Quantitative Spin-trapping ESR Investigation on Reaction of Hydroxyl Radical and Selected Scavengers by a Newly Developed Flow-injection ESR System

Rushiru Komatsu-Watanabe,[1] Yasuhiro Sakurai,[1] Chie Morimoto,[2] Shunji Sakamoto,[3] Kenji Kanaori,[1] and Kunihiko Tajima*[1]
[1]*Department of Biomolecular Engineering, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585*
[2]*Faculty of Pharmaceutical Science, Yasuda Women's University, Asaminami-ku, Hiroshima 731-0153*
[3]*Department of Nutritional Science, Yasuda Woman's University, Asaminami-ku, Hiroshima 731-0153*

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A new flow-injection ESR system (FI-ESR) special for the quantitative spin-trapping ESR measurements is developed to estimate the second-order rate constants (k_s) of the reaction between hydroxyl radical and selected scavengers, glycine, mannitol, and 4-hydroxycoumaric acid. The k_s values of these scavengers show reasonable agreement with those evaluated by pulse radiolysis method.

Spin-trapping ESR is a technique used to identify reactive oxygen species (ROS) generated in biological, medical, and chemical reaction systems.[1] A cyclic 5-membered nitrone, 5,5-dimethyl-1-pyrroline 1-oxide (DMPO), has been well established to be the most useful spin trap reagent for hydroxyl and superoxide radicals.[2] The spin-trapping technique using DMPO has frequently been applied to estimate the rate constants of ROS scavenging molecules,[3,4] however, the accuracy of ESR is still insufficient for the kinetic investigations.

Recently, several types of flow-ESR techniques, such as a stopped-flow-[5] and a flow-injection-ESR (FI-ESR),[6,7] have been utilized for quantitative analysis of radical reactions. It becomes clear that a flow-ESR cell fixed in the cavity successfully improves the reproducibility of ESR spectrometer. In the present study, attempts are made to develop FI-ESR system special for the spin-trapping ESR measurements focusing on detection of hydroxyl radical adduct of DMPO (DMPO/OH). This system is applied for estimation of the second-order rate constants (k_s) of the reaction of hydroxyl radical and selected scavengers, glycine (Gly),[8] mannitol (Man),[9] and 4-hydroxycoumaric acid (4CA).[10] The k_s values were obtained by means of the competitive kinetic treatment.[3,4] In comparison of k_s values, evaluated by the FI-ESR system and by pulse radiolysis method, the accuracy of this system will be discussed.

The FI-ESR was composed of an HPLC pump (TOSOH), an injection valve (Rheodyne), and a flat-ESR cell (Wilmad). An external flow-UV-irradiation cell (JEOL, $50.0 \times 4.0 \times 0.2$ mm i.d.), exposed to UV light (Sanei Electronic, UV-203S) for photolysis of hydrogen peroxide, was incorporated upstream of the ESR cell. The FI-ESR system was assembled using an X-band ESR spectrometer (JEOL, TE-100), operating with modified IPRIT (JEOL) system. Hydrogen peroxide (Wako Pure Chemical), DMPO (Labotec), and 2,2,6,6-tetramethyl-4-hydroxylpiperidine-1-oxyl (TEMPOL, Sigma Aldrich) were used without further purification. NaH_2PO_4–Na_2HPO_4 buffer (pH7.4, 0.1 M, M = mol/dm^3) was used for both sample preparation and carrier solution. The concentration of DMPO was verified by extinction coefficient 7800 M^{-1} cm^{-1} at 228 nm.[11]

As depicted in inset of Figure 1, ESR signal ascribed to be the DMPO/OH radical ($g = 2.0046$, $a^N = a^H = 1.49$ mT)[3] was observed for the control solution, which was composed of DMPO (50.0 μM) and hydrogen peroxide (100 μM). Then, the magnetic field was adjusted at the peak top of the second line, FI-ESR signals were recorded by time sweep ESR mode.[12] When the control solution was injected (200 μL) to the continuous flow of carrier solution (1.0 mL/min), the time-dependent ESR signal due to DMPO/OH radical was recorded, as shown in Figure 1. The concentration of DMPO/OH radical (defined as $[DMPO/OH]_c$) was estimated to be 1.0 μM by using TEMPOL as a primary standard. In order to estimate 50% inhibitory doses (ID_{50}) of 4CA, for example, sample solutions of 4CA (5.0–200 μM) and control solution were reciprocally injected. The signal intensity of DMPO/OH radical concomitantly decreased with increase of the concentration of 4CA (Figure 1). This is attributed to the competitive reaction between DMPO and 4CA to hydroxyl radical. Similar FI-ESR measurements were continued for 4CA by adjusting $[DMPO]_0$ at 75, 100, and 150 μM.

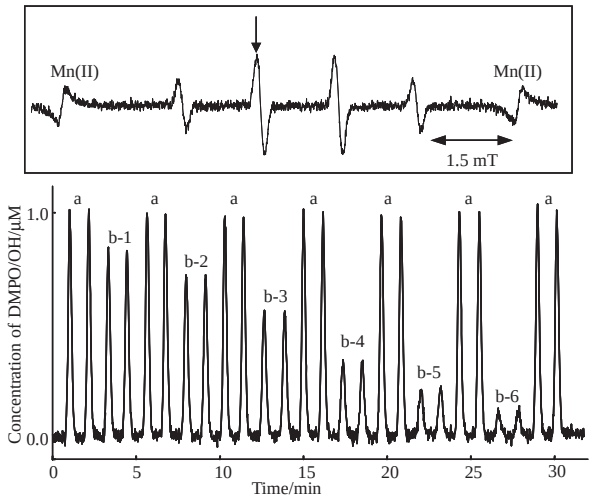

Figure 1. FI-ESR signals of DMPO/OH radical observed for duplicate injection of sample (200 μL) solutions to the continuous flow of carrier solution (pH 7.4, 1.0 mL/min); a) control solution containing DMPO (50 μM) and H_2O_2 (100 μM), b-1) sample solution containing 4CA 5.0 μM, b-2) 10.0 μM, b-3) 20.0 μM, b-4) 50.0 μM), b-5) 100 μM, and b-6) 200 μM. **Inset**, ESR spectrum of DMPO/OH radical. The downward arrow indicates the static field for FI-ESR measurements.

It is noted here that the signal intensity of the control solutions observed during 30 min for 14 injections showed excellent accordance. In the present system, the Ex-UV cell contributes to improve the stability of FI-ESR system. Since UV irradiation to the ESR cavity, which was the case of the trail FI-ESR stsystem,[7] causes a serious drift of ESR sensitivity probably due to the tem-

Table 1. The ID_{50}, $[DMPO]_0/ID_{50}$, γ_a, and k_s values of selected scavengers estimated by FI-ESR method

		$[DMPO]_0$							
		50[a]	75[a]	100[a]	150[a]	γ_a[b]	k_s[c]/$M^{-1}\,s^{-1}$	k[d]/$M^{-1}\,s^{-1}$	ref
Gly	ID_{50}[e]	8.60×10^3	1.22×10^4	1.59×10^4	2.56×10^4	—	—	—	
	$[DMPO]_0/ID_{50}$	5.8×10^{-3}	6.1×10^{-3}	6.3×10^{-3}	5.9×10^{-3}	5.8×10^{-3}	1.6×10^7	1.7×10^7	8
Man	ID_{50}[e]	1.26×10^2	1.69×10^2	2.06×10^2	2.95×10^2	—	—	—	
	$[DMPO]_0/ID_{50}$	4.6×10^{-1}	4.7×10^{-1}	5.0×10^{-1}	5.0×10^{-1}	5.3×10^{-1}	1.7×10^9	1.5×10^9	9
4CA	ID_{50}[e]	21.2	28.3	38.0	57.0	—	—	—	
	$[DMPO]_0/ID_{50}$	2.4	2.7	2.6	2.6	2.8	7.7×10^9	9×10^9	10

a) Initial concentration of DMPO in μM. b) Averaged $[DMPO]_0/ID_{50}$ value. c) Present study. d) Rate constants evaluated by pulse radiolysis. e) 50% inhibitory dose in μM.

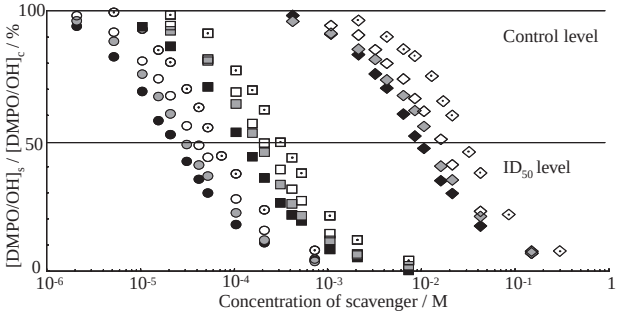

Figure 2. Inhibitory curves of Gly (◇), Man (□), and 4CA (○) obtained by adjusting $[DMPO]_0$ at 50.0 μM (black), 75.0 μM (gray), 100 μM (white), and 150 μM (dot).

perature change occurring in the ESR cavity.

Then, the concentration of DMPO/OH radical in the presence of scavenger ($[DMPO/OH]_s$) was also calibrated, and the ratio of $[DMPO/OH]_s/[DMPO/OH]_c$ was plotted against the concentration of 4CA. With increase of $[DMPO]_0$, the inhibitory curves showed stepwise shift to the right hand side (Figure 2). In addition, ID_{50} values were concomitantly increased from 21.2 to 57.0 μM, but the ratio of $[DMPO]_0$ and ID_{50} ($[DMPO]_0/ID_{50}$) seems to be almost constant (Table 1). In fact, plots of ID_{50} vs. $[DMPO]_0$ value (data not shown) gave a straight line. After a least-squares treatment, the slope of straight line is evaluated as 2.8, which corresponds to the averaged value of $[DMPO]_0/ID_{50}$ (defined as γ_a). These observations provide that eq 1 is applicable for estimation of k_s value of 4CA using γ_a and k_1 values.[4]

$$k_s = k_1[DMPO]_0/ID_{50} = k_1\gamma_a \quad (1)$$

Based on the reported k_1 value ($2.8 \times 10^9\,M^{-1}\,s^{-1}$), defined for the reaction between DMPO and hydroxyl radical,[13] k_s value of 4CA is evaluated to be $7.7 \times 10^9\,M^{-1}\,s^{-1}$ (Table 1), which is consistent with that estimated by the fast pulse radiolysis ($9 \times 10^9\,M^{-1}\,s^{-1}$).[10] On the other hand, Zang et al. reported k_s value of 4CA to be $1.8 \times 10^{11}\,M^{-1}\,s^{-1}$ by means of DMPO spin-trapping ESR applied to the Fenton reaction system.[14] This indicates that this method tends to overestimate k_s value unless the ESR measurements were conducted under the optimum reaction condition.

Similar FI-ESR measurements were carried out for Gly, and Man, so as to obtain the inhibitory curves (Figure 2). The line shape of the inhibitory curves was resembled to 4CA, but the ID_{50} values were characteristically different. By the same procedures to the case of 4CA, γ_a and k_s values were evaluated, as summarized in Table 1. The estimated k_s values showed reasonable agreement with those by the fast pulse radiolysis method. This result supports the adequacy for estimation of k_s value based on the experimentally determined γ_a values (eq 1). After the trial and error FI-ESR measurements by changing the $[DMPO]_0$, the initial concentration of hydrogen peroxide was optimized to 100 μM, thereby the $[DMPO]_0/ID_{50}$ value reached to constant. As the results, the $[DMPO/OH]_c$ was remarkably decreased to about 1.0 μM, which was close to the detection limit of the ESR spectrometer. By means of FI-ESR system, having the pronounced sensitivity and reproducibility, quantitative ESR analysis was precisely conducted for such a low concentration of DMPO/OH radical. Consequently, the newly developed FI-ESR system, designed for the quantitative spin-trapping ESR measurements, is demonstrated to be the reliable technique for kinetic investigation on the reaction between hydroxyl radical and naturally occurring scavengers.

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