Reevaluation of Quantitative ESR Spin Trapping Analysis of Hydroxyl Radical by Applying Sonolysis of Water as a Model System

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Electron spin resonance (ESR) spin trapping using 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) is commonly applied for quantitative analysis of hydroxyl radical. For better understanding of the analysis, we investigated kinetics related to formation and decay of hydroxyl radical spin adduct of DMPO compared with that of 3,3,5,5-tetramethyl-1-pyrroline *N*-oxide (M4PO) and 5-(diphenylphosphinoyl)-5-methyl-1-pyrroline *N*-oxide (DPPMPO). In our study where hydroxyl radical was generated by sonolysis of water, we found that (1) DMPO-OH formation was saturated even though hydroxyl radical was generated continuously and (2) the concentration of DMPO-OH decreased in inverse proportion to time after cessation of ultrasound irradiation, suggesting that the decay is a second-order reaction. Similar results were obtained in an experiment of DMPO-OH generated by photolysis of H_2O_2. Other than DMPO, M4PO, and DPPMPO also trapped hydroxyl radical but the spin trap efficiency was less than that of DMPO. Furthermore, M4PO-OH and DPPMPO-OH decayed more quickly than DMPO-OH. From this, we conclude that DMPO is more suitable for quantification of hydroxyl radical than M4PO and DPPMPO but results from the quantitative analysis must be interpreted with consideration of the kinetics related to formation and decay of DMPO-OH, especially in quantification of large amount of hydroxyl radical generation.

Hydroxyl radical, a free radical as well as reactive oxygen species (ROS), has been studied in various fields, such as physics, biochemistry, pharmacology, and medical science because it reacts non-specifically with most organic molecules causing various oxidative reactions.[1,2] Because of its short lifetime (less than 10 ns in liquid),[3,4] it is difficult to detect hydroxyl radical directly. Consequently, spin trapping using electron spin resonance (ESR) spectrometry is commonly used for measurement of hydroxyl radical.[5,6] Among various spin trap agents, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) has been regarded as the most useful and powerful spin trap used in determination of hydroxyl radical and other free radicals.[7–9] ESR spin trapping can detect very low concentrations of hydroxyl radical trapped by DMPO (2-hydroxy-5,5-dimethyl-1-pyrrolidinyloxyl; DMPO-OH) which is regarded as a stable free radical called a spin adduct. Since the ESR spectrum of DMPO-OH shows a quartet with 1:2:2:1 signal intensity and hyperfine coupling constants $a_N = a_H = 1.49$ mT, it is possible to identify and quantify it indirectly.[10–12]

It has been reported that the rate constant of the reaction between hydroxyl radical and DMPO is approximately 2 to $4 \times 10^9\,M^{-1}\,s^{-1}$[13,14] suggesting that the reaction is quite fast. However, since the lifetime of hydroxyl radical is very short, sufficient concentration of DMPO is necessary to trap all of the generated hydroxyl radical.[12] Hence, DMPO-OH formation will be greatly dependent on the concentration of DMPO in the reaction system. Unless sufficient concentration of DMPO exists in the reaction system, only a limited amount of generated hydroxyl radical will be trapped by DMPO. Optimal concentration of DMPO should be evaluated for each hydroxyl radical generation system because optimal concentration of DMPO might vary with the generation constant of hydroxyl radical of each generation system.

Among several generation systems for hydroxyl radical such as sonolysis of water,[12,15,16] photolysis of hydrogen peroxide (H_2O_2),[6,17] and Fenton reaction,[18] sonolysis of water is the simplest because it involves only one substance, water including dissolved oxygen. When the quantitative analysis of hydroxyl radical formation is considered in the ultrasound system as an example, formed DMPO-OH will react with other chemicals and will decay both during ultrasound irradiation and after cessation of ultrasound irradiation. During the ultrasound irradiation, there is a possibility that not only hydroxyl radical but also a slight amount of other ROS such as H_2O_2, superoxide anion and other radicals such as hydrogen trioxide[19] are generated and might react with DMPO-OH. In addition, both during and after cessation of ultrasound irradiation, dissolved oxygen, remaining substances (water and DMPO), and product of the reaction may also react with DMPO-OH. Although these factors should be studied in detail to quantify hydroxyl radicals precisely, little is known so far. Regarding the lifetime of DMPO-OH, a few previous studies

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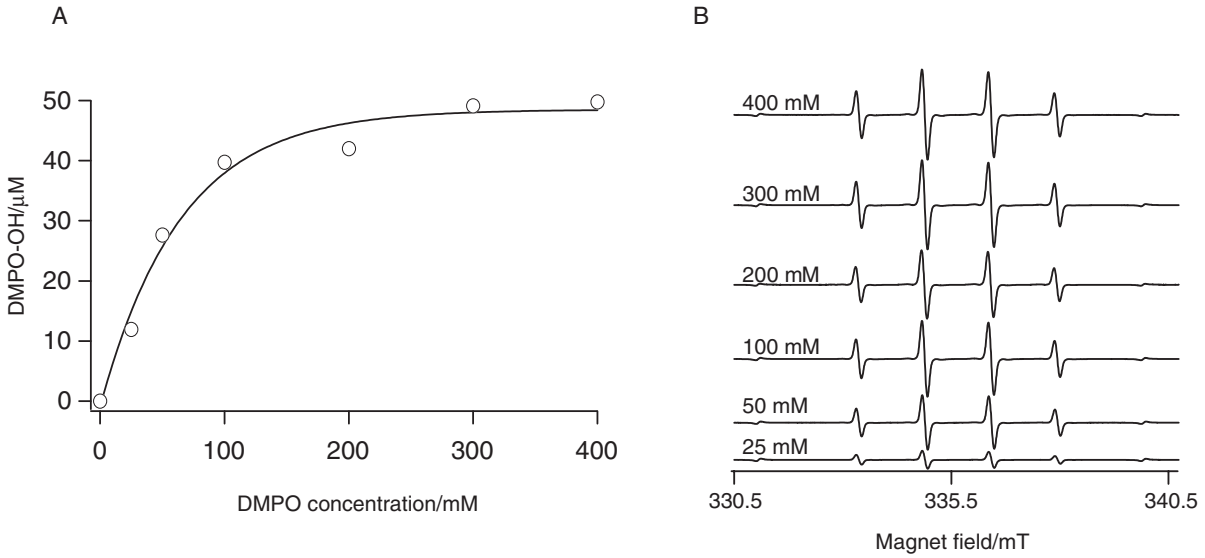

Figure 1. Saturation curve of DMPO-OH. (A) DMPO-OH increased with the concentration of DMPO and then was saturated. (B) The representative ESR spectrum of DMPO-OH generated at different concentrations of DMPO. Each value in Figure 1A represents the mean of triplicate determinations.

reported that the half-life of DMPO-OH was in the range of 20 to 60 min in aqueous solutions.[20,21] The lifetime of DMPO-OH will considerably influence the quantitative analysis of hydroxyl radical. If a large amount of DMPO-OH spontaneously decays in short time, it is difficult to quantify the concentration of DMPO-OH correctly.

Besides DMPO, other spin trap agents are also used for quantification of hydroxyl radical. For instance, 3,3,5,5-tetramethyl-1-pyrroline *N*-oxide (M4PO) sometimes is used for quantification of hydroxyl radical in biological samples because oxidation of M4PO by ferric ions is much slower than that of DMPO-OH.[22] In addition, 5-(diphenylphosphinoyl)-5-methyl-1-pyrroline *N*-oxide (DPPMPO) has been newly developed as a spin trap agent with a higher rate constant for hydroxyl radical than that of DMPO.[23] However, few studies have been conducted to compare the kinetics of these three spin trap agents in terms of hydroxyl radical trapping ability, and stability of the spin adducts.

In the present paper, we discuss the influences of kinetics related to formation and decay of DMPO-OH generated by the simple generation system of hydroxyl radical, sonolysis of water, on quantitative analysis by ESR spin trapping. Furthermore, spin trapping efficiency and the decay rate of spin adduct were compared among DMPO, M4PO, and DPPMPO.

Results

Optimal Concentration of DMPO for Quantification of Hydroxyl Radical. When ultrapure water containing different concentrations of DMPO was irradiated for 2 min by ultrasound with an output power of 30 W and a frequency of 1650 kHz, DMPO-OH ($a_N = a_H = 1.49$ mT) increased with the concentration of DMPO to a certain extent and then was saturated at the DMPO concentration of around 300 mM (Figure 1A). The changes in representative ESR spectra are shown in Figure 1B. Under the condition used in this study, neither DMPO-OOH, which is superoxide anion spin adduct of DMPO, nor DMPO-H, which is hydrogen atom (hydrogen radical) spin adduct of DMPO, was detected (Figure 1B).

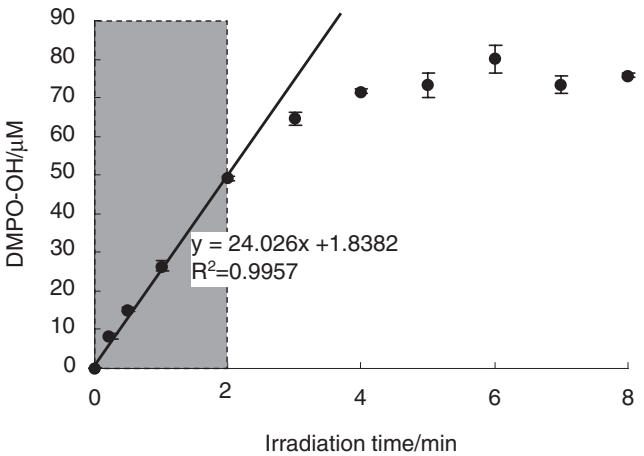

Figure 2. Saturation curve of DMPO-OH formation versus ultrasound irradiation time. DMPO-OH increased linearly up to 2 min with high correlation coefficient of over 0.997. Then, DMPO-OH was gradually saturated with the increase of irradiation time. Each value represents the mean of triplicate determinations with standard deviation.

Formation of DMPO-OH. DMPO-OH increased in a time dependent manner, and a linear relationship with a correlation coefficient of over 0.99 between the concentration of DMPO-OH and the irradiation time was observed up to 2 min (Figure 2). Then the DMPO-OH was saturated at around 80 μM (Figure 2). However, ultrasound irradiation to ultrapure water without DMPO for 8 min in advance did not affect the DMPO-OH formation after the addition of DMPO (data not shown). Therefore, it is suggested that hydroxyl radical is generated in accordance with the linear proportion ($y = 24.026x + 1.8382$) as shown in Figure 2 even though the DMPO-OH is saturated. In other words, it is unlikely that

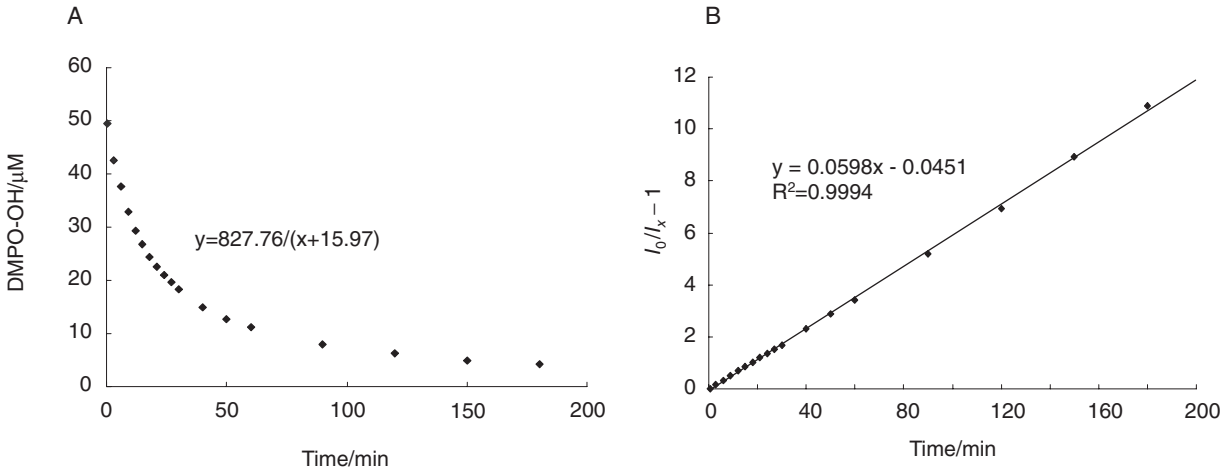

Figure 3. Decay curve of 50 μM DMPO-OH generated by ultrasound irradiation of ultrapure water. (A) DMPO-OH decreased inversely with time. The half-life was 16.6 min. (B) The inverse proportion was linearized by the equation $I_0/I_x - 1 = ax + b$, where I_0 is the initial concentration of DMPO-OH and I_x is the concentration of DMPO-OH at each time interval. Each value represents the mean of triplicate determinations.

DMPO trapped the entire hydroxyl radical generated by ultrasound for over 2 min.

It was observed that addition of H_2O_2 to the reaction system decreased DMPO-OH in a concentration dependent manner (data not shown). The IC_{50} of H_2O_2 against DMPO-OH formation increased with the concentration of DMPO. The IC_{50}s against 1, 2, and 4 mM DMPO were 80, 230, and 540 mM H_2O_2, respectively. The rate constant was calculated for each concentration of DMPO and then the mean rate constant was calculated. The mean rate constant was $3.3 \times 10^7\,M^{-1}\,s^{-1}$, which is lower by two orders than that of the reaction between DMPO and hydroxyl radical ($3.4 \times 10^9\,M^{-1}\,s^{-1}$).[24] However, addition of SOD, which was used to examine if superoxide anion and products derived from superoxide anion affect the decay of DMPO-OH, to the reaction system neither increased nor decreased the concentration of DMPO-OH.

Decay of DMPO-OH. The decay curve of 50 μM DMPO-OH is shown in Figure 3A. The concentration of DMPO-OH decreased in inverse relation to time. The values of $I_0/I_x - 1$, where I_0 is the initial concentration of DMPO-OH and I_x is the concentration of DMPO-OH at each time interval, were plotted to confirm the inverse relationship between DMPO-OH and time. The equation of the plot calculated by the least squares is $I_0/I_x - 1 = 0.0598 \times -0.0451$ ($r = 0.9997$) (x is time in the equation) (Figure 3B). Then, the actually measured initial concentration of DMPO-OH, 49.5 μM, is inserted to I_0 of the equation. The eq $49.5/I_x - 1 = 0.0598 \times -0.0451$ where I_x is y in Figure 3A is converted to $y = 827.76/(x + 15.97)$ showing inverse proportion between DMPO-OH and time. Hence, the decay of DMPO-OH proved to be a second-order reaction. The half-lives of 20, 30, 40, and 50 μM DMPO-OH are calculated using the equation. For instance, to calculate the half-life of 40 μM DMPO-OH, the x values, when y values are equal to 40 and 20, are calculated to be 4.7 and 25.4, respectively. Hence, 25.4–4.7 = 20.7 min represents the half-life of 40 μM DMPO-OH. When the calculated half-lives were compared with the actually measured half-lives of each concentration of DMPO-OH, the calculated values corresponded highly to the actually measured values (Table 1). Even when dissolved oxygen was replaced by Argon (Ar), the decay curve of DMPO-OH was not affected. The half-life of the 50 μM DMPO-OH in the deoxidized sample was 16.4 min. Addition of H_2O_2 to the sample after ultrasound irradiation did not affect the decay curve of DMPO-OH, either. The half-lives of 40 μM DMPO-OH in samples containing 1 and 2 M of H_2O_2 were 21.6 and 20.9 min, respectively. Those values corresponded highly to that of 40 μM DMPO-OH from a sample without H_2O_2. Furthermore, addition of superoxide dismutase (SOD) to the sample did not affect the decay curve of DMPO-OH. The half-lives of the 50 μM DMPO-OH in samples containing 10 and 20 $U\,mL^{-1}$ SOD were 15.8 and 17.0 min, respectively. Those values were similar to that of 50 μM DMPO-OH from the sample without SOD.

Table 1. Actually Measured and Calculated Half-Lives at Different Initial Concentrations of DMPO-OH[a)]

DMPO-OH/μM	Half-life/min	
	Actually measured	Calculated
50	16.6	—
40	21.0	20.7
30	29.4	27.6
20	41.8	41.4

a) The actually measured half-lives were compared with the calculated half-lives using the equation of the decay curve obtained from the 50 μM DMPO-OH experiment ($y = 827.76/(x + 15.97)$). Each value of the actually measured half-life is expressed as the mean of duplicate determinations.

Effect of Temperature on Decay of DMPO-OH. The temperature affected the DMPO-OH decay curve (Figure 4). The half-life of the 50 μM DMPO-OH decreased in a temperature dependent manner and the rate constants were calculated using the half-life of the DMPO-OH (Table 2).

Decay of DMPO-OH Generated by Visible Light Laser Irradiation to H_2O_2. When 1 M H_2O_2 containing different concentrations of DMPO was irradiated with a laser (an output

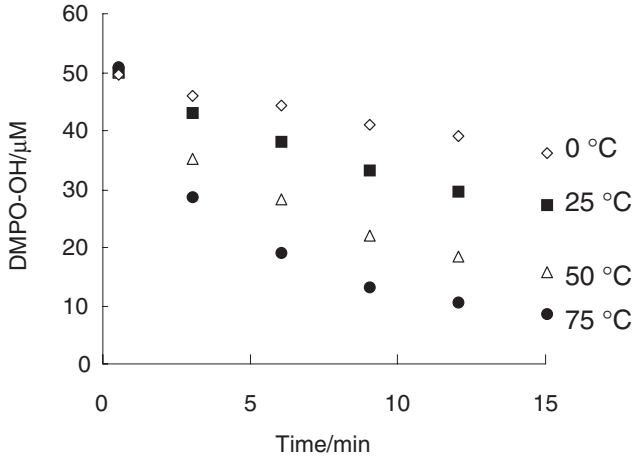

Figure 4. The influence of temperature on the decay curve of DMPO-OH. The half-life decreased with the increase of temperature. Each value represents the mean of triplicate determinations.

Table 2. Half-Lives of 50 μM DMPO-OH and Rate Constants of the Bimolecular-Reaction of DMPO-OH at 273, 298, 323, and 348 K[a)]

Temp/K	Half-life/min	Rate constant/$M^{-1}\,s^{-1}$
273	39.8	9
298	16.9	20
323	6.5	52
348	2.8	118

a) Each value is expressed as the mean of triplicate determinations.

power of 300 mW and a wavelength of 405 nm) for 30 s, DMPO-OH increased with the concentration of DMPO to a certain extent and then was saturated at the DMPO concentration of around 300 mM which was considered to be an optimal concentration (data not shown). The saturated level of DMPO-OH was around 50 μM which was coincident with that of DMPO-OH generated by the sonolysis of water. The 50 μM DMPO-OH generated by photolysis of H_2O_2 decreased with time similarly to the 50 μM DMPO-OH generated by ultrasound irradiation to ultrapure water (Figure 5). The equation of inverse relation between DMPO-OH and time was found to be $y = 931.86/(x + 18.89)$. The half-life of the 50 μM DMPO-OH was 18.6 min which was nearly equal to the half-life (16.6 min) of 50 μM DMOP-OH generated by ultrasound irradiation to ultrapure water.

Comparison of DMPO, M4PO, and DPPMPO. When ultrapure water containing M4PO was irradiated for 1 min by ultrasound, M4PO-OH ($a_N = 1.53$ mT and $a_H = 1.68$ mT)[25] was detected. M4PO-OH increased with the concentration of M4PO and then was saturated at around 16 μM. On the other hand, although DPPMPO-OH ($a_N = 1.39$ mT, $a_H = 1.39$ mT, and $a_P = 3.58$ mT)[23,26] was also detected and saturated around 14 μM, then it became undetectable at the DPPMPO concentration of over 40 mM. Since 1.0 M DPPMPO was prepared in 99% dimethyl sulfoxide (DMSO) and then diluted to the appropriate concentration with ultrapure water, it was considered that DMSO in the preparations of DPPMPO at concentrations of 40 mM or more scavenged hydroxyl radical, resulting in disappearance of the ESR spectrum. The optimal concentrations of M4PO and DPPMPO for this hydroxyl radical generation system were over 300 mM and between 10 and 30 mM, respectively.

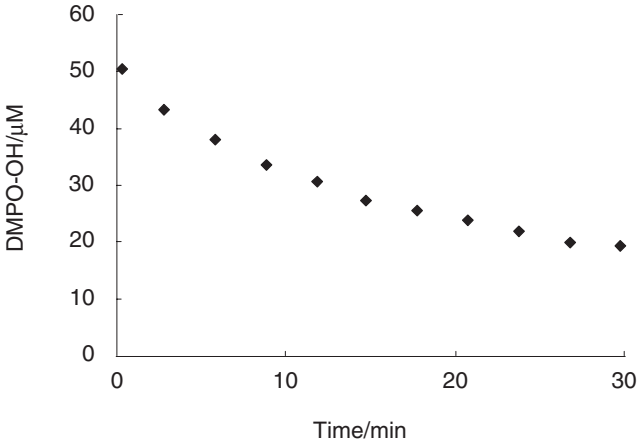

Figure 5. Decay curve of 50 μM DMPO-OH generated by laser irradiation of 1 M H_2O_2. DMPO-OH decreased inversely with time and the half-life almost corresponded to that of 50 μM DMPO-OH generated by ultrasound irradiation of ultrapure water. Each value represents the mean of duplicate determinations.

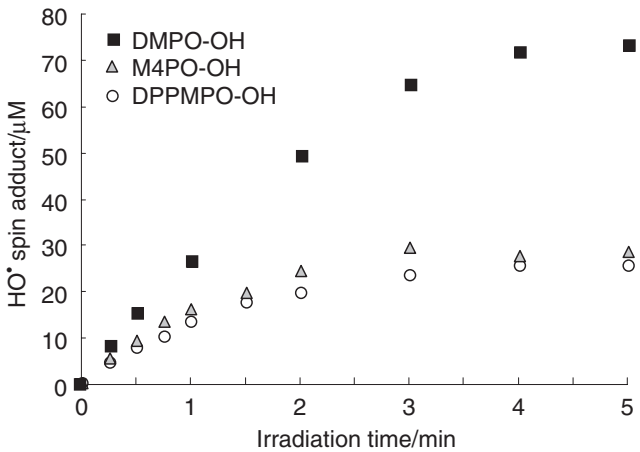

Figure 6. Relationship between spin adducts formation and ultrasound irradiation time. Each spin trap agent was used at their optimal concentration. i.e., 300 mM for DMPO and M4PO, and 20 mM for DPPMPO. Although spin adduct of hydroxyl radical increased with irradiation time, the amount of hydroxyl radical trapped by M4PO and DPPMPO was less than half of that trapped by DMPO. Each value represents the mean of duplicate determinations.

M4PO-OH and DPPMPO-OH increased in an irradiation time dependent manner, and a linear relationship with a correlation coefficient of over 0.99 between the spin adducts of hydroxyl radical and the irradiation time was observed up to 1 min (Figure 6). Then the M4PO-OH was saturated at around 30 μM and the DPPMPO-OH was at 25 μM when they were used at their optimal concentrations, 300 mM for M4PO and

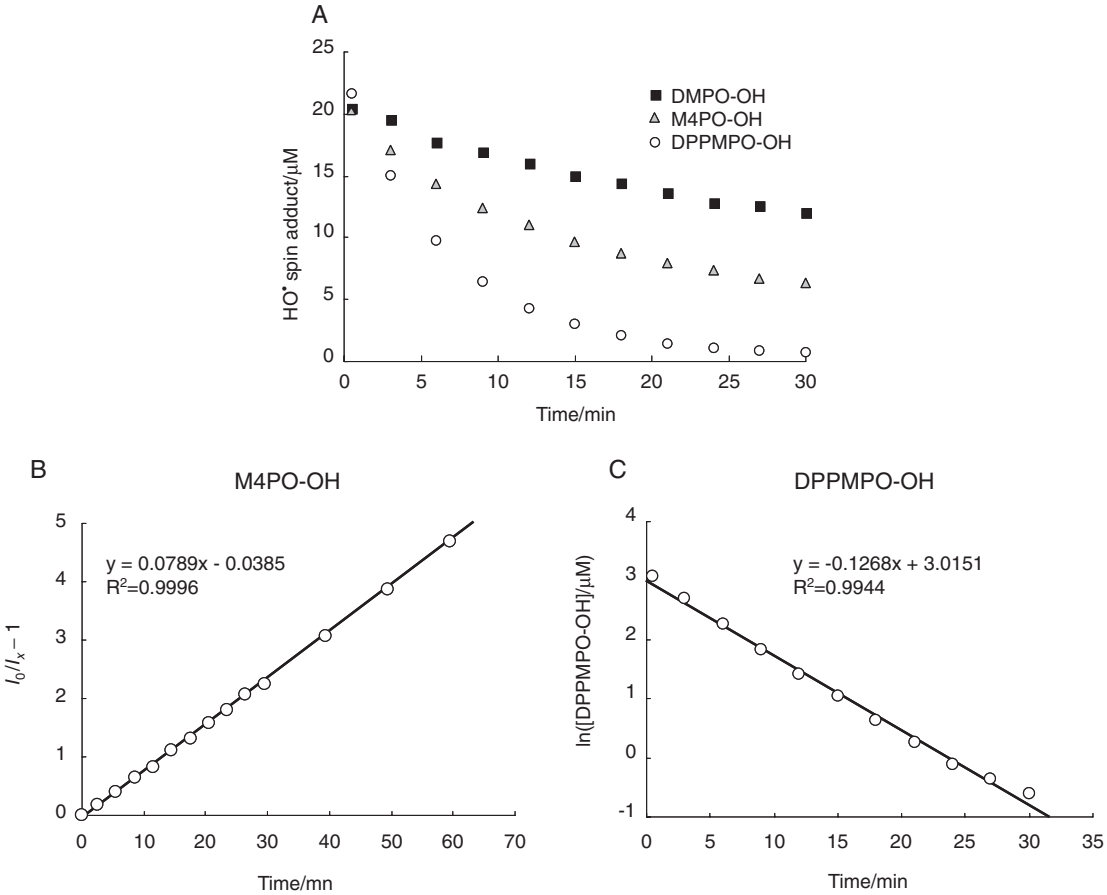

Figure 7. Analysis of decay curve of 20 μM spin adduct of hydroxyl radical. (A) DMPO-OH was the most stable followed by M4PO-OH and DPPMPO-OH. The half-lives of DMPO-OH, M4PO-OH, and DPPMPO-OH were 41.8, 12.7, and 5.5 min, respectively. (B) M4PO-OH decayed in inverse proportion to time suggesting second-order reaction. The inverse proportion was linearized by the equation $I_0/I_x - 1 = ax + b$, where I_0 is the initial concentration of M4PO-OH and I_x is the concentration of M4PO-OH at each time interval. (C) On the other hand, since plots of natural logarithm of DPPMPO-OH against time showed a linear relationship, it was suggested that DPPMPO-OH decayed by first-order reaction. Each value represents the mean of duplicate determinations.

20 mM for DPPMPO (Figure 6). Thus, it was demonstrated that hydroxyl radical was trapped more efficiently by DMPO than M4PO and DPPMPO in this experimental model.

When the decay rates of 20 μM spin adducts of hydroxyl radical were compared at 25 °C, DMPO-OH showed the longest half-life followed by M4PO-OH and DPPMPO-OH (Figure 7A). The half-lives of DMPO-OH, M4PO-OH, and DPPMPO-OH were 41.8, 12.7, and 5.5 min, respectively. DMPO-OH and M4PO-OH decayed by a second-order reaction (Figure 7B) while the decay of DPPMPO-OH was first-order (Figure 7C). The first-order reaction was confirmed by plotting the natural logarithm of DPPMPO-OH against time. The linear relationship between them confirmed that the decay was a first-order reaction.

It was observed that the addition of M4PO or DPPMPO to DMPO aqueous solution decreased DMPO-OH generated by the sonolysis in a concentration dependent manner. Since it was possible to differentiate the ESR spectrum of M4PO-OH and DPPMPO-OH from that of DMPO-OH in a certain magnet field, the IC_{50} for them against DMPO was evaluated. The IC_{50} of M4PO against 30 mM DMPO was 1.5 mM and that of DPPMPO was 6.1 mM. The calculated rate constants using the IC_{50}s were $6.8 \times 10^{10}\,M^{-1}\,s^{-1}$ for M4PO and $1.7 \times 10^{10}\,M^{-1}\,s^{-1}$ for DPPMPO.

Discussion

In the present study, we demonstrated that (1) the optimal concentration of DMPO for sonolysis of water and photolysis of H_2O_2 under the condition used was 300 mM, (2) DMPO-OH formation was saturated even though hydroxyl radical was generated continuously, (3) the concentration of DMPO-OH decreased in inverse proportion to time after generation of DMPO-OH, and (4) DMPO was more suitable for the quantification of hydroxyl radical than M4PO and DPPMPO in terms of spin trapping efficiency and stability of spin adduct. We further examined the effects of possible factors on the kinetics of DMPO-OH. H_2O_2 and SOD, which was used to examine if superoxide anion and products derived from superoxide anion affect the decay of DMPO-OH, did not or very slightly affected DMPO-OH formation under the condition used in the present study. Although the addition of H_2O_2 to the reaction system decreased DMPO-OH, the rate constant,

$3.3 \times 10^7\,M^{-1}\,s^{-1}$, which is approximately consistent with that reported in a previous paper,[27] was lower by two orders than that of the reaction between DMPO and hydroxyl radical ($3.4 \times 10^9\,M^{-1}\,s^{-1}$). It is considered that H_2O_2 in the presence of 300 mM of DMPO scavenged only a small amount of hydroxyl radical even though H_2O_2 was generated by ultrasound irradiation of water. Furthermore, since the concentration of H_2O_2 used for photolysis (1 M) was only 3.4 times higher than that of DMPO (300 mM), the generation of DMPO-OH by photolysis of 1 M H_2O_2 was unlikely affected by the presence of H_2O_2. SOD did not or very slightly affected the decay of DMPO-OH, so that there are two possibilities considered. One is that superoxide anion did not interact with DMPO-OH, and the other is that very small amount of superoxide anion was produced by ultrasound irradiation to water. Since it was reported that superoxide anion produced by xanthine–xanthine oxidase reaction induced the depletion of DMPO-OH,[28] it is likely that the decay of DMPO-OH observed in this study was free from the effect of superoxide anion. Indeed, in the present study, DMPO-OOH was not detected as shown in Figure 1B while DMPO-OH was detected in several dozen μM. Similarly, DMPO-H was not detected, either. It was reported that hydrogen atom as well as hydroxyl radical were generated by sonolysis of water when low-frequency ultrasound (several dozen to hundred kHz) was used.[12,29,30] However, the 1650 kHz ultrasound used in this study generated almost no hydrogen atom as reported in our previous study.[16]

The half-life of DMPO-OH depended on its initial concentration and varied from 16 to 42 min in this study. Although we investigated potential factors affecting lifetime of DMPO-OH, such as dissolved oxygen and H_2O_2, those factors did not seem to influence the DMPO-OH decay curve. In addition, the decay of DMPO-OH after the cessation of irradiation represents likely spontaneous decay or mutual interaction with DMPO-OH molecules themselves because the sample used in this study contained only the ultrapure water, the remaining DMPO, and DMPO-OH after ultrasound irradiation. Therefore, one possibility is that the DMPO-OH decayed by second-order reaction of two molecules of DMPO-OH so that the half-life of DMPO-OH was in inverse proportion to the initial concentration of DMPO-OH. If the second-order reaction was attributed to two molecules of DMPO-OH, the activation energy of the reaction was low ($26\,kJ\,mol^{-1}$) according to an Arrhenius plot using the values in Table 2. Thus, we presumed that the product from the second-order reaction might be a dimer of DMPO-OH.

Considering these findings, the difference of half-life of DMPO-OH between the previous reports[26,20,21] and the present study was mainly due to the difference of the initial concentration of DMPO-OH generated from different hydroxyl radical generation systems. According to the equation ($y = 827.76/(x + 15.97)$) used in this study, the 60 min half-life means that the initial concentration of DMPO-OH was about 14 μM. Similarly, as reported in a previous study on radiolysis of water, DMPO-OH exhibited second-order radical termination kinetics at initial concentrations of 13 μM or more, followed by first-order termination kinetics at lower concentrations,[31] which is in agreement with another study.[32] Therefore, it is suggested that the initial concentration of DMPO-OH is an important factor affecting quantitative analysis of hydroxyl radical.

As opposed to the potential factors discussed above (dissolved oxygen and H_2O_2), the DMPO-OH decay curve was affected by temperature. Since higher temperature causes faster decay of DMPO-OH, it was confirmed that the second-order reaction was accelerated by temperature. If the second-order reaction for two molecules of DMPO-OH occurred, the rate constants were quite low compared to those in the reactions of hydroxyl radical and DMPO even though the temperature was 75 °C, suggesting that the reaction was slow. On the other hand, the influence of the temperature on the quantitative analysis is not negligible as shown in Figure 4.

When the actually measured half-life of each concentration of DMPO-OH and the calculated value using the equation ($y = 827.76/(x + 15.97)$) of the decay curve of 50 μM DMPO-OH were compared, there is little difference between the two. This finding suggests that concentration of DMPO-OH decreases according to the equation. For instance, the calculated half-life of each concentration of DMPO-OH is as follows; 8.3 min for 100 μM DMPO, 4.1 min for 200 μM DMPO-OH, and 2.1 min for 400 μM DMPO. The short lifetime of several hundred μM DMPO-OH might be one of the reasons for the saturation phenomenon of the actually measured DMPO-OH. However, the spontaneous decay of DMPO-OH is too slow to explain the whole reaction of saturation of the actually measured DMPO-OH. Therefore, hydroxyl radical generated constantly by ultrasound irradiation might degrade DMPO-OH, which makes difficulty in measurement of several hundred μM DMPO-OH correctly. Indeed, hydroxyl radical is generated linearly with time (Figure 2) but the actually measured concentration of DMPO-OH is saturated at 80 μM. This finding is in accordance with previous reports.[12,16] Those studies investigated the relationship between ultrasound irradiation time and concentration of DMPO-OH. Iwasawa et al.[16] reported that the concentration of DMPO-OH was saturated at around 15 μM. The difference of the concentration of saturation between the previous study and ours might be due to the concentration of DMPO added to the reaction system and the power of the ultrasound device. The factors which cause the saturation of actually measured DMPO-OH should be studied in the future for better understanding of a generation system which could produce a large amount of hydroxyl radical.

Optimal concentration of DMPO for photolysis of 1 M H_2O_2 was 300 mM under the conditions in which 50 μM DMPO-OH was generated. Although the optimal conditions for photolysis of H_2O_2 were coincident with those for sonolysis of water in this study, optimal conditions should be evaluated for each experiment because they are probably affected by the amount and the generation rate of hydroxyl radical in each generation system. As to decay of DMPO-OH generated by photolysis of H_2O_2, a decay curve similar to that generated by sonolysis of water was observed. Therefore, it is thought that the kinetics related to the decay of DMPO-OH generated by sonolysis of water and photolysis of H_2O_2 is almost the same. However, kinetics of DMPO-OH may vary in other hydroxyl radical generation systems, such as Fenton reaction or Haber–Weiss

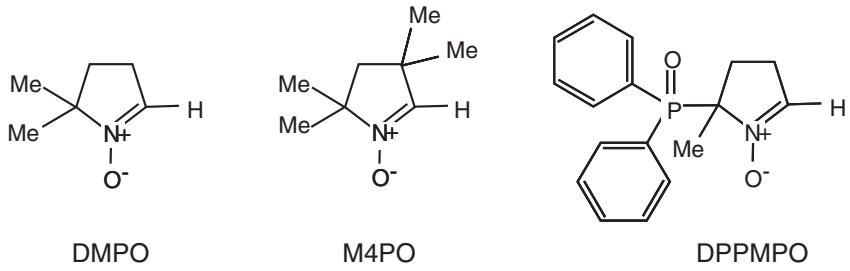

Figure 8. Structures of DMPO, M4PO, and DPPMPO.

reaction because they involve more chemicals than in sonolysis and photolysis used in this study.

It was confirmed that M4PO and DPPMPO could detect hydroxyl radical as reported previously. Both of the spin trap agents also showed the saturation of hydroxyl radical spin adduct as did DMPO (Figure 6). Since hydroxyl radical was continuously generated unless ultrasound irradiation was ceased as discussed above, the saturation of spin adducts was likely due to the same reason as in the case with DMPO. Hence, it was suggested that the hydroxyl radical spin adducts would not reflect the true generation of hydroxyl radical after certain ultrasound irradiation time irrespective of the types of spin trap agents used in this study. Although the rate constants of those two spin trap agents for hydroxyl radical were relatively higher than that of DMPO, the spin trapping efficiency of them was less than half (Figure 6). This finding probably means that M4PO and DPPMPO reacted to hydroxyl radical but could not trap the spin effectively. In other words, partial structures such as methyl and diphenylphosphinoyl moieties interact with hydroxyl radical, which in turn interferes with trapping. Concerning the kinetics of decay curve, M4PO-OH and DPPMPO-OH showed shorter half-lives than DMPO-OH. Interestingly, it was demonstrated that DPPMPO-OH decayed in the first-order reaction while M4PO-OH and DMPO-OH decayed in the second-order reaction. DPPMPO-OH might not react with another molecule of DPPMPO-OH but decompose by autolysis. As for the chemical structure of DPPMPO, a major difference from those of DMPO and M4PO is existence of diphenylphosphinoyl moiety in its structure (Figure 8). Therefore, one of the possibilities is that the diphenylphosphinoyl moiety is attributable to fragility of DPPMPO-OH. These findings suggest that DMPO is more suitable for quantification of hydroxyl radical than M4PO and DPPMPO at least in simple reaction systems, such as sonolysis of water and photolysis of H_2O_2.

Hydroxyl radical has a bilateral character. The negative character is thought to be an important factor of oxidative damage in cells and tissues causing many diseases.[33,34] In addition to host immune defense by polymorphonuclear leukocytes,[35,36] the positive character is applied medically, such as cancer treatment,[37] antibiotics,[38] and bactericidal (fungicidal) treatment.[16] For medical application of hydroxyl radicals, controlling the amount of production is one of the most important factors. Otherwise, hydroxyl radicals will kill not only bacteria or undesired cells but also normal cells causing adverse reactions to the body. We believe that the present study can give basic knowledge to quantitatively analyze hydroxyl radical production as the first step of radical control. On quantitative analysis using generation systems which generate a large amount of hydroxyl radical (up to several hundred μM in a short time) as seen in this study, the following factors for ESR spin trapping using DMPO must be considered; (1) the optimal concentration of DMPO, (2) the decay rate of DMPO-OH depending on the initial concentration of DMPO-OH, and (3) temperature effect on the kinetics of DMPO-OH. If these factors are taken into consideration, the true amount of hydroxyl radical generation can be speculated by the linear relationship between the DMPO-OH level and irradiation time of ultrasound or laser observed in the short period of time even though the actually measured DMPO-OH is saturated.

Experimental

Reagents. Reagents were purchased from the following sources: DMPO, M4PO, and DPPMPO from Labotec (Tokyo, Japan); H_2O_2 from Santoku Chemical Industries (Tokyo, Japan); 4-hydroxy-2,2,6,6-tetramethylpiperidine (TEMPOL) and SOD from Sigma Aldrich (St. Louis, MO, USA); DMSO from Wako Pure Chemical Industries (Osaka, Japan). All other reagents used were of analytical grade.

Optimal Concentration of DMPO for Quantification of Hydroxyl Radical. An experimental device which generates 1650 kHz ultrasound with an output power of 30 W was made. A glass tube (15 mm in diameter and 85 mm long) containing 500 μL of sample was set into the device. To evaluate an optimal concentration of DMPO for ESR measurement of hydroxyl radical generated from this system, the relationship between the concentration of DMPO and the concentration of DMPO-OH was investigated. Immediately after mixing of ultrapure water and given concentration of DMPO, the test tube was set in the device and was irradiated by ultrasound for 2 min. During the irradiation, the temperature of the water in the bath was maintained at 22 ± 1 °C. The DMPO aqueous solution was then transferred to a quartz cell for ESR spectrometry 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, 0.1 mT; amplitude, 80; sweep time, 2 min; time constant, 0.03 s; microwave frequency, 9.420 GHz: microwave power, 4 mW. To calculate the concentration of DMPO-OH, 20 μM TEMPOL was used as a standard sample for quantitative analysis and the ESR spectrum of manganese (Mn^{2+}) which was equipped in the ESR cavity was used as an

internal standard. The concentration of hydroxyl radical was determined using Digital Data Processing (JEOL, Tokyo, Japan) and was expressed as that of DMPO-OH in μM.

Formation of DMPO-OH. The relationship between the ultrasound irradiation time and the concentration of DMPO-OH was investigated. According to the experiment described above, DMPO was used in the final concentration of 300 mM. The 300 mM of DMPO aqueous solution was irradiated for 15 s to 8 min. The ESR measurement and data analysis were performed in the same way as described above. Then, equations indicating the linear relationship between DMPO-OH and the ultrasound irradiation time prepared by least squares fit to calculate the total amount of hydroxyl radical.

The change in the hydroxyl radical generation from ultrapure water was evaluated. In brief, ultrapure water was placed in a glass tube and then set in the ultrasound device without DMPO. After 8 min of irradiation, DMPO was added to make a final concentration of 300 mM. Immediately after addition of DMPO, the sample was irradiated for 2 min again. The amount of DMPO-OH generated was compared to that from 2 min ultrasound irradiation to DMPO aqueous solution without prior ultrasound irradiation. The ESR measurement and data analysis were performed in the same way as described above.

The effect of H_2O_2 on DMPO-OH formation in the hydroxyl radical generation system was investigated. H_2O_2 and DMPO were mixed to make a final concentration of 8 mM to 8 M for H_2O_2 and 1, 2, and 4 mM for DMPO. The mixture was then irradiated for 2 min. The inhibition curve of DMPO-OH against the concentration of H_2O_2 was made and the half maximal inhibitory concentration (IC_{50}) of H_2O_2 was calculated for each concentration of DMPO. The rate constant of the reaction between H_2O_2 and hydroxyl radical was also calculated on the assumption that the reaction proceeded with the following equations;

$$\mathrm{DMPO} + \mathrm{HO^{\bullet}} \rightarrow \mathrm{DMPO\text{-}OH} \quad (\text{rate constant: } k_1 = 3.4 \times 10^9\ \mathrm{M^{-1}\,s^{-1}})^{13} \quad (1)$$

$$\mathrm{H_2O_2} + \mathrm{HO^{\bullet}} \rightarrow \mathrm{X_1}\ (\text{rate constant: } k_2) \quad (2)$$

where $HO^{\bullet}$ is hydroxyl radical and X_1 is defined as a product produced by the reaction.

From eq 1 and eq 2, the following equations were derived;

$$\mathrm{d[DMPO\text{-}OH]}/\mathrm{d}t = k_1[\mathrm{HO^{\bullet}}][\mathrm{DMPO}] \quad (3)$$

$$\mathrm{d[X_1]}/\mathrm{d}t = k_2[\mathrm{HO^{\bullet}}][\mathrm{H_2O_2}] \quad (4)$$

If the concentration of H_2O_2 corresponded to the IC_{50}, the following equation was derived;

$$k_2 = k_1[\mathrm{DMPO}]/[\mathrm{IC_{50}}] \quad (5)$$

The value of k_2 was compared to k_1 to evaluate the influence of the hydroxyl radical scavenging of H_2O_2 in the radical generation system.

To investigate the influence of superoxide anion on DMPO-OH formation, SOD was added to the reaction system to make a final concentration of 10 and 20 U mL^{-1}. One unit of SOD was defined as the amount of SOD required to inhibit reduction of cytochrome *c* by 50% in a coupled system with xanthine oxidase. The concentration of DMPO-OH with or without SOD was compared.

Decay of DMPO-OH. The concentration of DMPO-OH was recorded by ESR using the same conditions as described above. Fifty μM DMPO-OH was generated from 300 mM of DMPO aqueous solution using the ultrasound device. The measurement of DMPO-OH was conducted up to 3 h using the same sample after cessation of irradiation. The mean values of DMPO-OH at each time interval were plotted. The equation of the decay curve was calculated to fit to the inverse curve ($y = a/(x - b)$). The half-lives of 20, 30, and 40 μM DMPO-OH generated in the same way as described above but shorter irradiation time than that required for 50 μM DMPO-OH were also evaluated. The actually measured half-lives were compared with the calculated half-lives using the equation of the decay curve obtained from the result of the 50 μM DMPO-OH experiment.

Since dissolved oxygen of the sample might affect the DMPO-OH decay curve, the dissolved oxygen was replaced by Ar gas bubbling. The initial measurement of the DMPO-OH was performed under the same conditions as described above but the remaining sample was bubbled by Ar gas in the glass tube during the initial measurement of the DMPO-OH. Then, the concentration of DMPO-OH was measured-up to 15 at 3 min intervals.

The influence of H_2O_2 on the decay curve of DMPO-OH was evaluated. Fifty μM DMPO-OH was generated from 400 μL of ultrapure water containing DMPO (final concentration of 300 mM) using the ultrasound device. Immediately after irradiation, 100 μL of ultrapure water or 100 μL of H_2O_2 was added to be 0, 1, and 2 M of the final concentration of H_2O_2. Then, the measurement of DMPO-OH was conducted up to 15 at 3 min intervals.

To investigate the influence of oxidative products which might be generated through superoxide anion on the decay curve of DMPO-OH, SOD was added to the reaction system. SOD and DMPO were dissolved in ultrapure water to make a final concentration of 0, 10, and 20 U mL^{-1} for SOD and 300 mM for DMPO. Fifty μM DMPO-OH was generated from the sample using the ultrasound device and the measurement of DMPO-OH was conducted up to 15 min at 3 min intervals. The half-life of DMPO-OH under each condition was calculated and compared with that of DMPO-OH without any additional treatment.

Effect of Temperature on Decay of DMPO-OH. The influence of temperature on DMPO-OH decay curve was studied. Fifty μM DMPO-OH was generated using the ultrasound device in the same way as described above. The samples were kept in crushed ice or in a water bath to maintain water temperature at 0, 25, 50, and 70 °C, and was used for subsequent measurement of DMPO-OH. The half-life of DMPO-OH under each condition was calculated. The rate constant of the reaction between mutual DMPO-OH was also calculated on the assumption that the reaction was second-order as shown in the following equations;

$$\mathrm{DMPO\text{-}OH} + \mathrm{DMPO\text{-}OH} \rightarrow \mathrm{X_2}\ (\text{rate constant: } k) \quad (6)$$

where X_2 is defined as a product produced by the mutual DMPO-OH reaction. If the reaction is second-order, the reaction rate was obtained from the following equation;

$$\mathrm{d[DMPO\text{-}OH]}/\mathrm{d}t = -k[\mathrm{DMPO\text{-}OH}]^2 \quad (7)$$

The eq 7 was converted to eq 8 by indefinite integral.

$$1/[\text{DMPO-OH}] - 1/[\text{DMPO-OH}]_0 = kt \quad (8)$$

where $[\text{DMPO-OH}]_0$ means the initial concentration of DMPO-OH. The eq 9 was delivered from eq 8 using the half-life ($t_{1/2}$).

$$t_{1/2} = 1/(k[\text{DMPO-OH}]_0) \quad (9)$$

Decay of DMPO-OH Generated by Visible Light Laser Irradiation to H_2O_2. A commercially available laser device (RV-1000, RICHO OPTICAL INDUSTRY, Hanamaki, Japan) was used. DMPO-OH was generated by means of irradiation of a laser diode with an output power of 300 mW and a wavelength of 405 ± 5 nm to 1 M H_2O_2. Optimal concentration of DMPO for photolysis of 1 M H_2O_2 was investigated under the conditions in which 50 μM DMPO-OH was generated, i.e., the irradiation time was 30 s. Then, the kinetic analysis of decay of 50 μM DMPO-OH was performed. In brief, DMPO and H_2O_2 were mixed in a 96-well plate to make a final concentration of 300 mM for DMPO and 1 M for H_2O_2. Fifty μM DMPO-OH was generated by 30 s irradiation and was measured by ESR along under the same conditions as described above. The measurement of DMPO-OH was conducted up to 30 min after laser irradiation. The DMPO-OH decay curve and the half-life of DMPO-OH were compared to those of DMPO-OH generated by ultrasound irradiation to ultrapure water.

Comparison of DMPO, M4PO, and DPPMPO. Structures of DMPO, M4PO, and DPPMPO are shown in Figure 8. M4PO (1.0 M) was prepared in ultrapure water while 1.0 M DPPMPO was prepared in 99% DMSO. Optimal concentration of M4PO and DPPMPO for the quantification of hydroxyl radical was investigated as performed for DMPO. The spin trap agents were then diluted with ultrapure water and were irradiated by ultrasound for 1 min. ESR spectrum was recorded by the same way as described above except that for the ESR measurement of M4PO, field modulation width was set at 0.05 mT.

The relationship between the ultrasound irradiation time and the concentration of M4PO-OH and DPPMPO-OH were also investigated as performed for DMPO. According to the experiment described above, M4PO and DPPMPO were used in the final concentration of 300 and 20 mM, respectively. The aqueous solutions containing the spin trap agents were irradiated for 15 s to 5 min. The ESR measurement and data analysis were performed in the same way as described above.

The decay curve of 20 μM spin adducts was investigated. To obtain 20 μM DMPO-OH, M4PO-OH, and DPPMPO-OH, each aqueous solution containing the spin trap agent at the optimal concentration was irradiated for 45, 90, and 120 s, respectively, depending on their spin trapping efficiency. The concentration of each spin adduct of hydroxyl radical was recorded up to 30 min by ESR in the same way as described above. Then, the half-lives of each spin adduct of hydroxyl radical were evaluated.

To evaluate the rate constant of M4PO and DPPMPO for hydroxyl radical, the competitive reactions of M4PO and DMPO, and of DPPMPO and DMPO to hydroxyl radical were investigated. Given concentration of M4PO or DPPMPO was mixed with DMPO to make a final concentration of 30 mM for DMPO. The mixture was then irradiated for 1 min. According to the inhibition curve of DMPO-OH against the concentration of the spin trap agents, IC_{50} of each spin trap agent was determined and then the rate constant was calculated.

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