

## Production of the free radicals $O_2^-$ and $\cdot OH$ by irradiation of the photosensitizer zinc(II) phthalocyanine

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Received 31 May 1996; accepted 12 August 1996

### Abstract

Zinc(II) phthalocyanine (ZnPC) is a new photosensitizer currently undergoing phase I and II clinical trials at Lausanne's CHUV hospital for the photodynamic therapy (PDT) of early cancer in the upper aerodigestive tract.

Activated oxygen species other than singlet oxygen produced during the photosensitization of ZnPC in liposomes have been examined by electron paramagnetic resonance (EPR) spin trapping and by the cytochrome *c* reduction method. Visible light irradiation of ZnPC associated with liposomes in the presence of the spin trap 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) gives an EPR spectrum characteristic of the DMPO-hydroperoxyl radical spin adduct (DMPO- $\cdot OOH$ ). Superoxide anion attains a level of  $1 \mu M \text{ min}^{-1}$  20 min after the start of irradiation as determined by the superoxide dismutase (SOD)-inhibitable reduction of cytochrome *c*. The yield of  $O_2^-$  is strongly enhanced by physiological electron donors. An EPR spectrum characteristic of the DMPO-hydroxyl radical spin adduct (DMPO- $\cdot OH$ ) is also observed. The addition of dimethyl sulphoxide or ethanol produces additional hyperfine splittings due to the respective hydroxyalkyl radical products, indicating the presence of free  $\cdot OH$ . DMPO- $\cdot OH$  is significantly inhibited by desferrioxamine or catalase. Conversely, this adduct is enhanced by hydrogen peroxide.

These data demonstrate the ability of ZnPC in liposomes to photoreact effectively by an electron transfer mechanism. Such type I processes may add to the effects of singlet oxygen in ZnPC-mediated PDT. © 1997 Elsevier Science S.A.

**Keywords:** Cytochrome *c*; Electron paramagnetic resonance; Hydroxyl radical; Liposome; Photodynamic therapy (PDT); Photosensitization; Superoxide anion radical; ZnPC

### 1. Introduction

Photodynamic therapy (PDT), which involves the use of photosensitizing chemicals combined with light, is a rapidly expanding modality for cancer treatment [1–3]. Currently, in our research for new PDT agents, we have focused our attention on zinc(II) phthalocyanine (ZnPC) (Fig. 1). Indeed, ZnPC in liposomes, developed by Ciba Geigy [4], is being tested in phase I and II clinical trials at Lausanne's CHUV hospital (ENT Department) for the PDT of early cancer in the upper aerodigestive tract, the tracheobronchial tree and the oesophagus.

With regard to the phototherapeutic potential of this molecule, it was thought to be of interest to determine the photosensitization mechanisms which may be involved. Although a photodynamic process has been demonstrated to

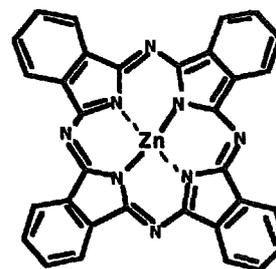


Fig. 1. Structural formula of ZnPC.

play an important role in the photobiological activity of ZnPC, the mechanism of this process (type I and/or type II) is still in question. It is possible that the photodynamic activity of ZnPC occurs via a type II mechanism in which singlet oxygen is produced. Indeed, in previous work, Rosenthal et al. [5] have shown that photoactivated ZnPC generates singlet oxygen in phosphate-buffered saline solution with a good quantum yield ( $\Phi_{\Delta} = 0.4$ ). The involvement of  $^1O_2$  has also

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been probed in ethanol and in liposomes, indirectly using 1,3-diphenylisobenzofuran (DPBF) and directly by recording the  $^1\text{O}_2$  IR phosphorescence [6]. Nevertheless, Firey et al. [7] failed to detect  $^1\text{O}_2$  IR luminescence with ZnPC in cellular media following flash illumination, but observed ZnPC triplet states. Moreover, different workers have suggested that singlet oxygen plays no major role in the photodynamic activity of phthalocyanines [5,8,9]. In order to contribute to the advancement of our knowledge of the photochemistry of ZnPC, we have examined the production of activated oxygen species other than singlet oxygen during ZnPC-mediated photosensitization. We have employed electron paramagnetic resonance (EPR) as well as spectrophotometric methods. EPR spin trapping is one of the most powerful analytical methods available for investigating the production of superoxide anion radicals ( $\text{O}_2^-$ ) and hydroxyl radicals ( $\cdot\text{OH}$ ) in photochemical systems [10–14]. Moreover, to quantify the formation of  $\text{O}_2^-$  during ZnPC–liposome-mediated photosensitization, we used the cytochrome *c* reduction method.

## 2. Materials and methods

### 2.1. Chemicals

1,4-Diazabicyclo[2,2,2]octane (DABCO), 5,5-dimethyl-1-pyrroline-1-oxide (DMPO), glutathione (GSH), reduced cysteine (CysH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), superoxide dismutase (SOD), catalase, histidine, cytochrome *c* and phosphate-buffered saline (PBS) were purchased from Sigma Company and desferrioxamine from Ciba-Geigy. Deuterated water (99.9% pure deuterium) was purchased from Aldrich Chemicals and was used immediately after opening. DMPO from the same source was purified before use via the procedure described by Buettner and Oberley [15]. The photosensitizer ZnPC in liposomes was a gift from Ciba Geigy, Basle, Switzerland.

### 2.2. The liposomal formulation of ZnPC

The liposomal ZnPC formulation CGP 55847, developed by Ciba Geigy [4], was prepared from a mixture of the phospholipids 1-palmitoyl-2-oleoyl-*syn*-glycero-3-phosphocholine and 1,2-dioleoyl-*syn*-glycero-3-phospho-L-serine in a ratio of 9:1. The dye to lipid ratio was 1:100. The manufacturing method for this dosage form is basically a controlled solvent dilution method reproducibly yielding small liposomes of about 70–100 nm in diameter in which ZnPC is completely incorporated as the monomer [4]. The liposomal ZnPC formulation CGP 55847 contains 0.4 mg ZnPC and 40 mg phospholipid in each vial and is freeze dried for storage. The working solutions were prepared immediately before use.

### 2.3. EPR studies

The EPR spectra were obtained using an aqueous flat cell at 9.4 GHz in a Bruker ECS 106 spectrometer at room temperature (22–24 °C). Unless otherwise indicated, the following instrumental settings were employed: microwave power, 20 mW; modulation amplitude, 0.63 G; time constant, 0.2 s; scan time, 4 min; scan width, 150 G. Photoinduced EPR spectra were obtained from samples (150  $\mu\text{l}$ ) injected into quartz capillaries designed especially for EPR analysis. In all of these experiments, solutions were irradiated outside the cavity in cuvettes with a Storz lamp (Karl Storz, Germany) at an intensity of 20 mW  $\text{cm}^{-2}$ . A long pass filter removed the emission wavelengths shorter than 610 nm. The samples were immediately transferred to quartz capillaries after exposure and the EPR spectrum was recorded within 4 min. Anaerobic samples were prepared in cuvettes which allowed the purging of the reactive volume with purified  $\text{N}_2$  for 30 min in the dark.

### 2.4. Measurement of superoxide anions by the cytochrome *c* method

The absorbance of cytochrome *c* was monitored at 550 nm using a Uvikon 860 spectrophotometer (Kontron Instruments, Zurich, Switzerland) and was recorded at 5 min intervals following a method described previously [16]. The molar extinction coefficient was  $0.89 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  for ferricytochrome *c* (Cyt.  $\text{Fe}^{3+}$ ) and  $2.99 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  for ferrocyanochrome *c* (Cyt.  $\text{Fe}^{2+}$ ) at 550 nm, as described previously [17,18]. Anaerobic samples were prepared in cuvettes which allowed the purging of the reaction volume with purified  $\text{N}_2$ . All data shown are the mean values of measurements repeated at least three times.

## 3. Results and discussion

### 3.1. Formation of superoxide anion radical ( $\text{O}_2^-$ ) by ZnPC

#### 3.1.1. Production of DMPO– $\text{O}_2^-$ adduct

To assess the possible contribution of a type I mechanism in the photosensitization process of ZnPC–liposomes, the resulting generation of superoxide anion radical ( $\text{O}_2^-$ ) and/or hydroxyl radical ( $\cdot\text{OH}$ ) was investigated. Recently, we have successfully applied EPR spectroscopy using the spin trap DMPO to characterize superoxide and hydroxyl radicals formed during photochemical reactions in homogeneous solutions as well as in liposomes [10–14]. Although the methodology is straightforward, the approach has some limitations. Indeed, in aqueous solution at pH 7, the rate constant of DMPO with  $\text{O}_2^-$  is very low ( $10 \text{ M}^{-1} \text{ s}^{-1}$ ) in comparison with that of  $\cdot\text{OH}$  ( $2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ ) [19]. However, previous reports have clearly shown that, at pH 5, the hydroperoxyl radical ( $\text{HO}_2$ ) forms the DMPO–OOH adduct, i.e. the

DMPO-trapped hydroperoxyl radical, at a higher rate:  $6.6 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$  [20]. Since superoxide- and peroxy-DMPO adducts have similar hyperfine splittings [21], a buffered solution at pH 4.65 was used to test for the presence of superoxide.

Irradiation of ZnPC-treated liposomes with visible light in the presence of DMPO and desferrioxamine produces an EPR signal with hyperfine splittings ( $a_N = 14.2 \text{ G}$ ,  $a_H^\beta = 11.2 \text{ G}$  and  $a_H^\alpha = 1.25 \text{ G}$ ) characteristic of the hydroperoxyl radical adduct of DMPO (DMPO- $\cdot\text{OOH}$ ) (Fig. 2(B)). The DMPO- $\cdot\text{OOH}$  adduct is unstable in aqueous solution, especially in the presence of transition metals, and decomposes to give many products of which DMPO- $\cdot\text{OH}$  is presumably only a fraction [15,19]. Desferrioxamine was included to chelate any residual adventitious iron. Moreover, a significant effort was made to eliminate the metallic impurities present in buffers and commercial preparations. Control experiments showed that no signal was obtained without light or oxygen, indicating that the formation of the radical intermediates leading to DMPO- $\cdot\text{OOH}$  is a photodynamic process (Fig. 2(A)). When the experiment was prolonged, a significant decrease in the intensity of the DMPO- $\cdot\text{OOH}$  adduct occurred as expected from its short half-life. The addition of SOD ( $45 \mu\text{g ml}^{-1}$ ) prior to illumination prevents the formation of the DMPO- $\cdot\text{OOH}$  adduct (Fig. 2(C)), whereas thermally denatured SOD has no effect on the EPR spectrum. This lack of effect of denatured SOD provides proof for the involvement of  $\text{O}_2^{\cdot-}$  in the formation of the DMPO- $\cdot\text{OOH}$  adduct during the ZnPC photosensitization process.

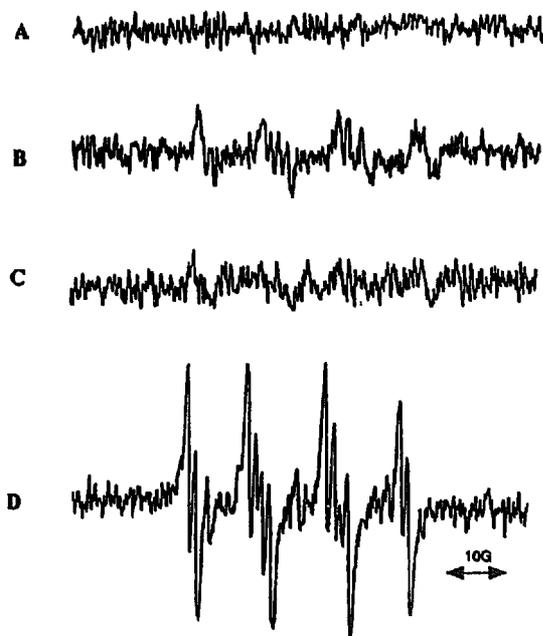


Fig. 2. EPR spectra of the DMPO- $\text{O}_2^{\cdot-}$  adduct in aqueous solution (pH 4.65) containing ZnPC-liposomes and DMPO (20 mM): (A) with ZnPC ( $1 \mu\text{M}$ ), except that light or oxygen or ZnPC was omitted; (B) with ZnPC ( $1 \mu\text{M}$ ) after 3 min of irradiation ( $\lambda > 610 \text{ nm}$ ,  $20 \text{ mW cm}^{-2}$ ); (C) same as (B), except that SOD was added ( $45 \mu\text{g ml}^{-1}$ ); (D) same as (B), except that GSH was added (3 mM). Gain,  $5.0 \times 10^5$ ; modulation amplitude, 0.63 G.

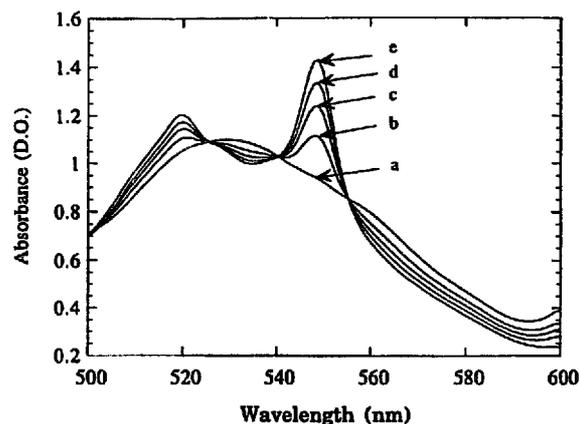


Fig. 3. Absorption spectra of an aqueous solution (pH 7.0) containing ZnPC-liposomes ( $1 \mu\text{M}$ ) and ferricytochrome *c* ( $43 \mu\text{M}$ ): (a) before continuous irradiation ( $\lambda > 610 \text{ nm}$ ,  $20 \text{ mW cm}^{-2}$ ); (b) after 5 min of continuous irradiation; (c) after 10 min of continuous irradiation; (d) after 15 min of continuous irradiation; (e) after 20 min of continuous irradiation. In the presence of SOD ( $45 \mu\text{g ml}^{-1}$ ), spectrum (a) was observed after 20 min of continuous irradiation.

### 3.1.2. Reduction of cytochrome *c*

Although the spin trapping method is at least 20 times more sensitive than the reduction of cytochrome *c* for the measurement of superoxide anions [22], the cytochrome *c* method appears to be more applicable for the quantitative analysis of  $\text{O}_2^{\cdot-}$  relative to DMPO spin trapping [22]. Fig. 3 shows the visible absorption spectrum of cytochrome *c* produced during irradiation of ZnPC in liposomes with visible light in the presence of cytochrome *c*. The increase in absorbance at 550 nm is characteristic of the reduction of cytochrome *c* (Cyt.  $\text{Fe}^{3+}$ ) [17,18]. Control experiments indicated that ZnPC, oxygen and light are essential for the reduction of cytochrome *c*. In the presence of  $45 \mu\text{g ml}^{-1}$  active SOD, under the same conditions of irradiation, no alteration of the initial spectrum was observed (Fig. 3). The rate of reduction of cytochrome *c* was unchanged after the addition of  $50 \mu\text{g ml}^{-1}$  catalase, ruling out hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) as a significant factor.

Superoxide anion radicals may be quantified by determining the amount of reduced cytochrome *c* (Cyt.  $\text{Fe}^{2+}$ ) produced by  $\text{O}_2^{\cdot-}$  that is inhibitable by SOD [16–18]. Hence the  $\text{O}_2^{\cdot-}$  concentration photoproduced by ZnPC was expressed as the amount of SOD-inhibitable reduced cytochrome *c* (Cyt.  $\text{Fe}^{2+}$ ) as a function of the illumination time (Fig. 4). The rate of  $\text{O}_2^{\cdot-}$  generation increases with increasing time of irradiation and the yield of  $\text{O}_2^{\cdot-}$  20 min after the start of irradiation is  $1 \mu\text{M min}^{-1}$  under our experimental conditions. While the amount measured is small, it may be sufficient over time to damage a variety of essential cellular components [23]. Indeed,  $\text{O}_2^{\cdot-}$  dismutation and the resulting production of  $\text{H}_2\text{O}_2$  and  $\cdot\text{OH}$  are early steps in cellular active oxygen species-driven reactions [23].

### 3.1.3. Proposed mechanisms for the production of $\text{O}_2^{\cdot-}$

To provide further evidence concerning the nature of the superoxide formation mechanism(s) during ZnPC-mediated photosensitization, the influence of physiological electron

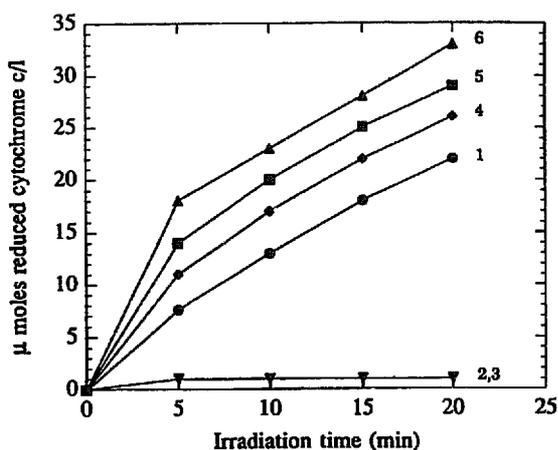
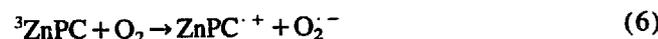
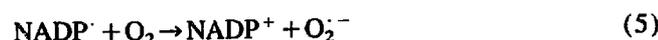
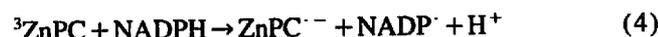
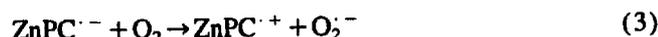


Fig. 4. Line 1: reduction of cytochrome *c* mediated by ZnPC-liposomes (1  $\mu\text{M}$ ) in aqueous solution (pH 7.0) with ferricytochrome *c* (43  $\mu\text{M}$ ) as a function of the illumination time ( $\lambda > 610$  nm, 20  $\text{mW cm}^{-2}$ ). Line 2: same as line 1, except that ZnPC or light or oxygen was omitted. Line 3: same as line 1, except that SOD was added (45  $\mu\text{g ml}^{-1}$ ). Line 4: same as line 1, except that NADPH was added (2.50 mM). Line 5: same as line 1, except that NADPH was added (5.0 mM). Line 6: same as line 1, except that NADPH was added (7.5 mM).

donor compounds, such as GSH, reduced CysH and NADPH, has been investigated. As shown in Fig. 2(D), the EPR intensity of DMPO- $\cdot\text{OOH}$  is strongly enhanced in the presence of electron donors, e.g. GSH. We also observed an enhancement in a concentration-dependent fashion of the reduction of cytochrome *c* in the presence of NADPH relative to similar conditions without a reducing agent (Fig. 4). Alternatively, it is possible for a donor such as NADPH to react directly with  $^1\text{O}_2$  to produce  $\text{O}_2^{\cdot-}$  [24]. Hence the possible contribution of  $^1\text{O}_2$  to the formation of  $\text{O}_2^{\cdot-}$  during the ZnPC photosensitization process has been investigated using the effects of singlet oxygen quenchers (DABCO and histidine) [25] as well as that of deuterium oxide ( $\text{D}_2\text{O}$ ) [25]. Our results clearly support the conclusion that the reduction of  $^1\text{O}_2$  to  $\text{O}_2^{\cdot-}$  can be ignored. From these data, the formation of superoxide which occurs in the absence (processes (2)/(3) and process (6)) or presence (processes (4)/(5)) of electron donors may be rationalized as follows



In processes (2)/(3), triplet ZnPC formed on irradiation (Eq. (1)) collides with another ZnPC molecule in the ground state, leading to electron transfer (Eq. (2)). The resulting  $\text{ZnPC}^{\cdot-}$  anion radical then donates its electron to dioxygen, producing  $\text{O}_2^{\cdot-}$  (Eq. (3)). This mechanism is supported by

the enhancement of the formation of  $\text{O}_2^{\cdot-}$  in the presence of electron donors. Indeed, it is well known that the addition of electron donors promotes the formation of photosensitizer radical anions, and thus the consistent environmental effects of the formation of  $\text{O}_2^{\cdot-}$  with the ZnPC anion radical ( $\text{ZnPC}^{\cdot-}$ ) suggest that  $\text{ZnPC}^{\cdot-}$  may be the precursor for the formation of  $\text{O}_2^{\cdot-}$ . However,  $\text{O}_2^{\cdot-}$  can also be produced by processes (4)/(5) in which the radicals formed in the electron transfer reaction between, for example, NADPH and  $^3\text{ZnPC}$  can reduce dioxygen to  $\text{O}_2^{\cdot-}$ . Indeed, at higher concentrations of NADPH (2.5 mM), we postulate that the majority of  $\text{O}_2^{\cdot-}$  produced probably arises from the chemistry of NADP $\cdot$  (or GS $\cdot$ ) (Eq. (5)). Similar mechanisms have also been observed with other sensitizers, such as haemato-porphyrin derivative [26], rose bengal [27] and merocyanine 540 [28]. Alternatively, the possibility that the ZnPC triplet may transfer its electron directly to the oxygen molecule to form the superoxide radical (process (6)) cannot be excluded, although there are no data on this process.

### 3.2. Formation of hydroxyl radical $\cdot\text{OH}$ by ZnPC

#### 3.2.1. Production of DMPO- $\cdot\text{OH}$ adduct

As shown in Fig. 5(A), the illumination of an aerobic aqueous solution at pH 7.0 containing ZnPC-treated liposomes and DMPO with visible light leads to the formation of a large amount of DMPO- $\cdot\text{OH}$  spin adducts. The EPR spectrum is characterized by hyperfine coupling constants of  $a_{\text{N}} = 14.9$  G and  $a_{\text{H}}^{\beta} = 14.9$  G, showing that the radical species is the DMPO- $\cdot\text{OH}$  adduct [19]. The intensity of the signal significantly increases on prolonged irradiation of the solution. No radical can be trapped when any component in the system is missing, i.e. ZnPC, oxygen, light and DMPO are all necessary (Fig. 5(B)). On the other hand, the singlet-oxygen-mediated oxidation of DMPO to radical species has been reported in aqueous solutions [29]. Experimental verification of such a mechanism was carried out by examining the effects of singlet oxygen quenchers (DABCO and histidine). The EPR signal intensity of the DMPO- $\cdot\text{OH}$  adduct does not decrease, indicating that  $^1\text{O}_2$ , which can be produced by ZnPC [5,6], is not involved in the formation of DMPO- $\cdot\text{OH}$ . The lack of a significant increase due to  $\text{D}_2\text{O}$  also indicates that DMPO- $\cdot\text{OH}$  formation is not due to the trapping of  $^1\text{O}_2$ .

The observed increase in the DMPO- $\cdot\text{OH}$  signal may be due to the decomposition of the superoxide radical adduct of DMPO (DMPO- $\text{O}_2^{\cdot-}$ ) or, alternatively, to direct trapping of  $\cdot\text{OH}$  radicals produced by ZnPC-mediated photosensitization, or from a combination of both processes. In order to determine the source of the DMPO- $\cdot\text{OH}$  adduct, we added dimethyl sulphoxide (DMSO) to the system. The  $\cdot\text{OH}$  radicals are very short lived and highly reactive. They react with DMSO to give  $\cdot\text{CH}_3$  radicals which can be spin trapped and identified [19]. When DMSO is added (5% v/v), the intensity of the DMPO- $\cdot\text{OH}$  signal decreases and the intensity of the DMPO- $\cdot\text{CH}_3$  signal increases as a function of the illu-

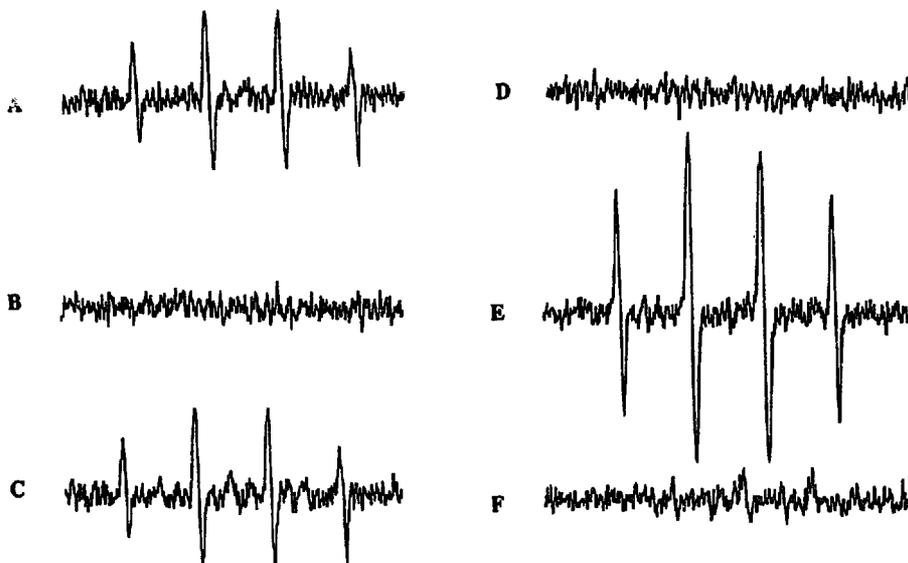


Fig. 5. EPR spectra of the DMPO- $\cdot$ OH adduct in aqueous solution (pH 7.0) containing ZnPC-liposomes (1  $\mu$ M) and DMPO (20 mM): (A) after 2 min of irradiation ( $\lambda > 610$  nm, 20  $\text{mW cm}^{-2}$ ); (B) same as (A), except that light, ZnPC or oxygen was omitted; (C) same as (A), except that SOD was added (45  $\mu\text{g ml}^{-1}$ ); (D) same as (A), except that catalase was added (50  $\mu\text{g ml}^{-1}$ ); (E) same as (A), except that  $\text{H}_2\text{O}_2$  was added (10  $\mu\text{M}$ ); (F) same as (A), except that desferrioxamine was added (5 mM). Gain,  $5.0 \times 10^5$ ; modulation amplitude, 0.63 G.

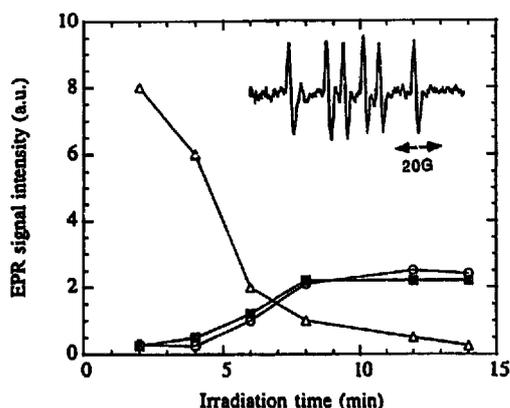


Fig. 6. Time course of the EPR signal intensities of the DMPO adducts. Production of the DMPO- $\cdot$ OH adduct ( $\Delta$ ) during irradiation of an aqueous solution (pH 7.0) containing ZnPC-liposomes (1  $\mu$ M) and DMPO (20 mM). Production of the DMPO- $\cdot$ CH(OH)CH $_3$  adduct ( $\blacksquare$ ) in the presence of 5 vol.% ethanol during irradiation of an aqueous solution (pH 7.0) containing ZnPC-liposomes (1  $\mu$ M) and DMPO (20 mM). Production of the DMPO- $\cdot$ CH $_3$  adduct ( $\circ$ ) in the presence of 5 vol.% DMSO during irradiation of an aqueous solution (pH 7.0) containing ZnPC-liposomes (1  $\mu$ M) and DMPO (20 mM). Inset: a typical EPR spectrum of DMPO- $\cdot$ CH(OH)CH $_3$  14 min after the start of irradiation. The spectra were acquired as described in Fig. 5.

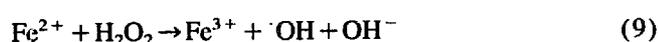
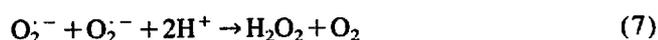
mination time (Fig. 6). The spectrum exhibits no trace of the DMPO- $\cdot$ OH signal 14 min after the start of irradiation, but contains features corresponding to the DMPO- $\cdot$ CH $_3$  adduct ( $a_N = 16.4$  G,  $a_H = 23.3$  G). These observations suggest that the DMPO- $\cdot$ OH signal observed in the absence of DMSO originates from the trapping of  $\cdot$ OH radicals and not from the decomposition of DMPO- $\text{O}_2^-$ . Further evidence on the source of the DMPO- $\cdot$ OH signal was obtained by carrying out the reaction in the presence of 5% (v/v) ethanol [19]. A signal with  $a_N = 15.8$  G and  $a_H = 23.0$  G, corresponding to the  $\alpha$ -hydroxyethyl adduct of DMPO (DMPO-

$\cdot$ CH(OH)CH $_3$ ) is observed (Fig. 6). This demonstrates that  $\cdot$ CH(OH)CH $_3$  is generated via the reaction between  $\cdot$ OH and CH $_2$ (OH)CH $_3$  and hence confirms the presence of  $\cdot$ OH radicals. It must also be emphasized that the DMPO- $\cdot$ OH signal is still observed 90 min after the end of irradiation. These data are consistent with the production of ZnPC hydroxyl radicals and not with the formation of DMPO- $\cdot$ OH from the decomposition of DMPO- $\text{O}_2^-$ . Indeed, the half-life of DMPO- $\text{O}_2^-$  is approximately 60 s at pH 7, whereas the DMPO- $\cdot$ OH adduct is significantly more persistent (half-life, 2 h) [19]. Thus it is evident that photoactivated ZnPC in liposome membranes generates both hydroxyl and superoxide radicals.

### 3.2.2. Transformation of superoxide radical ( $\text{O}_2^-$ ) into hydroxyl radical ( $\cdot$ OH)

In order to determine the possible involvement of either superoxide or the DMPO- $\text{O}_2^-$  adduct in the generation of hydroxyl radicals or DMPO- $\cdot$ OH, experiments were performed in the presence of the  $\text{O}_2^-$  scavenging enzyme SOD (45  $\mu\text{g ml}^{-1}$ ). The DMPO- $\cdot$ OH signal is still observed (Fig. 5(C)), suggesting that  $\cdot$ OH generation is not mediated by  $\text{O}_2^-$  radicals, and the observed DMPO- $\cdot$ OH adduct is not generated from the breakdown of DMPO- $\text{O}_2^-$ . There is concern that SOD may be ineffective due to localization in the aqueous phase, since ZnPC is intercalated into the bilayer [4]. However, DMPO partitions primarily into the aqueous phase [30], so that reactive species must diffuse across the membrane bilayer before they can react with DMPO to any appreciable extent. To determine whether hydroxyl radical generation proceeds via the reduction of hydrogen peroxide, experiments were performed in the presence of the hydrogen peroxide scavenging enzyme catalase. The addition of catalase (50  $\mu\text{g ml}^{-1}$ ) completely inhibits the formation of the

DMPO- $\cdot$ OH signal (Fig. 5(D)). This may suggest that  $\cdot$ OH is generated from  $\text{H}_2\text{O}_2$ . Similarly, the enhancement of DMPO- $\cdot$ OH adduct formation observed on addition of exogenous  $\text{H}_2\text{O}_2$  confirms the hypothesis that  $\cdot$ OH is generated via the reduction of hydrogen peroxide in the ZnPC-liposome photosensitization process (Fig. 5(E)). In addition, we checked that the formation of  $\cdot$ OH was fully inhibited by desferrioxamine (Fig. 5(F)), which is a well-known chelator of iron preventing further reaction with  $\text{H}_2\text{O}_2$  [31]. These findings support the view that photosensitized ZnPC-liposomes can generate  $\cdot$ OH via the Fenton reaction (Eqs. (8) and (9)). Overall, this result can be explained by the following additional reactions



Alternatively, since the addition of SOD ( $45 \mu\text{g ml}^{-1}$ ) has no obvious effect on the intensity of the DMPO- $\cdot$ OH adduct (Fig. 5(C)), the following reaction may also take place in our system



#### 4. Conclusions

Much evidence suggests a role for singlet oxygen in the mechanism of ZnPC-mediated photosensitization. However, the present work demonstrates unequivocally the ability of ZnPC in liposomes to mediate type I reactions with the involvement of both superoxide and hydroxyl radicals. These reactions are enhanced in the presence of physiological electron donors. This is particularly interesting since many electron donors are endogenously present in the human body. The results described here warrant further studies of the generation of the ZnPC anion radical under anaerobic conditions to evaluate fully the potential of ZnPC in PDT.

#### Acknowledgements

This work was mainly supported by a contribution from the CHUV-EPFL-UNIL Fund for collaboration in the area of Biomedical Technology. Contributions from the Swiss Priority Programme in Optics are also gratefully acknowledged. The gift of ZnPC from Ciba Geigy (Basle, Switzerland) is greatly appreciated. The authors thank Professor J.C. Bünzli from ICMA-UNIL (Lausanne) for the EPR facilities.

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