

# Is There a Simple and Easy Way to Detect Singlet Oxygen? Comparison of Methods for Detecting Singlet Oxygen and Application to Measure Scavenging Activity of Various Compounds

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## Abstract

Reactive oxygen species (ROS) are known to exert both beneficial and harmful effects in the human body. Singlet oxygen ( $^1O_2$ ), is highly reactive and considered as one of the ROS, although it is not a radical molecule.  $^1O_2$  reacts with many kinds of biological components such as lipids, proteins, and nucleic acids.  $^1O_2$  is short lived because it reacts with biomolecules and collisions with water molecules rapidly causing the return of  $^1O_2$  to the ground state, and is therefore not easy to quantify. Antioxidants, such as  $\beta$ -carotene, lycopene and astaxanthin, are quenchers of  $^1O_2$ . To compare the  $^1O_2$  quenching activity of new test compounds with these typical quenchers, it is desirable to measure  $^1O_2$  specifically, speedily, and easily. In this paper, various methods for the measurement of  $^1O_2$ , such as UV-Vis spectroscopy, fluorescence spectroscopy, and electron spin resonance, were compared for the purpose of evaluating the  $^1O_2$  quenching activity of various test compounds.

**Keywords:** Singlet oxygen ( $^1O_2$ ),  $^1O_2$  detection probe, Quenching activity, UV-Vis spectroscopy, Fluorescence spectroscopy, Near-infrared spectroscopy, Electron spin resonance

## Introduction

Reactive oxygen species (ROS) are known to exert both beneficial and harmful effects in the human body. The beneficial aspects of ROS are, for example, roles in signaling cascades and immunological defense. In contrast, overproduction of ROS has been reported to be involved in various diseases such as cancer, cardiovascular disease, and diabetes. To prevent the harmful effects of ROS, organisms have antioxidant enzymes and various antioxidative compounds. Various antioxidative compounds are present in foods, beverages, agricultural products, plants, as well as in the human body [1]. Measurement of antioxidant activity is important for developing new drugs, health foods, and nutritional supplements.

Atmospheric oxygen (triplet oxygen,  $^3O_2$ ) is a stable form of oxygen molecule. The other form, singlet oxygen

( $^1O_2$ ), is highly reactive and considered as one of the ROS, although it is not a radical molecule.  $^1O_2$  reacts with many kinds of biological components such as lipids, proteins, and nucleic acids [2-4].  $^1O_2$  is short lived because it reacts with biomolecules and collisions with water molecules rapidly causing the return of  $^1O_2$  to the ground state. Thus, it is not easy to quantify  $^1O_2$ .  $^1O_2$  is thought to be a cause of some disorders such as photo-aging, skin damage, and erythropoietic porphyria [5]. On the other hand, cell death and tissue destruction induced by  $^1O_2$  are used for photodynamic therapy (PDT), a promising cancer treatment [6-8].

It is known that antioxidants, such as  $\beta$ -carotene, lycopene, and astaxanthin, are quenchers of  $^1O_2$ . In order to compare the  $^1O_2$  quenching activity of new test compounds with these typical quenchers, it is desirable to specifically, speedily, and easily measure  $^1O_2$ . In addition, since light and heat may decompose the structure and

affect the quenching activity of the test compound, it is not ideal to use light or heat for  $^1\text{O}_2$  measurement. In this paper, we summarize and compare various methods for the measurement of  $^1\text{O}_2$  for the purpose of evaluating the  $^1\text{O}_2$  quenching activity of various test compounds. Artificial  $^1\text{O}_2$  generation is usually performed either by photosensitization of photosensitizers, such as rose bengal and porphyrins, or thermal decomposition of 4-methyl-1,4-etheno-2,3-benzodioxin-1(4*H*)-propanoic acid (EP).

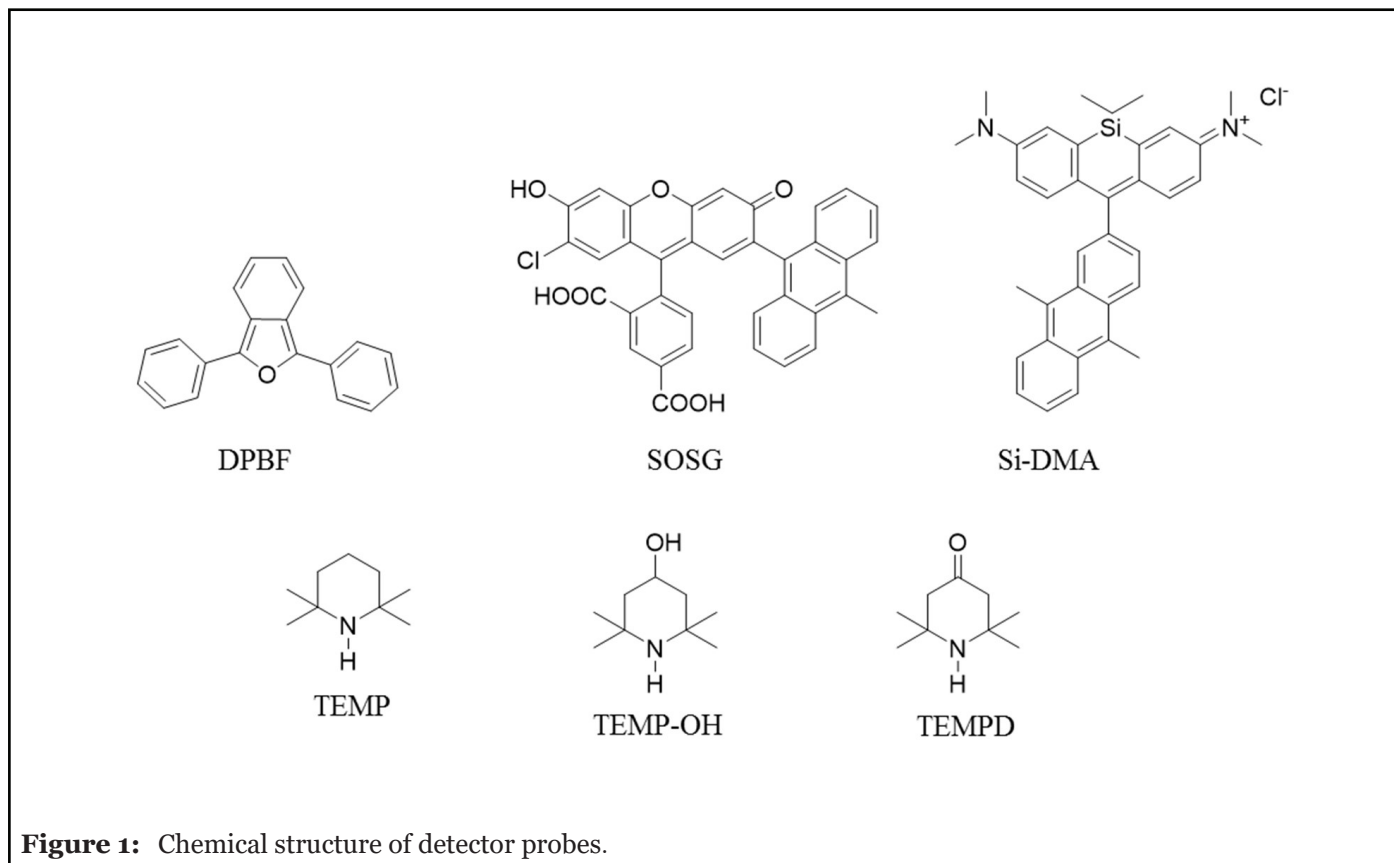
## UV-Vis Spectroscopy

UV-Vis absorbance measurement is simple, speedy, and the most commonly used method. Anthracene and benzofuran are known as compounds that selectively scavenge  $^1\text{O}_2$ . The absorbance of these aromatic molecules decreases when they are oxidized by  $^1\text{O}_2$ , and the amount of  $^1\text{O}_2$  can be estimated semi-quantitatively from the decrease in absorbance. UV-Vis spectroscopy is somewhat less sensitive than fluorescence spectroscopy. However, UV-Vis spectroscopy is less prone to artifacts, since the absorption spectrum of a compound with high absorption coefficient cannot be dominated by small amounts of contaminants. One of the most widely used  $^1\text{O}_2$  probes for UV-Vis spectroscopy is 1,3-diphenylisobenzofuran (DPBF, Figure 1), which is commercially available at a low cost. DPBF reacts with  $^1\text{O}_2$  in an essentially diffusion-limited reaction rate and thus has very high sensitivity. It traps up to 50% of  $^1\text{O}_2$  produced in alcohol/water or

micellar solution. In addition, replacing  $\text{H}_2\text{O}$  with  $\text{D}_2\text{O}$  (a  $^1\text{O}_2$  lifetime extender), is an option when only small amounts of  $^1\text{O}_2$  are produced [9]. However, DPBF is not specific to  $^1\text{O}_2$  and readily reacts with other ROS such as hydroxyl radical, alkoxy radical ( $\text{RO}\cdot$ ), and  $\text{H}_2\text{O}_2$  [10-12]. Furthermore, if the test compound is colored, the color affects the absorbance measurement with DPBF.

## Fluorescence Spectroscopy

Comparing the quantitative measurements of absorbance and fluorescence, the detection limit of fluorescence is generally three orders of magnitude lower. To detect  $^1\text{O}_2$ , a fluorescent sensor called Singlet Oxygen Sensor (SOSG, Figure 1) [13] has been used widely, ranging from material studies to medical applications, although the probe is expensive. SOSG exhibits weak blue fluorescence with excitation peaks at 372 nm and 393 nm and emission peaks at 395 nm and 416 nm. Upon reaction with  $^1\text{O}_2$ , endoperoxide, the immediate product of SOSG, exhibits green fluorescence with excitation and emission peaks at 504 nm and 525 nm, respectively. Therefore, SOSG can readily detect  $^1\text{O}_2$ . However, it was demonstrated that SOSG can generate  $^1\text{O}_2$  under exposure to UV (355 nm) or visible light (532 nm) in the absence of photosensitizers [14]. It was confirmed that the irradiation of visible light induces SOSG transitions to a triplet excited state, which converts  $^3\text{O}_2$  to  $^1\text{O}_2$  [15]. Therefore, a positive error can occur with respect to the amount of  $^1\text{O}_2$  measured by SOSG. In



**Figure 1:** Chemical structure of detector probes.

addition, since many photosensitizers used to generate  $^1\text{O}_2$  have a pronounced absorption band in the visible region, if the absorption overlaps with the emission peak of the fluorophores of probes, the signal produced by the reaction with  $^1\text{O}_2$  will be diminished. Photosensitizers, such as rose bengal and eosin Y, exhibit an intense absorption band at 525 nm, which coincides with the emission peak of SOSG endoperoxide [16]. Incorrect use of SOSG may lead to misinterpretation of the data on the yield of  $^1\text{O}_2$  production by photosensitization reactions. For biological application, a new type of fluorescence probe for  $^1\text{O}_2$  (Si-DMA, Figure 1) was developed [17]. Si-DMA has an emission peak at 680 nm and is permeable through cell membranes. It is now commercially available and can be used for imaging of  $^1\text{O}_2$  produced in mitochondria and by PDT.

### Near-infrared Spectroscopy

$^1\text{O}_2$  can be directly measured by detecting weak light emission in the near infrared region. Phosphorescence at 1270 nm emitted by  $^1\text{O}_2$  can be detected specifically at a low quantum yield [18]. By using an improved near-infrared-sensitive photomultiplier tube, time-resolved  $^1\text{O}_2$  luminescence was measured in various solutions of aluminum tetrasulphonated phthalocyanine and Photofrin [19]. Time-resolved analysis showed a strongly reduced lifetime of  $^1\text{O}_2$  and an increased lifetime of triplet state of the photosensitizer in the intracellular component [19]. This method is often used for imaging in cells and animals. However, the method requires specialized instruments because of the low quantum yield.

### Singlet Oxygen Absorption Capacity

Recently, a new assay method to quantify  $^1\text{O}_2$  absorption capacity (SOAC) was developed [20-23]. The SOAC values were measured in ethanol/chloroform/ $\text{D}_2\text{O}$  (50:50:1, v/v/v) solution at 35°C using a UV-Vis spectrophotometer equipped with a six-channel cell positioner and a temperature control unit. The reaction of antioxidants with  $^1\text{O}_2$  was measured using the competition reaction method, where EP was used for  $^1\text{O}_2$  generation and DPBF was used as an absorbance probe. In the SOAC method,  $^1\text{O}_2$  quenching activity is evaluated kinetically, and the activity of various phenolic antioxidants can be compared. The method is useful for evaluating the  $^1\text{O}_2$  quenching activity of both lipophilic and hydrophilic antioxidants. The method allows for measurement of activity that differs in five orders of magnitude (different rate constants of  $10^{10} \text{ M}^{-1}\text{s}^{-1}$  for lycopene to  $10^5 \text{ M}^{-1}\text{s}^{-1}$  for ferulic acid). However, in the SOAC assay, decrease in the absorbance is observed in 120 to 180 min, during which the test compound may become photodegraded.

### Electron Spin Resonance

Electron spin resonance (ESR) is a non-destructive

method that detects unpaired electrons and is specific to free radicals. Since  $^1\text{O}_2$  is a ROS but is not a free radical, it cannot be detected directly by ESR. A  $^1\text{O}_2$  detector probe, 2,2,6,6-tetramethylpiperidine (TEMP, Figure 1), was introduced in 1976 [24]. TEMP reacts with  $^1\text{O}_2$  to produce a stable nitroxide radical, 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO), which can be quantified by ESR spectroscopy. Other sterically hindered secondary amines, such as 2,2,6,6-tetramethyl-4-piperidinol (TEMP-OH, Figure 1) and 2,2,6,6-tetramethyl-4-piperidone (TEMPD, Figure 1), are also used as  $^1\text{O}_2$  detector probes [25-27]. Photoirradiation of rose bengal is often used as a  $^1\text{O}_2$  generation method. When  $^1\text{O}_2$  generated in the rose bengal system is detected by ESR using a hindered secondary amine probe, the signal of the nitroxide radical derived from the secondary amine probe is measured. However, rose bengal reportedly generates ROS other than  $^1\text{O}_2$ , such as super oxide anion radical and hydroxyl radical [26,28], which may affect the ESR signal. Indeed, when visible light was irradiated to the mixed solution of TEMP-OH and rose bengal, obvious splitting was observed in the ESR spectrum. This observation suggested the overlap of 4-oxo-2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO) and 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPOL) [25]. It was reported that the exposure of TEMPOL to hydroxyl radical led to the appearance of a new triplet ESR signal attributable to TEMPO, along with a reduction in the intensity of TEMPOL signals [29]. The following is a possible explanation for the ESR signal splitting observed in the mixed solution of rose bengal and TEMP-OH. TEMP-OH reacts with  $^1\text{O}_2$  to form TEMPOL, which is oxidized to TEMPO by hydroxyl radicals generated from rose bengal. On the other hand, when EP was used for  $^1\text{O}_2$  generation and TEMP-OH was used as a detector probe, no splitting of the three-line ESR spectrum was obtained, although the signal intensity was very small [25]. When the mixture solution of EP and TEMP-OH was reacted at 35°C for 40 min, the ESR signal intensity was smaller than that obtained with irradiation of rose bengal with visible light for 3 min, supporting that  $^1\text{O}_2$  production from EP is smaller than that from rose bengal. For the detection by ESR, if EP is used as a  $^1\text{O}_2$  generator, it is recommended to add  $\text{D}_2\text{O}$  in the system to increase the signal intensity. Although the amount of  $^1\text{O}_2$  generated from EP is small, it is possible to test the  $^1\text{O}_2$  scavenging activity of test compounds with a reaction time of 30~40 min in the presence of  $\text{D}_2\text{O}$  using TEMP-OH as a detector probe.

### Conclusion

Our opinions with respect to the pros and cons of the detection methods for  $^1\text{O}_2$  are summarized in Table 1. When measuring the scavenging activity of various compounds to  $^1\text{O}_2$ , it is desirable to avoid light irradiation, because the scavenging activity may decrease due to the decomposition of the test compounds. Since the ESR method does not

Methods	Pros	Cons	Probes
UV-Vis spectroscopy	Easy	Low sensitivity Disturbance by color	DPBF
Fluorescent spectroscopy	High sensitivity	Disturbance by color	SOSG Si-DMA
Near-infrared spectroscopy	Direct measurement High specificity	Low sensitivity Needs special equipment	
Singlet oxygen absorption capacity	Multi sample measurement	Photodegradation of sample Disturbance by color	DPBF
Electron spin resonance	High specificity No disturbance by color	Needs ESR spectrometer	TEMP TEMP-OH TEMPD

**Table 1:** Comparison of the methods for detecting  $^1\text{O}_2$ .

require light irradiation, it is suitable for measuring the  $^1\text{O}_2$  scavenging activity of various compounds. In addition, EP is better for the  $^1\text{O}_2$  generation system than the photosensitization method, because EP does not require light irradiation. In the present paper, we only focused on the  $^1\text{O}_2$  measurement system. However, development of a new  $^1\text{O}_2$  generation system may also contribute to improving the methods for measuring the  $^1\text{O}_2$  scavenging activity of various compounds. Because the methodologies described so far may detect other ROS together, it is necessary to develop specific probes capable of detecting  $^1\text{O}_2$  only, or specific reagents or systems that generate only  $^1\text{O}_2$  with moderate amounts. Finding compounds or plants with new  $^1\text{O}_2$  scavenging capacity by measuring scavenging activity correctly and easily should be useful to prevent or treat photoaging, skin damage, and erythropoietic porphyria caused by  $^1\text{O}_2$ .

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