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The use of nitroxides in viable biological systems: an opportunity and challenge for chemists and biochemists

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Abstract - This article attempts to provide an overview of recent developments in the use of nitroxides to study viable biological systems by magnetic resonance, especially electron spin resonance (ESR or EPR) techniques. These magnetic resonance approaches include studies in intact animals and imaging in up to four dimensions. The goal of this article is to stimulate chemists and biochemists to participate in such studies in order to enlist their creative input into the optimized development of nitroxides for these applications.

EXPERIMENTAL CONSIDERATIONS

Most biological systems of interest do not have sufficient amounts of paramagnetic materials to permit the full range of possible direct studies by ESR. In order to take advantage of the capabilities and sensitivity of ESR, therefore, stable paramagnetic species often are introduced into the system of interest. In most cases the paramagnetic species used have been nitroxides because of their stability and the sensitivity of their ESR spectra to environmental factors of interest such as motion, redox potential, etc. Over the last 30 years an extensive literature has been built up on the synthesis, properties, and application of nitroxides. Recently there also has been a large number of studies on their properties in functional biological systems. Consequently, as techniques have developed that enable ESR studies to be carried out in macroscopic objects such as tissues and intact animals, investigators also usually have used nitroxides for these studies. In general the uses of nitroxides in functional biological systems have been very productive, although, as indicated in more detail later in this article, the metabolic and physical interactions of nitroxides with cells and tissues pose some formidable challenges. For reasons of convenience, this article is keyed to publications of our laboratory; the references within these publications should provide access to the full literature on the subject. As the nature of the interactions between biochem systems and nitroxides have become understood better, the interactions often have been able to be exploited to gain new and sometimes, unique information (refs. 1,2).

One of the most noticeable experimental constraints on the use of nitroxides in functional biological systems is the ability of cells to reduce nitroxides to the corresponding non-paramagnetic hydroxylamines. Further study has shown that this is an enzymatic reaction and that the reverse reactions also can occur enzymatically (ref. 3). The rates of these reactions are affected by biological variables of considerable interest, especially the concentration of oxygen (ref. 4), the redox state of the cell (ref. 5), and the intactness of the membranes (ref. 6). Consequently, although metabolism of nitroxides can cause great experimental difficulties, when understood and controlled this metabolism can be used to obtain information that cannot readily be obtained by other means.

Another major experimental constraint is that functional biological systems usually have large amounts of water and therefore cause non-resonant absorption of the electromagnetic radiation that usually is used for the ESR experiments (ref. 7). This constrains the size and shape of the sample severely at the usual frequency (9GHz or "X-band"), limiting samples to a maximum diameter of about 1 mm. At lower frequencies (eg. 1.1 GHz or "L-band") this is less constraining but there is a loss of sensitivity.

Techniques to obtain ESR images have been developed recently, usually using nitroxides as the species that is imaged. This capability provides many new and potentially important experimental approaches using nitroxides and ESR. The imaging can be done at 1-4 dimensions including a spectral dimension (ref. 8). The technical demands for imaging hardware and software do not appear to be limiting and solutions to problems often can be found in prior work done with NMR. The principal technical constraints on imaging in biological systems are the amount of nitroxide that can be introduced without causing unacceptable line broadening and/or perturbing the biological system: and the ability to obtain sufficient penetration of the electromagnetic field. As implied above, this may limit imaging to high frequencies in order to have sufficient sensitivity, and the use of high frequencies will

limit the size of the sample that can be studied. Within these restraints, however, there are some very useful and important experiments that can be carried out.

Another major new advance has been the development of techniques to obtain ESR spectra from whole animals (ref. 9). These techniques usually require the use of lower frequencies (exception - the study of nitroxides in the blood or tissues of the tail of mice (ref. 10)). At lower frequencies spectra from small animals can be obtained by the use of large diameter resonators; for animals or tissues these usually are about 20 mm in diameter but in principle could be larger. Surface probes with sensitivity that extends to depths of 10 mm or more have been developed recently (ref. 9). These have been applied to mice, where they have been used successfully to obtain localized spectra from various areas within the mouse. In principle this technique could be applied to any size animal, including patients; the magnetic field required for these experiments, 300-400 gauss, is readily obtained with sufficient size and homogeneity. The potential limiting factors would be how deep from the surface spectra could be obtained and whether the nitroxides are sufficiently nontoxic for use in humans. Even if no further depth of sensitivity can be achieved, there are a number of potentially valuable clinical studies that could be carried out on tissues within 10 mm of the surface.

Although less spectacular, another important experimental advance has been the development of techniques to obtain high quality ESR spectra from viable cells as suspensions, concentrates, etc under conditions that permit adequate control of the environment of the cells. The techniques include the use of perfusion systems and gas permeable tubing to provide well characterized and controlled physiological conditions.

SUMMARY OF EXPERIMENTAL RESULTS IN FUNCTIONAL BIOLOGICAL SYSTEMS

This section attempts to provide a brief overview of the most promising uses of nitroxides in functional biological systems. The original motivation for using functional biological systems in ESR experiments was to study physical-chemical phenomena such as motion in membranes or electrostatic potentials in the more complex environments of living systems. These studies employed nitroxides as spin labels to probe biophysical phenomena in ways that closely parallel their use in model systems. This remains a very important set of applications and, as noted above, such measurements now may be possible in intact animals. It gradually has become clear that these techniques also could provide information on the status of complex biological functions, such as intactness of membranes, viability of cells, and redox metabolism.

The observation that the spectra of nitroxides could be used to follow the concentrations of oxygen ("$[O_2]$") in biological systems has led to an extensive and growing use of nitroxides for this purpose (ref. 11). Such ESR oximetry appears to be a method of choice for the measurement of $[O_2]$ in small samples where size of the sample and/or the problems from stirring make the use of Clark electrodes problematic. Of potentially even greater importance, is the use of nitroxides to measure $[O_2]$ in tissues and intact animals; ESR may provide the best way to make such measurements and this has great clinical as well as experimental potential. ESR oximetry also has provided some of the best data on intracellular $[O_2]$ (refs. 12,13).

The use of nitroxides as labels to follow the metabolism and distribution of spin-labeled drugs and structures such as antibodies and liposomes is another area of application that already has been productive but which might, by useof in vivo ESR techniques, become of widespread importance.

The use of nitroxides as imaging agents for ESR has rapidly become established as the method of choice. Uses include the measurement of rapid processes such as diffusion (ref. 14), imaging in 1-4 dimensions, including a spectral dimension (ref. 8), and a closely related technique -- in vivo ESR spectroscopy from localized regions of animals (and, potentially, patients) (ref. 9).

Another use with considerable potential is as contrast agents for NMR. With the development of NMR imaging ("MRI") as an important clinical tool, the use of contrast agents has become a routine clinical procedure. Nitroxides have some potential advantages as contrast agents, especially for experimental studies but perhaps also for clinical uses. Unlike the paramagnetic metal ions that are usually used for this purpose, nitroxides offer considerably more chemical versatility (enable them to be targeted more readily to specific areas) and the possibility of obtaining images that reflect redox metabolism and $[O_2]$(ref. 1). Such uses of nitroxides have the potential of combining the diagnostic capabilities of MRI with the versatility and biological sensitivity of the nitroxides. The use of nitroxides as specific contrast agents (relaxing agents) for in vivo NMR spectroscopy ("MRS") is a closely related use with equally great potential.

CHALLENGES FOR CHEMISTS AND BIOCHEMISTS – NEEDS FOR THE DEVELOPMENT OF NITROXIDES WITH PROPERTIES OPTIMIZED FOR USES IN COMPLEX BIOLOGICAL SYSTEMS

In this section I attempt to outline the key areas where new chemical ideas are needed, and, where appropriate, to provide possible bases for approaching the problems. It seems likely, however, that experienced chemists will come up with more plausible and effective approaches to solving the problems than the suggestions that are offered here!

1. Resistance to reduction

One of the most bothersome and limiting factors in the use of nitroxides is that they often undergo rapid reduction to the corresponding hydroxylamine. While much remains to be learned about this phenomenon, many of the critical aspects now are known (refs. 4,5,15). The reversible reduction to the hydroxylamine is essentially the only metabolism of the nitroxide group in cells and tissues; the reaction is enzymatic, proceeds more rapidly in very hypoxi cells, and probably is due primarily to reducing equivalents transferred from the electron transport chain. The development of nitroxides that are resistent to bio-reduction would be a major step forward in the use of nitroxides in functioning biological systems.

This problem of course, has been recognized by a number of investigators and some good starts have been made towards the development of such nitroxides but much more can and should be investigated. Nitroxides based on pyrrolidine and pyrroline rings are more resistant to reduction than those based on piperidine or doxyl rings (ref. 15). The resistance to bioreduction or reduction in model systems (eg. reduction by ascorbate) does not appear to correlate with measurements of electropotential (ref. 16). Accessibility of the nitroxide group may be a more likely basis for bioreduction; for example in the more readily reducible piperidines molecular models indicate that the nitroxide group can be well out of the plane of the ring and away from the adjacent methyl groups.

These considerations lead to the following suggestions for approaches to the problem of bioreduction. One obvious approach is to increase the steric blocking by having more bulky groups on the alpha carbons. Some such syntheses have been made and the results are encouraging (ref. 17). It may be useful to look for ways to provide a more extensive cage around the nitroxide group. A second approach might be to try to enhance delocalization of the unpaired electron by appropriate resonance stabilizing structures. A third approach might be to try to enhance the reverse reaction: oxidation of the hydroxylamine.

2. Development of hydroxylamines that undergo facile oxidation to nitroxides, especially at rates that are proportional to the concentration of oxygen (or other pertinent biological factors)

The desirability of developing hydroxylamines that can be readily oxidized follows naturally from the discovery of biological oxidation of hydroxylamines and the growing importance of being able to reverse the reduction of nitroxides to hydroxylamines. In cells the enzymatic oxidation of hydroxylamines appears to take place in a lipophilic environment, perhaps on cytochrome c (refs. 4,18). Importantly, the rate of enzymatic oxidation is related to the concentration of oxygen over a wide range of biologically important concentrations of oxygen. Therefore it appears desirable to develop two different types of hydroxylamines: those that will very rapidly be oxidized to nitroxides in order to overcome the problems of bioreduction of nitroxides and hydroxylamines that will provide sensitive indicators of the concentration of oxygen by their rate of oxidation to the paramagnetic nitroxides. It seems reasonable to explore ways to optimize both spontaneous and enzymatic oxidation of hydroxylamines; it seems likely that the mechanisms and hence the structural relationships for the two types of oxidation will differ. The rates of reduction of the resulting nitroxides will be another important factor in the optimal development of these hydroxylamines.

3. Development of nitroxides that localize into specific biological locations

In complex biological systems such as cells or tissues, the value of the information obtained with nitroxides can be increased significantly if the specific location of the nitroxides is known. In simple systems this often is less of a problem because the number of environments or compartments is small and often there are straightforward means to differentiate between these, using techniques such as the addition of paramagnetic broadening agents. Such approaches, however, usually cannot be applied readily to complex systems such as tissues. Using appropriate chemical and biochemical techniques it should be possible to develop nitroxides that would go selectively to the environments of interest. There have been some effective starts in these directions. For example, the use of a permanently charged nitroxide can enable one to probe the extracellular environment in a suspension of cells or the vascular system in a whole animal (ref. 13). Such localization may be enhanced further by putting the nitroxide on a macromolecule that does not readily cross membranes.

A somewhat more sophisticated approach has been to develop nitroxides that selectively concentrate in the intracellular compartment; this approach is based on the use of esterified derivatives that become converted intracellularly to polyanions by esterases and the resulting charged species cannot cross the membranes to leave the cell (ref. 19).

Such studies, however, are only a very incomplete beginning of the types of development that are needed and possible. It would be very desirable to develop nitroxides that would concentrate selectively in the following locations and therefore report on phenomena within them: overall intracellular compartment; extracellular compartment - total, intravascular, or extravascular; plasma membranes; microsomes; mitochondria; nuclei; specific organs or types of tissue including the brain (blood-brain barrier), various organs, macrophage-rich regions, tumors, atheromata. Although this is a very long list, it probably is incomplete and the successful development of nitroxides that would selectively sample any of these environments would almost certainly result in the immediate and productive use of the new nitroxides.

The means to develop selectively localizing nitroxides are likely to differ for each type of localization that is sought. One of the keys may be to use biological processing that occurs predominantly in the area of interest (eg., to localize in lysosomes one might use a step that requires activation by the uniquely low pH of the lysosome). In some locations there may be physical properties that could be used (eg., binding to the helix of DNA to obtain localization in nuclei). There seems to be a good number of potentially useful approaches and the real limiting step probably is to obtain sufficient interest to develop such probes, rather than the technical difficulties of their development.

4. Development of nitroxides with spectral features that very sensitively respond to the concentration of oxygen

As noted above, one of the most important uses of nitroxides in functional biological systems is likely to be the measurement of the concentration of oxygen. Consequently, there already has been considerable progress made in the development of suitable nitroxides for such measurements but it is not likely that all of the optimal probes have been developed. There are several different effective ways to use nitroxides to measure the concentration of oxygen and each of these may be the method of choice in different situations and therefore there are several different types of optimization of nitroxides that are possible for measuring $[O_2]$.

The initial and still quite useful approach is to use the effect of oxygen on the resolution of the "superhyperfine" spectra of nitroxides (ref. 11). This effect depends in part on the amount of localization of the unpaired spin on protons on the ring. The localization probably depends on both geometric and resonance factors, but this subject does not appear to have been treated systematically for this particular application. A very effective new approach has been made by Halpern and co-workers (ref. 20) in which they selectively deuterated the ring of a pyrrolidine nitroxide, leaving a single proton which gave a very measurable splitting that is quite sensitive to $[O_2]$; it would be very desirable to have additional nitroxides using this approach.

The second major approach has been the use of the effect of $[O_2]$ on the width of the principal nitrogen hyperfine lines, usually in nitroxides in which the ring protons have been replaced with deuterons in order to narrow the lines (ref. 13). It would be very useful if more such nitroxides were available and if there were means further to decrease the intrinsic line widths in order to increase sensitivity for the measurement of oxygen.

The third major approach has been to use the effect of $[O_2]$ on microwave power saturation. Although this approach may be less sensitive than the line broadening approaches, it may be more applicable in intact animals and tissues or in environments such as membranes where the motion of the nitroxide is hindered.

The fourth major approach, the use of oxygen dependent metabolism of nitroxides already has been discussed.

5. Development of nitroxides that effectively cause relaxation of protons of water or other nuclei studied by NMR techniques

The use of nitroxides as contrast agents for NMR is very attractive because of the potential of using nitroxides to reflect $[O_2]$ and redox metabolism (ref. 1), and the chemical versatility of the nitroxides that enables them to be used to obtain more specific localization. These applications, however, have ben limited by the problems of bioreduction and relatively low relaxivity. The former has already been discussed. There are two keys to enhance relaxivity of water protons by nitroxides: binding of water to the nitroxides and bringing the protons as close as possible to the unpaired electron. The general approaches to obtain such interactions with water seem straightforward but so far there have not been any significant developments reported. Some increased relaxivity has been obtained by

binding nitroxides to macromolecules, thereby achieving more optimum correlation times for the relaxation of water protons (refs. 21,22). Such binding, in principle, also could place a nitroxide in a favorable orientation to water molecules associated with the macromolecule (ref. 23). In addition to the useof nitroxides for contrast for MRI, they potentially also could be used to provide contrast for NMR spectroscopy (MRS). These uses may be especially valuable because the chemical versatility of the nitroxides could be exploited to obtain selective editing of spectra. For example, using a lipophilic nitroxide, we were able selectively to remove the resonances from methyl and methylene protons in serum and therefore were able to see the underlying resonances due to lactate (ref. 24). There is a need to develop nitroxides that will have the necessary localization and relaxivity to broaden away NMR spectra in specified regions.

6. Development of nitroxides that combine the properties that are appropriate for the particular biological applications

In order to emphasize the particular needs and opportunities for the various types of nitroxides the discussions have focused on the particular characteristics associated with each application. In reality, of course, often nitroxides will be needed that combine several different types of properties. This is especially true in regard to including structural features that control the distribution and metabolism of the nitroxides.

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