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Dual fluorophore-nitronyl probe for investigation of superoxide dynamics and antioxidant status of biological systems

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Abstract

A nitronyl nitroxide radical covalently linked to an organic fluorophore, pyrene, was synthesized and used to detect superoxide radicals and to quantitatively estimate the antioxidant activity of biological compounds in a wide range of antioxidant redox potential. This approach is based on the phenomenon of intramolecular fluorescence quenching of the pyrene fragment by the nitroxide in the dual fluorophore-nitronyl (FNO) molecule. The nitroxide fragment of the dual non-fluorescence molecule can be reduced by a variety of free radicals (e.g. superoxide) and antioxidants (e.g. ascorbic acid, quercetin, galangin, etc.). The reduction of FNO is accompanied by a drastic increase in fluorescence intensity and a corresponding decrease of the EPR signal. The results show that steady-state fluorescence measurements enable the detection of a very low flux of superoxide, starting from 10 nM/min. The fluorescence technique, which has been developed, allows detection of antioxidants at the submicromolar concentration scale while the EPR and light absorption techniques are limited to several micromolar. This method has also been applied to the determination of hydrogen peroxide in submicromolar concentrations. The specificity of this approach can be enhanced by the addition of superoxide dismutase and catalase.

Keywords: Superoxide; Antioxidants; Fluorophore-nitronyl nitroxide; Fluorescence

1. Introduction

The superoxide anion radical, a product of a one-electron reduction of molecular oxygen, has received considerable attention in connection with oxygen toxicity in biological systems. Superoxide is able to damage biological membranes and tissues directly or can act as a precursor of more reactive oxygen species. Thus in the presence of hydrogen peroxide it yields hydroxyl radicals via the Haber–Weiss reaction, while in the presence of nitric oxide, another reactive species peroxynitrite is formed [1–3]. To shed light on the reactivity of superoxide and also to account for its toxic effects, it is necessary to learn both how rapidly superoxide reacts with biological molecules and how rapidly it is scavenged by antioxidants.

The direct reaction of superoxide with an indicator to form a stable product is currently employed to measure superoxide generation in biological systems. These reactions can involve both oxidation and reduction of superoxide by the indicator. There are three commonly used techniques based on: light absorption [4–9], the electron spin resonance (ESR) signal [10–14] and chemiluminescence [9–15]. The principle disadvantage of the first two techniques is their relatively low sensitivity. Sensitivity of light absorption spectroscopy is strongly dependent on the sensitivity limit of the indicator itself in the micromolar range. This technique does not permit measurements of superoxide flux at a rate of a few nanomolar per minute. There are several precautions for applying various chromophores as indicators (e.g. cytochrome *c* analogs, nitro-substituted aromatics, epinephrine, etc.) to detect superoxide. The cytochrome *c* analogs assay reaction is inhibited by some enzyme systems and low-molecular-weight reductants such as thiols, ascorbates and other compounds. The reduced form of cytochrome *c* can be reoxidized by cytochrome oxidase, peroxidases and oxidants, including H_2O_2 and $ONOO^-$ [6–8]. For the nitro-substituted aromatics, such as nitroblue tetrazolium (NBT), reaction with superoxide is also nonspecific.

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Numerous other substances including cellular reductases are capable of donating an electron to NBT, forming NBT radicals [9]. Spin trapping methods are less efficient in the detection of superoxide in comparison with absorption spectroscopy. This conclusion is based on the fact that interaction of spin traps with superoxide is slow, but more selective. 5-Diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) is one of the most specific and stable superoxide spin traps and has proved to be increasingly useful in measurements of superoxide formation efficiency in cells and tissues [13,14]. However, the sensitivity limit of this method on the micromolar concentration scale, interference by tissue metabolism, and the high cost of ESR spectrometers make this technique more problematic for detecting oxygen reactive species.

The chemiluminescence methods of superoxide detection in living cells and tissues have been used frequently, because they are able to access to intramolecular sites of superoxide generation, and are relatively specific to the reaction of probes with superoxide. Several compounds such as luceginin, anthracene, luciferin, paraquat and their analogs, have been widely employed for the detection of superoxide [15–19]. Unfortunately, the chemiluminescence method is limited by the principal problem of chemiluminescence assay—the exact mechanism and quantum yield of bioluminescence are still unknown. According to reported data [17], the rate of superoxide generation detected so far by the chemiluminescence method does not exceed a micromolar range per minute.

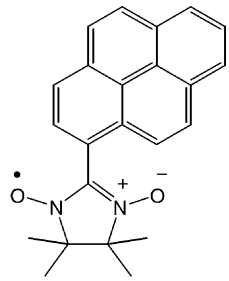
After elucidation of the decisive role of free radicals in cell aging and the development of many diseases, the difficult problem of the quantification of an antioxidant status has arisen ([2,3,20] and references therein). The effective reductive ability of nitroxide radicals and the mechanism of the nitroxide reaction with superoxide have been studied and accepted [10–12,21–30]. The quantitative characterization of quenching and redox processes is based upon the use of two subfunctional fragments (a fluorescent chromophore and a stable nitroxide radical) tethered together by a spacer. The nitroxide is a strong intramolecular quencher of the fluorescence from the fluorophore fragment. Both reduction of the nitroxide fragment to hydroxylamine and oxidation to nitronyl are accompanied by decay of the nitroxide fragment ESR signal and parallel enhancement of the fluorophore moiety fluorescence. On the basis of the unique property of the dual molecules to change drastically both fluorescence and the ESR signal with the modification of nitroxide moieties, several analytical methods have been developed [11,27–30]. The ability of these methods to measure the concentration of ascorbic acid in biological liquids (human blood plasma, fruit juices), that is to say, to establish the reducing status of these materials, has been demonstrated [28–30]. The principle advantage of the dual fluorophore-nitroxide assay procedure compared to other methods is the very high sensitivity of the fluorescence technique, which allows measurement of the concentration of fluorescent species in the nanomolar concentration scale. Nevertheless [11,28–30], molecules with piperidine and pyrrolidine nitroxide fragments cannot be directly used for analyzing superoxide radicals and antioxidants, with low reducing potential (less than that for hydrogen of ascorbic acid ± 0.08 V).

In the current study we apply the fluorophore-nitronyl (FNO) fluorescence method to detecting reactive oxygen species, and analyzing quantitatively the antioxidant status of biological systems with widely ranging antioxidant redox potential. The method involves one-electron reduction of the indicator molecule yielding a stable product with enhanced fluorescence. Since the described probe does not specifically react with superoxide, additional measurements are required in the presence of superoxide dismutase and catalase in order to estimate the contribution of superoxide radicals. The rate of superoxide formation by xanthine/xanthine oxidase system is found to be in the nanomolar per minute rate scale. At the same time the concentration of antioxidants with low reducing ability, such as flavones, is found in the submicromolar range.

2. Materials and methods

2.1. Reagents

Milk xanthine oxidase, xanthine, ascorbic acid, catalase, hydrogen peroxide and ferrous sulfate were purchased from Sigma, Israel. Flavonic compounds 3,5,7-trihydroxyflavone (galangin) and 3,3′,4′,5,7-pentahydroxyflavone (quercetin) were obtained from ROTH. Fluorescence nitroxide 4, 4,5,5-tetramethyl-2-(1-pyryl)-2-imidazoline-1-oxyl-3-oxide, compound **1** (FNO) was synthesized according to the modified published procedures [31] along with small amounts of 4,4,5,5-tetramethyl-2-(1-pyryl)-2-imidazoline (PI) as a synthesis by-product [28].



Compound **1,** (FNO)

A 0.1 M phosphate buffer solution (pH 7.4) was prepared from sodium hydrophosphate and potassium dihydrophosphate. Deionized and three times distilled water from the EASYpure LC system was used. Aqueous stock solutions of xanthine (0.1 mM), ascorbic acid (0.1 mM) and catalase (2 mg/ml) were freshly prepared before experiments and were used immediately. FNO and flavonic stock solutions were prepared by dissolving them in commercial DMSO

(spectrophotometric grade, Aldrich) to obtain a final concentration of 0.1 mM. Final solutions were obtained by further dilution in PBS (pH 7.4). All kinetic experiments were performed at room temperature ($T = 300$ K.).

2.2. Superoxide generation

In a typical experiment xanthine oxidase was added to a phosphate buffer solution containing xanthine (40 μM) and 280 U/ml catalase to avoid side effects of H_2O_2. The reaction was started by adding about 10^{-5} to 10^{-2} U/ml of xanthine oxidase, providing a steady flux of 10^{-8} to 10^{-6} M $O_2^{\bullet-}$/min. $O_2^{\bullet-}$ formation was monitored by reaction with cytochrome *c* as described in [32].

2.3. ESR experiments

ESR spectra were recorded on a Bruker EMX-220 digital X-band spectrometer equipped with a Bruker EP 4241VT temperature control system at $T = 297$ K. The ESR measurements were conducted with the following parameters: 9.40 GHz microwave frequency, 20.12 mW nonsaturated microwave power, and 100 kHz field modulation of 1 G amplitude. The kinetics of the ESR signal decay were obtained by fixing the magnetic field at the position of the top peak of the $M = 0$ hyperfine line and the consequent recording in time scan mode. Absolute concentration of FNO was obtained by the numerical double integration of ESR signal versus the 1 mM water Tempol solution, used as an external standard.

2.4. Fluorescence steady-state experiments

A Fluorolog-3 model FL3-22 with pulsed lamp HgXe and a cooled PMT IR CCD detector was used for measuring fluorescence intensity and steady-state kinetics of chemical reduction. All fluorescence measurements were done in PBS (pH 7.4) and $T = 300$ K at excitation wavelength of 340 nm with excitation slits of 5 nm, and an emission wavelength of 440 nm with emission slits of 10 nm and a voltage of 900 V. The total error in signal amplitude determination during the kinetic measurements was about 10%. Concentrations of reagents and other spectral parameters are given in the legends of the figures. Data from fluorescence kinetics were processed with KaleidaGraph software.

3. Kinetic analysis

Reactions of superoxide, generated by the xanthine/oxidase system (XOS), with the dual fluorophore probe (FNO) in the presence of an antioxidant (AH) occur by the following steps:

$$\mathrm{XOS} \xrightarrow{\omega_i} \mathrm{O_2}^{\bullet-} \tag{1}$$

$$\mathrm{O_2}^{\bullet-} + \mathrm{O_2}^{\bullet-} + 2\mathrm{H}^+ \xrightarrow{k_{\mathrm{dis}}} \mathrm{O_2} + \mathrm{H_2O_2} \tag{2}$$

$$\mathrm{O_2}^{\bullet-} + \mathrm{FNO} \xrightarrow{k_1} \mathrm{FNO}^* + \text{products} \tag{3}$$

$$\mathrm{FNO} + \mathrm{AH} \xrightarrow{k_2} \mathrm{FNO}^* + \text{products} \tag{4}$$

where FNO^* and FNO are the fluorescent and non-fluorescent forms of the probe. In the absence of an antioxidant the rate of superoxide decay is represented by the following expression:

$$\frac{\mathrm{d}O_2^{\bullet-}}{\mathrm{d}t} = \omega_i - k_{\mathrm{dis}}[O_2^{\bullet-}]^2 - k_1[\mathrm{FNO}][O_2^{\bullet-}] \tag{5}$$

where ω_i is the rate of superoxide formation in reaction (1), k_{dis} is the second-order rate constant for spontaneous dismutation of superoxide (2) and k_1 is the second-order rate constant for reaction (3). The rate of reaction of FNO with superoxide is expressed as follows:

$$\frac{\mathrm{d}[\mathrm{FNO}^*]}{\mathrm{d}t} = k_1[\mathrm{FNO}][O_2^{\bullet-}] \quad \text{or}$$
$$\frac{\mathrm{d}[\mathrm{FNO}^\bullet]}{\mathrm{d}t} = k_{\mathrm{app}}[\mathrm{FNO}] \tag{6}$$

where $k_{\mathrm{app}} = k_1[O_2^{\bullet-}]$ is the apparent pseudo first-order rate constant for the FNO^* appearance. At a steady flux of superoxide, the rate of superoxide formation is $\mathrm{d}[O_2^{\bullet-}]/\mathrm{d}t \approx 0$, and we can calculate the stationary superoxide concentration $[O_2^{\bullet-}]_{\mathrm{st}}$ from Eq. (5). In this condition the product $k_{\mathrm{app}}[\mathrm{FNO}]$ should be written as follows:

$$\frac{\mathrm{d}[\mathrm{FNO}^*]}{\mathrm{d}t} = k_{\mathrm{app}}[\mathrm{FNO}] = k_1[\mathrm{FNO}]\frac{k_1[\mathrm{FNO}] \pm \sqrt{(k_1[\mathrm{FNO}])^2 + 4k_{\mathrm{dis}}\omega_i}}{2k_{\mathrm{dis}}} \tag{7}$$

If the k_{dis} and ω_i are known and the value of the experimental apparent pseudo first-order rate constant k_{app} is observed at a definite rate of superoxide formation, the second-order rate constant k_1 for interaction of superoxide with FNO can be obtained from Eq. (7).

Taking into consideration that $[\mathrm{FNO}] = [\mathrm{FNO}]^0 - [\mathrm{FNO}^*]$, where $[\mathrm{FNO}]^0$ is the initial concentration of the non-fluorescence dual probe, and fluorescence intensity I is proportional to $[\mathrm{FNO}^*]$ ($I = \alpha[\mathrm{FNO}^*]$), the following equation emerges:

$$\frac{\mathrm{d}I}{\mathrm{d}t} \propto \alpha\frac{\mathrm{d}[\mathrm{FNO}^*]}{\mathrm{d}t} = A - k_{\mathrm{app}}I \tag{8}$$

where $A = I\alpha[\mathrm{FNO}]^0 k_{\mathrm{app}}$ and $k_{\mathrm{app}} = k_1[O_2^{\bullet-}]$. In this case, the plot $\mathrm{d}I/\mathrm{d}t$ versus I for the pseudo first-order reaction is described as a straight line with a slope corresponding to k_{app} and intercept A.

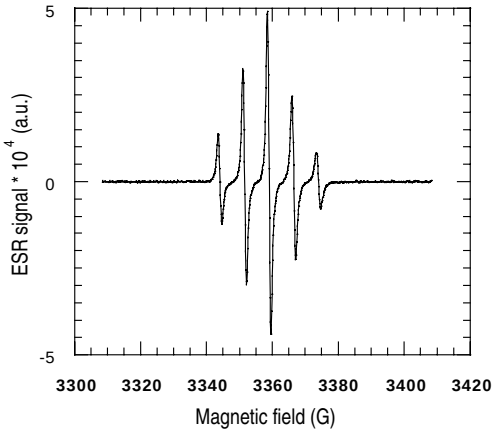


Fig. 1. ESR spectra of the FNO (10^{-4} M) in PBS (pH 7.4, $T = 297$ K).

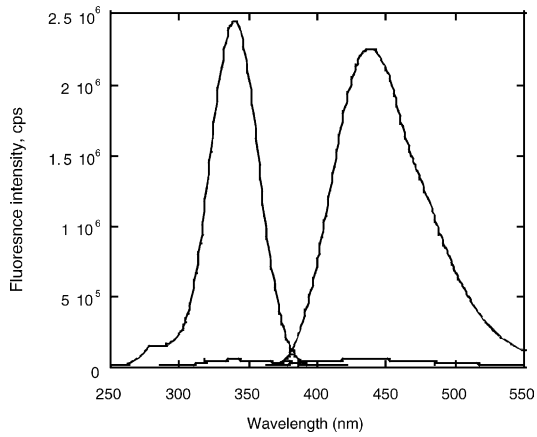


Fig. 2. Fluorescence emission and excitation spectra of the FNO (5×10^{-5} M) before (_) and after (—) reduction by quercetin (5×10^{-7} M) in PBS (pH 7.4, $T = 300$ K).

Based on the expressions outlined above, three different cases of the rate constant behavior are possible depending on a combination of rate constants and concentration of reagents.

1. At $k_1[\mathrm{FNO}][\mathrm{O_2}^{\bullet-}] \gg k_{\mathrm{dis}}[\mathrm{O_2}^{\bullet-}]^2$ when the majority of superoxide radicals disappear during reaction with FNO:
$$\frac{\mathrm{d}I}{\mathrm{d}t} \propto \alpha \frac{\mathrm{d}[\mathrm{FNO}^*]}{\mathrm{d}t} = \alpha\omega_i \tag{9}$$
and the reaction is a zero-order process.
2. At $k_{\mathrm{dis}}[\mathrm{O_2}^{\bullet-}]^2 \gg k_1[\mathrm{FNO}][\mathrm{O_2}^{\bullet-}]$, when spontaneous disproportion of $\mathrm{O_2}^{\bullet-}$ prevails over reactions of $\mathrm{O_2}^{\bullet-}$ with other components, the reduction rate of FNO should be expressed as
$$\frac{\mathrm{d}[\mathrm{FNO}^*]}{\mathrm{d}t} = k_1[\mathrm{FNO}]\sqrt{\frac{\omega_i}{k_{\mathrm{dis}}}} \tag{10}$$
3. For general cases the kinetic process is described by Eq. (7). If the concentration of FNO during the process is changed only slightly, $[\mathrm{FNO}] \approx [\mathrm{FNO}]^0$, the reaction is approximately of pseudo first-order.

In the case of a zero-order reaction, the plot of dI/dt versus ω_i should give a straight line with a slope α. Eq. (9) is useful for calibrating the parameter α for any fluorimeter at a given rate of superoxide generation ω_i. This coefficient can then be used for determination of ω_i and the [FNO*] concentration in biological systems under investigation.

4. Results and discussion

4.1. Reaction of FNO with superoxide

The EPR spectrum of nitronyl nitroxide FNO in buffer solutions shows five lines governed by the interaction of an unpaired electron with two equivalent nitrogen nuclei (^{14}N, 99.63% natural abundance, $I = 1$) (Fig. 1). FNO does not exhibit fluorescence spectra because of the nitronyl fragment, which is a strong intramolecular quencher of the chromophore fluorescence. Reactions between FNO and superoxide radicals or antioxidants lead to fluorescence enhancement (Fig. 2), and simultaneous decay of the nitroxyl ESR signal.

The generation of superoxide radicals by XOS results in an increase of the FNO fluorescence intensity (Fig. 3). In the absence of catalase, the rate of reaction appears to be lower. Hence, we conclude that hydrogen peroxide produced by dismutation of superoxide is also involved in the reaction with FNO, which is typically, the Fenton reaction. At relatively low rates of superoxide formation ($\omega_i < 2 \times 10^{-7}$ M/min), the reaction has zero-order character. Therefore the dependence of dI/dt on ω_i is linear (Fig. 4) and dI/dt is a constant value (Fig. 5A). Thus under such conditions the reaction of FNO with superoxide prevails

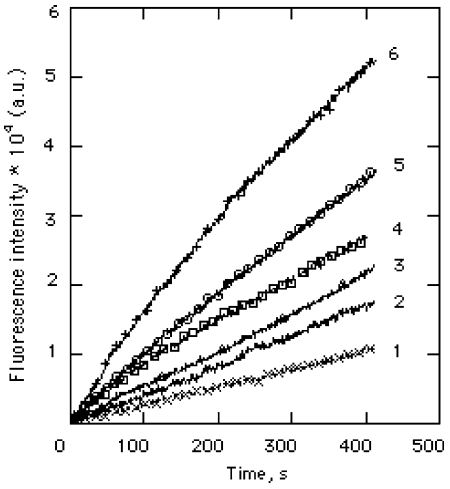


Fig. 3. Experimental fluorescence kinetics from the FNO by its interaction with superoxide in PBS (pH 7.4, $T = 300$ K). A different concentration of xanthine oxidase was added to the solution containing FNO (5×10^{-5} M), xanthine (4×10^{-4} M), and catalase (280 U/ml) to obtain following rates of superoxide formation: (1) 2×10^{-8} M/min; (2) 5×10^{-8} M/min; (3) 7×10^{-8} M/min; (4) 10^{-7} M/min; (5) 5×10^{-7} M/min; (6) 10^{-6} M/min.

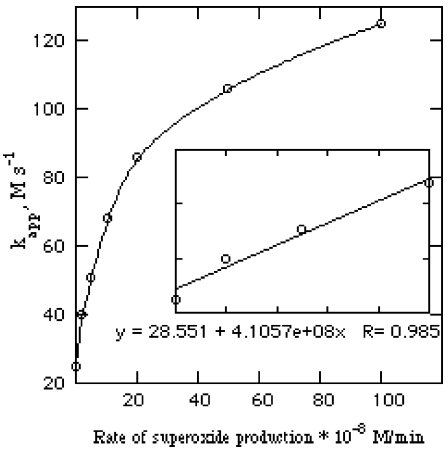


Fig. 4. Dependence of the apparent rate constant of FNO reduction on the rate of superoxide formation; PBS (pH 7.4, $T = 300$ K). Procedures are as described in the legend of Fig. 3. The insert shows the linear segment of the curve for low rates of superoxide formation corresponding to the zero-order process ($\omega_i < 2 \times 10^{-7}$ M/min).

over the dismutation reaction. Taking into consideration the fact that the experimental fluorescence intensity is proportional to the product concentration ($dI/dt \propto \alpha\, d[FNO^*]/dt$), the empirical coefficient $\alpha = 4.1 \times 10^8$ can be used in subsequent experiments for determination of the [FNO*] concentration. When the rate of the $O_2^{\bullet -}$ generation increases ($\omega_i > 2 \times 10^{-7}$ M/min), the reactions kinetic deviate from the zero-order and follow the first-order behavior. The second-order rate constant k_{dis} for spontaneous dismutation of $O_2^{\bullet -}$ at pH 7.4 is found to be $2 \times 10^5\ M^{-1}\ s^{-1}$ [33]. Using this value, the second-order rate constant of the reaction between FNO and $O_2^{\bullet -}$ $k_1 = 1.54 \times 10^6\ M^{-1}\ s^{-1}$ is obtained from the kinetic analysis of data presented in Fig. 3 (curve 6).

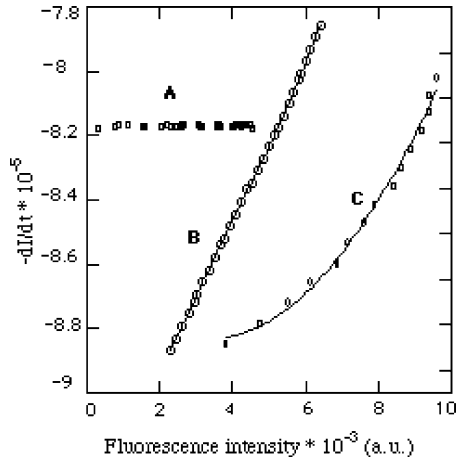
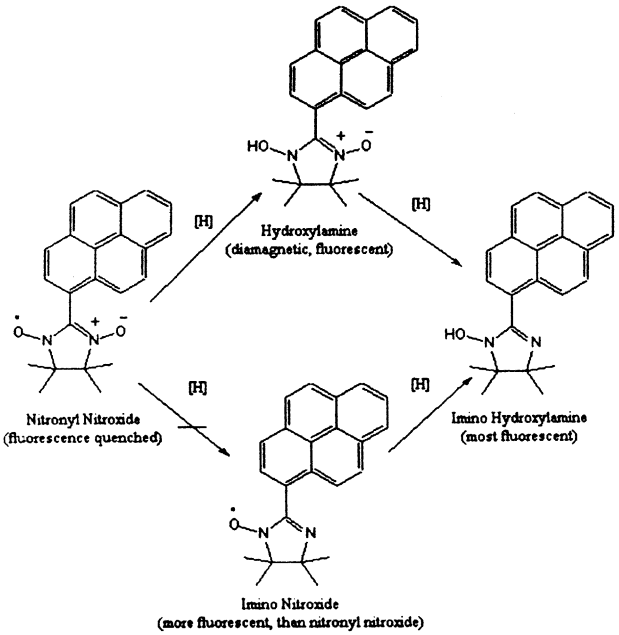


Fig. 5. Schematic presentation of the FNO fluorescence increases rate vs. fluorescence intensity: (A) zero-order reduction rate of FNO (5×10^{-5} M) by superoxide ($\omega_i = 7 \times 10^{-8}$ M/min); (B) pseudo first-order reduction rate of FNO (5×10^{-5} M) by ascorbic acid (5×10^{-6} M); and (C) second-order reduction rate of FNO (5×10^{-5} M) by ascorbic acid (5×10^{-5} M). The fluorescence kinetics was measured in the presence of catalase (280 U/ml) in PBS (pH 7.4, $T = 300$ K).

The reaction between FNO and $O_2^{\bullet -}$ can occur via two alternative mechanisms, i. e., oxidation and reduction. It has been previously reported [11,34] that in the case of the oxidation mechanism involving electron transfer from the nitroxide to the superoxide, an intermediate product is produced, which can then oxidize the second molecule of superoxide, and thus regenerate the nitroxide.



The addition of reductants (NADH, cysteine), which are capable of reducing the intermediate product of the nitroxyl oxidation, results in nitroxyl destruction. In our experiments, the addition of the cysteine reducing agent did not affect the kinetics of reaction between FNO and $O_2^{\bullet -}$. Therefore, we conclude that this reaction occurs by the reduction mechanism. It has been reported [35] that upon addition of reducing agent, nitronyls are effectively reduced to hydroxylamine derivatives.

The alternative mechanism of reduction to an imino-form can be excluded in our system, since no ESR signal corresponding to the imino-form has been detected. Nitronyl and imino nitroxide radicals have distinctly different hyperfine splitting patterns in EPR spectra. The EPR spectrum of nitronyl nitroxide shows five lines governed by the interaction of unpaired electron with two equivalent nitrogen nuclei (^{14}N, 99.63% natural abundance, $I = 1$). On the other hand, two nitrogen nuclei in the imino nitroxide molecule are not equivalent that, taking into account the isotropic hyperfine parameter for one nucleus is just a half of the same for another one, results in the distinctive seven-line pattern.

4.2. Reaction of FNO with antioxidants

It was determined that the FNO fluorescence assay is suitable for the submicro concentration scale of antioxidants.

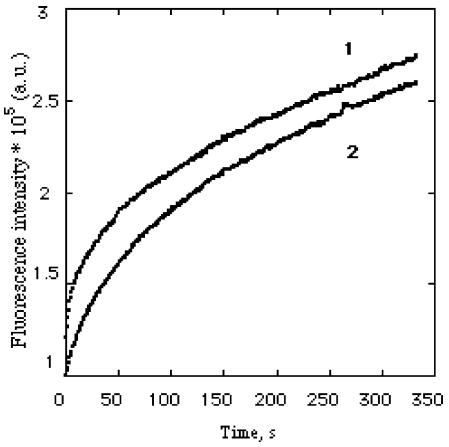


Fig. 6. Effect of catalase (280 U/ml) on the fluorescence emission of FNO (5×10^{-5} M) in its reaction with ascorbic acid (5×10^{-5} M); PBS (pH 7.4, $T = 300$ K): (1) and (2) fluorescence kinetics in both the presence and absence of catalase, respectively.

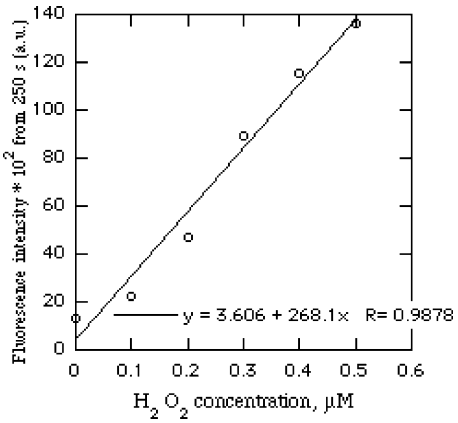


Fig. 7. Effect of the Fenton reagent on the intensity of FNO fluorescence emission spectra. Spectra were measured at 250 s after the addition of hydrogen peroxide to the reaction mixture containing 5×10^{-5} M FNO and ferrous sulfate, PBS (pH 7.4, $T = 300$ K). The concentration ratio (hydrogen peroxide:ferrous sulfate) was (1:1).

Hence the following well-known biological reducing agents were chosen—ascorbic acid, quercetin and galangin [36,37]. If FNO is taken in excess, the kinetic analysis suggests the pseudo first-order reaction takes place (Fig. 5B). Linear dependencies of the experimental pseudo first-order rate constant on antioxidants concentration in the range 5×10^{-8} to 10^{-6} M in the absence of catalase show slopes equal to 63.2, 5.9, 0.3 $M^{-1}\,s^{-1}$ for the ascorbic acid, quercetin and galangin, respectively.

Fig. 6 shows the kinetics of the reaction of FNO with ascorbic acid in both the presence and the absence of catalase monitored by the fluorescence technique. The reaction is the second-order process (Fig. 5C) with the rate constant 48 $M^{-1}\,s^{-1}$. As seen from Fig. 6, catalase essentially affects the reaction rate, probably because of dioxygen being involved in the process. The values of rate constants for reduction of FNO with flavonic compounds, quercetin and galangin in the presence of catalase were found to be 7.6×10^{-1} and 1.8×10^{-2} $M^{-1}\,s^{-1}$, respectively. These values are close to those obtained independently by following the decay of the FNO ESR signal.

4.3. Reaction of FNO with Fenton reagent

It was found that hydrogen peroxide does not react with FNO if the hydrogen peroxide concentration is less than 0.1 mM. In order to investigate the reaction of FNO with one of the most reactive oxygen species, namely the hydroxyl radical, various amounts of Fenton reagent were added to the FNO solution. The fluorescence intensity was measured 250 s after the samples were mixed, and was found to be linearly dependent on the submicromolar concentration of Fenton reagent (Fig. 7). Therefore, the above-mentioned technique is receptive to submicro concentrations of hydrogen peroxide.

5. Conclusion

Theoretical and experimental aspects of a new fluorescence-nitroxide approach for the analysis of superoxide radicals in the nanomolar concentration scale have been studied. This approach is based on the phenomenon of intramolecular fluorescence quenching of the fluorophore fragment by the nitroxide radical in the dual pyrene-nitronyl (FNO) molecule. The nitroxide fragment of the dual non-fluorescence molecule reacts with superoxide generated by XOS and antioxidants (ascorbic acid, quercetin and galangin). These reactions of FNO are accompanied by a drastic increase in the fluorescence intensity and a decrease of the EPR signal. When the rate of superoxide formation is slow ($\omega_i < 2 \times 10^{-7}$ M/min), the reaction follows the zero-order kinetics. Hence, the rate of fluorescence rise (dI/dt) is proportional to the rate of superoxide formation (ω_i). The dependence of dI/dt on ω_i was used as a calibration curve in the subsequent experiments. At elevated concentrations, the fluorescence kinetics follows the first-order character. The kinetic analysis allowed calculation of the rate constant of FNO reduction by superoxide $k_1 = 1.54 \times 10^6$ $M^{-1}\,s^{-1}$ (pH 7.4, $T = 300$ K).

The dual probe reacts with micromolar concentrations of the ascorbic acid, quercetin, and galangin with rate constants 48, 7.6×10^{-1}, and 1.8×10^{-2} $M^{-1}\,s^{-1}$, respectively. The possibility of using FNO for the detection of hydroxyl radicals generated from hydrogen peroxide in a less than micromolar concentration scale was also demonstrated. This method can also be used for detection of hydrogen peroxide.

Due to its high sensitivity and simplicity, the proposed method will, under certain conditions, have advantages over conventional light absorption, ESR and chemiluminescence techniques and will enable monitoring of processes in real time. The contribution of other reactive oxygen species could be estimated quantitatively by previous treatment with

appropriative inhibitors. Thus, the contribution of superoxide radical can be estimated in the presence of superoxide dismutase and catalase (NO does not react with FNO in aerobic conditions).

Application of this new methodology can afford insights into the biochemical significance of superoxide and hydrogen peroxide and can create a basis for the development of new methods for biomedical research and medical diagnostics.

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