Comment

Comment on “Water-Soluble Fluorescent Probe with Dual Mitochondria/Lysosome Targetability for Selective Superoxide Detection in Live Cells and in Zebrafish Embryos”

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Comment on "Water-Soluble Fluorescent Probe with Dual Mitochondria/Lysosome Targetability for Selective Superoxide Detection in Live Cells and in Zebrafish Embryos"

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ABSTRACT: Recently, a new water-soluble, fluorescein-based probe for the detection of superoxide radical anion in aqueous media was developed by Lu et al. (*ACS Sens.* **2018**, 3, 59-64). The probe was proven to be selective for superoxide and was used successfully also in cells and zebrafish embryos. To characterize the response of the probe to superoxide, Lu et al. used KO_2 dissolved in deionized water as surrogate. In testing this probe in different applications we repeated some of these experiments and came to realize that the fluorescence signal observed by the Authors in their experiments with KO_2 was incorrectly attributed to reaction of the probe with superoxide and is due instead to its reactions with HO^- and HO_2^-. We show that indeed under the conditions used in these assays KO_2 undergoes very fast reaction with water to form HO^- and HO_2^-. On the other hand, by using a proper surrogate, namely KO_2 dissolved in DMSO, and spin trapping experiments, we confirmed the ability of the probe to detect superoxide.

Recently, X. Lu et al. have reported the synthesis and characterization of a new water-soluble fluorescent probe for the selective detection of superoxide ($O_2^{-\cdot}$) in aqueous media.[1] The probe is a fluorescein derivative in which the two phenolic hydroxyls have been converted into N-methylpyridinium-3-yl sulfonate groups (Scheme S1). Thanks to these charged substituents the probe is highly soluble in water,[1] in contrast with the majority of related fluorescein-based probes reported in the literature.[2–11] It is stable within a wide pH range (1 - 9) and highly selective towards superoxide, with no significant interference by most oxidants and reductants of interest in biological systems. The probe was also successfully tested in cells and live zebrafish embryos.[1] In keeping with the general consensus on the reactivity of related probes,[3] the proposed mechanism involves nucleophilic attack by superoxide on sulfur and a one (probe) to two (superoxide) stoichiometry for the release of fluorescein.[1]

We are interested in applying this probe to determine superoxide in advanced oxidation processes induced by air non thermal plasma.[12–17] In repeating some of the experiments used by Lu et al.[1] to characterize the probe's behavior, we realized that the procedure they applied to perform experiments with KO_2 as a surrogate for superoxide is not appropriate and led them to misinterpret their results. The present comment addresses this issue and provides and discusses experimental evidence of interest for users of this and all related probes for superoxide.

Use of KO_2 as a surrogate for superoxide, is commonly adopted for testing a probe's ability to react with superoxide.[2,3,7–11,18] Such tests are usually carried out by adding the desired amount of KO_2 either as pure reagent (solid) or dissolved in an aprotic solvent. Indeed, superoxide is stable in aprotic solvents like, for example, DMSO, as is appreciated by the color of the solution. KO_2 is a pale yellow salt. When dissolved in DMSO, the solution maintains the yellowish color (Fig. S1). Lu et al. instead prepared stock solutions of KO_2 in deionized water (1 mM) and added aliquots of these solutions to the probe containing medium.[1] The problem with this procedure is that, upon dissolution in water, KO_2 reacts vigorously to give oxygen, hydrogen peroxide and hydroxide via a disproportionation reaction which can be described by equations (1) and (2).[19,20]

$$O_2^{\cdot -} + H_2O \leftrightarrow HO_2^{\cdot} + OH^- \qquad K_a(HO_2^{\cdot}) = 1.6\cdot10^{-5}\ \text{mol L}^{-1} \tag{1}$$

$$O_2^{\cdot -} + HO_2^{\cdot} + H_2O \rightarrow O_2 + H_2O_2 + OH^- \qquad k_{obs} = 9.7\cdot10^{7}\ \text{mol}^{-1}\ \text{s}^{-1} \tag{2}$$

Hydrogen peroxide, in turn, decomposes more slowly via its conjugate base (the pK_a of hydrogen peroxide is 11.6) to give oxygen and hydroxide (eq. 3). The rate of this reaction is extremely sensitive to experimental variables, including the material of the reaction vessel and trace amounts of impurities.[21]

$$HO_2^- \rightarrow \frac{1}{2}O_2 + OH^- \tag{3}$$

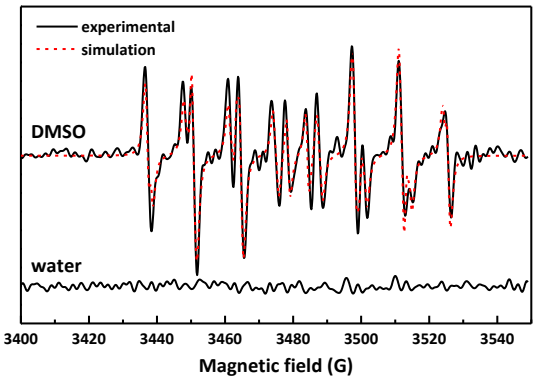

Figure 1. Cw-EPR spectra of a 10 mM solution of DEPMPO in deionized water (450 μL) recorded immediately after addition of 50 μL of freshly prepared 10 mM solutions of KO_2 in DMSO or deionized water. The red dashed line is the simulated spectrum, obtained as sum of two species. Species 1: a_N = 13.14 G, a_{H1} = 10.82 G, a_{H2} = 0.91 G, a_P = 50.05 G; Species 2: a_N = 14.10 G, a_H = 13.51 G, a_P = 47.23 G.

Indeed, when KO_2 is added to water, oxygen is immediately released and the resulting solution is colorless. It is therefore expected that during the time required to prepare the stock solution and to add aliquots thereof to the probe containing media, all superoxide should have decomposed according to reaction (2) releasing in solution HO^- and H_2O_2. Thus, the procedure adopted by Lu et al.[1] for using KO_2 as surrogate for superoxide appeared not to be appropriate and prompted the investigation reported here.

To prove unequivocally that when KO_2 is dissolved in water superoxide cannot survive for a time long enough to be transferred and used other processes we performed spin-trapping experiments and EPR analyses. We used DEPMPO, a well-known spin trap for superoxide, and treated it (450 μL aliquots of a 10 mM solution in deionized water) in parallel experiments with solutions of KO_2, (50 μL of a 10 mM solution) prepared immediately before use, one in deionized water, the other in DMSO. The EPR spectra recorded immediately after addition of the KO_2 solution are shown in Figure 1. The spectra show that no radical spin-adduct between DEPMPO and superoxide was formed when the aqueous KO_2 solution was used. In contrast, we detected the typical signals of the DEPMPO-OOH adducts when the solution of KO_2 in DMSO was used. The simulation of the spectrum formed in DMSO with a proper simulation software[22] and the comparison of the hyperfine coupling constants with those reported in the literature[23,24] confirm the formation of the DEPMPO-OOH adducts. These results prove that when KO_2 is preliminarily dissolved in water and next added to the probe solution, superoxide does not survive long enough to be detected by reaction with the probe. The fluorescence signals observed by Lu et al.[1] in their experiments with aqueous KO_2 are thus to be ascribed to reaction of the probe with species derived from superoxide, namely HO^- and HO_2^-, formed as shown in equations (2) and (3). We then

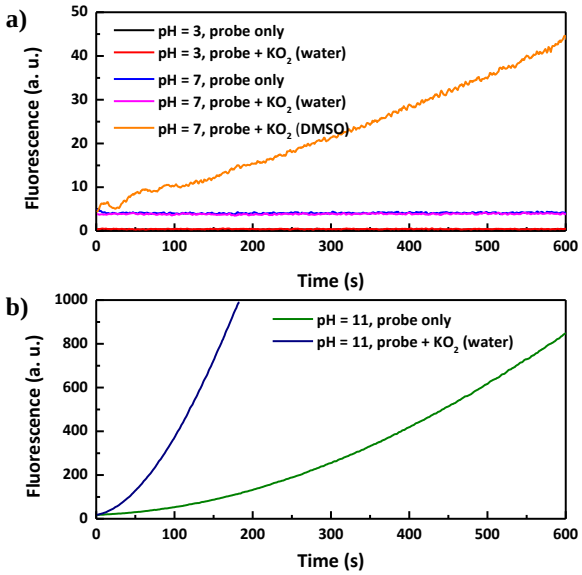

Figure 2. Time course fluorescence response of the probe (10 μM, final concentration) in phosphate buffer (200 mM) with and without addition of aqueous KO_2 (50 μM, final concentration). (a) in solution at pH 3 and 7; (b) in solution at pH 11. In panel (a) the time course fluorescence in buffered solution at pH 7 with addition of KO_2 dissolved in DMSO (100 μM, final concentration) is also reported for comparison. See SI for details.

further investigated the source of the fluorescence emission in these experiments.

The stability of the probe as a function of the solution pH was investigated by Lu et al. within the range 1-12.[1] We confirmed their results by measuring the time course fluorescence response of the probe (10 μM) in buffered aqueous solutions (200 mM phosphate buffer) at pH 3, 7 and 11 (Fig. 2). The results show that the probe is very stable in acidic solutions, that it decomposes slowly at pH 7 (the fluorescein signal increases with a doubling time of about 3 hours, Fig. S2), and that under basic conditions it reacts rapidly with HO^- anions to give fluorescein, with a doubling time of about 4 min. This poor stability in alkaline solutions explains the dependence of the fluorescence intensity on the amount of KO_2 reported by Lu et al. in figure 1 of their paper.[1] Their data are based on experiments in which the desired amount of KO_2 was transferred to the probe solution by adding proper aliquots of a 1 mM KO_2 stock solution in deionized water. By this procedure, however, the probe was not exposed to superoxide but rather to HO^- and HOO^-, generated upon reaction of superoxide with water. Specifically, the solution becomes basic and the probe releases fluorescein via base-induced hydrolysis and by reaction with hydroperoxide ion (vide infra). Indeed, it is expected from reactions (2) and (3) that the pH of aqueous KO_2 solutions should depend on the amount of KO_2 dissolved. This expectation was verified by measuring the pH of aqueous solutions obtained by dissolving various amounts of KO_2 in deionized water. A linear relationship was obtained between the HO^- concentration measured in solution and the amount of KO_2 dissolved (Fig. S3).

Next we tested the effect of additions of KO_2 on the fluorescence response by the probe in aqueous solutions buffered at the same pH values as used in the experiments described above (3, 7 and 11). The concentrations of probe and KO_2 in these experiments were 10 μM and 50 μM respectively. The results are reported in Figure 2. They can be usefully compared with those, also shown in the same figure, obtained under the same pH conditions but in the absence of KO_2. It is seen that addition of 5 equivalents of aqueous KO_2 did not produce any significant effect on the fluorescence observed under acidic or neutral pH. In contrast, at pH 11, the production of fluorescein was significantly faster when aqueous KO_2 was added to the probe solution. Considering that the concentration of HO^- was not affected because the solution pH was well buffered, the stronger fluorescence observed in the experiment with KO_2 indicates that fluorescein was produced also by reaction of the probe with HO_2^-, produced alongside with HO^- as shown in equation (2).

To confirm the response of the probe to superoxide we repeated the experiment at pH 7 by adding KO_2 dissolved in DMSO instead of water (100 μM final concentration). In this experiment an increase of the fluorescence signal in time was clearly evident (Fig. 2a, orange trace) which cannot be due to HO^-, since the pH was buffered, nor to HO_2^-, because, at pH 7, hydroperoxide is protonated (the pK_a of hydrogen peroxide is 11.6). Thus, we conclude that the species that reacts with the probe under these conditions is indeed superoxide. This experiment confirms the ability of the probe to detect selectively superoxide in non-alkaline aqueous media.

Last, we report and discuss the fluorescence emission measured when the probe (10 μM) was treated in various experiments with KO_2, HO^- and HO_2^- in deionized water (Fig. 3). Notably, we could reproduce quite well the time course signal obtained in the experiment with aqueous KO_2 (100 μM final concentration, black line), by that observed in an analogous experiment in which the probe was exposed to H_2O_2 and KOH (50 μM each, green line). These experiments confirm that the probe response to additions of KO_2 dissolved in aqueous solution is to be attributed to its reactions with both HO^- and HO_2^- which are formed by reactions (2) and (3). Reaction of the probe with HO_2^- appears to be important, since the fluorescence signal obtained in experiments run with only HO^- (either 50 μM or 100 μM, purple and blue lines, respectively) was appreciably lower.

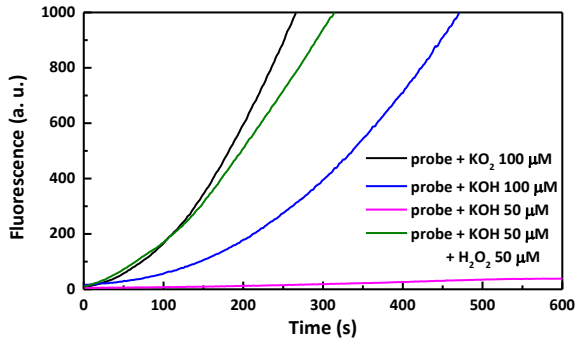

Figure 3. Time course fluorescence response of the probe in deionized water (10 μM) in experiments in which the following reagents were added, all previously freshly dissolved in deionized water: KO_2 (100 μM, final concentration, black line); KOH (100 μM, final concentration, blue line); KOH (50 μM, final concentration, purple line); KOH plus H_2O_2 (50 μM each, final concentration, green line). See SI for details.

In summary with this comment we provide an explanation of the results published by Lu et al.[1] which is consistent with a very fast reaction of KO_2 in water to produce HO^- and HO_2^-. We prove that the fluorescence signal observed by the Authors in their experiments with aqueous KO_2, incorrectly attributed to reaction of the probe with superoxide, is due instead to its reactions with HO^- and HO_2^-. The comment will hopefully contribute to avoid improper use of KO_2 aqueous solutions as a surrogate for superoxide, which appears to be a spreading malpractice. We also successfully tested the response and selectivity of the probe to superoxide by using as surrogate a KO_2 solution in DMSO. These results confirm the great potential of the probe developed by Lu et al.[1] in sensing superoxide produced in water-based solutions and in biological media.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at xxxxxx.

Experimental Section: materials, synthesis of the probe, spin-trapping experiments, time course fluorescence experiments, pH measurements.

Chemical structure of the probe. KO_2 solutions in water and in DMSO. Stability of the probe in water solution buffered at neutral pH. Generation of HO^- ions in water solution by dissolution of solid KO_2

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Notes

The authors declare no competing financial interests.

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