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

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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 D₂O, when excited with a 28 ns laser pulse at 412 nm. Moreover, unlike with sulfonated TPP (TPPS₄), 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 D₂O, and by direct TPP triplet state monitoring, detecting triplet-triplet absorption at 440 nm in H₂O. 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...
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 photosensitizer 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 photosensitizers 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 (TPPS₄, Winkelman 1962). Jirsa and Kakáč (1990) have synthesized TPPS₄ 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 TPPS₄ 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 TPPS4 were studied, and it has been proven that D2O 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 TPPS4 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 TPPS4- 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 TPPS4 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 buffer (125 mmol/l KCl, 20 mmol/l KPi, pH 7.3) were added and the lipids were hydrated while rotating the vessel. The obtained multilamellar vesicles were freeze/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 D2O

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)
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 D2O 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 TPPS4 in aqueous KP, 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 TPPS4. 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 TPPS4 (650 nm vs. 640 nm for TPPS4).

Fig. 2 illustrates a typical experiment with singlet oxygen produced by the photosenzitizers (TPPS4, Fig. 2B; liposomal TPP, Fig. 2C,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 TPPS4 in KP-D2O 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 (TPPS4, Fig. 2B) and 60 μs (TPP, Fig. 2C,D) represents the mentioned phosphorescence. When the liposomal TPP in KP-D2O was bubbled with nitrogen, no phosphorescence was observed (Fig. 2A). After illumination of the TPPS4 sample with a Xenon lamp for 18 min, the monoexponential decay was retained and the
Figure 1. Absorption, excitation and emission spectra of TPPS₄ and TPP in liposomes. 

A) Absorption spectra, B) fluorescence excitation (left) and emission spectra of TPP dissolved in lecithin liposomes – SUV (solid lines) and TPPS₄ in KP₇ buffer (dotted lines) were measured as described in Materials and Methods. Approx. 20 µmol/l TPP and exactly 1 µmol/l TPPS₄ 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. 2C vs. 2B). However, when illuminating of the TPP sample with the Xenon lamp for 18 min, the monoexponential decay became bi-phasic (Fig. 2D). 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-
result 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 2C 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 KP₂-D₂O. The points are averages of 4 independent measurements (standard deviations are indicated by the error bars).

Absorption 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 TPPs₄ (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 TPPs₄ 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 TPPs₄ 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 TPPs₄ in chloroform : methanol (0.43 ms). Measurements in dimethylformamide yielded 0.38 ms (TPPs₄) 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. 2D), the
Figure 4. Triplet-triplet absorption spectra of TPPS₄ and liposomal TPP. Left panel: 3 μmol/l TPPS₄ in aqueous solution; Right panel: 2 μmol/l TPP in liposomes (2.5 mg lipid/ml) solubilized in KP₇-H₂O 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 TPPS₄, 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 TPPS₄. The main parameters were similar, even when comparing aqueous solutions of TPPS₄ 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 TPPS₄ (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 KP₁-H₂O, top - the same sample after 150 s irradiation, B) 1 μmol/l TPPS₄ in KP₁-H₂O. Fits using monoexponential model (not shown) yielded time constants of 511,000 s⁻¹ for TPPS₄, 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 TPPS₄ when compared to TPP. The lifetime of TPP in liposomes was even much longer than for TPPS₄ in aqueous solution. The triplet-triplet absorption spectra for TPPS₄ 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 TPPS₄, 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 TPPS₄ 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 KP₂H₂O, open circles – 1 μmol/l TPPS₄ in KP₂H₂O.

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.
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