Application Notes # MT-72

Dedicated to Spin Labels: The ER 4123D CW-Resonator

The numerous parameters and hardware features associated with EPR imaging can be quite daunting at first. This guide was created to help facilitate image acquisition and processing and provide information relating to the gradient configuration and transformations.

Introduction

Nitroxide spin labels and spin traps together with CW EPR spectroscopy have found a wide range of applications for the study of chemical and biological systems. Due to the high sensitivity of the EPR parameters to the local environment, spin labels are used extensively as reporter molecules to probe pH, polarity, and local motion of complex structures. Spin traps are typically included in chemical and biochemical systems to form stable radical adducts and allow the detection and identification of short lived radical intermediates.

Though not limited to use in biological systems, spin labels have found an increased use for probing the structure of proteins, biological membranes, and DNA[1, 2]. Through the use of site directed spin labeling (SDSL) techniques, spin labels are attached at specific sites within the biological structure. The resulting EPR spectrum is then analyzed in terms of line position and line width. Changes in line position indicate the polarity of the environment and the participation of the spin label in hydrogen bonding, and changes in the line width

provide information on mobility and relaxation processes.

Mobility studies take advantage of the sensitivity of the CW EPR spectrum to motional narrowing of the hyperfine features. In the fast motion limit, the CW EPR spectrum of a typical nitroxide spin probe exhibits three narrow lines. As motion is decreased the high field feature begins to broaden and at subsequently slower motions the low field and center field features broaden. By simulating the CW EPR spectrum, the tumbling correlation time can be determined, giving a time scale for the motion of the spin probe and therefore the biological structure.

Another method for analysis of structure is based on relaxation enhancement by another paramagnetic molecule in solution, for example molecular oxygen or various Ni(II) complexes. Collisions with the relaxing species allows more microwave energy to be absorbed resulting in an increase in the power necessary for saturation, quantified by the power saturation parameter, $P_{1/2}$ (*vide infra*). An increase of $P_{1/2}$ in the presence of the relaxing agent indicates an exposed region, whereas a small change indicates an enclosed region. This method is also used for studying proteins in lipid bilayers to determine the portions of the protein within the lipid bilayer[3, 4]. The membrane bound protein is selectively spin labeled and then exposed to relaxation enhancement species with different solubilities in hydrophilic and hydrophobic

environments, chromium oxalate (hydrophilic) and molecular oxygen (hydrophobic). By measuring $P_{1/2}$, the accessibility of the spin label to the relaxant is determined and interpreted as a location facing a hydrophilic region (aqueous phase) or a hydrophobic region (lipid phase or protein interior).

All of these methods are powerful tools for the exploration of the native structure of proteins and rely upon the CW EPR resonator. Due to the limited sample size and the types of experiments to be performed, the EPR resonator design is critical. The sample in many biological systems typically involves a small volume, a low concentration, and an aqueous or a lossy medium. Effective measurements thus requires a resonator with a high sensitivity, determined by the B_1 field at the sample position and the Q-factor. For power saturation experiments, a maximum in the microwave magnetic field, B_1, in the sample volume is desired, therefore the construction must provide a high conversion factor. A resonator design that meets these requirements is the loopgap resonator. However, this resonator suffers from a low Q when loaded with an aqueous sample and instabilities that make tuning difficult. With the above experimental requirements in mind, the ER4123D resonator was designed.

In addition to providing superior sensitivity for lossy samples, the ER4123D features reliable, fully automatic tuning and matching. This facilitates use and allows an increased number of samples to be measured with a minimal amount of time spent retuning the resonator. When used with the automatic fine tuning features available in WinEPR and Xepr, 2D experiments require no user intervention to insure that proper tuning and matching are maintained during the course of the experiment.

This application note demonstrates the utility of the ER4123D resonator to measure the saturation curves of spin labels in the presence of oxygen and after purging with nitrogen. These measurements utilize a capillary sample tube fabricated from TPX® (polymethylpentene). TPX® has a high gas permeability and is ideally suited for sample deoxygenation and oxygen accessibility experiments.

Experimental Protocol

Data Acquisition and Processing

Room temperature, CW-EPR spectra were acquired on the ELEXSYS E500 spectrometer with the ER4123D dielectric resonator using the supplied TPX® capillary setup. For power saturation measurements, the ER4123D resonator comes equipped with two TPX® capillaries, two capillary supports, and a holder. The total active sample volume of the capillary was 3 µL. Saturation measurements were performed by collecting the field swept EPR spectrum as a function of microwave power attenuation in 1dB steps (0.01 mW - 87 mW). Saturation curves were then constructed by measuring the amplitude of the lowest field EPR line and normalizing

with respect to maximum amplitude and the square root of the power.

This facilitates the determination of the power required to reach half saturation, $P_{1/2}$, from a plot of log IN vs. log P.

$$I_N = \frac{I}{I_{MAX} \sqrt{P}} \tag{1}$$

At low microwave powers, the CW EPR signal intensity is proportional to the square root of the power. As the power is increased, a point is reached where the signal intensity decreases with increasing power. This saturation of the signal is dependent on the relaxation process present in the system being studied. A parameter which characterizes the relaxation is the power at which half the signal intensity is present in the absence of saturation, $P_{1/2}$, where Π is a proportionality constant, T_1 is the transverse (spin-lattice) relaxation time and T_2 is the longitudinal (spin-spin) relaxation time.

$$P_{1/2} = \frac{\alpha}{T_1 T_2} \tag{2}$$

The value for $P_{1/2}$ can be further used to determine the accessibility to oxygen through the oxygen accessibility parameter, Π. To compare Π for different species, the relaxation ($T_1 T_2$ from Eq. 2) and the motional narrowing (line width) must be normalized to a reference system, crystalline DPPH.

where $\Delta P_{1/2} = P_{1/2}^{O2} - P_{1/2}^{N2}$ and ΔH is the peak to peak line width.

$$\Pi = \frac{\Delta P_{1/2}(SL\text{-}NAD^+)}{P_{..}(DPPH)} \frac{\Delta H(DPPH)}{\Delta H(SL\text{-}NAD^+)} \tag{3}$$

Use of the TPX® capillary

First, the TPX® capillary was carefully attached to the TPX® tube support by screwing it in all the way to insure reproducible positioning. The capillary was filled by attaching either a pipette pump or a Pasteur pipette bulb to the support. The sample was then aspirated into the capillary. The bottom of the TPX® capillary was sealed with sealing wax to prevent sample leakage and evaporation of the sample during gas flow. After removing the pipette pump or pipette bulb from the support, the TPX® tube holder was attached and the entire assembly was inserted into position in the probe. For gas exchange, the gas flow was adjusted prior to connecting to the probe's gas inlet port. Due to the small sample volume and excellent permeability of the TPX® material, a slow gas flow of ~10 mL/min was sufficient for a gas exchange time of 10 minutes. The TPX® capillary was cleaned by removing

the support from the prove, removing the tube holder, and attaching a pipette pump or pipette bulb to the support. A forced air flow dislodged the sealing wax and removed the sample. The capillary and support were then further cleaned using the pipette pump or pipette bulb to flush with water. To avoid damage to the TPX® material, aromatic and aliphatic hydrocarbon solvents were avoided.

Results and Discussion

Three systems were selected to demonstrate the use of the ER4123D resonator and the TPX® system for measurement of the power saturation behavior in the presence and absence of oxygen: 1) TEMPOL (4-Hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl) in water, 2) the Trityl radical (Nycomed Innovations, Malmö, Sweden) in glycerol (90%) and in 1:1 glycerol: water, and 3) l-lactate dehydrogenase (LDH) and spin labeled nicotinamide adenine dinucleotide (SL-NAD⁺) (courtesy of Prof. Wolfgang Trommer, University of Kaiserslautern, Germany).

A typical example of a spin probe in a lossy solvent is TEMPOL dissolved in water. The improved sensitivity of the ER4123D resonator is clear, for $2*10^{13}$ spins (10 µM solution in 3 µL active sample volume), a signal to noise ratio of 300 was achieved at 21 mW with a scan time of 80 seconds. This is a significant improvement over the standard high sensitivity resonator (ER4119HS) using a 50 µL pipette ($18*10^{13}$ total spins, S/N=560).

The results of power saturation measurements in the presence and absence of air are presented in Figure 1. A significant change in $P_{1/2}$ is observed on degassing the sample with nitrogen. This indicates a high collision rate with dissolved oxygen leading to relaxation of the excited spin state. The
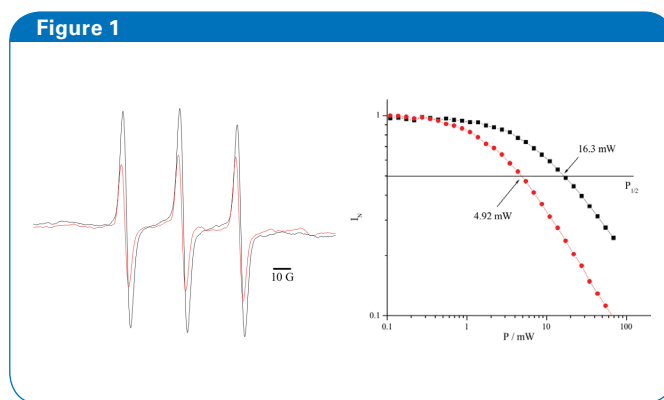

Figure 1

10 µM TEMPOL in Water. Right: Saturation curves in air (black) and nitrogen (red). Left: CW-EPR spectra at P = 17 mW, number of scans = 1, scan time = 80 s, time constant = 655 ms, and modulation amplitude = 2 G.

more rapid relaxation of the excited state then allows the system to absorb more microwave power which is evidenced by the change in $P_{1/2}$. The oxygen accessibility parameter for this system ($\Pi = 0.055$) can be used in comparison with the other examples to account for changes in oxygen diffusion and collision rates.

The effect of the environment on the power saturation behavior of a spin probe is demonstrated using the Trityl spin probe. Two environments were chosen: a high viscosity solvent (90% glycerol) and a lower viscosity solvent (50% glycerol). Saturation measurements in air and nitrogen of these solutions are presented in Figure 2. The high viscosity of the 90% glycerol solution leads to a lower diffusion rate, which results in a decrease in collisions with the spin probe ($\Pi = 0.002$). In the lower viscosity glycerol:water mixture, the collisions between oxygen and the spin probe are more
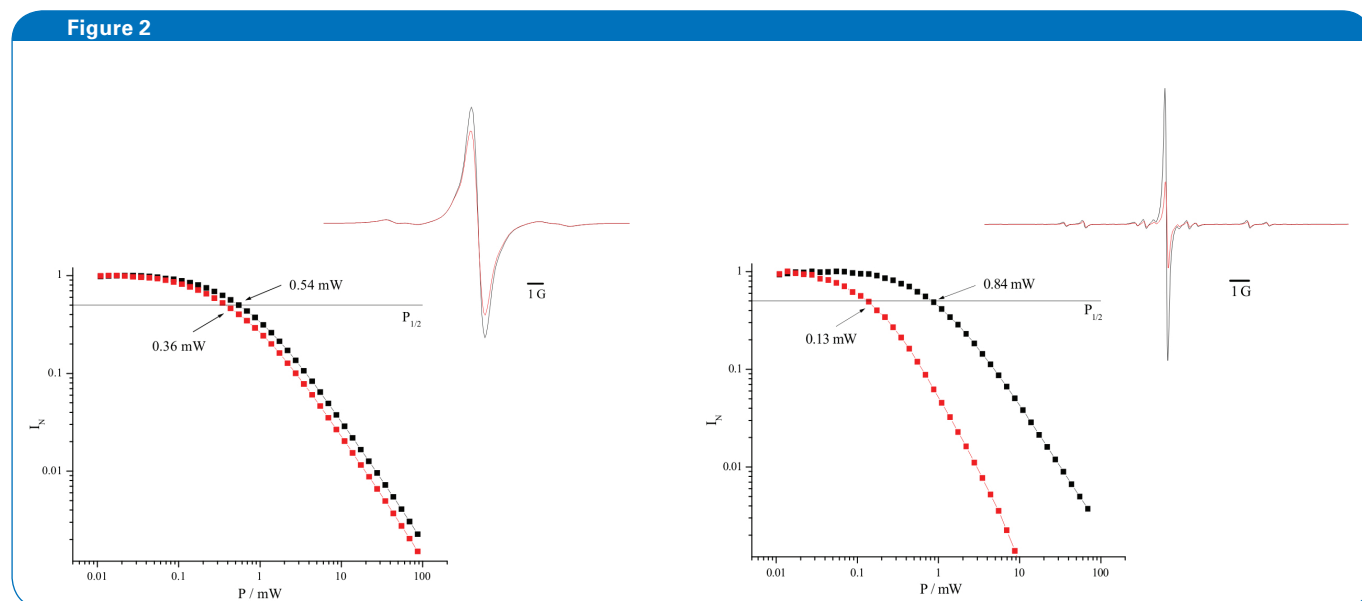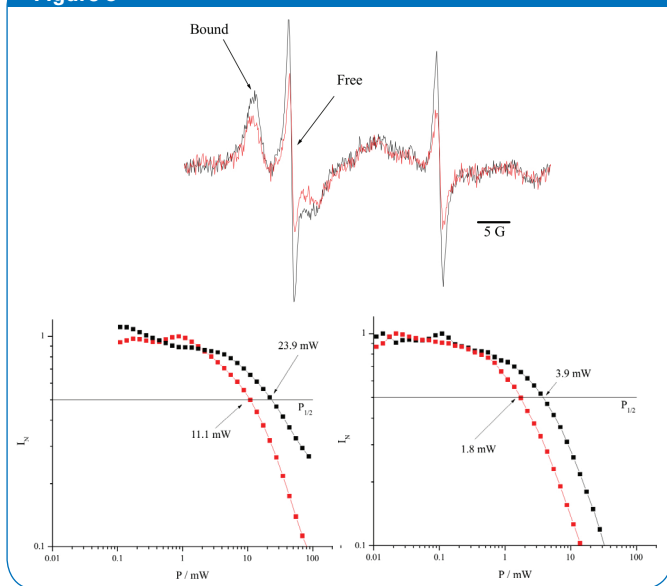
Figure 2

15 mM Trityl (Nycomed Amersham) in Glycerol-d_6 (Left) and 7 mM Trityl in 1:1 Glycerol-d_6:Water (Right). Bottom: Saturation curves in air (black) and nitrogen (red). Top: CW-EPR spectra at P = 0.5 mW and P = 0.875 mW, number of scans = 1, scan time = 170 s, time constant = 1.28 ms and modulation amplitude = 0.5 G.

Figure 3

300 µM l-Lactate Dehydrogenase and 50 µM ^{15}N-spin labeled-NAD$^+$. Top: CW-EPR spectra at P = 27.1 mW, number of scans = 1, scan time = 42 s, time constant = 1.28 ms, and modulation amplitude 0.7 G. Bottom: Saturation curves of the two components, bound SL-NAD$^+$ (left) and free SL-NAD$^+$ (right), in air (black) and nitrogen (red).

frequent as evidenced by the higher accessibility parameter (Π = 0.038). The fact that the $P_{1/2}$ in deoxygenated glycerol solution is three times that of the deoxygenated glycerol:water solution indicates other factors affect the relaxation in these two systems. Other studies[5] indicate that as the viscosity increases T_2 decreases, which would lead to a higher $P_{1/2}$ (see Eq. 2).

The influence of structure and motion on the CW EPR spectrum is demonstrated with l-lactate dehydrogenase (LDH) and its spin labeled coenzyme, ^{15}N-SL-NAD$^+$ (Figure 3). Two different signals are clearly observed in the CW EPR spectrum of the enzyme/coenzyme solution as a narrow set of peaks and a broad set of peaks. The line widths of these two signals directly provide which forms of the SL-NAD$^+$ are present and indicate the relative rotational freedom of each species. The more a molecule rotates in solution, a higher degree of averaging of the anisotropic components of the g and A matrices takes place giving rise to sharper EPR lines. Therefore, the sharp set of signals indicate a rapidly rotating molecule consistent with the free, solution phase SL-NAD$^+$ and the broad set of signals indicate a rotationally hindered molecule such as the enzyme bound SL-NAD$^+$. Analysis of the saturation behavior of each species in terms of the oxygen accessibility can be translated into structural information about the NAD$^+$ binding site. However, due to the overlap of the two species, an accurate determination of ΔH_{bound} and thus Π_{bound} is not possible. In the range of reasonable line widths (ΔH = 5.8 ± 1.0 G), the accessibility to the solvent of the bound species is found to be slightly lower than that of the free species (Π_{bound} = 0.024 ± 0.004

Figure 4
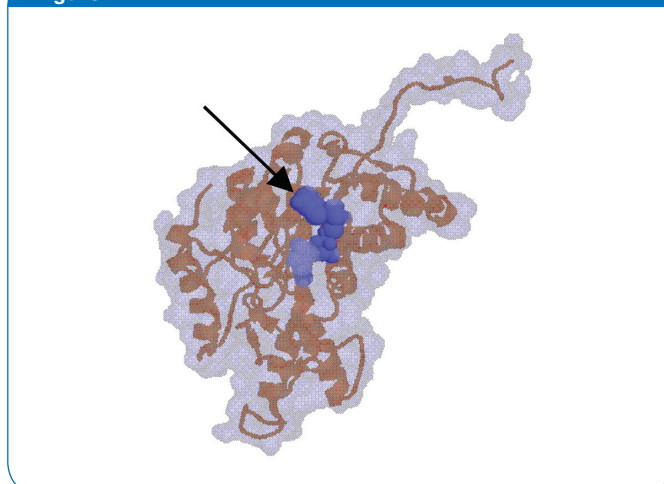
l-Lactate Dehydrogenase from pig heart complexed with NAD$^+$. The light blue area is the calculated surface accessible area based on the X-ray crystal structure and NAD$^+$ is represented by the blue spheres.

and Π_{free} = 0.029). This indicates the binding site of NAD$^+$ is located at the surface of the protein with part of the NAD$^+$ molecule exposed to the solvent. The CW EPR data analysis is consistent with what is observed in the crystal structure of the LDH/ NAD$^+$ complex (figure 4)[6]. The binding site of NAD$^+$ is indeed not deep within the enzyme structure and the adenine ring of NAD$^+$ is exposed to the solvent, where the spin label is attached as marked by the arrow in figure 4.

Summary

As the application of CW EPR techniques grows, so does the need for resonators designed to match the requirements of the application. The use of spin labeling and spin trapping techniques for the study of biological systems has increased the demands on the resonator. The ER4123D has therefore been designed to match these requirements. The resonator offers a high sensitivity to easily measure low concentrations and a high B_1 conversion factor to overcome dielectric losses due to the solvent. To further facilitate the measurements involving sample deoxygenation (saturation studies and line width determination), a support system for capillaries of the highly gas permeable TPX® are included. The TPX® capillary with designed support system greatly simplifies sample deoxygenation and sample handling, while lowering the risk of damage to the capillary. Used in conjunction with the fine tuning option available in WinEPR and Xepr, 2D field vs. power measurements can be performed reliably without the need of user intervention. These characteristics combine to make the ER4123D a valuable resonator for the study of spin label and spin trap systems.

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