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## **Use of spin traps to detect superoxide production in living cells by electron paramagnetic resonance (EPR) spectroscopy**

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### **Abstract (<250 words)**

Detection of superoxide produced by living cells has been an on-going challenge in biology for over forty years. Various methods have been proposed to address this issue, among which spin trapping with cyclic nitrones coupled to EPR spectroscopy, the gold standard for detection of radicals. This technique is based on the nucleophilic addition of superoxide to a diamagnetic cyclic nitron, referred to as the spin trap, and the formation of a spin adduct, i.e. a persistent radical with a characteristic EPR spectrum. The first application of spin trapping to living cells dates back 1979. Since then, considerable improvements of the method have been achieved both in the structures of the spin traps, the EPR methodology, and the design of the experiments including appropriate controls. Here, we will concentrate on technical aspects of the spin trapping/EPR technique, delineating recent breakthroughs, inherent limitations and potential artifacts.

**Keywords:** EPR spectroscopy, ESR spectroscopy, Spin trapping, Macrophage, Free radical, Cyclic nitron.

## Abbreviations

2-OH-E<sup>+</sup>, 2-hydroxyethidium; AMPO, 5-carbamoyl-5-methyl-1-pyrroline *N*-oxide; BMPO, 5-tert-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide; CD, cyclodextrin; CD-DEPMPO, 6-monodeoxy-6-mono-4-[(5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide)-ethylenecarbamoyl-(2,3-di-*O*-methyl) hexakis (2,3,6-tri-*O*-methyl)]-β-cyclodextrin; CD-DIPPMPO, 6-monodeoxy-6-mono-4-[(5-diisopropoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide)-ethylenecarbamoyl-(2,3-di-*O*-methyl) hexakis (2,3,6-tri-*O*-methyl)]-β-cyclodextrin; CFU, colony-forming unit; CYPMPO, 5-(2,2-dimethyl-1,3-propoxycyclophosphoryl)-5-methyl-1-pyrroline *N*-oxide; CW, continuous wave; DCFH<sub>2</sub>, dihydro-2',7'-dichlorofluorescein; DEPMPO, 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide; DFT, density functional theory; DIPPMPO, 5-diisopropoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide; DMPO, 5,5-dimethyl-1-pyrrolidine *N*-oxide; DMPOX, 5,5-dimethyl-2-pyrrolidone-*N*-oxyl; DTPA, diethylene triamine pentaacetic acid; E<sup>+</sup>, ethidium; EMPO, 2-ethoxycarbonyl-2-methyl-3,4-dihydro-2*H*-pyrrole *N*-oxide; EPR, electron paramagnetic resonance (equivalent to ESR, electron spin resonance); [Fe-S] clusters, iron-sulfur clusters; fMLP, *N*-formylmethionyl-leucyl-phenylalanine; GPx, glutathione peroxidase; GSH, glutathione; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; HE, hydroethidine; HO<sup>•</sup>, hydroxyl radical; Hp, hemeprotein; HRP, horseradish peroxidase; Mito-DIPPMPO, (4*R*<sup>\*</sup>, 5*R*<sup>\*</sup>)-5-(diisopropoxyphosphoryl)-5-methyl-4-[[[2-(triphenylphosphonio)ethyl]carbamoyl]oxy)methyl]pyrroline *N*-oxide bromide; PEG, polyethylene glycol; Nox, NADPH oxidase; NBT, nitro blue tetrazolium; NMR, nuclear magnetic resonance; NO<sup>•</sup>, nitric oxide; O<sub>2</sub><sup>•-</sup>, superoxide radical anion; ONOO<sup>-</sup>, peroxynitrite; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; PMNs, polymorphonuclear leukocytes; ROS, reactive oxygen species; SCE, saturated calomel electrode; SOD, superoxide dismutase; TAMs, triarylmethyl radicals.

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## 1. Introduction

### 1.1 Superoxide as a key intermediate in oxidative stress and a mediator of cell signaling

Superoxide radical anion ( $O_2^{\bullet-}$ ) is a major by-product of aerobic metabolism as a result of monoelectronic reduction of molecular oxygen by enzymes in the mitochondrial respiratory chain [1]. Other important sources, either endogenous or exogenous, include enzymes in the NADPH oxidase family (Nox), xanthine oxidase, microsomes, autoxidation reactions of reduced flavines, quinones, metal ions, metalloproteins, and exposition to ionizing radiation or photochemical irradiation. Characterized by both mild oxidizing and reducing properties, the reactivity of  $O_2^{\bullet-}$  results mainly from its interaction with radicals and cationic iron-sulfur clusters ([Fe-S] clusters). It is a precursor to more toxic species. Hydrogen peroxide ( $H_2O_2$ ) arises from spontaneous and enzyme-catalyzed disproportionation or from reduction of  $O_2^{\bullet-}$ . The highly reactive hydroxyl radical ( $HO^{\bullet}$ ) quickly follows. Peroxynitrite ( $ONOO^-$ ) results from the direct reaction of  $O_2^{\bullet-}$  with nitric oxide ( $NO^{\bullet}$ ). In cells,  $O_2^{\bullet-}$  is efficiently removed by the antioxidant enzyme superoxide dismutase (SOD) [2]. The instability of  $O_2^{\bullet-}$  and its poor ability to cross cell membranes due to its negative charge do not favor its role as a signaling molecule. However the SoxR transcription factor is an established example of specific  $O_2^{\bullet-}$  sensing through [Fe-S] clusters [3].

Monitoring production of  $O_2^{\bullet-}$  in cells with ever increasing sensitivity is thus an important issue in biomedical research on inflammation, cancer, aging, or atherosclerosis among other fields. Several techniques have been developed to answer this question, some of which based on spectrophotometric or fluorescent probes or on HPLC detection [4,5]. Here we will concentrate on technical aspects of the spin trapping/EPR technique, delineating recent breakthroughs, inherent limitations and potential artifacts.

### 1.2 Principle of spin trapping

EPR spectroscopy is the most direct method for characterizing and measuring free radicals in chemistry and biology, despite its rather low sensitivity. Using conventional X-band ( $\nu \approx 9$  GHz) spectrometers, the detection threshold is in the range of 2-3  $\mu$ M for a 25  $\mu$ L sample (however it highly depends on the experimental setup and the acquisition parameters). Most free radicals are highly reactive and have short half-life in biological environment, and thus their concentration does not reach measurable level under such conditions. Therefore, direct observation is not possible; free radicals are observed indirectly by using spin traps. A *spin trap* is a diamagnetic molecule that readily reacts with a reactive radical to form a more stable radical product called a *spin adduct*, the concentration of which can reach measurable level (Figure 1). This radical stabilization technique is called *spin trapping*. Spin trapping experiments with 5,5-dimethyl-1-pyrrolidine N-oxide (DMPO, Figure 1) in chemical systems were first reported in 1967 by Iwamura and Inamoto [6]. The method was further developed by Janzen and Blackburn [7], who coined the terms *spin trap* and *spin trapping*, and its first application to cellular systems dates back 1979 with detection on suspensions of stimulated human neutrophils [8].

The best spin traps for  $O_2^{\bullet-}$  are compounds with a cyclic nitron functional group that form persistent aminoxyl (nitroxide) radicals after addition of the reactive radical species. Though the spin traps are not selective of one radical, the resulting nitroxide spin adduct has a distinctive EPR spectrum which gives information about the trapped radical species.

Indeed, both nitrogen and  $\beta$ -hydrogen hyperfine coupling constants in spin adducts derived from cyclic nitrones are sensitive to the nature of the trapped radical. A careful selection of the spin trap is however necessary to unequivocally distinguish oxygen-centered and thiyl radical adducts [9–11]. In the following, we will append -OOH and -OH to the spin trap name to refer to the  $O_2^{\bullet}$  and the  $HO^{\bullet}$  adducts, respectively.

### 1.3 Spin traps developed for cell studies

DMPO was the first cyclic nitron used in spin trapping studies in cells and is still most commonly used (average of 13 articles published each year since 1990 according to Web of Science™), despite well identified pitfalls. Indeed, detection of DMPO-OH following incubation with stimulated neutrophils was first considered as an evidence for  $HO^{\bullet}$  production in these cells [8,12], before it was proved, both experimentally and by computation, that DMPO-OOH spontaneously (non-enzymatically) decomposes to several species including DMPO-OH with a half-life under 1 min [13–15], and that stimulated neutrophils do not produce  $HO^{\bullet}$  [16]. Thanks to the very active research in the field of spin trap design, several derivatives of DMPO with tuned lipophilicity have been synthesized since, bearing electron-withdrawing groups at position 5 of the ring, either alkoxycarbonyl (e.g., EMPO [17], BMPO [18], AMPO [19]) or dialkoxyphosphoryl substituents (DEPMPO [20], DIPPMPPO [21], CYPMPPO [22]) (Figure 2). Not only do these spin traps offer a dramatically enhanced intrinsic stability of the corresponding  $O_2^{\bullet}$  spin adducts in buffer, but no spontaneous decomposition to the  $HO^{\bullet}$  adduct is observed with these traps. The thermodynamically-preferred pathways for the spontaneous decomposition of  $O_2^{\bullet}$  spin adducts and the decomposition of their one-electron oxidation and reduction products were explored by computational studies, showing the need of a "loose" bond acceptor in the structure but also of both a low electron affinity and a low ionization potential for improved adduct stability [15,23,24]. Most of these traps now are commercially available and demonstrate extended shelf-life compared to DMPO, although they remain expensive and can be difficult to obtain. Phosphorylated nitrones allow easier identification of the trapped species because the large hyperfine coupling to  $^{31}P$  is highly dependent on the structure of the trapped radical. This can however be slightly detrimental to the sensitivity of detection due to a doubling of the number of lines in the EPR spectrum, which reduces the signal-to-noise ratio. The reaction of  $O_2^{\bullet}$  with DEPMPO derivatives leads to a mixture of diastereoisomers with slightly different EPR spectra. Rapid equilibrium between conformers of the predominant *trans* adduct also impacts the EPR spectra [25]. Further substitution at position 4 of the pyrroline ring in a *cis* position compared to the dialkoxyphosphoryl group in DEPMPO or DIPPMPPO simplifies the EPR spectrum by favoring the formation of one diastereoisomer and stabilizing one conformation of the ring in the adduct [26]. Moreover, conjugation of the pyrroline N-oxide ring with a permethylated  $\beta$ -cyclodextrin (CD), as in CD-DEPMPO or CD-DIPPMPPO [27,28], allows selective detection of  $O_2^{\bullet}$  over  $HO^{\bullet}$ , probably due to competition for  $HO^{\bullet}$  between the methoxy groups in the CD and the nitron function. Conjugation with a CD also guarantees extracellular localization of the spin trap, by contrast with DMPO and DEPMPO which are assumed to readily permeate cell membranes [29,30]. These newly developed compounds are unfortunately not commercially available, which limits their application.

A determinant characteristic of an efficient spin trap for  $O_2^{\bullet}$  is the kinetic rate of the spin trapping reaction ( $k_T$ ). Kinetics studies of this reaction are however difficult to perform and lots of published absolute values of  $k_T$  are still controversial due to biased methods of

determination [31,32]. Comprehensive analysis of the spin trapping reaction by Villamena *et al.* using the density functional theory (DFT) approach greatly contributed to rationalize the design of improved spin traps [23,33,34]. The nucleophilic nature of  $O_2^{\bullet-}$  addition to nitrones explains why increasing the positive charge at position 2 of the ring through electron-withdrawing groups at C-5 is beneficial to the spin trapping rate, provided excessive steric repulsion on position 5 is avoided. Also, initial H-bond interaction of  $O_2^{\bullet-}$  with an H-bond donor, such as an amide function, at position 5 further enhances its nucleophilicity towards the nitronone function, yielding higher  $k_T$ . Whatever the spin trap, the reaction with  $O_2^{\bullet-}$  remains a slow process ( $k_T < 100 \text{ M}^{-1} \text{ s}^{-1}$ ) [35] and large concentrations of spin traps (10-100 mM) are required to compete with  $O_2^{\bullet-}$  spontaneous disproportionation. Fortunately, most cyclic nitrones demonstrate low toxicity towards cellular systems at these concentrations, with the exception of BMPO which was moderately toxic in CHO cells and various cancer cell lines at 50 mM [29,36-38].

#### 1.4 Spin adduct stability and metabolism

A major limitation to the application of spin trapping with cyclic nitrones in cell studies is the instability of the spin adducts in biological systems which leads to rapid loss of EPR signal. The rate of cell-induced spin adduct decay is highly dependent on the cell type and on the activation state of the cells [39,40]. Modification of the flux of  $O_2^{\bullet-}$  production can also influence spin adduct stability [41], since reduction of the adducts by  $O_2^{\bullet-}$  is thermodynamically favored and supposed to play an important role in spin adduct decay [24]. Investigations, using redox agents, cells, or cellular fractions, of the biochemical mechanisms responsible for signal decay and the structure of diamagnetic products enabled to distinguish between oxidative and reductive pathways involved on this phenomenon [28,29,42-46]. Ferric hemeproteins with sufficient access to the iron cofactor, such as cytochrome P450, hemoglobin, or horseradish peroxidase (HRP), but not cytochrome *c*, could induce homolytic or heterolytic cleavage of the hydroperoxyde function in the  $O_2^{\bullet-}$  adducts of small-size cyclic nitrones, yielding ring-opened diamagnetic compounds or the  $HO^{\bullet}$  adduct, which upon further oxidation of the aminoxyl (nitroxide) function would lead to a highly unstable oxoammonium cation as an intermediate (Figure 3A) [46]. In fact, the reduction by intracellular reductants usually accounts for the largest contribution to EPR signal loss (Figure 3B). Reaction with cytosolic ascorbate forms EPR silent hydroxylamines, which are prone to fast elimination of small molecules, yielding the parent nitronone [44,46]. Glutathione peroxidase in the presence of glutathione (GPx/GSH) has been shown to catalyze the reduction of the  $O_2^{\bullet-}$  adduct to the  $HO^{\bullet}$  adduct [43]. Analogues of DMPO and DEPMPO displayed similar behavior when submitted to these different pathways [28]. Only when the spin trap structure ensures extracellular localization, are the adducts stabilized against cell-induced decomposition, as observed with the permethylated  $\beta$ -cyclodextrin conjugate CD-DIPPMPO [29].

#### 1.5 Comparison to other detection methods

Detailed reviews for comparing reactive oxygen species (ROS) detection methods have been published elsewhere and are beyond the scope of this work [4,47-49]. We will thus focus only on general conclusions here to highlight differences in specificity and reliability of each method compared to spin trapping.

Among the oldest means of detection, spectrophotometric probes, i.e. substances with optical properties that change upon reaction with  $O_2^{\bullet-}$ , are usually simpler to implement



and are rather specific of  $O_2^{\bullet-}$  but they are less sensitive than spin traps [4]. Common approaches include monitoring of the reduction of cytochrome *c* by  $O_2^{\bullet-}$  (increase of light absorption at 550 nm) and of the reduction of various tetrazolium salts (among which nitro blue tetrazolium, NBT). Known limitations of NBT method are  $O_2^{\bullet-}$ -independent reduction of the probe by cellular reductases and subsequent  $O_2^{\bullet-}$  production by interaction with oxygen.

Fluorescent probes, i.e. non fluorescent (or weakly fluorescent) reagents that yield fluorescent products upon reaction with ROS, such as dihydrorhodamines or the diacetate and free form of dihydro-2',7'-dichlorofluorescein (DCFH<sub>2</sub>), have been most frequently used to measure oxidative stress in cells due to their good sensitivity and versatility. However they are not specific of  $O_2^{\bullet-}$  and actually have a very low reactivity with  $O_2^{\bullet-}$  in absence of peroxidase-like catalyst [47]. Besides, a major artifact has been described with 2',7'-dichlorofluorescein derivatives in that they generate ROS themselves within cells [50,51]. Recently, a new series of fluorescent probes, that afford a specific non-redox detection of  $O_2^{\bullet-}$  and overcome interference with cellular reductants (glutathione and Fe<sup>2+</sup>), has been proposed [52]. The lead compound HKSOX-1 is a trifluorosulfonate derivative of 5-carboxy-2',4',5',7'-tetrafluorofluorescein. Clearly, more work needs to be done to fully characterize these promising probes.

Less frequently used, chemiluminescent probes for  $O_2^{\bullet-}$  based on lucigenin or luminol offer attractive high detection sensitivity. However, just as with NBT and DCFH<sub>2</sub>, redox cycling of the probes is known to generate  $O_2^{\bullet-}$  in cells and the detection is prone to many interferences with cellular reductants and oxidants, which are difficult to control [4,47]. As a consequence, there is a growing consensus against their use in living cells.

Hydroethidine (HE) and analogues are much more reactive towards  $O_2^{\bullet-}$  than reduced fluoresceins and rhodamines, specifically yielding 2-hydroxyethidium (2-OH-E<sup>+</sup>). Using HPLC but not fluorescence, 2-OH-E<sup>+</sup> can unequivocally be identified and separated from ethidium (E<sup>+</sup>) and dimerization products arising from the reaction with various biologically relevant oxidants [5,53]. This is thus considered a sound qualitative method of detection. The formation of 2-OH-E<sup>+</sup> is however a two-step process, the first oxidation step being non-specific, by opposition to spin trapping. The response of HE to  $O_2^{\bullet-}$  could thus be altered if another oxidant initially comes into play. Moreover, the rate of formation of 2-OH-E<sup>+</sup> within cells is dependent upon both HE cellular uptake and depletion by competing oxidation pathways. Quantitative monitoring of intracellular  $O_2^{\bullet-}$  could therefore require measurement of the ratio between 2-OH-E<sup>+</sup> and HE but this strategy needs further validation [54,55].

The low sensitivity of most commonly used spin traps is a major limitation of spin trapping. More sensitive EPR probes for ROS exist, such as diamagnetic hydroxylamines which yield nitroxide compounds upon oxidation by ROS [56–58]. They are designated as spin probes and should not be confused with spin traps where a covalent bond is formed with  $O_2^{\bullet-}$ . The rate constants of the reaction with  $O_2^{\bullet-}$  are at least a hundred times higher than with cyclic nitrones [4]. However, hydroxylamines do not afford unambiguous identification of the involved ROS, since they react with  $O_2^{\bullet-}$ , ONOO<sup>-</sup>, peroxy radicals, and H<sub>2</sub>O<sub>2</sub> in the presence of metal ions. Use of specific scavengers can help overcoming this drawback, provided it is proved that they access the site of detection. Hydroxylamines are also sensitive to autoxidation in air, which may lead to high background signals. Their reaction products, nitroxides, are sensitive to reduction in cellular systems (mainly by ascorbate or reductases) [59], though to a lesser extent than nitron spin adducts.

Monitoring the decay of spin probes such as triarylmethyl radicals (TAMs) is another EPR method for ROS detection. However loss of EPR signal occurs upon reaction with both  $O_2^{\bullet-}$



and peroxy radicals [60,61], and even if TAMs are not sensitive to reduction by ascorbate and NAD(P)H, reduction by reductases is quite efficient and can interfere with the detection.

In the end, it seems that the ideal, flawless detection method for  $O_2^{\bullet-}$  within cells is still out of reach. Yet, combination of well-characterized methods can help develop a more in-depth analysis of ROS produced in cellular systems, spin trapping with cyclic nitrones coupled to EPR being one of them.

## 2. Material and methods

### 2.1 Spin trap stock solutions

It is essential that spin trap solutions are free of contamination by hydroxylamine and nitroxide impurities that can impair  $O_2^{\bullet-}$  detection and give artifacts. Contaminating hydroxylamines can be tested by treatment with ferricyanide which converts them to EPR-visible nitroxides [62]. Most commercially available DMPO contains paramagnetic impurities. More recently described spin traps are usually more stable upon long-term storage at  $-20^{\circ}C$  or lower. However, DEPMPO and EMPO are colorless to slightly yellow oils that can also be contaminated. Distillation of the neat oils is difficult on a small scale. Purification methods of contaminated solutions of spin traps by sequential treatment by activated charcoal and ferricyanide have been proposed but can be rather tedious [31,63–66]. By contrast, BMPO and CYPMPO are crystalline solids and are easily purified by recrystallization [18,22].

Stock solutions of spin traps should be prepared in an appropriate buffered saline containing a metal chelator such as diethylene triamine pentaacetic acid (DTPA, 0.1 - 1 mM) when metal ions are not required in the assay and should be stored at  $-20^{\circ}C$  or below. A sufficient amount of the spin trap should be introduced in the assay before the start of  $O_2^{\bullet-}$  production to ensure satisfactory accumulation of the spin adduct above EPR detection limit. Enhancement of spin adduct decay by excessive amounts of spin trap has been described [32], however in the presence of cells this effect might be masked by cell-induced rapid spin adduct decay. Typical final concentrations used in the literature are 100 mM for DMPO, 50 mM for BMPO, and 5-20 mM for DEPMPO and CYPMPO.

Use of phenol red- and serum-containing culture media in spin trapping experiments should be avoided since they contain many components susceptible to generate or scavenge free radicals or to reduce spin adducts [67,68]. If these are used for the culture, cells should be carefully washed with buffered saline before addition of the spin trap.

### 2.2 EPR experimental setups and sample preparation

In this section, we will only describe experimental setups requiring commercial X-band continuous wave (CW) spectrometers and resonators.

#### 2.2.1 Detection on cell suspensions

Most spin trapping experiments in the literature have been performed on cell suspensions using either quartz flat cells or glass capillaries as sample holders. Phagocytic cells represent the most studied cell type with DMPO [8,12,16,69–72], because high level of  $O_2^{\bullet-}$  production can be conveniently triggered by incubation with various stimulants. Besides, results have been obtained on menadione-treated endothelial cells or enterocytes [43,73], endothelial cells exposed to anoxia-reoxygenation cycles [74,75], or

*N. gonorrhoeae* bacteria upon incubation with paraquat [76]. More recent spin traps were also individually tested in the same phagocytic cell systems [36,39,44,57]. The quartz flat cell is however a rather insensitive technique, poorly suited to low radical production (especially basal conditions), and requires large amounts of spin trap and cells. Artifacts due to cross-contamination between runs can occur if thorough cleaning of adherent proteins is not performed after analysis of each individual sample. More importantly, in such closed systems, oxygen supply is limited and hypoxia can impair detection of  $O_2^{\bullet-}$ , either because the production of oxygen-derived radicals themselves is lowered or because reductases become activated under these conditions enhancing spin adduct decay – a process that has been investigated using sterically-shielded nitroxides [77,78]. Use of a disposable length of gas-permeable PTFE tubing (Extruded Sub-Lite-Wall, inside diameter 0.635 mm, wall thickness 0.051 mm; Zeus Industrial Products Ltd., Ireland) folded twice in a W shape and inserted in a 4-mm EPR quartz tube solves many of these problems. The small sample size (100  $\mu$ L) allows analysis of multiple conditions from the same stock of cells, comparison of spin trap efficiency of detection, and avoids artifacts of contamination.

Shi *et al.* determined the optimal conditions for detection of  $O_2^{\bullet-}$  produced in human mammary epithelial cells, MCA-10A, upon treatment with a polycyclic aromatic hydrocarbon, 1,6-benzo[a]pyrene quinone (20  $\mu$ M) in phenol red- and serum-free cell growth medium containing 0.1 mM DTPA [40]. Only the  $HO^{\bullet}$  spin adducts derived from reduction of the  $O_2^{\bullet-}$  adducts in cells were detected in this case, as evidenced by inhibition with SOD. In this cellular system, the signal intensity was maximum using  $2.5 \times 10^6$  cells/mL, 50 mM spin trap, normoxia conditions, and physiological temperature of 37°C. From the comparison of four spin traps (one of which did not give any signal), the authors concluded that the maximal intensities (measured as low-field peak height of EPR spectra) obtained with DMPO, BMPO, and DEPMPO were similar, but that detection was more transient with DMPO. The maximum intensity was reached later and lasted longer with DEPMPO.

In a study using suspensions of RAW 264.7 macrophages ( $3.6 \times 10^6$  cells/ 100  $\mu$ L) in phosphate-buffered saline (PBS), we compared the spin trapping efficiencies of six spin traps (DMPO, BMPO, DEPMPO, DIPPMPPO, Mito-DIPPMPPO, and CD-DIPPMPPO) at 5-10 mM final concentration and 21°C upon stimulation with phorbol 12-myristate 13-acetate (PMA, 5  $\mu$ M, 20 min) [29] (Figure 4). Optimal detection was not obtained at such low concentrations with DMPO and BMPO, while 50 mM afforded satisfactory signal with both spin traps. Besides, with this cellular system, mixtures of  $O_2^{\bullet-}$  and  $HO^{\bullet}$  adducts were observed. The SOD-inhibitable signal of the  $HO^{\bullet}$  adduct was preponderant with DMPO, BMPO, DEPMPO, and DIPPMPPO, while Mito-DIPPMPPO and CD-DIPPMPPO enabled a greater detection of the  $O_2^{\bullet-}$  adduct, with a low (if any) amount of  $HO^{\bullet}$  adduct in the case of CD-DIPPMPPO (Figure 5). The sensitivity of detection was dramatically increased with CD-DIPPMPPO compared to all other spin traps, making detection on unstimulated macrophages possible for the first time. The superiority of CD-DIPPMPPO over other spin traps used was attributed to its extracellular localization which protects the spin adducts from intracellular reductants. It appears that the larger linewidth of CD-DIPPMPPO-OOH, arising from a higher molecular tumbling correlation time, which could have reduced the signal-to-noise ratio compared to DEPMPO-OOH, is largely outbalanced by the increase in adduct stability.

These last two examples show how dependent on the cell type the detection is, which means that optimal experimental conditions can vary from one study to another.

Results obtained on cell suspensions may occasionally differ from physiological responses. Cell harvesting methods, such as scraping or trypsinization, can undermine cell integrity. Physical damage to cells induced by mechanical detachment may reduce viability [79,80] or alter cell metabolism [81], while partial enzymatic digestion of membrane-expressed proteins may impair cell function [82–86]. Therefore different alternative setups have been proposed to perform detection of  $O_2^{\bullet-}$  produced by adherent cells. In the next sections, we will review detection on the supernatant and detection directly performed on adherent cells.

### 2.2.2 Detection on supernatant and distant analysis

When adherent cells are incubated with spin traps, the spin adduct formed by trapping of extracellular  $O_2^{\bullet-}$  accumulates in the supernatant and can be detected by EPR, though this method has been used in few studies. Optimal incubation times and spin trap concentration depend both on the level of  $O_2^{\bullet-}$  production, on the spin trapping rate, and on the stability of the adduct in the presence of cells.

Endothelium-derived  $O_2^{\bullet-}$ , a key player in atherosclerosis and vascular aging, is particularly difficult to measure because of its low level of production. Pre-confluent and post-confluent cultured bovine aortic endothelial cells ( $1.5\text{-}2.5 \times 10^6$  cells) in 25 cm<sup>2</sup> flasks were incubated with DMPO (150 mM in a mix containing 1g/L glucose, 0.2 g/L CaCl<sub>2</sub>, 0.0059 g/L DTPA, 0.15 g/L NaCl, 0.37 g/L KCl in sodium phosphate buffer at pH 7.4) for 15 min. The supernatant transferred to a quartz cell was used to assess production of  $O_2^{\bullet-}$  under stimulation by the calcium ionophore A23187 in a dose-dependent manner (3-10  $\mu$ M) in PBS [65]. The signal, identified as resulting from the DMPO-OH adduct, was completely inhibited by SOD (30 U/mL), whereas it was unaltered by addition of catalase (2000 U/mL), confirming it originated from the trapping of extracellular  $O_2^{\bullet-}$ . Production by unstimulated cells was barely detectable under these conditions.

Use of a spin trap that leads to more stable spin adducts, such as BMPO, allows distant analysis of the supernatant after freezing in liquid nitrogen when an EPR spectrometer is not available at the site of experiment [87]. Dysfunctional porcine aortic endothelial cells obtained by successive passages were thus shown to produce significantly more  $O_2^{\bullet-}$  than functional cells using this method [88]. There, one-hour incubation of the cells in the presence of 50 mM BMPO in phosphate buffer under agitation at 37°C, with or without addition of SOD (300 U/mL), was performed and the supernatant was rapidly frozen in liquid nitrogen. The samples were allowed to thaw just before analysis and were rapidly transferred to a 4-bore AquaX® (Bruker, Wissembourg). This specific sample cell, compatible with most standard EPR cavities, is made of closely spaced quartz capillaries with fittings that make it easy to fill and rinse with a syringe while installed within the spectrometer. Series of samples can be analyzed in a short period of time using this system. Adherent proteins can be removed by flushing the system with sodium hydroxide solution followed by buffer between runs since the cell is chemically-resistant.

Detection on the supernatant with BMPO was also successfully applied to the detection of  $O_2^{\bullet-}$  produced extracellularly by Nox family enzymes overexpressed at the plasma membrane of human embryonic kidney HEK 293 cells (Figure 6). NADPH oxidase 5 (Nox5) and dual oxidase 2 (Duox2) are large homologues sharing a plasma membrane associated flavoprotein and an N-terminal extension comprising four and two EF-hand motifs, respectively, implicated in their Ca<sup>2+</sup> activation. Duox2 has an additional transmembrane helix and a peroxidase homology ectodomain with several glycosylated

sites. While Nox5 and immature Duox2 catalyze the production of  $O_2^{\bullet-}$  [89], mature Duox2 is involved in thyroid hormones biosynthesis as the  $H_2O_2$  generator associated with thyroperoxidase. Mutations in Duox2 produce congenital hypothyroidism in humans. SOD-inhibitable and  $Ca^{2+}$ -dependent accumulation of BMPO-OH in the supernatant following release of  $O_2^{\bullet-}$  by Nox5 but not by mature Duox2 at the plasmic membrane was observed (Figure 7). Besides, it confirmed the selectivity of detection of  $O_2^{\bullet-}$  versus  $H_2O_2$  with BMPO since extracellular  $H_2O_2$  productions by both cell lines in the presence of ionomycin were similar (as assayed by the increase in homovanilic acid fluorescence in the presence of HRP). On the contrary, BMPO did not allow detection of  $O_2^{\bullet-}$  produced by immature Duox2 at the endoplasmic reticulum in cell lines lacking the human Duox2 maturation factor hDuoxA2 (i.e. in HEK-hDuox2/pcDNA5FRT cells).

Damage to the cell layer and presence of cells in the supernatant upon freezing should be avoided because leakage of intracellular components will reduce the stability of the spin adducts in the sample, a problem which can be identified when fast signal decay occurs during EPR acquisition. Despite its simple implementation, a significant drawback of the detection on supernatant is the large volume of solution used to cover the cell layer in culture wells and the consequent large amount of spin trap required for the experiments.

### 2.2.3 Detection on undetached cells

As an alternative to detection on the supernatant, measurement can be performed directly on adherent cells introduced inside the EPR cavity by different tricks.

Reinke *et al.* allowed polymorphonuclear leukocytes (PMNs) to adhere to the walls of a quartz flat EPR cell which was placed horizontally for 3 min before recording [90]. They could monitor a large and long-lasting *N*-formylmethionyl-leucyl-phenylalanine (fMLP)-induced  $O_2^{\bullet-}$  release by adherent PMNs using DMPO, while no signal was detected when spin trapping was performed on PMNs in suspension. Unlike fMLP-, PMA-induced  $O_2^{\bullet-}$  release by PMNs was not influenced by cell adhesion.

Britigan *et al.* performed spin trapping of  $O_2^{\bullet-}$  with DMPO (100 mM), in Hank's balanced salt solution without phenol red and containing 0.1 mM DTPA, using endothelial cells cultured on microcarrier beads (Cytodex 3, Pharmacia, Piscataway, NJ) [91]. The final cell concentration in the quartz flat EPR cell with 25 vol% microcarrier beads reached  $0.5\text{--}1 \times 10^7$  cells/mL. Upon exposure to paraquat (1 mM), DMPO-OH signal was observed. It was inhibited by SOD (30 U/mL), while it remained unchanged in the presence of catalase (400 U/mL) or DMSO, which is consistent with  $O_2^{\bullet-}$  trapping.

However, the problems of oxygen supply inside flat quartz cells and of potential cross-contamination between runs already mentioned in previous sections remain with these techniques. To overcome these issues, we recently proposed to perform detection on cells attached to coverslip glasses initially designed for microscope studies [92]. RAW 264.7 macrophages were incubated for 20 min with four spin traps (DEPMPO, DIPPMPO, Mito-DIPPMPO, or CD-DIPPMPO, 5-10 mM) and PBS containing DTPA (1 mM) in 24-well culture plates (150  $\mu$ L/well). Two coverslips were then mounted (cell layer facing outwards) on a quartz slide using EPR silent silicon vacuum grease (Bluestar silicones, Caronno Pertusella, Italy) and introduced inside the EPR TM cavity equipped with a flat quartz protection sleeve (Figure 8). All spin traps gave satisfactory detection upon macrophage stimulation with PMA and the results correlated those obtained with cell suspensions. Again, only the  $O_2^{\bullet-}$  adduct was observed with CD-DIPPMPO. This setup requires a reduced number of cells for one condition (500 000 cells on two coverslips) and a limited



amount of spin trap. Moreover, sample preparation is straightforward which allows medium-throughput EPR detection of  $O_2^{\bullet-}$  production on adherent cells.

### 2.3. Choice of acquisition parameters

As mentioned in previous examples, the choice of EPR parameters for the experiments can be critical because the spin adducts usually form at low and transient concentrations. It is good practice to pretune the cavity with some control sample (buffer or untreated cells) in order to save time when analyzing samples containing short-lived spin adducts. Microwave power between 10 and 20 mW is widely accepted as optimal value to obtain the maximal EPR intensity while avoiding saturation of the signal. However undesired heating of the sample can occur even at 20 mW, which can influence both cell metabolism, spin trapping rate, and spin adduct decay [35]. It is therefore important to keep this value constant and to be aware that results cannot be compared when different EPR sample cells are used (glass capillaries, flat cells, AquaX, gas-permeable tubing...).

Modulation of the magnetic field is used in conventional EPR spectrometers in order to enhance the sensitivity, which results in the EPR spectrum being recorded as the first derivative of the absorption signal with respect to magnetic field. Recommended modulation amplitude is generally around 0.1 mT for nitroxides. However it can be increased up to 0.25 mT in order to fathom very weak signals of spin adducts from the baseline, with only negligible distortion of the EPR spectra.

When looking at very weak signals, EPR spectroscopists usually increase signal-to-noise ratio by using large time constants to filter noise or by repeatedly acquiring the spectrum and subsequently adding each spectrum together. As a result, the signal increases proportionally with the number of scans  $N$ , random noise with  $\sqrt{N}$ , and thus signal-to-noise ratio with  $\sqrt{N}$ . However, modern spectrometers also allow recording of large series of spectra as a function of time and this function can be used as an advantage to further increase the sensitivity of detection with spin traps. By keeping the time constant low and recording large series of spectra that are then treated with statistical data analysis methods such as singular value decomposition (SVD), one can extract kinetic information in addition to performing noise filtration [31,32]. We used this method to unravel unstimulated  $O_2^{\bullet-}$  production in RAW 264.7 macrophage suspensions with CD-DIPPMPO [29].

## 3. Interpretation of the results

### 3.1 Identification of the spin-trapped species and of the site of radical production

According to the theory of spin trapping, the trapped radical can easily be identified by the characteristic EPR spectrum of the spin adduct. Hyperfine coupling constants of  $O_2^{\bullet-}$  and  $HO^{\bullet}$  adducts of common spin traps have been tabulated [9,28]. In the case of phosphorylated spin traps, significant lines for  $O_2^{\bullet-}$  and  $HO^{\bullet}$  adducts do not overlap, which makes interpretation more straightforward. Computer-assisted simulation of the EPR spectra with different softwares (such as WinSim, Roki, or Easyspin) helps in the determination of the relative proportions of overlapping species [93–95].

Interpretation of spin trapping results can sometimes be difficult due to the instability of the spin adducts in biological systems and the conversion of  $O_2^{\bullet-}$  to  $HO^{\bullet}$ -adduct (see section 1.4). However, relatively straightforward procedures exist to determine whether  $O_2^{\bullet-}$  or  $HO^{\bullet}$  radicals have been trapped [96]. Most importantly, to prove extracellular  $O_2^{\bullet-}$

radical formation, incubation with SOD or polyethylene glycol-conjugated SOD (SOD-PEG) (30 U/mL) should prevent the formation of the  $O_2^{\bullet-}$  adduct, while catalase (200 U/mL) should have no effect. (Heat-inactivated SOD can be used to rule out interference of preservatives in commercial preparations of the enzymes.) Neither SOD nor SOD-PEG can cross the cell membrane at short incubation times and thus these controls cannot be used to track down intracellular production of  $O_2^{\bullet-}$ .

The origin of  $HO^{\bullet}$  adduct can be identified by further controls (Figure 9). In the circumstances of  $HO^{\bullet}$  formation, addition of small amounts (1 vol%) of ethanol or DMSO will lead to an inhibition of the  $HO^{\bullet}$  adduct and will result in appearance of a new species due to the trapping of secondary  $\alpha$ -hydroxyethyl or methyl radicals, respectively.  $HO^{\bullet}$  adduct resistant to both SOD- and ethanol/DMSO-inhibition likely originates from the reduction of  $O_2^{\bullet-}$  adduct by intracellular components and can thus be regarded as a sign of intracellular  $O_2^{\bullet-}$  production, provided nucleophilic addition of water under the experimental conditions has been excluded (see section 4.1).

Besides, intracellular versus extracellular localization of spin adducts can be probed by using membrane-impermeant paramagnetic salts, such as tri(oxalato) chromiate(III) (30 mM)[97] or tris(ethylenediamine)nickel(II) chloride (100 mM) [29]. Concentration- and distance-dependent magnetic relaxation enhancement mechanisms (Heisenberg exchange) between the paramagnetic metal ions and the organic radicals lead to line-broadening and diminution of intensity of the EPR signal of spin adducts present in the extracellular space, while intracellular species are unaffected. Passive diffusion of the spin adducts away from their sites of production is however a possibility that must be taken into account for long-lived adducts.

### 3.2 Quantification

The EPR spectrum, being the first derivative of the absorption signal, contains information about the number of spins in the sample. Assuming the recording parameters have been properly chosen to avoid saturation of the signal, the intensity of the EPR spectrum – defined as the area under the absorption curve – is proportional to the number of spins and the concentration of the spin adduct easily follows from the comparison with an external standard of known concentration (usually a stable nitroxide). Can this quantity be used to derive a quantitative estimation of  $O_2^{\bullet-}$  production from cells? Roubaud *et al.* suggested that DEPMPO has ideal properties for such quantitative measurements [39]. Considering the poor spin trapping rate and the low spin adduct stability in the presence of biological systems, any attempt to quantify  $O_2^{\bullet-}$  production by cells using spin trapping is however extremely difficult and should be considered with caution, especially when the concentration of oxygen varies during the experiment, altering both the  $O_2^{\bullet-}$  production rate and the reducing potency of cells. In our opinion, only CD-DIPPMPO, with its high intrinsic adduct stability and its extracellular localization that prevents cell-induced decay, when used in combination with gas-permeable tubing, would make quantitative estimates more reliable.

## 4. Troubleshooting

### 4.1 False positives

Nitrones are very reactive organic compounds and various reactions that do not involve radical trapping may lead to nitroxides and undesired EPR signals. Specific warnings against misinterpretations appeared in the literature [96,98]. Possible artifacts include: i)



oxidation of DMPO and related nitrones to the corresponding hydroxamic acid by metal ions or other oxidizing systems, followed by oxidation to a nitroxide characterized by a 7-line EPR spectrum [99]; ii) inverted spin trapping [100,101], and iii) the Forrester-Hepburn mechanism [102] (Figure 10). These last two mechanisms lead to radicals that are identical to spin adducts but through combinations of mono-electronic oxidation of the spin trap and nucleophilic addition, in either order. Inverted spin trapping, where the oxidation precedes the nucleophilic addition, is a rather limited source of artifacts with cyclic nitrones in biological systems since their oxidation potentials are very high ( $E_{\text{DMPO}^{\bullet+}/\text{DMPO}} \approx E_{\text{DEPMPO}^{\bullet+}/\text{DEPMPO}} \approx 1.9 \text{ V vs. SCE}$  in acetonitrile [101,103]) compared to biologically-relevant oxidation potentials, but it should be considered in the presence of high valency metal ions ( $\text{Fe}^{3+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Mn}^{4+}$ ). These species can oxidize the nitron to the radical cation and make it susceptible to hydration to form the  $\text{HO}^{\bullet}$  adduct (water being the most abundant nucleophile in biological systems). The Forrester-Hepburn mechanism, where the first step involves nucleophilic attack of the  $\beta$ -carbon of the nitron by nucleophiles such as water, sulfite, or thiols and formation of a readily-oxidizable hydroxylamine product, is potentially a greater source of artifacts, especially with nitrones bearing a higher positive charge than DMPO on this position like in DEPMPO, and in the presence of trace metal ions that further activate the electrophilic character of the nitron function by complexation [104,105]. Nucleophilic addition of water leading to the  $\text{HO}^{\bullet}$  adduct is the main concern in the absence of metal chelators. Timmins *et al.* proposed a technique involving pre-incubation with isotopically labeled spin traps to identify such artifacts [106]. This was further refined by Leinisch *et al.* [62,107] and completed by auxiliary experiments by Rangelova *et al.* [98].

## 4.2 False negatives

Unsuccessful EPR detection using spin traps should not be considered as a proof of absence of radical production by the cellular system. The actual spin trap concentration at the site of  $\text{O}_2^{\bullet-}$  production inside cells may be limiting if the rate of cell membrane crossing by DMPO and DEPMPO is slow [30]. The low spin trapping rate and fast cell-induced decay of spin adducts can prevent accumulation of the spin adduct above the detection limit of EPR when low fluxes of  $\text{O}_2^{\bullet-}$  are produced. Incubation with preformed adducts or stable nitroxides can be used to probe the metabolic potential of the cells and test this hypothesis [29]. Simultaneous production of  $\text{NO}^{\bullet}$  may also prevent trapping of  $\text{O}_2^{\bullet-}$  with cyclic nitron spin traps [108].

## 5. Conclusions and perspectives

Since the first application of the technique of spin trapping in cellular systems in the 1970s, many improvements were achieved regarding the structures and properties of the spin traps designed for  $\text{O}_2^{\bullet-}$  detection. The specificity of detection remains the greatest strength of this methodology, together with the absence of redox cycling of the probes within cells. Nowadays extracellular detection of  $\text{O}_2^{\bullet-}$  production can be performed with excellent sensitivity using standard spectrometers and CD-DIPPMPO, however commercial unavailability of the new-generation spin traps is a serious brake to further spreading. On the other hand, low sensitivity for the detection of intracellular  $\text{O}_2^{\bullet-}$  production is still a major shortcoming of the technique, which has up-to-now prevented detection of mitochondrial  $\text{O}_2^{\bullet-}$  within cells for example, despite progresses in CW EPR spectrometer sensitivity and in chemical design of cyclic nitron spin traps. Future

breakthroughs will certainly come from the use of new detection methodologies such as rapid-scan EPR [109]. In rapid-scan EPR, a dramatic increase in sensitivity (> 40 times compared to CW EPR) is obtained by scanning the magnetic field through resonance at a rate that is high compared to electron spin relaxation rates. With this method, 0.5  $\mu\text{M}$  of BMPO-OOH was detected in suspensions of *Enterococcus faecalis* with  $2 \times 10^6$  CFU/mL at a rate of  $\text{O}_2^{\cdot-}$  production of 0.2  $\mu\text{M}/\text{min}$  [110], and imaging of spin trapped radicals at low and physiologically-useful concentrations has become possible [111].

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## Figure legends:

**Figure 1:** Principle of spin trapping with DMPO.  $R^\bullet$  is a short-lived radical, such as  $O_2^{\bullet-}$  or  $HO^\bullet$ .

**Figure 2:** Structure of cited spin traps. The seven-glucopyranose unit of the permethylated  $\beta$ -cyclodextrin in CD-DEPMPO and CD-DIPPMPO is shown as a cylinder and drawing is not to scale.

**Figure 3:** Proposed mechanisms for the disappearance of  $O_2^{\bullet-}$  and  $HO^\bullet$  spin adducts of BMPO: A) in the presence of ferric heme proteins (Hp), B) in the presence of intracellular reductants. (Adapted from reference [46])

**Figure 4:** Procedure for sample preparation using RAW 264.7 macrophage suspensions in gas-permeable tubing as described by Abbas *et al.* [29].

**Figure 5:** Representative EPR spectra obtained by Abbas *et al.* [29] after 3-min incubation of BMPO (10 mM), DEPMPO (10 mM), or CD-DIPPMPO (5 mM) in the presence of PMA-stimulated RAW 264.7 macrophages ( $3.6 \pm 0.3 \times 10^6$  cells/100  $\mu$ L) in PBS (pH 7.1-7.4) and 1 mM DTPA. The y-axis was expanded 5-fold for BMPO and 2-fold for DEPMPO for the sake of clarity. Only BMPO-OH is observed in the upper spectrum. The middle spectrum shows superimposition of DEPMPO-OOH ( $\star$ ) and DEPMPO-OH ( $\bullet$ ). Only CD-DIPPMPO-OOH is seen in the bottom spectrum. EPR parameters: microwave frequency, 9.85 GHz; microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 0.2 mT; receiver gain, 60 dB; time constant, 40.96 ms; conversion time, 41.04 ms; data points, 1024; sweep width, 15 mT; sweep time, 42.02 s. Noise was filtered using the SVD method (see section 2.3).

**Figure 6:** Procedure for spin trapping experiments with HEK 293 cell lines, when EPR detection was performed on the supernatant. Stable human Nox5b-transfected HEK 293 cells (HEK-hNox5) and wild-type HEK 293 cells (HEK-WT) were cultured as previously described [89]. Human Duox2-transfected HEK 293 cells were transiently transfected with human Duox2 maturation factor (hDuoxA2) that allows expression of the mature enzyme at the plasma membrane [112]. The Duox2 cell lines transfected with the empty expression vector (pcDNA5FRT) were used as controls in this case. Cells ( $\sim 1 \times 10^6$  cells) in 6-well culture plates were washed with HEPES buffered Earle's solution (pH 7.2) and incubated for 30 min at 37°C in the same buffer containing EDTA,  $CaCl_2$ , ionomycin, and BMPO. The  $Ca^{2+}$  dependence of the activity was evaluated with 30-min pre-incubations with BAPTA-AM (1,2-bis(2-aminophenoxy)ethane- $N,N,N',N'$ -tetraacetic acid tetrakis(acetoxymethyl ester), an intracellular calcium chelator), followed by incubations with the ionomycin-free mixture.

**Figure 7:** Representative EPR spectra obtained with BMPO on the supernatant of HEK-hNox5 cells, HEK-hDuox2/hDuoxA2 cells, and respective control cells, or without cells, upon stimulation by ionomycin or after incubation with BAPTA-AM. 27 EPR spectra were sequentially recorded over 18 min, the sum of which is shown. Same EPR parameters as in Figure 5 except: microwave frequency, 9.82 GHz; microwave power, 5.1 mW; sweep

width, 6.6 mT. (Unpublished results)

**Figure 8:** Procedure for sample preparation with undetached RAW 264.7 macrophages on microscope coverslip glasses as described by Abbas *et al.* [92].

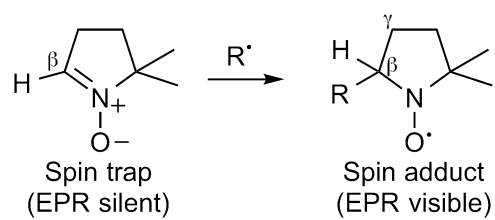
**Figure 9:** Flow chart for determining the origin of HO• adduct of cell-permeant spin traps such as DMPO, DEPMPO, or BMPO.

**Figure 10:** Different pathways leading to artifactual detection of nitroxides in biological systems. DMPO is used as a model spin trap in this figure but similar mechanisms exist for other cyclic nitrones. The EPR spectrum of 5,5-dimethyl-2-pyrrolidone-*N*-oxyl (DMPOX) is characterized by 7 lines and hyperfine coupling constants  $A_N = 0.72$  mT and  $A_{H\gamma} = A_{H\gamma'} = 0.41$  mT in water [99]. Nu: stands for nucleophiles such as H<sub>2</sub>O, sulfite or thiols.

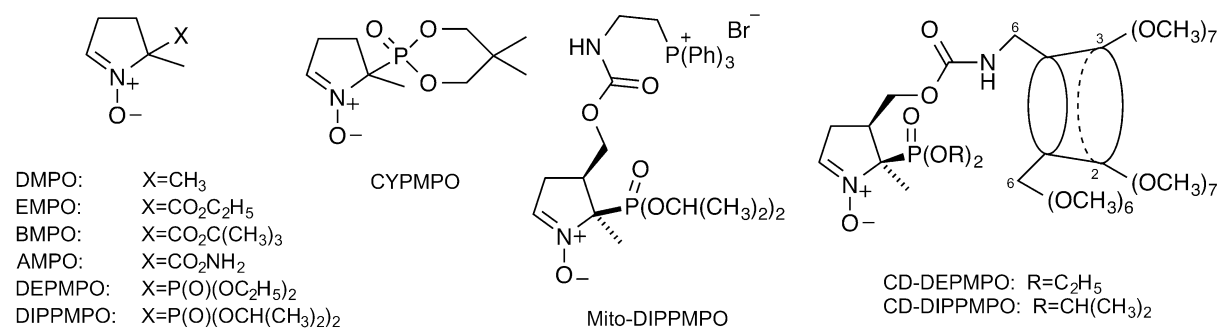


## Figures

Figure 1



**Figure 2**



**Figure 3**

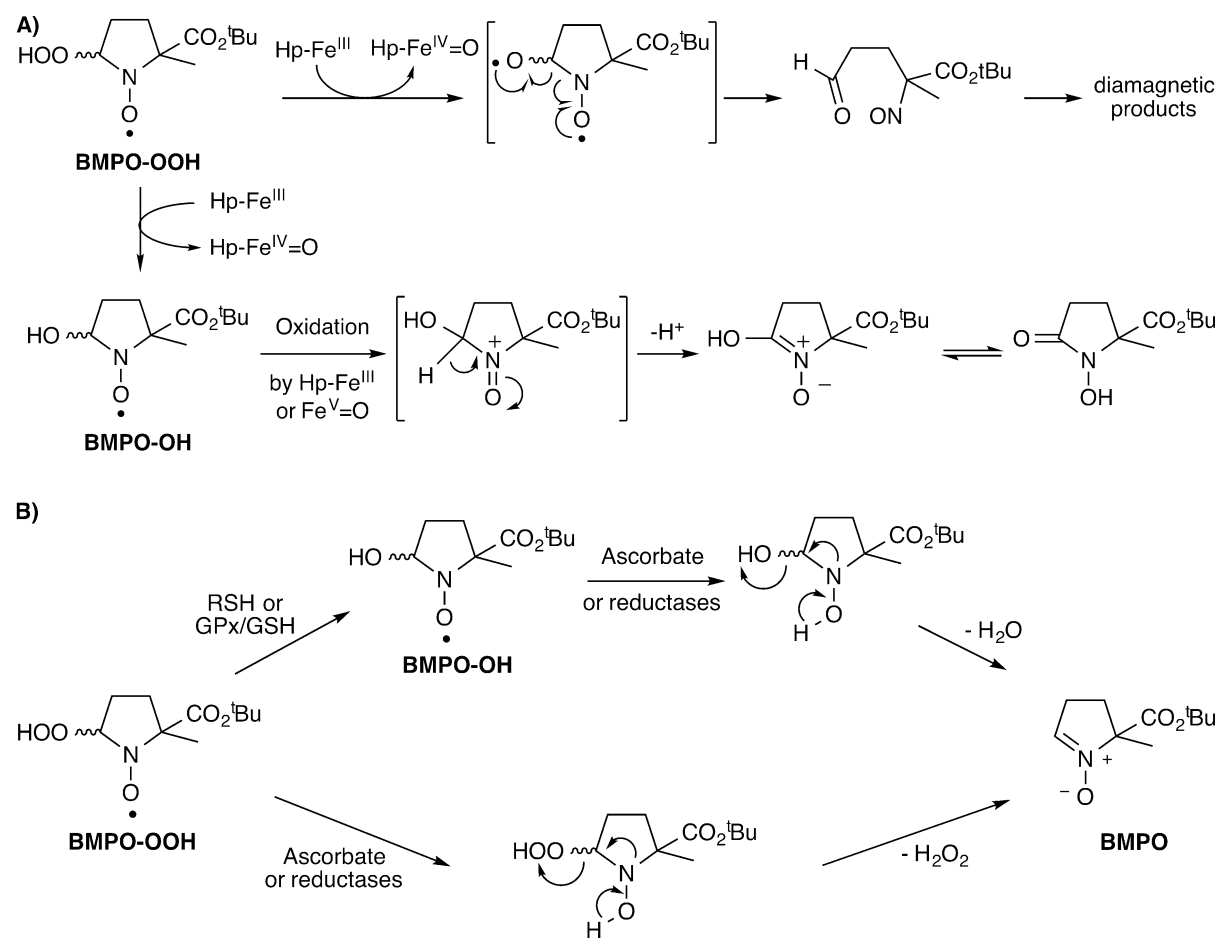


Figure 4

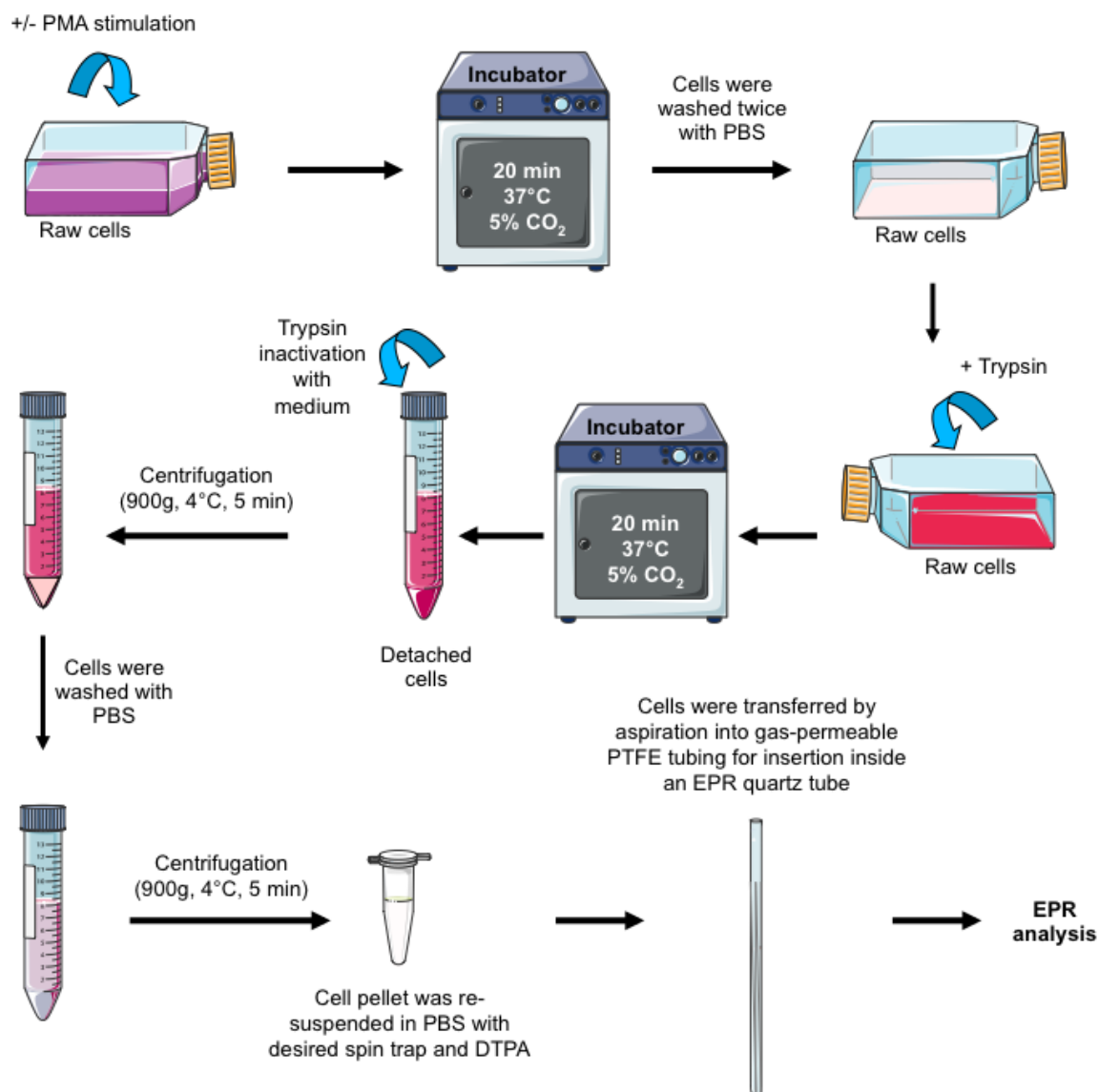


Figure 5

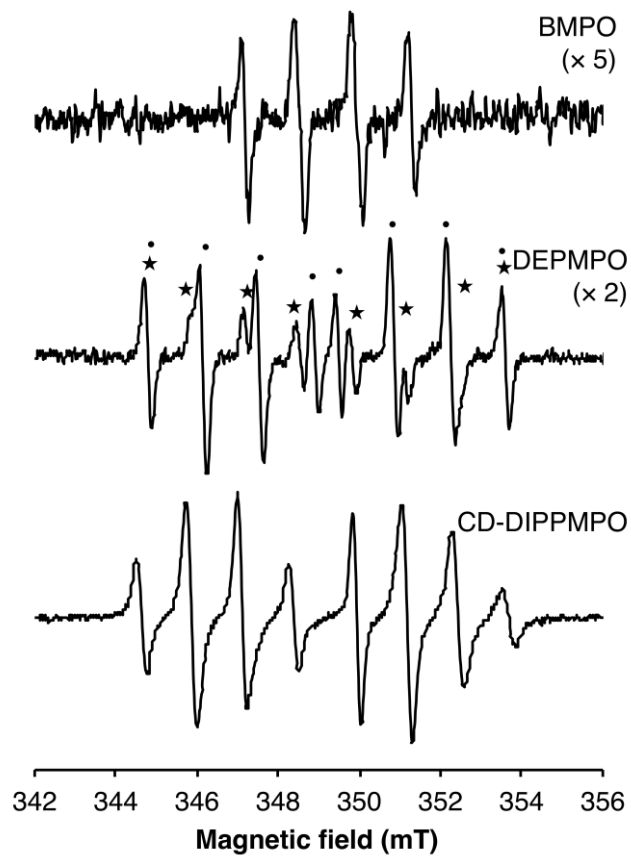


Figure 6

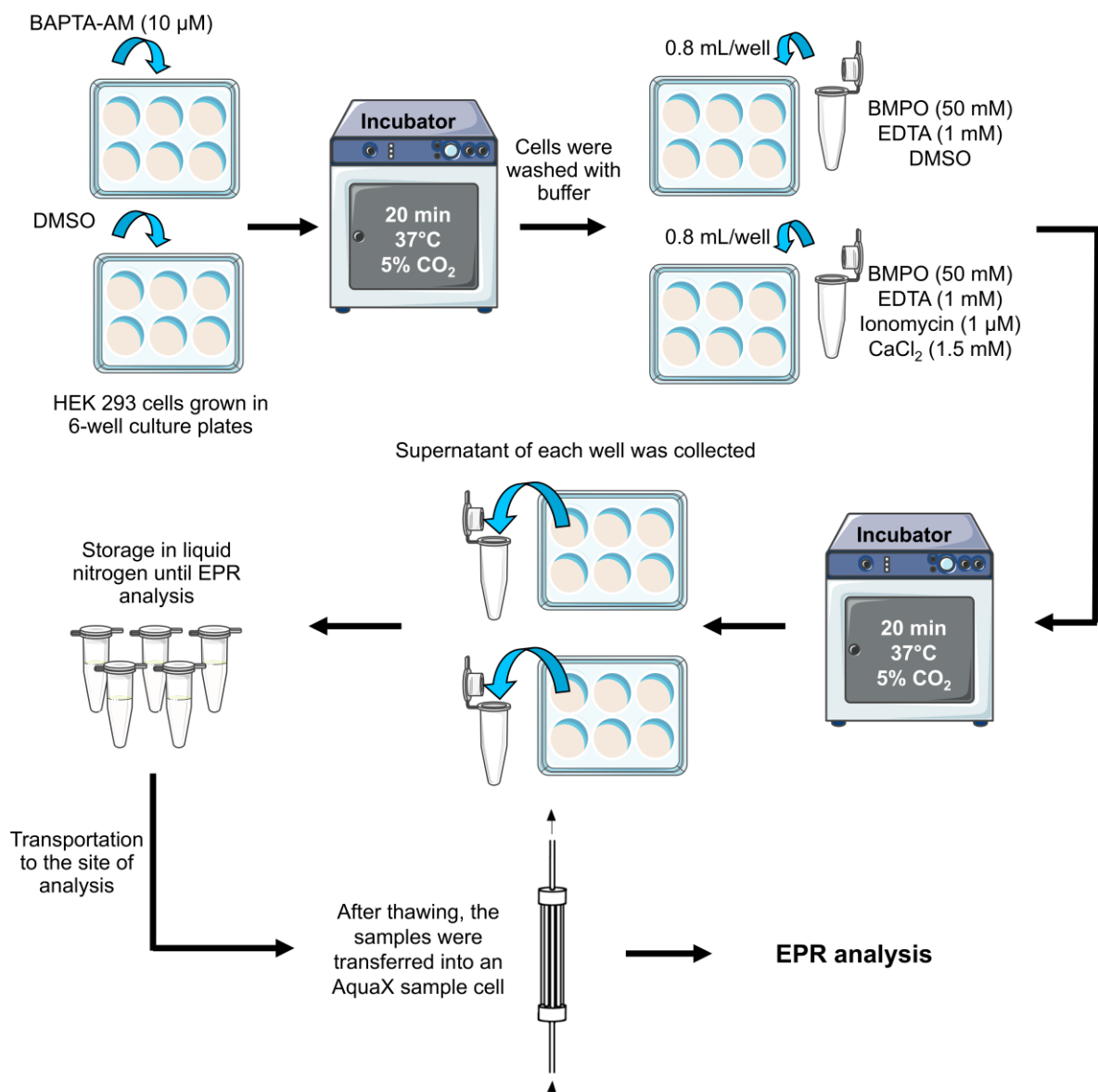
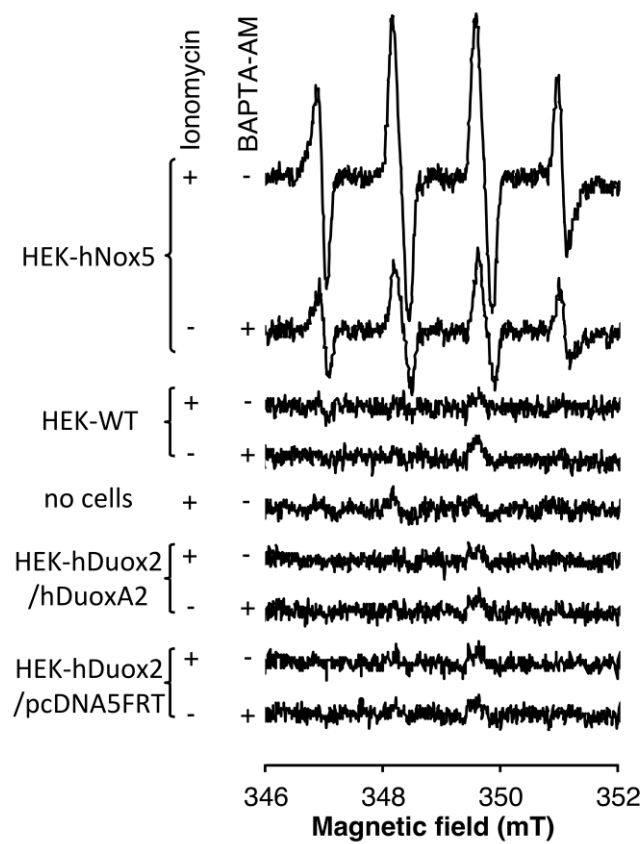




Figure 7



**Figure 8**

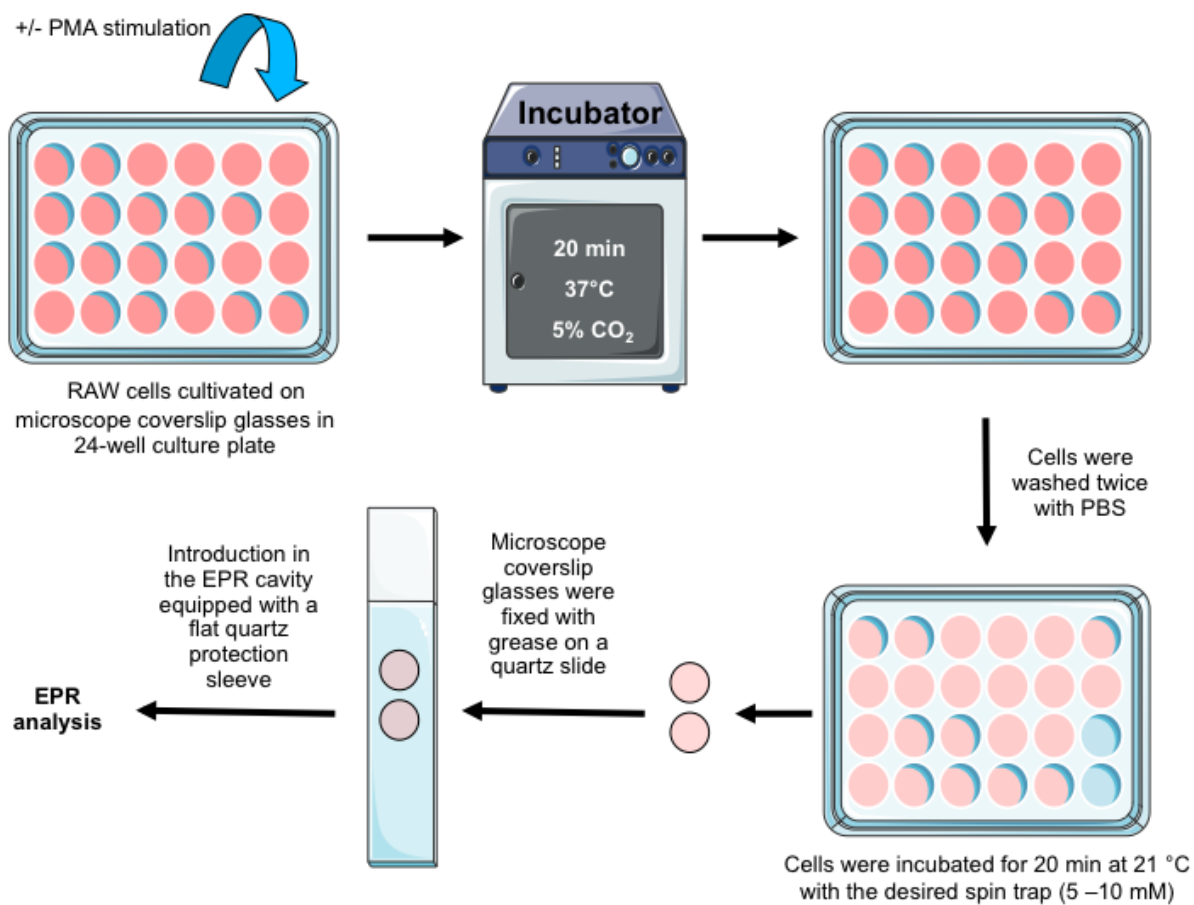


Figure 9

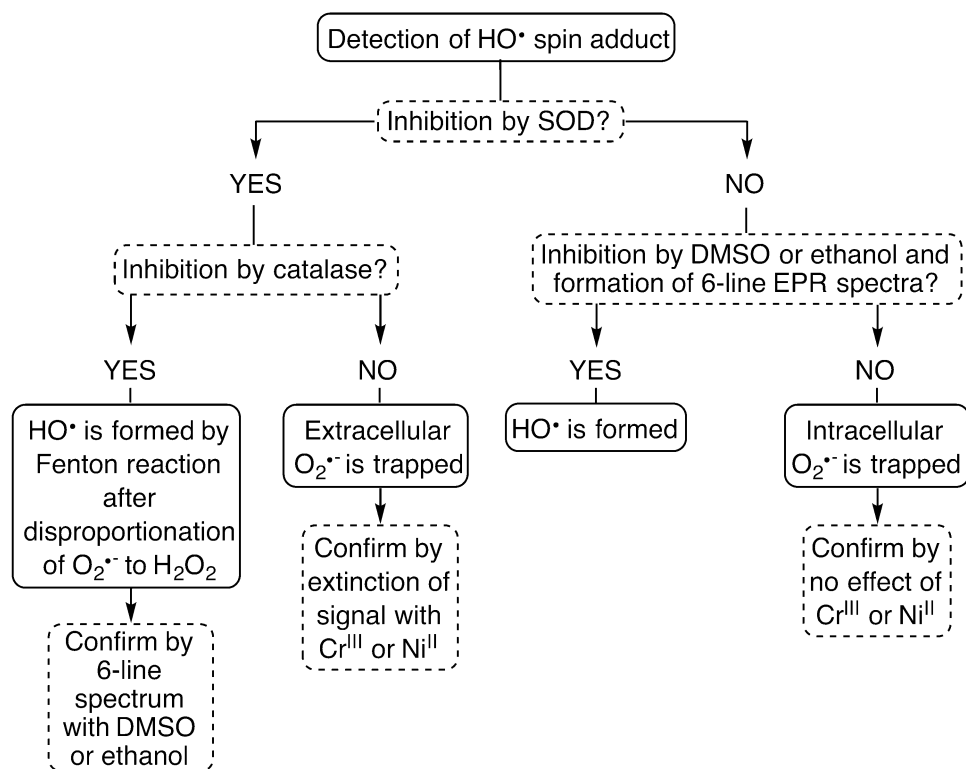
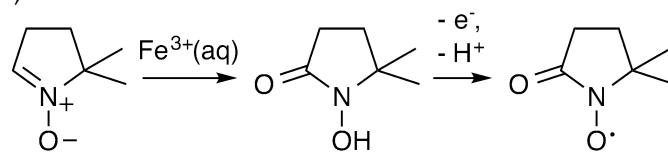
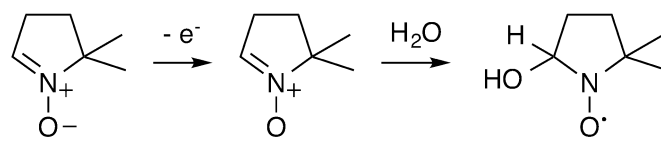


Figure 10

i) Formation of DMPOX



ii) Inverted spin trapping



iii) Forrester-Hepburn mechanism

