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Detection of superoxide in cells, tissues and whole organisms

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1. ABSTRACT

The radical anion of dioxygen superoxide ($O_2^{\cdot-}$) is a physiological free radical formed in various enzymatic processes. On the one hand superoxide is a precursor of reactive oxygen and nitrogen species (hydroxyl radicals, peroxy radicals, hydrogen peroxide, peroxynitrite, etc.), - the initiators of cellular damage; on the other hand it is a signaling molecule regulating numerous physiological processes including apoptosis, aging, and senescence. Therefore, the detection and measurement of superoxide in cells, tissues, and whole organisms is of a vital importance for *in vitro* and *in vivo* studies of many physiological and pathophysiological processes. At present different efficient methods were developed, which allow to identificate and measure superoxide in biological systems. In present review the creditability and efficiency of principal mostly applied methods of superoxide detection based on one-electron transfer and nucleophilic reactions are discussed, and spectrophotomentrical, chemiliminescent, fluorescent, and ESR spin trapping methods are compared.

2. INTRODUCTION

Detection of superoxide, a major precursor of reactive oxygen species, is one of the most important problems of free radical biology. Despite numerous works, its correct identification and quantitative measurements still are uncertain due to the use of not enough selective assays. In spite of its name superoxide is not a "super"-oxidant but a moderate reductant and an extremely reactive nucleophile. Therefore, successful methods for superoxide detection are based on the reactions of one-electron reduction and nucleophilic addition or substitution. Below, the mechanisms and the reliability of major widely used experimental methods are considered.

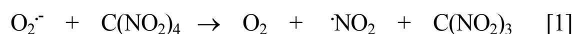
3. SUPEROXIDE ASSAYS BASED ON ONE-ELECTRON TRANSFER REACTIONS

One-electron transfer reactions are the most rapid chemical processes with the rate constants of up to 10^9 $M^{-1}s^{-1}$. Therefore, superoxide assays based on the one-electron

reduction of various molecules should be the very effective methods of its detection. The reduction potential of the dioxygen/superoxide pair $(O_2)/(O_2^{\cdot-})$ E^o = -0.16 V. It means that electron acceptors with the one-electron reduction potentials more than –0.16 V (the difference between the one-electron reduction potentials of reactants $\Delta E^o > 0$) can be effectively applied for the detection of superoxide. Typically, spectroscopic, luminescent, and fluorescent methods are applied for superoxide detection.

3.1. Spectroscopic detection of superoxide

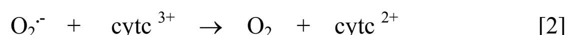
In 2000 Hodges, *et al.* proposed to measure superoxide spectrophotometrically by detection of the nitroform anion formed by the reduction of tetranitromethane (Reaction 1) (1):



$$O_2^{\cdot-} + C(NO_2)_4 \rightarrow O_2 + {}^{\cdot}NO_2 + C(NO_2)_3^- \quad [1]$$

As the rate constant of this reaction is very high (2×10^9 $M^{-1}s^{-1}$), this method could be the most effective assay for superoxide detection. Unfortunately, the highly explosive properties of tetranitromethane make it difficult to use this method in laboratory.

One of the oldest superoxide assays is the spectrophotometrical detection of reduced cytochrome *c* formed by Reaction 2 (2):



$$O_2^{\cdot-} + \text{cytc}^{3+} \rightarrow O_2 + \text{cytc}^{2+} \quad [2]$$

The equilibrium of this one-electron transfer reaction is completely shifted to the right because ΔE^o is about 0.4 V. A major limitation of this method is the inability of cytochrome *c* to penetrate cellular membranes. Therefore, this method can be used only in the experiments with cell components or for the detection of superoxide released from cells. The additional shortcoming of this method is the possibility to underestimate superoxide production due to a relatively low value of its rate constant ($(2.6\pm0.1)\times10^5$ $M^{-1}s^{-1}$) incomparable to typical values for exothermic one-electron reduction reactions of 10^8–10^9 $M^{-1}s^{-1}$. In addition, some oxidants such as quinones can artificially enhance the SOD-inhibitable reduction of cytochrome *c* (3-5).

To diminish the side reduction of cytochrome *c* in mitochondria by mitochondrial electron carriers, it has been proposed to increase the selectivity of cytochrome *c* assay by its acetylation or succinoylation (6,7). It was thought that acetylation and succinoylation might suppress the reduction of cytochrome *c* by NADPH cytochrome P-450 reductase. However, the rate constant for the most selective succinoylated cytochrome *c* was only about 10% of native cytochrome, making this assay less sensitive (8).

Another compound widely applied for superoxide detection is nitro blue tetrazolium (NBT, (3',3'-(3',3'-dimethoxy-1,1'-biphenyl-4,4'-ditl)bis 2-(4-nitrophenyl-2*H*-tetrazolium dichloride). It has been shown that superoxide reduced NBT by a one-electron transfer mechanism with the formation of colored monoformazan anion (9). Unfortunately, it is difficult to estimate the selectivity of this superoxide assay on the grounds of the published experimental data. However the NBT water-soluble analog WST-1 (4-(3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio)-1,3-benzene disulfonate sodium salt) is apparently a more selective superoxide scavenger than cytochrome *c* because SOD inhibited the reduction of WST-1 by 86-96% in the Maillard reaction comparing to the inhibition of cytochrome *c* reduction in this reaction only by 20-25% (10).

3.2. Chemiluminescent detection of superoxide

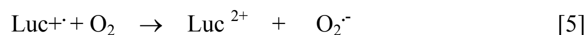
3.2.1. Lucigenin-amplified chemiluminescence

Lucigenin (bis-N-methylacridinium) is a selective chemiluminescent probe, which is widely used for detection of superoxide in cells and tissues. To the present time about 1200 articles quote the use of lucigenin for superoxide detection. It has been proposed that the mechanism of lucigenin-amplified chemiluminescence (CL) is described by Reactions 3 and 4:

$$O_2^{\cdot-} + \text{Luc}^{2+} \rightarrow O_2 + \text{Luc}^{+\cdot} \quad [3]$$

$$O_2^{\cdot-} + \text{Luc}^{+\cdot} \rightarrow \text{LucO}_2 \rightarrow \text{decomposition} \rightarrow \text{CL} \quad [4]$$

CL is produced during decomposition of the excited dioxetane intermediate $LucO_2$ as it was recently confirmed by Okajima and Ohsaka (11). The one-electron reduction potential of Luc^{2+})/ $Luc^{+\cdot}$) pair is by 0.4-0.6 V more positive than that of the $(O_2)/(O_2^{\cdot-})$ pair; therefore the equilibrium of Reaction 3 is completely shifted to the right and back Reaction 5 is practically impossible.



$$\text{Luc}^{+\cdot} + O_2 \rightarrow \text{Luc}^{2+} + O_2^{\cdot-} \quad [5]$$

(In 1969 Legg and Hercules measured the difference between the one-electron reduction potentials of lucigenin and dioxygen in DMF, ΔE^o = 0.6 V (12). Our estimate of this difference in aqueous solution yielded ΔE^o = 0.35 V (13). Until now, the attempts of direct measurement of dioxygen one-electron reduction potential in aqueous solution were unsuccessful and led to the determination of two-electron potential, which cannot be used for estimation of the equilibrium of Reaction 3 (14)).

Since 1960, when Totter *et al.* suggested that lucigenin-amplified CL produced in the reaction catalyzed by xanthine oxidase was generated by oxygen radicals, this method was widely applied for superoxide detection in cell-free systems, cells, and tissue (15). The method has several advantages: a high selectivity (the reduction of lucigenin by the other reductants does not produce CL due to the inability of the forming semiquinone $Luc^{+\cdot}$ to react with dioxygen by Reaction 5), a high sensitivity (the rate constant of Reaction 3 is of about 10^8 $M^{-1}s^{-1}$), and the ability of lucigenin to penetrate cell membranes. (The efficiency of different assays for superoxide detection is considered below). However, this method has been criticized because lucigenin-amplified CL might artificially overestimate superoxide production or even lead to false conclusions about superoxide formation in various enzymatic processes. It was suggested that Reaction 3 is a reversible one and therefore its back reaction (Reaction 5) might be responsible for the artificial enhancement of superoxide formation (16-19). As it was showed above,

thermodynamic consideration shows that Reaction 4 cannot be responsible for artificial superoxide overproduction; therefore, higher values estimated on the basis of lucigenin assay must have different explanation.

Creditability and advantages of lucigenin assay have been already discussed earlier (13, 20-23). Achieved conclusions were supported by experimental studies. For example Murphy *et al.* showed that lucigenin CL was not only the most sensitive assay for superoxide detection in the Rose cell plasma membranes but in contrast to the cytochrome *c* and NBT methods, only lucigenin CL was completely inhibited by SOD (24). Correspondingly, lucigenin-amplified CL was successfully applied for superoxide detection in many new publications (23-28). The most convincing experimental proof of creditability of lucigenin assay is an excellent correlation between findings received by different methods. It was showed that the correlation coefficients are equal to 0.932 – 0.994 for the correlations between lucigenin and cytochrome *c* assays (22,23).

If the "redox cycling" of lucigenin is impossible, then while there is the difference between lucigenin assay and other methods? The simplest explanation is that lucigenin reacts with superoxide with the highest rate constant (10^8 $M^{-1}s^{-1}$), whereas the reaction rates for cytochrome *c* or NBT are much lower. Therefore, these compounds cannot successfully compete with cellular substrates and enzymes (such as NO, SOD) and catch quantitatively superoxide. (Efficiency of different methods of superoxide detection is considered below). Since redox cycling of lucigenin can stimulate the dioxygen consumption by Reaction 5, the effect of lucigenin on dioxygen consumption has been studied. Trush and co-workers demonstrated that dioxygen consumption was negligible in the most of systems at lucigenin concentrations smaller than 100 μM and increased only at higher concentrations (20). But it does not mean that an increase in dioxygen consumption could always originate from the redox cycling of lucigenin. Thus Souza *et al.*found that superoxide production by aorta rings and aorta homogenates in the presence of NADPH or NADH was about 3 - 6 times lower than dioxygen consumption (29). Therefore there should be the other origins of enhanced dioxygen consumption.

3.2.2. Luminol-amplified chemiluminescence

To my knowledge the one-electron reduction potential of luminol (5-amino-2,3-dihydro-1,4-phthalazine-dione) is unknown. It is usually accepted that luminol is reduced to the semiquinone by some reductants and that the semiquinone reacts with superoxide to form an intermediate hydroperoxide, which is decomposed producing CL (9). Superoxide can probably also reduce luminol to semiquinone, although such a process is possible if only the reduction potential of luminol is more positive than that of dioxygen.

It is widely accepted that luminol-amplified CL is not a selective method of superoxide detection because the other reactive oxygen and nitrogen species react with luminol producing even a more intensive CL than superoxide does. In 1984 Aniansson, *et al.*showed that the myeloperoxidase-hydrogen peroxide system stimulated intensive luminol-amplified CL in cells (30). (It ought to be reminded that lucigenin-amplified CL is not affected by myeloperoxide system). Even a more intensive CL is generated by the interaction of luminol with peroxynitrite (31). Furthermore, hemoglobin and other iron heme-containing compounds are able to enhance luminal-amplified CL (32). Therefore luminol-amplified CL apparently characterizes the total formation of all reactive oxygen and nitrogen species.

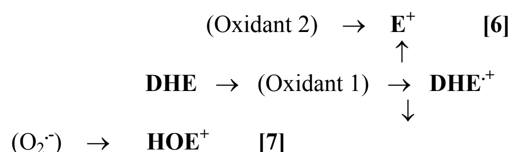
3.2.3. Luminescence amplified by coelenterazine and L-012

At present some new chemiluminescent probes are described to detect superoxide such as coelenterazine, a luminescent enzyme substrate and "luminol analogue" 8-amino-5-chloro-7-phenylpyrido (3,4-d)pyridazine-1,4 (2H,3H)dione (L-012). Their structures suggest that these molecules are able to react not only with superoxide by a one-electron transfer mechanism but also with hydroxyl radicals or peroxynitrite *via* the reactions of H-abstraction. Nishinaka, *et al.* demonstrated that L-012 generated SOD-inhibitable CL by human eosinophilic leukemia cells (33). However subsequent works (34,35) showed that peroxynitrite even more efficiently stimulated L-012-amplified CL than superoxide did. Lucas and Solani showed that coelenterazine-amplified CL by neutrophils was inhibited by SOD (36). San Martin, *et al.* recently confirmed the high efficiency of coelenterazine-amplified CL as superoxide assay (37).

3.3. Fluorescent detection of superoxide

Several fluorescent probes are now used for fluorescent detection of superoxide. These assays are successfully applied for the study of reactive oxygen species in cells and tissue, but their selectivity for superoxide detection is questionable. Dihydroethidium (DHE) is a one of the most frequently used fluorescent probes. Unfortunately its oxidation product ethidium (E^+) is formed not only in the reaction with superoxide but also in the reactions with the other redox molecules and first of all peroxynitrite. However it has been recently found that 2-hydroxyethidium (HOE^+) a final product of reaction of DHE with superoxide has fluorescent spectrum different from that of ethidium (38). Subsequent works confirmed that the SOD-inhibitable formation of HOE^+ can be used for the selective detection of superoxide (39-41). For detection of superoxide in mitochondria Robinson, *et al.*suggested to use a new derivative of ethidium obtained by covalent binding of hexyl triphenylphosphonium cation to DHE (Mito-DHE) (41).

Thus major directions of DHE interaction with redox biomolecules might be presented as follows:



Unfortunately, the mechanisms of these reactions remain unclear. The rates of DHE or mito-DHE oxidation by superoxide are high enough (k_7 = (2-4)×10^6 $M^{-1}s^{-1}$; therefore they should be the rapid reactions of one-electron transfer or free radical recombination (41,42). It was suggested that Oxidant 1 is the pair [$O_2^{\cdot -}$/$HOO^{\cdot}$] (41). Although $O_2^{\cdot -}$ is not an oxidant despite its famous name and is unable to oxidize any organic compound, its protonated form hydroperoxyl radical $HOO^{\cdot}$ can do it. However a pK value of $O_2^{\cdot -}$/$HOO^{\cdot}$ is very low (4.2), and [$HOO^{\cdot}$] concentration must be < 1% at physiological pH. It makes doubtful the participation of $HOO^{\cdot}$ in DHE oxidation.

The formation of hydroxylated product HOE^+ suggests the participation of hydroxyl radicals in this reaction. Actually, there are experimental findings supporting such a proposal: the treatment of DHE with the Fenton system resulted in the formation of HOE^+ (40,42). There are various possible ways for the transformation of superoxide into hydroxyl radical, for example by reducing ferric ions into ferrous ions and starting by this the Fenton reaction or by the interaction with nitric oxide to form peroxynitrite decomposing to hydroxyl and nitrate radicals. Nonetheless the fact that SOD inhibits the formation HOE^+ indicates that the DHE assay might be used for superoxide detection despite some possible complications (43,44).

lular superoxide is able to penetrate the endothelial cell plasma membrane through ClC-3 channels and induce SOD-inhibitable DHE fluorescence (45). Interestingly, that these authors believe that DHE fluorescence depends only on superoxide and not hydrogen peroxide generation. Peshavariya, *et al.* suggested that DHE-derived fluorescence can be used for detection of intracellular and extracellular superoxide produced by phagocytic and vascular NADPH oxidases (46). At the same time Meany, *et al.* recently found that 2-hydroxyethidium (HOE^+), a product of hydroethidine oxidation by superoxide is unable to penetrate the mitochondrial membrane (47). (In the last work Zelonka, *et al.* pointed out that the detection of superoxide in mitochondria by Mito-HE or MitoSOX Red could be severe compromized due to oxidation by cytochrome *c*) (60).

2',7'-Dichlorofluorescin diacetate (DCFH-DA) is another fluorescent probe frequently used for the detection of superoxide in cells and tissue (48). DCFH-DA is a non-fluorescent compound which freely penetrates cellular membranes where its acetate moiety is hydrolyzed by esterases to fluorescent 2',7'-dichlorofluorescein (DCF). DCFH-DA is therefore widely applied for intracellular detection of superoxide. Unfortunately, in addition to superoxide the other reactive oxygen and nitrogen species such as hydrogen peroxide, peroxynitrite, nitric oxide, and even cellular labile iron also effectively oxidize DCFH and its derivatives (49-51). In conclusion it should be noted that some new chemiluminescent and fluorescent probes have been recently synthesized and applied for superoxide detection (52-54).

4. ASSAYS BASED ON NUCLEOPHILIC REACTIONS OF SUPEROXIDE

"Super"-nucleophilic properties of superoxide suggest that some nucleophilic reactions of superoxide for can be used for its detection (Ref. 9, page 50). Basically, nucleophilic reactions have much greater activation energies comparing to the one electron transfer reactions, and therefore superoxide assays based on these reactions will be always less effective than those based on the reactions of electron transfer. In 1974 Harbour *et al.* demonstrated that superoxide reacted with 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) forming free radical DMPO-OOH (spin-adduct) which was easily identified by its ESR spectrum (55). This method is of greatest importance for the identification of superoxide in biological systems and is widely used in experiments.

A major limitation of spin trapping is the low rates of reactions of superoxide with nitroxides such as DMPO (10-15 $M^{-1}s^{-1}$) that casts doubts on the application of spin trapping assays for quantitative detection of superoxide in cells (see, below). Another shortcoming is a short life- time of spin-adducts. Therefore, numerous new spin trapping agents were synthesized with a supposedly longer life-time and more informative ESR spectra, for example, (diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide (DEPMPO) or the ethyl-substituted derivatives of 5-ethoxycarbonyl-5-methyl-1-pyrroline N-oxide (EMPO) (56,57).

Superoxide assays considered above are not the only ones described in literature, but they are the most frequently used reliable methods. Undoubtedly, new assays could be of interest, but all of them must be considered from the two most important points of view: (1) the efficiency of superoxide detection and (2) the selectivity of methods. Now we will compare the efficiency of major methods of superoxide detection taking into account their competitiveness with endogenous biomolecules in cells.

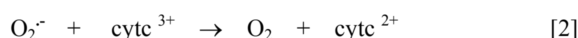
5. COMPARISON OF DIFFERENT SUPEROXIDE ASSAYS

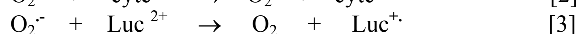
Quantitative detection of superoxide in cells and tissues is impeded by the presence of many endogenous redox-active compounds. Therefore, in order to measure correctly superoxide formation, we need scavengers able to compete with these biomolecules. It was earlier thought that the use of 50 microM – 100 microM cytochrome *c* (k_2 = (2.6±0.1)×10^5 $M^{-1}s^{-1}$) is sufficient for the quantitative detection of superoxide produced by phagocytes outside of cells (9). However, recent discovery of two additional effective endogenous superoxide scavengers nitric oxide and extracellular SOD (exSOD) reacting with superoxide with the diffusion rate constants of 2×10^9 $M^{-1}s^{-1}$ (Reactions 9 and 10) points out at the existence effective endogenous competitors of superoxide outside and inside of cells. (Table 1). (There are of course many other endogenous

Table 1. Rate constants for reactions of superoxide with exogenous and endogenous scavengers ($M^{-1}s^{-1}$)

	k_2 (5)	**k_3 (13)**	**k_8 (9) (p.147)**	**k_9**	**k_{10}**
Cyt. C	$(2.6\pm0.1)\times10^5$				
Lucigenin		10^8			
DMPO			10-15		
NO				10^9	
ExSOD					10^9

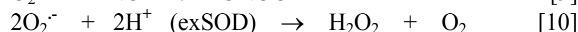
superoxide scavengers such as SODs, cytochromes, numerous heme-containing enzymes, etc. in cells, but it will be sufficient to consider just two of them). Below, we consider the ability of major superoxide scavengers DMPO, cytochrome *c*, and lucigenin traditionally used for superoxide detection (Reactions 2, 3, and 8) to compete with reactive endogenous biomolecules nitric oxide and exSOD (Reactions 9 and 10).


$O_2^{\cdot-}$ + cytc $^{3+}$ → O_2 + cytc $^{2+}$ [2]


$O_2^{\cdot-}$ + Luc $^{2+}$ → O_2 + $Luc^{+\cdot}$ [3]

$O_2^{\cdot-}$ + DMPO → DMPO-OOH [8]

$O_2^{\cdot-}$ + NO → $ONOO^-$ [9]


$2O_2^{\cdot-}$ + $2H^+$ (exSOD) → H_2O_2 + O_2 [10]

Using typical experimental concentrations of DMPO equal to 100 mM and NO of 10^{-3} mM (57), we have:
V_9/V_8 = k_9 (N0)/k_3 [DMPO] = (1-2) x10^3 [11]

Thus 100 mM DMPO will scavenge about 0.1%-0.2% of a total superoxide amount if the NO concentration is equal to 1 microM. Similar equations can be written for cytochrome *c* and lucigenin:

$V_9/V_2 = k_5[NO]/k_2[cytc^{3+}] = 15{,}4$ [12]
$V_9/V_3 = k_5[NO]/k_3[Luc^{2+}] = 2x10^{-5}/[Luc^{2+}]$ [13]

Therefore, 50 microM cytochrome *c* will scavenge about 6% of superoxide. For $[Luc^{2+}]$ = 5 microM, 50 microM, and 250 microM, V_9/V_3 ratio is equal to 4, 0.4, and 0.08, and the efficiency of superoxide detection by lucigenin-amplified CL will be equal to 20%, 71%, and 93%. Similar calculations were made for superoxide detection in the presence of exSOD.

Thus, it seems that only the lucigenin assay allows to measure quantitatively superoxide in the presence of NO and SOD. Of course, their real concentrations in cells might be smaller and correspondingly the efficiencies of superoxide detection by the above scavengers might be higher. Therefore, the above calculations should be considered only as a rude estimate of the problems of superoxide quantitative detection in biological systems because NO and exSOD do not the only reactive redox-active biomolecules in cells. For example, ubiquinones, glutathione, iron-containing enzymes can react with superoxide with the rate constants of 10^5-10^9 $M^{-1}s^{-1}$. Therefore, our calculations only show a relative efficiency of traditional scavengers for quantitative superoxide detection. However, it is obvious that despite its importance for the direct identification of superoxide in cells and tissue, spin trapping with DMPO and the other spin trapping agents cannot be recommended for the quantitative measurement of superoxide due to a very low rate constant of Reaction 8.

In conclusion, two recent experimental works should be mentioned, which demonstrated the failure of various spin traps and cytochrome *c* to measure superoxide quantitatively in cells. Pignitter, *et al.* has shown that traditionally used spin trapping agents cannot trap all superoxides produced simultaneously with NO by uncoupled rat neuronal NOS (58). The measurement of superoxide by cytochrome *c* and the spin traps EMPO and DEPMPO in human lymphoblast cells gave 3-5 times smaller values comparing to spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) (59). However, it should be noted that the mechanism of the reaction of superoxide with CMH remains unclear.

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