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Forum Editorial

EPR Spectroscopy in Biology and Medicine

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DURING THE LAST COUPLE OF DECADES the role of free radicals has been well recognized in the pathogenesis of many diseases, including cancer, atherosclerosis, ischemia-reperfusion injury, stroke, diabetic vascular disease, and a variety of inflammatory diseases. The radicals, collectively termed as "reactive oxygen/nitrogen species," are generated in the biological milieu and propagated through a cascade of reactions leading to various physiological and pathological states. Much progress has been made in the detection, characterization, and quantification of these radicals. Electron paramagnetic resonance (EPR) [also known as electron spin resonance (ESR)] spectroscopy detects the free radicals and paramagnetic molecules. By definition, the paramagnetic molecules contain one or more unpaired electrons, *e.g.,* nitric oxide (NO), oxygen (O_2), nitroxyls, or $CuSO_4$. Free radicals are paramagnetic, but the term is limited to the short-lived fragments or redox intermediates that possess unpaired electron(s). Examples of free radicals are superoxide anion ($O_2^{\bullet -}$), hydroxyl ($^{\bullet}OH$), alkyl ($R^{\bullet}$), lipid peroxyl ($LOO^{\bullet}$), or ascorbyl. The free radicals, in general, are unstable and reactive. Most of the reactive oxygen species (ROS) are free radicals, with some exceptions including hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and peroxynitrite ($ONOO^-$).

EPR spectroscopy plays a major role in the detection of oxygen and most of the oxidants. The magnetic field-based EPR detection enables nondestructive (*in vitro*) and noninvasive (*in vivo*) measurements of biological samples. EPR data provide a wealth of information (fine, hyperfine, and superhyperfine structures; *g*-factor; line-shape; saturation) that serve as fingerprints of the paramagnetic species for unequivocal identification. Furthermore, the detection is direct, definitive, and quantitative. The purpose of this Forum Issue is to accentuate the usefulness of the EPR technique for the study of free radicals in biology and medicine.

This Forum Issue contains seven review articles and four original research articles that comprise a broad range of applications of EPR spectroscopy, from radicals to redox measurements, in biological systems. While the biological applications of EPR spectroscopy became prominent only during the past 2 decades after the spurt in the study of oxygen free radicals in the pathogenesis of disease processes, the technique has been known and used in numerous applications in other branches of science for over 5 decades. Until recently, the biological applications were limited to *in vitro* or excised frozen tissue biopsy specimens that could be analyzed at X-band (9–10 GHz) frequencies. The technological advances in low-frequency (2 GHz or less) instrumentation and imaging capabilities have enabled the use of this technique for measurements of free radicals in intact organs and whole-body animals, *in vivo* (6). Recently, measurements have been performed in the human body, and efforts are underway towards the development of the first EPR system for clinical use (3, 4, 12).

As mentioned above, most of the biological applications of the EPR technique involve the measurement of oxygen free radicals. Free radicals such as $O_2^{\bullet -}$ and $^{\bullet}OH$ have very short half-lives (nanoseconds to microseconds) and hence are usually detected using stabilizer molecules called spin-traps. The spin-trap molecules form adducts (1:1) with the short-lived free radicals, resulting in a more stable (half-life of several seconds to minutes) paramagnetic species that can be conveniently detected by EPR spectroscopy under ambient conditions. A notable advantage of the spin-trapping technique is that the free radical species can be specifically identified from the multiplet structure of the spectrum. Even better, multiple species in a given sample can be individually identified and quantified. A variety of spin-trap molecules customized for the detection of specific radicals as well as under a variety of experimental conditions is available. In addition to the primary oxygen radicals, secondary radicals such as alkyl ($R^{\bullet}$), alkoxyl ($RO^{\bullet}$), and alkylperoxyl ($ROO^{\bullet}$) as well as lipid peroxyl ($LOO^{\bullet}$) radicals can be measured using spin-trapping EPR. Villamena and Zweier have done an up-to-date review of spin-trapping (14). Venkatraman *et al.* provide an overview of how the spin-trapping EPR can be used to detect the free radicals formed during lipid peroxidation in cells and tissues (13).

The detection of NO, the ubiquitous paramagnetic molecule in biological systems, by EPR is complicated for a different reason. Although NO is a fairly stable molecule in physiological environments, its EPR spectrum is too broad to be observed. This is because of its very short half-life in the excited spin state (fast relaxing!). Hence, NO too requires a

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tranquilizer molecule (spin-trap) such as iron-dithiocarbamate for its detection by EPR. Yoshimura and Kotake present a brief review of the chemical and biological aspects concerning spin-trapping of NO with the iron-dithiocarbamate complex as a spin-trap (16). Berliner and Fujii discuss the extension of the capability of the detection of NO non-invasively in living animals or excised organs using specialized EPR methods (1). Despite the need to infuse the iron-dithiocarbamate into the animal for the determination of NO production in animals, highly sensitive localized concentrations of NO may be observed *in vivo* by both low-frequency EPR and contrast-enhanced magnetic resonance imaging (1). An alternate approach is to measure NO in body fluids such as urine, blood, excised tissue, or cerebrospinal fluid. Bratasz *et al.* demonstrate the potential value of detection of NO level in the cerebrospinal fluid collected from neurological patients as a prognostic marker in human brain diseases (2). The authors note that the EPR determination of the level of NO is much faster and more accurate as compared with the time required for the bacteriological or other assays.

While oxygen is a critical component of oxidative damage that is involved in pathophysiological processes, its measurement is often ignored. Molecular oxygen is paramagnetic, but is not amenable to direct detection by EPR under ambient conditions. EPR spectroscopy, coupled with the use of oxygen-sensitive paramagnetics (probes), has become a potential technique for accurate and precise determination of oxygen concentrations in a variety of biological samples, including tissues and cells. The technique, referred to as "EPR oximetry," uses soluble molecular spin probes for the determination of dissolved oxygen concentration and particulate spin probes for targeted determination of local oxygen tension (partial pressure of oxygen) in tissues and cells. The later methodology has the capability of making repeated measurements from the same site non-invasively. Swartz, a pioneer in the development of the technique, describes the principles and applications of EPR oximetry to viable systems, including cell suspensions and intact animals (11). Kutala *et al.* report on the simultaneous determination of intra- and extracellular oxygen concentrations in bovine lung microvascular endothelial cells using EPR oximetry (8). The method utilizes dual spin probes, one exclusively internalized in cells and the other placed extracellularly. which are capable of reporting oxygenation simultaneously from the two distinct regions. Some of the recent developments by Swartz and co-workers at Dartmouth College and in our laboratory indicate that the EPR oximetry has the potential of accurate and reliable measurements of intracellular and tissue oxygen concentration in humans.

EPR has made important contributions in our understanding of the paramagnetic metal ions such as iron, copper, manganese, and chromium in a variety of biological systems. Unlike the oxygen radicals and NO, the metal ions can be detected directly at ambient conditions or at low temperatures. In this special issue, Rifkind *et al.* highlight the contributions of EPR in the study of hemoglobin redox reactions and autoxidation as well as the reactions of hydrogen peroxide generated by superoxide dismutation, redox reactions associated with NO produced in the circulation (9). They show how EPR not only identifies the paramagnetic species formed but can also be used to provide insights into the mechanism involved in the redox reactions.

The application of EPR is not limited to the determination of free radicals and paramagnetic molecules alone. Instead of "spying on" free radicals, one can also use the free radicals as "spying agents" to obtain physiologically pertinent (functional) information such as tissue redox status, metabolic information, and pH. This approach uses known paramagnetic probes as reporter molecules to gain insights into the biochemical, biophysical, or metabolic processes in the tissue of interest. Nitroxyl molecules are commonly used as redox probes (7). Stable nitroxides of imidazoline and imidazolidine types provide the unique possibility to measure tissue thiol content and local values of pH in various biological systems, including *in vivo* studies (5). For example, free radical-mediated alterations in the brain tissue redox status were investigated in diabetic rats, *in vivo,* by measuring the pharmacokinetics of nitroxyl decay using EPR spectroscopy (15). Although the nitroxyl probes have been extensively used in several studies, the mechanism by which they are metabolized in cells and tissues is not well understood. Samuni *et al.* investigate the most widely used nitroxyl probe, Tempol, and reported that while the bioreduction of the nitroxyl is influenced by a number of factors, the hexose monophosphate shunt appears to be involved in both nitroxyl reduction as well as cytotoxicity induced by high levels of exposure to Tempol (10).

In conclusion, the EPR technique offers unique advantages in the determination of free radicals and paramagnetic species in biological samples. The method is direct, definitive, non-invasive, sensitive, and quantitative. It is an indispensable tool in the study of oxidants, oxygen, and oxidative stress in free radical biology and medicine.

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ABBREVIATIONS

EPR, electron paramagnetic resonance; ESR, electron spin resonance; NO, nitric oxide; ROS, reactive oxygen species.

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