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Investigation of the anthracene–nitroxide hybrid molecule as a probe for hydroxyl radicals

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A new method for the determination of hydroxyl radicals is proposed. The method is based on the use of a hybrid molecule consisting of a fluorescent chromophore, anthracene, and a nitroxide radical. In the hybrid molecule, the nitroxide quenches the fluorescence of anthracene strongly. The reaction of hydroxyl radicals with dimethyl sulfoxide generates quantitatively methyl radicals, which then combine with the nitroxide moiety of the hybrid molecules to result in an increase in the fluorescence intensity. The fluorescence increase is proportional to the concentration of hydroxyl radicals. The proposed method is capable of detecting hydroxyl radicals generated in the Fenton system. It is a simple and sensitive technique for the determination of hydroxyl radicals.

Introduction

Oxygen-derived free radicals are potent agents that cause many pathological effects, as well as aging.[1] Among the various radicals, the hydroxyl radical (·OH), which is formed non-enzymatically from hydrogen peroxide (H_2O_2) in a metal-dependent reaction, is the most reactive and toxic oxygen radical known to date.[2] It can initiate radical chain reactions, such as lipid peroxidation,[2,3] and has been suggested to play a critical role in many pathological processes. As ·OH is highly reactive (second-order rate constants of 10^7–10^{10} M^{-1} s^{-1})[4] and short-lived (lifetime of about 2 ns in aqueous solution), it is rather difficult to monitor its concentration and action in normal conditions.

In order to establish the role of free radicals in toxicology and in human disease, it is necessary to be able to probe them accurately in real time. Hence, many methods have been developed for the detection of ·OH in recent years. The two most commonly used methods, in chemical terms, are probably electron spin resonance (ESR)[5–7] and aromatic hydroxylation. The ESR method measures the electron paramagnetic resonance spectrum of a spin adduct derivative after spin trapping. Although the use of spin traps, such as a-phenyl-*N*-*tert*-butylnitrone (PBN), is a powerful technique,[8] this method is rather insensitive and cannot be employed readily to acquire quantitative estimates of ·OH production due to numerous secondary reactions of the ·OH spin trap adduct.[9] In addition, a highly costly instrument system makes it unsuitable for routine analysis. Aromatic hydroxylation, another commonly used method, is based on the measurement of the hydroxylated products arising from the reaction of ·OH with salicylic acid,[10–13] phenylalanine,[14] 4-hydroxybenzoate[15] and benzoic acid,[16] either electrochemically or spectrophotometrically after high-performance liquid chromatography (HPLC) separation. This method has the advantage of being easy to use, but the multiple hydroxylated products make the quantitative detection of ·OH complicated. Dimethyl sulfoxide (DMSO) has also been used as a molecular probe for ·OH, and the methanesulfinic acid produced *via* the reaction of ·OH with DMSO is detected electrochemically[17] or by HPLC of its dye-binding derivative.[18,19]

Numerous studies have shown that paramagnetic nitroxides are efficient quenchers of excited singlet states of aromatic hydrocarbons,[20–22] presumably through an intermolecular electron exchange interaction between the ground state nitroxide and the excited state compound within a collision complex. In a series of studies, Blough and coworkers[23–25] covalently linked a nitroxide with a fluorophore to constrain them to reside in a permanent and easily accessible 'collision complex'. Because of the proximity of the fluorophore, the fluorescence quenching by nitroxide within the compound is highly efficient compared with that of traditional intermolecular quenching arising from the nitroxide. In such a hybrid molecule, both the fluorescence quenching and the radical trapping properties of the nitroxide are advantageous in a technique for monitoring radicals. Fluorescence emission from the fluorophore of the hybrid molecule is greatly quenched, presumably through an electron exchange mechanism, which both reduces the fluorescence quantum yield and shortens the fluorescence lifetime. However, the reaction of such a hybrid molecule with a carbon-centred radical, or chemical reduction to its corresponding hydroxylamine, leads to the formation of a diamagnetic product, thereby eliminating the intramolecular quenching pathway and resulting in a large enhancement in fluorescence emission.[24,25] The fluorescence quantum yields of the diamagnetic products are much greater than those of the corresponding paramagnetic derivatives. The research work of Pou *et al.*[26] has shown that there is a direct relationship between the fluorescence enhancement and the decrease in nitroxide concentration. The research work of Moad *et al.*[27] has shown that the fluorescence increase is proportional to the increase in the concentration of O-alkoxyamine, which, in turn, is proportional to the concentration of carbon-centred radicals generated in the experiment.

Herein, the synthesis and application of a new anthracene–nitroxide hybrid molecule (**I**) as a potential probe for ·OH is reported. 4-Hydroxy-2,2,6,6-tetramethylpiperidinyloxyl (4-hydroxy-TEMPO) was chosen as the radical label, and 9-anthracenecarboxylic acid (9-ACA) as the fluorophore. The synthesized anthracene–nitroxide hybrid molecule (**I**) showed an indirect sensitive response to ·OH. The response was based on the reaction of ·OH with DMSO to produce quantitatively a methyl radical ($\cdot CH_3$), which then combined with **I** to produce a stable O-methylhydroxylamine (**II**) (Scheme 1). Because of the highly efficient intramolecular quenching of the excited single states by the stable nitroxide radicals within the compound, the fluorescence emission of **I** was very weak. The combination of **I** with $\cdot CH_3$ led to the formation of its diamagnetic product (**II**), thereby eliminating the intramolecular quenching within the

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hybrid molecule (**I**), and resulting in a large enhancement in fluorescence intensity. The fluorescence increase was proportional to the amount of $\cdot CH_3$ generated from the reaction of DMSO with $\cdot OH$, which, in turn, was proportional to the concentration of $\cdot OH$. Based on this mechanism, a new method for the determination of $\cdot OH$ was developed. The proposed method has been demonstrated to be simple and sensitive and does not require expensive instrumentation; it offers a potentially powerful tool to study $\cdot OH$ in biological materials.

Experimental

Apparatus

The fluorescence spectra and relative fluorescence intensity were measured with a Shimadzu RF-5000 spectrofluorimeter (Kyoto, Japan) with a 10 mm quartz cuvette. The excitation wavelength was set at 377 nm and the emission wavelength was set at 427 nm. The excitation and emission bandpasses were both set at 5 nm. Absorption spectra were obtained on a Beckman DU-7400 ultraviolet–visible spectrophotometer. All pH values were measured with a pHS-301 digital ion meter.

Reagents

A 1.0×10^{-3} mol l^{-1} Fe(II) solution was prepared by dissolving an appropriate amount of ammonium ferrous sulfate in distilled water. The Fe(II) solution was prepared daily. A stock solution of H_2O_2 (0.10 mol l^{-1}) was prepared by diluting 1.1 ml of 30% (v/v) H_2O_2 to 100 ml with water. The concentration was standardized by titration with potassium permanganate. The stock solution (5.0×10^{-4} mol l^{-1}) of **I** was prepared in 40% (v/v) methanol–water solution and stored in a refrigerator at 4 °C. A 1.0×10^{-3} mol l^{-1} anthracene solution was prepared by dissolving 0.0178 g of anthracene in 100 ml of methanol. The following solutions were prepared in double distilled water: DMSO, 2.0 mol l^{-1}; EDTA, 0.01 mol l^{-1}; ascorbic acid, 0.01 mol l^{-1}; 4-hydroxy-TEMPO, 0.01 mol l^{-1}; NaH_2PO_4–Na_2HPO_4 (pH 7.4) buffer solution. 4-Hydroxy-TEMPO was a gift from Beijing Huashan Auxiliary Factory.

All the chemicals used were of analytical reagent grade and double distilled water was used throughout.

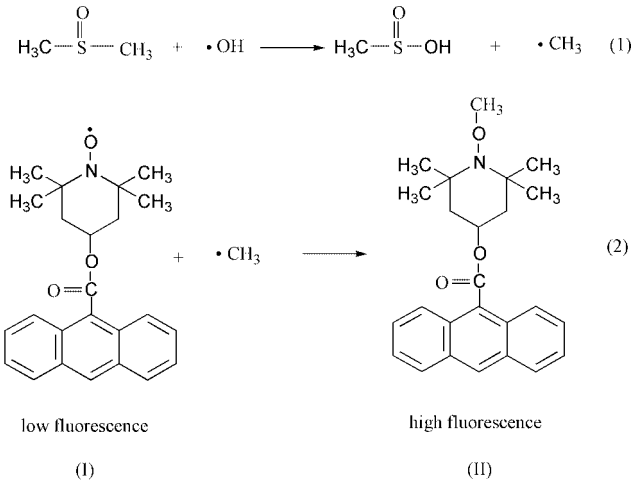
Synthesis of 4-(9-anthroyloxy)-2,2,6,6-tetramethylpiperidine-1-oxyl (I)

I was synthesized (Scheme 2) using 4-hydroxy-TEMPO (**IV**) and 9-ACA (Aldrich, 99%;). To 100 mg of 9-ACA, 2.0 ml of thionyl chloride was added and refluxed for 3 h; it was then evaporated to dryness to yield **III**. **III** was dissolved with chloroform, and added dropwise to a stirred solution of **IV** (120 mg, dissolved in 4 ml of chloroform). An appropriate amount of carefully powdered anhydrous sodium carbonate was added to neutralize the hydrochloric acid generated in the reaction solution. The reaction mixture was stirred overnight. This solution was then diluted with chloroform, washed with water, dried with anhydrous sodium sulfate and evaporated to a yellow solid. This solid was dissolved with methanol and loaded on a silica gel column. Elution with chloroform yielded 62 mg of **I** as an orange solid (R_f = 0.5). The desired product was stored in the dark in a refrigerator. Analysis by UV–visible spectrophotometry, λ_{max} (phosphate buffer, pH 7.4): 385 nm ($\varepsilon = 1.05 \times 10^4$ M^{-1} cm^{-1}), 366 nm (1.2×10^4 M^{-1} cm^{-1}), 348 nm (8.5×10^3 M^{-1} cm^{-1}); by MS (CI): 378.6 ($[M + 2H]^+$, 50), 362.9 ([M − 14], 20), 360.8 ([M − 16], 8), 205.6 (100).

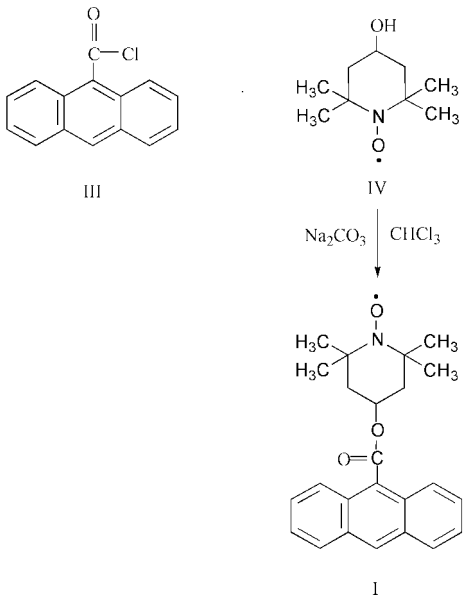
Results and discussion

Comparison of the intermolecular and intramolecular fluorescence quenching

Collisional quenching of fluorescence by stable nitroxide radicals has been studied extensively over the past 20 years. The goal of much of this work has been to understand the mechanism through which excited states are quenched by paramagnetic species. Recently, however, it has been demonstrated that fluorescence quenching by nitroxide radicals can be used as a very sensitive optical probe of radical/redox reactions. In the present study, we covalently linked a paramagnetic nitroxide radical to anthracene and compared the intermolecular and intramolecular fluorescence quenching by paramagnetic nitroxide in the same conditions. Fig. 1 shows the fluorescence spectra of anthracene, the hybrid molecule (**I**) and a mixture of anthracene with different concentrations of 4-hydroxy-TEMPO. It can be seen that the fluorescence of **I** is very weak, while that of anthracene is strong. If anthracene and 4-hydroxy-TEMPO are mixed in a ratio of 1 : 1 [the concentrations of both being the same as that of the hybrid molecule (**I**)], no fluorescence quenching is observed. Significant fluorescence quenching of anthracene is observed when the concentration of 4-hydroxy-TEMPO is 1000 times higher than that of anthracene. The results show that the efficiency of intramolecular



Scheme 1



Scheme 2

quenching of the hybrid molecule (**I**) is much higher than that of intermolecular quenching produced in the solution mixture of anthracene and 4-hydroxy-TEMPO. This is understandable, as in the former conditions, nitroxide and anthracene are linked by an ester bond and remain easily accessible, facilitating the fluorescence quenching by the paramagnetic nitroxide.

Spectral characteristics

The absorption and fluorescence emission spectra of anthracene and its radical-labelled species were compared in the present study. When a paramagnetic nitroxide radical was attached to the anthracene ring *via* an ester bond, the sharp structural absorption of anthracene was almost lost; instead, a broad structureless absorption was found in the same position, while the fluorescence emission maximum of **I** was red-shifted by about 8 nm compared with that of authentic anthracene. The fluorescence emission of the hybrid molecule, however, was quenched dramatically. To test whether the UV absorption or fluorescence spectra varied when the paramagnetism of **I** was lost, ascorbic acid was used to reduce **I** to its corresponding hydroxylamine; the results showed that the fluorescence emission maximum remained the same, while the fluorescence intensity increased dramatically. The UV absorption spectra of **I** were the same before and after reduction (as shown in Fig. 2).These results demonstrate that the elimination of the paramagnetism of **I** has no significant influence on the spectral characteristics of **I**, except for the large increase in fluorescence intensity. The same conclusion was drawn when **I** was converted into **II** *via* reaction with $\cdot CH_3$.

Detection of hydroxyl radical generated in the Fenton system

The Fenton system was used as the source of $\cdot OH$ to test the applicability of the proposed method towards the detection of $\cdot OH$. H_2O_2 (1.0×10^{-5} mol l^{-1}) was added to a reaction mixture containing 7.0×10^{-5} mol l^{-1} of Fe(II)–EDTA, 0.1 mol l^{-1} of DMSO and 3.0×10^{-6} mol l^{-1} of **I** in pH 3 sulfuric acid medium; the reaction solution was kept at room temperature (20 °C) for 45 min, phosphate buffer solution (pH 7.4) was added and the fluorescence intensity was measured.

Some preliminary experiments were performed to ensure that the enhancement of the fluorescence intensity was indeed due to the reaction of $\cdot CH_3$ with compound **I**. The experimental results demonstrated that the fluorescence intensity of the reaction mixture showed no significant increase when Fe(II)–EDTA, H_2O_2 or DMSO was added separately, or when two were added in combination, to the solution of **I** (as shown in Fig. 3). There was a slight increase in the fluorescence intensity in the absence of H_2O_2; this is understandable as the Fe(II)–EDTA chelate can

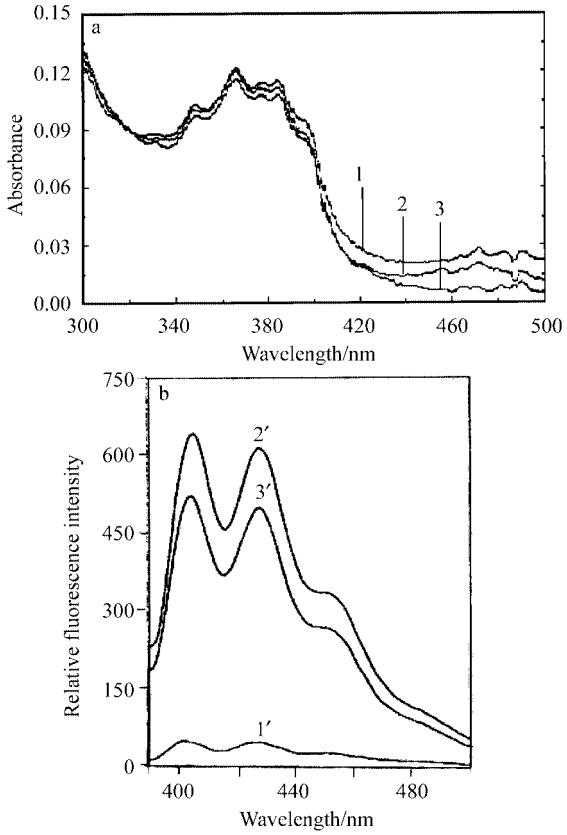


Fig. 2 Absorption (a, **I** = 1.0×10^{-5} mol l^{-1}) and fluorescence emission (b, **I** = 3.0×10^{-6} mol l^{-1}) spectra of **I** and its analogues. 1, 1′, spectra of **I** in pH 7.4 phosphate buffer solution; 2, 2′, spectra of **I** 15 min after the addition of ascorbic acid (5.0×10^{-4} mol l^{-1}) in pH 7.4 phosphate buffer solution; 3, 3′, spectra of **I** after the addition of Fe(II)–EDTA (7.0×10^{-5} mol l^{-1}), H_2O_2 (1.0×10^{-5} mol l^{-1}) and DMSO (0.1 mol l^{-1}) in pH 3 sulfuric acid for 45 min, then pH 7.4 phosphate buffer solution was added and the spectra were recorded.

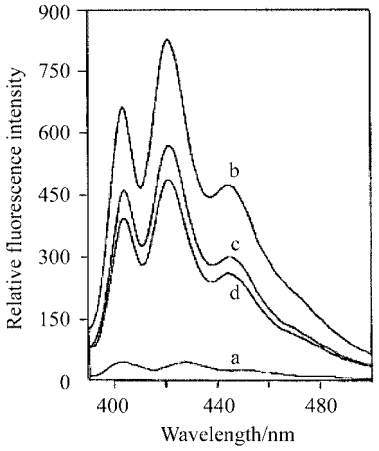


Fig. 1 Comparison of the fluorescence spectra of the hybrid molecule (**I**), anthracene and a mixture of anthracene with different concentrations of 4-hydroxy-TEMPO. (a) **I**, 3.0×10^{-6} mol l^{-1}; (b) anthracene, 3.0×10^{-6} mol l^{-1}; (c) same as (b), except for the addition of 3.0×10^{-3} mol l^{-1} of 4-hydroxy-TEMPO; (d) same as (b), except for the addition of 6.0×10^{-3} mol l^{-1} of 4-hydroxy-TEMPO.

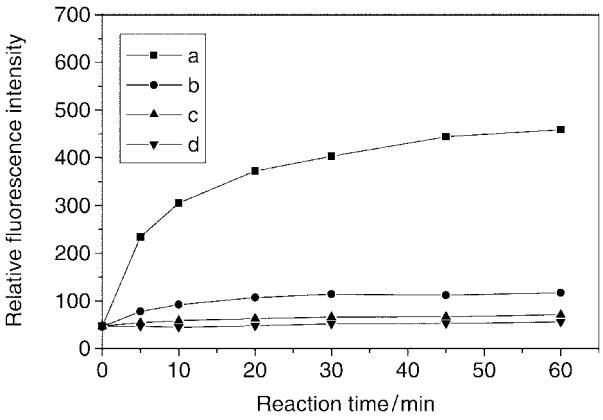


Fig. 3 Fluorescence intensities as a function of reaction time. (a) Reaction mixture contained: **I**, 3.0×10^{-6} mol l^{-1}; Fe(II)–EDTA, 7.0×10^{-5} mol l^{-1}; H_2O_2, 1.0×10^{-5} mol l^{-1}; DMSO, 0.1 mol l^{-1}; and pH 3 sulfuric acid. (b) Same as (a), except for the absence of H_2O_2. (c) Same as (a), except for the absence of Fe(II)–EDTA. (d) Same as (a), except for the absence of DMSO. The reaction solution was kept at room temperature for a period of time, and then pH 7.4 phosphate buffer solution was added and the fluorescence intensity of the reaction solution was recorded.

be oxidized by molecular oxygen rapidly in weakly alkaline media and can generate a small amount of ·OH *via* a series of reactions.[28–30] When Fe(II)–EDTA, H_2O_2 and DMSO were added simultaneously, a remarkable increase in fluorescence intensity occurred due to the formation of **II**, a highly fluorescent product. These results demonstrate that it is $\cdot CH_3$ that reacts with **I** to form the diamagnetic product (**II**), thereby eliminating the intramolecular quenching pathway and leading to a large increase in fluorescence emission.

Optimization of the general procedure

Kinetic characteristics. The kinetic characteristics of the reaction were investigated to obtain the optimal experimental conditions of the proposed method. To do this, the fluorescence signal was recorded as a function of reaction time and the results are shown in Fig. 3(a). It can be seen that the fluorescence intensity increased with increasing reaction time. Compared with reactions (1) and (2) in Scheme 1, which are nearly diffusion controlled with rate constants of 7.0×10^9 M^{-1} s^{-1},[31] and 7.8×10^8 M^{-1} s^{-1},[32] respectively, the Fenton reaction is the rate-determining step, with a small rate constant of $\sim 10^4$ M^{-1} s^{-1}.[28] As a compromise between high sensitivity and short analysis time, a 45 min reaction time was selected. In fact, biological samples presented with ·OH ready-made would need very much shorter reaction times, less than 1 s in some cases. Thus, if the present method was applied to biological systems, the reaction time would be greatly reduced.

Effect of media and pH. Considering the highly oxidizing nature of ·OH [E^0(·OH/OH^-) = 1.9 V],[33] which can oxidize organic substrates, the study of the generation of ·OH is usually performed in inorganic medium. Thus, sulfuric acid was selected to investigate the generation of ·OH by the Fenton reaction at different pH values. Fig. 4 shows the effect of pH on the production of **II** in the range of pH 1.5–5.0. It can be seen that the fluorescence intensity of the reaction mixture increased with increasing pH, was optimal at pH 3.0 and thereafter decreased, which was consistent with the values observed in the Fenton system.

Effect of DMSO concentration. DMSO has become of interest as a trap for ·OH formation owing to its unique chemical and biological properties, such as its benign biological effects and its appreciable rate of interaction with ·OH.[31] To establish the appropriate conditions for the quantitative reaction of DMSO with ·OH, the effect of the DMSO concentration on the fluorescence signal was examined and the results are shown in Fig. 5. Unlike the other compounds utilized in this study, the concentration of DMSO can be substantially increased without much adverse effect on the fluorescence emission. It can be seen that the fluorescence signal increased with increasing concentration of DMSO up to 0.1 mol l^{-1}, with a slight decrease in signal above this value. To trap ·OH effectively, the concentration of DMSO should be as high as possible. Thus, the concentration of DMSO was selected as 0.1 mol l^{-1}.

Effect of Fe(II)–EDTA concentration. It has been reported that EDTA complexes with Fe(II) to produce ·OH *via* the Fenton reaction at a faster rate than Fe(II) itself,[34] which could enhance the sensitivity of the proposed method by decreasing the concentration of Fe(II) needed and hence decreasing the fluorescence quenching by Fe(III). Thus, Fe(II)–EDTA was employed in the following experiment. It was desirable to keep the concentration of Fe(II)–EDTA as high as possible to accelerate the rate of the Fenton reaction, but higher concentrations of Fe(II)–EDTA give rise to a large amount of Fe(III)–EDTA, which will lead to a decrease in the signal. Thus, we attempted to find the concentration of Fe(II)–EDTA that would maximize the rate of the Fenton reaction, while minimizing the fluorescence quenching by Fe(III)–EDTA at the same time. The dependence of the fluorescence signal on the concentration of Fe(II)–EDTA is shown in Fig. 6. It can be seen that the maximum fluorescence signal was obtained at 7.0×10^{-5} mol l^{-1} of Fe(II)–EDTA. Hence, 7.0×10^{-5} mol l^{-1} of Fe(II)–EDTA was employed.

Effect of H_2O_2 concentration. As ·OH is a short-lived species, it is difficult to determine its exact concentration generated by the Fenton reaction. However, the ·OH concentration is proportional to the concentration of H_2O_2 used to generate ·OH *via* the Fenton reaction when the Fe(II)–EDTA concentration remains the same. The fluorescence increase is linearly related to the H_2O_2 concentration in the range 2.5×10^{-7}–2.0×10^{-6} mol l^{-1}, which proves indirectly that the fluorescence increase is proportional to the concentration of ·OH over a certain range. The fluorescence signal increased slowly when the concentration of H_2O_2 was above 2.0×10^{-5} mol l^{-1}.

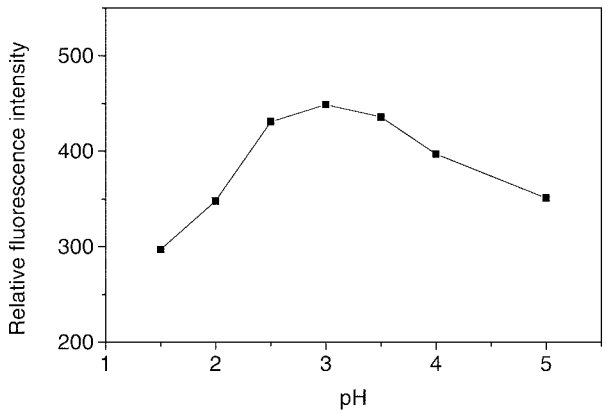


Fig. 4 Effect of pH on the fluorescence intensity. Reaction mixture contained: **I**, 3.0×10^{-6} mol l^{-1}; Fe(II)–EDTA, 7.0×10^{-5} mol l^{-1}; H_2O_2, 1.0×10^{-5} mol l^{-1}; DMSO, 0.1 mol l^{-1}. The different pH values of the reaction mixtures were adjusted with sulfuric acid. The reaction solution was kept at room temperature for 45 min, and then pH 7.4 phosphate buffer solution was added and the fluorescence intensity of the reaction solution was measured.

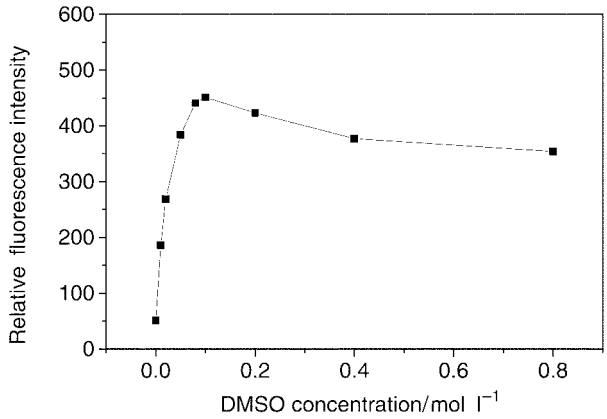


Fig. 5 Effect of DMSO concentration on the fluorescence intensity. Reaction mixture contained: **I**, 3.0×10^{-6} mol l^{-1}; Fe(II)–EDTA, 7.0×10^{-5} mol l^{-1}; H_2O_2, 1.0×10^{-5} mol l^{-1}; DMSO (at different concentrations); and pH 3 sulfuric acid. The reaction solution was kept at room temperature for 45 min, and then pH 7.4 phosphate buffer solution was added and the fluorescence intensity of the reaction solution was measured.

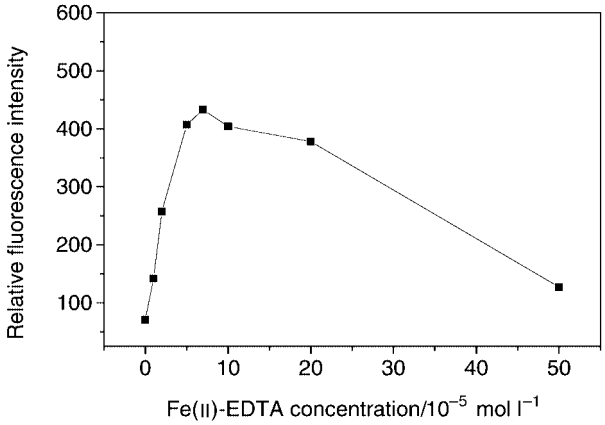


Fig. 6 Effect of Fe(II)–EDTA concentration on the fluorescence intensity. Reaction mixture contained: **I**, 3.0×10^{-6} mol l^{-1}; H_2O_2, 1.0×10^{-5} mol l^{-1}; DMSO, 0.1 mol l^{-1}; Fe(II)–EDTA (at different concentrations); and pH 3 sulfuric acid. The reaction solution was kept at room temperature for 45 min, and then pH 7.4 phosphate buffer solution was added and the fluorescence intensity of the reaction solution was measured.

Conclusions

In summary, a novel technique for the detection of ·OH is presented in this paper. The proposed method is simple, specific, easy to operate and has a relatively high sensitivity. Unlike the aromatic hydroxylation method, only one quantitative product (compound **II**) is produced in the detection system, thus making the quantitative analysis simple. Compared with the spin adduct, the product of **II** is stable and does not undergo significant loss *via* second reactions; thus, its accumulation with reaction time provides a quantitative estimate of ·OH production, which will be of great value in many biological studies. Because DMSO is highly water soluble, can be tolerated by living systems at up to 1 mol l^{-1} concentration[35] and has an appreciable rate of interaction with ·OH, it has been proved to be an ideal trap for ·OH. The quantitative reaction of ·OH with DMSO can be obtained at a relatively high DMSO concentration with little adverse effect on the fluorescence signal.

The proposed method also suffers from some limitations. One is that the nitroxide moiety of the hybrid molecule (**I**) would be metabolized to its corresponding hydroxylamine in the presence of cellular reductants [*e.g.* ascorbic acid, glutathione (GSH), nicotinamide adenine dinucleotide hydrophosphate (NADPH)], and hence cause an overestimate of ·OH production. This limitation can be alleviated when a suitable five-membered ring (pyrrolidine) nitroxide is used with anthracene instead of a six-membered ring (piperidine) nitroxide, as the former are generally known to be more resistant towards reduction than the latter.[36] Another drawback is that other carbon-centred radicals will also couple with the hybrid molecule (**I**) and hence give rise to a fluorescence increase in the detection system. This effect, however, can be overcome when the present method is coupled with HPLC to detect O-methylhydroxylamine (**II**) post-column. Thus, we believe that the proposed method is broadly applicable to the detection of ·OH in a variety of systems.

Acknowledgements

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