Reevaluation of analytical methods for photogenerated singlet oxygen

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The aim of the present study is to compare different analytical methods for singlet oxygen and to discuss an appropriate way to evaluate the yield of singlet oxygen photogenerated from photosensitizers. Singlet oxygen photogenerated from rose bengal was evaluated by electron spin resonance analysis using sterically hindered amines, spectrophotometric analysis of 1,3-diphenylisobenzofuran oxidation, and analysis of fluorescent probe (Singlet Oxygen Sensor Green®). All of the analytical methods could evaluate the relative yield of singlet oxygen. The sensitivity of the analytical methods was 1,3-diphenylisobenzofuran < electron spin resonance < Singlet Oxygen Sensor Green®. However, Singlet Oxygen Sensor Green® could be used only when the concentration of rose bengal was very low (<1 μM). In addition, since the absorption spectra of 1,3-diphenylisobenzofuran is considerably changed by irradiation of 405 nm laser, photosensitizers which are excited by light with a wavelength of around 400 nm such as hematoporphyrin cannot be used in the 1,3-diphenylisobenzofuran oxidation method. On the other hand, electron spin resonance analysis using a sterically hindered amine, especially 2,2,6,6-tetramethyl-4-piperidinol and 2,2,5,5-tetramethyl-3-pyrroline-3-carboxamide, had proper sensitivity and wide detectable range for the yield of photogenerated singlet oxygen. Therefore, in photodynamic therapy, it is suggested that the relative yield of singlet oxygen generated by various photosensitizers can be evaluated properly by electron spin resonance analysis.

Key Words: singlet oxygen, rose bengal, electron spin resonance, DPIBF, fluorescent probe

It is well known that singlet oxygen, one of reactive oxygen species (ROS), is highly oxidative and exerts strong cytotoxic effects.(1) Singlet oxygen is thought to be a cause of some disorders, such as light-induced skin disorder, oxidative lung injury, and erythropoietic porphyria.(2) On the other hand, the cytotoxicity of singlet oxygen is applied for cancer treatment and antimicrobial therapy, which is known as photodynamic therapy (PDT).(2–5) In terms of effect of PDT, the larger amount of singlet oxygen the target is exposed to, the more effectively undesired cells such as cancer cells and bacteria are killed. The yield of singlet oxygen, however, should be minimum for the treatment to avoid excessive oxidative damage on normal cells.(3) Since singlet oxygen has such bilateral characters, it is important to evaluate the yield of singlet oxygen photogenerated from various photosensitizers and to control the yield.

Singlet oxygen is generated by energy transfer to triplet oxygen from a photosensitizer excited by light with specific wavelength in PDT.(6,7) Many studies suggest that singlet oxygen plays a central role for cytotoxicity in PDT.(8–10) However, the relationship between the yield of singlet oxygen and the bactericidal effect has not been fully understood. One of the reasons for that is the difficulty to analyze the yield of singlet oxygen precisely. Although several indirect analytical methods for measurement of singlet oxygen, such as electron spin resonance (ESR) technique,(9,10) analysis of 1,3-diphenylisobenzofuran (DPIBF) oxidation,(11,12) and application of fluorescent probes,(13) have been widely used in various fields of research, it has not been demonstrated that which analytical method is appropriate for evaluation of the yield of singlet oxygen generated in PDT. To evaluate the yield of singlet oxygen generated in PDT, there are some interference factors. For instance, it is known that colored chemicals including photosensitizers sometimes interfere with spectrophotometric and/or fluorescent analysis. So far, there have been no comparative studies for the determinations of photogenerated singlet oxygen.

The aim of the present study is to compare the different kinds of indirect analytical methods for photogenerated singlet oxygen and to discuss an appropriate way to evaluate photosensitizers for PDT.

Materials and Methods

Reagents. Reagents were purchased from the following sources: 2,2,6,6-tetramethylpiperidine (TEMP), 2,2,6,6-tetramethyl-4-piperidinol (4-oxo-TEMP), 2,2,6,6-tetramethyl-4-piperidinol (4-hydroxy-TEMP), 2,2,5,5-tetramethyl-3-pyrroline-3-carboxamide (TPC), 4-hydroxy-2,2,6,6-tetramethylpiperidine N-oxyl (TEMPOL), 4-oxo-2,2,6,6-tetramethyl-1-piperidinoyl (4-oxo-TEMPO), DPIBF, hypoxanthine (HPX), sodium azide and superoxide dismutase (SOD) from Sigma Aldrich (St. Louis, MO); Singlet Oxygen Sensor Green® (SOSG) from Molecular Probes (Eugene, OR); xanthine oxidase (XOD) and 5,5-dimethyl-1-pyrroline N-oxide (DMO) from Labotec (Tokyo, Japan); rose bengal, astaxanthin, dimethyl sulfoxide (DMSO), D-mannitol and catalase from Wako Pure Chemical Industries (Osaka, Japan); H2O2 from Santoku Chemical Industries (Tokyo, Japan); All other reagents used were of analytical grade.

An experimental laser device for photoactivation of rose bengal. An experimental laser device equipped with the second harmonic of Nd-YAG laser (wavelength: 532 nm) and a laser power meter was made (PAX Co. Ltd., Sendai, Japan, Fig. 1). The wavelength of laser light for excitation of rose bengal was determined by spectrophotometric analysis of the absorbance of rose bengal. An output power of the laser was set at 20 mW in the present study. When a semi-micro cuvette containing 200 μL of sample is set in the experimental device, the area of the sample irradiated by the laser is almost 5 × 5 mm resulting in the energy dose of 80 mW/cm². The light path of the cuvette was 10 mm.
ESR analysis of nitroxide radical generated through oxidation of sterically hindered amine by singlet oxygen. Four different sterically hindered amines, TEMP, 4-oxo-TEMP, 4-hydroxy-TEMP and TPC, were used without further purification (Fig. 2). TEMP was dissolved in 99.5% ethanol while the other amines were dissolved in ultrapure water to make stock solutions with a concentration of 1 mM. To examine the concentration effect of the amines on the yield of nitroxide radical, two-fold serial dilutions of each stock solution were prepared using each solvent. Rose bengal known as a photosensitizer for generation of singlet oxygen was dissolved in ultrapure water to make a stock solution with a concentration of 100 µM. Then, 180 µL of the amine and 20 µL of the rose bengal was mixed in a disposable plastic semi-micro cuvette to make final concentrations of 28, 56, 112, 225, 450 and 900 mM for the amine, and 0, 1, and 10 µM for rose bengal. Immediately after mixing, the cuvette was set in the experimental laser device. The sample in the cuvette was irradiated by the laser light for 60 sec. After the laser irradiation, the sample was transferred to a quartz cell and ESR spectrum was recorded on an X-band ESR spectrometer (JES-FA-100, JEOL, Tokyo, Japan). The measurement conditions for ESR were as follows: field sweep, 330.50–340.50 mT; field modulation frequency, 100 kHz; field modulation width, 0.05 mT; microwave frequency, 9.420 GHz; microwave power, 4 mW. To calculate the spin concentration of each nitroxide radical, 20 µM TEMPOL was used as a standard sample and the ESR spectrum of manganese (Mn^2+) which was equipped in the ESR cavity was used as an internal standard. The spin concentration was determined using Digital Data Processing (JEOL, Tokyo, Japan).

Based on the result of above experiment, further experiments were performed using 4-hydroxy-TEMP and TCP to investigate the relationship among the concentration of rose bengal, the laser irradiation time, and the yield of nitroxide radical. Each amine and rose bengal were mixed to make final concentrations of 450 mM for the amine, and 0, 1, 5, 10, 20, and 40 µM for rose bengal. The sample was then irradiated by the laser light for 60 sec. Similarly, the sample containing 450 mM amine and 10 µM rose bengal was irradiated for 0, 15, 30, 45, and 60 sec. The yield of nitroxide radical was measured and calculated by the same way as described above. 

Spectrophotometric analysis of DPIBF oxidation caused by singlet oxygen. The relationship between the concentration of rose bengal and absorption peak of DPIBF at 420 nm was analyzed. DPIBF was dissolved in 99.5% ethanol to make a stock solution with a concentration of 4 mM. Fifty µL of the DPIBF stock solution and 150 µL of rose bengal were mixed in a cuvette to make final concentrations of 1.0 mM for DPIBF, and 0, 1, 5, 10, 20, or 40 µM for rose bengal. The sample was then irradiated by the laser light for 60 sec. Immediately after irradiation, the absorption peak was analyzed using spectrophotometer (Nanovue plus, GE Healthcare, Buckinghamshire, UK). Furthermore, the relationship between the laser irradiation time and the absorption peak of DPIBF was also investigated. The sample containing 1.0 mM DPIBF and 10 µM rose bengal was irradiated by the laser light for 0, 15, 30, 45, and 60 sec. The changes in absorption peak at 420 nm were analyzed by the same way as described above.

Analysis of fluorescent probe oxidized by singlet oxygen. SOSG, a commercially available fluorescent probe for detection of singlet oxygen, was used. According to the manufacturer’s instruction, SOSG was dissolved in methanol to make a stock solution with a concentration of 5 mM and then was diluted with ultrapure water to given concentrations. To examine the concentration effect of SOSG on fluorescence response, 20 µL of SOSG and 180 µL of rose bengal were mixed in a cuvette to make final concentrations of 1, 2, 5 or 10 µM for SOSG and 1 mM for rose bengal. The sample was irradiated by the laser light for 60 sec and then 100 µL of the sample was transferred to a black 96 well microplate. Fluorescence was measured with a microplate reader (DTX 880, Beckman Coulter, Brea, CA) using excitation/emission of 485/535 nm. According to the manufacturer’s instruction, SOSG was dissolved in methanol to make a stock solution with a concentration of 5 mM and then was diluted with ultrapure water to given concentrations. To examine the concentration effect of SOSG on fluorescence response, 20 µL of SOSG and 180 µL of rose bengal were mixed in a cuvette to make final concentrations of 1, 2, 5 or 10 µM for SOSG and 1 mM for rose bengal. The sample was irradiated by the laser light for 60 sec and then 100 µL of the sample was transferred to a black 96 well microplate. Fluorescence was measured with a microplate reader (DTX 880, Beckman Coulter, Brea, CA) using excitation/emission of 485/535 nm. The fluorescence response was examined. SOSG and the rose bengal were mixed to make final concentrations of 10 µM for SOSG and 0, 0.06, 0.13, 0.25, 0.5, 1.0 or 2.0 µM for rose bengal. The sample was then irradiated by the laser light for 60 sec. Similarly, the sample containing 10 µM SOSG and 1.0 µM rose bengal was irradiated by the laser light for 0, 15, 30, 45, and 60 sec. The fluorescence response was measured by the same way as described above.

Specificity of each analytical method for singlet oxygen. Since it was considered that the reagents used in this study would be oxidized by not only singlet oxygen but also other ROS, such as superoxide anion, H_2O_2, and hydroxyl radical, we investigated how much the other ROS oxidized the reagents. The amines (4-hydroxy-TEMP and TCP), DPIBF and SOSG were used at a final concentration of 450 mM, 1 mM and 10 µM, respectively. Each analytical method was performed by the same way as described above. Superoxide anion was generated by HPX/XOD reaction system. HPX and XOD were mixed with each reagent (the amines, DPIBF and SOSG) to make final concentrations of 100 and

![Fig. 1. Schematic illustration of experimental laser device.](image)

![Fig. 2. Chemical structures of sterically hindered amines.](image)
1,000 µM for HPX, and 0.25 U/mL for XOD. H₂O₂ diluted with ultrapure water was mixed with each reagent to make a final concentration of 50 or 500 mM. Hydroxyl radical was generated by photolysis in which the sample containing 50 and 500 mM H₂O₂ and each reagent was irradiated by the laser light with a wavelength of 405 nm and an output power of 20 mW for 60 sec. Furthermore, hydroxyl radical was also generated by sonolysis in which the ultrapure water mixed with each reagent was irradiated by ultrasound with a frequency of 1,650 kHz and an output power of 30 W for 15 and 30 sec. The yields of superoxide anion and hydroxyl radical were quantitatively analyzed by an ESR spin trapping technique using DMPO. In brief, DMPO instead of each reagent was added to the reaction system of HPX/XOD, photolysis, or sonolysis to make a final concentration of 300 mM for DMPO. The reaction mixture was treated in the same way as described above and then ESR spectrum was recorded.

Astaxanthin and sodium azide known as authentic singlet oxygen quenchers were added to the reaction system of aforementioned ESR analysis to examine the effect on the yield of nitroxide radical. Astaxanthin was dissolved in DMSO while sodium azide was dissolved in ultrapure water. They were mixed with the amine (4-hydroxy-TEMP and TCP) and rose bengal to make final concentrations of 2.5 mM for astaxanthin or sodium azide, 450 mM for the amine, and 10 µM for rose bengal. Since ROS other than singlet oxygen might be involved in the generation of nitroxide radical in the photoactivation of rose bengal, the effect of the ROS scavengers on ESR analysis was examined. Mannitol, SOD, and catalase which are well-known scavengers against hydroxyl radical, superoxide anion, and hydrogen peroxide, respectively, were used. They were mixed with the amine and rose bengal to make final concentrations of 2.5 mM for mannitol, 10 U/mL for SOD and catalase, 450 mM for the amine, and 10 µM for rose bengal. Each mixture was irradiated by the laser light for 60 sec and then analyzed using the ESR by the same way as described above. Furthermore, since astaxanthin has an absorption peak at around 490 nm, it can absorb the excitation light for rose bengal. Hence, we examined if astaxanthin absorbs the excitation light for rose bengal, which in turn causes a decrease in the yield of nitroxide radical. The power of transmitted light through the reaction mixture with or without astaxanthin was measured by a power meter equipped in the experimental laser devise. Since astaxanthin reduced the power of the light by 3 mW, the mixture without astaxanthin was irradiated with the same laser except that an output power was 15 mW instead of 20 mW. On the other hand, sodium azide, mannitol, SOD and catalase did not absorb the light with a wavelength of 532 nm.

The yields of the other ROS which might be generated by photoactivation of rose bengal were analyzed by the ESR spin trapping technique as described above. Rose bengal was mixed with DMPO to make final concentrations of 10 µM for rose bengal and 300 mM for DMPO. Then, the sample irradiated by the laser light for 60 sec was subjected to the ESR analysis.

Results

ESR analysis of nitroxide radical generated through oxidation of sterically hindered amine by singlet oxygen. Representative ESR signals of nitroxide radical generated during the oxidation of each amine by singlet oxygen are shown in Fig. 3. When 4-hydroxy-TEMP was oxidized by singlet oxygen, two different ESR signals were observed. They were confirmed as TEMPOL and 4-oxo TEMP as a result of comparison with authentic TEMPOL and 4-oxo-TEMPO.

When the amines used in this study were dissolved in each solvent, ESR signal of each nitroxide radical was observed even if singlet oxygen did not exist. The amount of nitroxide radical increased with the concentration of amines (Fig. 4). Of the amines, TEMP and 4-oxo-TEMP showed the relatively large amount of nitroxide radical even when singlet oxygen was not produced. However, when the sample containing each amine and rose bengal was irradiated by the laser light, the spin concentration of nitroxide radical increased much more than that of the amine alone (Fig. 4). According to the aforementioned experiment, 4-hydroxy-TEMP and TCP, both of which showed only small amount of background nitroxide radical, were used in the subsequent experiments. The yields of nitroxide radical increased with the concentration of rose bengal and then reached a maximum at 10 to 20 µM rose bengal (Fig. 5A). When the effect of laser irradiation time was examined, the yield of nitroxide radical linearly increased with the time (Fig. 5B). The generation rates of nitroxide radical which corresponded to the slope values were 0.91 µMs⁻¹ and 0.46 µMs⁻¹ for 4-hydroxy-TEMP and TCP, respectively. Concerning sensitivity for detection of singlet oxygen, both 4-hydroxy-TEMP and TCP could detect the yield of singlet oxygen photogenerated from 1 µM rose bengal.

Spectrophotometric analysis of DPIBF oxidation caused by singlet oxygen. Neither addition of rose bengal to DPIBF without laser irradiation nor laser irradiation to DPIBF without rose bengal affected the absorption peak of DPIBF. On the other hand, when DPIBF was mixed with rose bengal and irradiated by the laser light, the absorption peak of DPIBF decreased with the concentration of rose bengal and then reached a minimum at 20 µM rose bengal (Fig. 6A). In the experiment where the effect of laser irradiation time was examined, the absorption peak of DPIBF linearly decreased in a time dependent manner (Fig. 6B). The analysis using DPIBF could hardly detect the yield of singlet oxygen photogenerated from 1 µM rose bengal.

Analysis of fluorescent probe oxidized by singlet oxygen. The fluorescence response of SOSG linearly increased with the concentration of SOSG. Although the fluorescence of SOSG without oxidation was detected, SOSG oxidized by singlet oxygen photogenerated from rose bengal showed much higher fluorescence intensity (Fig. 7A). When 10 µM SOSG was used, the fluorescence increased with the concentration of rose bengal and then gradually saturated at around 0.5 to 1.0 µM rose bengal (Fig. 7B). Therefore, SOSG could not detect the difference in the yield of singlet oxygen when the concentration of rose bengal was 1.0 µM or more. On the other hand, SOSG could detect the yield of singlet oxygen photogenerated from rose bengal even at a concentration as low as 0.06 µM suggesting that SOSG has the highest sensitivity for detection of singlet oxygen among the analytical methods used in this study. When the effect of laser irradiation time was examined, the fluorescence response linearly increased with the time (Fig. 7C).

Specificity for singlet oxygen of each analytical method. In ESR analysis, 4-hydroxy-TEMP and TPC were converted to nitroxide radical through the oxidation by 500 mM H₂O₂ and/or hydroxyl radical generated by photolysis of H₂O₂ (Fig. 8 A and B). On the other hand, superoxide anion generated in HPX/XOD reaction system and hydroxyl radical generated by sonolysis hardly oxidized the amines. The yields of the ROS were as follows; hydroxyl radical generated by photolysis of 50 mM and 500 mM H₂O₂ were 1 and 7 µM, hydroxyl radical generated by sonolysis of water for 30 and 60 sec were 8 and 15 µM, and superoxide anion generated by HPX/XOD reaction system using 100 and 1,000 µM HPX were 3 and 8 µM, respectively. When 4-hydroxy-TEMP was oxidized by ROS other than singlet oxygen, the ESR signal of TEMPOL increased but the signal of 4-oxo-TEMPO, which was detected when singlet oxygen was produced, was not observed. Thus, it is demonstrated that the amines will be oxidized by not only singlet oxygen but the other ROS. Spectrophotometric analysis of DPIBF oxidation was not or only little affected by H₂O₂ and hydroxyl radical (Fig. 8C). However, since the absorption spectrum of DPIBF was considerably changed in the HPX/XOD reaction system, it was not possible to evaluate the DPIBF oxidation by superoxide anion. Furthermore, it was also impossible.
to evaluate the DPIBF oxidation by hydroxyl radical generated by photolysis of H$_2$O$_2$ because the laser light with a wavelength of 405 nm was absorbed by DPIBF resulting in a large reduction of the peak of DPIBF at 420 nm without oxidation. SOSG was also slightly oxidized by ROS other than singlet oxygen resulting in a small increase in the fluorescence response (Fig. 8D).

Either astaxanthin or sodium azide with a concentration of 2.5 mM suppressed the yield of nitroxide radical derived from the reaction between the amines and singlet oxygen (Fig. 9 A and B). To confirm that astaxanthin directly quenched singlet oxygen, the reaction mixture without astaxanthin was irradiated by the laser light with an output power of 15 mW which was set to minimize the absorption effect of astaxanthin on the laser light. Although the yield of nitroxide radical was reduced by decreased laser output power, the yield was still larger than that generated by irradiation of 20 mW laser light to the reaction mixture containing astaxanthin. This finding suggests that astaxanthin quenched not only laser light for excitation of rose bengal but also singlet oxygen. On the other hand, mannitol, SOD, and catalase did not affect the yield of nitroxide radical generated by photoactivation of rose bengal. In the analyses of DPIBF oxidation and fluorescence response of SOSG, it was not possible to evaluate the quenching effect of astaxanthin because astaxanthin affected the absorption spectrum of DPIBF and it also interfered with the measurement of fluorescence response.

To examine if rose bengal generates ROS other than singlet oxygen when irradiated by the laser light, ESR spin trapping analysis using DMPO was performed. As a result of the analysis, photostimulated rose bengal generated hydroxyl radical but did not or did only slightly generate superoxide anion. The spin concentration of DMPO-OH photogenerated from the rose bengal was, however, only 0.48 µM.

Discussion

ESR analysis used in the present study demonstrated that the four different sterically hindered amines were converted to nitroxide radicals when they were oxidized by singlet oxygen photogenerated from rose bengal as reported in previous studies. (9,10,21,22) Interestingly, when 4-hydroxy-TEMP was oxidized by singlet oxygen, ESR signals of TEMPOL and 4-oxo-TEMPO were observed. It was reported that TEMPOL was converted to 4-oxo-TEMPO when TEMPOL was oxidized by hydroxyl radical generated by photolysis of H$_2$O$_2$ using 500 W ultraviolet (UV) light. (23) However, in the present study, hydroxyl radical generated by either photolysis of H$_2$O$_2$ using 20 mW visible light (405 nm) or sonolysis of water using ultrasound with a frequency of 1,650 kHz did not produce 4-oxo-TEMPO from 4-hydroxy-TEMP. Furthermore, the ESR signal of 4-oxo-TEMPO did not disappear when mannitol, a hydroxyl radical scavenger, was added to the reaction system (data not shown). These findings suggest that singlet oxygen abstracts not only hydrogen of imino group but also that of hydroxyl group in 4-substitution in the reaction system used in the present study. The discrepancy between the previous

Fig. 3. Representative ESR spectra of nitroxide radical generated from TEMP (A), 4-hydroxy-TEMP (B), 4-oxo-TEMP (C), and TPC (D). Each amine with a concentration of 450 mM mixed with 10 µM rose bengal (RB) was irradiated by the laser light with a wavelength of 532 nm for 60 sec. When 4-hydroxy-TEMP was used, two different ESR signals were detected. They were 4-hydroxy-2,2,6,6-tetramethylpiperidine N-oxyl (closed circle, ●) and 4-oxo-2,2,6,6-tetramethyl-1-piperidinyloxy (open triangle, △) were observed (C).
Figure 4. Effect of the concentration of amines on the yield of nitroxide radical. TEMP (A), 4-hydroxyl-TEMP (B), 4-oxo-TEMP (C), and TPC (D) were used. The yields of nitroxide radical photogenerated from samples containing 0, 1 and 10 µM rose bengal (RB) in 60 sec were compared. Each value represents the mean of triplicate measurements with standard deviation.

Figure 5. The yields of nitroxide radicals in relation to concentration of rose bengal (RB) (A) and laser irradiation time (B). The amines, 4-hydroxyl-TEMP and TPC, were used at 450 mM. In general, the yields of both nitroxide radicals increased with the concentration of RB and then reached a maximum (A). The yields of both nitroxide radicals generated by photooxidation of 10 µM RB linearly increased in a time dependent manner (B). Each value represents the mean of triplicate measurements with standard deviation.

study where hydroxyl radical was not quantitatively determined and the present study would probably be due to the yield of hydroxyl radical. In the present study, the yields of hydroxyl radical generated by photolysis of H₂O₂ and sonolysis of water were 1–15 µM. However, since a high power UV light was used to photolyze H₂O₂ in the previous study, larger amount of hydroxyl radical might be generated than that in the present study. Thus, it is strongly suggested that the emergence of ESR signal of 4-oxo-TMEPO converted from 4-hydroxy-TEMP can be an evidence of generation of singlet oxygen if it is confirmed that only a small
Fig. 6. Changes in absorption peak of DPIBF at 420 nm. The absorbance (Abs) decreased with the concentration of rose bengal (RB) when the sample containing 1 mM DPIBF and various concentration of RB was irradiated by the laser light for 60 sec (A). The inset shows the representative absorption spectra of DPIBF which was oxidized by singlet oxygen photogenerated from 0, 1, 5, 10, 20 and 40 μM RB sequentially from the top. The Abs decreased in a time dependent manner when DPIBF and RB were used at 1 mM and 10 μM, respectively (B). Each value represents the mean of triplicate measurements with standard deviation.

Fig. 7. Fluorescence response of SOSG oxidized by singlet oxygen. The fluorescence measurement was analyzed using excitation/emission of 485/535 nm. The fluorescence intensity linearly increased with concentrations of SOSG when the samples containing rose bengal with a concentration of 0, 0.06 and 1 μM were irradiated by the laser light for 60 sec (A). The fluorescence intensity also increased with concentrations of RB but then reached a maximum when the samples containing 10 μM SOSG was irradiated by the laser light for 60 sec (B). When SOSG and RB were used at 10 and 1 μM, respectively, the fluorescence intensity linearly increased in a time dependent manner (C). Each value represents the mean of triplicate measurements with standard deviation.
amount of hydroxyl radical (e.g., 15 µM or less hydroxyl radical as observed in the present study) is generated in the reaction system.

Of sterically hindered amines, TEMP and 4-oxo-TEMP have been often used for ESR analysis of singlet oxygen. \cite{10,21,24,25} In the present study, however, several dozen to hundreds µM nitroxide radical was detected when TEMP and 4-oxo-TEMP are dissolved in each solvent without oxidation. On the other hand, only less than 10 µM nitroxide radical was detected in the 4-hydroxy-TEMP and TPC solutions. Thus, it is considered that 4-hydroxy-TEMP and TPC are more suitable to analyze the yield of nitroxide radical induced by singlet oxygen because of the clear difference from background value of nitroxide radical. The yield of nitroxide radical generated from 4-hydroxy-TEMP was larger than that from TPC under the same conditions suggesting that the 4-hydroxy-TEMP would probably have higher reactivity to singlet oxygen than TPC. However, both amines did not seem to react with entire amount of singlet oxygen photogenerated from rose bengal because the yield of nitroxide radical increased with the concentration of the amines but did not reach a maximum. In ESR quantitative analysis of hydroxyl radical and superoxide anion using DMPO, it is fundamental to use sufficient concentration of DMPO which will be determined as a concentration to give a maximal yield of DMPO-OH.\cite{17,26} Therefore, it is considered that the yield of nitroxide radical measured by ESR analysis using the amines reflects the relative yield of singlet oxygen but not the true yield. However, the yield of nitroxide radical increased with the concentration of rose bengal and the irradiation time. The findings suggest that it is possible to compare the characteristic of various photosensitizers in relation to the relative yield of singlet oxygen.

Regarding the specificity of ESR analysis using 4-hydroxy-TEMP and TPC for singlet oxygen, it was demonstrated that both amines could be converted to nitroxide radical by not only singlet oxygen but also 500 mM H₂O₂ and hydroxyl radical generated by photolysis of 500 mM H₂O₂ (HO• 7 µM (p)). In the analysis of oxidation of DPIBF, H₂O₂ and hydroxyl radical did not or only slightly affect the absorption peak of DPIBF (C). However, since the absorption spectrum of DPIBF was interfered with HPX/XOD reaction system, the influence of superoxide anion was not evaluated (C). Furthermore, the laser light with a wavelength of 405 nm was absorbed by DPIBF resulting in decrease of the peak of DPIBF at 420 nm without oxidation (C). SOSG was slightly oxidized by ROS other than singlet oxygen (D). In each analytical method, ultrapure water was used as control. Each value represents the mean of triplicate measurements with standard deviation. O₂⁻: superoxide anion, HO• (p) and (s): hydroxyl radical generated by photolysis and sonolysis, respectively, RB: rose bengal irradiated by the laser light for 60 sec, NA: Not applicable.
of catalase to the reaction system did not affect the yield of nitroxide radical, suggesting that H₂O₂ was not involved in the generation of nitroxide radical. Although hydroxyl radical generated by photolysis of 500 mM H₂O₂ converted the amines to nitroxide radical, as is the case with the 500 mM H₂O₂, it is also negligible in PDT because addition of mannitol to the reaction system did not affect the yield of nitroxide radical. Photolysis of H₂O₂ and sonolysis are the well-known systems for hydroxyl radical generation. Nevertheless, the former oxidized the amines but the latter did not. We assumed that the concentration of H₂O₂ used in photolysis experiment was 500 mM, so that H₂O₂ but not hydroxyl radical likely associated with the oxidative reaction of the amines.

In ESR spin trapping analysis, photoactivation of rose bengal generated hydroxyl radical but did not or did only slightly generate superoxide anion. However, since the yield of DMPO-OH photogenerated from the rose bengal was very small (0.48 µM), hydroxyl radical is hardly involved in the photoactivation of rose bengal. Further experiment, in which astaxanthin and sodium azide known as singlet oxygen quenchers were added to the reaction system, was performed to confirm that the generation of nitroxide radical was derived from oxidative reaction with singlet oxygen. When colored substance such as astaxanthin is added to a photoactivation system, absorption effect of the colored substance on the excitation light should be considered. In the present study, we used the laser with decreased output power based on the measurement of light energy absorbed by astaxanthin. Since the addition of astaxanthin reduced the yield of nitroxide radical much more than did the decreased laser power, it was confirmed that the singlet oxygen was considerably associated with the generation of nitroxide radical from the amines. This finding was also confirmed by the experiment using sodium azide, in which the yield of nitroxide radical was also suppressed.

When the mixture of DPIBF and rose bengal was irradiated by the laser light, the absorption peak of DPIBF decreased with the concentration of rose bengal and reached a minimum at around 20 µM for rose bengal. This finding corresponded to ESR analysis where the yield of nitroxide radical was saturated with 10 to 20 µM rose bengal. These results suggest that the absorption of laser light by rose bengal increases with the concentration of rose bengal resulting in a saturation of the yield of singlet oxygen. Since the analysis using DPIBF could hardly detect the yield of singlet oxygen photogenerated from 1 µM rose bengal, sensitivity was less than that of ESR analysis using the amines. When rose bengal was used at 10 µM and laser irradiation was performed for 0 to 60 sec, the absorption peak of DPIBF decreased linearly with the irradiation time. These findings suggested that the yield of singlet oxygen increased with the concentration of rose bengal and the irradiation time, which corresponded to the results of ESR analysis. Therefore, it is considered that the relative yield of photogenerated singlet oxygen can be evaluated by the spectrophotometric analysis of DPIBF oxidation. However, when the yield of singlet oxygen generated from various photosensitizers used in PDT, it should be verified in advance if each photosensitizer affects the absorption spectrum of DPIBF. Furthermore, photosensitizers which are excited by light with a wavelength of around 400 nm such as hematoporphyrin cannot be used in the analysis. Indeed, the absorption spectrum of DPIBF was considerably changed by irradiation of 405 nm laser (data not shown).

Analysis of fluorescence response of SOSG showed the highest sensitivity for detection of singlet oxygen among the analytical methods used in this study. SOSG could detect the yield of singlet oxygen photogenerated from rose bengal even at a concentration as low as 0.06 µM. On the other hand, the fluorescence response reached a maximum with 1 µM rose bengal, which was much lower than that in ESR analysis and analysis of DPIBF oxidation. This would be due to the absorption of fluorescence from SOSG oxidized by singlet oxygen. Thus, it is suggested that the analysis of fluorescence response of SOSG oxidized by singlet oxygen can be achieved when a photosensitizer, which interferes with the fluorescence from SOSG such as rose bengal, are used at very low concentration (<1 µM).

The results of the present study demonstrated that all of the analytical methods used in this study could detect singlet oxygen and could evaluate the relative yield. However, it was suggested that applications of DPIBF oxidation and of fluorescent probe for measurement of singlet oxygen photogenerated in PDT have
limitations as discussed above. On the other hand, ESR analysis using a sterically hindered amine, especially 4-hydroxy-TEMP and TPC, has proper sensitivity and wide detectable range for the yield of photogenerated singlet oxygen. Furthermore, ESR analysis has an advantage that it is not interfered with the color of photosensitizers. Therefore, it is considered that the relative yield of singlet oxygen generated in PDT using various photosensitizers can be evaluated properly by ESR analysis.

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**Abbreviations**

DMPO 5,5-dimethyl-1-pyrrole N-oxide
DMSO dimethyl sulfoxide
DPIBF 1,3-diphenylisobenzofuran
ESR electron spin resonance
HPX hypoxanthine
4-hydroxy-TEMP 2,2,6,6-tetramethyl-4-piperidinol
4-oxo-TEMP 2,2,6,6-tetramethyl-4-pyrrolidone
4-oxo-TEMPO 4-oxo-2,2,6,6-tetramethyl-1-piperidinol
PDT photodynamic therapy
ROS reactive oxygen species
SOD superoxide dismutase
SOSG Singlet Oxygen Sensor Green®
TEMP 2,2,6,6-tetramethylpiperidine
TEMPOL 2,2,6,6-tetramethylpiperidiner-N-oxyl
TPC 2,2,5,5-tetramethyl-3-pyrrole-3-carboxamide
XOD xanthine oxidase