Original Article

Reevaluation of analytical condition using highly singlet oxygen sensitive/selective reagent in aqueous-/non-aqueous solution

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Abstract

The spin-trapping ESR (ST-ESR) method, which observes unstable radicals as stable spin adducts using electron spin resonance (ESR), is an effective experimental method for evaluating chemical reactions involving radical reactions. For instance, reactive oxygen species (ROS) such as hydroxyl radicals and so on is trapped well by spin-trapping reagent like as DMPO. In this research, we considered the analytical conditions of generation and detection of singlet oxygen. All conditions were decided based on the viewpoints of high usability for the singlet oxygen scavenging/quenching evaluating method. In addition, all experimental conditions were examined with a solvent-independent measurement method. In this study, a nonaqueous solution is *N*,*N*-dimethylformamide (DMF), and an aqueous solution is phosphate buffer solution (PBS, 100 mM pH 7.4). In each solvent $CDCl_3$ or D_2O was added at a 10 vol.% ratio respectively. Because heavy solvent was previously reported as an extending lifetime of singlet oxygen. The experimental conditions of singlet oxygen were examined by two different generation/detection methods. Photosensitization method using organic dye and thermal decomposition method by naphthalene derivative endoperoxide were studied as generation method, and 4,4′-bis (1-p-carboxyphenyl-3-methyl-5-hydroxyl) pyrazole (DRD156) and 2,2,5,5-tetramethyl-3-pyrroline-carboxamide (TPC) were studied as detection reagent, respectively. As a result, DRD156 and thermolysis method was good combination for singlet oxygen generation and detection in aqueous solution (around neutral condition) and nonaqueous solution.

Key words

spin-trapping ESR, singlet oxygen, antioxidant capacity evaluation, carotenoid compounds, competition reaction

1. Introduction

The oxygen radical absorption capacity (ORAC) assay established by USDA as official method was among the most used for antioxidant capacity evaluation. (Niki, 2012) Similarly, the multiple free-radical scavenging capacity (MULTIS) (Endo et al., 2009; Kamogawa and Sueishi, 2014; Oowada et al., 2012; Sueishi and Takemoto, 2015) assay has been reported as the post-ORAC assay. But CYPMPO (Cyclic phosphorous-combining DMPO derivative or improved-DEPMPO analogue) as a spin-trapping reagent have to need to consider on very few experimental knowledges.

Additionally, the singlet oxygen absorption capacity (SOAC) employing UV–Vis spectrometry was recently reported as one of singlet oxygen scavenging and/or quenching activity evaluation. (Itoh et al., 1994: Mukai et al., 2017). But the low sensitivity of TMPD (4-oxo-TEMP) for singlet oxygen has to consider. A present singlet oxygen scavenging and/or quenching activities had been evaluated by employing UV–Vis spectrometry using the decolorization speed of DPBF (Sachindra et al., 2007; Tada et al., 2010), or employing chemiluminescence (CL) measurements signal intensity changes in very weak near-infrared luminescence originating from singlet oxygen at around 1270 nm. (Nishida et al., 2007: Wang et al., 2012) The endoperoxide of a naphthalene derivative 3-(1,4-epidioxy-4-methyl-1,4-dihydro-1-naphthyl) propionic acid (MNP-O_2) and 1,4-dimethyl-naphthalene-1,4-endoperoxide (DMN-O_2) was reported as a useful pure singlet oxygen generating reagent and employed. (Aubry et al., 1989; Liu et al., 2000; Mascio P. D. and Sies H., 1989, Nishida et al., 2007) TMPD has often been used by spin-trapping ESR (ST-ESR) (Kim et al., 2009; Shimizu et al., 2010), but has not been used because of its low selectivity and low sensitivity, now. On the other hand, 2,2,5,5-tetramethyl-3-pyrroline-carboxamide (TPC, sterically hindered amines) have been used to detect singlet oxygen in photodynamic therapy. (Matsumura et al., 2013; Nakamura et al., 2011). According to this article, the combination of TPC and rose bengal (RB) showed the best performance for singlet oxygen detection and generation. (Figure 1) Originally, spin-trapping reaction and radicalizing reaction should be separate and use on strictly meanings. But, conforming to the conventions, we treat singlet oxygen scavenging/quenching reactions as one of evaluating a

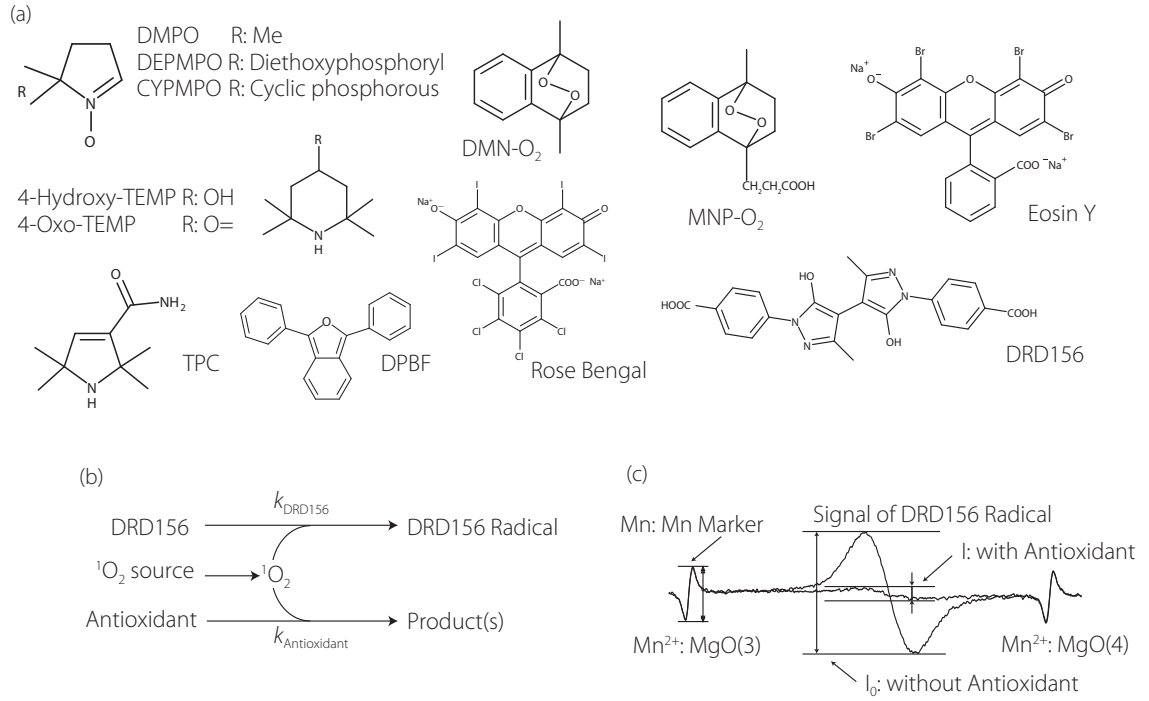


Figure 1: (a) Chemical structure of well-known antioxidant capacity evaluation reagent with use of ESR spectroscopy. DMPO, DEPMPO, and CYPMPO are spin-trapping reagent for $O_2^{\bullet-}$, •OH, $R^{\bullet}$, $RO^{\bullet}$, and $ROO^{\bullet}$. TMPD (4-oxo-TEMP), 4- Hydroxy-TEMP, and TPC are spin-trapping reagent for singlet oxygen. DPBF is a chemical probe of UV-Vis spectroscopy for SOAC assay. MNP-O_2 and DMN-O_2 are thermolysis singlet oxygen generating reagent, and eosin Y and rose bengal are photolysis singlet oxygen generating reagent. (b) Outline of evaluation mechanism using competition reaction of DRD156 and carotenoid compounds against singlet oxygen., and (c) ESR spectra of DRD156. (anti-phase signal of both sides means 3rd and 4th Mn^{2+} Marker in ESR)

method based on the spin-trapping technique, and DRD156 treat as the spin-trapping reagent.

In our previous reports, we found that 4,4′-bis (1-p-carboxyphenyl-3-methyl-5-hydroxyl) pyrazole (DRD156) and MNP-O_2 was the best singlet oxygen detecting reagent and pure source of singlet oxygen, with high-selectivity and high-sensitivity. (Igarashi et al., 1999; Liu W et al., 2000) Hence, in this paper, based on our previous result of DRD156 and MNP-O_2 as singlet oxygen detector and generator, we identified and considered a good combination of singlet oxygen detecting/generating reagents using two different methods each. That is, TPC and DRD156 as detecting reagent, a photosensitized method of organic dyes (Eosin Y as photosensitizer) (Harbour et al., 1980; Igarashi et al., 1999; Sueishi et al., 2014) and thermal degradation of MNP-O_2 (Aubry et al., 1989; Igarashi et al., 1999; Jung and Min, 2009; Liu et al., 2000; Mascio 1989). (Figure 1) And, the effect of heat and/or light clarified by comparing its result respectively. Our evaluating method was based on a competition reaction (Koide et al., 2000; Liu et al., 2001) and ST–ESR (Figure. 1(b) and (c)), and we believe that our method has high-possibility and it will have become as a standard method.

2. Materials and methods

2.1 Reagents

N,*N*-Dimethylformamide (DMF), chloroform-d ($CDCl_3$), phosphate buffer powder (1/15 mol/L, pH 7.4), deuterated water (D_2O), Eosin Y (EY), methylene blue trihydrate (MB), hematoporphyrin (HP), phosphoric acid, acetic acid, and rose bengal (RB) were purchased from Wako Pure Chemicals. Boric acid was obtained from Kanto Chemical Co., Inc. TPC and Protoporphyrin IX (PPIX) were taken from Sigma-Aldrich Co. Ltd. DRD156 (Igarashi et al., 1999) and MNP-O_2 (Liu et al., 2000) was prepared using literature procedures. Britton- Robinson buffer (BRB) solution was prepared from the equal volumes of diluted phosphoric acid, acetic acid, and boronic acid, to afford a final concentration of 40 mM of each. A pH 7.4 phosphate buffer solution (PBS) was prepared from phosphate buffer powder and deionized water to give a concentration of 100 mM. All reagents were of analytical grade and used without further purification.

2.2 Equipment

A JES-FR30 X-band ESR spectrometer was obtained from. JEOL Resonance Inc. A V-630 UV–Vis spectrophotometer and THERMO mini F-0010D thermostat bus were obtained from

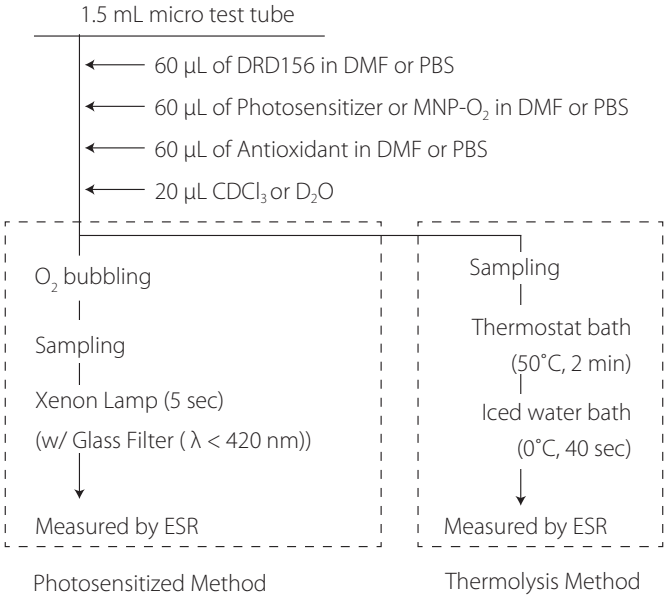


Figure 2: Experimental procedure

JASCO Co., Ltd., and Tokyo Glass Kiki (TGK) Co. Ltd., respectively. A Luminar Ace LA-1000UV xenon lamp was from HAYASHI WATCH-WORKS CO., LTD. A gas flow meter (Superlabo B1-1NR-1G8G-P1N1-F72) was taken from Tanaka Factory Co., Ltd. Micro test tubes (1.5 mL, Eppendorf-type, graduated with cap) were purchase from Kartell S.p.A. Colored-glass optical filter L-42 (optical glass filter that cuts wavelengths under 420 nm) was obtained from Toshiba Co., Ltd. Hematocrit capillary tubes (VC-H075P, l = 75 mm, φ = 1.45-1.65 mm) and its putty (XX-VCS35) were shopped from Thermo Corporation.

2.3 Measurement of UV-Vis spectra

UV–Vis spectra were recorded using a V-630 UV–Vis spectrometer at concentrations of 10 μM for EY, 10 μM for PPIX, 6.8 μM for HP, 6.8 μM for MB, and 1.0 mM for DRD156 in DMF. RB was dissolved in BRB at a concentration of 10 μM, and solutions with pH values of 9, 10, and 11 were prepared. Under desirable conditions, absorption spectra between organic dyes and spin-trapping reagents, no overlapped area should be observed (a minimum requirement for the photosensitized method). From the UV-Vis spectra, the main peaks for PPIX and HP were overlapped in the main absorption of DRD156. Therefore, PPIX and HP were not optimal photosensitizers for the photosensitized method. In contrast, MB did not have a large absorption at a concentration of 6.8 mM, while RB, under irradiation with visible light, generated both singlet oxygen and superoxide ($O_2^{\bullet-}$). Therefore, in this research, the photosensitized method was conducted using EY.

2.4 Measurement conditions and spin-trapping ESR procedure

ESR was conducted using the following parameters: Microwave power, 4 mW; center field, 336.7 mT; sweep width, ±5 mT; sweep time, 1.0 min; modulation width, 0.5 mT; time constant, 0.1 s; gain, arbitrary; Mn marker in ESR, arbitrary, and temperature, r.t.

DRD156, photosensitizer, and MNP-O_2 were dissolved in DMF at a suitable concentration. Then, 60 μL of each solution and 20 μL of $CDCl_3$ were mixed in a 1.5-mL micro test tube. $CDCl_3$ was added to the mixed solution to achieve long-life singlet oxygen. (Lion et al., 1976; Moan and Wold, 1979) For the photosensitized method, O_2 gas was bubbled through the mixed solution for 10 s at a flow speed of 30 mL/min. The mixed solution was then sampled using a hematocrit capillary and sealed with putty. The sample was irradiated with a xenon lamp (from vertically above, distance = 40 mm) for 5 s through an optical glass filter that cut wavelengths under 420 nm. After irradiation, ESR spectra were measured immediately. In the same manner, a thermolysis method was operated, in which the solution mixture was heated using a thermostat bus for 0-5 min at 50 ℃, and then cooled in iced water for 40 s. The cooled solution was sampled using a hematocrit capillary and sealed with putty. ESR spectra were then measured shortly after. The procedures and antioxidants are summarized in Figure 2. Similarly, a thermolysis method employing an aqueous solvent (PBS) was also investigated using the procedure outlined in Figure 2.

3. Result and discussion

All graphs are illustrated using final concentration. S/M ratio indicates the ratio of signal intensity to manganese marker in ESR.

3.1 Considering photosensitization conditions

An artificial dye, natural dye and biological-origin dye can

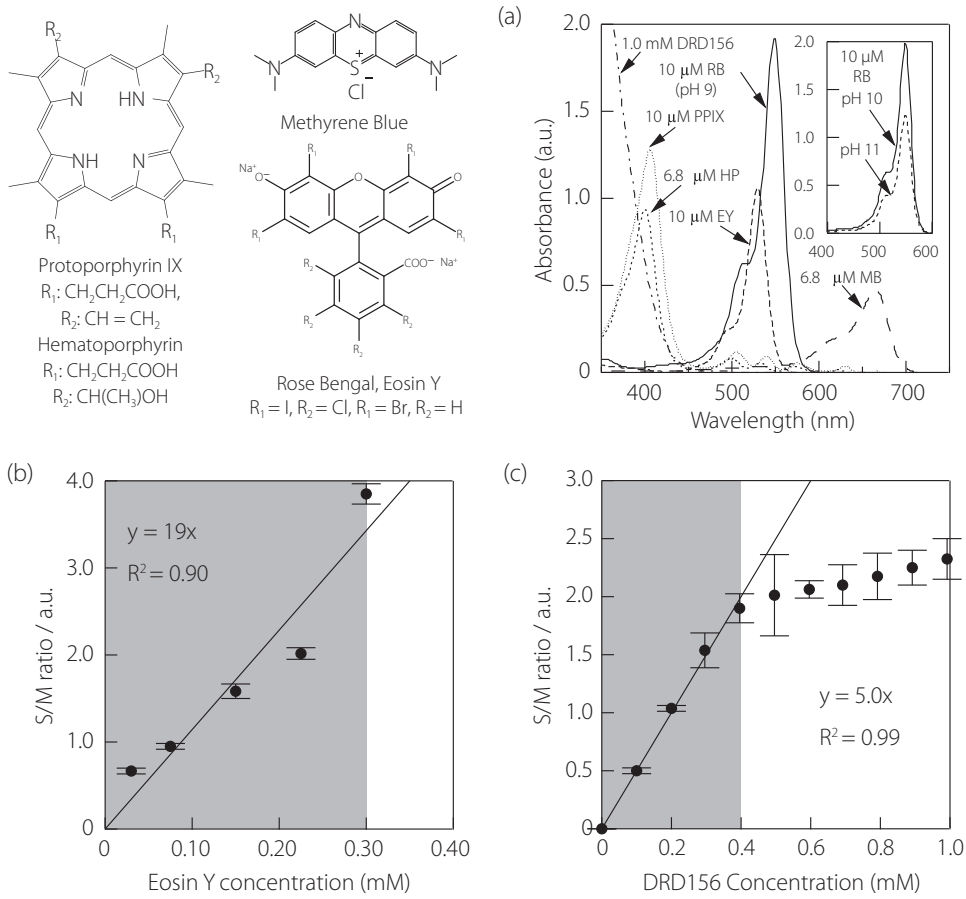
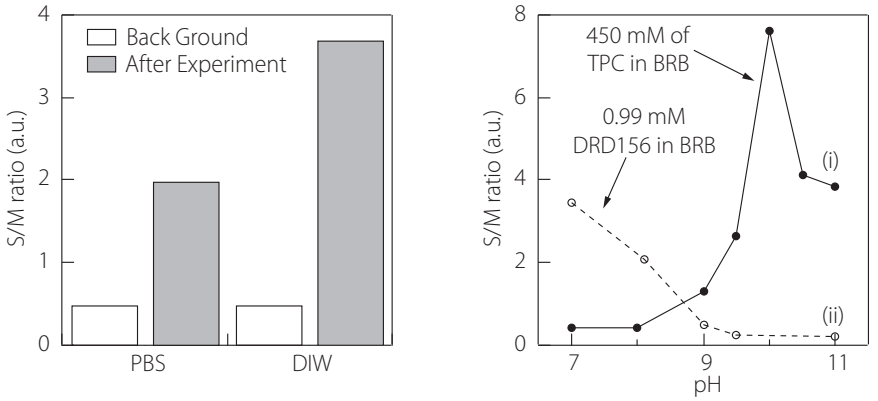


Figure 3: Structure of organic dyes and (a) absorption spectra. Consideration of experimental condition on (b) Eosin Y concentration, (c) DRD156 concentration

be purchased commonly and were used generating singlet oxygen in photosensitized method. A suitable photolysis condition using MB, PPIX, HP, Eosin Y and RB was considered in absorption spectra. Additionally, a photolysis condition of RB had further considered on pH-dependence spectra using BRB. Because the TPC sensitivity changes depending on solution pH. Spectrum of EY and RB was showed similar shape and was not overlapped on spectra of DRD156. (Figure 3 (a)) According to previous reports, RB has been suggested become superoxide and singlet oxygen generator (Nakano et al., 1988), so we employed EY for singlet oxygen generator of photolysis. Using the photosensitized method shown in Figure 2 (a), the dependence of EY concentration was studied in the concentration range 0.01-0.1 mM, using 3.3 mM DRD156 in DMF and $CDCl_3$ (Figure 3 (b)). A report of Nakamura et al. (2011) except pH dependence of RB. A xenon–lamp irradiation time was fixed 5 s due to limitation of equipment flexibility. Similarly, the dependence of DRD156 was studied in the concentration range 0.33-3.3 mM, using 1.0 mM DRD156 in DMF and $CDCl_3$ (Figure 3 (c)).

Increasing signal intensity was dependent on increasing concentrations of EY or DRD156. As EY has generated singlet oxygen in DMF/$CDCl_3$ (9/1, v/v), and the signal intensity increased linearly. On a dependence of EY concentration, a coefficient of determination shown not high, but reproducibility was not so bad. On the other hand, a DRD156 concentration-dependent showed opposite trend; coefficient of determination showed high, but reproducibility of signal intensity was not high. Additionally, signal intensity over 0.4 mM was showed plateau (a small signal increase was observed) in high DRD156 concentration. EY and DRD156 that mixed at a concentration 0.33-mM and 0.99-mM, respectively, in DMF/$CDCl_3$ soln. showed enough large S/M ratio over 3.0. According to the results above, the most suitable concentrations of EY and DRD156 were 1.0 mM and 3.3 mM, respectively. Additionally, the experimental conditions in PBS/D_2O soln. were considered in a similar manner, however, experimental results showed in large errors that required improvement (data not shown). Therefore, suitable experimental conditions cannot have been established.

According to Nakamura et al., (2011) TPC is a more sensitive spin-trapping reagent than TMPD in singlet oxygen detection. Therefore, a combination of TPC and RB was considered in aqueous soln. In addition, the signal intensity of TPC dissolved in deionized water (DIW) showed twice higher signal intensity against than that in PBS. A TPC solution dissolved in DIW was



(a) Signal intensity of TPC in PBS and DIW (b) pH dependence of TPC and DRD156

Figure 4: Signal intensity of TPC and DRD156

showed strongly alkaline (pH 10) than that dissolved in PBS (pH 7.4). A pH dependence was clarified at ranging from neutral (pH 7.0) to alkaline (pH 11.0) using TPC dissolved in Britton–Robinson buffer (BRB). The pH value of BRB was controlled by the addition of concentrated NaOH solution (5.0 M). In addition, a pH dependence of DRD156 dissolved in BRB was also considered similar fashion. (detail of experiments on DRD156 follows in supplementary materials) Those results ensured that TPC was not usable in PBS solution, because TPC was showed large signal intensity in alkaline condition. DRD156 showed sensitivity 8.05 times higher than that of TPC at pH 7, despite having a concentration 58.8 times lower (Figure 4).

Based on the structural characteristics of TMPD, TPC, and DRD156, piperidine (a six-membered ring containing one nitrogen substituent) showed lower sensitivity than pyrroline and pyrazole (five-membered rings containing one and two nitrogen, respectively). Therefore, pyrroline or pyrazole-based compounds showed potential as singlet oxygen detecting reagents, with the future research required.

3.2 Consideration of experimental conditions of thermolysis in aqueous and non-aqueous solutions

As mentioned above, the thermolysis method in non-aqueous solution was also studied using mixture solution consisting of DRD156 (60 µL), MNP-O_2 (60 µL), DMF (60 µL), and $CDCl_3$ (20 µL) (totally DMF/$CDCl_3$ = 9/1, v/v) (Figures 5 (a)-(c)). A thermal degradation of MNP-O_2 was started in thermostat bus at 50 ˚C and stopped by 40 sec cooling in the iced bus. A heating time was studied in the range 1-5 min (Figure 5 (a)), and DRD156/MNP-O_2 concentration were studied in the range 1.3-3.3 mM DRD156 (vs MNP-O_2 25 mM)/2.5-25 mM (vs. 3.3 mM DRD156) (Figure 5 (b), (c)).

The ESR signal intensity increased with increasing heating time and MNP-O_2 concentration. The ESR signal intensity of DRD156 adduct was saturated, and a slight an increase in ESR signal intensity was observed at over 2 min. Similarly, the ESR signal intensity of DRD156 adduct was an increase in the experimental concentration range. On the other hand, a similar DRD156 signal intensity (S/M-1.9) was observed in Figure 5 (b). Signal intensity appearing as a plateau denotes saturated signal intensity in the experimental concentration range.

Additionally, the thermolysis method in aqueous solution (PBS/D_2O = 9/1, v/v) was studied using a solution consisting of MNP-O2 (60 µL, 17 mM), DRD156 (60 µL, 10-20 mM), PBS (60 µL), and D_2O (20 µL). The experimental conditions were considered as follow; a heating time, DRD156 concentration, and MNP-O_2 concentration was studied in the range of 1-5 min (Figure 5 (d)), 10-20 mM (Figure 5 (e)), and 2.5-25 mM (Figure 5 (f)). The ESR signal intensity of DRD156 was saturated at over 2 min heating and at over 5 mM MNP-O_2 concentration, after that a slight increase in ESR signal intensity was observed. The ESR signal intensity of DRD156 was saturated at over 2 min heating and at over 5 mM MNP-O_2 concentration, after that a slight increase in ESR signal intensity was observed. The trend of reproducible results in an aqueous solution was almost similar to those in a non-aqueous solution, but the range of signal intensity showed variable largely. Decided and considered experimental conditions in aqueous-/non-aqueous solution as mentioned above were summarized in Table 1.

Conclusion

We showed that this protocol was adaptable in aqueous and non-aqueous solutions using the same reagent in the thermolysis method. Meanwhile, the photosensitized method requires further investigation and improvement regarding experimental conditions, which are currently in progress. We believe this protocol has good potential for antioxidant capacity evaluation. To evaluate more accurately, the second-order rate constant of DRD156 should be determined in aqueous and non-aqueous solutions.

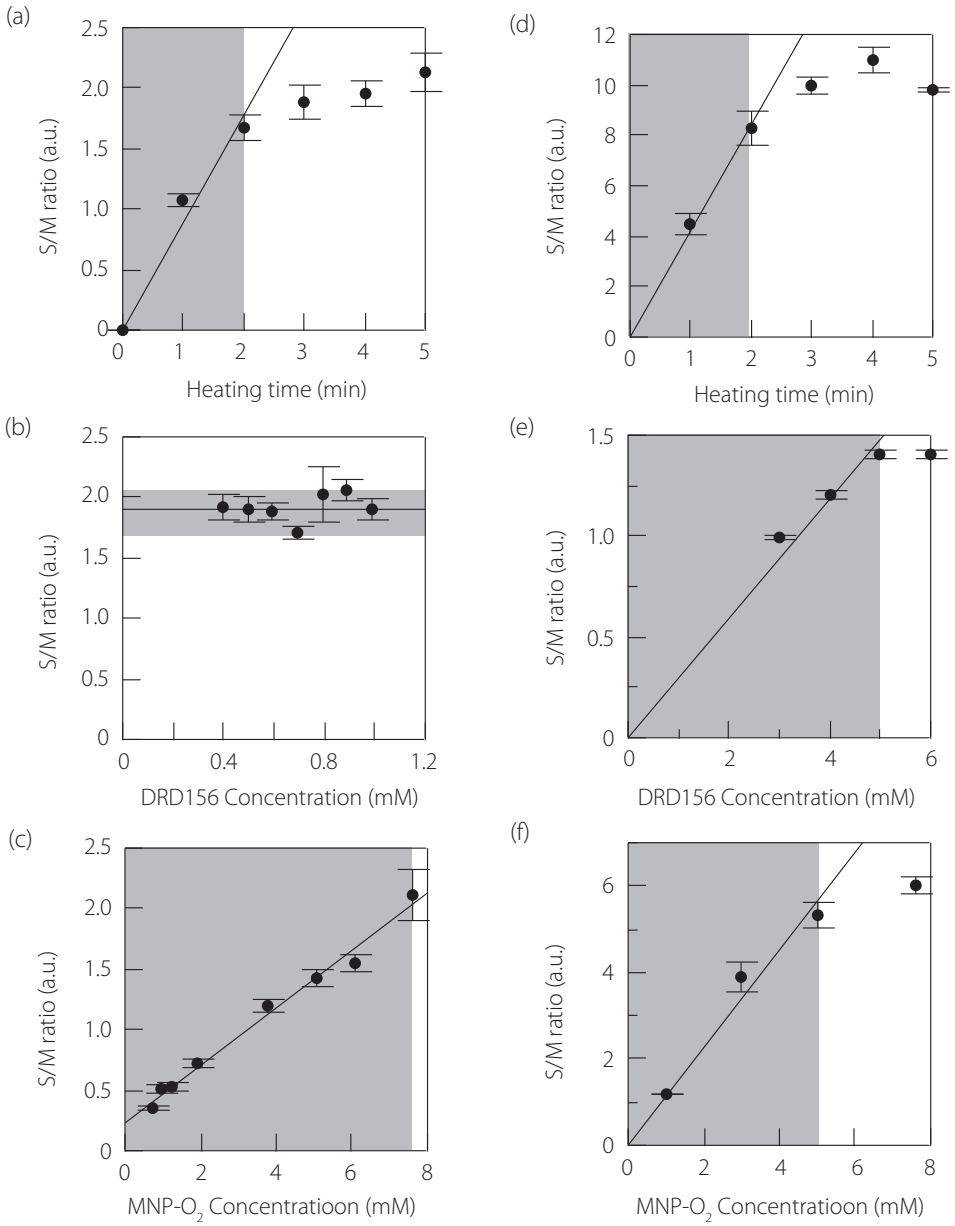


Figure 5: Consideration of experimental condition in DMF/$CDCl_3$ about (a) Heating time, (b) DRD156 concentration, and (c) MNP-O_2 concentration. (d)-(f) Similar consideration in PBS/D_2O

Table 1: Decided experimental conditions for singlet oxygen evalating [a]

Solution	Concentration / mM			
	hv [a]		Δ [a]	
	EY	DRD156	MNP-O_2	DRD156
DMF/$CDCl_3$ [b]	1.0	3.3	25	3.3
PBS/D_2O [b]	N.E. [c]	N.E. [c]	17	1.7

Notes: [a] Photolysis (hv): Xenon lamp 5 sec with optical glass filter, Thremolysis (Δ): Heating 50 °C, 2 min / Cooling 0 °C, 40 sec; [b] 9/1 (v/v); [c] N.E.: No Establishment.

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