# A new EPR methodology for the study of biological systems

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Advances in EPR during the past five years or so have been phenomenal. There has been explosive growth in EPR imaging and in vivo EPR (1), and in the range and power of pulsed EPR techniques (2). High-field EPR is also becoming accessible (3).

The contributing advances that separately and jointly revolutionize CW and pulsed EPR include low-noise microwave sources (4–6), loop-gap resonators (7–9), low-noise GaAsFET microwave preamplifiers (10, 11), and pseudomodulation for resolution enhancement (12, 13).

The most striking advance is the development of a totally new branch of EPR, multiquantum EPR (MQEPR), by James S. Hyde and co-workers at the National Biomedical ESR Center at the Medical College of Wisconsin (14–18). With this new advance, we should now think of EPR in terms of three modes: CW (continuous wave), pulse (spin echo, fourier transform, saturation recovery, etc.), and multiquantum.

Two main intellectual themes converge in the development of MQEPR. First, there are so many problems with magnetic field modulation in EPR that the search for an alternative to magnetic field modulation is imperative (and has a long history, e.g., 14, 19). Second, in other fields, such as NMR and nonlinear laser spectroscopy, there has been extensive development of multiquantum techniques, which resulted in new insights.

The fundamental equation is due to Anderson (20):

$$(\omega_2 - \omega_1)^2 = (\gamma H_0)^2 + \gamma^2 H_1^2.$$

$\omega_1$ and $\omega_2$ are the two microwave frequencies applied to the sample. $\gamma H_0$ is the resonant frequency if just one microwave frequency is applied. $H_1$ is the amplitude of microwave magnetic field at the higher-power microwave frequency.

The EPR spectrometer bridge for MQEPR uses two sources locked a specific frequency apart. The use of a LGR for the sample is important, because the low $Q$ and high microwave efficiency of the LGR make it possible to irradiate simultaneously with two microwave frequencies of varying separations. Numerous combinations of powers and sweeps of magnetic field or of one or both frequencies are conceptually possible. Irradiation with two microwave frequencies is equivalent to irradiating with a single frequency which has been sinusoidally modulated. There is no explicit instrumental modulation since this would be exceptionally difficult using the available microwave technology. Nonlinear response of the spin system can result in intermodulation sidebands, which can be detected. The outputs are the multiquantum transitions, which can be combined in various ways to get useful displays. Two of the frequencies, for example, are $2\omega_1 - \omega_2$ and $2\omega_2 - \omega_1$, which correspond to three-quantum transitions. Transitions involving as many as 13 quanta have been observed. At this early stage (the paper in this issue is only the fifth on MQEPR) the "optimum" experiment awaits application tests on a variety of spin systems.

The MQEPR display is independent of the separation between the two microwave frequencies so long as they are both within the homogeneously broadened (i.e., $T_1$ and $T_2$ determined) EPR linewidth. The amplitude of the $3Q$ EPR signal is proportional to $T_1 T_2^2$. Individual linewidths can be determined by $T_2$ in some displays and by $T_1$ in others. As the frequency difference is increased beyond the homogeneous linewidth the display becomes a function of the spin relaxation time. If $T_1 > T_2$, then the amplitude of the frequency difference swept MQEPR signal is dependent on $T_1$. Recall that in the "normal" CW EPR display the signal intensity is dependent on $T_2$. The different information contents of MQEPR, saturation recovery EPR, and progressive saturation CW EPR will surely enrich understanding of each of the displays, but the comparison experiments have not been done yet.

Note that no magnetic field modulation is used in the MQEPR experiment, and that the spectral display is an absorption signal, not a derivative signal. Both of these features have advantages, independent of the spin relaxation information content of the MQEPR display.

Magnetic field modulation is a severe technical problem for the design of EPR resonators. For the future one should consider multiquantum EPR as a practical alternative to magnetic field modulation. One could pseudomodulate to get the normal derivative display. Indeed, MQEPR is proposed for many types of experiments, such as high pressure, low temperature, etc., where it is technically difficult to get modulation to the sample. At the present stage of development, MQEPR appears to have about half the signal-to-noise of a CW EPR display of the same sample, at least for cases in which the modulation amplitude has to be limited to avoid distorting the linewidth. For very broad lines, where the available modulation amplitude is much less than optimal for the line-

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width, MQEPR may yield better $S/N$ than field-modulated CW EPR. This case includes metal ions in biological systems. For example, the EPR signals observed in photosynthesis research are spread over wide magnetic field ranges due to $g$-anisotropy and exchange coupling. Much of biological spectroscopy has to be done at very low temperature, where it is often difficult, or inconvenient to get magnetic field modulation to the sample. A scheme to obtain pure absorption EPR signals would also greatly enhance CW EPR imaging. An important question is the extent to which the pulsed EPR techniques, such as those developed in the Freed and Schweiger laboratories can be combined with the MQEPR Hyde is developing to get even better insights.

In this issue, Mchaourab et al., (21) apply MQEPR to the study of the copper site in nitrous oxide reductase. MQEPR yields a spectrum different from that obtained by normal CW EPR. The difference is attributed to suppression of forbidden transitions in the MQEPR display. The $T_1$ dependence of the MQEPR display was used to measure $T_1$. A series of magnetic-field-swept MQEPR spectra as a function of the frequency difference between the two irradiation frequencies yields $T_1$ for all parts of the spectrum simultaneously. The similarity of $T_1$ for copper in nitrous oxide reductase to that previously observed for copper in cytochrome $c$ oxidase argues in support of the thesis that the EPR detectable copper centers in the two enzymes are similar. Beyond its contribution to the understanding of nitrous oxide reductase, this paper is important for the research community because of the major technological development that underlies the performance of this study. Other researchers may now design new experiments modeled on this work.

Multiquantum EPR is an exciting new opportunity. At the moment, the only spectrometer capable of performing MQEPR is in the National Biomedical ESR Center. However, since this is an NIH-supported Research Resource, potential users are encouraged to contact the ESR Center with problems to which they would like to apply MQEPR.

The references to topics other than MQEPR merely provide an introduction to the literature. In addition to the MQEPR references cited, this essay is based on presentations by Hyde and co-workers at the International EPR Symposium in Denver in recent years, and especially at the second Workshop on the Future of EPR, held in Denver, 7 August 1992. Several other papers on MQEPR are in press, and an important one on the theory of intermodulation sidebands has just been published (18). The photosynthesis example is due to comments by Professor Melvin Klein.

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