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REVIEW

From spin-labeled proteins to in vivo EPR applications

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Abstract This is a historical overview of the advent of applications of spin labeling to biological systems and the subsequent developments from the perspective of a scientist who was working as a Ph.D. student when the technique was conceived and was fortunate enough to participate in its development. In addition, the historical development of in vivo applications of EPR on animals and other living systems is described from a personal perspective.

Keywords Nitroxides · Aminoxyl radicals · Spin labels · Proteases · Imaging · Bioreduction

Introduction and dedication

It is a great honor to contribute to this special issue recognizing my longtime friend and colleague, Marcus Hemminga, on the celebration of his retirement from Wageningen University. When Marcus invited me to contribute, he graciously suggested that I cover my research adventures from the 'beginning' in Harden McConnell's Stanford University laboratory to our contributions to the in vivo electron paramagnetic resonance (EPR) field while at Ohio State University and beyond. The work that I will describe is indeed a tribute to Marcus as his contributions have both complemented and enhanced the field as well.

The more you see: spectroscopy in molecular biophysics.

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The scope of this article will start from the period of the initial conception and applications of spin labeling, some of the seminal advances in the field, followed by coverage of the early days of in vivo EPR and imaging into the future. While the perspective is from a personal viewpoint, I attempt throughout to point out the pitfalls and challenges that have arisen in these exciting fields of research. I also attempt to provide a deeper view on the new scientific challenges and opportunities that await the next generation of EPR spectroscopists.

The early years at Stanford

I started my Ph.D. studies at Stanford University with a specialization in physical chemistry. Two of us from the entering graduate class in 1963 postponed by a few months choosing our research mentor in order to join Professor Harden McConnell upon his arrival from Caltech in the summer of 1964. My first research project involved chemical physics, particularly triplet excitons in charge-transfer crystalline complexes. At the same time, the group was just starting to look at EPR applications of labeling macromolecular systems, particularly proteins, in a fashion analogous to the fluorescence labeling approaches of Gregorio Weber at the University of Illinois (Weber 1953). After 1 year, we published a *J Chem Phys* paper that involved the construction of a homemade 400-MHz EPR spectrometer operating at 100 p.s.i. hydrostatic pressure and its application to the properties charge-transfer crystalline complexes (Merkl et al. 1965). I decided to change my project and found my love of biochemistry and protein structure. Prof. McConnell was amenable to my wishes and assigned me simply to 'work on' α-chymotrypsin and see whatever came up as an interesting research avenue.

The early days of spin labeling

The spin-label technique is specifically a reporter group technique, a concept coined by Dan Koshland in the 1960s, as depicted in Fig. 1 (Burr and Koshland 1964). Fortunately, there were stable paramagnetic compounds developed in Russia at the Institute of Chemical Physics in Moscow containing *aminoxyl* (or iminoxyl or nitroxyl or nitroxide) 'reporter' groups. While these compounds were not commercially available, their synthesis was fairly straightforward (from commercially available phorone or triacetoneamine). The Russian group was headed by the physical chemist, Moisei Neiman, and organic chemist Eduard Rozantsev, the latter of whom propelled the syntheses into a broader range of versatile protein modification reagents (Rosantzev and Neiman 1964; Rozantsev 1970).

The nomenclature confusion: IUPAC versus common usage

The compounds used in spin labeling have been commonly called 'nitroxides,' although the terms iminoxyl or nitroxyl have been used in the literature as well as an occasional use of the term *aminoxyl*. IUPAC RNRI Rule RC-81.2.4.D rules define compounds having the structure $R_2NO^{\cdot}$ as 'radicals derived from hydroxylamines by removal of the hydrogen atom from the hydroxy group, and they are in many cases isolable.' Nitroxide is the parent name used by Chemical Abstracts Service for $H_2N–O^{\cdot}$, e.g., $(ClCH_2)_2N–O^{\cdot}$ bis(chloromethyl) nitroxide. The IUPAC name is bis(chloromethyl)*aminoxyl*. Nitroxides should not be used as a class name for *aminoxyl* radicals. As for iminooxy/iminoxyl radicals, this term has been used improperly for alkylidene*aminoxyl* radicals, also called iminoxyl radicals, R2C=N–O$^{\cdot}$, and its use is strongly discouraged. If one goes to the Sigma/Aldrich catalog, the spin probe TEMPO is listed as 2,2,6,6-tetramethylpiperidine 1-oxyl. The most inappropriate term for these radicals, nitroxides, is the most widespread and has been cited about 115,000 times, nitroxyl about 29,500 times, iminoxyl (initiated by E.G. Rozantsev) about 4,150 times, and *aminoxyl*, the most correct, just 3,910 times. It is clear that the correct nomenclature that the spin-label community should be using is to classify these compounds as *aminoxyl* radicals. Let us, from this point forward, attempt to correct this error in the future and abide by the IUPAC rules.

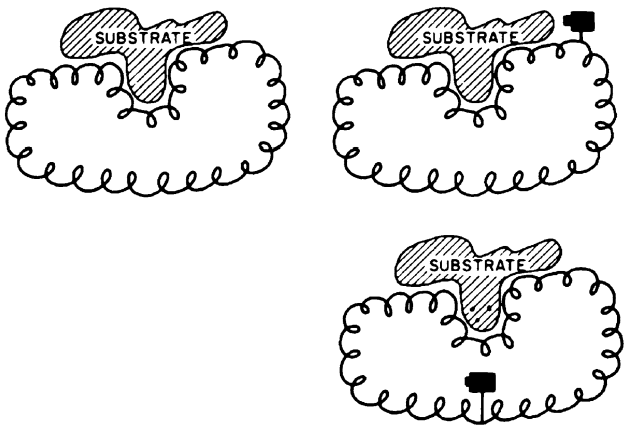

Fig. 1 Schematic representation of enzyme–substrate complex in native protein, protein-containing reporter group (*solid black area*) adjacent to substrate binding area and reporter group distant from substrate-binding area [from Burr and Koshland (1964) with permission]

Spin-labeled proteins

To say the least, the protein labeling field really 'took off' in McConnell's group with a large team of group members assigned to a range of important proteins and enzymes (Stone et al. 1965; Griffith and McConnell 1966). We literally bought every enzyme and protein in the Worthington Biochemicals catalog and applied the maleimido and similar protein-modifying spin-label reagents. At the time, we had no convenient cold room in the Stanford chemistry department, yet we needed a place to run our experiments in the cold. One of the laboratory members modified a refrigerator to accommodate a magnetic stirrer. In order to keep the AC power on inside the fridge, he drilled a hole in the side of the door opposite the plunger type switch so that the light and other AC power stayed on inside the fridge. What a mishap when the cooling did not function very well and the protein solutions eventually significantly denatured from the heat of the light! We had to reorder the Worthington catalog again! Nonetheless, several interesting results were found, including some fascinating results with my enzyme, α-chymotrypsin, related to its mechanism.

Alpha-chymotrypsin

After reading a plethora of papers, particularly related to its mechanism as an esterase and a protease by Carl Neiman at Caltech and others, my mission was to synthesize spin-label *aminoxyl* radical analogs of active site-specific, directed labels (either substrate or inhibitors). Never really having been an avid organic chemist (but having a synthetic chemist roommate also working on his Ph.D.), I had the sufficient support system and advice to proceed ahead. The classical textbook example with chymotrypsin, introduced by Myron Bender and colleagues, was the *p*-nitrophenylacetate reaction, where a stoichiometric enzyme-acyl intermediate is formed in the mechanism since esterase hydrolysis by chymotrypsin (Bender and Kezdy 1965). We synthesized an *aminoxyl* radical nitrophenylester analog as shown below in Fig. 2. The enzyme took a 'bite' of the bait,

particularly at low pH where the acyl intermediate was very stable, due to the pH dependence of the next step (i.e., hydrolysis of the ester link between Ser195 of the enzyme and the acyl*aminoxyl* radical moiety). These results were found just before the holiday period in late autumn 1965, and our goal was to measure the kinetics of deacylation to see how this correlated with previous experiments on various substrate analogs (Berliner and McConnell 1966). This work was, in reflection, a seminal advance as it was the first time that the spin-labeling technique was applied to a 'real' enzyme mechanism/structure problem, where one could observe both the dynamics and binding of a 'substrate' with the enzyme. Reflecting back on the original maleimide spin-label screening of various proteins and enzymes, we also found an interesting result, i.e., there were two overlapping spectra: a minor weakly immobilized spectrum that probably reflects partial, non-specific amino group labeling and a strongly immobilized component that, upon raising the pH above 7.0, underwent a first-order decay reminiscent of the same type of acyl-enzyme hydrolytic decomposition found with the spin-labeled nitrophenyl ester. Figure 3 shows the structure of the maleimide spin label as well as the isomaleimide derivative that also results in the synthesis (Barratt et al. 1971). Note that both cyclic structures can be cleaved at the ester (or amide) linkage to yield the same acyl-enzyme intermediate. Consequently, we were convinced that we had found a new aspect of α-chymotrypsin specificity and reaction mechanism from a spin-labeling experiment.

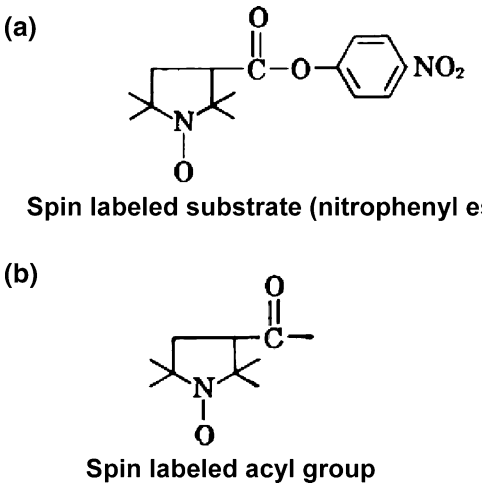

Fig. 2 Spin-labeled substrate (*R,S)*-2,2,5,5-tetramethyl-3-carboxy-pyrrolidine-*p*-nitrophenyl ester (**a**). The acyl-*aminoxyl* radical group that is covalently linked to Ser 195 of α-chymotrypsin (**b**). It was later found that the "specificity" for a spin-labeled acyl-chymotrypsin was the *S*-enantiomer, although both enantiometers can be isolated as acyl enzymes (Flohr and Kaiser 1972)

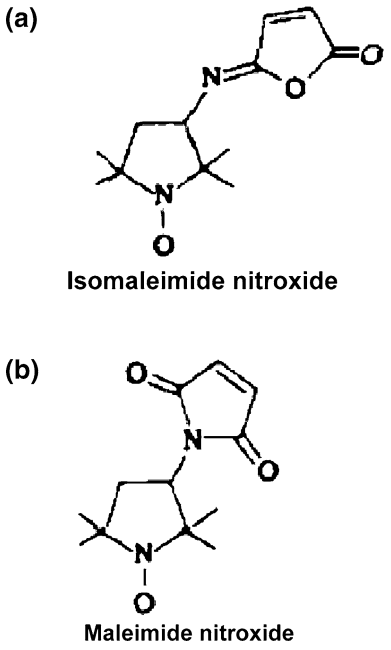

Fig. 3 Structures of isomaleimide (**a**) and maleimide (**b**) *aminoxyl* radicals (Barratt et al. 1971)

Spin-labeled protein crystals

Since the X-ray crystal structure of α-chymotrypsin had been recently reported, we had the potential to extrapolate the techniques of paramagnetic radicals in host crystals to a biological system, where the host crystal was the enzyme structure and the paramagnet was the strategically placed spin label analog. My mission was to learn how to grow protein crystals, spin label a crystal and then do a full hyperfine tensor analysis in order to learn something about symmetry in a protein crystal where the crystallographic (crystal form or space group) symmetry did not reveal these relationships. Briefly, α-chymotrypsin forms a monoclinic crystal system with 4 molecules per unit cell (2 dimers/unit cell) where the dimer was found to have twofold symmetry after the X-ray structure was revealed. This was revealed by a method called the rotation function, developed by Michel Rossman and coworkers (Tollin et al. 1966). Our goal was to confirm the same non-crystallographic symmetry by single crystal EPR where an enzyme molecule was the host for a bound paramagnetic analog. To the best of our knowledge this was the fist time that the spin-labeling technique had been applied to an oriented, 'active' enzyme intermediate (Berliner and McConnell 1971). As we shall note later, the particular pyrrolidinyl spin label (shown in Fig. 2) was enantiomeric, and the acyl enzyme intermediate was formed from a racemic mixture of two, *R,S*, *p*-nitrophenyl esters (Flohr and Kaiser 1972). Since we were fairly sure that we were observing only one of the enantiomeric acylated forms (although we did not know which one at the time), the result was a

confirmation that the enzyme preferred one enantiomer over the other. Later, at Ohio State University, Roger Bauer separated and prepared both enantiomers and carried out single crystal EPR studies of each labeled derivative. He then translated the spin-label orientation into the published crystal structure, a method that we should have coined as spin label isomorphous replacement. It was clear from the results why the *R*-enantiomer was turned over more rapidly than the *S*-enantiomer as the carbonyl moiety of the acyl group in the latter was poorly oriented for nucleophilic attack by the hydrolytic water molecule (Bauer and Berliner 1979).

Serine proteases

It appeared that one of my callings was to synthesize enzyme-specific spin labels (although I was a physical chemist by training, not really a practicing synthetic organic chemist). We learned from the initial enzymology and crystal structure work that sulfonylfluoride-containing compounds were very specific irreversible inhibitors for α-chymotrypsin and other serine- and cysteine-containing pretenses. With the help of the Eastman, Aldrich and Sigma chemicals catalogs, we undertook a broad synthetic scheme involving every *aminoxyl* radical compound commercially available at the time with every sulfonyl fluoride analog and studied them with α-chymotrypsin and then later with other enzymes in this family: trypsin, elastase, some bacterial proteases such as subtilisin and eventually the blood clotting enzyme, thrombin. The aim was to 'compare' their active site structures and conformational environments from the perspective of the catalytic triad surrounding Ser195 in their respective, highly homologous three-dimensional structure(s). While the X-ray structures were known for almost all of the enzymes in this family, it is important to point out that having a method that can 'look' at solution structure is invaluable in that dynamic aspects are clearly revealed. Our work also took us into the other analogs, particularly the phosphofluoridates, found more commonly in chemical warfare agents targeted for acetycholineesterase, which were, nonetheless effective irreversible inhibitors for the serine proteases. In later years, at Ohio State University, we looked at urea-induced protein unfolding of this intermediate where one could monitor the intimate active site structure and interactions from a local perspective. The correlation with CD and other spectroscopic techniques as monitors of this unfolding was interesting as each technique measures a different region of the topography many time, both global and local.

Nitroxides are not toxic

There were also some preliminary 'in-vivo' experiments run in the laboratory in the very early years. In order to show that *aminoxyl* radicals were not toxic, several grams of something like di-*tert*-butylnitroxide were dissolved in a goldfish tank, and the mortality of the occupants was monitored on a daily basis. Needless to say, while the goldfish encountered another fate, it was clear that it survived the relatively high *aminoxyl* radical concentrations in its environs. Since we were frequently carrying out GC separations of *aminoxyl* radical precursors and products, as well as subjecting ourselves to the possibility of ingesting these materials, one of my laboratory mates (heretofore unnamed) checked the EPR spectra of his urine over several days to monitor its accumulation. While there were no published data (and I believe to this date, no documented human studies have been published), he did find detectable concentrations.

Scientific colleagues

While my venture in the field involved moving on (postdoctoral years and then an academic position), it is important to note several people, specifically my laboratory mates at Stanford, who eventually established themselves as key players in the biological magnetic resonance field. One was Seiji Ogawa, who joined the McConnell group just a year after me, working on the hemoglobin problem. Seiji eventually went to Bell Telephone Laboratories where he 'discovered' and developed susceptibility imaging that was the key to functional MRI. Brian Hoffman worked on the superconductivity problem at Stanford, but after moving to Northwestern established himself as a key player in ENDOR studies of metalloproteins. I also cannot omit the fact that Varian had a staff of key EPR scientists, many of whom left for academic positions prior to the EPR division closing down; in particular, Jim Hyde and Larry Piette were people that I would encounter in my later career, both as colleagues, collaborators or friendly competitors. Lastly, Wayne Hubbell and I studied similar problems and worked together closely over the years. Besides teaching me how to fix cars, Wayne loved to do organic synthesis and was of great help and encouragement to my synthetic ventures. As we shall see later, Wayne pioneered an important advance in the spin-labeling field about a decade later after we developed perhaps one of the most versatile spin labels ever reported (Berliner et al. 1982).

The Ohio State years

Spin labeling as a technique was increasing in popularity, and its applications were spreading. It was important during this era to properly represent and defend the technique with the purists, who would continually utter the words: *perturbant, perturbant, perturbant*. The NMR spectroscopists in

particular would note that, after all, a stable isotope on a probe was no perturbant. We were continually bombarded with proving that this technique was not *making the news* instead of *reporting the news.* Yet the field fortunately flourished, both in the protein arena, nucleic acid arena and especially membranes and synthetic bilayers. In fact, the NMR community started to employ spin labels as convenient surveying tools for intramolecular distance measurements in proteins (Battiste and Wagner 2000). The other misconception was that, since an *aminoxyl* radical is a free radical, it was carcinogenic and would never have any potential in in vivo studies. Little did those people know that *aminoxyl* radicals, particularly TEMPOL, would eventually be shown to be a tumor protectant and life enhancer (Mitchell et al. 2003)!

Spin labeling: theory and applications

As the field expanded, it became clear to me that there was not a comprehensive textbook or manual on spin labeling from the theoretical, instrumental, synthetic and biochemical perspectives. While Eduard Rozantsev had published a nice tome on synthesis and chemical properties of aminoxyl radicals (Rozantsev 1970), the full picture oriented at the graduate student level was not available. Since I wanted something written that I could fully understand, I decided to edit a volume entitled *Spin labeling: theory and applications* and was fortunate enough to find Tom Lanigan, then at Academic Press, to sponsor my volume. The criterion for the book was that it had to be at a student level and talk about the methodology, successes and pitfalls of the method, similar to the philosophy of the Methods in Enzymology series. My friends Hayes Griffith and Pat Jost also inspired me, as they had written a chapter on instrumental methods in EPR that they nicknamed 'EPR for Idiots,' which we adapted and reprinted in *Spin labeling*: *theory and applications* (Jost and Griffith 1971). The most important hint, when editing a major book, is to enlist well-known experts (who are also close friends if at all possible). After some brutal editing and prodding (and almost losing a few close friends!), we produced a book in 1976 that somehow became the 'bible' in the field (Berliner 1976). I was very honored and touched when Renad Zhdanov initiated and organized translating into Russian by Mir Press and probably sold more volumes in the Soviet Union than outside, mostly due to the price of 6 rubles versus $49.95 (Berliner 1979a). This eventually spawned three more volumes over the years (Berliner 1979b; Berliner and Reuben 1989; Berliner 1998), and a fifth volume is planned by the end of the current decade. While the first volume was published with a selfish, but important, motive, i.e., a book that I could understand, it was amazing how often it was cited, which meant that other people had the same need for this type of textbook. My 'dog-eared' copy still exists, one with the cover almost totally ripped away by people in the laboratory who do not treat books with as much respect as do I.

Since I value high quality people over business, when my editor and friend Tom Lanigan moved to Plenum Publishing, I followed him there, and, together with magnetic resonance pioneer Jacques Reuben, initiated *Biological magnetic resonance.* The series will soon approach 30 volumes of critical reviews in the NMR and EPR arena, including a coedited volume with Marcus Hemminga (Hemminga and Berliner 2007). Tom Lanigan left Plenum later to start Humana Press with his wife Julia, creating phenomenal success that Springer (Plenum's eventual parent company) recently acquired. Unfortunately, Tom passed away about 3 years ago, but the Humana philosophy of excellence remains.

New protein-specific spin labels and the origins of site-directed spin labeling

We found that our series of serine protease sulfonyl fluoride inhibitors were valuable reagents for comparing and contrasting the family of homologous serine proteases that eventually led us into the field of thrombosis via the blood clotting enzyme, thrombin (Berliner 1990). An important accomplishment for the spin-label method was finding important hydrophobic binding regions that were coupled to the active site when the thrombin X-ray structure was not available until several years later. In later years, X-ray structures of spin-labeled thrombins were reported, which remains one of the few spin-labeled protein X-ray structures available (Nienaber and Berliner 2000; Nienaber et al. 2000). In the interim we were working on the enzyme, galactosyl transferase, which was difficult and expensive to isolate, but contained a key sulfhydryl group near the active site. Our aim was to spin label this cysteine group in order to learn more about the structure and conformation of the enzyme; the protein was so valuable that the label needed to be both reversible and specific. To date, the only spin labels that are somewhat more specific for thiols are the maleimide *aminoxyl* radicals that result primarily in irreversible labeling (Berliner 1983). The advantage of reversibility would also allow us to double check the labeling stoichiometry upon release of the free *aminoxyl* radical. A colleague, George Kenyon, who had developed a reversible thiol blocking reagent called methylthiomethanesulfonate for creatine kinase and other enzymes (Kenyon and Bruice 1977), suggested to me that we substitute the methyl group with an *aminoxyl* radical ring. After several months of amateur synthetic organic chemical approaches, my colleague Kalman Hideg made one of his annual lecturing visits from Hungary to the US where he regularly visited our laboratory, displaying the hundreds of new reagents that he, Olga

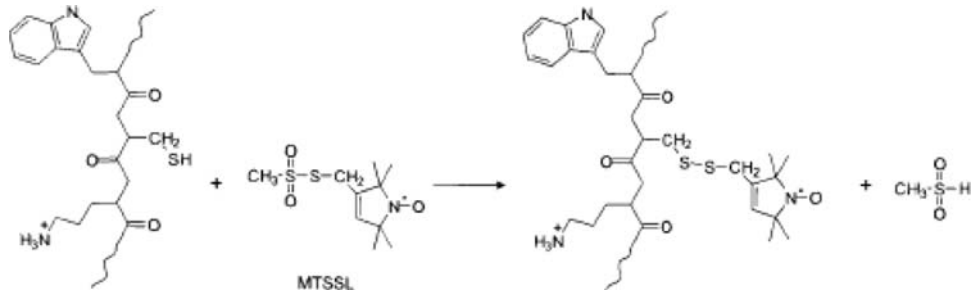

Fig. 4 Labeling chemistry of MTSSL with cysteine side chains. Note that the MTSL moiety can be removed with DTT or mercaptoethanol to yield the thiol *aminoxyl* radicals and free cysteine. The molecular volume of the MTSL moiety is similar to that of the tryptophan side chain [Adapted from Feix and Klug (1998) with permission]

and his laboratory group had created during the interim. Amazingly, I saw just the precursor that we needed for a pyrollidinylmethanethiosulfonate spin label, later abbreviated as MTSL or MTTSL. I asked Kalman to synthesize this compound product by one additional step beyond his precursor. In a story of international conspiracy that would not occur during current times, we arranged an exchange at Toronto airport in the summer of 1980. He and I were attending different scientific meetings in the area, and Kalman was en route back to Hungary. The key experiments were actually done during a minisabbatical in Haifa with Jacob Grunwald, a former postdoctoral student. Our results were eventually published in 1982 in *Anal Biochem* (Berliner et al. 1982). An initial submission to *Biochemistry* was not found 'appropriate' for their journal. Wayne Hubbell called me a few months later with intense interest in synthesizing and utilizing this spin label with site-directed mutagenesis of strategically placed cysteine groups in proteins as shown in Fig. 4.

Site-directed spin labeling

The technique, which quickly became known as site-directed spin labeling, or SDSL, has seen a profusion of publications since 1990 and is frequently the subject of specialized symposia at scientific conferences (Altenbach et al. 1990; Hubbell and Altenbach 1994). This was a real renaissance in spin labeling of proteins and spawned some additional thiol-specific, reversible spin labels. As to the journal that initially did not find this spin label appropriate, it appears that the largest numbers of SDSL studies are published in *Biochemistry*. However, it is important to note that scientific discoveries involve serendipity, connections and also friends. We would have never embarked on the synthesis were it not for the advice and collaboration with George Kenyon, Kalman Hideg and especially Wayne Hubbell, who had the foresight to see the enormous potential with molecular biological techniques. Needless to say, SDSL is truly a major advance in the field of protein structure in solution, particularly for membrane proteins that frequently elude crystallization. The major challenges, as well as pitfalls, have been finding spin labels that do not adopt multiple orientations or conformations that may contribute to ambiguities in the structural interpretations. Some progress has been made with devising 'multipoint' attachment labels, either by covalent or hydrogen bonding interactions, where the number of conformations are minimized. The ultimate challenge for the synthetic organic chemists is finding other stable paramagnetic molecules that may be adapted to the spin labeling/reporter group technology. To date, the challenge has been daunting, with only trityl-based radicals showing some potential.

In-vivo EPR

The most recent area for us involved in vivo EPR methods, i.e., the possibility of carrying out experiments directly on animals or possibly humans. It seemed clear to us that, if MRI/MRS could apply NMR to living systems, why could one not image paramagnetic materials in living animals? Fortunately, there had been some courageous pioneers in this field who preceded us, including Barry Commoner at the Washington University in the 1950s and 1960s and some unpublished studies by Rowlands and colleagues at the Southwest Research Institute on whole animals (Commoner and Hollocher 1960). But the key publications that inspired our laboratory to move forward were from Larry Piette's laboratory in Hawaii where an in vivo spectrum of an *aminoxyl* radical in the liver of a living rat was demonstrated at X-band (Feldman et al. 1975) and from Stan Lukiewicz (on sabbatical at the time in Jim Hyde's laboratory) who observed the melanin radical signal in a tumor implanted in the tail of a mouse in an S-band cavity (Lukiewicz and Lukiewicz 1984). Our goal was to expand upon these early discoveries with the aim of also imaging the paramagnetic material(s) in vivo. It quickly became clear to us that the microwave heating observed by Piette at X-band would be an unsurmountable obstacle; in addition, S-band was not suitable for highly aqueous tissue with the exception of the tail of a mouse or rat. It became clear that

L-band (1 GHz and below) would not induce microwave heating, and, modifying the helix coil resonator technology from Larry Piette's laboratory (Feldman et al. 1975), Hiroyasu Nishikawa in our laboratory developed the single-turn L-band flat loop coil (which later was used as a surface coil). The work blossomed even more with the arrival of Nishikawa's good friend and colleague, Hirotada Fujii, who together showed over the years that we could both observe and image nitroxyl radicals in plants, tissue and living systems at L-band (Nishikawa et al. 1985). This culminated with an extremely important collaboration with Stan Lukiewicz, who brought us an excellent murine tumor model. In a very short time, we demonstrated that we could obtain a two-dimensional image of *aminoxyl* radicals in the circulation of a large tumor implanted in the tail of a mouse (Berliner et al. 1987). We expanded our resonator portfolio to loop gap resonators after several visits to Milwaukee and Krakow, by copying the designs of Froncisz and Hyde (1982), both of whom personally helped us immensely. The technological sophistication in our laboratory was greatly enhanced by the arrival of Janusz Koscielniak, sent to us by Stan Lukiewicz from Poland. It was pleasing to see the work from several other laboratories expanded on in vivo EPR imaging technology in the 1990s and beyond with much more sophisticated technology developments. All of these laboratories were collaborators and good friends who frequently exchanged visits with us. Some of the most impressive developments were the implementation of oxymetry in EPR imaging (Halpern et al. 1994), oximetric images of a beating heart (Zweier et al. 1998) and the design of pulsed FT imaging techniques at NIH (Subramanian et al. 2003). When our funding ran out, Janusz moved to the NIH where he worked with the FT EPR imaging group. As to accomplishing the ultimate goal of carrying out EPR on humans, my close friend and prolific colleague Hal Swartz demonstrated the efficacy of monitoring oxygen tension below the skin, particularly in cases of deep vein thrombosis in diabetics (Swartz et al. 2004). The list of contributors has now become so extensive that I apologize for any names omitted by oversight; however, we have thoroughly covered the subject in a recent Biological Magnetic Resonance volume (Berliner 2003).

The Denver connection

After almost 32 years at Ohio State, I decided to go west, like the pioneers, to confront the challenge of heading a small department in a medium-size, private university in Denver, a city where free radical-related research was active at several other institutions (Webb-Waring Institute for Antioxidant Research, University of Colorado Health Science Center, National Jewish Hospital Research Center). My goal was to establish a Free Radical Research Center with state-of-the-art equipment and techniques. While this was not feasible at my home institution, University of Denver, I was invited to join the graduate toxicology program at the University of Colorado School of Pharmacy and initiated a thematic, pedagogical free radical seminar series and established CO ORS, the Colorado Oxygen Radical Society.

As to new research avenues, we had just finished developing some new methods at Ohio State for observing free radical chemistry via NMR and MRI. Since most reactive oxygen species are short lived and the sensitivity limitations of EPR were frequently too restrictive, the concept was to observe the (bioreductive) chemical fate of nitrone-based trapped radicals since it was well known that the nitrone adduct EPR signal decayed fairly rapidly (seconds to 10–20 min) in-situ and even faster in vivo. By utilizing 31-P based nitrones, i.e., commercial DEPMPO, or 19-F based nitrones from Valery Khramtsov's colleagues in Novosibirsk, we together with Tom Clanton (Department of Pulmonary and Emergency Medicine, Ohio State) and Hirotada Fujii developed two techniques called NMR spin trapping and MRI spin trapping (Khramtsov et al. 1999; Fujii et al. 1999). We also discovered that some nitrone adducts could be formed not from radical reactions, but from nucleophilic addition side reactions that resulted in diamagnetic 'adducts' that were easily oxidized to a paramagnetic product (Fujii and Berliner 1999). These 'artifacts' had been observed in vitro and noted earlier by others, but we were finding quite rich signals in living animals (Berliner 2003). In one case, with the antihypertensive drug, nifedipine, we found it resulted in strong lipid radical adduct signals in the liver, heart and other organs after administering the drug to mice (Fujii and Berliner 1999). While the paramagnetic state was created 'after the addition,' there was an FDA warning to patients taking fast-acting nifedipine with relation to a much higher susceptibility to myocardial infarction (Psaty et al. 1995). Our current, on-going studies are to determine whether this drug enhances lipid peroxidation steps that eventually lead to cardiac-related disease states. The other exciting results were showing how one could visualize nitric oxide in vivo in septic rats, in collaboration with Fujii, graduate student Xiaoming Wan and collaborators and then later at Pierre Robitaille's 8-T MRI site at Ohio State (Fujii et al. 1999).

Scientific challenges awaiting the next generation of EPR spectroscopists

From this author's perspective, it is imperative that other spectroscopic techniques be combined with EPR. As to addressing the great frontiers in biology, free radical biology

and medicine have great potential. Nitrone spin traps, which are precursors to *aminoxyl* radicals (i.e., spin labels), yield *aminoxyl* radical adducts upon reaction with a variety of biological and organic radicals. The resultant EPR spectrum can be characterized as identifying the radical that combined with the nitrone. By strategically placing nitrone 'spin labels' at particular sites in a biological system, one has the possibility of trapping radicals at some local site of interest.

The other major problem remains with sensitivity. While the EPR signal of a radical is upwards of 600–1,000 times more sensitive than, e.g., a proton in NMR, the minimum concentration limits for EPR are still too high for most biological problems. By reducing the linewidth, e.g., with 15-N perdeuterated *aminoxyl* radicals, the signal to noise can be increased by several fold, but still less than an order of magnitude. Where the future will lead us depends almost entirely on synthetic organic chemistry and, as mentioned earlier, the development of yet other stable paramagnetic species beyond *aminoxyl* radicals. Hence, the development of other radical centers that afford the same synthetic versatility, sensitivity and dynamic specificity as *aminoxyl* radicals remains the challenge. The previously mentioned trityl-based radicals have line widths that are narrower than *aminoxyl* radicals by one or two orders of magnitude.

The SDSL method allows one to determine distances to be determined from the dipole–dipole interaction between two paramagnets that are strategically placed within 8–25 Å of each other. This methodology involves incorporating a spin label at two specific cysteines followed by lineshape analysis for dipolar contributions (Rabenstein and Shin (1995). The distances obtained are fairly accurate (to within 10%). However, more recent instrumental and methodological advances have extended these distances up to 50 Å and beyond. The Freed laboratory at the NIH Center at Cornell University (ACERT) has developed capabilities for higher sensitive pulsed EPR methods that preclude the need for freezing samples in order to enhance the dipolar interactions (Borbat and Freed 2000; Borbat et al. 2001). They utilize double quantum coherence (DQC), a powerful application of pulsed EPR that permits detection of weak dipolar interactions between two *aminoxyl* radicals. Oscillations in echo amplitude versus echo time are due to this dipolar interaction. A detailed analysis of DQC indicates that distances can be measured to ~80 Å, comparable to the capabilities of fluorescence energy transfer (Borbat and Freed 1999). A recent, exciting approach utilizes a method based on double electron–electron resonance (DEER) spectroscopy where it was shown that one could measure forces between small protein domains using a doubly labeled model peptide from the α-helical coiled-coil leucine zipper (Gullà et al. 2009). Hence, this methodology can be extended into molecular assemblies and molecular motors where accurate distance changes are important. The future is bright, albeit the equipment is more complex and expensive than commercial continuous wave spectrometers.

Lastly, combining EPR with NMR, fluorescence and other physical techniques may open other more powerful probes of biological systems. As mentioned earlier, Wagner has fostered a renaissance in this area (Battiste and Wagner 2000). Several laboratories have taken advantage of the dynamic nuclear polarization effect for enhancing nuclear spin signals and structure/distance/mechanism studies in several protein systems by irradiating strategically placed *aminoxyl* radicals with strong microwave radiation in an NMR experiment, either by solid-state NMR (Mak-Jurkauskas et al. 2008) or hyperpolarizing just the substrate nuclei in an enzyme–substrate reaction. (Bowen and Hilty 2008) With fluorescence spectroscopy, the creation or disappearance of a radical center that is close to certain fluorophores can 'turn off' or 'turn on' the fluorescence signal (Lozinsky et al. 2004) as well as measure distances, allowing for a more sensitive probe of radical centered events (Zhu et al. 2005).

Prospective

Since the EPR equipment and expertise available at the University of Denver are not suitable for in vivo work, we maintain our connections with the premier laboratories at NIH, a new center at Ohio State and other close academic friends in the region. As I complete this article, I am transitioning to spend a sabbatical year abroad learning and pursuing new biological EPR problems that I cannot accomplish at University of Denver. The other relief is stepping down from almost 8 years as department head, a job that severely taxed my time and prevented me from pursuing new research challenges in what was a fairly limited environment. We are also embarking on some new *BMR* volumes for Springer, as well as some unique publishing projects that are still in the conceptual stage.

While I have more than once concluded that spin labeling had run its course and had reached a dead-end (although we are quite familiar with fields and technologies where people continue to beat a dead horse; i.e., like measuring a relaxation time at every conceivable frequency and temperature or a physical parameter to the fourth decimal place). Spin labeling continues to flourish. A fifth volume (precise volume title to be decided) will be conceived over the next year as a revival of the first, textbook, volume with the latest in state-of-the-art approaches and methods and details. As to the *BMR* series, we follow the science and new approaches wherever it leads us, and I have a strong intuition that some new, highly sensitive approaches will 'come over the horizon' in the next 5–10 years, particularly coupled to optical methods.

My other endeavors relate to the future of the magnetic resonance research area and the future of science support by government agencies in the future. This has been steadily eroding in most countries (on a per capita basis) and can politically be a sacrificial lamb when the economy worsens. Since funding is an investment in future technology and economic growth in any country, it is imperative that our legislators and parliamentarians are continually educated about advances in science and the benefits to society. Over the past 5 years, I have been actively engaged in lobbying the US Congress and visiting our federal science agencies to discuss priorities for the future. This is an important activity that, although requiring an indeterminable amount of patience, is crucial to the survival of our establishment. I encourage other scientists to participate in similar advocacy.

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