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Review

Detection and characterisation of radicals in biological materials using EPR methodology☆

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ABSTRACT

Background: Electron paramagnetic resonance (EPR) spectroscopy (also known as electron spin resonance, ESR, spectroscopy) is widely considered to be the "gold standard" for the detection and characterisation of radicals in biological systems.
Scope of review: The article reviews the major positive and negative aspects of EPR spectroscopy and discusses how this technique and associated methodologies can be used to maximise useful information, and minimise artefacts, when used in biological studies. Consideration is given to the direct detection of radicals (at both ambient and low temperature), the use of spin trapping and spin scavenging (e.g. reaction with hydroxylamines), the detection of nitric oxide and the detection and quantification of some transition metal ions (particularly iron and copper) and their environment.
Major conclusions: When used with care this technique can provide a wealth of valuable information on the presence of radicals and some transition metal ions in biological systems. It can provide definitive information on the identity of the species present and also information on their concentration, structure, mobility and interactions. It is however a technique that has major limitations and the user needs to understand the various pitfalls and shortcoming of the method to avoid making errors.
General significance: EPR remains the most definitive method of identifying radicals in complex systems and is also a valuable method of examining radical kinetics, concentrations and structure. This article is part of a Special Issue entitled Current methods to study reactive oxygen species — pros and cons and biophysics of membrane proteins. Guest Editor: Christine Winterbourn.

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1. Introduction

Free radicals are believed to play a critical role in normal cell function, in cell signalling, in host defence against invading pathogens and in a large number of important human diseases. Whilst the evidence for a role for free radicals is compelling in some scenarios, the evidence for radical formation is less certain in other cases. In some situations it is clear that radical formation and reactions are critical to the biological event (either positively or negatively), but in other cases, the formation of these species may be a secondary (downstream) event and irrelevant to the overall biological process. Given this situation, there is a pressing need for definitive information as to whether radicals are present, what these species are, how they are involved, and whether their effects are causative in the biological phenomenon under study. As such there is considerable interest in methods that allow the unequivocable detection and identification of radicals in biological systems.

As there are very few situations where radical formation occurs rapidly and to a high concentration (i.e. acute massive fluxes), the detection of radicals is typically fraught with problems. One of the few methods that allow direct detection of radicals in complex biological systems such as intact cells and tissue samples, is EPR spectroscopy. However, the (typically) low rates and extents of radical generation in biological systems make detection challenging, particularly as it is now well established that there are many processes that can give rise to artifactual radical formation (e.g. elevated temperature, light and radiation exposure, presence of redox active metal ions, cell

Abbreviations: BMPO, 5-*tert*-butoxycarbonyl 5-methyl-1-pyrroline *N*-oxide; CYPMPO, 5-(2,2-dimethyl-1,3-propoxycyclophosphoryl)-5-methyl-1-pyrroline *N*-oxide; DBNBS, 3,5-dibromo-4-nitrosobenzene sulfonic acid; DEPMPO, 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide; DETC, diethyldithiocarbamate; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DTCS, *N*-(dithiocarboxy)sarcosine; EMPO, 5-(ethoxycarbonyl)-5-methyl-1-pyrroline *N*-oxide; ESR, electron spin resonance; EPR, electron paramagnetic resonance; Hb, haemoglobin; MGD, *N*-methyl-D-glucamine; MNP, 2-methyl-2-nitrosopropane; MS, mass spectrometry; $NO^{\bullet}$, nitric oxide; $O_2^{\bullet-}$, superoxide radical anion; PBN, *N*-*tert*-butyl-α-phenylnitrone; POBN, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone; SOD, superoxide dismutase

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necrosis, sample manipulation). Given the complex nature of these systems, a number of different approaches have been developed to maximise the amount of useful information that can be obtained from a given system, and it is also now clear that data obtained from multiple techniques is often required to obtain a definitive picture as to the formation and effects of radicals in biological systems. The data and methods outlined below are intended to provide guidance, and suggest approaches, that can maximise useful data and minimise artefacts and poor experimental design for this complex and challenging task. It cannot cover all of the detailed experimental information required to tackle particular problems, but will hopefully provide guidance and leading references to such information.

2. General principles of EPR spectroscopy

EPR is a technique that is based on the absorption of electromagnetic radiation (usually in the microwave frequency region of the electromagnetic spectrum) by a paramagnetic sample (i.e. a species with one of more unpaired electrons) when placed in a magnetic field. These absorptions only occur at very well defined frequencies and magnetic field combinations, which depend on the type of paramagnetic species present and its characteristics, described by Eq. (1):

$$h\upsilon = g\beta H \quad (1)$$

where h = Planck's constant, υ = frequency, g = a constant that is dependent on the nature of the radical, equal to 2.0023 for a free electron, β = Bohr magneton, and H = applied magnetic field. As such absorptions (resonances) can only occur with paramagnetic samples, the technique is entirely specific for radicals, and certain metal ions with unpaired electrons; this is one of the great beauties of the technique — if there are no radicals/unpaired electrons present, there are no absorptions, thereby eliminating the background absorptions that are so problematic with other spectroscopic techniques used to examine biological materials. The combination of frequency and magnetic field (determined by Eq. (1)) that give rise to absorptions by a radical is critically dependent on the nature of the radical, and hence different types of radicals (and also metal ions) will produce lines at very different regions of the scanned magnetic field.

The absorptions arising from a single unpaired electron are further complicated by the presence of other neighbouring magnetic nuclei (e.g. ^{1}H, ^{13}C, ^{14}N, ^{19}F, ^{32}P, ^{35}Cl, ^{37}Cl), resulting in highly distinctive patterns of lines (hyperfine couplings), which depend on the exact structure, conformation and environment (physical state, solvent) and motion of the radical. The number of lines arising from interaction with a given magnetic nucleus is determined by Eq. (2):

$$\text{Number of lines} = 2nI + 1 \quad (2)$$

where I is the nuclear spin of the nucleus in question, and n is the number of such nuclei. Each unique (inequivalent) magnetic nucleus that is associated with significant spin density generates such couplings with this *multiplying* the number of lines detected. In some cases these lines occur at identical frequency and field values resulting in superposition of lines, which can complicate analysis.

The value of g in Eq. (1) provides valuable data on the nature of a radical species. Most carbon-centred radicals have g values close to those of the free electron (2.0023), but these values can show distinctive changes and be diagnostic of structure. Thus, the presence of various neighbouring groups that donate or accept electron density alters the g value in a distinctive and predictable manner. Radicals with extensive spin delocalisation, especially on to heteroatoms, have higher g values, and metal ions can have very markedly different g values. Whilst such data is of great use in identifying radicals and metal ions in direct EPR experiments, the g value is of less diagnostic use in spin trapping as the unpaired electron density in many spin adducts is primarily confined to the nitroxide bond, resulting in relatively small, if any, changes with the added radical.

Eq. (2) allows the prediction of the number and intensity of the spectral lines observed. Thus for nitroxide radicals such as $(^{t}Bu)_2N-O^{\bullet}$ the spectrum consists of a 1:1:1 triplet of equally spaced lines, arising from interaction of the unpaired electron with the single (n = 1) nitrogen (I = 1) of the nitroxide bond. The hydrogen atoms present on the ^{t}Bu groups in this radical are too far removed from the spin density to give detectable couplings in most experimental conditions. For many spin traps additional couplings are also detected, with the number and size of these providing useful diagnostic information. Typically these include couplings to a single (n = 1) additional hydrogen atom (with I = 1/2), with this resulting in the splitting of each of the nitrogen couplings in to an additional 1:1 doublet (pair of lines). Thus many spin adducts detected in spin trapping experiments contain 6 lines arising from the couplings to the nitroxide nitrogen and a single hydrogen [1,2]. This is not always the case however, and additional small couplings are sometimes present with these often being highly distinctive and diagnostic. A classic example is the case of the superoxide radical adduct to the spin trap DMPO, which has a 12 line spectrum [1]. This pattern of lines was originally assigned to an additional small coupling to one further hydrogen from the ring structure, though the presence of two different conformers of DMPO–OOH has also been suggested [3].

The area beneath an absorption line (or its double integral if the spectrum is recorded as the first derivative, as is usually the case), is directly proportional to the spin concentration present in the sample, thereby allowing absolute radical concentrations to be determined. Integration of spectral lines can be carried out either manually or automatically by spectral analysis software, but often the intensity of one or more absorption lines (i.e. peak-to-peak line heights) is used as a surrogate indicator of concentration, making the assumption that the line width remains constant. EPR spectroscopy is therefore a technique that can yield a wealth of information not only on the presence of radical species, but also their exact structure, concentrations, environment, motion, and distance from, and orientation to, other magnetic nuclei. A full description as to how such information can be obtained and a detailed explanation of the origins of these hyperfine couplings and the structural information that they impart is beyond the scope of this review, and the interested reader is referred to a number of excellent reviews, books and monographs on this topic (e.g.[4], and the specialist periodical series Electron Paramagnetic Resonance, formerly Electron Spin Resonance, published by the Royal Society of Chemistry, UK).

3. Direct EPR detection of reactive radicals in vivo and ex vivo

As many of the processes that give rise to radicals in biological systems generate radicals at (relatively) slow rates, and the subsequent reactions of these species are rapid, the *steady state* concentration of many radicals in vivo is rather low (usually micromolar at best). The detection limit of most commonly available EPR spectrometers is in the range 10^{-7}–10^{-9} mol dm^3, therefore *direct* EPR detection of radicals is limited to a relatively small number of examples, with these usually limited to radicals which are long-lived (i.e. stabilised by electron delocalisation) and where a slow rate of radical formation is compensated for by a slow rate of decay, allowing their steady state levels to rise to detectable levels. Examples include: a) the ascorbyl radical — generated on oxidation of ascorbic acid (vitamin C) a key cellular and plasma antioxidant; b) the α-tocopheroxyl radical — generated as a result of the oxidation of the key membrane and lipoprotein antioxidant α-tocopherol (a component of vitamin E); c) phenoxyl radicals generated from hindered phenols — structures which are present in a number of synthetic antioxidants (e.g. butylated hydroxytoluene and butylated hydroxyanisole), a large number of natural products (e.g. flavanoids) which have antioxidant properties, and Tyr-residues on proteins; d) nitroaromatics that yield long-lived radical anions on reduction; and e) semiquinone radicals (e.g. those

generated from the important membrane electron transport component, coenzyme Q and a number of important drugs, such as adriamycin and doxorubicin) (e.g. [5–9]).

In cases where high yields of radicals are (or can be) generated in a transient fashion (e.g. via enzyme burst kinetics, by addition of high concentrations of an oxidant, or during reperfusion injury after ischaemia etc.), it is possible to obtain valuable data from low-temperature EPR studies on rapidly frozen samples. This can be achieved by freeze-clamping tissue samples with tongs cooled in liquid nitrogen, or use of freeze-quenching apparatus for enzyme reactions. Trapping the radical in a solid matrix, enhances the lifetimes of these species by preventing radical–radical or radical–molecule reactions, enabling EPR spectra (either singly or multiple) to be acquired over an extended period of time. The interpretation and assignment of the resultant EPR spectra can however be problematic due to the anisotropy (multiple different orientations of the radical) induced by freezing. This is not necessarily a problem in systems with only a single or few components (e.g. studies on isolated enzymes) but it can be a major problem in biological systems where radicals may be formed or generated at multiple sites. There are other drawbacks also associated with carrying out low-temperature studies not least of which are the increased number of samples that are needed to allow time course data to be examined (a particular problem with animal studies), problems associated with introducing tissue samples into EPR tubes whilst keeping the material frozen, and the inevitable bias towards the detection of long-lived species over short-lived radicals, resulting from the finite time required for freezing (which is often >10–100 ms). Manipulation of tissue samples should be avoided if at all possible — cutting, grinding, homogenisation and lyophilisation are all known to introduce artefacts that are difficult to control for [10].

A number of continuous generation methods have been used to circumvent the short lifetimes of many radical species. Rapid flow systems can be used to detect radicals in isolated chemical systems and in a few cases with isolated enzymes, though the large amounts of reagents required can be prohibitively expensive (e.g. [11,12]). Stopped flow systems have been employed in studies on isolated enzymes with some success, but again such studies are limited to systems where relatively high reagent concentrations are achievable and to systems that can withstand the stresses imposed by the conditions employed (e.g. the shear stress induced by rapid mixing which can lyse cells and unfold proteins). In situ radical generation methods have also been employed in radiation studies (high-energy radiation, UV, visible light) with the radical generated inside the EPR spectrometer cavity (e.g. via transmission gratings).

4. Direct EPR detection of transition metal ions in vivo and ex vivo

A significant number of biologically important metal ions (iron, copper, manganese, molybdenum, chromium) can be detected directly by EPR in biological systems when present in certain defined oxidation states. These metal ions occur in a number of widely-distributed and critical enzymes and hence EPR is a valuable tool for investigating the nature of these species and the enzymatic/functional activity of the metal ions [13]. Data has also been obtained on the role of some of these metal ions in tissue damage and toxicity (e.g. the roles of iron and copper in tissue damage resulting from metal ion overload and chromium ions in carcinogenicity) [14,15]. Due to the relatively long lifetimes of many metal ion oxidation states (e.g. resting state species, or transients generated by stopped flow or by use of rapid freezing techniques), a considerable amount of information that can be obtained from direct studies.

5. Spin trapping of reactive radicals in vivo and ex vivo

5.1. General principles

Spin trapping was developed in the late 1960s to facilitate the detection of reactive radicals in chemical systems (reviewed in [2,16–19]). This method involves the addition of a spin trap, typically a nitrone or nitroso compound (Fig. 1), to the system being studied, at a concentration sufficient to ensure significant extents of trapping of any radicals present to give stable, detectable, nitroxide radical adducts (Fig. 2).

The use of spin traps to detect radicals in biological systems has been reviewed extensively (e.g. [2,20,21] and the specialist periodical series Electron Paramagnetic Resonance, formerly Electron Spin Resonance, published by the Royal Society of Chemistry, UK). Although spin trapping was initially developed to examine radicals from low-molecular mass compounds, including the biologically relevant superoxide ($O_2^{\bullet-}$) and hydroxyl radical ($HO^{\bullet}$) [16–19], this methodology has also been used extensively to examine radical formation on proteins, lipids, DNA/RNA and polysaccharides in both isolated and complex biological systems (e.g.[22–24]).

Nitrone spin traps are the most widely used traps, with these including the cyclic species 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and related traps, 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO), 5-(ethoxycarbonyl)-5-methyl-1-pyrroline *N*-oxide (EMPO) 5-*tert*-butoxycarbonyl 5-methyl-1-pyrroline *N*-oxide (BMPO), 5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline *N*-oxide (CYPMPO) and the acyclic compounds *N*-*tert*-butyl-α-phenylnitrone (PBN), and α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN) (shown in Fig. 1).

Nitrone spin traps form long-lived adducts with a wide range of radicals (e.g. carbon-, oxygen-, sulphur-, and nitrogen-centred species), unlike nitroso spin traps (see below) (reviewed in [25,26]). However, a disadvantage to nitrone spin traps is that the reactive radical adds to the carbon atom adjacent to the incipient nitroxide group, and is therefore distant from the nitrogen–oxygen orbitals of the nitroxide group that contains the unpaired electron. As a result, couplings to magnetic nuclei present in the added radical tend to be small (except in a few specialised cases) and it is often not possible to resolve these hyperfine couplings; this makes definitive assignment of the hyperfine couplings to a specific spin adduct problematic. However, the *magnitude* of the hyperfine couplings arising from the spin trap-derived nitroxide nitrogen (which yields a 1:1:1 triplet splitting) and particularly the β-hydrogen (which splits each of these lines further into 1:1 doublets), are markedly dependent on the nature and structure of the added radical as a result of the influence of this species on the conformation of the nitrone; this is particularly marked with cyclic nitrones such as DMPO, DEPMPO and related compounds. Whilst the magnitude of the nitrogen coupling in many spin traps is relatively invariant, the size of the hydrogen couplings in cyclic nitrone adducts varies widely and provides valuable information on the nature of the radical that has been trapped [1,17,19].

Nitroso spin traps (e.g. 2-methyl-2-nitrosopropane, MNP; 3,5-dibromo-4-nitrosobenzene sulfonic acid, DBNBS) have a distinct advantage over the nitrone family of traps in that the reactive radical attaches *directly* to the nitroso nitrogen atom, and is therefore in close proximity to the unpaired electron present on the nitroxide function. This typically results in the detection of additional distinctive hyperfine couplings from magnetic nuclei present in the added radical. The distinctive nature and size of these couplings provide valuable data on the identity of the added radical. However, unlike the nitrones, nitroso spin traps give long-lived adducts with a much narrower range of radicals (often restricted to carbon-centred species and a few others), which clearly limits the type of information that can be obtained. The choice of spin trap for a particular experiment is therefore a critical factor, as an incorrect choice may result in an absence of signals even though high radical concentrations may be present, simply because the spin trap does not yield long-lived adducts that can be detected by EPR (discussed further below). A further disadvantage of this family of traps is that many of these species are thermally and photochemically unstable (due to the presence of relatively weak carbon-nitrogen bonds), which can result in significant generation of artifactual EPR signals that complicate spectral analysis [27]. A number of compilations of radical adduct data are available to

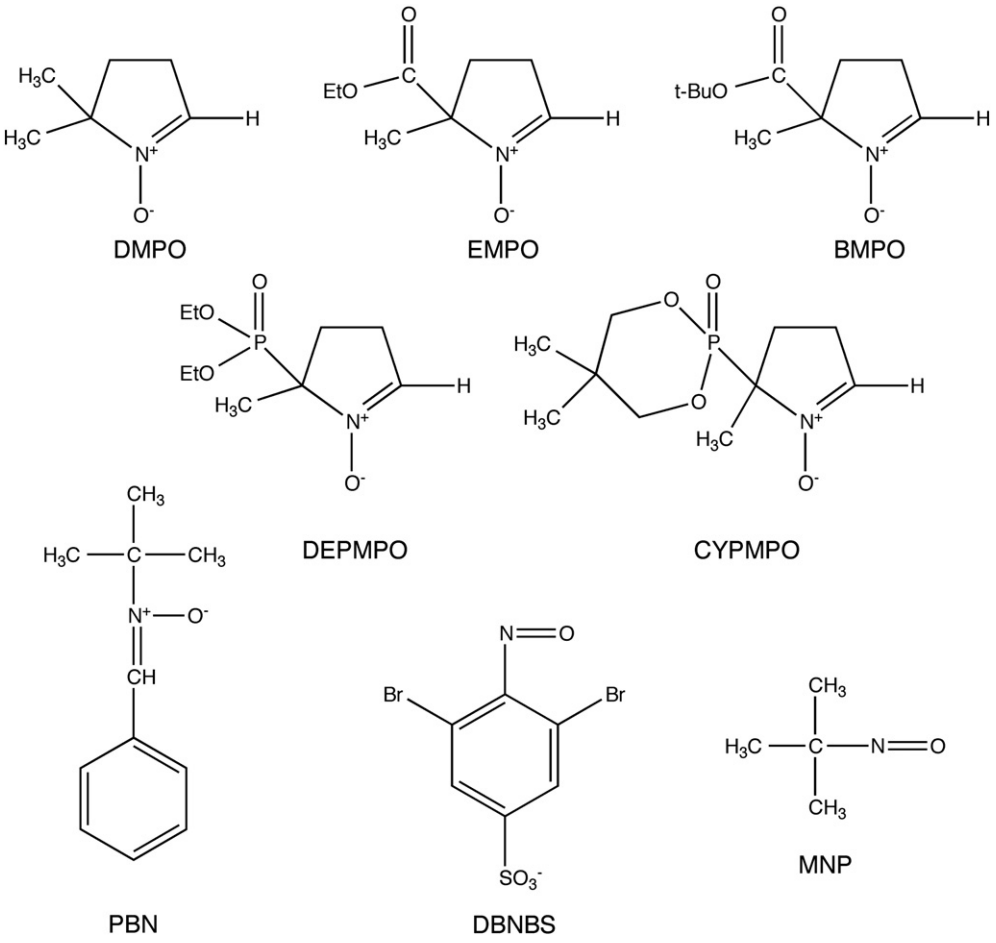

Fig. 1. Structures of commonly used spin traps.

aid analysis of EPR spectra obtained in spin trapping experiments (e.g. [1] and the NIEHS website: http://tools.niehs.nih.gov/stdb/index.cfm).

As indicated above, choice of spin trap can be a critical factor in determining the success of an experiment. Data regarding the nature of the species that might be formed, or a clear idea of what information is required can aid such choices. Thus nitrone traps are likely to be the traps of choice for oxygen-, nitrogen, sulphur-centred radicals, whereas if detailed information is required regarding carbon-centred species, nitroso traps may be the most sensible choice due to the highly characteristic nature of the hyperfine coupling constants obtained [25,26]. If such traps are not possible for other reasons, cyclic nitrones (e.g. DMPO, DEPMPO, BMPO) are also a reasonable choice, as these tend to provide more distinctive data than the linear nitrones such as PBN and POBN, owing to the large variation in the magnitude of the β-hydrogen couplings seen with the cyclic nitrones.

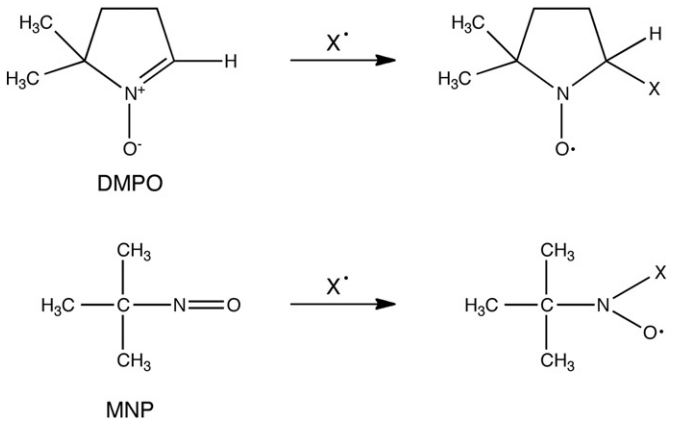

Fig. 2. Formation of radical adducts from nitrone (DMPO) and nitroso (MNP) spin traps.

The purity of the spin trap is also a factor that cannot be ignored. Even though spin traps obtained from commercial suppliers can be >99% pure, the high concentrations of trap used in many EPR experiments (often up to hundreds of mM) to ensure competitive reaction of the initial radical with the trap, also increases the concentration of impurities that may be present. If these impurities are radicals, or species that can be readily oxidised (e.g. hydroxylamines) or reduced to radicals, then these can give rise to artefact signals [28–30].

A number of methods have been developed or utilised to introduce spin traps in to experimental systems. This is (usually) readily achieved for in vitro studies though it should be noted that spin traps may take time to equilibrate into cell and tissue slices, and it is essential that the spin trap be present *before* the insult (whatever this may be) is applied/or the experiment started. In studies where media is used (e.g. cell culture experiments) binding of the trap to extracellular proteins may be a problem, with this potentially reducing the effective concentration; this is most likely to be a problem with hydrophobic traps. It is also important to consider the toxicity of the spin trap, which is discussed in detail below.

For animal studies, a number of approaches have been developed including oral dosing via a stomach tube (oral gavage), intraperitoneal injection (a useful way of getting spin traps to the liver and into the circulation), and intravenously (which ensures rapid systemic

dosing) (reviewed in [20,31,32]). Micro-dialysis has been employed in some studies with the spin trap introduced via a semi-permeable membrane, with the resulting spin adducts diffusing out via the same route for sampling [33]. A large number of avenues are also available for sampling of the spin adducts including urine, bile and blood collection, and tissue biopsies; the cost of animals and the principle of reduction of animal usage, make the former methods preferable, though not always possible.

For human studies, ex vivo addition of a spin trap (e.g. to blood or tissue samples) is currently the only method that can be employed [34–37]. The interpretation of data from such studies is fraught with problems; even with the fastest sampling procedure (drawing blood directly in to a syringe containing a solution of the spin trap) the time frame is likely to be too great to detect any radicals that were originally present in the circulation. Thus any species detected are likely to arise from secondary reactions (e.g. decomposition of pre-existing peroxides present on lipids [34]). Unfortunately, blood sampling often results in some cell lysis and the resulting release of haemoglobin and other cellular materials that can confound data.

5.2. Kinetics of adduct formation and decay

Kinetic data are available for the rate constants for addition of a number of radicals to spin traps (e.g. [38,39]). Generally, the rate constants are higher for nitroso traps than nitrones. In some cases, these reactions are slow (e.g. the trapping of superoxide radicals, $O_2^{\bullet-}$, by DMPO [40,41]), which is clearly a major disadvantage when generation of this radical is subject of study. Selected rate constant data for a range of radicals with nitrone and nitroso spin traps are provided in Tables 1 and 2, respectively. It should be noted that pH and solvent can often have a dramatic effect on the magnitude of these values [21,38]. An example of relevance to biological systems, is the reactivity of $O_2^{\bullet-}$ with cyclic nitrone spin traps (e.g. DMPO). This rate constant is highly dependent on pH, with faster reaction rates (by an order of magnitude) observed under mildly acidic conditions [41,42]. This is attributed to the faster rate of reaction of DMPO with the neutral species $HO_2^{\bullet}$ compared to $O_2^{\bullet-}$ [41], though a deshielding of the methine hydrogen and nitronyl carbon at pH 6 (the pK_a of DMPO) may also play a role [42].

Detailed information relating to the rates of decay of spin trap radical adducts is rather more limited (e.g. [38]). Again, decay rates are highly dependent on the reaction conditions, with the presence of metal ions and reductants, pH, temperature and solvent all known to play a role (reviewed in [25,26]). The stability of $O_2^{\bullet-}$ adducts with cyclic nitrones has been investigated in detail, owing to the interest in quantifying this radical in biological systems. The decomposition kinetics and half-lives ($t_{1/2}$) of the most commonly used cyclic nitrone spin traps are collected in Table 3 (see also [43,44]).

5.3. Potential transformations of spin trap and adducts

The transformation of both the spin trap and/or its adducts to other species can be a significant and important factor. Rapid reduction of the spin adducts to the corresponding hydroxylamines is a particularly important route to loss of adduct signals in intact biological systems [45]. Oxidation to a nitrone can also occur, though this is far less common (but of considerable significance as it forms the basis of immuno-spin trapping: see below, and is also of potential importance in mass spectroscopic studies) [46].

Nucleophile addition to the β-position of nitrones (often called the Forrester–Hepburn mechanism), oxidation of the spin trap and subsequent reaction with a nucleophile (inverted spin trapping) or "ene" addition of a nitroso group to a double bond and subsequent oxidation of the hydroxylamines to a nitroxide are all potential sources of artefacts, which are sometimes very difficult to distinguish

Table 1
Selected rate constants (k, $M^{-1}\ s^{-1}$) for the addition of radicals to nitrone spin traps (room temperature, aqueous solution, unless stated otherwise).

Spin trap	Radical	k ($M^{-1}\ s^{-1}$)	Method	Ref.
DMPO	$^{\bullet}OH$	1.9×10^{9}[a]	Competition	[175]
		3.2×10^{9}	Competition	[40]
		3.3×10^{9}[c]	Direct	[41]
	$^{\bullet}OOH$	6.6×10^{3}	Competition	[40]
	$O_2^{\bullet-}$	50[a]	Competition	[176]
		10[b]	Competition	[40]
		2.4[c]	Competition	[177]
		170[c]	Direct	[41]
		2[d]	Competition	[178]
	$^{\bullet}CO_2^-$	6.6×10^{7}	Direct	[179]
		1.2×10^{8}	Direct	[41]
	Glutathionyl-S$^{\bullet}$	2.6×10^{8}	Competition	[180]
	Cysteinyl-S$^{\bullet}$	2.1×10^{8}	Competition	[180]
	$^{\bullet}OC(CH_3)_3$	9.0×10^{6}	Competition	[181]
	$^{\bullet}OOC(CH_3)_3$	$<10^{6}$	Competition	[181]
	$^{\bullet}CH_3$	1.4×10^{7}	Direct	[179]
	$^{\bullet}C(CH_3)_3$	5.0×10^{7}[b]		[21]
	Ph$^{\bullet}$	7.0×10^{7}[b]		[182]
	$^{\bullet}CH_2OH$	2.2×10^{7}	Direct	[179]
		4.4×10^{7}	Direct	[41]
	$^{\bullet}CH(OH)CH_3$	6.1×10^{7}	Direct	[41]
	$^{\bullet}C(OH)(CH_3)_2$	6.8×10^{7}	Direct	[179]
		6.6×10^{7}	Direct	[41]
	PhC(O)O$^{\bullet}$	8.0×10^{7}[e]		[21]
DEPMPO	$^{\bullet}OH$	4.8×10^{9}	Competition	[175]
		7.8×10^{9}	Competition	[50,183]
	$O_2^{\bullet-}$	58[a]	Competition	[176]
		90[a]	Competition	[183]
		0.53[c]	Competition	[177]
		0.66[c]	Competition	[184]
		3.95[d]	Competition	[178]
BMPO	$^{\bullet}OH$	2.5×10^{9}[c]	Direct	[41]
		4.5×10^{9}[a]	Competition	[175]
	$O_2^{\bullet-}$	7[a]	Competition	[176]
		77[c]	Competition	[185]
		0.24[c]	Competition	[177]
		<3[c]	Direct	[41]
	$^{\bullet}CH_3$	2.8×10^{7}	Direct	[41]
	$^{\bullet}CH_2OH$	5.4×10^{7}	Direct	[41]
	$^{\bullet}C(OH)(CH_3)_2$	1.4×10^{8}	Direct	[41]
	$^{\bullet}CO_2^-$	1.8×10^{8}	Direct	[41]
EMPO	$^{\bullet}OH$	5.0×10^{9}	Competition	[175]
	$O_2^{\bullet-}$	10.9[d]	Competition	[178]
		74.5[a]	Competition	[186]
PBN	$^{\bullet}CH_3$	4.0×10^{6}[f]		[182]
	$^{\bullet}C(CH_3)_3$	<10[e]	Competition	[187]
	$^{\bullet}CH_2Ph$	2.0×10^{7}[e]		[182]
	$^{\bullet}Ph$	1.2×10^{7}[g]	Competition	[39]
	$^{\bullet}CH_2OH$	4.3×10^{7}		[39]
	$^{\bullet}CH(OH)CH_3$	1.6×10^{7}		[39]
	$^{\bullet}C(OH)(CH_3)_2$	1.0×10^{7}		[21]
	$^{\bullet}C(O)Ph$	8.1×10^{5}		[21]
	$^{\bullet}H$	5.5×10^{8}[i]		[39]
	$^{\bullet}OH$	8.5×10^{9}		[39]
	$^{\bullet}OOH$	50		[39]
	$O_2^{\bullet-}$	0.1[h]	Competition	[188]
	$^{\bullet}OC(CH_3)_3$	7.9×10^{7}		[21]
	$^{\bullet}CO_2^-$	1.5×10^{7}[i]		[39]
	$^{\bullet}OCH_3$	1.2×10^{8}[i]		[39]
	PhC(O)O$^{\bullet}$	5.0×10^{6}[e]		[21]
	$^{\bullet}SPh$	1.1×10^{7}		[21]
	Glutathionyl-S$^{\bullet}$	7.5×10^{7}		[21]
	$Et_3Si^{\bullet}$	7.1×10^{7}[j]		[21]

[a] At pH 7.0.
[b] At pH 7.8.
[c] At pH 7.4.
[d] At pH 7.2.
[e] At 40 °C in benzene.
[f] At room temperature in organic solvents (usually benzene).
[g] At −45 °C in methanol.
[h] In DMF using KO_2.
[i] At 20 °C, solvent not given.
[j] At 27 °C in di-tert-butylperoxide/triethylsilane.

Table 2
Selected rate constants (k, M^{-1} s^{-1}) for the addition of radicals to nitroso spin traps (room temperature, aqueous solution, unless stated otherwise).

Spin trap	Radical	k (M^{-1} s^{-1})	Method	Ref.
MNP	$^{\bullet}CH_3$	1.7×10^7	Direct	[189]
	$^{\bullet}CH_2CH_3$	5.3×10^7	Direct	[189]
	$^{\bullet}CH_2COO^-$	7.0×10^6	Direct	[189]
	$^{\bullet}CH(CH_3)_2$	4.6×10^7	Direct	[189]
	$^{\bullet}CH_2OH$	1.4×10^8	Direct	[189]
	$^{\bullet}CH(OH)CH_3$	3.2×10^8	Direct	[189]
	$^{\bullet}C(CH_3)_3$	3.3×10^{6a}	Competition	[187]
	$^{\bullet}CCl_3$	1.9×10^{6b}	Competition	[39]
	$^{\bullet}SO_3^-$	4.3×10^7	Direct	[189]
	$^{\bullet}CO_2^-$	1.7×10^9	Direct	[189]
	e^-_{aq}	6.2×10^9	Direct	[189]
	$^{\bullet}H$	9.1×10^8	Direct	[189]
	$^{\bullet}OH$	2.5×10^9	Direct	[189]
	$^{\bullet}OC(CH_3)_3$	1.3×10^8	Competition	[181]
	$^{\bullet}OCH_3$	1.3×10^{8c}	Competition	[38]
	$^{\bullet}SPh$	1.7×10^{8b}	Direct	[190]
DBNBS	$^{\bullet}CH_3$	1.5×10^9	Competition	[191]
	$^{\bullet}CH_2OH$	2.5×10^8	Competition	[191]
	$^{\bullet}CH(OH)CH_3$	6.3×10^7	Competition	[191]
	$^{\bullet}OH$	3.9×10^9	Competition	[191]
	$^{\bullet}CO_2^-$	1.5×10^9	Direct	[191]
	$^{\bullet}N_3$	2.4×10^8	Direct	[191]
	$O_2^{\bullet-}$	4.3×10^7	Direct	[191]

[a] At 40 °C in benzene.
[b] At room temperature in organic solvents (usually benzene).
[c] At −45 °C in methanol.

from "real" spin trapping [18,47–49]. These processes are outlined in Fig. 3.

A number of studies have shown that the activity of some enzymes and biological co-factors can compromise the interpretation of spin adduct data, with the decay of the $O_2^{\bullet-}$ radical adduct to cyclic nitrones such as DMPO being a classic example. The adduct formed by addition of $O_2^{\bullet-}$ is a peroxide species, and it has been established that this can undergo rapid reduction to the corresponding alcohol (i.e. would appear as the hydroxyl radical adduct) [27,42]. This process does not occur (in the absence of transition metal ions) with other cyclic nitrone traps, e.g. DEPMPO [50], BMPO or EMPO [51]. Thiol adducts can also decompose via this pathway (though direct reduction of the nitroxide function to the hydroxylamine also occurs: this results in complete loss of the EPR signals, rather than a transformation of one adduct to another), and may also be induced by reductase enzymes that remove peroxides (e.g. glutathione peroxidase) [51–53].

Table 3
Half-life (min) of various superoxide cyclic nitrone spin adducts at room temperature and pH 7.0–7.4.

Spin trap	$t_{1/2}$/min	Ref.
DMPO	0.9	[176]
	0.8	[50]
	1.3	[53]
DEPMPO	14	[176]
	14.2	[50]
	14.8	[178]
	15.3	[92]
	24.4	[53]
EMPO	8	[57]
	8.6	[85]
BMPO	8.5	[176]
	15.7	[91]
CYPMPO	12.3	[55]
	30.4	[53]
Mito-DEPMPO	40.4	[92]
Mito-BMPOBr	10.2	[91]

5.4. Pharmacokinetics of trap and adducts

The properties of a spin trap, and particularly its solubility in different environments, need to be appropriate for the planned study, and a lack of spin adducts signals may be a consequence of poor localisation of the spin trap at the site of radical formation. Partition coefficient data, which give an estimate as to how a spin trap distributes between aqueous and organic phases, are available for many common spin traps (Table 4) [54–57]. It should be noted however that multiple other factors (e.g. binding to proteins) may modulate such distribution. Penetration across membranes is also not readily predicted from these data, and requires experimental verification, but generally it is a reasonable assumption that small neutral amphiphilic molecules will equilibrate rapidly across membranes. Relatively little is known about the partitioning of spin adducts once formed (though a considerable amount of data are available on stable nitroxides, which may be structurally related and provide a guide as to behaviour, as these have been extensively used as MRI contrast agents and oxygen probes [45]). Rapid translocation of adducts from their site of generation may result in erroneous conclusions about the site of radical generation, but post-formation mobility can be beneficial as, spin adducts may partition into plasma, or be excreted in bile or urine, which are often more readily sampled than tissues. A number of studies have used such excretion to investigate radical formation in vivo (see, e.g. [58,59]).

5.5. Toxicity

As spin traps are often used at very high concentrations (mM–M) toxicity and interference in the pathways under study are potentially of critical importance. The hydrophilic nitrone traps such as DMPO appear to be relatively non-toxic, and have been given to animals at high millimolar concentrations in acute experiments [54,60,61]. The toxicity of nitrone spin traps has been examined in several in vitro studies with various cell lines and test systems, generally with toxicity in the order DMPO < DEPMPO ≈ EMPO ≈ CYPMPO < BMPO < PBN [44,55,62,63]. Toxicity is related to hydrophobicity, with the more lipid-soluble nitrone traps (e.g. BMPO and PBN) inducing cell death via necrotic pathways [63]. Nitroso spin traps are significantly more toxic than nitrones, which may again be related to hydrophobicity and membrane perturbation effects, though enzyme inhibition may also play a role [31,54,60]. The toxicity of nitroso compounds is such that these traps are generally best restricted to cell-free, model systems [62]. Clearly such effects should be avoided as confounding toxicity make data analysis problematic, and it is therefore of great importance that studies on the direct effects of the spin trap on the system under study are carried out. Unfortunately, there is a paucity of information on this topic area.

5.6. Aids to spectral analysis

As most radicals react to give further radicals, the possibility of trapping multiple radicals with any spin trap is high, which can make spectral analysis problematic. Furthermore, as the rate constants for the trapping of radicals and spin adduct decay vary significantly (cf. Table 1 and comments above), the most intense EPR signals detected may not necessarily arise from the radical generated in the highest yield, nor be from the most important radical in terms of biological action/effect. Thus a minor species that reacts rapidly with a spin trap, and which gives a persistent adduct, may give much more intense EPR spin adduct signals, than a major radical that reacts slowly with a trap and/or gives a short-lived (or undetectable) adduct. As such, it is of critical importance to determine whether the radicals detected are central to the observed biological phenomenon, which often requires complimentary functional/endpoint studies. A common method of examining this is to determine whether radical scavenging by the spin trap correlates with alterations to the biological

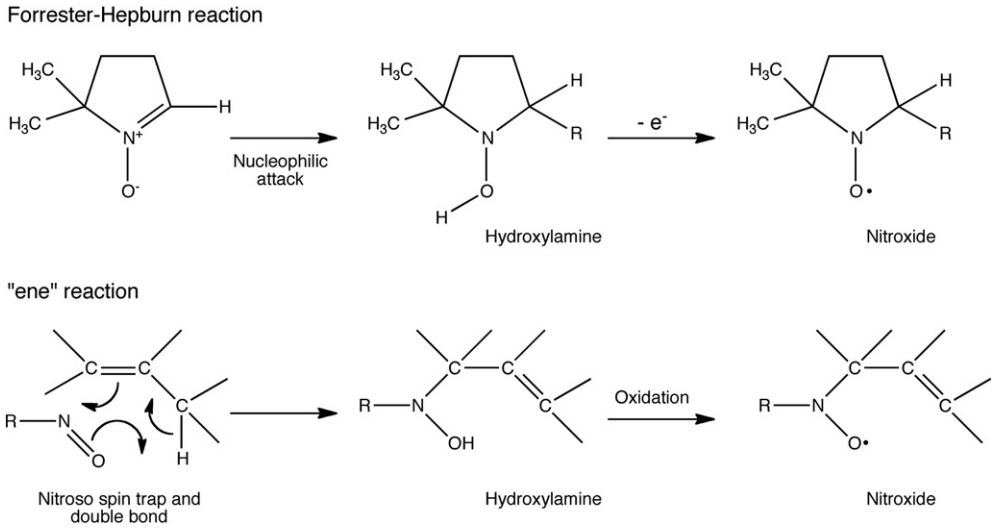

Fig. 3. Reactions resulting in artifactual spin adduct generation.

endpoint. Analysis of mixtures of spin adducts can be challenging and in some cases, definitive assignments are not possible. A number of approaches have been developed to aid this process, which are discussed further below.

Modification or blocking of a presumed site of radical formation can be a powerful tool to determine the nature of trapped radicals, though this is not always definitive. This method has been used extensively with macromolecules such as proteins and enzymes, where techniques to chemically modify, or block, side-chains groups have been developed (reviewed in [64,65]). Examples include confirmation of the generation of thiyl radicals on proteins by use of alkylating agents, such as *N*-ethylmaleimide, iodoacetic acid and iodoacetamide, that block the thiol group of Cys residues [66,67], reductive methylation which blocks reactions at Lys residues [68], iodination which prevents detection of phenoxyl radicals derived from Tyr residues [69,70]), treatment with *N*-bromosuccinimide, which destroys Trp residues [69]), and treatment with diethylpyrocarbonate, which blocks His residues [71]. Confirmation of modification of particular sites (e.g. by loss of parent amino acid or generation of products) is important, as there are multiple reasons why modification may not be efficient, including steric and electronic interactions that prevent modification of inaccessible residues. It should also be noted that many of the reagents used in such modification methods are only partially selective and alteration of other (unplanned) sites may also occur particularly when high concentrations of the modifying agent need to be employed [64,65].

Isotopic modification of the suspected site of radical formation has proved to be a valuable method in identifying sites of radical generation. This approach relies on the fact that the isotopes with different nuclear spins can alter the observed spectrum of the radical adduct in defined and predictable ways. This may be due to removal of an observed splitting (by substitution of the desired atom with a nonmagnetic, $I = 0$, isotope), by reducing or altering an existing coupling (e.g., by substitution of a hydrogen atom with a deuterium, which results in the conversion of a large 1:1 doublet coupling into a much smaller 1:1:1 triplet, or substitution of a ^{14}N nucleus with ^{15}N, which results in the conversion of a 1:1:1 triplet pattern into a larger 1:1 doublet pattern), or by generating an additional coupling (e.g., substitution of a ^{12}C atom with a ^{13}C atom ($I = 0.5$), which results in an additional doublet coupling). This type of approach is relatively easy to achieve with low-molecular-mass compounds (e.g. drugs, solvents, chemicals), and has provided useful information in relation to the site of radical production on biological molecules of interest, including amino acid, carbohydrate and nucleoside-derived radicals (e.g. [72–75]).

This approach is more challenging with macromolecules such as proteins, DNA, lipids or complex carbohydrates. This approach has been used extensively with isolated, well-characterised, proteins with the label introduced at defined sites using a labelled amino acid and a bacterial or cell-free expression systems [76,77]. It should be noted that this approach generally results in the labelling of *all* residues of a particular type, and hence does not provide exact identification of the radical site, but does provide information on the *type* of amino acid involved. Of the various possible substitutions, those that give additional large couplings (e.g. ^{12}C to ^{13}C) are most likely to be definitive, as these give pronounced spectral changes.

Site-directed mutagenesis is an alternative, more selective approach, where a specific suspected radical site is altered to a residue with different chemistry (or which yields spectrally-distinct species). This approach has been used successfully to examine radical formation at Tyr, Trp and Cys residues in myoglobin and various peroxidases treated with H_2O_2; in several cases both single and multiple mutants have been examined (e.g. [76–80]). Such studies have provided important information on the transfer of "positive holes" or electrons within 3-D structures, and the role of particular residues as 'sinks' for oxidising species. As with chemical modification, verification of the modification and an absence of other changes (e.g. changes in structure and stability) are important for interpretation of the data. It should however be noted that both chemical modification and site-directed mutagenesis may not prevent radical formation: it may merely alter the radical site from one residue to another [81], and hence EPR signals may still be detected.

Table 4
Partition coefficients for spin traps in 1-octanol:phosphate buffer (10 mM, pH 7.5).
Data from [54–57,63].

Spin trap	Partition coefficient
DMPO	0.02–0.09
DEPMPO	0.06–0.17
POBN	0.09–0.15
CYPMPO	0.11–0.14
DBNBS	0.15
EMPO	0.15–0.19
BMPO	0.8
MNP	8.2
PBN	10.4–15
Nitrosobenzene	73.0

The resolution of the additional couplings from an added radical can provide very valuable data as to the nature of added radicals and this can sometimes be achieved by use of isotope-substituted (typically-deuterated) spin traps where the intrinsic line-width of the adduct signals derived from these traps is decreased relative to the hydrogen-containing species (see, e.g., [82]). Thus wholly (d14) or partly (d9) deuterated PBN; wholly (d9) deuterated MNP; wholly deuterated (d2) DBNBS and deuterated DEPMPO have been synthesised and employed to provide greater spectral resolution (e.g. [83,84]). ^{15}N-labelled spin traps have also been developed as this results in a large (1:1) doublet coupling from the nitroxide nitrogen in place of the 1:1:1 triplet coupling from ^{14}N, with this resulting is an increased spectral width (thereby decreasing potential overlap of spectral lines) and a greater signal intensity due to the smaller number of couplings [84,85].

Spectral simulation is a powerful tool to confirm proposed assignments, but it should be noted that a satisfactory outcome does not *prove* that an assignment is correct. It is therefore important that this approach is used carefully, and that the inputs make chemical sense. Simulations can also provide valuable data on the relative concentrations of different adducts when multiple species are present, though it should be noted that this data only applies to the *adducts* and not the parent radicals from which they were formed (cf. the discussion above regarding the kinetics of radical trapping and decay). This approach has been used for both low-molecular-mass, rapidly-tumbling species which yield isotropic spectra and also, more recently, anisotropic spectra arising from the trapping of protein-derived radicals (e.g. from DMPO and DEPMPO [24,86]). The large number of inputs required for the latter type of simulations is likely to limit this approach to systems where only a single, or a small number of adducts are formed. Considerable progress has been made in this area and it is possible to distinguish between the trapping of oxygen-, sulphur-, or carbon-centred species with reasonable confidence [24,86].

Considerable effort has been expended in the last few years on the development of spin traps that facilitate the detection of particular radicals (e.g. $O_2^{\bullet-}$) or species generated at particular sites [25]. Major advances have been made in developing traps that react rapidly with $O_2^{\bullet-}$, and give adducts with longer half-lives compared to the traditional cyclic nitrone trap, DMPO (see Table 3). Some of these traps are commercially available (e.g. DEPMPO, EMPO, BMPO, CYPMPO) and may allow more ready detection in complex systems, including intact cells [43,53]. DEPMPO, EMPO and related traps also have the advantage a decreased propensity of the superoxide radical adduct to undergo decay to the hydroxyl radical adduct, a particular problem with DMPO, which has made determining whether superoxide radical formation occurs exclusively or in concert with hydroxyl radicals, rather difficult (see above). Although many of the newer traps do not undergo spontaneous interconversion at significant (noticeable) rates, enzymatic reactions may still occur and hence this problem may still persist, albeit to a less significant extent [52,53].

Spin traps have been developed that localise to particular organelles within cells, including cell membranes [87,88], mitochondria (e.g. mitoPBN, mitoBMPO, mitoDEPMPO) which localise to this organelle due to the inclusion of a triphenylphosphonium substituent [89–92]) and which bind to specific targets [93,94]. Unfortunately the limited market for these materials may limit their commercial availability.

6. Spin scavenging of reactive radicals in vivo and ex vivo

The term spin scavenging has been used to denote two different processes: trapping of a radical with another radical (typically a nitroxide) and subsequent product analysis; and secondly the use of hydroxylamines that are readily oxidised to a nitroxide radical on reaction with another radical. The former approach has been used less frequently than the latter.

In the first of these methods, the initial radical can be "trapped" using a stable nitroxide (e.g. TEMPO) to give non-radical products, which can then be characterised by a range of techniques, with mass spectrometry (MS) a common choice. This method has been employed to characterise radical formation on myoglobin treated with H_2O_2 [95,96]. This approach has some advantages over conventional spin trapping, in that the rate constants for reaction of the "trap" nitroxide with the initial radical are often near, or at, the diffusion limit (as they are radical–radical rather than radical–molecule reactions) making the trapping reaction more efficient [97]. A recent study has reported kinetic data on the rates of reaction of a number of peptide and protein radicals with TEMPO and related species with these values being in the range 10^6–10^8 M^{-1} s^{-1} [98]. Although some of the resulting adducts appear to be sufficiently stable to undergo subsequent mass spectroscopic analysis (thereby eliminating a potential source of artefacts [95]), it is unclear whether *all* of the species survive, and it would not be surprising if some of the adducts (particularly those with weak N–O–X bonds, where X = S, N, OR, OOR, OH, and OOH, arising from the trapping of sulphur, nitrogen, alkoxyl, peroxyl, hydroxyl, and superoxide radicals) were unstable and hence not readily detected. This technique may therefore be primarily limited to carbon-centred species. In addition, the spin scavenging approach may be complicated by the ability of nitroxides to shuttle between different oxidation states, particularly in the presence of heme-containing proteins, generating adducts independently of spin scavenging reactions (e.g. [96]). With appropriate controls, spin scavenging can provide a powerful method of directly examining radical formation on macromolecules and give accurate data on not only the type of radical trapped, but also its exact location within a macromolecule.

In the second approach, a hydroxylamine, which is EPR-silent and hence does not give rise to any background signals, is oxidised to an EPR-detectable nitroxide (e.g. [99]). This process can be both rapid, and efficient, if high levels of hydroxylamine are used. Highly substituted 5- or 6-membered ring species are most commonly used as these yield persistent nitroxide radicals [99]. These hydroxylamines typically react with $O_2^{\bullet-}$ with much higher rate constants than traditional spin traps such as DMPO and DEPMPO, and as such may be useful markers of $O_2^{\bullet-}$ formation [100–102]. However, as hydroxylamine oxidation can occur with multiple radicals, and also be induced by peroxidase enzymes, this approach needs to be used with care, and its limitations understood. The issue of lack of specificity can be addressed by parallel experiments, for example, by the addition of superoxide dismutase (SOD) to implicate $O_2^{\bullet-}$, as this should decrease the intensity of the nitroxide EPR signal if $O_2^{\bullet-}$ (or species derived from this) are causative in the oxidation [102]. This does however require the enzyme to have access to the site of $O_2^{\bullet-}$ formation, which may not always be easy to achieve.

A number of hydroxylamines that have desirable properties (low artifactual oxidation, rapid kinetics of reaction with radicals, persistent nitroxides once oxidised) have been developed and are commercially available. Addition of specific groups has allowed these materials to be localised at particular sites (external or internal to cells) and in specific sub-cellular organelles (e.g. mitochondria) [103]. One clear limitation of this approach is the lack of data relating to the nature of the oxidising radical. Although SOD can be used to examine the role of $O_2^{\bullet-}$, there are limited options when other radicals or oxidants are involved. Hydroxylamines that can be detected by other spectroscopic means (e.g. by fluorescence) have also been developed [104], together with pro-fluorescent nitroxides as markers of oxidation processes in vitro and in vivo (reviewed [105]).

7. Spin trapping of nitric oxide ($NO^{\bullet}$)

Nitric oxide ($NO^{\bullet}$) is a key intracellular signalling molecule, and an important regulator of numerous biological processes (e.g. vascular

dilation, blood pressure regulation, platelet aggregation, neurotransmission). As such, there is therefore widespread interest in the detection and quantification of $NO^{\bullet}$ in complex systems. As the unpaired electron in $NO^{\bullet}$ is delocalised across both the oxygen and nitrogen, it does not react rapidly with traditional EPR spin traps, and consequently alternative approaches are required to detect this species.

One well-established, biologically-important, reaction of $NO^{\bullet}$ is its reaction with Fe(II) heme species, a process that occurs with a high rate constant. This process allows the detection of $NO^{\bullet}$ in situations where heme proteins are endogenous or can be easily introduced, as nitrosyl-heme species can be readily detected by EPR, with the spectra having highly distinctive triplet 1:1:1 couplings arising from the interaction of the unpaired electron with the nitrogen atom [106]. Moreover, the origin of the observed spectra can be verified by using ^{15}N-labelled precursors, which results in conversion of the triplet coupling to a large doublet [107]. Endogenous Fe(II) heme proteins, in particular haemoglobin (Hb) and myoglobin, have been widely used in this respect allowing measurements to be made on humans, and the prospect of in vivo quantification using L-band spectrometers [108,109]. The detection limit of Hb-NO in human blood has been reported to be ~200 nM, but basal levels (in both arterial and venous blood) appear to be below this limit. Exposure to $NO^{\bullet}$ results in dramatic increases in blood concentrations with levels in the range 0.25–2.5 μM having been reported, with these levels remaining elevated for considerable periods (half-life ~ 40 min) [110]. These concentrations are similar to those detected by tri-iodide chemiluminescence, but lower than those reported for other methods (e.g. some chemiluminescence techniques) [110]. This approach of trapping with endogenous heme proteins has been extended to plants, where $NO^{\bullet}$ formation has been detected using leghaemoglobin (a myoglobin analogue) in the symbiotic root nodules on leguminous plants [111].

Nitronyl nitroxides (e.g. 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; carboxy-PTIO) react with $NO^{\bullet}$ to yield iminonitroxides (e.g. 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl), which have highly distinctive EPR spectra that are markedly different to the parent nitroxide, thereby allowing the two radicals to be readily discerned from each other. This therefore provides a method that allows the formation of $NO^{\bullet}$ to be examined [112–114]. The stoichiometry of the reaction of $NO^{\bullet}$ with carboxy-PTIO is theoretically 1:1 [113], but later work suggests that the true figure is closer to 0.6:1 as a result of the occurrence of competing reactions [114]. It has also been reported that the stoichiometry of conversion varies with the rate of $NO^{\bullet}$ generation, making quantification of $NO^{\bullet}$ by this method difficult [114]. As might be expected, these nitronyl nitroxides also react rapidly with other radicals such as $O_2^{\bullet-}$ (with $k \sim 8 \times 10^5\ M^{-1}\ s^{-1}$) but this process does not generate the iminonitroxide [115]. However, reactivity with $O_2^{\bullet-}$, together with other reduction pathways that remove nitroxides (e.g. reaction with GSH and ascorbate [115]), may significantly perturb the concentration of both the parent nitroxide and the product iminonitroxide making quantification of $NO^{\bullet}$ by this means problematic. Nitronyl nitroxides have recently used as an agent to distinguish between $NO^{\bullet}$ and HNO [116].

Water-soluble Fe(II) dithiocarbamate complexes (e.g. those derived from diethyldithiocarbamate (DETC), *N*-methyl-D-glucamine (MGD), *N*-(dithiocarboxy)sarcosine (DTCS)) have been used extensively both in vitro and in vivo to quantify $NO^{\bullet}$ (reviewed [117–120]). This results in the formation of EPR-detectable Fe–NO complexes at room temperature, with distinctive nitrogen hyperfine couplings and g values (e.g. $a_N \sim 1.25$ mT, $g \sim 2.04$ for the $(MGD)_2$–Fe(II) species). As with other $NO^{\bullet}$ trapping methods, the identity of the complex can be confirmed using ^{15}N-labelled materials which result in the appropriate changes in numbers of absorptions, hyperfine coupling constants and line intensities [118,119]. A range of ligands can be used, in order to provide persistent $NO^{\bullet}$ adduct complexes that accumulate, even with relatively low rates of $NO^{\bullet}$ generation. It should be noted that the Fe centre needs to be kept in the +2 state (or to be readily reduced to this [121,122]) for the reaction to occur. Variation in the ligands also allows the solubility of the complex to be altered, allowing examination of different environments, and modulated penetration into cell, plant or animal systems [122]. However it should be borne in mind that high levels of iron complexes can be toxic, and that (auto)oxidation of the complex is likely to result in oxidant formation, thereby perturbing the system under study. As such there is a need for a balance between having sufficient complex to trap any $NO^{\bullet}$ generated, and the possibility of the trapping agent generating significant damage to the system under study. Although $(MGD)_2$–Fe(II) and $(DCTS)_2$–Fe(II) complexes fulfil some of these criteria (e.g. long lifetimes in blood — up to 12 h [108]), which has allowed them to be used in studies on animals (e.g. in studies on brain, liver, kidney, heart and urine) and plants [122] and detected non-invasively using L-band EPR [118], their potential toxicity is a significant potential drawback. No toxicity has been reported at levels of up to 2.5 g kg^{-1} for MGD itself [123]), but this would be expected to be system specific and perturbations may occur at lower levels. Furthermore, the stability of the complexes in water, particularly at low pH values, and the poor water solubility of the DETC species can limit their use [120].

Another approach that has been used to study both O_2 and $NO^{\bullet}$ concentrations is broadening of the EPR lines arising from solid carbon-based paramagnetic species (chars) [124]. This approach has been used extensively to study O_2 levels in vivo, but a similar line-broadening effect occurs with $NO^{\bullet}$, and hence this could be used potentially to measure $NO^{\bullet}$ concentrations [125]. However, the levels of $NO^{\bullet}$ required to detect significant changes are in the pharmacological range rather than the physiological, and hence this method may not be sensitive enough to measure the concentrations present in vivo in animals. Changes in O_2 levels may also confound measurements, as this will also contribute to the overall linewidth [125].

8. Complementary techniques that can be used together with EPR to provide additional data

Whilst detection and identification of radicals and paramagnetic metal ions can be a goal in itself, many current research programmes are more broadly focused, with the goal of examining the role of radicals in a particular biological/medical context. It has therefore become increasingly necessary to pair EPR measurements with other methods (e.g. product analysis) in order to provide a more in depth picture of the role and consequences of radical generation. A wide range of quantitative techniques have therefore been developed that allow additional information pertinent to radical generation and reactions to be obtained. These include quantification of loss of parent materials, formation of specific protein (e.g. side-chain) oxidation products (reviewed [126]), products of DNA damage (reviewed [127,128]) and lipid oxidation products (isoprostanes, hydroperoxides, alcohols, epoxides, hydrocarbons, aldehydes [129–131]) and detection of generic markers of such reactions (e.g. protein carbonyls [126,132], DNA strand breaks [133], protein thiol levels [126]). These methods depend on the sensitivity and resolving power of techniques such as HPLC and GC, with detection of materials by UV, visible and fluorescence spectroscopy, electrochemistry and various MS techniques. MS in particular has become a powerful tool in the search for, and quantification of, low levels of oxidation products in biological samples (e.g. [130,134]).

A wide range of other semi-quantitative techniques have also been developed to further study radical processes. These include the use of dyes that have altered fluorescence properties (gain, loss, or altered wavelength) on oxidation (e.g. [135–138]. However, results from experiments with many fluorescent dyes need to be interpreted with care, as often fluorescence changes are not specific to particular radicals [139,140]). Other methods include the use of specific

electrodes (e.g. for $NO^{\bullet}$, O_2, H_2O_2), and ELISA and Western-blotting methods using antibodies raised against specific oxidation products (e.g. protein carbonyls, 3-nitrotyrosine, methionine sulphoxide, isoprostanes [126]).

Many of these techniques have been coupled with EPR to aid radical identification and characterisation of the resulting products. Direct MS analysis has been attempted for spin adducts, primarily with well-characterised systems. For example, MS has been coupled with MNP spin trapping to detect radicals generated from liver metabolism of halocarbons [141,142], and the formation of thiyl radicals [143], with various low-molecular-mass radicals trapped by DEPMPO also quantified in this manner [144,145]. For complex systems with multiple radicals, and hence many radical adducts, the MS approach has been combined with HPLC or UPLC separation to resolve the products prior to analysis. Gas chromatography (GC) for such studies is more limiting, as separation conditions tend to promote adduct degradation, in contrast to HPLC for example, where water-based eluents, and ambient temperatures that can be used. The HPLC-MS approach has been used extensively, and successfully, to examine a number of systems [146–157]. In many cases, the species detected by MS are not the radical adducts themselves, but species derived from them, either by oxidation or reduction (e.g. [158,159]). This is not necessarily a disadvantage, as long as the chemistry that results in the formation of these species is well understood. Indeed the ability of a number of techniques to detect nitrones resulting from oxidation or disproportionation of initial radical adducts has resulted in the development of novel approaches to radical detection in biological systems.

Kagan and colleagues have developed an assay for glutathione thiyl radicals in cells, where the nitrone formed from decay of the initial (short-lived) $GS^{\bullet}$ adduct to DMPO is detected by LC-MS [160]. Whilst this method can yield valuable data, absolute quantification of $GS^{\bullet}$ by this approach may be limited by the presence of alternative removal pathways for the initial adduct (e.g. rapid nitroxide reduction to the hydroxylamine). As the ratio of these two routes is likely to be system dependent, the yield of GS-DMPO nitrone may vary significantly, and hence be misleading.

The detection of nitrones arising from adduct decay has also been exploited in the in the technique of immuno-spin trapping [46]. In this (non-EPR) technique, antibodies raised against a DMPO nitrone derivative are used to probe for the presence of DMPO adducts to macromolecules. These antibodies, which can be used in ELISA or Western blotting experiments, detect the nitrone present on the macromolecules, and by linking this recognition (which is highly specific as cyclic nitrones are very rare in nature) to highly sensitive detection systems (e.g. enhanced chemiluminescence), the initial presence of radical adducts can be detected with great sensitivity and specificity [46,161–163]. This approach has been used with great success to detect radicals in both isolated systems and in cells formed on a range of proteins [164–170] and DNA [161,171]. The use of fluorescently-tagged secondary antibodies and confocal microscopy has allowed sites of radical adduct formation to be determined in intact cells, though it is not possible to identify specific types of radical formed, as the antibodies recognise only the nitrone function and not the group through which the nitrone is linked [170]. This approach also has utility for the visualisation of radical formation in vivo, when used in combination with magnetic resonance imaging [172] or immunohistochemistry [173]. In each case, immuno-spin trapping should be seen as a highly sensitive and specific complimentary technique to more traditional EPR studies where information is obtained on the nature of the intermediate species.

9. Constraints and pitfalls of using EPR

EPR is a highly specific and powerful tool for detecting radicals and some metal ions in biological systems and is, and is likely to remain, the "gold standard" for the detection and identification of radicals in biological systems. When used correctly, it has an unparallelled potential to provide detailed information on the identity, structure, concentration, motion and environment of radicals and paramagnetic metal ions, without the common problem of complex interfering background signals, which complicate many other spectroscopic techniques. However, it is a far from a faultless technique, and it is critically important that the user is aware of the shortcomings and limitations of this technology, which are a ready trap for the unwary.

Whilst the sensitivity of modern spectrometers is a great asset in detecting the low levels of radicals that are present in biological systems, this also brings with it attendant problems particularly with regard to determining whether radical formation is a major process and responsible for observed effects, or merely a minor side reaction that appears to be important because it generates significant concentrations of persistent radicals that can be readily detected. Furthermore the greater sensitivity of modern EPR spectrometers allows the detection of minor impurities in reagents and supposedly pure spin traps which are a significant nuisance.

Although there is a significant body of data available in the literature on hyperfine coupling constants and g values for many radicals, there remain a number of significant omissions that hinder the development of the technique. In particular, the field would benefit from further data in the following areas: a) the rate constants for spin trapping and spin adduct decay processes and kinetics which prevent true quantification of spin trapping data; b) methods for the analysis of complex mixtures of radical adducts particularly those present on biological macromolecules, which yield highly complex spectra due to the slow rate of motion of these species resulting in broad overlapping spectral lines; c) the eternal problem of deciphering which signals are true radical adduct and which are artefacts in spin trapping studies; d) spin traps that have both high rate constants for trapping and slow rates of radical adduct decay (i.e. give persistent adduct species; e) additional spin traps that can be compartmentalised or localised at particular sites in intact systems (though immuno-spin trapping offers promise in this area); f) additional spin traps of low toxicity and that are inert in the systems under study; and finally (and possibly most importantly), a spin trap that can be used safely in humans, though there are an increasing number of promising potential therapeutic applications of nitrones (reviewed [174]).

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