Meso-Tetraphenylporphyrin in Liposomes as a Suitable Photosenzitizer for Photodynamic Therapy of Tumors

M Lovčinský[1], J Borecký[1], P Kubát[2] and P Ježek[1]

1 *Institute of Physiology, Department of Membrane Transport and Biophysics, Academy of Sciences of the Czech Republic, Prague, Czech Republic*
2 *J Heyrovsky Institute of Physical Chemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic*

Abstract. The suitability of a liposomal form of hydrophobic nonsulfonated *meso*-tetraphenyl porphyrin (TPP) for the photodynamic therapy of tumors was investigated TPP was solubilized in small unilamellar lipid vesicles prepared by extrusion on a LIPOSOFAST apparatus These samples were studied by laser-excited time resolved luminescence and triplet-triplet absorption spectroscopy In this lipid environment TPP was still an efficient singlet oxygen producer, as indicated by the characteristic singlet oxygen phosphorescence at 1270 nm in D_2O, when excited with a 28 ns laser pulse at 412 nm Moreover, unlike with sulfonated TPP ($TPPS_4$), liposomal TPP showed the reduced decay rates of TPP triplet-states with the increasing time of pre-illumination by a Xenon lamp This was shown in an indirect way, based upon the appearance of a second component of the luminescence decay at 1270 nm in D_2O, and by direct TPP triplet state monitoring, detecting triplet-triplet absorption at 440 nm in H_2O The deactivation of higher triplet states was delayed upon pre-illumination This reflects an irreversible interaction of singlet oxygen with membrane lipids, thus demonstrating the potential of the liposomal form of TPP to efficiently disintegrate tumor cell membranes and to be a suitable preparation for the photodynamic therapy

Key words: *Meso*-tetraphenylporphyrin (TPP) — Small unilamellar vesicles — Photodynamics therapy of tumors — Singlet-oxygen phosphorescence — Triplet-triplet absorption

Introduction

Photodynamic therapy (PDT) is widely used in the world to cure suitable malignancies (Van der Meulen 1996) The high success rate of PDT has led to its high

Correspondence to Dr Petr Ježek, Dept No 375, Institute of Physiology, Videňska 1083, 142 20 Prague 4, Czech Republic E-mail jezek@sun1 biomed cas cz

appreciation and consideration as an alternative to cytostatics and radiotherapy. The main principle of PDT is formation of cytotoxic species (singlet oxygen, triplet states of photosensitizer) resulting from irradiation of a photosensitiser-loaded tumor. This causes an irreversible damage to the neoplastic tissue. The photosensitizer is usually preferentially accumulated in tumor tissues and can be administered intravenously or applied locally. The tumor is then illuminated with a light source of an appropriate wavelength (600–800 nm), having a significant penetration into the tissue. The efficiency of PDT is dependent on the penetration depth, local dye concentration and photophysical properties of the photosensitizer, and the presence of oxygen. The indication range for the use of PDT for the therapy of malignancies is just dependent on these factors. All these aspects are currently subject of intensive studies, and the first commercial photosensitizer preparations are available on the market, including liposomal formulations (Love et al. 1996).

To direct the photosensitizer to tumors more selectively and thus enhance the efficiency of PDT, liposomes may be used as carriers delivering the hydrophobic (lipophilic) photosensitizer either locally or intravenously to tumor tissue. This proceeds via low-density-lipoproteins (LDL) and improves photosenzitizer penetration of the tumor cells due to an increased internalization of LDL by hyperproliferating cells (Jori 1989). Lipophilic photosensitizers then associate with cellular membranes and photoactivation results in direct cell killing. In contrast, water-soluble photosensitizers act in PDT predominantly by disrupting the tumor microvasculature, resulting in vascular stasis, anoxia and necrosis of the tissue. Hence, their mechanism is rather indirect. Two major problems might exist in the liposomal therapy: fast clearance by the RES cells and the inability of larger vesicles to cross the capillary endothelium and the basal membrane (Renade 1989). To achieve the latter, vesicles of around 100 nm in size with a rigid bilayer matrix are required (Gabizon and Papahadjopoulos 1988). Moreover, accumulation of vesicles in tumors might result from extravasation into the interstitial tumor space or from endocytosis by the capillary endothelial cells of the tumor microvasculature.

From the photophysical point of view, a good photosensitizer should have a high quantum yield of fluorescence for visualisation of the tumor. It should generate singlet oxygen with a high quantum yield and/or easily accept (donate) electrons in its triplet states. Currently, the most frequently used photosenzitizers are hematoporphyrin derivatives – Photophrine II (USA) and Photosan 3 (FRG), complex and rather unstable compounds; followed by phthalocyanines and synthetic porphyrins such as *meso*-tetrakis-(4-sulfonatophenyl)porphyrin ($TPPS_4$, Winkelman 1962). Jirsa and Kakáč (1990) have synthesized $TPPS_4$ in a pure form which, unlike the old preparation, exhibited no side neurotoxicity (Jirsa et al. 1991, 1996; Lapeš et al. 1996). The efficient production of the singlet oxygen in processes sensitized by $TPPS_4$ has been directly quantified using a variety of techniques (Mosinger and Mička 1997; Wilkinson et al. 1993, 1995). Also the effects of heavy water on the

triplet states of $TPPS_4$ were studied, and it has been proven that D_2O increases triplet lifetime similarly as it is in the case with the singlet oxygen (Engst et al 1994) Some metal complexes (Zn, Cd and Mg) of $TPPS_4$ were found to be more efficient producers of triplet states (Kubát and Mosinger 1996) and singlet oxygen (Mosinger and Mička 1997)

In this work, we employed the hydrophobic precursor of $TPPS_4$- nonsulfonated TPP-*meso*-tetraphenylporphyrin solubilized in small unilamellar lipid vesicles (SUV), prepared by the extrusion technique on a LIPOSOFAST apparatus We studied the basic photophysical properties of this system and found that the liposomal form of TPP makes a suitable and promising preparation for PDT

Materials and Methods

IVS type L-α-phosphatidylcholine (soya bean lecithin) and dimethylformamide were purchased from Sigma (St Louis, USA), all other chemicals and $TPPS_4$ were of analytical grade from Lachema (Brno, Czech Republic) TPP was kindly donated by Prof M Jirsa who synthesized it as described by Jirsa and Kakáč (1990)

Preparation of liposomes containing TPP

The extrusion method using the LIPOSOFAST extruder (Avestin Inc , Canada) was adopted (MacDonald et al 1991) for the preparation of small unilamellar vesicles (SUV) with a diameter of less than 100 nm, while incorporating TPP into the lipid membranes 10 mg TPP and 100 mg of soya bean lecithin were dissolved in chloroform methanol (2 1) and evaporated in a rotatory evaporator (Buchi, Switzerland) 2 ml of KP_i buffer (125 mmol/l KCl 20 mmol/l KP_i, pH 7 3) were added and the lipids were hydrated while rotating the vessel The obtained multilamellar vesicles were freezed/thawed five times in a dry ice/ethanol mixture A 100 nm filter membrane was inserted into the LIPOSOFAST middle chamber and eleven extrusion cycles were performed, thus producing SUV For the purpose of measuring singlet oxygen production the buffer was made in D_2O

Absorption and fluorescence spectra

Absorbance was measured using a Spectronics 3000 diode array spectrophotometer (Milton Roy, USA) With the liposomal TPP preparations, the contribution of light scattering had to be subtracted (scattering fitted by a hyperbolic function) Uncorrected steady-state fluorescence excitation and emission spectra were measured on a RF-5301 PC fluorometer (Shimadzu, Japan)

Triplet triplet absorption spectra

Triplet-triplet absorption spectra were measured on a laser kinetic spectrometer (Applied Photophysics, U K), described elsewhere (Lang et al 1992) Transient

species were generated by a laser pulse at 416 nm. The source of the excitation radiation was a Lambda Physik FL 3002 dye laser (pulse length 28 ns, output energy 1–3 mJ) pumped by LPX 205 XeCl laser (both Lambda Physik, Germany). Changes in the solution absorbance over time at the selected wavelength were detected using an R928 photomultiplier (Hamamatsu). The spectra were measured point-by-point by choosing absorbance values at selected detection time and wavelength. A 250 W Xenon lamp (Optical Radiation Corporation, USA) was used as the source of light for absorption spectroscopy and for continuous irradiation of some samples.

Singlet oxygen phosphorescence

Singlet oxygen was monitored by means of its characteristic phosphorescence at 1270 nm. This radiation was separated from the red fluorescence by a 1270 nm band-pass filter (Laser Components, Olching, Germany) and detected with a Ge diode (Judson J16-8SP-R05M-HS, USA). The signal was amplified with a home-made amplifier and recorded on an oscilloscope. All traces were accumulated at least 16 times. The experiments were carried out in D_2O to increase the lifetime of singlet oxygen (Wilkinson et al. 1995).

Results

Fig. 1 compares absorption (panel A), fluorescence emission and excitation spectra (panel B) of $TPPS_4$ in aqueous KP_i buffer solution and TPP, solubilized in lipid vesicles of less than 100 nm in diameter (SUV). TPP absorption, corrected for light scattering has a slight red shift to a maximum at 419 nm *vs.* 412 nm for $TPPS_4$. The same shift was found in the excitation spectra (417 nm *vs.* 411 nm). The corresponding emission spectra of liposomal TPP were also slightly red-shifted when compared to spectra of $TPPS_4$ (650 nm *vs.* 640 nm for $TPPS_4$).

Fig. 2. illustrates a typical experiment with singlet oxygen produced by the photosenzitizers ($TPPS_4$, Fig. 2*B*; liposomal TPP, Fig. 2*C,D*) detected by phosphorescence at 1270 nm. The photosenzitizers were excited with a 28 ns laser pulse at 412 nm, inducing their triplet states which subsequently interact with the triplet (natural) oxygen contained in the solution (air saturated). Thus, the singlet oxygen was produced. Subsequently, the singlet oxygen was spontaneously deactivated by phosphorescence, detected at 1270 nm. Its lifetime for the sample of $TPPS_4$ in KP_i-D_2O buffer was approx. 50 μs. This value is comparable with the data (44–70 μs) of Wilkinson et al. (1995). Experimental records up to 5 to 10 μs contain a contribution of photosensitizer fluorescence, while the delayed emission up to 120 μs ($TPPS_4$, Fig. 2*B*) and 60 μs (TPP, Fig. 2*C,D*) represents the mentioned phosphorescence. When the liposomal TPP in KP_i-D_2O was bubbled with nitrogen, no phosphorescence was observed (Fig. 2*A*). After illumination of the $TPPS_4$ sample with a Xenon lamp for 18 min, the monoexponential decay was retained and the

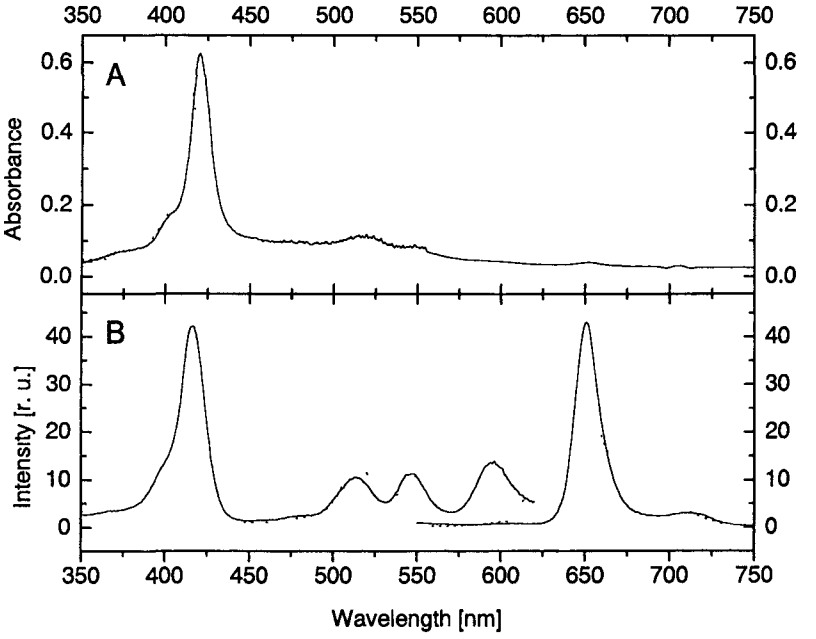

Figure 1. Absorption, excitation and emission spectra of $TPPS_4$ and TPP in liposomes *A*) Absorption spectra, *B*) fluorescence excitation (left) and emission spectra of TPP dissolved in lecithin liposomes – SUV (solid lines) and $TPPS_4$ in KP_i buffer (dotted lines) were measured as described in Materials and Methods Approx 20 μmol/l TPP and exactly 1 μmol/l $TPPS_4$ were used, and the lipid concentration of vesicles amounted to 0 5 mg/ml Note that the higher background absorbance of liposomal TPP was due to light scattering

lifetime was the same as without pre-illumination (not shown).

TPP solubilized in liposomes produced similar amounts of singlet oxygen, as judged upon nearly the same initial phosphorescence intensity of singlet oxygen (Fig. 2*C vs.* 2*B*). However, when illuminating of the TPP sample with the Xenon lamp for 18 min, the monoexponential decay became bi-phasic (Fig. 2*D*). The two phases could be fitted as a convolution of two exponentials with time constants 20 000 and 140 000 s^{-1}, respectively. The first component thus represents a spontaneous deactivation of singlet oxygen by phosphorescence, whereas the second component reflects the TPP triplet state lifetime. Absence of this component in the measurements without pre-illumination is due to the extremely short triplet lifetime in the existing oxygen-rich environment. On the contrary, the prolonged triplet lifetime indicates a reduced quenching of the TPP triplet states, which is most probably due to the decreasing oxygen concentration, the latter being a re-

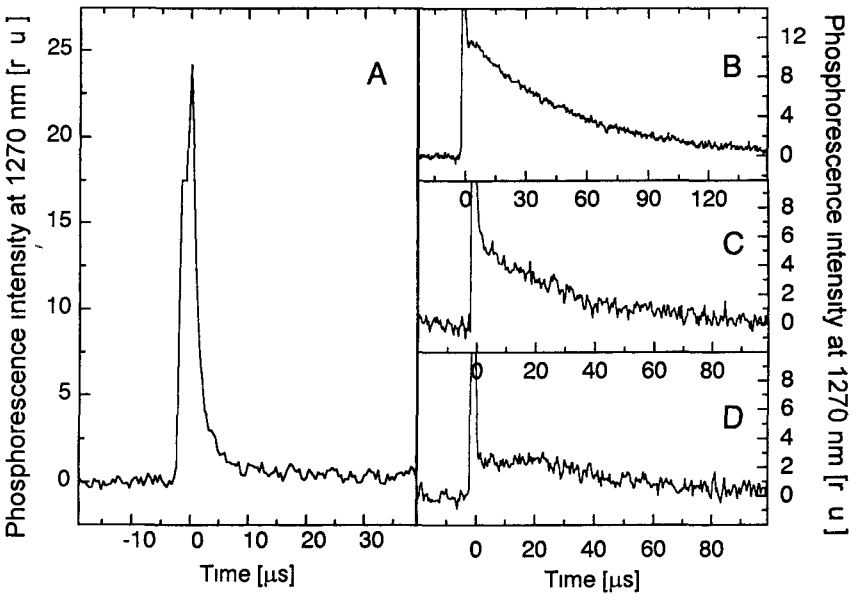

Figure 2. Singlet oxygen production of $TPPS_4$ and liposomal TPP Intensity of singlet oxygen phosphorescence at 1270 nm in relative units (r u) plotted *vs* time related to a laser pulse for *A*) 20 mmol/l $TPPS_4$ added to lecithin liposomes SUV(4 mg/ml) suspended in KP_i D_2O, bubbled with nitrogen, *B*) 1 μmol/l $TPPS_4$ in KP_i-D_2O buffer *C*) approx 1 μmol/l TPP solubilized in liposomes (1 mg lipid/ml) suspended in KP_i D_2O measured without any previous illumination with Xenon lamp, *D*) same as *C*), but after 15 min illumination with Xenon lamp

sult of the fact that singlet oxygen formed during the Xenon lamp irradiation irreversibly interacts with the membrane lipids The decreasing singlet oxygen concentration was also reflected by the reduced integral intensity of phosphorescence decay (Fig 2*C vs D*) Prolonged illumination time led to further decrease in the integral phosphorescence intensity (Fig 3) Thus, after 18 min illumination with the Xenon lamp, the concentration of singlet oxygen in the system decreased by 45%

Direct detection of TPP triplet states was achieved when TPP excited at 412 nm was simultaneously illuminated with a Xenon lamp and the pre and after pulse differences in absorbance at 440 nm (differential absorbance) were recorded The triplet-triplet absorption spectra were constructed from a series of experiments, with the differential absorbance at various time intervals after the excitation pulse plotted against the detection wavelength (Fig 4) The spectra of the liposomal TPP (right panel) show the negative absorption maximum representing the singlet ab-

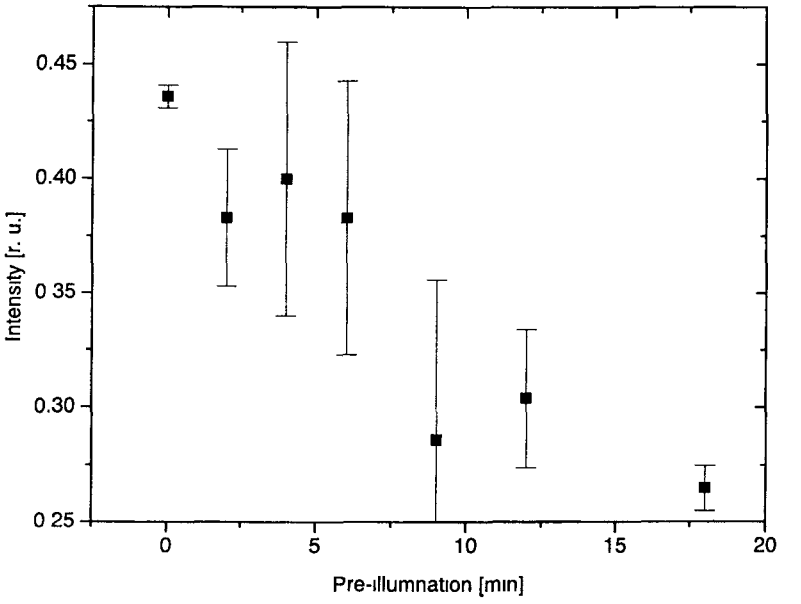

Figure 3. Faster decomposition of singlet oxygen in the liposomal system Integral singlet oxygen phosphorescence intensity in relative units (r u) is plotted vs time of pre-illumination with Xenon lamp Approx 22 mmol/l TPP was dissolved in liposomes (4 mg lipid/ml), suspended in KP_i-D_2O. The points are averages of 4 independent measurements (standard deviations are indicated by the error bars)

sorption of TPP, and a positive peak around 440 nm representing the triplet-triplet absorption. Both the negative and positive peak approach zero with the increasing time interval, due to deactivation of the excited triplet state into the ground singlet state. These results are almost identical to the T-T absorption spectra for $TPPS_4$ (Fig. 4, left panel), as reported previously (Engst et al. 1994).

TPP in liposomes exhibited a decay of triplet-states with the lifetime of 4.8 μs, whereas $TPPS_4$ in aqueous solution yielded 1.9 μs (Fig. 5). The latter value is in good agreement with the previously published data (Engst et al. 1994). The addition of liposomes to $TPPS_4$ solution did not significantly affect the lifetime of its triplet states. TPP in chloroform : methanol (1:1) mixture exhibited triplet state lifetime of 0.49 ms which is similar to the lifetime of $TPPS_4$ in chloroform : methanol (0.43 ms). Measurements in dimethylformamide yielded 0.38 ms ($TPPS_4$) and 0.46 ms (TPP). We also studied the dependence of the triplet-state time constant on the irradiation time (Fig. 6). Similarly as for the signal response of the triplet states derived from 1270 nm phosphorescence after Xenon lamp irradiation (Fig. 2*D*), the

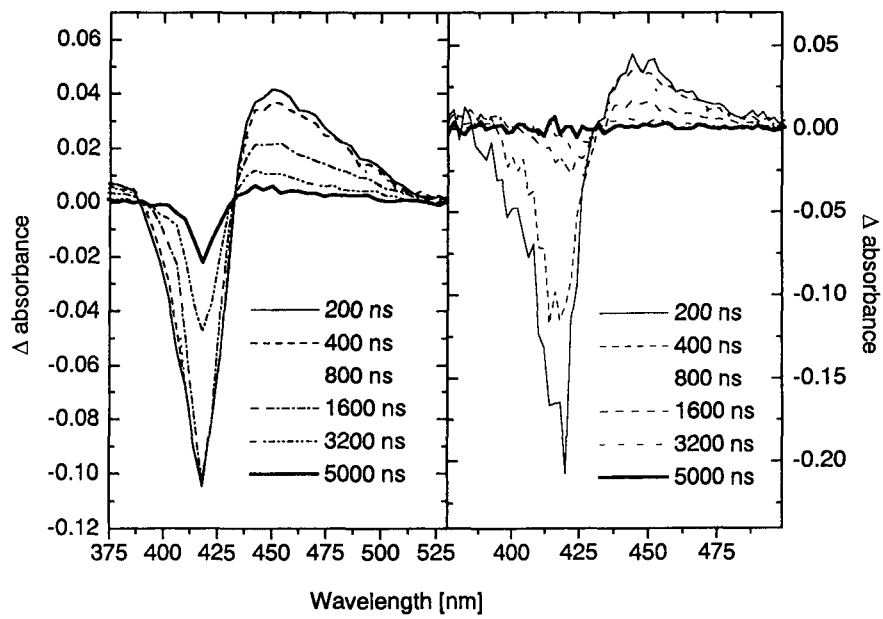

Figure 4. Triplet-triplet absorption spectra of $TPPS_4$ and liposomal TPP *Left panel* 3 μmol/l $TPPS_4$ in aqueous solution; *Right panel:* 2 μmol/l TPP in liposomes (2 5 mg lipid/ml) solubilized in KP_i-H_2O buffer. Samples were irradiated by a pulse dye laser at 412 nm A 250 W Xenon lamp, focused onto the cuvette perpendicularly to the laser beam, was used as the radiation source, while transmitted light was detected by a photomultiplier via monochromator set at 440 nm. The differential absorbance (absorbance before the laser pulse has been subtracted from that after the pulse) has been plotted *vs.* detection wavelength The legend indicates the time after excitation pulse for each trace

directly measured time constant of the triplet states of liposomal TPP shortened by 83% with the increasing irradiation time. In the case of $TPPS_4$, triplet states remained nearly constant (Fig. 6). In these direct measurements of the triplet state kinetics, the irradiation time required for the observed change was less than one minute. But when monitoring 1270 nm phosphorescence, several minutes were necessary. This is because the latter measurement is less sensitive.

Discussion

TPP exhibited basic photophysical properties very similar to the hydrophilic $TPPS_4$. The main parameters were similar, even when comparing aqueous solutions of $TPPS_4$ and TPP solubilized in liposomes. The only difference found was a 10 nm red shift in both absorption (excitation) and fluorescence emission spectra. This

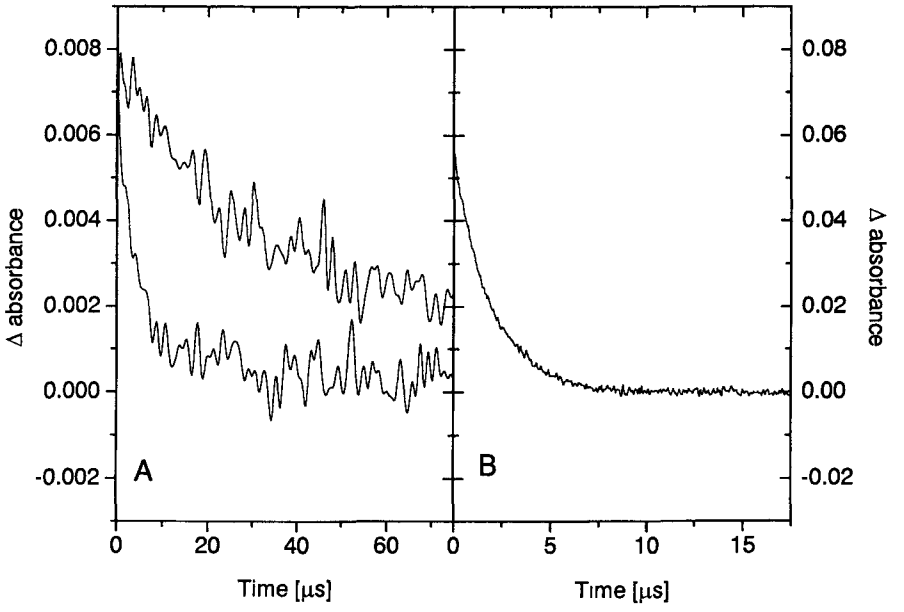

Figure 5. The decay of triplet-states of $TPPS_4$ (A) and TPP in liposomes (B) Measurements were performed as described in Legend to Fig 4 Differential absorbance at detection wavelength 440 nm plotted vs time *A*) bottom – 30 μmol/l TPP in liposomes (0 3 mg lipid/ml) solubilized in KP_i-H_2O, top – the same sample after 150 s irradiation, *B*) 1 μmol/l $TPPS_4$ in KP_i-H_2O Fits using monoexponential model (not shown) yielded time constants of 511,000 s^{-1} for $TPPS_4$, 284,000 s^{-1} for TPP before illumination, and 27,000 s^{-1} for TPP after illumination

might reflect a solvent effect of the phospholipid headgroups. In nonpolar solvents the lifetime of deactivation of higher triplet states was 20% shorter for $TPPS_4$ when compared to TPP. The lifetime of TPP in liposomes was even much longer than for $TPPS_4$ in aqueous solution. The triplet-triplet absorption spectra for $TPPS_4$ and liposomal TPP were also nearly identical.

From our data we can clearly conclude that TPP in lipid environment remained still the efficient singlet oxygen producer. Similarly as $TPPS_4$, TPP in the lipid environment forms sufficient amounts of triplet states (Fig 2). Even being shielded by lipid headgroups, TPP in the membrane interacts with native oxygen, solubilized in the surrounding aqueous space. A proof for singlet oxygen being formed comes from the observed phosphorescence at 1270 nm. Thus, the ability of TPP to produce singlet oxygen in the membrane system is a very important property for its potential use in PDT, and enables its usage in the liposomal form.

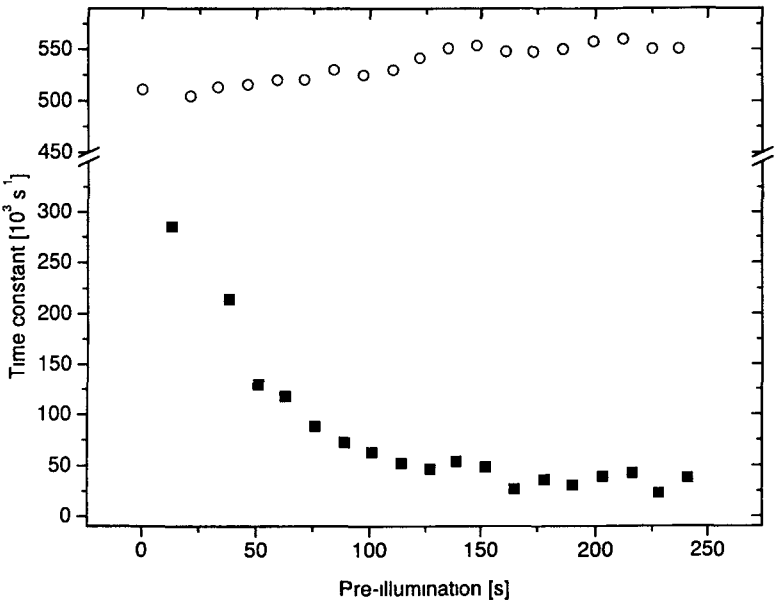

Figure 6. Changes in TPP and $TPPS_4$ triplet state deactivation after sample irradiation Measurements were performed as described in Legend to Fig 5 Time constant (obtained using monoexponential fit) plotted *vs* sample irradiation time Filled squares – 30 μmol/l TPP in liposomes (0 3 mg lipid/ml), solubilized in KP_i-H_2O, open circles – 1 μmol/l $TPPS_4$ in KP_i-H_2O

Moreover, performing experiments with pre-illumination by Xenon lamp, we could demonstrate the reducing decay rates of triplet-states in the membrane system with the increasing pre-illumination time, independent of whether the measurement was direct or indirect. As Fig. 6 clearly shows, the deactivation of higher triplet states was delayed upon illumination. Also, indirect measurements indicated the appearance of the second component in the time resolved luminescence decay at 1270 nm (Fig. 2). The observed faster consumption of singlet oxygen in the liposomal system pointed to the existence of the singlet oxygen interaction with membrane lipids The availability of natural oxygen with time had to be limited, since the triplets formed had longer lifetimes. This decrease in oxygen can be explained only by an irreversible interaction of singlet oxygen formed in the previous cycle with membrane lipids. Thus, upon steady state illumination, oxygen is depleted from near the surface of the membrane system. All these results demonstrate the potential of the liposomal form of TPP to efficiently disintegrate membranes of tumor cells Consequently, we suggest that necessary studies should be conducted with the liposomal form of TPP to allow clinical trials of PDT.

Acknowledgements. This work has been supported by the grant of the Czech Ministry of Health No NC/4810-3 and grant No 312/96/0518 from the Grant Agency of the Czech Republic The purchase of the fluorometer from grant No 94043 of the U S -Czech Science and Technology Program is gratefully acknowledged

References

Engst P , Kubát P , Jirsa M (1994) The influence of D_2O on the photophysical properties of *meso*-tetra(4-sulfonatophenyl) porphine, Photosan III and tetrasulfonated aluminium and zinc phthalocyanines J Photochem Photobiol A Chem **78**, 215—219

Gabizon A , Papahadjopoulos D (1988) Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumours Proc Natl Acad Sci USA **85**, 6949—6953

Jirsa M , Kakáč B (1990) Method of purification and desalting of meso-tetra-(para-sulfofenyl)-porphine Cs Patent P V 7488—78, (in Czech)

Jirsa M , Jirsa M Jr , Kubát P (1996) The effect of sensitizers on the rate constant of photodegradation of bilirubin ditaurate J Photochem Photobiol B **36**, 99—101

Jirsa M Jr , Poučková P , Doležal J , Pospíšil J , Jirsa M (1991) Hyperbaric oxygen and photodynamic therapy in tumour-bearing nude mice Eur J Cancer **27**, 109

Jori G (1989) *In vivo* transport and pharmacokinetic behaviour of tumour photosensitizing properties of phthalocyanines In Photosensitizing Compounds Their Chemistry, Biology, and Clinical Use (Eds G Bock and S Harnett), pp 17—26, John Wiley & Sons, Chichester

Kubát P , Mosinger J (1996) Photophysical properties of metal derivatives of tetrakis (sulphonatophenyl)porphine J Photochem Photobiol A **96**, 93—97

Lang K , Wagnerová D M , Engst P , Kubát P (1992) Influence of protonation on the reactions of triplet state sulphonated chloro-aluminium (III) phthalocyanine with dioxygen J Chem Soc Faraday Trans II **88**, 677–680

Lapeš M , Petera J , Jirsa M (1996) Photodynamic therapy of cutaneous metastases of breast cancer after local application of meso-tetra-(para-sulfophenyl)-porphine ($TPPS_4$) J Photochem Photobiol B **36**, 205—207

Love W G , Duk S , Biolo R , Jori G , Taylor P W (1996) Liposome-mediated delivery of photosenzitizers Localization of Zinc(II)-phthalocyanine within implanted tumours after intravenous administration Photochem Photobiol **63**, 656—661

MacDonald R C , Mac Donald R I , Menco B P M , Takeshita K , Subbarao N K , Hu L-R (1991) Small-volume extrusion apparatus for preparation of large unilamellar vesicles Biochim Biophys Acta **1061**, 297—303

Mosinger J , Mička Z (1997) Quantum yields of singlet oxygen of metal complexes of meso-tetrakis(sulphonatophenyl)porphine J Photochem Photobiol A **107**, 77—82

Renade V V (1989) Drug delivery systems 1 site-specific drug delivery using liposomes as carriers J Clin Pharmacol **29**, 685—694

Van der Meulen F (1996) PDT into the commercial era Int Photodynamics **1**, (5) 1

Wilkinson F , Helman P , Ross A B (1993) Quantum yield for the photosensitized formation of the lowest electronically excited singlet state of molecular oxygen in solution J Phys Chem Ref Data **22**, 113—261

Wilkinson, F , Helman, P , Ross, A B (1995) Rate constant for the decay and reactions of the lowest electronically excited singlet state of molecular oxygen in solution An expanded and revised version J Phys Chem Ref Data **24**, 663—1021

Winkelman J (1962) The distribution of tetraphenylporphine-sulfonate in the tumour-bearing rat Cancer Res **22**, 589—596

Final version accepted April 26, 1999