High-Molar-Mass Hyaluronan Behavior During Testing Its Radical Scavenging Capacity in Organic and Aqueous Media: Effects of the Presence of Manganese(II) Ions

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This study compares the radical scavenging capacity of high-molar-mass hyaluronan (HA) using standardized methods applying 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals and 2,2′-azinobis[3-ethylbenzthiazoline sulfonate] (ABTS) radical cations as oxidants. Additionally, spin-trapping technique combined with electron paramagnetic resonance (EPR) was used to evaluate the ability of HA to scavenge reactive radicals. The thermal decomposition of $K_2S_2O_8$ in pure H_2O or in a H_2O/dimethyl sulfoxide (DMSO) mixture at 333 K was used as a source of reactive paramagnetic species. We found that HA does not exhibit radical-scavenging activity when DPPH radicals or $ABTS^{\bullet +}$ radical cations are used as oxidant, but that HA is an effective radical scavenger at low concentrations, if the oxidation reactions are initiated by the decomposition of $K_2S_2O_8$. At higher HA concentrations, a more complex behavior and prooxidant HA action was observed. The influence of Mn^{II} ions on the reaction mechanisms of radical generation and termination in the $K_2S_2O_8/H_2O$/DMSO system in the presence of HA was studied in detail.

Introduction. – A special case of antioxidant/prooxidant behavior of antioxidants emerges in the presence of metal ions [1][2], and consequently the antioxidant and radical scavenging of natural compounds in the presence of metal ions attracts a high interest. Organisms contain a variety of radical-scavenging antioxidants, which delay lipid peroxidation by reacting with chain-propagating peroxyl radicals faster than these radicals can react with proteins or fatty-acid side chains [3]. One effective method for measuring the ability of samples to scavenge free radicals in the presence of metal ions is based on electron paramagnetic resonance (EPR) spectroscopy applying the spin-trapping technique, as was recently shown for carotenogenic yeasts stressed by Cu^{II}, Ni^{II}, and Zn^{II} ions, where completely different reactive radicals were detected under thermal decomposition of $K_2S_2O_8$ in the presence of Cu^{II} ions in comparison to the presence of Ni^{II} and Zn^{II} ions [4].

Recently, by evaluating the dynamic viscosity (η) of the high-molar-mass hyaluronan (HA) aqueous solution in dependence on the presence of $CuCl_2$ and ascorbate, we observed a decrease of the η value of the HA solution, indicating degradation of the biopolymer by the system containing Cu^{II} cations [5][6]. Hyaluronan is a polysaccharide constituent of almost all tissues in vertebrate organisms and is one of the functionally most essential components of synovial fluid [7].

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Therefore, the reactions of high-molar-mass HA, occurring under oxidative conditions and/or inflammation, are of high interest, focusing especially on the attack of $HO^{\bullet}$ radicals on HA macromolecules. There are numerous reports confirming radical-scavenging properties of biomacromolecules, particularly proteins, where the importance of proteins in defense against oxidation was studied (see, *e.g.*, [8–12]).

In the present study, we investigated the high-molar-mass HA behavior in tests covering determination of radical-scavenging activity in organic and aqueous media in the presence of Mn^{II} ions in order to follow the complex reaction mechanism of HA in the presence of reactive radicals and metal ions.

Results and Discussion. – The results from the two most common assays for determination of radical-scavenging capacity of samples on the basis of scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals and 2,2′-azinobis[3-ethylbenzthiazoline sulfonate] radical cations ($ABTS^{\bullet +}$) are shown in *Fig. 1*. In ABTS assay, the decrease in absorbance was followed to monitor the consumption of colored $ABTS^{\bullet +}$ radical cations, and absorbance changes at 730 nm were taken as a measure of scavenging activity. *Fig. 1,a*, shows a representative experiment for the sample HA (100 μl, 1 mM) plus $ABTS^{\bullet +}$ radical cation solution (2 ml). The UV/VIS spectra were recorded in 1-min intervals for 10 min. The UV/VIS spectrum of initial $ABTS^{\bullet +}$ radical-cation solution recorded against distilled H_2O was taken as a reference spectrum (black line in *Fig. 1,a*). The difference in the absorbance in the 10th min at 730 nm relative to the reference spectrum, ΔA, was used to calculate the percentage of scavenged $ABTS^{\bullet +}$ radical cations by the HA sample. It should be mentioned here that addition of the HA sample to aqueous $ABTS^{\bullet +}$ radical-cation solution led to a change of baseline due to turbidity effects. These effects were eliminated mathematically by the baseline correction of all UV/VIS spectra. To achieve this, the absorbance at 470 nm was set to the same value as observed for the initial spectrum recorded before adding the HA sample to the assay. Turbidity effects disappeared using DPPH EtOH solution as illustrated in *Fig. 1,b*. As seen in *Fig. 1*, both ABTS and DPPH tests showed a negligible antioxidant activity. This indicates that no such effect as obviously seen for 'hydrogen supplying' antioxidants (as polyphenolics, phenol acids, *etc.*) [13], and for some proteins and their complexes with polyphenolics [8–12] was observed for high-molar-mass hyaluronan (HA). Serum albumin is, *e.g.*, known as an effective antioxidative protein in mammals. Recently the antioxidant activities of selected macromolecules, including β-lactoglobulin, α-lactalbumin, β-casein, and α-casein, were determined by the ABTS radical-scavenging assay [11]. It was shown that β-casein and α-casein exhibit radical-scavenging activities in aqueous solution, whereas bovine serum albumin (BSA), α-lactalbumin, and β-lactoglobulin showed much weaker antioxidant activity when assessed by the ABTS test. However, albumin, mannitol, and dehydrins effectively scavenge $HO^{\bullet}$ radical, $HOO^{\bullet}$ radical, superoxide anion radical, and H_2O_2 [8–10]. Therefore, different tests applying generation of reactive radicals and monitoring of their scavenging were used for HA in various media.

In these experiments in aqueous solutions, HA (200 μl, 1 mM) in distilled H_2O was mixed with 3,4-dihydro-5,5-dimethyl-2*H*-pyrrole *N*-oxide (DMPO; 25 μl, 0.2M) in DMSO and with $K_2S_2O_8$ (25 μl, 0.01M), and filled into an EPR flat cell. A time course of EPR spectra of the DMPO spin-adducts was recorded in 1-min intervals for 20 min at

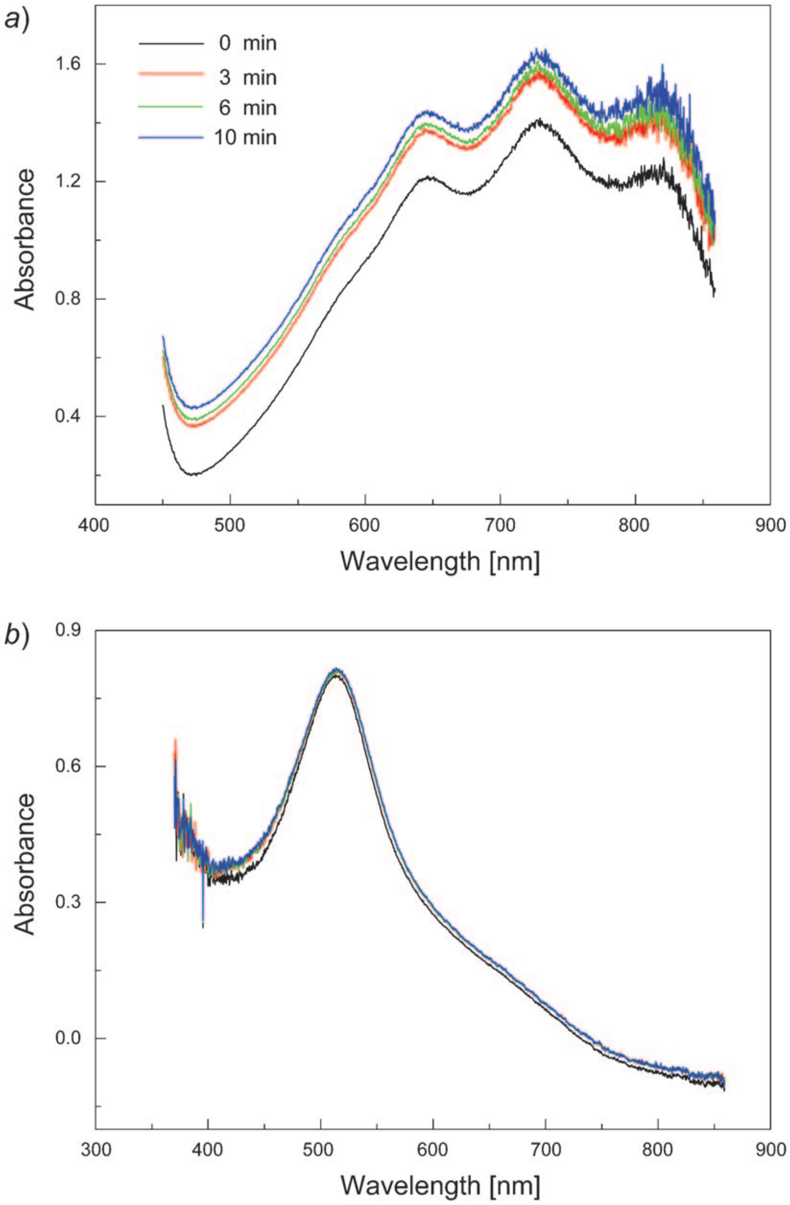

Fig. 1. a) *ABTS and* b) *DPPH tests for a 1-m*M *HA sample solution*

333 K (each spectrum was an accumulation of three scans). *Fig. 2* shows the time course of EPR spectra of DMPO adducts for the reference (200 μl distilled H_2O) and the HA sample. The difference between the integral intensities of the reference and the HA sample characterizes the amount of radicals scavenged by the compounds present in the corresponding sample. The value for the virtual conditions with all radicals scavenged

was set to 100% of the relative radical-scavenging capacity (RSC). The RSC value in the 20th min for the corresponding HA sample was determined to be 30% of scavenged radicals indicating medium reactivity of HA to HO$^•$ radicals.

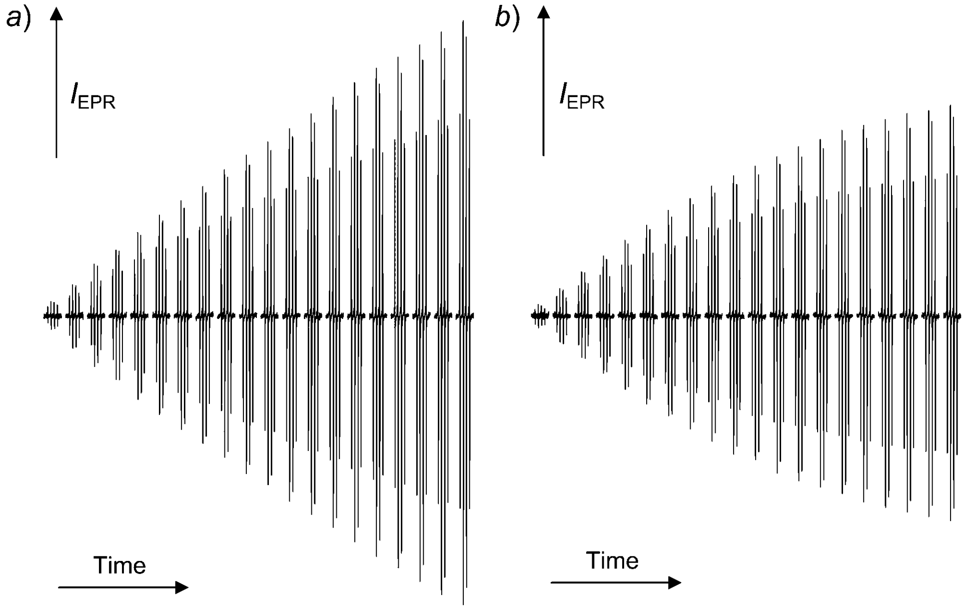

Fig. 2. *Time course of 20 EPR spectra of DMPO spin adducts for the reference* (*a*; 200 µl H_2O) *and HA sample* (*b*; 200 µl, 1 mM HA), *recorded during the first 20 min of the thermal decomposition of* $K_2S_2O_8$ *in the presence of DMPO spin trap at 333 K*

As seen in *Fig. 3*, a negligible radical-scavenging behavior of HA was observed in H_2O/DMSO, indicating low reactivity of HA to alkoxyl radicals. In this system, the EPR spectra with hyperfine splittings $a_N = 1.45$ mT and $a_H = 1.32$ mT fully dominate and correspond to DMPO/alkoxyl radical adducts formed by the reaction of HO$^•$ and $SO_4^{•-}$ radicals with neighborhood [4].

In our previous EPR spin-trapping experiments, we studied the influence of the presence of Zn^{II} and Ni^{II} ions on the time course of EPR signal of the reference sample [4]. As Mn^{II} ions are often present in synovial fluid as a trace metal, we investigated their influence on our system, including HA in different media. *Fig. 4* presents the comparison of the time evolution of the EPR intensity of EPR spectra for the reference sample in the absence or presence of Mn^{II} ions. In these experiments, either distilled H_2O (25 µl; *Fig. 4,a*) or aqueous solution of $MnCl_2$ (1 mM) was added to the standard reaction mixture containing DMSO (175 µl; *Fig. 4,b*). It is evident that the presence of Mn^{II} ions significantly decreases the quantity of radicals produced in the reaction system. Concerning the high reactivity of Mn^{II} to HO$^•$ radicals [14] and taking into account a possible reaction of Mn^{2+} with $SO_4^{•-}$ (*i.e.*, $SO_4^{•-} + Mn^{2+} \rightarrow Mn^{3+} + SO_4^{2-}$), the strong influence of Mn^{II} on the production of reactive radicals in our reaction system must have been mostly caused by the direct reaction of Mn^{II} with the reactive radicals

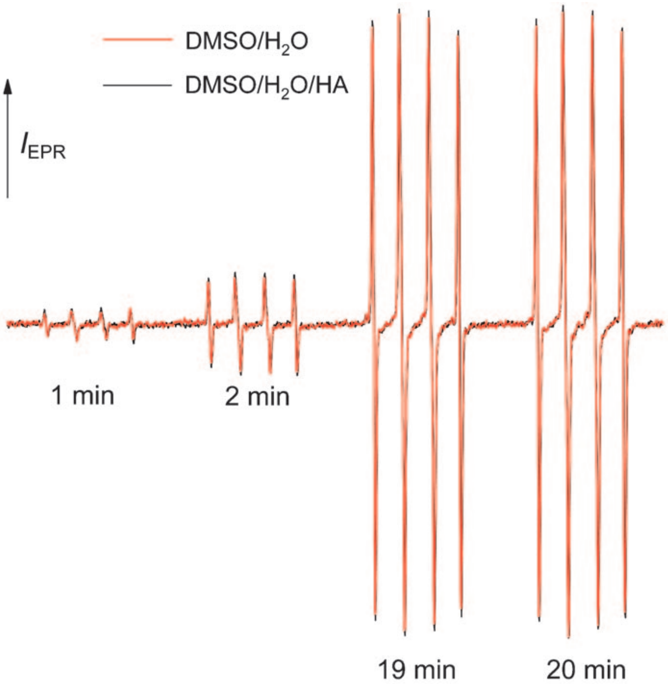

Fig. 3. *EPR Spectra of DMPO adducts for the reference* (DMSO/H_2O 1:1 (*v*/*v*)) *and HA* (1 mM) *samples, respectively.* Black lines: 100 µl H_2O + 100 µl DMSO, red lines: 100 µl 1 mM HA + 100 µl DMSO, recorded in the 1st, 2nd, 19th, and 20th min of the thermal decomposition of $K_2S_2O_8$ in the presence of DMPO spin trap.

generated in the reference system. An analogous reaction for Mn^{2+} ions in scavenging lipid peroxides ($LOO^•$) was proposed by *Coassin et al.* and *Sziraki et al.* [15–17]:

$$LOO^• + Mn^{2+} + H^+ \rightarrow LOOH + Mn^{3+}$$

A high effect of Mn^{II} ions was also observed for the HA samples in physiological solution, as illustrated in *Fig. 5*. A time course of EPR intensity (double integrals) of DMPO adducts for the reference system (200 µl, 0.15M NaCl) was substantially different compared to the concentrated HA sample in H_2O/NaCl solution (200 µl of HA, 2.5 mg HA/ml dissolved in 0.15M NaCl). Although the number of trapped radicals in the 20th min of the experiment was virtually the same, the kinetic behavior was different. The HA sample exhibited prooxidant behavior, and consequently a high increase of reactive radicals was found in the initial phase of the experiment. This indicates the concentration and environmental dependence of HA behavior concerning its radical-scavenging capability. At lower HA concentrations in pure H_2O solutions, an antioxidant behavior was observed (see *Fig. 2*). On the other hand, the presence of NaCl and application of higher HA content turned the antioxidant behavior to the prooxidant one. We propose that, in addition to the concentration dependence of

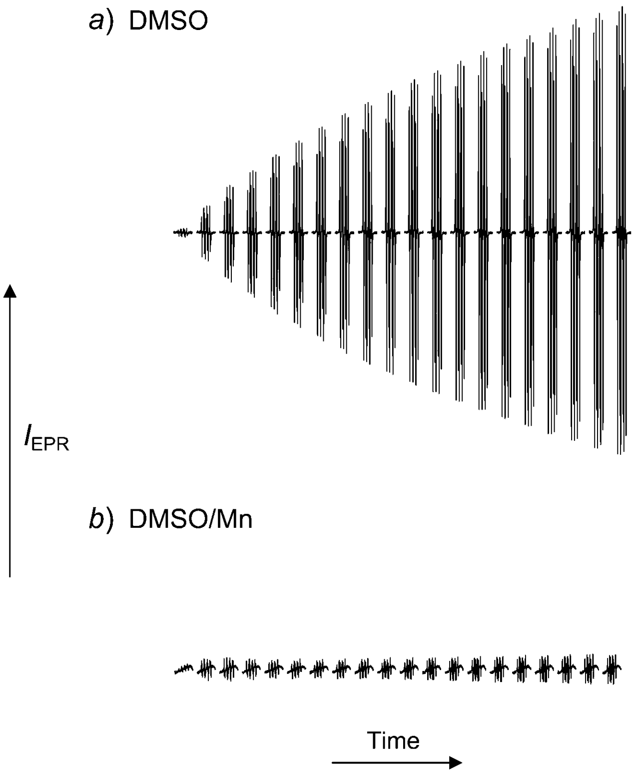

Fig. 4. *Time course of EPR spectra of DMPO adducts for the reference* (*a*; 175 μl DMSO + 25 μl H_2O) *and Mn* (*b*; 175 μl DMSO + 25 μl of 1 mM $MnCl_2$ in H_2O) *samples in DMSO/H_2O solutions*

radical-scavenging behavior of HA, the minor components or impurities (especially metal ions) play an important role in this case. The strong influence of Mn^{II}-ion presence both in the reference system (175 μl of 0.15M NaCl + 25 μl of 1 mM $MnCl_2$) and the HA sample in H_2O/NaCl (175 μl of HA, 2.5 mg HA/ml in 0.15M NaCl + 25 μl of 1 mM $MnCl_2$), demonstrates the special role of Mn^{II} ions as already observed in the field of lipid peroxidation. Mn^{II} Ions often take part in the complex reaction pathways of free radicals, and in many cases they substantially decrease their amount, thus increasing the radical-scavenging capability of the system.

Grants VEGA 2/0003/08 and 1/3579/06 of the *Slovak Academy of Sciences* and the *Ministry of Education of the Slovak Republic*, and APVV-51-017905, APVT-20-0045/04 from the *Slovak Research and Development Agency* are appreciated.

Experimental Part

Biopolymer and Chemicals. The high-molar-mass HA sample *Lifecore P9710-2A* from *Lifecore Biomedical Inc.*, Chaska, MN, USA (M_w = 808.7 kDa; M_w/M_n 1.63) was used. Anal. purity grade $MnCl_2 \cdot 4\,H_2O$ was from *Lachema*, Brno (CZ), and NaCl from *Slavus Ltd.*, Bratislava (SK). DMSO (UV-purity grade), spin-trapping agent DMPO were from *Sigma-Aldrich*. DMPO was distilled before use and stored

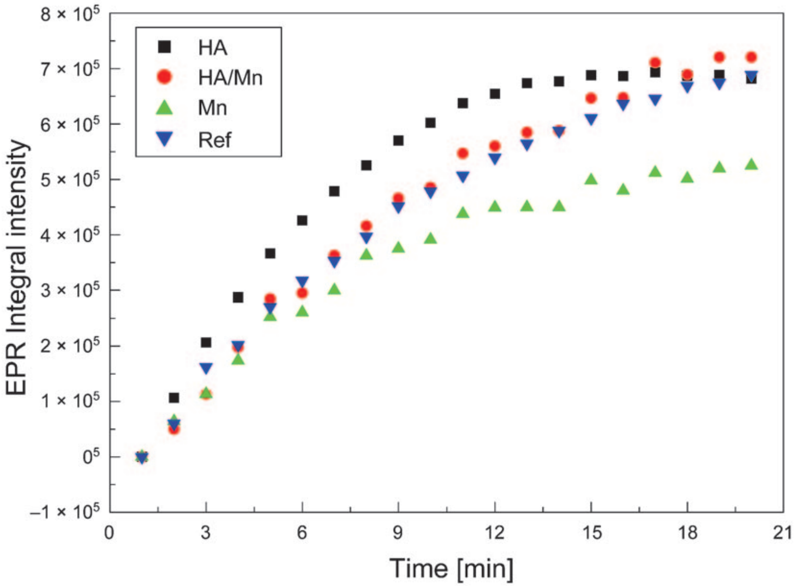

Fig. 5. *Time course of EPR spectra of DMPO adducts in different systems.* Ref: 200 µl of 0.15M NaCl; Mn: 175 µl of 0.15M NaCl + 25 µl of 1 mM $MnCl_2$; HA/Mn: 175 µl of HA (2.5 mg of HA/1 ml) in 0.15M NaCl + 25 µl of 1 mM $MnCl_2$; HA: 200 µl of HA (2.5 mg of HA/1 ml dissolved in 0.15M NaCl). The solutions of 25 µl of 0.2M DMPO in DMSO and 25 µl of 0.01M $K_2S_2O_8$ were added to all samples and filled into a EPR flat cell.

under Ar at −18°. Water used was of redistilled de-ionized quality grade. EtOH for UV/VIS spectroscopy was purchased from *Mikrochem*, Pezinok (SK). $K_2S_2O_8$ (*p.a.* purity, max 0.001% N, *Merck*, Germany), 2,2′-azinobis[3-ethylbenzthiazoline sulfonate] (ABTS; *purum*, >99%, *Fluka*, Germany), and 2,2-diphenyl-1-picrylhydrazyl (DPPH; 95%, *Aldrich*, Germany) were used as received.

Procedures. The HA soln. was prepared in two steps: first, NaCl (4 ml, 0.15M) was added to the *Lifecore P9710-2A* sample (20 mg), it became swollen, and, after 6 h, further NaCl (4 ml, 0.15M) was added and dissolved overnight in the dark at r.t. The HA soln. (2.5 mg/ml) was then diluted with dist. H_2O to the concentration of 1 mM. Radical-scavenging activity was measured according to a modified ABTS method [18] and DPPH test. To prepare $ABTS^{\bullet +}$ radical cations, an aq. soln. of $K_2S_2O_8$ (3.3 mg of $K_2S_2O_8$ in 5 ml of dist. H_2O) was added to ABTS (17.2 mg), and the resulting soln. was stored 14 h in the dark. Finally, the dark-green $ABTS^{\bullet +}$ radical cation soln. (1 ml) was diluted with dist. H_2O (60 ml) and used in ABTS tests. UV/VIS Spectra were recorded in 1-min intervals for 10 min using a *UV-VIS S2000* spectrophotometer (*Sentronic*, Germany) in 1-cm quartz UV cuvette after mixing the HA sample (100 µl) with $ABTS^{\bullet +}$ radical-cation soln. (2 ml). In the DPPH test, DPPH soln. (2 ml; 95% $DPPH^{\bullet}$; 120 mg of DPPH in 100 ml of 96% EtOH) was mixed with the HA sample (100 µl). In the reference sample, EtOH (1 ml) was used instead of HA soln., and all spectra were recorded against EtOH in 1-min intervals.

The thermal decomposition of $K_2S_2O_8$ in DMSO or in H_2O/DMSO at 333 K was used as a source of reactive radicals [19]. For measuring the radical-scavenging ability of the HA samples, the EPR/spin-trapping method employing the DMPO spin trap was used. The EPR measurements at the X-band were performed with a *Bruker EMX* EPR spectrometer equipped with a standard *TE102* (*ER 4102 ST*) rectangular cavity. Temp. control was achieved by using a *Bruker* temp. control unit *ER 4111 VT*.

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Received February 18, 2008