

ESR Measurement Using 2-Diphenylphosphinoyl-2-methyl-3,4-dihydro-2*H*-pyrrole *N*-Oxide (DPhPMPO) in Human Erythrocyte Ghosts

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Competitive spin-trapping behavior of 2-diphenylphosphinoyl-2-methyl-3,4-dihydro-2*H*-pyrrole *N*-oxide (DPhPMPO) and 2,2-dimethyl-3,4-dihydro-2*H*-pyrrole *N*-oxide (DMPO) indicated that the affinity of DPhPMPO for hydroxyl radicals was 1.7 times higher than that of DMPO. Human erythrocyte ghosts (HEGs) with encapsulated Fe^{II} catalysts (HEG–Fe) were prepared for ESR measurement. By trapping the hydroxyl radical ($OH^{\bullet}$) in the presence of polyethylene glycol (PEG-4000) outside the HEG–Fe, the generation of $OH^{\bullet}$ inside the catalyst was confirmed. The trapping of hydroxyl radicals generated in HEG was accomplished using DPhPMPO, and the permeability of the erythrocyte membrane to DPhPMPO was 2.4 times greater than that of DMPO.

Reactive oxygen species (ROS) are a prominent cause of aging and a number of diseases. Electron spin resonance (ESR) employing spin-trapping methodology has been widely used to detect ROS and related free radicals.[1] 2,2-Dimethyl-3,4-dihydro-2*H*-pyrrole *N*-oxide (DMPO) (Chart 1) has been widely used as a spin trap over the last thirty years.[2] However, the lifetime of its $O_2^{\bullet-}$ adduct is too short to be detected clearly. In contrast, the $O_2^{\bullet-}$ adducts of a number of 5-substituted DMPO analogues, such as 2-(diethoxyphosphinoyl)-2-methyl-3,4-dihydro-2*H*-pyrrole *N*-oxide (DEPMPO),[3] 2-(ethoxycarbonyl)-2-methyl-3,4-dihydro-2*H*-pyrrole *N*-oxide (EMPO),[4] and its derivatives, have been developed and found to have longer lifetimes than that of DMPO. In addition, the $O_2^{\bullet-}$ adduct of DEPMPO does not decompose to form hydroxyl radical ($OH^{\bullet}$) adducts.[3,5] Substitution at the 2-position of the 3,4-dihydro-2*H*-pyrrole ring plays an essential role in stabilizing of $O_2^{\bullet-}$ adducts, both sterically and electronically. These substituents not only extend the lifetime of the adducts, but also increase the lipophilicity of the spin traps. Recently, lipophilic spin traps have been widely used for the detection of lipid radicals generated from lipid peroxidation in vivo.[6] Upon detection of ROS using the spin-trapping method in vivo, the membrane permeability of the spin traps toward cell membranes becomes a vital factor. It is also thought that high lipophilicity of the spin traps prevents them from entering the cytosol, and thus, it remains within the cellular membrane.[7] Anzai et al. have observed the passive permeation rates of DMPO and DEPMPO through liposomal membranes.[8] To our knowledge, there are no other reports.

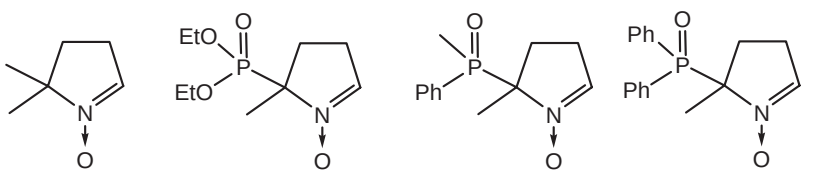
The erythrocyte membrane has an asymmetric lipid bilayer composed of an hydrophobic core with hydrophilic edges and multiple lipid species, a variety of skeletal proteins, such as spectrin, and integral proteins, such as Band 3.[9] The encapsulation of drugs, such as antibiotics,[10] antineoplastics agents,[11] angiotensin-converting enzyme inhibitors,[12] antioxidant drugs,[13] and iron chelators,[14] in erythrocytes is of current interest. By using erythrocyte ghosts, iron(II) ions as a reagent for generation of $OH^{\bullet}$ might be encapsulated.

Previously, we reported the synthesis of 2-diphenylphosphinoyl-2-methyl-3,4-dihydro-2*H*-pyrrole *N*-oxide (DPhPMPO).[15] The membrane permeability of DPhPMPO should be higher, because the lipophilicity of DPhPMPO should be higher with two phenyl group. In the present work, we evaluate the $OH^{\bullet}$ trapping ability in HEGs and the membrane permeability of DPhPMPO (Scheme 1).

Results and Discussion

Spin Trapping of Hydroxyl Radicals. Hydroxyl radicals were generated using the Fenton system (H_2O_2–$FeCl_2$) in phosphate buffer (pH 7.0). An ESR signal, which was split into double-quartets shown in Fig. 1, due to the trapping of a hydroxyl radical by DPhPMPO was observed ($A_P = 3.56$, $A_N = 1.38$, $A_H = 1.39$ mT). Additionally, the ESR signal of the stereoisomer was observed in a ratio of 96:4 ($A_P = 4.42$, $A_N = 1.41$, $A_H = 1.41$ mT). The appearance of its signal was inhibited by the addition of catalase to the incubation mixture.

The relative rate of hydroxyl radical trapping of DPhPMPO with respect to DMPO was measured using the competitive



DMPO DEPMPO MPhPMPO DPhPMPO

Chart 1.

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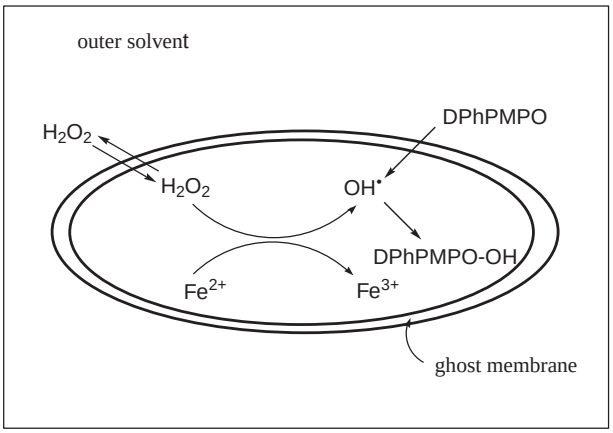


Scheme 1.

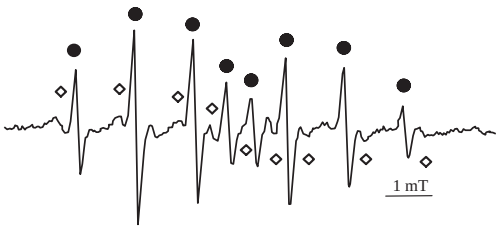


Fig. 1. ESR spectrum obtained using Fenton's reaction in the presence of DPhPMPO (10 mM) ●; major isomer DPhPMPO–OH ($A_P = 3.56$, $A_N = 1.38$, $A_H = 1.39$ mT) ◇; minor isomer of DPhPMPO–OH ($A_P = 4.42$, $A_N = 1.41$, $A_H = 1.41$ mT).

$$\text{DPhPMPO} + \text{OH}^\bullet \xrightarrow{k_1} \text{DPhPMPO-OH}$$

$$\text{DMPO} + \text{OH}^\bullet \xrightarrow{k_2} \text{DMPO-OH}$$

$$\frac{R_{\text{DPhPMPO}}}{R_{\text{DMPO}}} = \frac{v_{\text{DPhPMPO-OH}}}{v_{\text{DMPO-OH}}} = \frac{k_1[\text{DPhPMPO}]_0}{k_2[\text{DMPO}]_0}$$

Scheme 2.

spin-trapping method (Scheme 2).[16] From the Fenton reaction using $FeCl_2$ in phosphate buffer (pH 7.4) containing both DPhPMPO and DMPO, typical ESR signals due to two hydroxyl radical adducts were observed (Fig. 2a). Signals were assigned by comparing the signals obtained after the Fenton reaction using DPhPMPO or DMPO. The spectral lines marked as open circles represent the spin adduct DMPO–OH, while those marked as closed circles correspond to the spin adduct DPhPMPO–OH (Fig. 2a). The relative rate constants for the hydroxyl radicals of DPhPMPO and DMPO (k_1/k_2) can be calculated from the slope of the straight lines obtained by plotting the relative abundance of the two spin adducts ($R_{\text{DPhPMPO}}/R_{\text{DMPO}}$) against the ratio of initial concentration ($[\text{DPhPMPO}]_0/[\text{DMPO}]_0$), as indicated in Fig. 2b. Thus, the relative spin-trapping rate constant of DPhPMPO for the hydroxyl radicals as compared to that of DMPO was 1.68 ± 0.08. The spin-trapping rate constants for the hydroxyl radicals by DEPMPO and DMPO were 6.16×10^9 and 3.6×10^9 dm^3 mol^{-1} s^{-1}, respectively.[17] The relative rate constant calculated from these values was 1.71. Comparing with these values, the affinity of DPhPMPO for hydroxyl radicals is greater than that of DMPO and similar to that of DEPMPO. The X-ray crystallographic structure of DPhPMPO showed that the diphenylphosphinoyl group was protected by a *N*-oxide moiety. However, there was no steric effect on ionic addition to the C2 carbon. The difference in the reactivity between DPhPMPO and DEPMPO was caused by the strength of the electron-attractive group.[15]

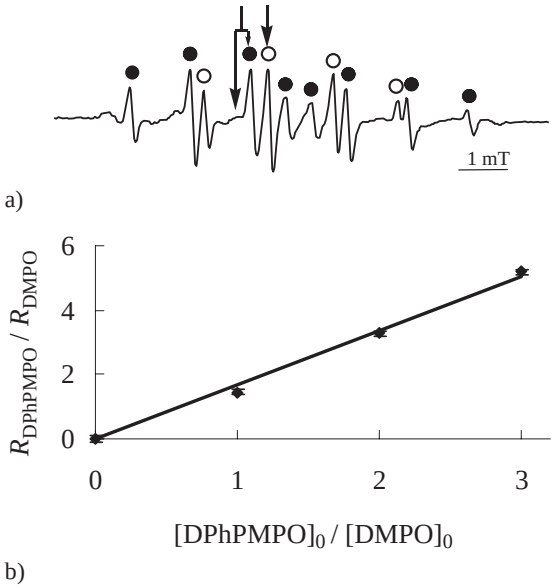


Fig. 2. a) ESR spectrum obtained using Fenton's reaction in the presence of DPhPMPO (1 mM) and DMPO (1 mM). ●; DPhPMPO–OH and ○; DMPO–OH. b) The ratio of OH• adduct formation rate for DPhPMPO and DMPO is plotted as a function of the ratio of the initial concentration of spin traps.

Spin Trapping of Hydroxyl Radicals in HEG. We encapsulated Fe^{II} within HEG and investigated the corresponding Fenton reactions (Scheme 1). Here, the catalyst should generate OH• was generated in the inner sphere. Thus, when membrane permeation was measured, the reaction was carried out without the use of an OH• scavenger in the outer solution. The encapsulation of Fe^{II} within HEG (HEG–Fe) was carried out by using the hypotonic dialysis method.[18] Excess Fe^{II} adsorbed on the HEG surface was removed with an iron chelator, diethylenetriamine pentaacetic acid (DTPA). After washing, the HEG was punctured using a 5P8 buffer solution and then centrifuged. When the Fenton reactions were carried out in the supernatant, OH• adducts of both DMPO and DPhPMPO were observed. These observations suggest that Fe^{II} was encapsulated in the HEG. ESR spin-trapping experiments using DPhPMPO were carried out in a phosphate buffer containing HEG–Fe. The appearance of the double-quartet spectrum was similar to that obtained from the standard Fenton reaction (Fig. 3). The peak width of the observed ESR spectra was similar to that observed in the spectra of the experiments performed without HEG. This observation indicated that DPhPMPO–OH existed in the water layer. However, it was not possible to determine whether DPhPMPO trapped OH• in the aqueous layer of the HEG or whether it trapped OH•

Fig. 3. ESR spectra of DPhPMPO–OH obtained in aqueous solution in the presence of HEG–Fe.

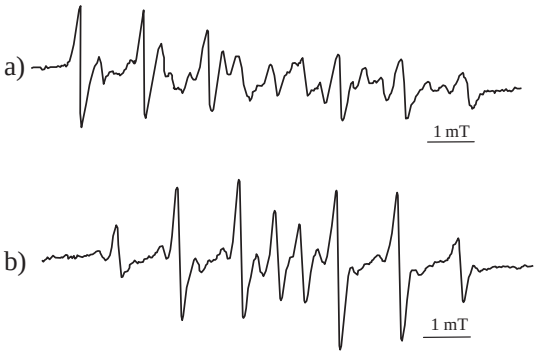
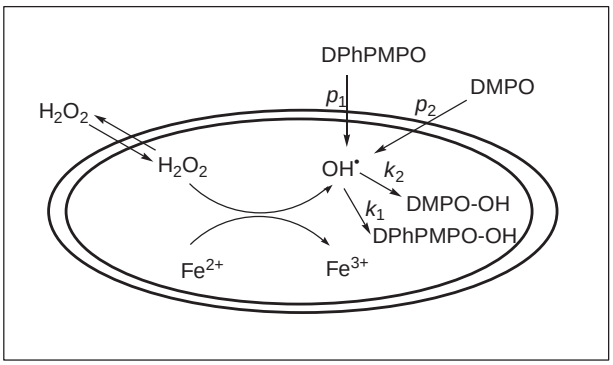


Fig. 4. ESR spectra of Fenton's reaction using DPhPMPO (10 mM) in the presence of PEG-4000 (20 mM). a) Fenton reaction using Fe^{II}. b) Fenton reaction using HEG–Fe.



$$\text{DPhPMPO} \xrightarrow{p_1} \text{DPhPMPO} + OH^\bullet \xrightarrow{k_1} \text{DPhPMPO-OH}$$

$$\text{DMPO} \xrightarrow{p_2} \text{DMPO} + OH^\bullet \xrightarrow{k_2} \text{DMPO-OH}$$

$$\frac{R_{\text{DPhPMPO}}}{R_{\text{DMPO}}} = \frac{v_{\text{DPhPMPO-OH}}}{v_{\text{DMPO-OH}}} = \frac{p_1 k_1\ [\text{DPhPMPO}]_0}{p_2 k_2\ \ [\text{DMPO}]_0}$$

Scheme 3.

in the lipid layer and DPhPMPO–OH subsequently seeped into the aqueous layer.

Polyethylene glycol (PEG-4000) has been used to scavenge $OH^\bullet$ to give carbon-centered radicals of PEG.[8] In the ESR spectra, the $OH^\bullet$ adduct in the presence of PEG-4000 yielded a signal associated with the carbon-centered radical adduct (Fig. 4). In the presence of HEG–Fe, however, this signal was not obtained. These results suggested that $OH^\bullet$ was only generated inside HEG, and that DPhPMPO was able to permeate the membrane, while $OH^\bullet$ was not.

The relative rate of DPhPMPO with respect to DMPO for the reaction with $OH^\bullet$ in HEG was measured using competitive spin trapping with HEG–Fe (Scheme 3). The observed

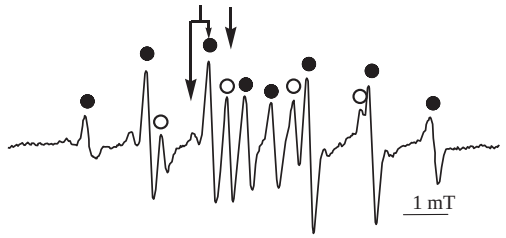


Fig. 5. ESR spectra obtained in aqueous solution using HEG–Fe in the presence of DPhPMPO (10 mM) and DMPO (10 mM).

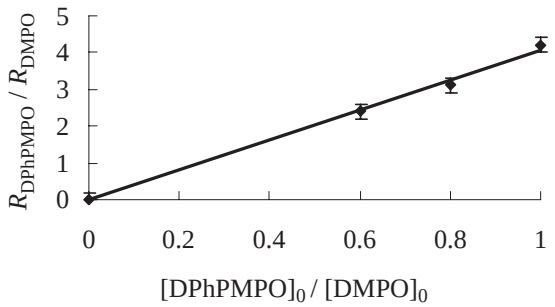


Fig. 6. The ratio of $OH^\bullet$ adduct formation rate for DPhPMPO and DMPO in HEG plotted as a function of the ratio of the initial concentration of spin traps.

Table 1. Relative Rate of Addition Reaction of Hydroxyl Radical

k_1/k_2	p_1k_1/p_2k_2	p_1/p_2
1.68 ± 0.08	4.08 ± 0.27	2.42

Table 2. Partition Coefficient of Spin Traps

Spin traps	DPhPMPO	MPhPMPO	DMPO	DEPMPO
K_p	4.0	0.7[a)]	0.11[b)]	0.06[c)]

a) Ref. 17. b) Ref. 2a. c) Ref. 3.

ESR spectra were similar to those without HEG (Fig. 5). Equilibrium between the outer and inner concentration of the spin traps through the membrane was observed. Therefore, the reaction rate ratio of the spin traps with $OH^\bullet$ was represented by $p_1k_1[\text{DPhPMPO}]_0/p_2k_2[\text{DMPO}]_0$. Here, p_1 and p_2 indicated the degree of permeation of DPhPMPO and DMPO, respectively. These values correspond to the permeability of the spin traps into the lipid layer and at the partition between water and the lipid layer. The plot of relative abundance ($R_{\text{DPhPMPO}}/R_{\text{DMPO}}$) against the ratio of initial concentration ($[\text{DPhPMPO}]_0/[\text{DMPO}]_0$) is shown in Fig. 6. The slope (4.08 ± 0.27) corresponds to the ratio of overall rate between DPhPMPO and DMPO. The obtained value was 2.4 times larger than that of the in vitro reaction. These values are summarized in Table 1.

The lipophilicity of DPhPMPO was estimated by measuring of the partition coefficients in 1-octanol and water.[19] In a previous paper, we have reported the partition coefficients of 2-methyl-2-[methyl(phenyl)phosphinoyl]-3,4-dihydro-2*H*-pyrrole *N*-oxide (MPhPMPO),[20] and the data is summarized in Table 2. The partition coefficient of DPhPMPO was larger

than those of MPhPMPO and DMPO. The enhancement in lipophilicity due to substitution of a phenyl group into the 2-position of the pyrroline ring has been reported by Tordo et al.[21]

The difference in permeability of the membrane to the lipophilic spin traps such as DMPO and DEPMPO, has not been referred. However, DMPO–OH and DEPMPO–OH has been reported to penetrated with the same ability.[8] From these partition coefficients, the membrane permeabilities toward DMPO and DEPMPO were assumed to be almost the same. A comparison of the values among DPhPMPO, DMPO, and DEPMPO suggested that the permeability of DPhPMPO was higher than that of DMPO. This is the first report of a direct measurement of the penetration rate of spin traps traversing the cell membrane.

Summary

We prepared human erythrocyte ghost cells with encapsulated Fe^{II} catalysts. In ESR measurements, the signal of the $OH^{\bullet}$ adduct in HEG could be observed using novel lipophilic spin traps. The permeability of the spin trap was higher than that of DMPO.

Experimental

Materials. DPhPMPO was synthesized according to literature methods. DMPO $FeCl_2$, hydrogen peroxide, catalase, polyethylene glycol (PEG-4000), diethylenetriamine pentaacetic acid (DTPA), and all other chemicals were purchased from commercial suppliers.

Preparation of Encapsulated Fe^{II} Ions in Human Erythrocyte Ghost Cells. A solution (3 mL) of PBS (10 mM (1 M = 1 mol L^{-1})) of phosphate buffer (pH 7.4) including 150 mM of NaCl was added to 2 mL of fresh blood and stirred gently. After centrifugation (4 °C, 1000 g, 10 min), the supernatant was removed. To prepare ghosts, the erythrocyte pellets were lysed in 5P8 buffer (5 mM phosphate buffer, pH 8.2). After centrifugation (4 °C, 10000 g, 10 min), the pellets were washed three times using 5P8. The obtained white ghosts were suspended in 5P8 buffer containing 100 mM of $FeCl_2$ and then incubated for 1 h at 37 °C under isotonic conditions produced by adding of 1/20 volumes of solution (2.8 M of KCl, 0.2 M of NaCl, 20 mM of $MgCl_2$). The resealed ghosts were washed three times with PBS and then with the buffer containing 10 mM of DTPA solution to remove Fe^{II} ions adsorbed on the membrane surface.

Spin-Trapping Studies. ESR Measurements: ESR spectra were recorded at room temperature using a spectrometer at 9.5 GHz, employing 100 kHz field modulation. The reaction mixture was prepared in a phosphate buffer.

Hydroxyl Radical Trapping: Hydroxyl radicals were generated by addition of H_2O_2 (10 mM) to a solution of DPhPMPO (1.0 mM), DTPA (2.0 mM), $FeCl_2$ (1.0 mM) in phosphate buffer (10 mM, pH 7.4). No ESR signals were observed when catalase (600 U mL^{-1}) was added to the incubation mixture. The reaction using HEG–Fe was carried out by mixing H_2O_2 (10 mM), DPhPMPO (10 mM), HEG–Fe were in PBS (10 mM, pH 7.4).

Hydroxyl Radical Trapping with DPhPMPO in the Presence of Polyethylene Glycol: Hydroxyl radicals were generated according to method (b). In brief, H_2O_2 (10 mM) was added to a solution of DPhPMPO (10 mM), DTPA (2.0 mM), $FeCl_2$ (1.0 mM) or HEG–Fe in phosphate buffer (10 mM, pH 7.4) in the presence of PEG-4000 (2.0 mM). The ESR spectra were recorded immediately.

Competitive Measurement of Relative Rates for Hydroxyl Radical Trapping of DPhPMPO and DMPO: A competitive spin-trapping method was used to determine relative spin-trapping rates of DPhPMPO and DMPO. For instance, 1.0 mM of $FeCl_2$ or HEG–Fe, DPhPMPO (1.0–3.0 or 6.0–8.0 mM), and DMPO (1.0 or 10 mM) were mixed in PBS buffer (pH 7.4). After 1 min, H_2O_2 (10 mM) was added to the reaction mixture, and the corresponding ESR spectra were recorded after 30 s. The relative abundance of the two components was calculated from a two-fold calculation of the peak area of 3rd peak to the left of DPhPMPO (and its stereoisomers), and from the area of the 2nd peak to the left of DMPO, respectively. In the reaction where HEG–Fe was used, these same peaks were increased continually for a full 6 min from the start of incubation. This observation indicated that the permeation of the spin traps did not reach equilibrium. A plot of the relative abundance of the spin adduct against the initial concentration of the spin traps afforded a line with slope k_1/k_2 for the reaction using $FeCl_2$, and p_1k_1/p_2k_2 for the reaction using HEG–Fe. In order to confirm that the spin adduct decay would not influence the trapping rate measurement. We monitored the peak area ratio obtained at different times. These ratios were taken after 30 s and 1 min, respectively. The difference was less than 2%.

Measurement of Partition Coefficient. A solution of DPhPMPO was prepared in 1-octanol at a concentration of 0.25 mM. The concentration of spin trap was measured at its optical absorption maximum using UV–vis spectrophotometry. Equal volumes (5 mL) of prepared 1-octanol solutions of DPhPMPO and distilled water were vigorously mixed at 37 °C during 1 h, and the two phases were separated. K_p was measured as absorbance ratios of DPhPMPO in 1-octanol compared to that in water.

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