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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

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 nitrone, 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.

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Abbreviations: 2-OH-E^+, 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; $DCFH_2$, 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^+, 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_2O_2, hydrogen peroxide; HE, hydroethidine; $HO^{\cdot}$, hydroxyl radical; Hp, hemeprotein; HRP, horseradish peroxidase; Mito-DIPPMPO, (4*R**, 5*R**)-5-(diisopropyloxyphosphoryl)-5-methyl-4-[({[2-(tripheny lphosphonio)ethyl]carbamoyl}oxy)methyl]pyrroline *N*-oxide bromide; PEG, polyethylene glycol; Nox, NADPH oxidase; NBT, nitro blue tetrazolium; NMR, nuclear magnetic resonance; $NO^{\cdot}$, nitric oxide; $O_2^{\cdot -}$, superoxide radical anion; $ONOO^-$, 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 ($O_2^{\cdot-}$) 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 $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$. The highly reactive hydroxyl radical ($HO^{\cdot}$) quickly follows. Peroxynitrite ($ONOO^-$) results from the direct reaction of $O_2^{\cdot-}$ with nitric oxide ($NO^{\cdot}$). In cells, $O_2^{\cdot-}$ is efficiently removed by the antioxidant enzyme superoxide dismutase (SOD) [2]. The instability of $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$ sensing through [Fe-S] clusters [3].

Monitoring production of $O_2^{\cdot-}$ 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 ($\nu \simeq 9$ GHz) spectrometers, the detection threshold is in the range of 2–3 μM for a 25 μL 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 (Fig. 1). This radical stabilization technique is called *spin trapping*. Spin trapping experiments with 5,5-dimethyl-1-pyrrolidine N-oxide (DMPO, Fig. 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].

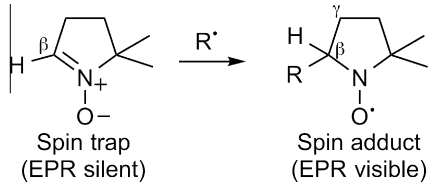

Fig. 1. Principle of spin trapping with DMPO. R$^{\cdot}$ is a short-lived radical, such as $O_2^{\cdot-}$ or $HO^{\cdot}$.

The best spin traps for $O_2^{\cdot-}$ are compounds with a cyclic nitrone 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 β-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 $O_2^{\cdot-}$ and the $HO^{\cdot}$ adducts, respectively.

1.3. Spin traps developed for cell studies

DMPO was the first cyclic nitrone 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 $HO^{\cdot}$ 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 $HO^{\cdot}$ [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], DIPPMPO [21], CYPMPO [22]) (Fig. 2). Not only do these spin traps offer a dramatically enhanced intrinsic stability of the corresponding $O_2^{\cdot-}$ spin adducts in buffer, but no spontaneous decomposition to the $HO^{\cdot}$ adduct is observed with these traps. The thermodynamically-preferred pathways for the spontaneous decomposition of $O_2^{\cdot-}$ spin adducts and the decomposition of