Quantitative kinetics of intracellular singlet oxygen generation using a fluorescence probe

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Singlet oxygen (1O2) is a type of reactive oxygen species involved in numerous physiological activities. We previously reported that 1O2-specific oxidation products are increased in patients with prediabetes, suggesting that measurement of 1O2 may be an important indicator of physiological and pathological conditions. The turnover in the generation and quenching of 1O2 is extremely rapid during biological activities owing to its high reactivity and short lifetime in solution. However, the dynamic changes in 1O2 generation in living cells have not been fully explored. In this study, we investigated whether the kinetics of 1O2 generation can be quantified using a far-red fluorescent probe for mitochondrial 1O2, Si-DMA, following addition of the 1O2 generator, endoperoxide, to mammalian cells. The kinetics of Si-DMA fluorescence intensity dose-dependently increased following treatment of mammalian living cells with endoperoxide. Alternatively, treatment with 1O2 quenchers decreased the fluorescence intensities following endoperoxide treatment. Our results indicate that the kinetics of intracellular 1O2 can be readily obtained using Si-DMA and time-lapse imaging, which provides new insights into the mechanism of 1O2 generation in mammalian cells and the exploration of 1O2 generators and quenchers.

Reactive oxygen species (ROS) play critical roles in host defence and the production of biologically essential substances, as well as in the regulation of physiological functions as redox signalling messenger. Singlet oxygen (1O2) is a type of ROS that is involved in numerous biological processes and has also been applied in water disinfection and photodynamic therapy (PDT) in cancer treatment. PDT can effectively kill cancer cells via the reaction between a photosensitiser and laser light, as 1O2 induces cytotoxicity due to the strong oxidation reaction. Our previous studies demonstrated that the levels of 1O2-specific oxidation products 10- and 12-(Z,E)-hydroxyoctadecadienoic acids (HODEs) transiently increased in a mouse model of type 2 diabetes mellitus (T2DM) and that plasma levels of 10- and 12-(Z,E)-HODEs were positively correlated with glucose levels in patients with prediabetes. In addition, the production of cytoplasmic 1O2 induces cell cycle progression in HeLa cells, whereas the nuclear production delays the cell cycle. These findings suggest that 1O2 may contribute to the regulation of physiological and pathological conditions in mammals.

Singlet oxygen is produced by the energy transfer of triplet oxygen, which constitutively occurs in plant leaves during the reaction between light and chlorophyll pigments in cells. In mammals, 1O2 is physiologically generated in the skin upon exposure to ultraviolet-A or as a product of the reaction between hypochlorous acid and hydrogen peroxide mediated by myeloperoxidase. As 1O2 generation in mammalian cells is essential for biological activity, methods for its precise detection and quantification are needed to facilitate research for better understanding its roles in physiological and pathological conditions. One of the primary methods for 1O2 detection is the measurement of near-infrared phosphorescence at 1270 nm; however, this phosphorescence signal is narrow due to weak emission intensities. Further, this platform requires the use of sophisticated instruments. Fluorescence probes with high sensitivity, fast response time, and high spatial resolution under microscopic imaging have also been developed for the detection of 1O2, several of which even enable 1O2

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imaging in living cells\textsuperscript{18–22}. Recently, a far-red fluorescent probe for \(^{1}\text{O}_2\) composed of 9,10-dimethylanthracene (DMA) and silicon-containing rhodamine (Si-rhodamine) moieties, namely Si-DMA, was developed for monitoring \(^{1}\text{O}_2\) at subcellular levels\textsuperscript{23}. One of the main advantages of Si-DMA over other real-time probes is its increased sensitivity to specifically detect mitochondrial \(^{1}\text{O}_2\) at the subcellular level\textsuperscript{18–22}. This probe can react with mitochondrial-originating \(^{1}\text{O}_2\)\textsuperscript{23} as the Si-rhodamine contained in Si-DMA accumulates in the mitochondria, and the diffusion distance of intracellular \(^{1}\text{O}_2\) reaches a maximum of approximately 300 nm in aqueous solution\textsuperscript{24}. Although the turnover in the generation and quenching of \(^{1}\text{O}_2\) is predicted to be very rapid for the maintenance of cellular functions, the detailed dynamic changes in intracellular \(^{1}\text{O}_2\) generation levels in living cells remain largely unknown.

In this study, we investigated the feasibility of using the \(^{1}\text{O}_2\) fluorescence probe Si-DMA to obtain kinetic data on intracellular \(^{1}\text{O}_2\) generation and quenching following treatment of cells with an \(^{1}\text{O}_2\) generator, endoperoxide. Toward this end, we treated the mouse fibroblast cell line, NIH3T3, and the human hepatocarcinoma cell line, HepG2, with the \(^{1}\text{O}_2\) generator endoperoxide, or the oxygen quenchers, sodium azide (NaN\textsubscript{3}) and astaxanthin, at various concentrations, and measured the resulting fluorescence intensities of Si-DMA with fluorescence time-lapse imaging. HepG2 cells were used as a common \textit{in vitro} liver model since \(^{1}\text{O}_2\) was reported to be produced in the liver\textsuperscript{25,26}. Astaxanthin is a carotenoid pigment with high \(^{1}\text{O}_2\)-quenching capacity \textit{in vitro}\textsuperscript{27–30}, and was therefore used as a model for evaluating the feasibility of detecting \(^{1}\text{O}_2\) quenching of food ingredients in living cells. This technique may offer a valuable tool to better understand the mechanism of \(^{1}\text{O}_2\) generation, while providing a practical method to rapidly and simply screen for candidate \(^{1}\text{O}_2\) generators or quenchers in mammalian cells.

**Results**

**Dynamic changes in Si-DMA fluorescence after endoperoxide treatment.** We first attempted to quantify the intracellular \(^{1}\text{O}_2\) concentration based on Si-DMA fluorescence intensities, which react exclusively with mitochondrial-originating \(^{1}\text{O}_2\)\textsuperscript{23} as the diffusion distance of intracellular \(^{1}\text{O}_2\) is less than 300 nm in aqueous solution\textsuperscript{24}, using fluorescence-activated cell sorting (FACS) (Fig. 1a). We used an \(^{1}\text{O}_2\) generator endoperoxide, which is commercially available from WakenbTech Co., Ltd. (Osaka Japan), contains an epidi- oxy group crosslinked in the aromatic hydrocarbon, and generates \(^{1}\text{O}_2\) during thermolysis without photosensitiser (Equation 1). As this material produces \(^{1}\text{O}_2\) via thermal decomposition at temperatures greater than 25 °C\textsuperscript{27}, we maintained the storage temperature of endoperoxide under 20 °C until just before use to avoid decomposition.

![Diagram of endoperoxide thermolysis](image)

\begin{equation}
\text{endoperoxide} \xrightarrow{\text{thermolysis}} \text{Si-DMA} + \text{O}_2
\end{equation}

The intensities were measured after the addition of endoperoxide at concentrations \(\leq 2\) mM because the use of higher concentrations resulted in insoluble endoperoxide in the culture medium at 37 °C. In FACS analysis, the fluorescence intensity in NIH3T3 cells clearly increased after treatment of 1 or 2 mM endoperoxide compared with that of the control cells treated with 1% dimethyl sulfoxide (DMSO) (Fig. 1b,c). Increase in fluorescence intensity was observed for endoperoxide, with a dose-dependent increase up to 2.0 mM, at which point it reached a plateau (correlation coefficient \(r = 0.9729\), Fig. 2c).

These results indicate that the relative \(^{1}\text{O}_2\) yield in living cells could be quantitatively measured using Si-DMA after treatment of 0.5–2 mM endoperoxide with FACS or 0.2–0.7 mM endoperoxide with fluorescence microscopy. Singlet oxygen becomes rapidly quenched in culture medium and mammalian cells as the lifetime of \(^{1}\text{O}_2\) is less than 3 μs in cells cultured in a \textit{H}_2\text{O}-based medium\textsuperscript{26}, and 15 μs in cells cultured in a \textit{D}_2\text{O}-based medium\textsuperscript{31}. In addition, \(^{1}\text{O}_2\) generated by endoperoxide treatment readily reacts with mitochondrial molecules, such as membrane lipids\textsuperscript{23} and glutathione\textsuperscript{32}. Thus, we considered that time-lapse imaging using fluorescence microscopy is an appropriate method for measurement of the dynamic changes in \(^{1}\text{O}_2\) yield in living cells.

**Measurement of Si-DMA fluorescence intensity after treatment of \(^{1}\text{O}_2\) quenchers.** To evaluate whether decreases in \(^{1}\text{O}_2\) generation can also be observed based on Si-DMA fluorescence, we treated HepG2 cells with the \(^{1}\text{O}_2\) quencher NaN\textsubscript{3} after confirmation of the increase in fluorescence intensity with 0.5 mM endoperoxide treatment. As shown in Fig. 3a, the fluorescence intensity dose-dependently decreased immediately after NaN\textsubscript{3} treatment. These decreases did not appear to be due to direct cellular damage, as the viability of HepG2 cells was not reduced at 24 h after 10 mM or 30 mM NaN\textsubscript{3}, whereas treatment with more than 60 mM NaN\textsubscript{3} decreased the viability of HepG2 cells (Supplementary Fig. S1). In addition, the relative trough intensity after
NaN₃ treatment significantly decreased compared with that of the control group (Fig. 3b). Interestingly, the kinetics of fluorescence intensity after NaN₃ treatment varied according to experimental conditions with a transient decrease in Si-DMA fluorescence intensity observed in NIH3T3 cells even after 0.1 mM endoperoxide and 100 mM NaN₃ treatment (Fig. 3c,d). With treatment of 30 mM NaN₃, the intensity tended to exceed the initial value after the transient reduction (Fig. 3c). These results indicate that quenching of ¹⁰⁰₂ in living cells can be observed by time-lapse imaging with Si-DMA and fluorescence microscopy.

As shown in Fig. 4a, the Si-DMA fluorescence in HepG2 cells clearly decreased after treatment with 50 μM astaxanthin (which was confirmed to have no cytotoxic effect in preliminary experiments; data not shown) following 0.5 mM endoperoxide treatment compared with that in the control cells treated with 1% DMSO. The peak values of the fluorescence intensity in the control and astaxanthin groups were 1.82 and 1.40, respectively (Fig. 4b). These results suggest that this method could be applied to investigate the ¹⁰⁰₂-quenching capacity of various materials in living cells.

Discussion
Due to its short lifetime and low concentration in mammalian cells, it is difficult to quantify intracellular ¹⁰⁰₂. Although some studies recently demonstrated that ¹⁰⁰₂ in living cells could be detected using fluorescent probes¹⁸⁻²⁰, the dynamic alterations in intracellular ¹⁰⁰₂ generation have remained unclear. In the present study, we developed and verified a method for the quantitative measurement of ¹⁰⁰₂ generation in living cells using the fluorescent probe Si-DMA.

Endoperoxide solution stably generated measurable amounts of ¹⁰⁰₂ in the culture medium, whereas hydrogen peroxide and hypochlorous acid produced non-reproducible amounts of intracellular ¹⁰⁰₂. Using the

Figure 1. Measurement of Si-DMA fluorescence using FACS analysis. (a) The difference in fluorescence intensities in NIH3T3 mouse fibroblasts with and without Si-DMA. The intensity is indicated as the mean value of the M1 range. (b) Relationship between the number of cells and Si-DMA fluorescence intensity after 1% DMSO or 2 mM endoperoxide (EP) treatment. (c) Averages of Si-DMA fluorescence intensity after EP treatment. Results are expressed as mean ± standard deviation (n = 2).
In the endoperoxide solution and Si-DMA, we observed an increase in $^1\text{O}_2$ generation that positively correlated with the endoperoxide concentration. In contrast, the intracellular $^1\text{O}_2$ generation decreased after $^1\text{O}_2$ quencher treatment. In particular, the fluorescence intensities were detectable despite the rapid changes in the intracellular $^1\text{O}_2$ generation and quenching, which occurred within a few minutes. These observations can be explained by the high selectively of the probe Si-DMA toward $^1\text{O}_2$. The structure of Si-DMA allows photoinduced electron transfer (PET) between dimethylanthracene (DMA, electron donor) and Si-rhodamine (electron acceptor) by light excitation. Si-rhodamine is a fluorescence pigment, while DMA acts as a quencher. Hence, the fluorescence of excited Si-rhodamine becomes quenched by intramolecular PET from DMA, resulting in dim fluorescence ($\phi_{fl} = 0.01$). The anthracene analogue specifically traps and reacts with $^1\text{O}_2$, which introduces an endoperoxide group to the centre ring of the DMA unit in Si-DMA leading to the formation of Si-DMEP (Equation 2). As a result, PET from DMA to Si-rhodamine is inhibited due to the reduced electron-donating capacity of DMA, and the formed Si-DMEP exhibits an approximately 18-fold higher fluorescence than Si-DMA in methanol23. The DMA moiety in Si-DMA does not release $^1\text{O}_2$ following endoperoxidation, which is in accordance with the endoperoxide form of Singlet Oxygen Sensor Green®, SOSG-EP35. In contrast, certain anthracene derivatives undergo a reversible reaction with $^1\text{O}_2$ that is, endoperoxide formation is followed by the release of $^1\text{O}_2$.36 As Si-DMA can selectively and stably react with $^1\text{O}_2$, we could monitor intracellular $^1\text{O}_2$ levels using time-lapse imaging.

Figure 2. Dynamic changes in Si-DMA fluorescence intensity using time-lapse imaging. (a) Representative fluorescence microscopic images in HepG2 cells incorporating Si-DMA before and after endoperoxide (EP) treatment, respectively. Scale bars = 50 μm. (b) Kinetics of relative Si-DMA fluorescence intensities after EP treatment. The intensities were obtained from each sequence of images (movie) and relative intensities were normalised to the control values (1% DMSO treatment). (c) Correlations between the inclination angle of Si-DMA fluorescence and EP concentration. The inclination angle represents the degree of the slope for the linear regression of Si-DMA fluorescence intensity against the EP concentration. Results are indicated as mean ± SE (n = 3). The strength of the association between two parameters was evaluated on the basis of Pearson’s correlation coefficient.
Figure 3. Effect of the \( ^1\text{O}_2 \) quencher NaN3 on Si-DMA fluorescence intensity. (a,c) Kinetics of the intensity in HepG2 (a) and NIH3T3 (c) cells after NaN3 treatment. Relative fluorescence intensity is presented as the average values relative to those obtained during 1 min before NaN3 treatment, set to 1. (b,d) Comparison of trough intensity in Si-DMA fluorescence within the first 3 min after NaN3 treatment. Relative intensities were determined by the ratio of trough intensity to the average obtained during 1 min before NaN3 treatment. Results are indicated as mean ± SE (b: n = 3, d: n = 1). *p < 0.05 compared with the control.
Considering that the lifetime of $^{1}$O$_2$ is approximately 3 $\mu$s in the nuclei of H$_2$O-incubated cells$^{24}$, the lifetime of $^{1}$O$_2$ in this study was probably shorter since the culture medium contained high levels of amino acids that also react with $^{1}$O$_2$. Moreover, since the diffusion distance of $^{1}$O$_2$ is less than 300 nm in 6 $\mu$s$^{24}$ and endoperoxides can diffuse to mitochondria, we speculated that only mitochondrial $^{1}$O$_2$ was detected by Si-DMA in this study. This is also supported by the fact that Si-DMA selectively localizes in the mitochondria at a concentration of 100 nM$^{23}$, which was used in this study. Hence, the results of this study deepen our understanding of the mechanisms underlying the generation of mitochondrial $^{1}$O$_2$. Furthermore, the half-life of endoperoxide is approximately 20 minutes in ethanol/chloroform/D$_2$O (50:50:1, v/v/v) at 35 °C$^{27}$; similarly, Si-DMA is considered to be photo-stable as it has been detected in HeLa cells in at least 15 minutes following treatment$^{23}$. These previous findings suggest that endoperoxide and Si-DMA are likely stable in cells and culture media. Therefore, although $^{1}$O$_2$ was rapidly quenched under the present experimental conditions, we were able to detect mitochondrial $^{1}$O$_2$ using time-lapse fluorescence imaging.

Specific food ingredients, such as astaxanthin and lycopene, are known to exhibit high $^{1}$O$_2$-quenching capacities under cell-free conditions$^{28,37,38}$. It is believed that these ingredients can exert similar quenching effects in living cells, contributing to the prevention of human chronic diseases, including cardiovascular diseases and diabetes$^{39,40}$. Although it was previously reported that the intracellular $^{1}$O$_2$ quenching capacity of $\beta$-carotene could not be observed using laser-based time-resolved photosensitised methods$^{41}$, we showed that the fluorescence intensities of Si-DMA in astaxanthin-treated cells significantly decreased, indicating that astaxanthin can quench $^{1}$O$_2$ in mammalian cells. On the other hand, we sought to evaluate whether $\beta$-carotene quenches intracellular $^{1}$O$_2$ generated by endoperoxide; however, reproducible results were not obtained, which might be because carotenoids preferentially accumulate in cell membranes. $\beta$-carotene is localized deep inside the hydrophobic core membranes and oriented parallel to the membrane surface, whereas astaxanthin, with two polar hydroxyl groups, is anchored across the cell membrane with the polar groups oriented outside the membrane$^{42}$. While the $\beta$-carotene reaction sites for $^{1}$O$_2$ are buried in the cell membrane, those of astaxanthin cross the membrane. We, therefore, speculate that astaxanthin localized in the mitochondrial membrane can quench mitochondrial $^{1}$O$_2$ produced by endoperoxide. This further confirms that the $^{1}$O$_2$-quenching capacity of various materials can be evaluated using Si-DMA in time-lapse imaging, offering a novel approach for exploring the modes of action of $^{1}$O$_2$ quenchers in living cells. Furthermore, the present approach might clarify whether some ingredients prevent chronic diseases via $^{1}$O$_2$-quenching.

As the biological function of $^{1}$O$_2$ in mammals remains poorly understood, we expect that the application of the fluorescence probe Si-DMA will help to elucidate the detailed mechanism underlying intracellular $^{1}$O$_2$ generation. Although the plasma levels of the $^{1}$O$_2$-mediated oxidation products 10- and 12-(Z,E)-HODEs correlated with fasting glucose levels in patients with prediabetes$^{47}$ and temporarily increased before the pathogenesis of T2DM in mice$^{4}$, the precise mechanisms behind this increase in a prediabetic state remain elusive. Singlet oxygen itself or its oxidation products, 10- and 12-(Z,E)-HODEs, may induce an adaptive response to ROS exposure$^{43}$.

![Figure 4. Effect of astaxanthin on Si-DMA fluorescence intensity. (a) Kinetics of the fluorescent intensity in HepG2 cells after 0.5 mM endoperoxide (EP) treatment. At 24 h after 50 $\mu$M astaxanthin treatment, Si-DMA was added, and the fluorescence intensity was measured. Relative fluorescence intensity was calculated against the value under EP treatment, set to 1 (grey bar = SE, n = 3). (b) Comparison of peak intensity in Si-DMA fluorescence after 0.5 mM EP treatment. Relative intensities were determined by the ratio of peak intensity to the value obtained just after EP treatment. Results are indicated as mean ± SE (n = 3). *p < 0.05 compared with the control.](image-url)
leading to enhanced cellular detoxification activities, against T2DM. Thus, it is important to elucidate the biological function of $^1\text{O}_2$ and the underlying generation mechanism. We also observed Si-DMA-positive cells in primary murine hepatocytes (data not shown), suggesting that Si-DMA is also applicable for the detection of $^1\text{O}_2$ in animals. Thus, the application of Si-DMA in patients and model mice with T2DM might contribute to a better understanding of the role of $^1\text{O}_2$ in the development of T2DM.

In conclusion, we developed a method to quantitatively measure of intracellular $^1\text{O}_2$ levels using a fluorescence probe. To the best of our knowledge, this is the first study assessing the dynamic changes in $^1\text{O}_2$ generation and can offer a valuable tool for exploring the role of $^1\text{O}_2$ quenchers in living mammalian cells.

**Methods**

**Cell culture.** Human hepatic carcinoma HepG2 cells and mouse NIH3T3 fibroblasts were grown in Dulbecco’s modified Eagle medium (DMEM; Sigma Aldrich, MO, USA) supplemented with 10% foetal bovine serum (ICN Biochemicals, CA, USA) in a humidified atmosphere containing 5% CO$_2$ at 37°C.

**Measurement of Si-DMA fluorescence intensity.** The cells (5 × 10$^5$ per well or dish) were seeded in a 6-well plate (Nunc, MA, USA) for flow cytometry or in a 35-mm glass-based dish (Nunc) for live cell imaging. One day after incubation, each concentration (0.5–1.0 mM) of endoperoxide [3-(1, 4-epidioxo-4-methyl-1, 4-dihydro-1-naphthyl) propionic acid; WakenBtech Co., Ltd., Kyoto, Japan] dissolved in DMSO was added to the cells and incubated for 30 min at 37°C and 5% CO$_2$; control cells were treated with DMSO at the same concentrations. The endoperoxide solution was cooled below 20°C just before use, as endoperoxide stably generates $^1\text{O}_2$ above 35°C. The cells were washed with serum-free DMEM and incubated in Hanks’ balanced salt solution (HBSS) containing Si-DMA (Dojindo Laboratories, Kumamoto, Japan), which is a suitable fluorescent dye for $^1\text{O}_2$ imaging$^{22}$, for 30 min. Si-DMA was dissolved in DMSO according to the manufacturer’s instruction and stored at –30°C until used for the experiments. After washing with phosphate-buffered saline, the cells were harvested and analysed by flow cytometry with the FACS Calibur system (Becton Dickinson, NJ, USA). The laser amplitude was appropriately set to divide into two groups (with or without Si-DMA) and the median of the M1 marker was defined as the Si-DMA fluorescence intensity (Fig. 1a).

For live cell imaging, cells seeded onto the 35-mm glass-based dish were washed with HBSS after incubation in Si-DMA-containing solution. Subsequently, 1 ml HBSS was added to the cells and the dish was placed on the stand of the fluorescence microscope (BZ-X710 All-in-one, Keyence, Tokyo, Japan). Fluorescence images were obtained from snapshots of movies (around 1 s exposure) taken for 30 min. At 5 min after filming, endoperoxide or the negative control (1% DMSO) was added to the cell-seeded dish and the fluorescence intensities were continuously measured. The single oxygen quencher NaN$_3$ was added to the dish at 5 min after endoperoxide or 1% DMSO treatment. Astaxanthin (Sigma Aldrich) was dissolved in DMSO and added to the cells 1 day before endoperoxide treatment. The fluorescence intensity was analysed using BZ-X Analyzer (Keyence), and the results are presented as the relative fluorescence intensity to that measured just prior to the addition of each material.

**Measurement of cell viability.** HepG2 cells were plated in the wells of a 96-well microplate (Nunc) and incubated overnight at 37°C with 5% CO$_2$. DMEM containing each concentration of NaN$_3$ was prepared and incubated in a humidified atmosphere containing 5% CO$_2$ at 37°C.

**Statistical analysis.** The results are expressed as means ± standard deviation or standard error. Statistical analysis was performed using analysis of variance followed by Tukey’s test for multiple comparisons with Eksu-to-Toukei 2012 software (Social Survey Research Information Co., Ltd., Tokyo, Japan). The strength of correlation between two variables was analysed by Pearson’s correlation coefficient. Differences with a probability of 5% or less were considered statistically significant.

**Data availability**

The all data sets used and/or analysed during this study are available from the corresponding author on reasonable request.

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Author contributions
K.M. and Y.Y. initiated the research project and designed the research. K.M., A.U. and S.S. performed the experiments, and they analysed research data. K.M., A.U. and Y.Y. drafted the manuscript and designed the Figures. All authors discussed the results and commented on the manuscript.

Competing interests
The authors declare no competing interests.

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