singlet oxygen ($^1O_2$) production. The purpose of this study was to investigate the feasibility of a newly developed photomultiplier tube (PMT)-based singlet oxygen detection (SOD) system. The lowest and highest $^1O_2$ concentrations detectable by the PMT-SOD system were 15 nM and 10 μM, respectively. Dose-dependent quenching, by NaN_3, of the fluorogenic reaction was observed, which was negatively correlated with the $^1O_2$ level measured by the PMT-SOD system. The lifetime of $^1O_2$, as measured by the PMT-SOD system, was found to be lengthened when H_2O was replaced with deuterium oxide. $^1O_2$ photon counts were significantly and dose-dependently correlated with intracellular fluorescence intensity after photosensitizer treatments. *In vitro* cell viability test and *in vivo* xenografted-tumor mass shrinkage showed a positive association between PDT-induced cytotoxicity and $^1O_2$ production concomitantly measured by the PMT-SOD system. It was concluded that the PMT-SOD system is capable of measuring $^1O_2$ production directly and accurately, demonstrating that this system can be useful in the determination of dosimetry for PDT.

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1. Introduction

Photodynamic therapy (PDT) is a light-activated treatment modality [1], which selectively kills tumor cells mainly by direct cytotoxicity and indirect vascular destruction of blood supplying vessels [2,3]. The efficacy of PDT depends on the intra-tumoral uptake of different photosensitizers (PS), total light dose of different wavelengths used to activate varied PS, and intra-tumoral production of singlet oxygen ($^1O_2$) generated by PDT [4]. $^1O_2$ acts as the main cytotoxic agent in malignant tumor cells [5]. Therefore, the development of PS capable of generating more $^1O_2$ in tumors is important for effective PDT in cancer management.

Many researchers have attempted to achieve high-precision dosimetry for PDT against various cancers [6,7]. However, this has been challenging due to the dynamics of oxygen-independent photobleaching of the PS, as well as the complexity of photoproduct formation. Previous studies have used PDT dosimetry with monitoring systems based on indirect $^1O_2$ detection using $^1O_2$ quantum yield, with singlet oxygen sensor green, bilirubin ditaurate, or 1,3-diphenylisobenzofuran as chemical quenchers [8–10], as well as direct methods using HPLC, UV–Vis spectroscopy, and the photomultiplier-tube (PMT)-based singlet oxygen detection (SOD) system [11–18].

$^1O_2$ produced by light-excited PS emits very weak infrared (IR) light near 1.27 μm. Previous studies have shown that the PMT-SOD system detects $^1O_2$, both *in vitro* and *in vivo*, by using ultra-sensitive and non-imaging sensors combined with a near-IR sensitive PMT detector [13,14,17], which is a single systemic component with three different systems: a) the pulsed diode laser as a 670-nm wavelength excitation source, b) the coupled fiber with pulsed diode laser sources and tailoring of the excitation wavelength, to optimize coupling into the particular PS being studied, and c) a computer system that measures and calculates photon counting from PMT-based spectral discrimination, *via* a set of three optical filters (1.22, 1.27, and 1.32 μm) [19]. Our current PMT-SOD system is an improvement over this system in the following ways: 1) the high sensitivity of our system is derived from pulsation of the laser at high rates (10–20 kHz), and counting of the emitted $^1O_2$ photons at these rates, 2) the current system uses a monolithic fiber optic cable to both direct the diode laser to the target tissue or cell, and collect the $^1O_2$ emission, and 3) our system provides the only available real-time monitoring of $^1O_2$ production during PDT.

In this study, we validate the ability of the newly developed PMT-SOD system to directly and efficiently detect $^1O_2$ production from PDT.

Abbreviations: Che6, Chlorin e6; DMSO, dimethylsulfoxide; D_2O, deuterium oxide; IR, infrared; OD, optical density; $^1O_2$, singlet oxygen; PDT, photodynamic therapy; PMT, photomultiplier tube; PPa, pyropheophorbide-a; PS, photosensitizer; SOD, singlet oxygen detection.

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We also discuss its reliability and feasibility, which is demonstrated by the measurement of intracellular PS levels and their correlation with PDT-induced cytotoxicity.

2. Materials and methods

2.1. Cell cultures

Four human colon and pancreatic cancer cell lines, SW480, HT29, AsPC-1, and MiaPaCa-2, were obtained from Korean Cell Line Bank (KCLB, Seoul, Korea). Cells were prepared and resuspended in RPMI1640 medium (Gibco, Carlsbad, CA, USA), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Gibco). The cells were maintained in a humidified incubator (Sanyo, Tokyo, Japan) at 37 °C in an atmosphere containing 5% CO_2.

2.2. Chemicals

Chlorin e6 (Che6) and pyropheophorbide-a (PPa) were purchased from Frontier Scientific, Inc. (Salt Lake City, UT, USA) and dissolved in dimethylsulfoxide (DMSO; Sigma, Saint Louis, MO, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma and dissolved in PBS to a final concentration of 2 mg/ml. Deuterium oxide (D_2O) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA).

2.3. Measurement of $^1O_2$ by the new PMT-SOD system

$^1O_2$ was measured by direct detection of the near-IR luminescence emission of oxygen at 1.27 μm, corresponding to a singlet–triplet transition state. Samples were excited with 670 nm, generated by a diode laser (PMT-SOD system; Physical Sciences Inc., Andover, MA, USA). A fiber coupled diode laser with wavelength centered at 670 nm was operated at a repetition rate of 10 kHz, with a pulse width of 5 μs, and an average output power of ~230 mW during each pulse. Each pulse contained 1.15 μJ. $^1O_2$ luminescence was detected by a PMT detector (R5509-42; Hamamatsu Corp., Bridgewater, NJ, USA) by using a computer controlled slider containing three bandpass filters centered at 1.22, 1.27 and 1.32 μm, each with a full width at a half-maximum bandwidth of 15 nm. These filters were used to spectrally isolate $^1O_2$ emission at 1.27 μm from the long wavelength spectral background signal, such as PS fluorescence and/or phosphorescence, and autofluorescence. Emissions at 1.22 and 1.32 μm (out-of-the-band wavelengths) contained only PS fluorescence, while the emission at 1.27 μm contained contributions from both the $^1O_2$ and PS.

2.4. Detection of $^1O_2$

Fig. 1 shows the evaluation process for the measurement of $^1O_2$ production by the PMT-SOD system from various PSs and cell lines. We measured the $^1O_2$ concentration in 6-well and 96-well culture plates (Fig. 1). For a small-scale experiment to measure intracellular PS concentration, cells were seeded at 5×10^3–1×10^4 cells per well, in 100 μl of culture medium in 96-well plates and incubated in a cell culture incubator. After overnight incubation, they were treated with various concentrations of PS in 100 μl of medium. After 6–48 h, cell medium was washed with PBS and replaced with 100–200 μl of methanol per well. The plate was shaken for 10 s, and $^1O_2$ photon count was measured with the PMT-SOD system. The large-scale experiment to measure intracellular PS concentration was performed according to the same procedure as that of the small-scale experiment, except for the additional steps of washing the culture plate or dish with PBS and cell harvest. After transferring the cells to amber colored bottles or 96-well white plates, $^1O_2$ photon count was measured with the PMT-SOD system. The PS concentration was calculated as a standard curve, showing $^1O_2$ counts in a set of serially diluted solutions of PS in methanol. Briefly, Che6 or PPa were diluted to half serial concentration from 10 uM for high concentration and 1 uM for low concentration, respectively. After transferring the samples to 96-well plates with 200 μl per well, $^1O_2$ photon count was measured. For the $^1O_2$ near infrared luminescence measurements, luminescence data collection and analysis were made continuously during irradiation of the samples by selecting each of the 1220 nm, 1270 nm, and 1320 nm band-pass filters. This entire luminescence data collection process was automated in PMT-SOD system. $^1O_2$ counts or PS concentration in cells were adjusted by cell counts.

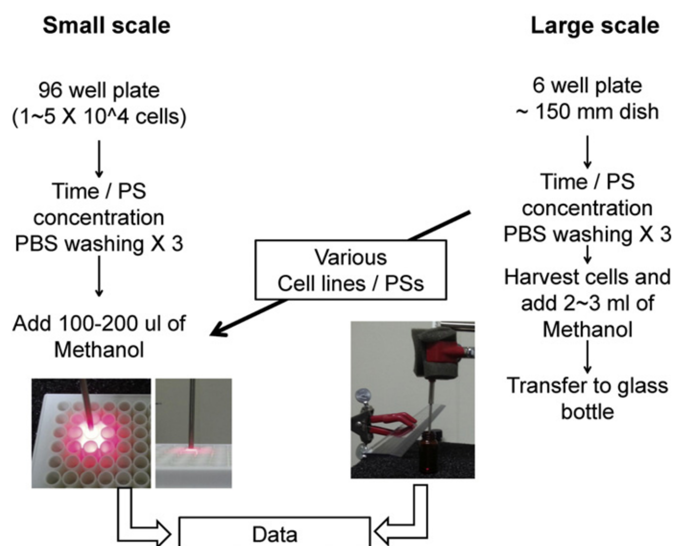

Fig. 1. Scheme of the experimental procedures. (Left) Cells were seeded onto a 96-well culture plate, treated with photosensitizers, incubated, and washed. Then, methanol (100–200 μl per well) was added to cells. The plate was shaken for 10 s, and the $^1O_2$ photon count was measured by the PMT-SOD system. (Right) The procedure used was similar to that used for the previous experiment, but additional steps such as washing the culture plate or dish with PBS and cell harvest were performed. $^1O_2$ photon count was measured by the PMT-SOD system after transferring samples to an amber-colored bottle or 96-well plate.

2.5. Photo-quenching test with sodium azide (NaN_3)

Measurement of PS fluorescence and $^1O_2$ emission at 1.27 μm was performed in the presence of an efficient $^1O_2$ quencher, NaN_3, using a near-IR PMT with a time response <5 ns. $^1O_2$ photon counts under Che6 and PPa treatment were measured with the PMT-SOD system. NaN_3 was then added to a final concentration of 0.1–10 mM in 10 μM of Che6 or PPa solution, including 200 μl methanol. Preliminary experiments were performed without NaN_3 to evaluate $^1O_2$ photon counts and lifetime under treatment with Che6 or PPa. Next, $^1O_2$ quenching was observed consecutively after adding 0, 0.1, 1.0, and 10 mM of NaN_3.

2.6. Intracellular level of Che6 or PPa

Intracellular Che6 or PPa level was calculated with $^1O_2$ counts by using PMT-SOD system and measured with a fluorescence meter (Synergy MX, BioTek, Winooski, VT, USA). $^1O_2$ photon counts were compared with the fluorescence intensity at various concentrations of Che6 (1.25, 2.5, 5.0, and 10 μM) and PPa (0.125, 0.25, 0.5, and 1 μM) in SW480, HT29, AsPC-1, and MiaPaCa-2 cells, which were seeded onto a 96-well culture plate, at 1×10^4 cells per well in 100 μl of RPMI1640 supplemented with 10% FBS. To detect fluorescence, the excitation and emission wavelengths were selected as 500 and 670 nm, respectively, for Che6, and 610 and 676 nm, respectively, for PPa. $^1O_2$ counts and fluorescence intensity in cells were corrected by cell counts.

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