Mechanism of action of sensors for reactive oxygen species based on fluorescein–phenol coupling: the case of 2-[6-(4′-hydroxy)phenoxy-3*H*-xanthen-3-on-9-yl]benzoic acid

Belinda Heyne, Vincent Maurel and J. C. Scaiano*

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We demonstrate the ability of a sensor containing a tethered fluorescein–phenol structure to react with peroxyl radicals and with an oxidizing agent such as potassium ferricyanide. This latter reaction yields the corresponding peroxyl radical as observed by EPR analysis. We propose that the reaction of the sensor with peroxyl and alkoxyl radicals is also initiated by the formation of the phenoxyl radicals, which is followed by radical–radical reactions and product hydrolysis responsible for the release of fluorescein. The proposed mechanism is based on results obtained by laser flash photolysis, HPLC and EPR studies of the reaction of peroxyl and alkoxyl radicals with 4-phenoxylphenol, a molecule used to mimic the behavior of the sensor.

Introduction

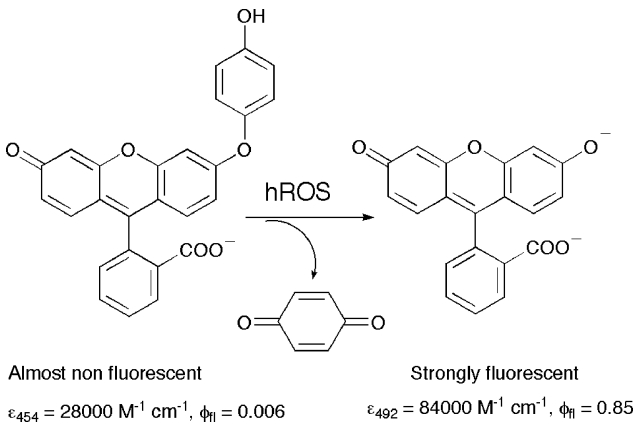
Although molecular oxygen is inert toward living organisms and required for many enzymatic activities of aerobic organisms, it can be reduced to form reactive oxygen species (ROS), such as the superoxide anion ($O_2^{\bullet}$), the hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2) and peroxyl radicals ($ROO^{\bullet}$).[1] Under normal conditions, the production of ROS is relatively weak and well controlled by the antioxidant defenses of the organism.[1] On the other hand, under pathological conditions (such as inflammatory processes, ischemia–reperfusion or cancer), these species are produced at a greatly increased rate, inducing a perturbation in the intracellular oxido–reducing balance, which leads further to cellular and tissular damage.[1–5]

Even if certain targets are more sensitive than others (unsaturated lipids, some amino acids and aromatic compounds), ROS are able to react with a large variety of molecules including proteins, lipids, carbohydrates and nucleic acids.[1] However, as reported in numerous studies, it is believed that different ROS species can have specific roles and reactivities in the organism. For example, whereas $O_2^{\bullet}$ is a good reducing agent characterized by a relatively short lifetime in aqueous medium, in cell membranes its lifetime increases and it is capable of deesterifying some lipids, such as phospholipids.[6]

Therefore, it appeared essential to develop tools to detect selectively the different ROS under *in vivo* and *in vitro* conditions. Among the various detection systems available, fluorescence is the most attractive due to its high sensitivity. For that reason, new fluorescence probes were developed such as 7-(4′-hydroxyphenoxy)coumarin[7] and 2-[6-(4′-hydroxy)phenoxy-3*H*-xanthen-3-on-9-yl]benzoic acid (HPF).[8] From an applications point of view, by reacting with ROS, these initially non-fluorescent probes release a highly fluorescent compound.

Department of Chemistry, University of Ottawa, Ottawa, K1N 6N5, Canada. E-mail: tito@photo.chem.uottawa.ca; Fax: +1 613 562 5633; Tel: +1 613 562 5896

Although HPF is commercially available, its mechanism of reaction is not completely understood. It has been proposed[8] that the interaction between HPF and ROS leads to the formation of benzoquinone and dianionic fluorescein according to the mechanism of Scheme 1.



Scheme 1 Mechanism of HPF action, adapted from the literature.[8]

However, the formation of benzoquinone was not demonstrated, and the mechanism of Scheme 1 does not reveal the true complexity of a mechanism that releases fluorescein. In other words, the simple unimolecular fragmentation of the phenoxyl radical formed following initial hydrogen abstraction (*e.g.*, by peroxyl radicals) is unlikely, but also insufficient to yield the proposed products. While the reported[8] release of fluorescein is not in question, it is clear that a better understanding of mechanistic and kinetic details would be useful as a starting point for the design of new probes.

Therefore, with the aim of designing new fluorescent probes to detect the different ROS, this study was devoted to investigate the mechanism of reaction of the commercially available fluorescent probe HPF with oxygen-centered radicals. For that purpose, different spectroscopic and chromatographic techniques were

employed. Further, 4-phenoxyphenol (4-PPOH) was also used in this study to mimic the behavior of the reactive center in HPF.

Experimental

Materials

2-[6-(4′-Hydroxy)phenoxy-3*H*-xanthen-3-on-9-yl]benzoic acid (3″-(*p*-hydroxyphenyl)fluorescein, HPF) was purchased from Molecular Probes (Canada). 4-Phenoxyphenol (4-PPOH), 2,2′-azobis-(2-methylpropionitrile) (AIBN), 2,2′-azobis-(2-methylpropionamidine) dihydrochloride (AAPH), sodium hydroxide (NaOH), di-*tert*-butyl-peroxide were purchased from Aldrich (Canada). Potassium ferricyanide (KFC) was supplied by Sigma (Canada). Acetonitrile from Omnisolv was HPLC grade.

Fluorescence and absorption measurements

Fluorescence emission spectra were recorded using an LS-50 fluorimeter (Perkin Elmer). The excitation of the HPF probe or its products was set at 490 nm. When necessary, the temperature was maintained at 40 °C using a cuvette holder equipped for circulating thermostated water.

Absorption spectra were recorded using a Cary 50 (Varian) single beam spectrophotometer.

For both measurements, the solutions were placed in a 1 × 1 cm quartz cuvette. Prior to the experiments, the samples were deaerated by bubbling argon through the solution. The oxygenated samples were obtained by maintaining an oxygen atmosphere above the solution. All gases were supplied by Proxair (Canada) with a high purity.

Laser flash photolysis

The phenoxyl radical form (4-PPO$^{\bullet}$) from 4-PPOH was generated by laser excitation of di-*tert*-butyl-peroxide in acetonitrile (50 : 50) using 355 nm pulses from a Continuum Nd-YAG Surelite. The instrument is a customized version of a Luzchem LFP-111 system (Luzchem Research, Ottawa, Canada). All experiments were carried out using static Luzchem cuvettes constructed from 7 × 7 mm fused silica tubing. Samples were deaerated with argon prior to exposure.

Chromatographic analysis

The characterization of the by-product resulting from the interaction of 4-PPOH and the peroxyl radical was performed by HPLC (Waters, model 2690) with UV–vis (Waters, model 996) and mass spectroscopy detection (Waters, Integrity system). Briefly, a solution of 4-PPOH (10^{-3} M) dissolved in acetonitrile in presence of AIBN (10^{-1} M) was kept under oxygen at 40 °C for 3 h in a water bath. The mixture was then injected without any treatment on a C-18 reverse phase column (Zorbax, SB-C18 4.6 × 250 mm, Agilent). An isocratic elution with a mixture of water and acetonitrile (50 : 50) was realized at a flow rate of 0.25 ml min^{-1} for 40 min, followed by a gradient to reach 100% acetonitrile in 20 min. Notably, *p*-benzoquinone (the expected product if 4-PPO$^{\bullet}$ underwent a cleavage analogous to that of Scheme 1), was not detected. All the solvents used as mobile phase were of HPLC grade.

Electron paramagnetic resonance (EPR) experiments

The interaction between HPF and KFC was studied in alkaline aqueous solution (NaOH 0.1 M). For that purpose, HPF (2×10^{-4} M) was added to a strongly oxidizing KFC solution (10^{-1} M), under air. The reactants were mixed at room temperature directly in an EPR tube and immediately after, the mixture was frozen in liquid nitrogen. The measurement was monitored at −160 °C.

The hydrogen abstraction from 4-PPOH (80 mM) was carried out in a mixture of acetonitrile–di-*tert*-butyl peroxide (1 : 1, v : v). The sample was deaerated with argon for 15 min then transferred to an EPR flat cell (JEOL, ES LC 12, 0.3 mm thick) under an inert atmosphere. The cell was then placed into the cavity of the spectrometer and irradiated *in situ* by the means of a xenon illuminator (Luzchem). A cut-off filter ($\lambda > 345$ nm) was placed between the lamp and the sample in order to avoid the direct excitation of 4-PPOH. The EPR signal was recorded at room temperature. All measurements were carried out on a JEOL (FA 100) EPR spectrometer. The different parameters used are reported in the legends of the different figures. The *g*-values were estimated by comparison with the 3rd and the 4th lines of the Mn^{2+} internal marker, calibrated with the standard DPPH radical ($g = 2.0036$).

Results

Although HPF was described as non-reactive towards the peroxyl radical in the literature,[8] we demonstrated the ability of the probe to react with this type of ROS. Indeed, as depicted in Fig. 1, an increase in the fluorescence is observed when HPF (0.8 μM) is in presence of AAPH (10 mM) at 40 °C under O_2. AAPH is a convenient water-soluble source of carbon-centered radicals that react with oxygen readily to yield peroxyl radicals.[9,10]

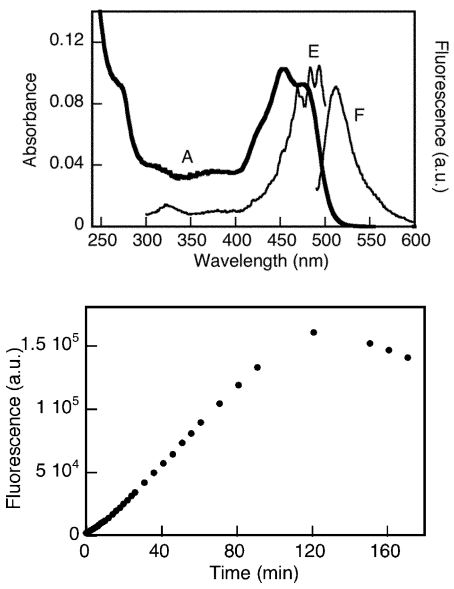
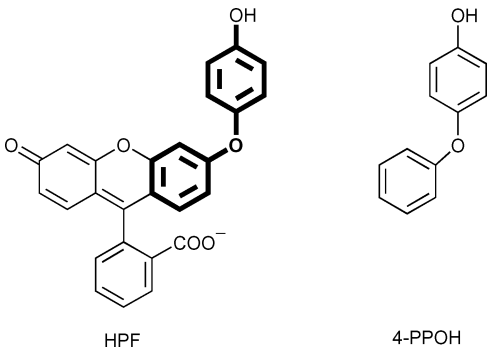


Fig. 1 Bottom: evolution of the maximum fluorescence at 511 nm of a HPF (0.8 μM) solution in phosphate buffer (pH 7.4) in the presence of AAPH (10 mM) under O_2 at 40 °C. Top: absorption (thick, A), the excitation (E) and the fluorescence (F, right axis) of a HPF (1.67 μM) solution in phosphate buffer (pH 7.4).

As shown in Fig. 1, the fluorescence intensity increases until it reaches a plateau and is then followed by a slow decrease, suggesting that the interaction between the fluorescein formed and peroxyl radicals leads to partial degradation. This reaction has already been reported in the literature.[11] The top of Fig. 1 shows the absorption and fluorescence spectra of HPF (1.67 μM) in phosphate buffer at pH 7.4, in the absence of an oxidant. The excitation and the absorption spectra of HPF are quite distinct (even if similar), suggesting that the fluorescence exhibited is not due to the probe itself. By comparison with the absorption and the excitation spectra of a solution of pure fluorescein, the initial fluorescence of HPF was attributed to the presence of a trace amount of free fluorescein in the solution.

In order to have more information about the mechanism governing the release of fluorescein from the HPF probe after interaction with a ROS, we used potassium ferricyanide (KFC) in alkaline solution (NaOH 0.1 M) as an oxidizing agent. Indeed, KFC has already been reported as a potent oxidant agent toward phenol[12] and as shown in Scheme 2, the chemical structure of HPF includes a phenolic moiety.



Scheme 2 The structure of 4-PPOH is shown contained as the key reactive moiety in HPF.

The interaction of HPF (3.3 μM) with KFC (8×10^{-4} M) in the absence of oxygen yields an increase in the fluorescence intensity at 511 nm (Fig. 2) due to the release of fluorescein as confirmed by HPLC with fluorescence detection (data not shown). Quinone products are usually not fluorescent and are not expected to be detectable under these analytical conditions. Moreover, as shown in Fig. 2, the kinetics of fluorescein release are not affected by the presence of oxygen, indicating clearly that the mechanism of the reaction is not oxygen dependent.

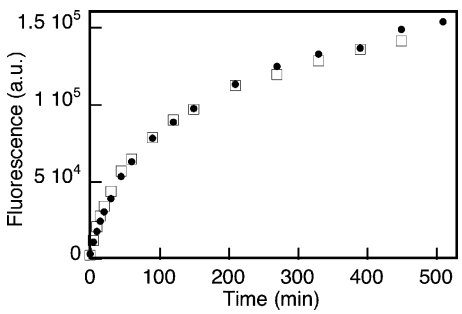


Fig. 2 Evolution of the maximum fluorescence at 511 nm of HPF (3.3 μM) in alkaline medium (NaOH 0.1 M) in the presence of KFC (8×10^{-4} M) under argon (open squares) and under oxygen (closed circles).

On the other hand, addition of ascorbic acid (1 mM) to the solution leads to a decrease in the amount of fluorescein released (Fig. 3). Note that the faster kinetics in that experiment are due to the use of a higher (6 times) concentration of KFC (5 mM). Thus, the latter result suggests that the mechanism of reaction of the probe involves a phenoxyl radical pathway. Indeed, it is well known that ascorbic acid is a potent reducing agent of the phenoxyl radical restoring the phenol.[13] To verify this result, the mode of interaction of KFC (10^{-1} M) and HPF (2×10^{-4} M) was analyzed by EPR spectroscopy. When the KFC and HPF were simultaneously present, a broad EPR signal with a *g*-value of 2.00445 was observed in frozen alkaline (NaOH 0.1 M) solution at −160 °C (Fig. 4). No radical was detected if KFC was absent. The observed radical signal has a *g*-value consistent with that expected for the phenoxyl radical moiety of the HPF probe formed from the interaction between HPF and KFC.[14]

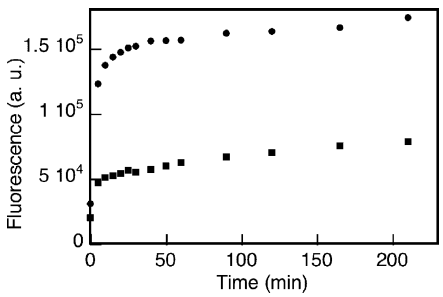


Fig. 3 Evolution of the maximum fluorescence at 511 nm of HPF (3.3 μM) in alkaline medium (NaOH 0.1 M) in presence of KFC (5×10^{-3} M) under aerobic conditions, in the absence (squares) and in the presence (circles) of ascorbic acid (10^{-3} M).

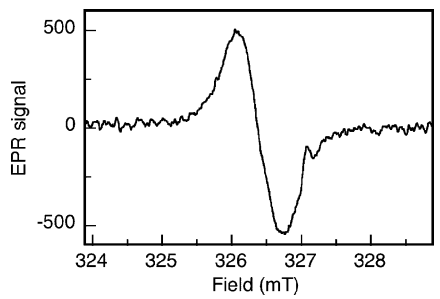


Fig. 4 EPR signal recorded at −160 °C of HPF-derived phenoxyl radical in alkaline medium (NaOH 0.1 M) obtained by the interaction of HPF (2.5×10^{-4} M) with KFC (10^{-1} M) under aerobic conditions. The experimental setting was as follows: microwave power 0.988 mW, center field 326.4 mT, sweep time 2 min, time constant 0.3 s, amplitude 2, number of scans 1. The modulation width used was 0.1 mT.

The use of 4-phenoxylphenol (4-PPOH) is suggested here as a probe mimicking the HPF reactivity at the phenolic position, see Scheme 2. The reactivity of 4-PPOH towards alkoxyl radicals, generated by the 355 nm photolysis of di-*tert*-butyl peroxide ((*t*-BuO)$_2$) in acetonitrile,[15] was evaluated by laser flash photolysis (inset Fig. 5). The rate constant (k_r) for this reaction was estimated from a Stern–Volmer plot of the experimental rate constant (k_{exp}) *vs.* the concentration of 4-PPOH. From it, we obtained $k_r = 5.1 \times 10^7$ M^{-1} s^{-1} (Fig. 5), in line with other substrates in hydrogen bond accepting media.[16] Fig. 6 represents the transient spectrum

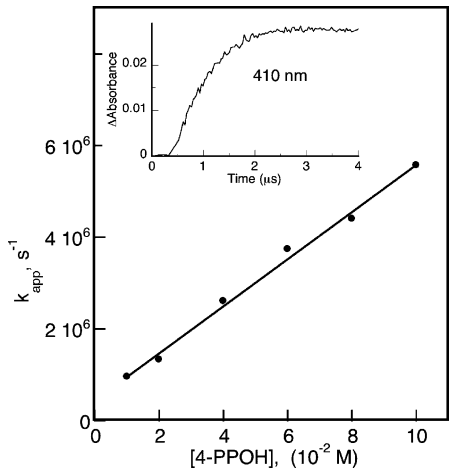


Fig. 5 Quenching data for the hydrogen abstraction from 4-PPOH by *tert*-butoxyl radicals (*t*-BuO$^{\bullet}$) generated by 355 nm laser pulse excitation of di-*tert*-butyl peroxide–acetonitrile (50 : 50) under argon. The inset represents the typical kinetics observed at 410 nm for 4-PPOH (20 mM).

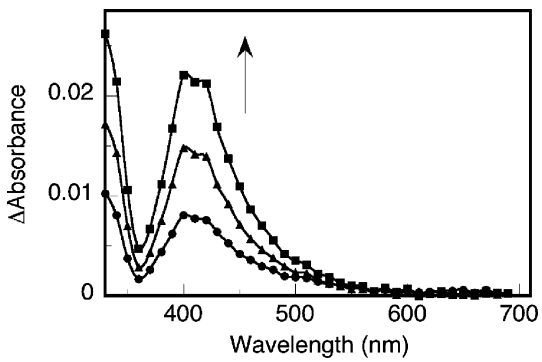


Fig. 6 Transient spectrum obtained after laser pulse excitation (355 nm) of di-*tert*-butyl peroxide in acetonitrile (50 : 50) under argon in the presence of 4-PPOH (20 mM); the delay times between the laser pulse and data recording are 176 ns (●), 402 ns (▲) and 2160 ns (■).

obtained after the hydrogen abstraction from 4-PPOH by the *tert*-butoxy radical (*t*-BuO$^{\bullet}$). This transient species characterized by a positive absorption band centered at 410 nm was attributed to the 4-phenylphenoxyl radical (4-PPO$^{\bullet}$) form of 4-PPOH.

We could not find the transient spectrum of 4-PPO$^{\bullet}$ in the literature. Indeed, the direct photolysis of 4-PPOH yields to a photocleavage of the molecule resulting in the formation of the *p*-hydroxyphenoxyl radical characterized by a maximum absorbance centered at 430 nm[17,18] Thus, to ensure that the transient spectrum obtained in our laser flash photolysis experiments is due to the phenoxyl radical 4-PPO$^{\bullet}$, the interaction between 4-PPOH and *t*-BuO$^{\bullet}$ was analyzed by EPR. As depicted in Fig. 7, a triplet of triplets EPR signal with a *g*-value of 2.00517 and with coupling constants of $a_{meta}(2H) = 0.105 \pm 0.005$ mT and $a_{ortho}(2H) = 0.58 \pm 0.05$ mT was observed. This signal is assigned to the phenoxyl radical 4-PPO$^{\bullet}$. Indeed, the EPR signal of the *p*-hydroxyphenoxyl radical obtained in the same conditions by abstraction of the hydrogen from the hydroquinone does not correspond to a spectrum showing the characteristic splitting of a triplet of triplets (data not shown), as that shown in Fig. 7. This result is in agreement with the data found in the literature about the

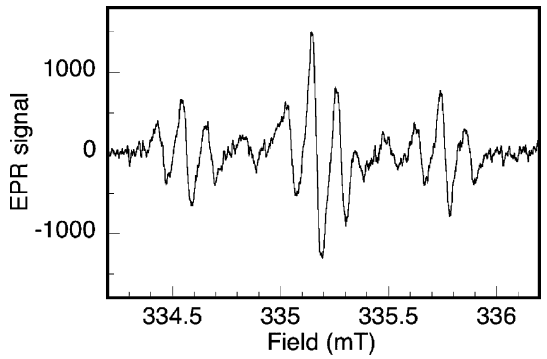


Fig. 7 EPR signal at room temperature of the 4-PPOH-derived phenoxyl radical (4-PPO$^{\bullet}$) obtained by the continuous irradiation ($\lambda > 345$ nm) of di-*tert*-butyl peroxide in acetonitrile (50 : 50) under argon in the presence of 4-PPOH (80 mM). The experimental settings were as follows: microwave power 5 mW, center field 335.2 mT, sweep time 30 sec, time constant 0.03, amplitude 5, number of scans 10. The modulation width used was 0.2 mT.

p-hydroxyphenoxyl radical.[19,20] Therefore, the transient spectrum observed in our laser flash photolysis (Fig. 6) and that observed by EPR (Fig. 7) correspond to the phenoxyl radical 4-PPO$^{\bullet}$. The extinction coefficient of 4-PPO$^{\bullet}$ was evaluated to 3730 M^{-1} cm^{-1} at 410 nm by comparison to the extinction coefficient of phenoxyl radical, according the method described by Das *et al.*[16] The decay of 4-PPO$^{\bullet}$ formed following hydrogen abstraction from 4-PPOH by *t*-BuO$^{\bullet}$ follows second order kinetics (data not shown), suggesting the dimerization of the radicals. The dimerization rate constant (k_{dim}) was estimated to be *ca.* 4×10^9 M^{-1} s^{-1}.

The interaction between 4-PPOH and the peroxyl radical formed by the thermal decomposition of AIBN in acetonitrile under O_2 at 40 °C was evaluated by HPLC with UV–vis and mass spectroscopy detection. As shown in Fig. 8, the HPLC chromatogram of a 4-PPOH (1 mM) solution heated at 40 °C during 3 hours in presence of AIBN (100 mM) and oxygen gives rise to the appearance of 4 different peaks. AIBN decomposes readily at this temperature with an activation energy of 31.5 kcal mol^{-1}.[21] By comparison to authentic samples, the two first peaks ($R_t = 18.01$ min and 26.81 min, respectively) were attributed to unreacted AIBN. The first one ($R_t = 18.01$ min) is probably due to a trace impurity present in AIBN because it is not detected at lower concentrations whereas the second one ($R_t = 26.81$ min)

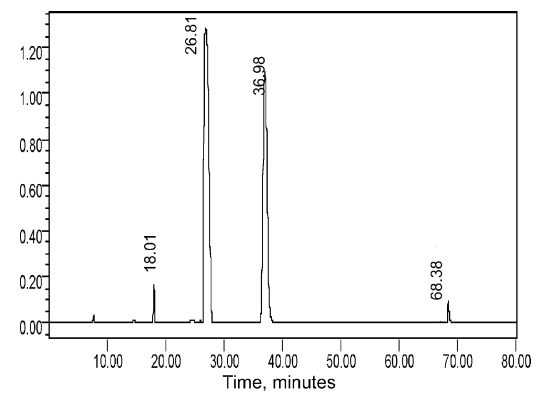


Fig. 8 HPLC chromatographic analysis at room temperature of an oxygenated 4-PPOH (1 mM) solution dissolved in acetonitrile in the presence of AIBN (100 mM) heated for 3 h at 40 °C.

corresponds to AIBN itself. The third peak with a retention time of 36.98 min corresponds to 4-PPOH. The last peak ($R_t = 68.38$ min) was assigned to a by-product of the reaction of 4-PPOH with the peroxyl radical. Indeed, its formation is dependent on the simultaneous presence of 4-PPOH, AIBN, oxygen and heat. By analysis of its mass spectrum (data not shown), this compound was assigned to a dimer form of 4-PPOH.

Discussion

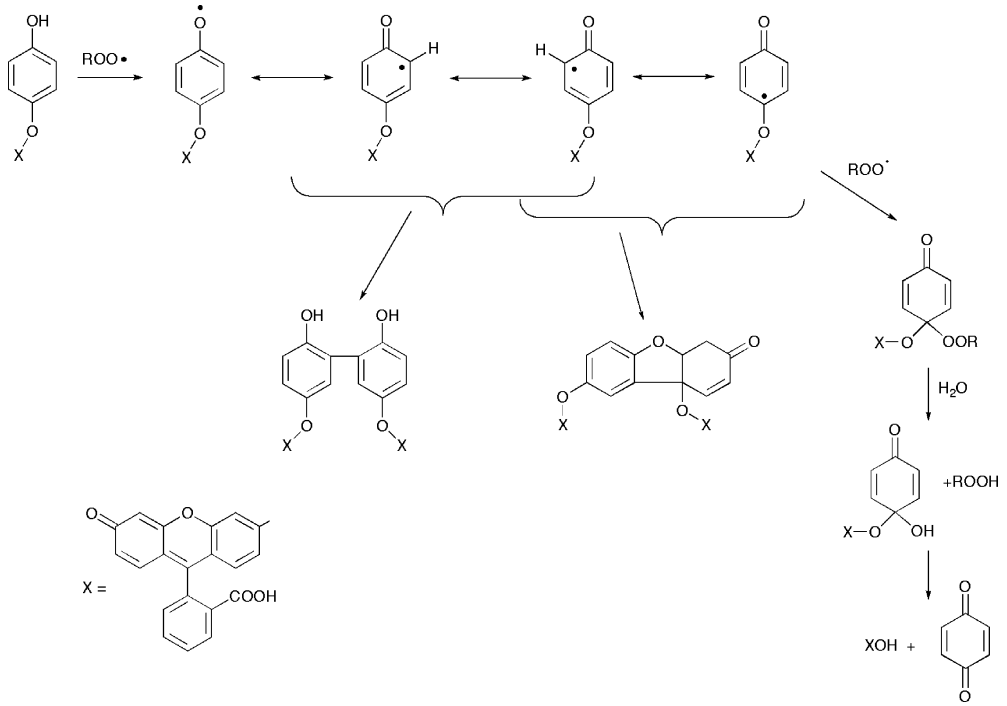
This paper reports new information regarding the mechanism of the reaction of the commercial fluorescent probe HPF in the presence of oxidants, especially oxygen-centered radicals. The reaction of the probe is shown to be initiated by oxidation of the phenol moiety of the probe resulting in the formation of a phenoxyl radical as demonstrated by the EPR results. Note that the *g*-value obtained for this phenoxyl radical is in the same order as the one reported in the literature.[14,22] The reaction triggers the release of fluorescein as shown by HPLC with fluorescence detection, and was reported to be independent of the presence of oxygen. However, hydrogen abstraction from the phenoxyl position by itself is not sufficient to lead to the release of fluorescein in solution. In order to explore that part of the mechanism, 4-PPOH was used to mimic the behavior of HPF.

We have shown that the interaction between 4-PPOH with alkoxyl radicals leads to the formation of the corresponding phenoxyl radical (4-PPO$^{\bullet}$), characterized by an absorption spectrum centered at 410 nm, and an EPR spectrum with a *g*-value of 2.00517. Although this *g*-value seems to be slightly high for this kind of radical, it remains close to those found for certain *para*-substituted phenoxyl radicals.[22] Moreover, it should be noted that this latter value was obtained in a very particular solvent (di-*tert*-butyl peroxide–acetonitrile; 1 : 1, v : v), making the comparison with the values found for the phenoxyl radical in water difficult. On the other hand, the coupling constants obtained by computer simulation ($a_{meta}(2H) = 0.105 \pm 0.005$ mT and $a_{ortho}(2H) = 0.58 \pm 0.05$ mT) are consistent with those expected for a phenoxyl radical moiety.[14]

As evidenced by laser flash photolysis, the 4-PPO$^{\bullet}$ radical decay follows second order kinetics indicating that radical–radical reactions are the dominant decay mode. The rate constant for this reaction was found in the same order as that for the phenol and phenol-substituted radicals.[23] Thus, this result suggests the possible formation of a dimer as a by-product. Indeed, as reported in this study, the interaction of 4-PPOH with the peroxyl radical in acetonitrile leads to dimer formation.

Therefore, by extrapolating these results to the fluorescent probe HPF, we propose that the phenoxyl radical form of HPF formed by interaction with an oxidizing agent can undergo self-reaction, or reaction with other radicals (particularly ROO$^{\bullet}$) leading to the formation of radical coupling products from which the release of the fluorescein can take place, probably by reaction with water, according to the mechanism of Scheme 3. This scheme is consistent with prior knowledge of the radical–radical reactions of phenoxyls, including self reaction and the possible reaction with peroxyl radicals.[12] Moreover, the hypothesis of the release of the fluorescein moiety by a hydrolysis reaction is not unprecedented. Indeed, this reaction is similar to the demethylation described in the literature for 3-*tert*-butyl-4-methylphenol and 2,5-di-*tert*-butyl-4-methylphenol.[12]

The results presented here are relevant from a biological point of view. Indeed, if the release of the fluorescein from the HPF probe results from radical–radical coupling products, the efficiency of the probe in a biological environment may be compromised. It is well known that the diffusion of radicals species is very limited *in vivo*, making the self-association of the phenoxyl radical form of



Scheme 3 Mechanism of the reaction proposed.

HPF highly improbable. Therefore, the release of the fluorescein moiety from the HPF probe in a biological environment should be mainly initiated by the association of the phenoxyl radical form of HPF and other radicals. This hypothesis is in agreement with the literature where the self-association of phenoxyl radicals has been described as predominant only at high concentration while the coupling with other radicals, such as peroxyl, takes place in the other cases.[12]

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