EPR Spin Trapping of Free Radicals with DMPO (5,5-Dimethyl-1-Pyrroline N-oxide) and BMPO (5-*Tert*-Butoxycarbonyl-5-Methyl-1-Pyrroline N-oxide)

Introduction

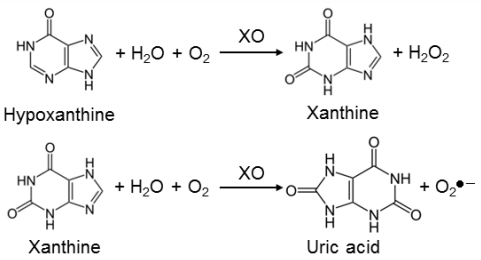
In general, the least ambiguous method for characterizing free radicals in chemistry, biology and medicine, is detection by EPR spectroscopy. However, direct detection of some free radicals (e.g., superoxide and hydroxyl radical) is very difficult or impossible in solution at room temperature. Spin trapping is a technique developed at late 1960s where a nitrone or nitroso compound reacts with a target free radical to form a stable and distinguishable free radical to be detected by EPR spectroscopy. The most popular spin trap is 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), which has been cited in Medline more than 1,000 times. DMPO has significant advantages over other nitrone spin traps. First, it is the most redox inactive. Second, common nitrone spin traps other than DMPO such as α-phenyl-*N*-*tert*-butylnitrone (PBN) and α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN) have ESR spectra of their radical adducts which show relatively little dependence on the structure of the trapped radical, whereas the assignment of DMPO radical adducts of small free radicals (O-, C-, N-, S-centered) can often be made from knowledge of the literature.

BMPO is a discovered nitrone spin trap from Dr. Kalyanaraman's lab at Wisconsin Medical College. It is most suitable for the specific *in vivo* or *in vitro* detection of short-lived superoxide, hydroxyl and thiyl radicals, by forming distinguishable adducts measurable with EPR spectroscopy. Other nitrone spin traps such as DMPO does not distinguish superoxide and hydroxyl radical easily because of spontaneous decay of DMPO-superoxide adduct ($t_{1/2}$ = 45 seconds) into the DMPO-hydroxyl adduct. BMPO-superoxide adduct does not decay into a hydroxyl adduct and has a much longer half-life ($t_{1/2}$ = 23 minutes). Also, BMPO-derived adducts exhibit a much higher signal-to-noise ratio in their EPR spectra, and it may be suitable for detection of sulfite, hydroxyl and methyl radicals in cell suspension. Additionally, the solid cyclic nitrone BMPO, highly purified by crystallization, can be handled and stored for extended periods of time without fear of decomposition.

Spin trapping of O-centered radicals by DMPO and BMPO with the Xenon software package

Superoxide

Oxygen-centered radicals are of particular interest because they have been implicated in many reactions in vivo. The EPR spin trapping of superoxide ($O_2^{\bullet-}$) with DMPO and BMPO is a widely used approach to study the production of $O_2^{\bullet-}$ in biological systems. The system xanthine/xanthine oxidase is commonly applied as a tool for generation of superoxide as well as a standard for the comparison of other sources of $O_2^{\bullet-}$ in EPR spin trapping experiments. Xanthine oxidase will oxidize hypoxanthine to uric acid; the electrons from this oxidation are passed to dioxygen to produce both H_2O_2 and $O_2^{\bullet-}$:



Unfortunately, the EPR detection of DMPO/$^{\bullet}$OOH is not without its pitfalls such as: interference of transition metals, short lifetime of DMPO/$^{\bullet}$OOH, reaction of $O_2^{\bullet-}$ with DMPO/$^{\bullet}$OOH and DMPO/$^{\bullet}$OH, and the reported possibility that DMPO/$^{\bullet}$OOH decays to form DMPO/$^{\bullet}$OH. The following experiment is used to verify the formation of superoxide- and hydroxyl radical adducts formation with DMPO (BMPO).

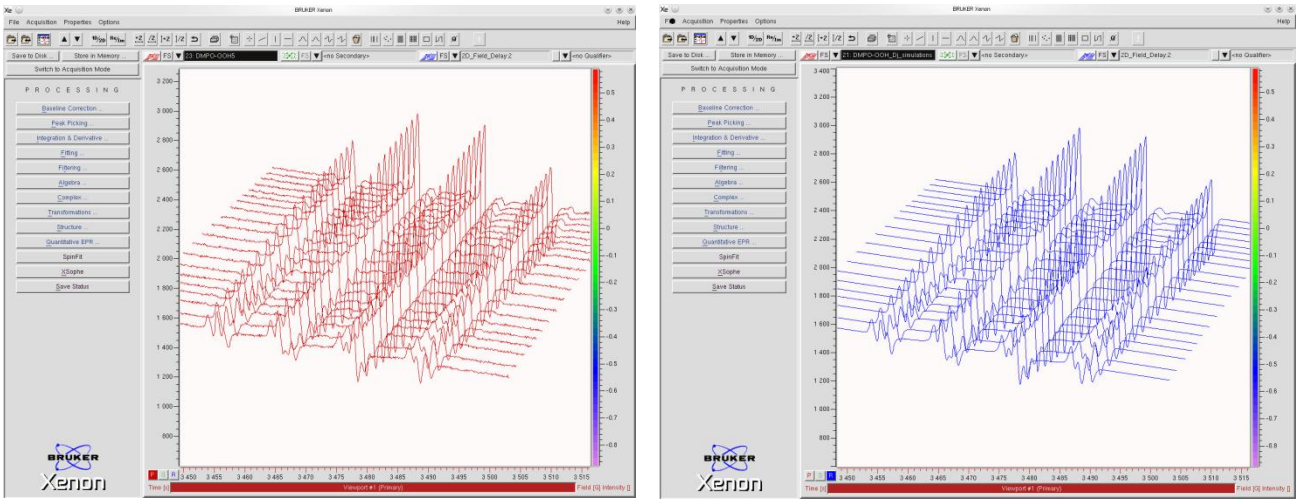
Experimental Protocol

1. Prepare a solution of 100 mM phosphate buffer (pH 7.4) containing 25 μM diethylenetriaminepentaacetic acid (DTPA) (Sigma) as transition metal chelator.
2. Make up a solution of 1 mM hypoxanthine (Sigma) in 100 mM phosphate buffer, pH 7.4.
3. Make up a solution of xanthine oxidase (Sigma) with concentration of 1 unit/ml
4. Make up a solution of DMPO (Dojindo) with concentration of 1 M. If you use BMPO (Dojindo) dissolve 10 mg of BMPO into 200 μl phosphate buffer (the final concentration should be 250 mM).
5. Prepare your reaction mixture to a total reaction volume of 200 μl. Add 70 μl of buffer to an Eppendorf tube. Add 20 μl DMPO of your 1 M DMPO solution (or 20 μl of your 250 mM BMPO stock) and 100 μl hypoxanthine of the stock 1 mM solution. Initiate the reaction with 10 μl xanthine oxidase, vortex the tube and transfer the solution to a flat cell. Insert the flat cell into the cavity, tune the spectrometer, and acquire the spectrum. The final concentrations of the components are: 100 mM DMPO (or 25 mM BMPO), 0.5 mM hypoxanthine, and 0.05 units/ml xanthine oxidase.

6. You should always perform control experiments in which one or more of the reagents are excluded. These experiments will reveal any paramagnetic impurities and will demonstrate that all the components were required to produce the EPR signal.

Spin trapping time course experiment

The spin trapping experiments were performed using DMPO or BMPO as spin traps. The formation of the radical adducts and their time evolution was monitored by a 2D experiment (B_0 vs. time) (Figure 1). After the experimental data were acquired each spectrum was simulated by SpinFit software to define the radicals adducts (Figure 2). The parameters can either be typed in or imported from the database. One fitting result is the area (double integral) for each adduct. This value is then used to calculate the adduct concentration with the spin counting module (Figure 3).



Figures 1 and 2. Experimental data (in red) and SpinFit simulations (in blue) of two sets of DMPO radical adducts at a given time in the 2D field versus time experiment.

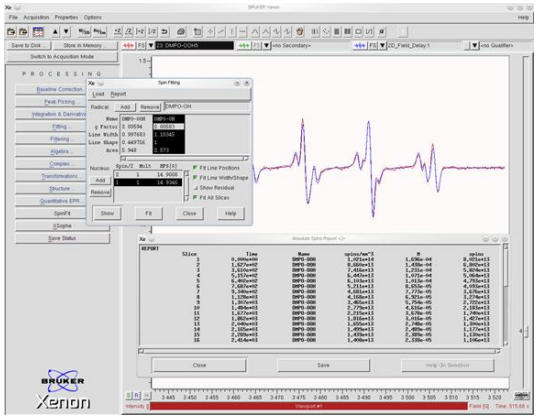
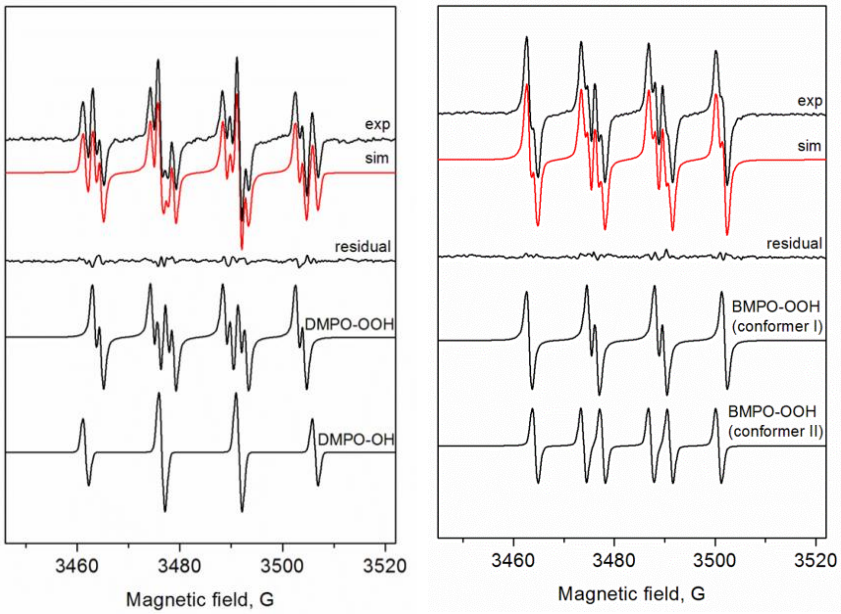
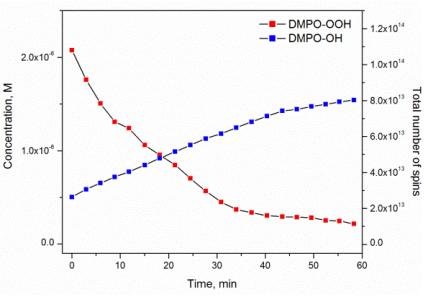


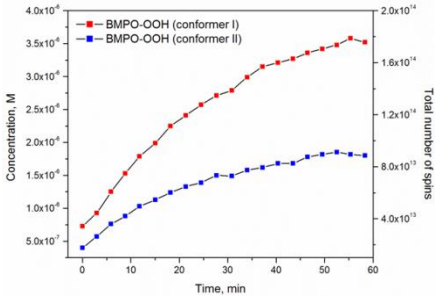
Figure 3. Defining the DMPO radical adducts in the SpinFit dialog by import from database or by manual entry. SpinCount provides a report of the fit species areas' during the time course of the experiment.

In the case of DMPO two spin adducts (DMPO-superoxide and DMPO-hydroxyl) were generated by the xanthine oxidase system (Figure 4). When BMPO was used two stereoisomers of BMPO/$^{\bullet}$OOH were formed without BMPO/$^{\bullet}$OH production (Figure 5). Outputs of the spin fitting routine are the fitted spectra (Figs. 4 and 5). Both figures show the experimental spin trap EPR spectra, the composite simulation, the fitted spectra of each radical adduct, and the residual at one particular time of the 2D experiment. The two DMPO adducts (Figure 4) were fitted with a_N = 14.2 G, a_H^{β} = 11.4 G, and $a_H^{\gamma 1}$ = 1.2 G for the DMPO/$^{\bullet}$OOH adduct and a_N = a_H^{β} =14.9 G for the DMPO/$^{\bullet}$OH adduct. The two BMPO/$^{\bullet}$OOH adducts were fitted with a_N = 13.4 G, a_H^{β} = 12.1 G for conformer I, and a_N = 13.4 G, a_H^{β} = 9.4 G for conformer II (Figure 5). The double integral values of the 2D spin fitting are used by the spin counting module to calculate the concentration of the DMPO- and BMPO- radical adducts (Figures 6 and 7).



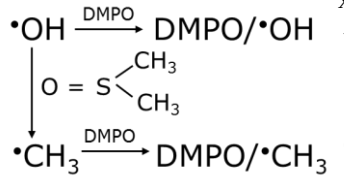
Figures 4 and 5. Resultant fits of two sets of species at a given time in the 2D field versus time experiment. The conditions in both panels were the identical except the choice of spin trap.





Figures 6 and 7. After feeding the results of SpinFit into SpinCount, the concentration changes over the time of the experiment are obtained.

In any case, to establish the existence of free hydroxyl radical in spin trapping experiments, it is necessary to perform kinetic-based competition experiments with hydroxyl radical scavengers. For example, dimethyl sulfoxide, ethanol, and formate can be used in these competition experiments because, upon hydroxyl radical attack, they form carbon-centered radicals that can subsequently be trapped by DMPO. The following experiment is used to study the origin of the hydroxyl radical in xanthine oxidase system. Follow the steps 1-6 from the described experiments above except perform the reaction in 10% DMSO (i.e. add 20 µl of DMSO to the reaction mixture before adding the other reagents.). The obtained spectrum (Figure 8) exhibited a negligible trace of DMPO/•OH signal, while it contained features corresponding to DMPO/•CH_3 (a_N = 16.4 G, a_H^β = 23.3 G), which was confirmed by SpinFit simulations. The above observations suggest that the majority of DMPO/•OH signal observed in the absence of DMSO originates from the trapping of •OH radicals and not from DMPO/•OOH break down.



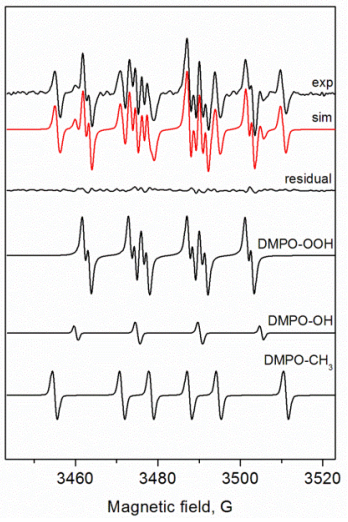


Figure 8. EPR spectrum of the DMPO radical adducts formed in xanthine-xanthine oxidase system in the presence of 10% DMSO.

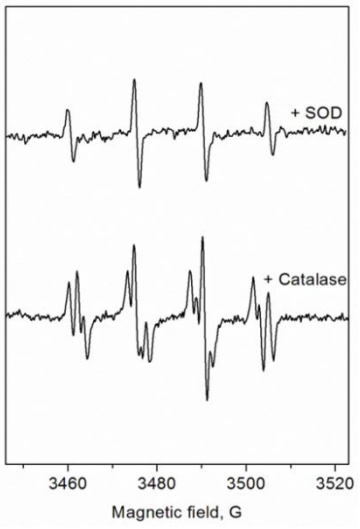
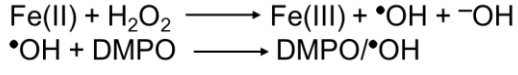


Figure 9. EPR spectra of the DMPO radical adducts formed in xanthine-xanthine oxidase system in the presence of 1000 units/ml SOD (the top spectrum) and 1000 units/ml catalase (the bottom spectrum).

Another approach to study the possible involvement of either superoxide or the DMPO/•OOH adduct in the generation of hydroxyl radicals or DMPO/•OH, experiments can be performed in the presence of superoxide scavenging enzyme superoxide dismutase (SOD) added before initiation of the reaction (Figure 9). The top spectrum in Fig. 9 was taken immediately after adding xanthine oxidase. As expected SOD totally scavenged the superoxide radicals, and only DMPO/•OH signal was detected suggesting that the •OH radical generation is not mediated by superoxide, and the observed DMPO/•OH is probably due to the further reduction of H_2O_2 by xanthine oxidase. This is also confirmed by addition of catalase (Fig. 9, the bottom spectrum) where both DMPO/•OOH and DMPO/•OH have decreased EPR intensity compared to the top spectrum in Figure 4.

Hydroxyl radical

Hydroxyl radical (•OH) has received a great amount of attention due to its high oxidation potential ($E_{\cdot OH/OH^-}$) and its ability to oxidize almost all biomolecules. It is important to realize that DMPO/•OH can also form by a slow process that involves the nucleophilic addition of water to DMPO. Because water is the most abundant nucleophile in biological systems, it is not surprising that the formation of hydroxylamine followed by easily oxidation leads to form DMPO/•OH. Traces of DMPO/•OH are always detectable in water containing DMPO, and this has long been assumed to be due to slow hydrolysis of DMPO. In fact, there are often small traces of DMPO/•OH in stock DMPO preparation that you receive from commercial vendors (except Dojindo). A much higher concentration of DMPO/•OH has been reported to occur through nucleophilic addition of water containing DMPO in the presence of the Lewis acid Fe(III). Due to the serious implications of these findings with respect to many spin-trapping studies, the suitability of DMPO as a hydroxyl radical spin trap was studied in typical Fenton systems. The following procedure is used to show the formation of DMPO/•OH and BMPO/•OH in Fenton system:



1. Make up a solution of 1 mM $FeSO_4$, 10 mM H_2O_2, and 1 M DMPO (or 250 mM BMPO) in water.

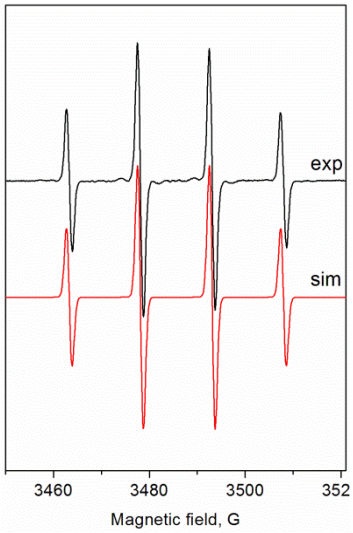


Figure 10. EPR spectrum of the DMPO/$^{\bullet}$OH radical adduct formed in Fenton system.

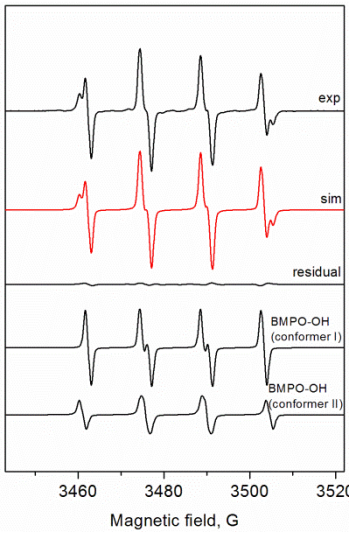


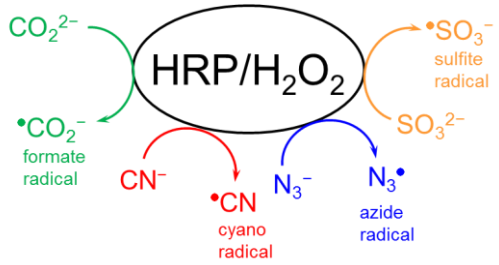
Figure 11. EPR spectra of the BMPO/$^{\bullet}$OH stereoisomers in Fenton system.

2. Prepare your reaction mixture to a total reaction volume of 200 µl. Add 140 µl of distilled water to an Eppendorf tube. Add 20 µl DMPO of your 1 M DMPO solution (or 20 µl of your 250 mM BMPO stock) and 20 µl $FeSO_4$ of the stock 1 mM solution. Initiate the reaction with 20 µl 10 mM H_2O_2. Mix the reactants and quickly transfer the solution to a flat cell. Insert the flat cell into the cavity, tune the spectrometer, and acquire the spectrum. The final concentrations of the components are: 100 mM DMPO (or 25 mM BMPO), 0.1 mM $FeSO_4$, and 1 mM H_2O_2.

Output spectra of the DMPO and BMPO spin trapping of free hydroxyl radical are presented in Figures 10 and 11, respectively. The DMPO/$^{\bullet}$OH adduct is the most reported radical adduct of DMPO and is easily distinguished by a typical 4-line spectrum with the relative intensity ratio 1:2:2:1 due to the equivalent hyperfine splitting constants (Figure 10). In the case of BMPO, two stereoisomers of BMPO/$^{\bullet}$OH were formed similar to BMPO/$^{\bullet}$OOH results. Figure 11 represents a typical EPR spectrum of BMPO/$^{\bullet}$OH radical adduct, the composite simulation (in red), the residual spectrum, and the simulations of each of the stereoisomers. The two BMPO adducts (Fig. 11) were fitted with a_N = 13.47 G, a_H^{β} = 15.31 G, and $a_H^{\gamma 1}$ = 0.62 G for conformer I and a_N = 13.56 G, a_H^{β} = 12.3 G, and $a_H^{\gamma 1}$ = 0.66 G for conformer II.

DMPO spin trapping of C-, N-, and S-centered radicals in biological system horseradish peroxidase (HRP)/hydrogen peroxide (H_2O_2)

As mentioned above, DMPO is capable of trapping not only O-centered radicals but also C-, N-, and S-centered ones. The following experiments are used to demonstrate how different types of radicals can be trapped by DMPO in a simple HRP/H_2O_2 system by using different protein substrates:



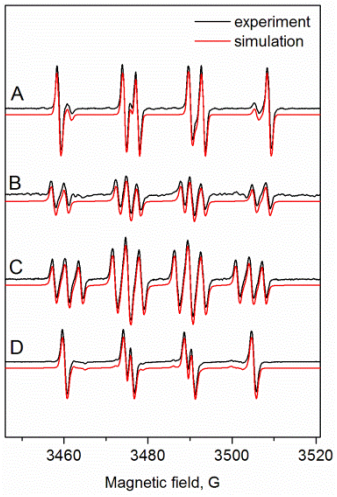


Figure 9. EPR spectra of: (A) DMPO/$^{\bullet}CO_2$, (B) DMPO/$^{\bullet}$CN, (C) DMPO/$N_3^{\bullet}$, and (D) DMPO/$^{\bullet}SO_3$.

Experimental Protocol

1. Prepare a solution of 100 mM phosphate buffer (pH 7.4) containing 25 µM diethylenetriaminepentaacetic acid (DTPA).
2. Make up a solution of the following peroxidase substrates: (A) 100 mM sodium formate (HCOONa); (B) 100 mM potassium cyanide (KCN); (C) 100 mM sodium azide (NaN_3); (D) 100 mM sodium sulfite (Na_2SO_3) in 100 mM phosphate buffer, pH 7.4.
3. Make up a solution of horseradish peroxidase (Sigma) with concentration of 4.0 mg/ml (~ 100 µM) and 1 mM solution of hydrogen peroxide (H_2O_2).
4. Make up a solution of DMPO (Dojindo) with concentration of 1 M.
5. Prepare your reaction mixture to a total reaction volume of 200 µl. Add 130 µl of buffer to an Eppendorf tube. Add 20 µl DMPO of your 1 M DMPO solution, 20 µl of one of the substrates' stock solutions, 10 µl of 1 mM H_2O_2, and initiate the reaction with 20 µl HRP. Vortex the tube, transfer the solution to a flat cell, and acquire the spectrum. The final concentrations of the components are: 100 mM DMPO, 10 mM substrate (formate, cyanide, azide, sulfite), 50 µM H_2O_2, and 10 µM HRP.

Summary

EPR spin trapping is a valuable tool in the study of transitient free radicals. Spin traps react with free radicals in solution to yield stable products, "spin adducts", which can be observed directly by EPR spectroscopy. The ideal spin trap should be reactive enough to scavenge the free radicals of interest while at the same time being relatively inert toward reactive but nonradical species. The nitrone spin trap 5,5-dimethyl-1-

pyrroline N-oxide (DMPO) is widely used to provide evidence for the involvement of free radicals in many chemical and biological reactions. It is particularly useful for identifying oxygen-centered radicals, e.g. superoxide and hydroxyl radicals, but also carbon-, nitrogen-, and sulfur-centered radicals because the resultant spin adducts have characteristic EPR spectra. BMPO is another very useful spin trap to detect and identify the structure of reactive oxygen-centered radicals with relatively long half-life of its superoxide radical adduct. Both spin traps (DMPO and BMPO) are cell permeable which makes them very useful for detecting extracellularly and intracellularly reactive oxygen species in vivo.

References

Eaton S.S., Eaton G.R., Berliner L.J. "Biomedical EPR", (2005) vol. 23, pp. 80-82

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