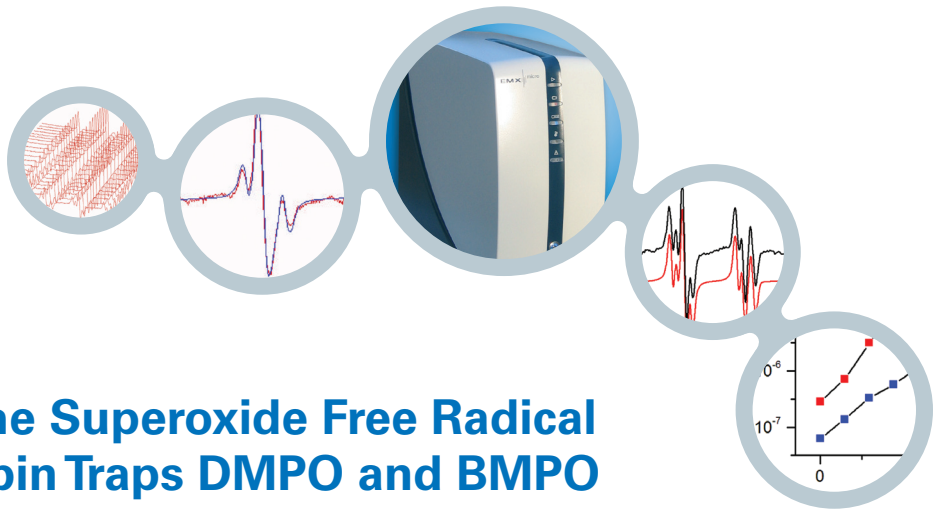
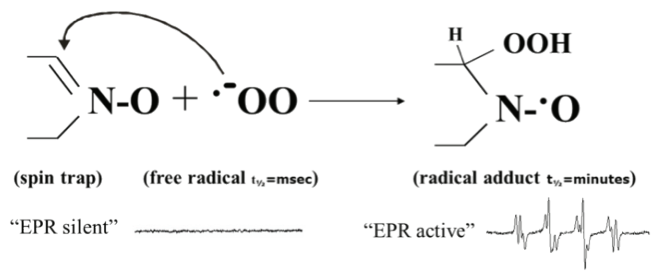


EPR Detection of the Superoxide Free Radical with the Nitrone Spin Traps DMPO and BMPO

In general, the most direct method for measuring and characterizing free radicals in chemistry, biology and medicine, is detection by EPR spectroscopy. However, due to their high reactivity and short half-lives, direct EPR detection of many free radicals *(e.g.*, superoxide, hydroxyl radical, alkyl radicals, *etc.)* is virtually impossible in solution at room temperature. Spin trapping is a technique developed in the late 1960s in which a nitrone or nitroso compound reacts with a target free radical to form a stable and identifiable free radical that is detected by EPR spectroscopy. The spin trapping technique involves the addition of the reactive free radical across the double bond of a diamagnetic ''spin trap'' to form a much more stable free radical (a ''radical adduct'') which can then be measured with EPR:



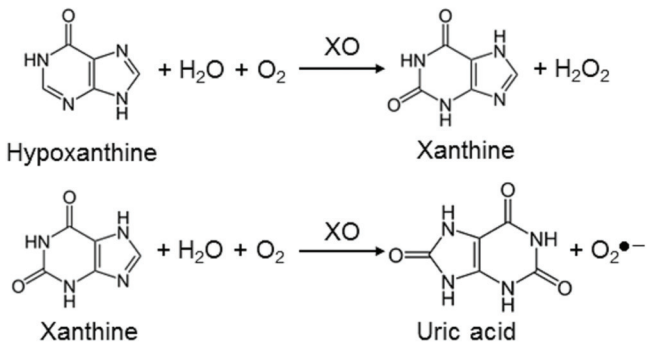
The most popular spin trap is 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), which has been cited in Medline more than 1,000 times. DMPO has significant advantages over other nitrone spin traps. It is very redox inert. DMPO forms radical adducts with O-, C-, N-, S-centered radicals that have very distinguishable EPR spectra. This allows the researcher to identify the type of free radical that was formed in a given reaction. This is not the case for other spin traps such as α-phenyl-*N*-*tert*-butylnitrone (PBN) where the EPR spectra for the radical adducts are nearly identical regardless of the radical trapped. The identity of a DMPO radical adduct is then identified using references from a web search of related scientific literature.

BMPO (5-tert-butoxycarbonyl 5-methyl-1-pyrroline N-oxide) is an analog of DMPO that was developed at the Medical College of Wisconsin with the intent of addressing some limitations that are encountered with DMPO. It is most suitable for the specific *in vivo* or *in vitro* trapping of short-lived superoxide, hydroxyl and thiyl radicals. Like DMPO, BMPO forms radical adducts with very distinguishable EPR spectra. However, BMPO provides a significant improvement over DMPO as BMPO forms much more stable radical adducts with superoxide (DMPO/•OOH $t_{1/2}$ = 45 seconds; BMPO/•OOH $t_{1/2}$ = 23 minutes.). BMPO-derived adducts also exhibit a much higher signal-to-noise ratio in their EPR spectra making it a useful trap for detection of radicals in cell suspensions. Additionally, BMPO is commercially available in a highly purified crystalline form that can be stored for extended periods of time.

Spin trapping of O-centered radicals by DMPO and BMPO (Analysis 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 enzyme/substrate system xanthine/xanthine oxidase is a common method used to generate superoxide and is a standard for comparing superoxide flux from other chemical or biological reaction systems. Xanthine oxidase will oxidize hypoxanthine to uric acid (Scheme 1); the electrons from this oxidation are passed to dioxygen to produce both H_2O_2 and $O_2^{\bullet-}$:



Scheme 1

Unfortunately, the EPR detection of DMPO/$^{\bullet}$OOH is not without its problems 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 possibility that DMPO/$^{\bullet}$OOH spontaneously converts to form DMPO/$^{\bullet}$OH. The following experiment is used to verify the formation of superoxide- and hydroxyl radical adducts formation with DMPO or BMPO.

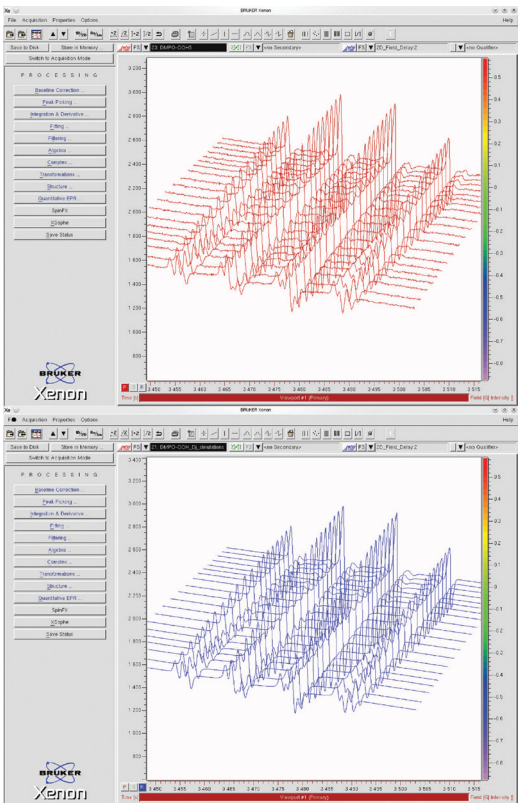
Experimental Protocol

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 in 100 mM phosphate buffer.
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 reveal any paramagnetic impurities and demonstrate that all the components are required to produce the EPR signal.

Spin trapping time course experiment

The spin trapping experiments were performed using DMPO or BMPO as spin traps on a Bruker EMXmicro 6/1 EPR system. The formation of the radical adducts and their time evolution was monitored by a 2D experiment (Field sweep vs. time) configured in Bruker's Xenon software (Figure 1). After the experimental data were acquired, each spectrum containing multiple species was simulated by Xenon's SpinFit module to identify the radical adducts (Figure 2).

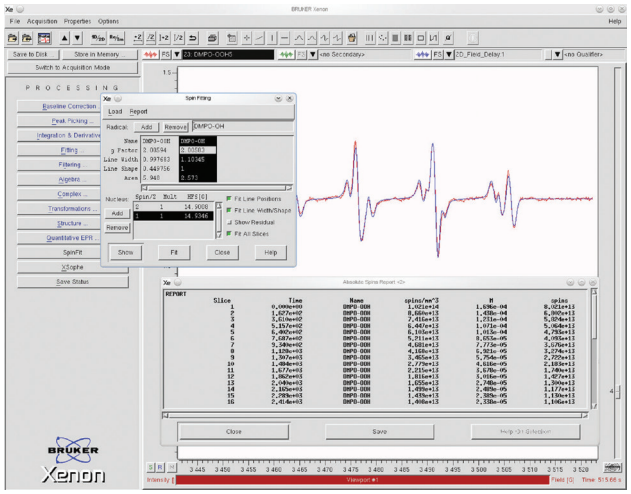
Figures 1 & 2



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.

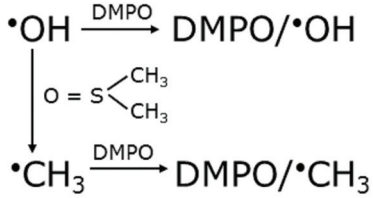
The simulation parameters can either be typed in or imported from a library contained within the Xenon software. One fitting result is the integrated intensity for each adduct. This value is then used to calculate the actual molar concentration of each radical adduct using Xenon's SpinCount module (Figure 3).

Figure 3



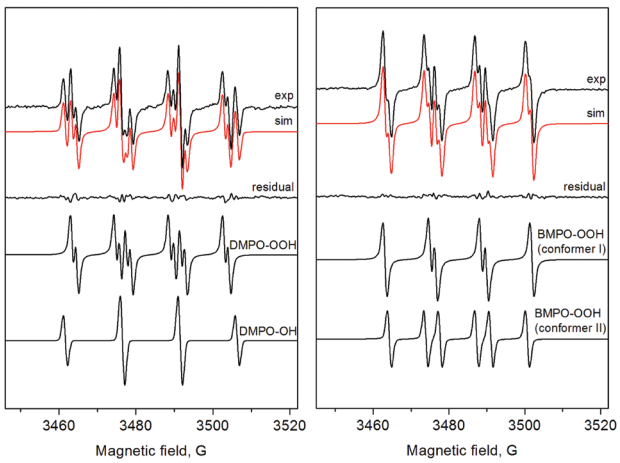
Defining the DMPO radical adducts in the SpinFit dialog by import from the Xenon radical adduct library or by manual entry. SpinCounting provides a report of the fit species areas' during the time course of the experiment.

In the case of DMPO, two spin adducts (DMPO/•OOH and DMPO/•OH) were generated by the xanthine oxidase system (Figure 4). When BMPO was used two stereoisomers of BMPO/•OOH were formed without BMPO/•OH production (Figure 5). SpinFit provides the simulated spectrum for each acquired data slice in the 2D experiment. (Figs. 4 and 5). For each time point in the 2D experiment, you can see the experimental spin trap EPR spectra, the composite simulation, the simulation for each radical adduct and the residual data. The hyperfine fit parameters for the two DMPO adducts (Figure 4) were: a_N = 14.2 G, a_H^{β} = 11.4 G, and $a_H^{\gamma 1}$ = 1.2 G for the DMPO/•OOH adduct and $a_N = a_H^{\beta}$ =14.9 G for the DMPO/•OH adduct. The two BMPO/•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 integrated intensity of the 2D spin fits are used by the SpinCount module to calculate the concentration of the DMPO and BMPO radical adducts (Figures 6 and 7).



Scheme 2

Figures 4 & 5

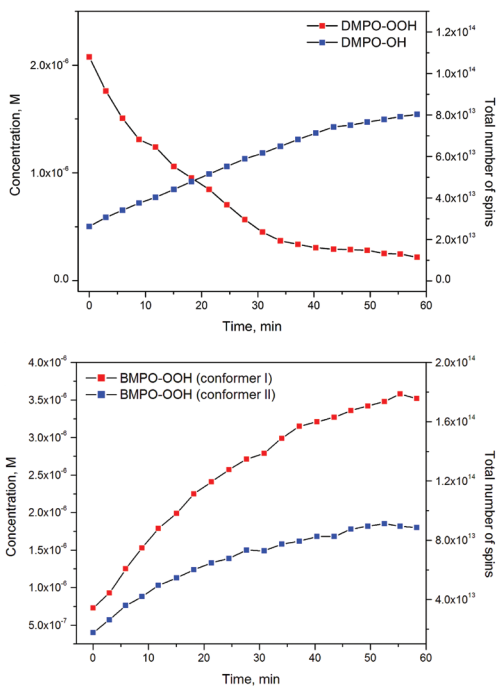


Resultant fits of two sets of species at a given time in the 2D field versus time experiment. The conditions in both panels were identical except for the choice of spin trap.

Example experiment: Verification of superoxide and hydroxyl radical production by xanthine/xanthine oxidase

To unequivocally establish the existence of free hydroxyl radical in spin trapping experiments, it is typical to perform kinetic-based competition experiments with hydroxyl radical scavengers. For example, dimethyl sulfoxide, ethanol, and formate can be used in these competition experiments.

Figures 6 & 7

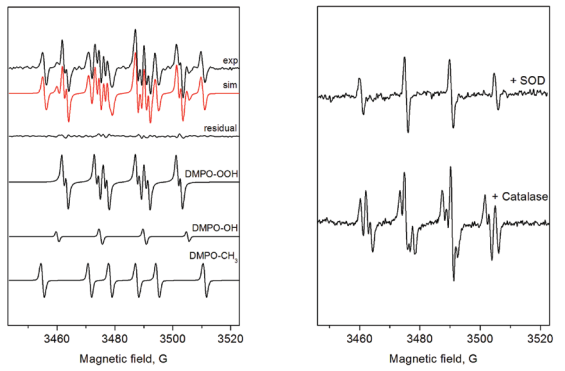


After feeding the results of SpinFit into SpinCount, the concentration changes over the time of the experiment are obtained.

Upon reaction with hydroxyl radicals, these reagents form carbon-centered radicals that can subsequently be trapped by DMPO (Scheme 2). The following experiment is used to study the origin of the hydroxyl radical in xanthine oxidase system. Follow the steps 1–6 from the experiments described 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 resulting spectrum (Figure 8) exhibited a negligible trace of DMPO/•OH signal, while the primary spectral component displays features from the DMPO/•CH_3 radical adduct (a_N = 16.4 G, $a_H\beta$ = 23.3 G). This was confirmed using SpinFit simulations. This result shows that the majority of DMPO/•OH signal observed in the absence of DMSO originates from the trapping of •OH radicals and not from the spontaneous conversion of DMPO/•OOH.

The superoxide scavenging enzyme superoxide dismutase (SOD) was added before initiation of the reaction (Figure 9) to confirm the trapping of •OH. If the DMPO/•OH we are measuring is actually from the conversion of DMPO/•OOH, we would expect the DMPO/•OH spectrum to totally disappear. The top spectrum in Fig. 9 was taken immediately after adding xanthine oxidase. As expected, SOD totally scavenged the superoxide radicals. However, the DMPO/•OH spectrum was still present. This demonstrates that the •OH radical generation is not mediated by superoxide, but is due to the further reduction of H_2O_2 by xanthine oxidase. This was also confirmed by addition of catalase (Figure 9, the bottom spectrum) where both DMPO/•OOH and DMPO/•OH have decreased EPR intensity compared to the top spectrum in Figure 4.

Figures 8 & 9



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

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

Summary

EPR spin trapping with DMPO and BMPO can be used effectively for mechanistic studies and kinetic analysis of superoxide radical generated in enzyme reactions. Properly controlled spin trapping experiments verify that the formation of radical adducts is due to free radical production in the reaction system being studied. The EPR spectra of superoxide radical adducts of DMPO and BMPO are very identified and easily distinguished. Both spin traps are also cell permeable which makes them useful for detecting extracellular and intracellular superoxide in tissues and cells. Bruker's simulation module SpinFit (included in the Xenon software package) makes it easy to accurately determine the hyperfine coupling values for the nitroxide nitrogen and β-hydrogen of the spin adducts. The major disadvantage of DMPO is that the reaction with superoxide is slow, the radical adduct is unstable ($t_{1/2}$ = 45 seconds) and spontaneously decays into the DMPO-hydroxyl adduct. In contrast, BMPO superoxide spin adduct has a much longer half-life ($t_{1/2}$ = 23 minutes) and does not decay into a hydroxyl adduct. Additionally, BMPO can be highly purified by crystallization and handled and stored for extended periods of time without fear of decomposition.

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