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Original scientific paper

Evaluation of the oxidative activity of some free base porphyrins by a chemiluminescence method

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Abstract: Due to their spectral characteristics, phototoxicity and high affinity for tumour tissues, porphyrins and their derivatives are widely used in modern medicine as contrast agents for cancer diagnostics and as sensitizers in photodynamic therapy, where they kill tumours *via* enhancement of tumour oxidative stress. The aim of this work was to simulate *in vitro* the effects caused by oxidation of two free base porphyrins, 5,10,15,20-tetraphenylporphyrin (TPP) and 5,10,15,20-tetra(4-methoxyphenyl)porphyrin (TMOPP). The kinetic study was monitored using spectral techniques and chemiluminescence. The effect of both porphyrins on an oxidation process was evidenced using the chemiluminescent system, luminal–hydrogen peroxide, in a phosphate buffer at pH 7. It was found that at low concentration, TPP exerts the anti-oxidative effect in the employed chemiluminescent system, while at higher concentrations, its effect is pro-oxidative. TMOPP exerts a pro-oxidant effect, which was more pronounced than TPP. The results are discussed with respect to oxidative stress.

Keywords: free base porphyrins; chemiluminescence; oxidative activity; luminol.

INTRODUCTION

Porphyrins, free base and metallocomplexes,[1,2] play an important role in many energy transfer processes from photochemistry,[3] photobiology[4] and photomedicine.[5,6] Porphyrins can be oxidized chemically, electrochemically, photochemically (photosensitization) to form porphyrin-ring centred oxidized products.[7]

Recently, importance was granted to oxidative stress (a common state in most pathological conditions, such as cancer, diabetes, radiation injury and disorders of the central nervous system), especially regarding the role of synthetic antioxidants, such as Mn porphyrins, in the treatment of oxidative stress.[8,9] Chemi-

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luminescence detection reactions have become popular in analytical biochemistry, essentially due to their high sensitivity. In this way, a synthetic chemiluminescent system (luminol/porphyrin) was conveniently employed to measure serum oxalate by determination of the hydrogen peroxide generated through oxalate oxidase.[10–12] In order to use metalloporphyrins as labels in immunoassays or in nucleic probes, a detection method based on luminol chemiluminescence (CL) at alkaline pH was developed.[13] The generation of the free radicals $HO^{\bullet}$ and $O_2^{\bullet-}$ was found to be the result of luminol oxidation by metalloporphyrins. In these respects, using 5,5'-dimethyl-1-pyrroline-*N*-oxide as a spin trap agent, electron spin resonance (ESR) evidenced the production of $HO^{\bullet}$ and $O_2^{\bullet-}$. The role of $O_2^{\bullet-}$ was confirmed by the almost complete inhibition of light emission when superoxide dismutase was added to the CL reaction. The contribution of oxygen was also confirmed by the large decrease in the CL emission when deaerated solutions were used.[7,13] Owing to their redox properties, FMN and NAD enhancers could act at this level through an increase of the exchange rate between Fe^{2+} and Fe^{3+}. In the presence of FMN, a significant red shift and shape change of the luminol emission spectrum was observed, which arise from an energy transfer phenomenon in the final luminescent step of the reaction.[13]

It is known that several synthetic metalloporphyrins associated with oxygen atom donors are potent catalysts for the chemiluminescent oxidation of luminol or isoluminol. In these respects, the luminescence produced at pH 7.5 in the presence of isoluminol, H_2O_2 and metalated water – soluble porphyrins (Fe^{3+} or Mg^{2+} derivatives of tetra-sodium *meso*-tetrakis(*p*-sulphonatophenyl)porphyrin and *meso*-tetrakis(4-*N*-methyl-pyridiniumyl)porphyrin] tetraacetate was of the same order of magnitude as that produced by horseradish peroxidase under the same conditions.[14,15] Many porphyrins catalyze luminol chemiluminescence at pH 13 without addition of peroxide. The most active catalyst was Mn-*meso*-tetrakis(p-sulphonatophenyl)porphyrin. It was found that Tween-20 enhanced the activity of this catalyst best at a Tween-20 to luminol ratio of 74:1. Dodecyl sulphate enhanced best at the optimum dodecyl sulphate to luminol ratio of over 1000:1 and both detergents enhanced the reaction when present below their critical micelle concentrations. Moreover, negatively charged aliphatic compounds, such as fatty acids, enhanced the reaction but positively charged aliphatic compounds inhibited it.[11]

The Mn and Fe porphyrins that were shown to have broad antioxidant properties are, in effect, analogues of naturally occurring haeme (iron protoporphyrin IX). It was shown that the reactivities of the synthetic metalloporphyrins were not constrained by the microenvironment of protein-bound haeme.[16] Moreover, Ferrer-Sueta *et al.*[17] and Crow[18] reported that some metalloporphyrins appear to be capable of scavenging the CO_2 adduct of peroxynitrite. In addition, metalloporphyrins were shown to be protective in a number of cell and animal models of

oxidative injury[19] and models of stroke.[20] Such evidence that these redox active porphyrins were effective *via* peroxynitrite scavenging was provided by showing that protein nitration was prevented.[21,22] In these regards, prevention of protein nitration strongly suggests that other deleterious reactions of peroxynitrite were also prevented. However, new evidence suggests that metalloporphyrins have protective effects independent of antioxidant activities, namely that they are potent inducers of haeme oxygenases and other heat shock proteins – proteins which are known to enhance survivability to oxidative stress.

Recently, the lipophilicity of potent porphyrin-based antioxidants, by a comparison between *ortho*- and *meta*-isomers of Mn^{3+}–*N*-alkylpyridylporphyrins was studied.[23]

This work follows a previous study[24] and deals with the effects of two free base porphyrins (5,10,15,20-tetra-*p*-phenylporphyrin (TPP) and 5,10,15,20-tetra--(*p*-methoxyphenyl)porphyrin (TMOPP)) in an oxidation process studied by a chemiluminescence method using the chemiluminescent system luminal–hydrogen peroxide in phosphate buffer at pH 7. It was found that at low concentrations, TPP exerted an anti-oxidative effect in the employed CL system, while its effect was pro-oxidative at higher concentrations. TMOPP exerted a pro-oxidant effect, which was more pronounced than TPP. These findings could be important regarding oxidative stress as a function of concentration. The studied free base porphyrins could have a protective effect against reactive oxygen species.

EXPERIMENTAL

Materials

The system luminol (LH_2) (8.80×10^{-5} M)–hydrogen peroxide (H_2O_2) (30 mM) in 50 mM phosphate buffer at pH 7 was considered the reference system. LH_2 and H_2O_2 were from Merck and the phosphate buffer from Sigma. TPP, in which R = C_6H_5 (5,10,15,20-tetraphenylporphyrin) and TMOPP, in which R = C_6H_5–OCH_3 (5,10,15,20-tetra(4-methoxyphenyl)-porphyrin) (Fig. 1) were synthesized by Prof. Ion's team and the solutions for this study were prepared in dimethyl sulphoxide (DMSO).[25]

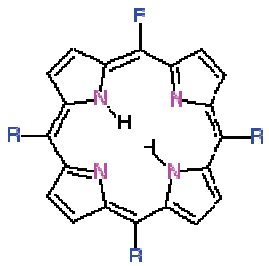

Fig. 1. Chemical structure of the studied porphyrins.

Methods and apparatus

The chemiluminescence (CL) measurements were realised with a chemiluminescence instrument TD 20/20 Turner Design, USA. The points on the plot were obtained by integrating the light signal at periods of 4 s. Five measurements were made and an average value

calculated, obtaining a maximum 10 % relative scattering of the results from the mean value. The working volume was 1000 µl.

The extinction of the CL emission, *S*, was calculated according to the equation:

$$S = 100\frac{I_0 - I}{I_0}$$

where I_0 and I represent the CL intensity measured for the reference system and for the reference system in the presence of antipyrine, respectively; both values were measured 5 s after the beginning of the reaction.

The $I_{CL} = f(t)$ variation enable the determination of the rate constant of the reaction, for the upward part of the plot, $-k_2$ (attributed to the consumption of free radicals reaction), as well as for the downward part of the curve, k_1 (attributed to the formation of free radicals reaction), considering that the CL reaction is first order. The values of k_1 and k_2 were determined using the following equation:

$$k = \frac{1}{\Delta t}\ln\frac{I_i}{I_0},\ \Delta t = t_i - t_0$$

in which t_i represents the time at the moment i, t_0 represents the initial time, I_i is the intensity of CL signal at the moment i and I_0 is the intensity of the CL signal at the initial moment. The value of the rate constants were calculated in time range 5–60 s ($-k_2$) for the LH_2/phosphate at pH 7/H_2O_2, at pH 7/H_2O_2/TPP and TMOPP systems.

The visible absorption spectra were recorded on a SPECORD M400 Carl Zeiss Jena spectrophotometer. The mass spectra were obtained with an HP 5985 spectrometer (Hewlett–Packard), using a silica column. The final product of the photodegradation reaction of TTP was identified with a Perkin Elmer spectrometer, from CCl_4 solution.

The FTIR spectra were acquired using a Jasco FT/IR-470 plus or a FT/IR-4200 spectrometer as KBr pellets.

Carlo Erba FTV 4160 gas chromatograph, incorporating Grob-type split/splitless and on-column injectors, was employed throughout this work. The same type of instrument was also linked to an AEI MS 30 magnetic sector mass spectrometer. A flexible silica interface was employed to prevent adsorption and decomposition of the porphyrins.

Several WCOT capillary columns, coated with apolar phases, were employed. A 20 m×0.34 mm i.d. glass capillary column coated with CP Sil 5 (Chrompak) was adequate for the analysis of the porphyrin derivatives. The less volatile porphyrin derivatives were analysed on a 6 m×0.3 mm i.d. Hewlett–Packard flexible fused silica capillary column coated with OV-1 stationary phase. Hydrogen was usually employed as the carrier gas, typically at an inlet pressure of 0.2 kg cm^{-2}, producing an average gas velocity of approximately 100 cm s^{-1}. All analyses were temperature programmed.

The ketone and peroxide concentrations were determined by colorimetric methods as indicated in the literature.[26]

RESULTS AND DISCUSSION

The chemiluminescence (CL) technique is based on generating free radicals ($HO^{\bullet}$, $O_2^{\bullet -}$, $^1O_2$, $ROO^{\bullet}$) in a luminescence system and is followed by the study of pro- and anti-oxidative actions on a specific molecule.[13,14] CL is a concerted chemical and physical process which occurs after an exergonic chemical reaction, releasing the energy as light. In this way, a molecule in the excited state un-

dergoes a structural arrangement process of an electronic level, showing physical and chemical properties that are different compared to those of the ground state. It is well known that LH_2–H_2O_2 in alkaline solution yields an excited aminophthalate dianion species, which leads to the appearance of oxygen free radicals, such as: the superoxide anion, singlet oxygen and hydroxyl radical.[13,14] The result of the multiple interactions is light emission, as luminol is consumed. In the presence of an antioxidant compound (which consumes free radicals), the CL intensity decreases, while the effect of a pro-oxidant compound (which increases the concentration of free radicals) is an increase in the CL intensity.

The CL measurements were recorded in order to evaluate the anti-oxidative ability of TPP and TMOPP in various systems. Chemiluminescence, already evidenced for some porphyrins by Wasser and Fuhrhop[27] was evaluated at 430–440 nm in this study. Usually, the degradation of porphyrins involves a macrocycle break with an energy release of 60–70 kcal/mol, sufficient for light emission. In DMSO and hydrogen peroxide, the proton from inside the porphyrin macrocycle can leave the macrocycle and the porphyrin becomes an anion with a strong electronic density at the methine carbon position.[28–30] Under such conditions, the anionic form of the porphyrin could aggregate (Fig. 2, 2), in good agreement with literature data,[31] Fig. 2.

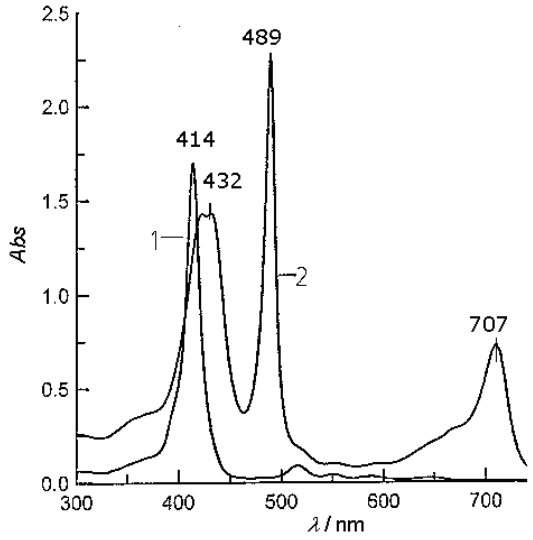

Fig. 2. UV–Vis spectra of TPP in the monomeric form (1) and the aggregated form (2).

The variation of the CL intensity as a function of time (Fig. 3) allows the determination of the quenching of the CL reaction for systems that contain porphyrins. It was found that the profiles of the CL in the presence of TPP and TMOPP porphyrins were almost the same and the peak intensities of the CL increased with increasing concentration of the employed porphyrin. At small concentrations, TPP

acts as a trap for free radicals produced in the system LH_2–H_2O_2. At higher concentrations, 0.50–1.5 μM, TPP leads to a strong increase of the CL intensity, which in fact could be the result of a process of aggregation of porphyrins in DMSO and in the presence of H_2O_2. An oxidation of luminol by the porphyrin occurs. This is a consequence of the photogeneration of hydrogen peroxide by the porphyrins.[32]

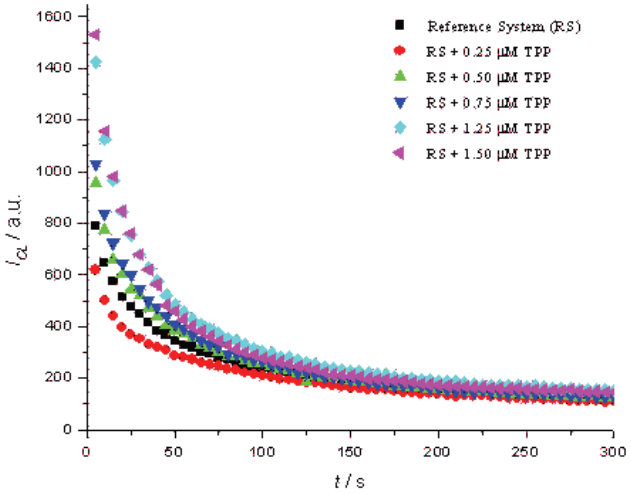

Fig. 3. The effect of TPP concentration on the CL intensity of the LH_2 (8.80×10^{-5}M)–H_2O_2 (30 mM) system in 50 mM phosphate buffer at pH 7 (reference system).

Similarly, in the case of TMOPP (the results are not shown), an increase in the CL intensity, more pronounced than in the case of TPP, was evidenced. These are according to the efficiency of CL quenching (S / %) and reaction rate values, Table I. Moreover, it can be observed that the results also corroborate with the concentration of ketones and peroxides.

The responsible species for chemiluminescence are dioxetane species, *via* ketone and peroxide species evidently, as determined by IR spectroscopy, the stretching vibrational bands ν(C=O) at 1600 and 980 cm^{-1} (Fig. 4), and by mass spectrometry (Fig. 5).

TABLE I. The kinetic parameters of the CL process (efficiency of CL quenching, reaction rate and rate constants) in the LH_2–H_2O_2–phosphate buffer at pH 7 system (reference system, RS), in the presence of TPP and TMOPP, 5 s after the beginning of the chemiluminescence reaction

System	S / %	v / s^{-1}	$k\times10^2$ / s^{-1}	Ketone concentration, μl ml^{-1}	Peroxide concentration, μl ml^{-1}
RS	–	150	3.1	–	–
RS + 1.50 μM TPP	–93.8	306	4.2	5.12	3.13
RS + 1.50 μM TMOPP	–130.1	364	4.0	23.46	17.45

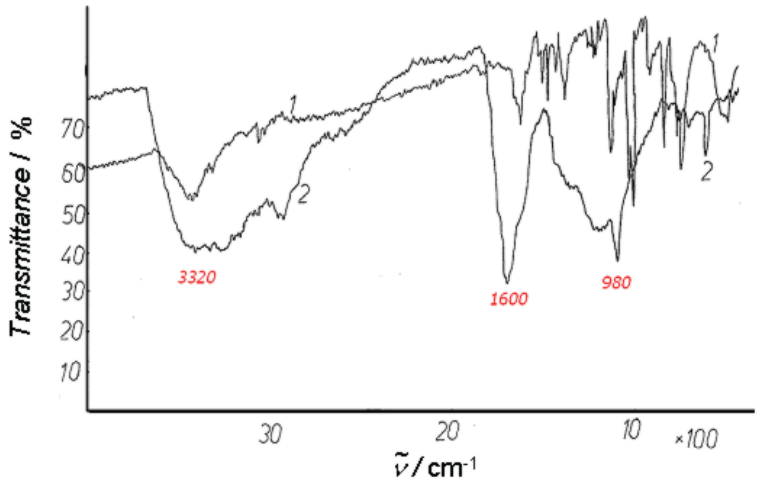

Fig. 4. IR spectra of TPP, initial (1) and final (2).

It was possible to determine the ketone and peroxide concentrations, as a proof for the above-mentioned porphyrin degradation, Table I. It can be observed that, in the case of TMOPP, the found concentration of ketone, 23.5 μM/ml was higher than that in the case of TPP porphyrin, 5.12 μM/ml. With regards to the peroxide concentration, a higher value was also found, 17.4 μM/ml in the case of TMOPP compared with 3.13 μM/ml in the case of TPP.

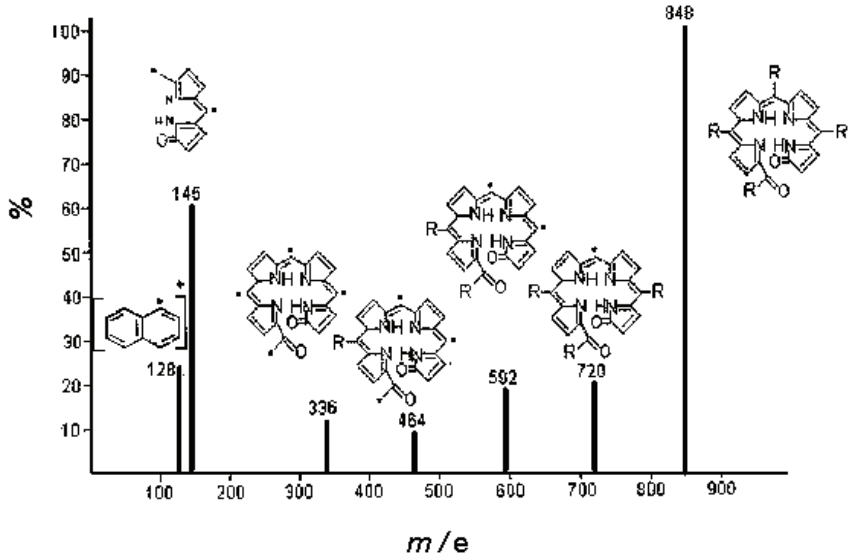

Fig. 5. The fragmentation diagram for TPP degradation.

CONCLUSIONS

The study was aimed at simulating *in vitro* the effects of 5,10,15,20-tetraphenylporphyrin (TPP) and 5,10,15,20-tetra(4-methoxyphenyl)porphyrin (TMOPP) in the oxidative system luminal–hydrogen peroxide, in phosphate buffer at pH 7. The kinetic study was monitored by the evolution of the chemiluminescence intensity as a function of time. It was found that at a low concentration (0.25 μM) TPP exerted an anti-oxidative effect in the employed chemiluminescent system while at higher concentrations (0.50–1.5 μM), its effect was pro-oxidant. TMOPP, for the same range of concentrations, exerted a pro-oxidant effect, which was more pronounced than that of TPP.

The ketone and peroxide concentrations were found to be higher in the case of TMOPP than in the case of TPP.

These aspects are important and could be an indication in future investigations with regard to the oxidative stress process. Moreover, the results are an additional tool to predict a possible protective effect of free base porphyrins in an oxidation process. These free base porphyrins may have a protective effect that could work independently of anti-oxidant activities.

И З В О Д

ОДРЕЂИВАЊЕ ОКСИДАТИВНЕ АКТИВНОСТИ НЕКИХ СЛОБОДНИХ БАЗА ПОРФИРИНА ХЕМИЛУМИНЕСЦЕНТНОМ МЕТОДОМ

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Порфирини и њихови деривати се, захваљујући својим спектралним карактеристикама, фототоксичности и великом афинитету према ткиву тумора, широко користе у модерној медицини као контрастни агенси у дијагнози тумора и терапији. Они убијају туморе повећавајући оксидативни стрес у њима. Циљ овог рада је да *in vitro* симулира ефекте које изазивају две слободне базе порфирина, 5,10,15,20-тетрафенилпорфирин (TPP) и 5,10,15,20-тетра(4-метоксифенил)порфирин (TMOPP). Кинетичка студија је праћена спектралним и хемилуминесцентним методама. Ефекат порфирина у процесу оксидације је одређиван у хемилуминесцентном систему, луминол–водоник-пероксид, у фосфатном пуферу, pH 7. При ниским концентрацијама TPP има антиоксидативни ефекат, док при вишим концентрацијама има прооксидативно дејство. TMOPP испољава прооксидативни ефекат и то израженије него TPP. Резултати су дискутовани у односу на оксидативни стрес.

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