The Application of ESR Spin-Trapping Technique to the Evaluation of SOD-*like* Activity of Biological Substances

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The SOD-*like* activity of several biological substances was evaluated by an ESR spin-trapping technique. Superoxide radicals ($O_2^{\cdot-}$) were supplied enzymatically from a hypoxanthine–xanthine oxidase reaction to the evaluating system. By using a spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), the generated $O_2^{\cdot-}$ was trapped stoichiometrically (1:1) as the spin adduct of $O_2^{\cdot-}$ (DMPO–O_2^-). When biological substances were added to the system, a decrease in the ESR signal intensities of the adducts was observed. This phenomenon could be explained as being an inhibition of adduct formation, and related to the reactivity of added biological substances with $O_2^{\cdot-}$, called an SOD-*like* activity. By the method of kinetic competition with a 50% inhibitory dose (ID_{50}), the second-order rate constant for the reaction between $O_2^{\cdot-}$ and biological substance was determined. These rate constants can be used as a measure of the reactivity.

Superoxide radicals ($O_2^{\cdot-}$) are generated from molecular oxygen or hydrogen peroxide by an one-electron transfer reaction.[1] This radical induces various injuries to the surrounding organism.[2,3] Therefore, removing $O_2^{\cdot-}$ is probably one of the most effective defences of a living body against oxidative stress.[2,3] In 1969, McCord and Fridovich found superoxide dismutase (SOD) to be a scavenger of $O_2^{\cdot-}$.[4]

Superoxide radical generations and SOD activities in biological systems are commonly measured by optical spectrometry using cytochrome *c*, tetranitromethane, epinephrine, nitroblue tetrazolium (NBT), pyrogallol, NADH+lactate dehydrogenase (LDH), ascorbate, hydroxylamine, and 6-hydroxydopamine.[4–10] However, there are some questions about these methods: the low selectivity against $O_2^{\cdot-}$ and/or an obstruction by the coexistence of insoluble particles and colored impurities, such as chromoproteins.[2,3]

Superoxide radicals are also detected at low temperatures by ESR spectrometry using a rapid-freezing technique or at room temperature by a spin-trapping technique. Especially, the spin-trapping technique[11] is useful for discriminating trapped radical species, and various short-lived radical intermediates are identified by this technique.[12] In the process of developing this technique, many workers have reported reactions between specific radical species and their scavengers.[13–17] Recently, part of these studies was applied to an assay of SOD activity.[18,19] This SOD assay method can be used to analyze crude samples without any purification, since the color and turbidity do not affect the measurements.[19]

There have been some reports that coexisting substances, such as L-ascorbic acid or celuroplasmin, hinder accurate measurements of SOD activity.[2] This means that they have an SOD-*like* activity in playing important roles, namely, biological defences against $O_2^{\cdot-}$.[8,20] On the other hand, it has been reported that in competition reactions for $O_2^{\cdot-}$ between 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and SOD, the formation of the adduct (DMPO–O_2^-) was suppressed by SOD, and the second-order rate constant of DMPO, itself, was estimated from the inhibitory effect of SOD.[13,14] Here, we expand this method to evaluate the SOD-*like* activity of various biological substances.

Experimental

Materials. A nitrone spin trap [5,5-dimethyl-1-pyrroline *N*-oxide (DMPO, Mitsui Toatsu Chemicals)], a chelator for trace metal impurities [diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DETAPAC, Wako Pure Chemical)], a superoxide radical source [hypoxanthine (HPX, Sigma Chemical) and xanthine oxidase (XOD, Boehringer Mannheim, cow milk)], and a primary standard of spin concentration [4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL, Sigma Chemical)] were used.

Radical scavengers [copper–zinc superoxide dismutase (Cu,Zn–SOD, Boehringer Mannheim, bovine erythrocyte), manganese superoxide dismutase (Mn–SOD, Sigma Chemical, *Escherichia coli*), iron superoxide dismutase (Fe–SOD, Sigma Chemical, *Escherichia coli*), ceruloplasmin (Alpha Therapeutic, human serum), ascorbate oxidase (Toyobo, type III, cucumber), ferricytochrome *c* (Sigma Chemical, type VI, horse heart), peroxidase (Toyobo, type I-C, horseradish), catalase (Boehringer Mannheim, type I, beef liver), and L-ascorbic acid (Daiichi Pure Chemical)] were also used. The other chemicals used were of the highest grade commercially available.

Instruments. ESR spectra were recorded on a JEOL JES-RE1X spectrometer using an aqueous quartz flat cell (a JEOL LC-12 ESR cuvette, inner size 60 mm×10 mm×0.31 mm, effective volume 160 μl). Optical absorption spectra were measured by an Otsuka Electronics MCPD-100 multi-channel photodetector.

Preparation of Samples. Superoxide radicals were generated from a hypoxanthine–xanthine oxidase (HPX–XOD) reaction system.

The sampling procedure was as follows: One hundred mM (1 M=1 mol dm^{-3}) sodium phosphate buffer solution (pH 7.8) was used for a solvent. A solution of 2.0 mM HPX (**a**),

5.5 mM DETAPAC (**b**), various concentrations of biological substance (**c**) and 0.33 unit/ml XOD (**d**) was prepared before use. The XOD solution was stored in an ice bath so as to prevent any inactivation of enzyme.

Fifty μl of **a**, 35 μl of **b**, 50 μl of **c** (or solvent) and 15 μl of DMPO were put into a test tube. To the mixed solution, 50 μl of **d** was added. After quick stirring, 200 μl of the mixture was taken into a flat cell. The mixture contained 0.50 mM HPX, 0.96 mM DETAPAC, 0.67 M DMPO,[21] 0.083 unit/ml XOD and an adequate concentration of the biological substance.

Measurements. The enzyme activity of Cu,Zn-, Mn-, and Fe-SOD was calibrated by the method of McCord and Fridovich.[4] The superoxide radical generation in our system was confirmed by the reduction of ferricytochrome *c* using the absorbance change at 550 nm.[22]

A quantitative analysis of DMPO-O_2^- by ESR spectrometry was performed under the following conditions for obtaining a high reproducibility of the spin adduct yields: Recording of the ESR spectrum started 40 s after the addition of XOD. The recording rate was 5 mT min^{-1}. After recording, the signal intensity of the lowest field peak of the spectrum (about 85 s after the addition of XOD) was normalized as a relative height against the standard signal intensity of the manganese oxide marker (MnO). An absolute concentration of DMPO-O_2^- was finally determined by a double-integration of the ESR spectrum. One μM TEMPOL solution was used for a primary standard of ESR absorption.

Results and Discussion

Reactivity of Several Biological Substances with O_2^-. When DMPO was added to a solution of the HPX-XOD reaction system, the spin adduct, DMPO-O_2^-, was formed.[13] Figure 1(a) shows a typical ESR spectrum of DMPO-O_2^- obtained under controlled conditions. Hyperfine coupling constants of the signal were analyzed as one nitrogen, a_N=1.41 mT, one hydrogen of β-position, $a_{H\beta}$=1.14 mT, and one hydrogen of γ-position, $a_{H\gamma}$=0.13 mT.[12] At the same time, a small amount of the hydroxyl radical adduct (DMPO-OH, $a_N=a_{H\beta}$=1.48 mT) was observed.[13,16] When Cu,Zn-SOD of various concentrations was added to the system, the signal intensities of DMPO-O_2^- decreased with an increase in the SOD concentration, as shown in Fig. 1(b) to (e). This phenomenon suggests that the reaction between O_2^- and DMPO is inhibited by Cu,Zn-SOD. According to Finkelstein et

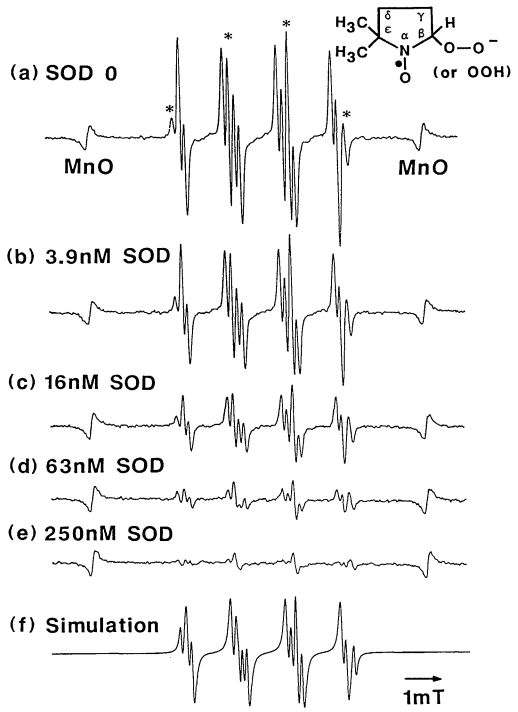

Fig. 1. ESR spectra of DMPO-O_2^- formed from the HPX-XOD system. The medium contained 0.50 mM HPX, 0.96 mM DETAPAC, 0.67 M DMPO, 0.083 unit/ml XOD, various concentrations of Cu, Zn-SOD and 100 mM sodium phosphate at pH 7.8, 23°C. A small amount of DMPO-OH (*) was found in the spectra. Modulation amplitude was 0.05 mT (100 kHz), recording range 10 mT, recording time 2 min, time constant 0.1 s, microwave power 8 mW (9.414 GHz). Simulation spectrum consisted of 90% DMPO-O_2^- and 10% DMPO-OH.

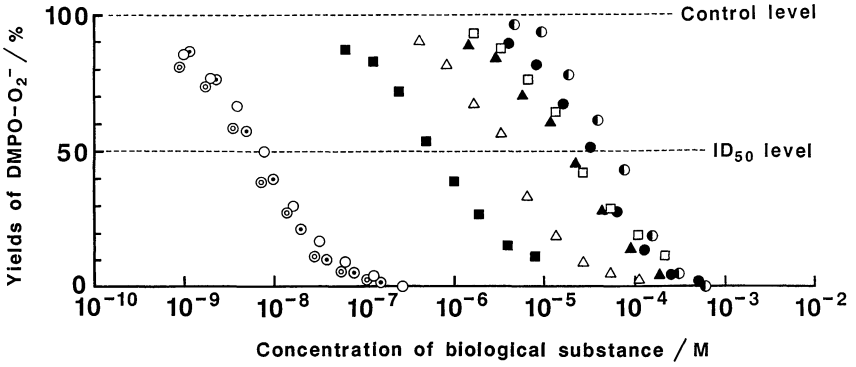

Fig. 2. Inhibitory effect of various biological substances on the formation of DMPO-O_2^-: Cu,Zn-SOD (○), Mn-SOD (⊙), Fe-SOD (◎), ceruloplasmin (□), ascorbate oxidase (△), ferricytochrome *c* (●), peroxidase (■), catalase (▲), and L-ascorbic acid (◐).

al.,[13,14] this phenomenon is a competition reaction between DMPO and SOD for $O_2^{\cdot-}$.

A phenomenon similar to the addition of Cu,Zn-SOD occurred widely in the addition of various biological substances such as Mn-SOD, Fe-SOD, ceruloplasmin, ascorbate oxidase, ferricytochrome *c*, peroxidase, catalase, and L-ascorbic acid. Figure 2 shows the relationship between the signal intensities of the DMPO-O_2^- observed and the concentrations of the biological substances added. Among the biological substances, the patterns of inhibitory effects were similar, but the effective concentration ranges were different.

The inhibitory effect of the biological substances was in the order: SOD > peroxidase > ascorbate oxidase > catalase ≧ ceruloplasmin ≧ ferricytochrome *c* ≧ L-ascorbic acid.

Stoichiometry between Generated $O_2^{\cdot-}$ and Formed Spin Adduct. When DMPO of various concentrations was added to the HPX-XOD system, the signal intensities of DMPO-O_2^- increased with an increase in the DMPO concentration when it was less than 0.5 M and were independent of the DMPO concentration when it was more than 0.5 M. Figure 3 shows the relationship between the concentrations of DMPO-O_2^- formed and DMPO added. The figure indicates that the DMPO concentration shown in Fig. 1(a) (0.67 M, the control condition, see Experimental) was at a saturating level for generated $O_2^{\cdot-}$. This observation indicates that almost all $O_2^{\cdot-}$ is trapped by DMPO under the conditions specified in Fig. 1(a).[13,14]

This result was compared with the reduction rate of ferricytochrome *c*. Figure 4 shows the reduction rate of ferricytochrome *c* (▲) when 0.53 mM ferricytochrome *c* was added to the HPX-XOD system instead of 0.67 M DMPO. The concentration of DMPO-O_2^- (●) measured under the conditions specified in Fig. 1(a) was overlapped on the plots. In the experiment, the concentration of ferricytochrome *c* was sufficient for scavenging $O_2^{\cdot-}$, because 0.53 mM ferricytochrome *c* can almost suppress adduct formation, as shown in Fig. 2. This fact means that the reduction rate of ferricytochrome *c* by $O_2^{\cdot-}$ is nearly equal to the generation rate of $O_2^{\cdot-}$ of the system.[23] In the figure, the concentrations of reduced cytochrome *c* (ferrocytochrome *c*) and formed DMPO-O_2^- increased to 18 μM after about 90 s, and the time course of DMPO-O_2^- within the period also agreed with that of ferrocytochrome *c*.

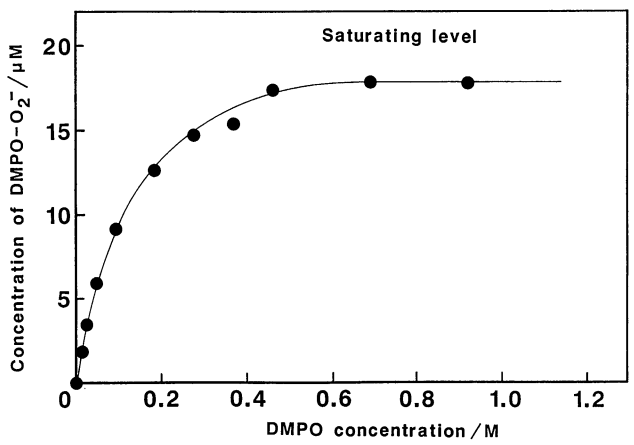

Fig. 3. Yields of DMPO-O_2^- as a function of DMPO concentration in the HPX-XOD reaction system.

Thus, the correlation between the concentrations of formed ferrocytochrome *c* and formed DMPO-O_2^- clearly showed that: (1) the $O_2^{\cdot-}$ generated from the HPX-XOD system changed rapidly and stoichiometrically (1:1) into the DMPO-O_2^- under this experimental condition, and (2) a kinetic study of generated $O_2^{\cdot-}$ using the signal intensities of DMPO-O_2^- is possible at an early stage of the reaction.

Evaluation of Second-Order Rate Constant for the Reaction between $O_2^{\cdot-}$ and Biological Substance. Based on the obtained results, we modified the kinetic competition models[8,13,14,19,23] for our experiments. It is useful to consider the first step of the scavenging reaction of $O_2^{\cdot-}$ as a contact reaction between $O_2^{\cdot-}$ and its reactant. Assuming that the contact reaction of $O_2^{\cdot-}$ to DMPO or scavenger (S) is of second-order, the first step of each reaction can be described as

$$O_2^{\cdot-} + \text{DMPO} \xrightarrow{k_1} \text{DMPO-}O_2^- \quad (1)$$

and

$$O_2^{\cdot-} + \text{S} \xrightarrow{k_2} \text{S-}O_2^-, \quad (2)$$

where k_1 and k_2 are second-order rate constants for

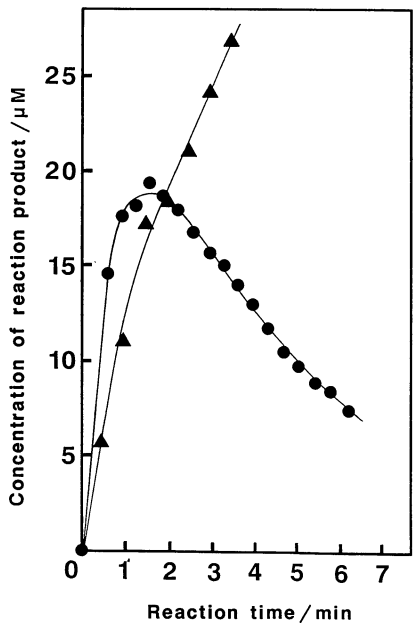

Fig. 4. Time course of product formation in the HPX-XOD reaction system: Ferrocytochrome *c* (▲) and DMPO-O_2^- (●).

reactions 1 and 2, respectively. Then, the initial rate of each reaction is given by

$$\frac{d[DMPO\text{-}O_2^-]}{dt} = k_1 \cdot [DMPO] \cdot [O_2^-] \quad (3)$$

and

$$\frac{d[S\text{-}O_2^-]}{dt} = k_2 \cdot [S] \cdot [O_2^-]. \quad (4)$$

A spontaneous disproportionation of O_2^- often causes a scavenger-independent decrease of DMPO-O_2^-. In our experiments, however, such a decrease was insignificant. The superoxide-scavenging effect caused by an added scavenger has its main contribution to the intensity change of DMPO-O_2^-, since the DMPO concentration (0.67 M) is sufficiently high to trap almost all of the O_2^- under our experimental conditions (as discussed in the preceding section). A similar consideration was first reported by Sawada and Yamazaki.[23] Thus, when reactions 1 and 2 compete with each other, the reaction rate in the initial stage can be expressed simply by

$$\frac{d[DMPO\text{-}O_2^-]}{dt} : \frac{d[S\text{-}O_2^-]}{dt} = (1 - X) : X, \quad (5)$$

where variable X ($0<X<1$) is the ratio of O_2^- consumption by the added scavenger. From Eqs. 3, 4, and 5, the following equation can be derived:

$$k_2 = k_1 \cdot \frac{X}{(1 - X)} \cdot \frac{[DMPO]}{[S]}. \quad (6)$$

In the case of 50% inhibition (X=0.5), a conventional measure of the inhibition, ID_{50} (inhibitory dose-fifty), can be used.[4-6,9,10,15,24] Equation 6 can be simplified as

$$k_2 = k_1 \cdot \frac{[DMPO]}{ID_{50}}. \quad (7)$$

In the above equation, [DMPO] and ID_{50} are the experimental values, and k_1 at pH 7.8 is obtained from Ref. 14 to be 18 M^{-1} s^{-1}. Therefore, by using [DMPO], ID_{50} and k_1 as known values, we can estimate an approximate value of unknown k_2.

On the other hand, ID_{50} can be related to [S] by using variable X (see Eqs. 6 and 7) as

$$[S] = \frac{X}{(1 - X)} \cdot ID_{50}, \quad (8)$$

where $X/(1-X)$ means the ratio of the competition between scavenger (S) and the spin trap (DMPO). In addition, from the definition of variable X, the spin concentration of DMPO-O_2^- in the early stage of the reaction is written by

$$I = (1 - X) \cdot I_0, \quad (9)$$

where I and I_0 are the spin concentrations of DMPO-O_2^- in the presence and absence of a scavenger, respectively. The I_0 is also a constant peculiar to the superoxide generating system.

Based on Eqs. 8 and 9, the inhibitory effect of biological substances can be simulated by computer. When simulations were carried out, I and [S] were

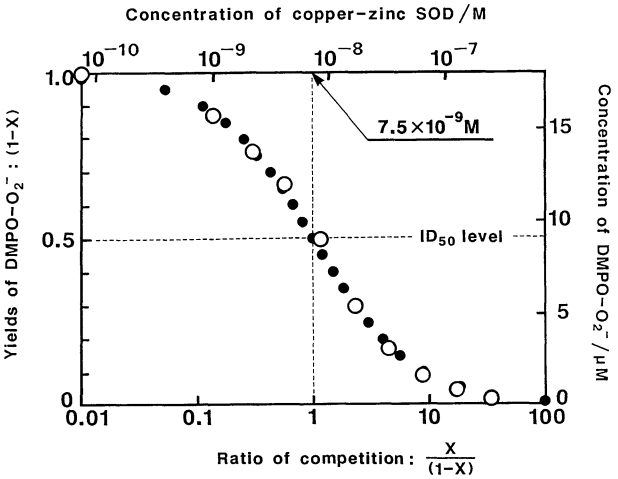

Fig. 5. Inhibitory effect of Cu,Zn-SOD on the formation of DMPO-O_2^-: Experimental (○) and simulated (●) values, where I_0 and ID_{50} used were 1.8×10^{-5} M and 7.5×10^{-9} M, respectively.

Table 1. ID_{50} and Second-Order Rate Constants of Various Biological Substances

Substance	ID_{50}	ID_{50}[b]	k_2	k_{ref}	pH_{ref}	Ref.
	M	mg ml^{-1}	M^{-1} s^{-1}	M^{-1} s^{-1}		
Cu,Zn-SOD[a]	7.5×10^{-9}	0.00024	1.6×10^{9}	ca. 2×10^{9}	5—9.5	25,26
Mn-SOD[a]	6.5×10^{-9}	0.00026	1.9×10^{9}	1.8×10^{9}	7.8	24
Fe-SOD[a]	5.1×10^{-9}	0.00020	2.4×10^{9}	1.6×10^{9}	7.8	24
Ceruloplasmin[a]	1.5×10^{-5}	2.0	8.1×10^{5}	3.04×10^{5}	7.8	27
Ascorbate oxidase[a]	2.5×10^{-6}	0.35	4.8×10^{6}			This work
Ferricytochrome c[a]	2.7×10^{-5}	0.33	4.5×10^{5}	6.2×10^{5}[c]	7.8	28
Peroxidase[a]	5.0×10^{-7}	0.020	2.4×10^{7}	1.6×10^{6}[d]	7—8.8	29
Catalase[a]	1.3×10^{-5}	3.0	9.6×10^{5}	ca. 2×10^{5}	7.4	30
L-Ascorbic acid	3.4×10^{-5}	0.0060	3.5×10^{5}	2.7×10^{5}	7.4	8

a) The following molecular weights were used: Cu,Zn-SOD (32000), Mn-SOD (40000), Fe-SOD (39000), ceruloplasmin (134000), ascorbate oxidase (140000), ferricytochrome c (12400), peroxidase (40000), and catalase (240000). b) The molarity of ID_{50} was calculated from this value. c) Calculated from the data of Ref. 28. d) Rate constant for the reaction between horseradish peroxidase Compound I and O_2^-.

replaced by $(1-X)\cdot I_0$ and $\{X/(1-X)\}\cdot ID_{50}$, respectively. The I_0 and ID_{50} were fixed at suitable values which led to a best fit to the experimental data. Simulation values were calculated by changing variable X from 0.01 to 0.99 at intervals of 0.05. Figure 5 shows both simulated (●) and experimental (○) values of Cu,Zn-SOD. Both values agreed well and the validity of our treatment was confirmed. By the simulation, the ID_{50}'s of Cu,Zn-SOD could be evaluated more exactly. The ID_{50}'s of the other substances were determined in the same manner.

Table 1 shows the ID_{50}'s, evaluated rate constants (k_2) and reported rate constants (k_{ref}) of several biological substances, where k_{ref} of Refs. 25—30 were the values obtained directly by a pulse radiolysis method; the others were indirectly obtained by the kinetic competition method. In the table, the rate constants of Cu,Zn-SOD, Mn-SOD, Fe-SOD, ceruloplasmin, ferricytochrome *c*, peroxidase, catalase and L-ascorbic acid agree with the reported values within one order of magnitude.[31]

Thus, we concluded that: (1) the original k_1 in Ref. 14 was really an exact value; (2) our method gave reasonable values as k_2; (3) the concentration of DMPO essentially did not affect the reactivity of above biological substances; (4) there was no reference of the reactivity between ascorbate oxidase and $O_2^{\cdot-}$, but the ascorbate oxidase showed a strong reactivity with $O_2^{\cdot-}$, and (5) these rate constants became a good measure of the reactivity.

In general, the ESR spin-trapping technique could scarcely be used for a kinetic investigation of the reactivity between an activated short-lived radical intermediate and its scavenger. This new kinetic analysis technique will be applied to an investigation of the SOD-*like* function of drugs.

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31) The disagreement of our values with the reported ones is seemed to be due to the difference between two superoxide generating methods (a hypoxanthine-xanthine oxidase reaction and a pulse radiolysis). It has been reported that the pulse radiolysis of water (usually containing sodium formate to be a scavenger of HO·) generated a variety of activated products, such as e_{aq}^-, H·, HO·, H_2, H_2O_2, $O_2^{\cdot-}$, and $CO_2^{\cdot-}$.[25,28,29] According to some reports, the $CO_2^{\cdot-}$ reduced the type 1 copper(II) of ceruloplasmin to the univalent state,[27] and the H_2O_2 react with catalase to form Compound I.[30] On the other hand, the hypoxanthine-xanthine oxidase system often produces HO· adduct of DMPO.[13,16] So, such side reactions may affect the reaction of biological substances with $O_2^{\cdot-}$.