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Invited Paper

Kinetic- and localization-properties of protoporphyrin dimethyl ester in fibrosarcoma cells

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ABSTRACT

Protoporphyrin-dimethylester (PP) is an amphiphilic porphyrin which shows a fast clearence and is therefore of interest for the photodynamic therapy. The localization of the sensitizer is one point of interest, since it has a strong influence on the phototoxic effect.

Localization of PP in fibrosarcoma cells was probed by time resolved and steady state spectroscopy. Dioleoyl L-α-phosphatidylcholine (DOPC) liposomes were used as a carrier system for the amphiphilic sensitizer with a molar ratio of PP/DMSO = 1/200. Since intermembrane exchange is responsible for the uptake of PP we expected a membrane bound localization. Therefore the characterization of the microenvironment was based on the comparison of the spectroscopic properties of PP in organic solvents, artificial membranes and in cells. Cells were incubated with a concentration of $3.5 \cdot 10^{-7}$ M in FCS free medium at T = 37°C for 4h.

Aprotic solvents show a bathochromic shift of the Soret-band with increasing dielectric constant ϵ whereas the Q-bands exhibit no spectral shift within the instrumental resolution ($\Delta\lambda$=3nm). Additionally, the fluorescence decay time of the monomer (τ=(11.5±1.0)ns) remains constant. However, in aprotic solvents like dimethylsulfoxide, 1-propanol or ethylenglycol which exhibit an amphiphilic character we observed no spectral shift of the Soret-band with increasing ϵ but an increase in the fluorescence decay time (τ=(16.8±1.2)ns). This decay time of PP is within the error margin in agreement with its monomer decay time in artificial membranes (τ=(16.8±1.0)ns) and in single cells (τ=(15.3±0.4)ns). Based on this comparison we suggest that PP is bound within the amphiphilic part of the membranes i.e. between the polar headgroups and the fatty acyl chains of the cytoplasm membrane.

1.INTRODUCTION

Photosensitive drugs as for example porphyrins have caused considerable interest in the photodynamic therapy of cancer.[1] Their efficiency is determined by their photophysical and photochemical properties as well as their specificity and localization of the photosensitizer. The distribution of photosensitizers within the neoplastic tissue depends on their transport properties. Since the sensitizers are assumed to react via the type II singlet oxygen mechanism, and the lifetime restricts the reactivity to a distance of approximately 100 nm,[1] the site of the photodynamic action is strongly influenced by the localization of the sensitizer.

Liposomes are of considerable interest as carriers for lipophilic photosensitizers. It has been shown that the transport in liposomal vesicles results in a larger and more selective transport.[2] Protoporphyrin dimethyl ester (PP) packed into L-α-phosphatidylcholine dioleoyl (DOPC) liposomes exhibits good tumor localizing properties (Wessels and Sroka, unpublished results), whereas protoporphyrin itself as a tumor localizing agent was not convincing.[3] This paper therefore presents kinetic and localization studies on fibrosarcoma cells incubated with a liposomal suspension of PP using steady state and time-resolved fluorescence spectroscopy.

2. MATERIALS AND METHODS

2.1 Chemicals

The organic solvents chloroform ($CHCl_3$) (ϵ = 4.8*, η = 0.58 mPas*), tetrahydrofuran (THF) (ϵ = 7.4*, η = 0.47 mPas*), acetone (ϵ = 20.7**, η = 0.31 mPas**), methanol (MeOH) (ϵ = 32.6**, η = 0.52 mPas*), acetonitrile (ϵ = 37.5*, η = 0.39 mPas*), dimethyl sulfoxide (DMSO) (ϵ = 48.9*, η = 1.98 mPas*), 1-propanol (ϵ = 20.1**, η = 2.75 mPas**) and ethylene glycol (ϵ = 37.7**, η = 21.0 mPas**) were obtained from Merck (Germany) and used without further purification (* T = 20 °C and ** T = 25 °C).[4] After column chromatography the

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purification grade of protoporphyrin dimethyl ester (PP) (Sigma, Germany) was 95%, which was verified by HPLC. L-α-phosphatidylcholine dioleoyl (DOPC) (Sigma, Germany) was used without further purification. PP loaded DOPC-liposomes were prepared with a molar ratio of PP:DOPC = 1:200, according to the protocol of Richert.[5]

2.2 Cell cultivation

Cultures of fibrosarcoma cells (SSK II) were grown in Petri dishes in Earle's MEM with 5% foetal calf serum (FCS). 250000 cells were seeded 24 h before incubation with the liposomal sensitizer suspension. For uptake studies the cells were incubated at T = 37 °C, T = 4 °C or at T = 10 °C without or with 5% FCS in the dark with a PP concentration of 0.35 μM in Earle's MEM. For retention studies, cells were incubated for 4h in a FCS free incubation medium. After incubation, cells were washed twice with PBS and reincubated in medium with 5%- or 10%-FCS. Upake and retention studies were done with a FACSStar$^+$ flow cytometer (Becton Dickenson). Fluorescence of PP as well as of polysterol beads (fluorescence standard particles) was excited at $\lambda = 488$ nm and detected at $\lambda > 590$ nm. For localization studies, cells were incubated for 4 h in FCS-free medium with 0.35 μM PP at T = 37 °C and 5% CO_2. The measurements were performed immediately after washing with phosphate buffered solution (PBS).

2.3 Absorption and steady state fluorescence measurements

The absorption measurements of the solutions (c = 3.5 μM) were performed using a spectrophotometer (Perkin Elmer, Lambda 5 UV/VIS) with a spectral resolution of $\Delta\lambda = \pm 1$ nm. Fluorescence spectra of the solutions (c = 0.35 μM) were measured with a spectrofluorimeter (Shimadzu, Kyoto, Japan) in the wavelength region between $\lambda = 600$ nm and $\lambda = 700$ nm. The spectral resolution of the instrument was $\Delta\lambda = \pm 2$ nm.

2.4 Time-resolved fluorescence measurements

Excitation pulses were provided by a tandem laser system, consisting of a dye laser which was synchroneously pumped by a modelocked argon ion laser (Spectra Physics Model 2040E, USA). The pulse repetition frequency was reduced by means of a cavity dumper to 400 kHz. Stilbene 3 was used as a laser dye and the wavelength was set at $\lambda = 420$ nm. At this rate autocorrelation traces showed a pulse duration of approximately 10 ps. The pulses were directed into an epifluorescence microscope (Leica, Germany). Fluorescence emission was detected with a photomultiplier (Hamamatsu R928, Japan) and a time-correlated single-photon counting apparatus.[6] The fluorescence emission was detected above $\lambda = 600$ nm. The fluorescence data were analysed using a non-linear least-square algorithm. The error of the measurement is given by the standard deviation of 10 measurements.

3.RESULTS

3.1 Kinetic measurements

Figure 1 shows the intracellular concentration of PP as a function of the incubation time for cells incubated at T = 37 °C and at T = 4 °C in either a serum free liposomal suspension of PP (c = 0.35 μM) (a) or at T = 37 °C and at T = 10 °C in the presence of 5% FCS (b).

The errors of the measurements are given by the standard deviation from 10 measurements. Within the error margin the uptake of PP in a serum free incubation medium at T = 37 °C and at T = 4 °C is comparable, whereas in the presence of serum the uptake at T = 37 °C is significantly different compared to the uptake at T = 10 °C. Additionally, in the presence of proteins the uptake of PP seems to be more effective but significantly slower compared to the uptake in a protein free incubation medium.

Figure 2 shows the remaining intracellular PP concentration as a function of the retention time in medium with 5%- or 10%-FCS, respectively. The cells were incubated at T = 37 °C for 4h in a serum free liposomal suspension of PP (c = 0.35 μM).

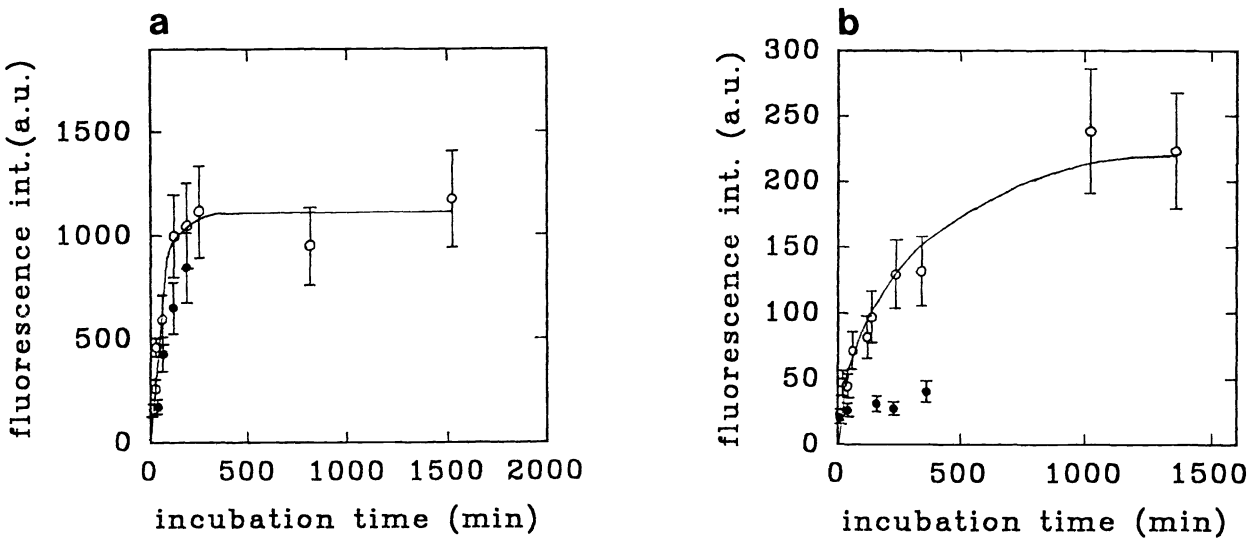

Figure 1: Intracellular concentration of PP as a function of the incubation time in FCS free medium at T = 37°C (o) and T = 4°C (●) (a) as well as in the presence of 5% FCS at T = 37°C (o) and T = 10°C (●) (b).

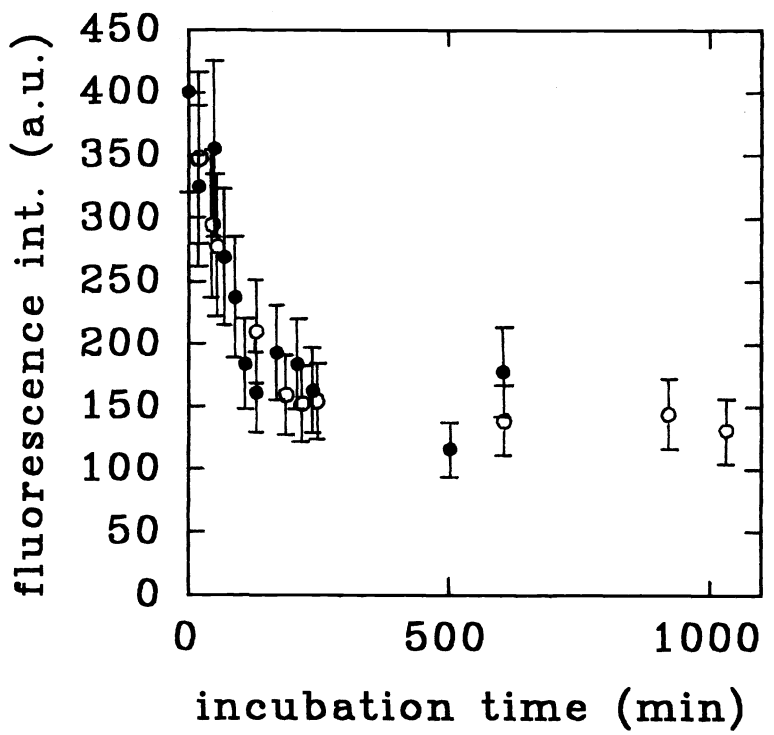

Figure 2: Intracellular concentration of PP as a function of the retention time in the presence of 5%-(●) or 10%-FCS (o).

3.2 Localisation measurements

Steady-state and time-resolved fluorescence measurements of PP in organized environments like liposomes and cells as well as in organic solvents with varying polarity and viscosity were performed in order to characterize the cellular microenvironment of PP. The organic solvents, their dielectric constants and viscosities are listed in 2.1.

Figure 3 shows the wavelength of the absorption maxima as a function of the polarity of the organic solvent. The error of the steady-state measurements is given by the wavelength resolution of the instrument. With three exceptions we observed a hypsochromic shift of the Soret-band with increasing polarity of the solvent. This can be attributed to changes in the non-specific dipol-dipol interactions with increasing polarity. In DMSO, 1-propanol and ethylene glycol the peak position of the Soret-band remains constant. Within the spectral resolution of the instrument, no shifts in the peak positions of the Q-bands could be detected.

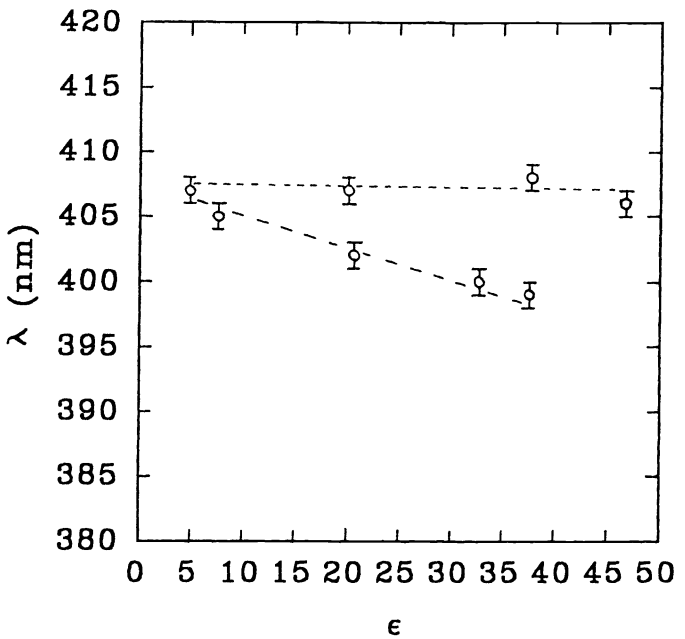

Figure 3: Peak-position of the Soret-band of as a function of solvent polarity (c = 3.5 μM).

Within the resolution of the instrument no changes with increasing polarity in the peak positions of the Q-bands could be observed. Since the fluorescence emission takes place from the lowest excited singlet state, this result agrees with the fluorescence emission measurements where no changes in the peak position of the 0-0 and 0-1 fluorescence emission bands could be detected. The peak positions of the 0-0 fluorescence emission band varied between 633 nm and 636 nm.

In Figure 4 the fluorescence emission spectra of PP in $CHCl_3$ and liposomes (a) as well as in cells after incubation in FCS-free medium for 4h with c = 0.35 μM (b) are shown, respectively. In order to detect the fluorescence from a confluent cell monolayer these spectra were recorded with an optical multichannel analyzer (O-SMA-Systems Spectroscopy, Gilching, Germany).

PP fluorescence was excited with the blue lines (λ = 407.5 nm and λ = 417.5 nm) from a Kr^+-laser. The resolution of the instrument was $\Delta\lambda = \pm$ 3 nm. The 0-0 transition in the emission spectrum of PP in liposomes and in cells peak at 634 nm and 635 nm, respectively. The background in the emission spectrum of cells is caused by autofluorescence. The small peak at approximately 671 nm can be attributed to the hydroxyaldehyde photoproduct isomers that are generated upon illumination of PP.[7,8]

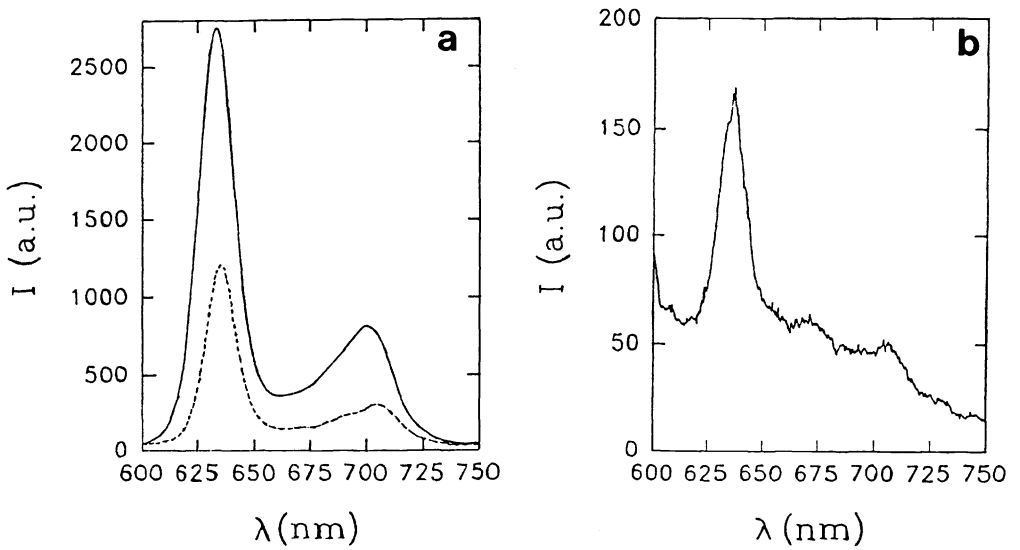

Figure 4: Fluorescence emission spectrum of PP in $CHCl_3$ (---), in DOPC-liposomes(- -) (c = 0.35 μM) (a) and in SSKII cells (incubated for 4h in a FCS free liposomal suspension with c = 0.35 μM) (b).

The time dependence of the PP fluorescence was detected at $\lambda_{det} > 600$ nm and is summarized in Table 3 for organic solvents, liposomes and SSKII-cells. In organic solvents two components could be discriminated. There was no evidence for aggregation. Besides the long decay time which can be attributed to the monomeric PP, an average decay time of τ_2 = (4.6 $\pm$ 0.6) ns was observed. This decay time lies in the range of the fluorescence life time of chlorines in organic solvents and is attributed to the hydroxyaldehyde product.[7] The photoproduct was detected with an integral part of I_2 = (8 $\pm$ 2) %. Within the error margins the decay times of the monomer τ_3 = (11.6 $\pm$ 0.9) ns did not vary with increasing solvent polarity. In 1-propanol, DMSO and ethylene glycol an increase in the decay time to τ_3 = (16.0 $\pm$ 1.1) ns for the PP-monomer was observed.

microen-vironment	τ_1(ns)	τ_2(ns)	τ_3(ns)
solvent type 1	-	4.4 $\pm$ 0.4	11.6 $\pm$ 0.9
solvent type 2	-	5.1 $\pm$ 0.8	16.0 $\pm$ 1.1
liposome	0.9 $\pm$ 0.6	5.3 $\pm$ 0.4	16.8 $\pm$ 1.0
cell	0.5 $\pm$ 0.2	4.2 $\pm$ 0.4	15.3 $\pm$ 1.8

Table 1: Time dependence of PP fluorescence in organic solvents type 1: $CHCl_3$, THF, acetone, MeOH, acetonitrile and type 2: 1-propanol, ethylene glycol, DMSO (c = 0.35 μM), liposomes (c = 0.35 μM) and SSKII-cells (incubated for 4h in FCS-free medium with c = 0.35 μM).

In liposomes and in cells (incubation with PP for 4h in FCS-free medium, c = 0.35 μM) we obtained three different decay times. Besides the PP-monomer and the photoproduct a short component was detected additionally with an average fluorescence-decay time of τ_1 = (0.9 ± 0.6) ns in liposomes and τ_1 = (0.5 ± 0.2) ns in cells and an average integral part of I_1 = (2 ± 1) % in liposomes and I_1 = (14 ± 6) % in cells. This could be attributed to an oligomeric fraction of the sensitizer. The integral part of the photoproduct contributed with I_1 = (14 ± 2) % in liposomes and with I_1 = (21 ± 4) % in cells to the fluorescence decay. Within the error margin, the average fluorescence decay time of the PP-monomer in cells (τ_3 = (15.3 ± 1.8) ns) and in liposomes (τ_3 = (16.8 ± 1.0) ns) are in agreement.

4. DISCUSSION

4.1 Kinetic measurements

According to Muir et al.[9] receptor mediated endocytosis and pinocytosis are inhibited at T = 4 °C. Since in FCS free incubation medium the uptake at T = 4 °C and at T = 37 °C are comparable within the error margin, unspecific pinocytosis or receptor mediated endocytosis can be excluded as mechanisms for the uptake of PP. A fusion controled uptake can be excluded, since fusion promoting lipids are necessary for this pathway. Therefore, a diffusion controled uptake mechanism was adopted for the uptake of PP . Essential for an interaction between both phases is a close approach of liposomes and cells.

For the evaluation of the time constants, the measurements were fitted with a single exponential approach, assuming that the intracellular concentration of PP is proportional to the fluorescence intensity

$$c_{intra} = c_0(1 - e^{-kt}).$$

c_{intra} is the intracellular PP concentration after the incubation time t, c_0 the concentration in the incubation medium and k the time constant. 50% of the maximal intracellular PP fluorescence intensity is reached after $t_{1/2}$ = (42 ± 14) min. In the case of liposome bound substances, the time constant for photosensitizers taken up by a diffusion controled pathway is influenced by the strength of the interaction between sensitizer and lipid and the probability of the substance to diffuse from the liposomes into the cells through the hydrophilic phase. There are significant differences in the kinetic properties of lipophilic sensitizers. For example the two lipophilic porphycences 2-hydroxyethyl-7,12,17-tetrakis(methoxyethyl)porphycene (HEPn) and 9-acetoxy-2,7,12,17-tetrapropylporphycene (ATPPn) exhibit in SSKII cells under the same incubation conditions and the same liposomal formulation time constants of $t_{1/2}$ = (2.3 ± 0.3) min and $t_{1/2}$ = (780 ± 480) min, respectively.[10]

In the presence of proteins the uptake of the sensitizer is slower compared to the uptake in serum free medium, whereas the intracellular PP concentration at the saturation level is about twice as high. From the measurements we obtained a time constant of $t_{1/2}$ = (213 ± 57) min. According to Gregoriadis,[11] the interaction of proteins and liposomes result in a moistening of the liposomes. On the one side this offers the possibility of a receptor mediated endocytosis of the opsonated liposomes. On the other side the proteins can simply increase the diffusion barrier between liposomes and cytoplasm membrane causing a slower uptake. Since the uptake at T = 10 °C is inhibited in the presence of serum proteins, it can be concluded that a receptor mediated pathway is responsible for the uptake of PP. The small remaining uptake at T = 10 °C might result from the uptake during the time interval the cells need for adjustment to the incubation temperature.

Within the resolution of the measurements there was no evidence for an influence of the serum concentration in the medium on the retention time of PP.

4.2 Localisation measurements

Based on the assumption that PP is taken up on a diffusion-controled pathway in protein-free medium and that specific or non-specific interactions with aprotic solvents show an influence on the luminescence properties of molecules[12], steady-state and time-resolved fluorescence measurements of PP in organized environments like liposomes and cells as well as in organic solvents with varying polarity and viscosity were performed in order to characterize the

cellular microenvironment of the sensitizer. The cellular membrane exhibits a dielectric constant of $\epsilon = 4$ within the hydrocarbon bondings and of $\epsilon = 8$ including the regions of the polar headgroups.[13]

With three exceptions we observed a hypsochromic shift of the Soret-band with increasing polarity of the solvent which can be attributed to changes in the non-specific dipol-dipol interactions with increasing polarity. This shift could be a result of a more pronounced stabilization of the ground state. Within the spectral resolution of the instruments no shifts in the peak positions of the Q-bands were observed. Therefore we assume that the sterical effects caused by an increase of the solvent polarity are comparable in the ground- and in the first excited singlet state. Since the fluorescence emission takes place from the lowest excited state, this is concomitant with the fact, that no significant changes in the peak positions of the 0-0 and 0-1 fluorescence emission bands could be observed with increasing polarity. Additionally, the fluorescence decay time of $\tau_3 = (11.6 \pm 0.9)$ ns with an integral part of $I_3 = (92 \pm 2)\%$ was found to remain constant.

In DMSO, 1-propanol and ethylene glycol, solvents which exhibit a somewhat higher viscosity, the peak position of the Soret-band in the absorption spectrum of PP remained constant and was comparable with the peak position in $CHCl_3$. This is direct evidence that in these solvents PP is somehow protected against sterical effects of solvents responsible for the hypsochromic shift. Additionally, we observed an increase of the fluorescence decay time in these solvents from $\tau_3 = (11.6 \pm 0.9)$ ns to $\tau_3 = (16.0 \pm 1.1)$ ns. Changes in the viscosity of a solvent could cause a reduced fluorescence quenching of the first excited singlet state and therefore an increase of the decay time. Since within the error margins no changes in the fluorescence decay time in DMSO ($\eta = 1.98$ mPas at $T = 37°C$) and ethylene glycol ($\eta = 21$ mPas at $T = 20°C$) were observed, the somewhat higher viscosity η of these solvents seems to be not responsible for the enhanced decay time. This is an additional hint that these solvents might protect PP from solvent substrate interactions. We attributed the increase of the monomeric decay time and the observations from the absorption measurements to the solvent mediating character of DMSO, 1-propanol and ethylene glycol which might result in the formation of a solvent cavity.

In liposomes and in cells (incubation with PP for 4h in FCS free medium $c = 1\ \mu M$) the average fluorescence decay time of the PP-monomer in cells ($\tau_3 = (15.3 \pm 1.8)$ ns)) and in liposomes ($\tau_3 = (16.8 \pm 1.0)$ ns) are in agreement within the error margin. Since the luminescence properties of PP in liposomes and in cells are comparable, we assumed that their microenvironment is comparable. As already suggested from Kessel[14] and Richelli[15] PP might exhibit a membrane bound localization. Since the fluorescence decay time of PP in organized environments as well as in DMSO, 1-propanol and ethylene glycol are comparable, the dynamic behaviour of the radiationless transitions of the PP-monomer in both microenvironments can be also assumed to be comparable. We therfore concluded that PP might exhibit a membrane bound localization within the solvent mediating part of the lipid bilayer, whereas Kessel and Richelli suggested a localization within the hydrocarbon bondings. Richelli at al.[14] investigated the microenvironment of PP in L-α-phosphatidylcholine dipalmitoyl (DPPC). They found at concentrations below $0.5\ \mu M$ (i.e. approximately 20 PP-molecules per liposome) a preferential distribution of PP within the hydrocarbon bondings, whereas at higher concentrations the outer monolayer also becomes populated, which might be the case for our liposomes. At a molar ratio of PP/DOPC = 1/200 we have approximately 60 PP-molecules per liposome.[16] Additionally, differences in the fluidity of DOPC and DPPC caused by a different amount of unsaturated double bonds might be responsible for differences in the microenvironment. However, we were able to show that time-resolved fluorescence spectroscopy is a highly sensitive method to study intracelluar localizations of photosensitizers in single living cells.

Photosensitizers taken up on a diffusion controled pathway exhibit a membrane bound localization. The pathway of the uptake from liposome-bound substances can be influenced in the presence of proteins in the incubation medium. In the case that the probability for an interaction between proteins and liposomes is higher compared to the interaction between liposomes and cytoplasm membrane, opsonated liposomes are taken up by receptor mediated endocytosis.

5. ACKNOWLEDGMENT

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