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Review article

Imaging free radicals in organelles, cells, tissue, and in vivo with immuno-spin trapping

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ABSTRACT

The accurate and sensitive detection of biological free radicals in a reliable manner is required to define the mechanistic roles of such species in biochemistry, medicine and toxicology. Most of the techniques currently available are either not appropriate to detect free radicals in cells and tissues due to sensitivity limitations (electron spin resonance, ESR) or subject to artifacts that make the validity of the results questionable (fluorescent probe-based analysis). The development of the immuno-spin trapping technique overcomes all these difficulties. This technique is based on the reaction of amino acid- and DNA base-derived radicals with the spin trap 5, 5-dimethyl-1-pyrroline *N*-oxide (DMPO) to form protein- and DNA-DMPO nitroxide radical adducts, respectively. These adducts have limited stability and decay to produce the very stable macromolecule-DMPO-nitrone product. This stable product can be detected by mass spectrometry, NMR or immunochemistry by the use of anti-DMPO nitrone antibodies. The formation of macromolecule-DMPO-nitrone adducts is based on the selective reaction of free radical addition to the spin trap and is thus not subject to artifacts frequently encountered with other methods for free radical detection. The selectivity of spin trapping for free radicals in biological systems has been proven by ESR. Immuno-spin trapping is proving to be a potent, sensitive (a million times higher sensitivity than ESR), and easy (not quantum mechanical) method to detect low levels of macromolecule-derived radicals produced in vitro and in vivo. Anti-DMPO antibodies have been used to determine the distribution of free radicals in cells and tissues and even in living animals. In summary, the invention of the immuno-spin trapping technique has had a major impact on the ability to accurately and sensitively detect biological free radicals and, subsequently, on our understanding of the role of free radicals in biochemistry, medicine and toxicology.

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I was trained in the physical sciences as a physical chemist. Especially in physics, inductive reasoning is commonly used where general principles are derived from particular facts. In my biomedical research, the first question I ask is whether free radical formation is possible. This is really a question of thermodynamics, which can be calculated, but I rely on my chemical intuition. The next step is detecting the free radical. Before I invented immuno-spin trapping, I relied on ESR for this. Once the free radical is detected by ESR, the same experiment can, in general, identify the free radical. The next question is what are the reactions and the rate constants of these free radicals with oxygen, antioxidants, biochemicals, and macromolecules. A vast array of these rate constants have been determined by pulse radiolysis and other techniques. The last question is the most difficult question. What are the critical biological targets of the free radicals? In practice, I work backwards using deductive logic from the biochemical, toxicological and pathological consequences to discern the critical target and, ultimately, the initiating free radical event.

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Many of the best understood human toxicities are generally accepted to be caused by free radicals. These toxicities include ionizing radiation, iron sulfate (the leading cause of pediatric poisoning), oxygen toxicity (common in premature infants), paraquat (classic pulmonary toxicant), daunorubicin (a cardiotoxicant), UVA radiation (skin cancer), and carbon tetrachloride (classic hepatotoxicant). Of these established free radical toxicities in humans, ESR experiments provided evidence that free radical formation is the fundamental, initiating event in all of them. On the other hand, the role of free radicals in human diseases is less definitive, largely because ESR has been unsuccessful in detection of free radicals in disease models. Presumably, in animal models of human diseases, free radical formation is characterized by lower rates of formation over a longer period of time than in acute toxicity models, and a technique much more sensitive than ESR was necessary to demonstrate free radical formation.

The advantages of free radical detection with ESR, which is without question the gold standard of free radical detection, are listed in Table 1. The disadvantages of ESR are listed in Table 2. In the biomedical sciences, the greatest limitation is the quantum mechanical basis of ESR. Quantum mechanics requires higher math and physics. People not trained in ESR have, in fact, been limited to repeating variations of experiments first done by people trained as ESR spectroscopists or, more commonly, totally excluded from the field. With the help of co-workers and collaborators, I have invented a technique that solves all these problems!

It starts with ESR and spin trapping.

$$R^{\bullet} + S{=}T \longrightarrow R{-}S{-}T^{\bullet}$$

Spin trapping is a technique in which a short-lived reactive free radical combines with a diamagnetic molecule ("spin trap") to form a more stable free radical ("radical adduct") which, historically, could only be detected by electron spin resonance (ESR). By extending the lifetime of the radical adduct, the concentration of the radical adduct is increased and, therefore, the signal-to-noise of the ESR spectrum. To an ESR spectroscopist, the conservation of the unpaired electron is the most important aspect of this reaction. To an organic chemist, the most unique feature of the reaction is the formation of a new chemical bond to the free radical in a way that is specific to free radicals.

Historically, my laboratory has used ESR, especially spin trapping, to detect free radicals. The most versatile and therefore most popular spin trap is DMPO (5,5-dimethyl-1-pyrroline *N*-oxide). The ESR spectrum of Mb$^{\bullet}$-DMPO is consistent with that of a partially immobilized nitroxide (Fig. 1A). Interpreting this ESR spectrum demonstrated that metmyoglobin reacts with hydrogen peroxide to produce a tyrosyl radical which is trapped by DMPO at the phenoxyl oxygen as demonstrated by O-17 isotope labeling [1,2]. The trapping of this myoglobin radical is the result of hydrogen peroxide-driven self-peroxidation, which forms a phenoxyl radical at tyrosine-103 as determined by ESR studies using site-specific mutants where phenylalanine was substituted for tyrosine [2]. This radical adduct decays with a half-life of one minute [3].

Table 1
Advantages of free radical detection with electron spin resonance (ESR) – The Gold Standard.

- Direct ESR is physics – **No assumptions**!
- ESR not only **detects** free radicals unambiguously, but can **identify** them
- ESR is as sensitive as optical spectroscopy, but free radicals are very reactive **and insufficient concentration** is a major limitation
- This **instability** of free radicals is **partially solved** by the organic chemistry trick of spin trapping
- ESR is without question the **best way** to detect free radicals of small organic or inorganic molecules, such as **drugs or antioxidants**

Table 2
Limitations of free radical detection with electron spin resonance (ESR) – The Gold Standard.

- **Not sensitive** enough, especially for cell studies
- **No subcellular location** of free radicals is possible
- **No tissue distribution** of free radicals is possible except nitroxides at mM in vivo concentrations
- **Very limited** number of free radicals detected in vivo
- No **DNA-derived radicals**, except with **radiation and chemical** generation
- ESR is relatively **expensive**
- **Quantum mechanical** – higher math and physics

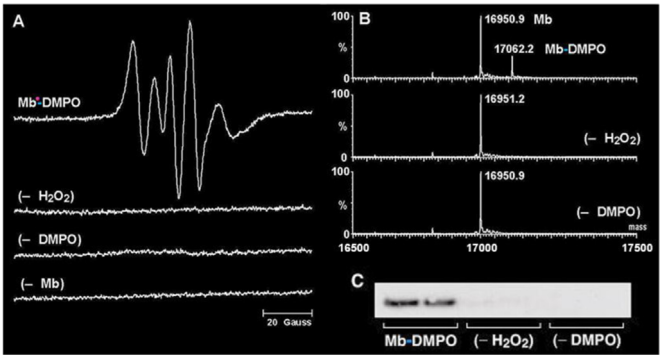
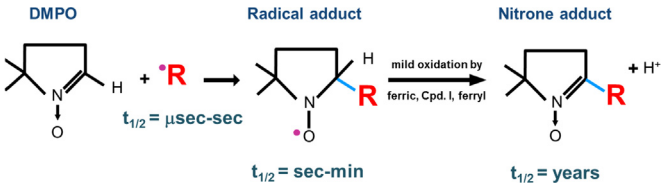


Fig. 1. DMPO spin trapping of the tyrosyl radical generated on horse metmyoglobin by hydrogen peroxide. Samples were analyzed using ESR, ESI/MS, and Western blot, respectively. (A) ESR analysis: Sample contained 50 μM metmyoglobin, 50 μM hydrogen peroxide, and 10 mM DMPO. (B) ESI/MS analysis: Sample contained 1 μM metmyoglobin, 1 μM hydrogen peroxide, and 10 mM DMPO. (C) Western blot: Sample contained 5 μM metmyoglobin, 5 μM hydrogen peroxide, and 10 mM DMPO (1).

After the ESR signal disappeared, analysis of the Mb-DMPO samples by electrospray ionization mass spectroscopy (MS) demonstrated the formation of a myoglobin-derived product with a mass increase of 111 Da, which is essentially the mass of DMPO (Fig. 1B). These data are consistent with the addition of DMPO as expected for the formation of a covalent bond between myoglobin and DMPO. Approximately one quarter of the myoglobin reacted to form the persistent DMPO adduct. This ion was not detected in the controls. This result always fascinated me, because this mass spectrum demonstrated that the ESR-silent species was still a chemical adduct of DMPO and myoglobin. These findings show that the DMPO remains covalently bound to the myoglobin (Mb-DMPO) after the ESR signal of the radical adduct decays. The oxidation of the radical adduct to a nitrone adduct is facile and expected on chemical grounds due to the ease of removal of the β-hydrogen to form the chemically stable nitrone adduct. The position of the DMPO nitrone adduct is a *specific marker or tag for where the radical was*, as determined by the new chemical bond formed during spin trapping.



I had the idea of making antibodies to the DMPO bound to proteins. In order to raise antiserum that specifically binds to a