[1]University of Oslo, School of Pharmacy, Department of Pharmacy – Division of Pharmaceutics, PCI Biotech AS[2], Oslo, Norway

Evaluation of physicochemical properties and aggregation of the photosensitizers $TPCS_{2a}$ and $TPPS_{2a}$ in aqueous media

M. Lilletvedt[1], H. H. Tønnesen[1], A. Høgset[2], S. A. Sande[1], S. Kristensen[1]

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Marianne Lilletvedt, School of Pharmacy, Department of Pharmacy, University of Oslo, P.O. box 1068 Blindern, N-0316 Oslo, Norway
marianne.lilletvedt@farmasi.uio.no

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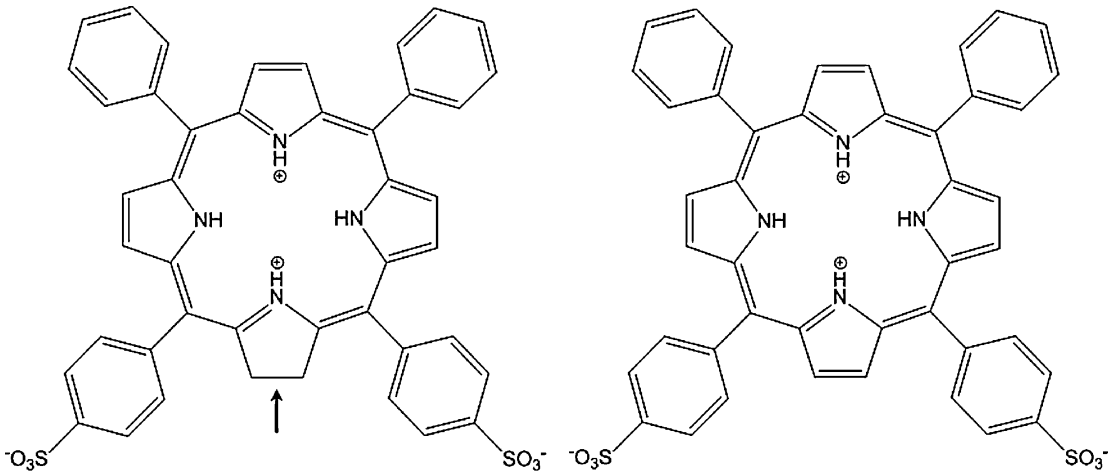
Physicochemical properties of the novel photosensitizer meso-tetraphenyl chlorin disulphonate ($TPCS_{2a}$) and the chemically related meso-tetraphenyl porphyrin disulphonate ($TPPS_{2a}$) were investigated in aqueous solutions as part of pharmaceutical preformulation. Inflection points were calculated to be 3.9 for both compounds based on spectral shifts of aqueous solutions in the pH range 2-12, which likely correlate with indistinguishable pK_a values of the imino nitrogens of the molecular cores. Accordingly, the fluorescence emission spectra showed pH dependent spectral shifts. Porphyrin-like compounds are known for aggregation in aqueous environments, and a small percentage of Tween 80 (0.006 % v/v = 4 x cmc) seemed to stabilize the aqueous samples of the two photosensitizers through hindrance of aggregation. The distribution coeffient of $TPCS_{2a}$ determined spectrophotometrically in 1-octanol/water is 0.4 (± 0.4 SD) and 1.5 (± 0.5 SD) for the reference $TPPS_{2a}$. This confirms amphiphilicity which indicates preferred distribution and further restrain of diffusion in membranes, which is relevant for the use of $TPCS_{2a}$ as a photosensitizer in the process of photochemical internalization *in vivo*.

1. Introduction

The novel photosensitizer meso-tetraphenyl chlorin disulphonate ($TPCS_{2a}$; Fig. 1A) is used in the patented technology of photochemical internalization (PCI) (Norum et al. 2009a; Berg et al. 2006; Høgset et al. 2004). PCI is at present applied in a clinical trial (phase I/II) concerning the medical treatment of cancer. Site-specific drug delivery is achieved by administering the photosensitizer, $TPCS_{2a}$, and a conventional antineoplastic agent, at present bleomycin, in addition to tumour-specific irradiation with red light (Berg et al. 1999). In detail, the PCI technology comprises first endocytosis of the photosensitizer and the chemotherapeutic by target cells. $TPCS_{2a}$ remains in the membrane of the endocytic vesicles, while bleomycin is trapped in the inside of the vesicles. During tumour illumination, $TPCS_{2a}$ will absorb red light which is followed by creation of reactive oxygen species (ROS), primarily singlet oxygen ($^1O_2$). The highly reactive $^1O_2$ has a short lifetime, 0.01–0.04 μs, and the range of action is only 10–20 nm (Moan and Berg 1991). Therefore, $TPCS_{2a}$ only acts in the membrane of the vesicles. Intact bleomycin is released before lysosomal degradation and can reach its intracellular target (Berg et al. 2005). In total, PCI promotes site-specific cellular delivery and consequently more efficient utilization of membrane-impermeable drugs, like bleomycin (Norum et al. 2009b). As a result, reduced side effects are obtained.

The chemically related photosensitizer, meso-tetraphenyl porphyrin disulphonate ($TPPS_{2a}$; Fig. 1B), is used as a reference substance in this study. The tetrapyrrole nucleus present in both $TPCS_{2a}$ and $TPPS_{2a}$ consists of two different sorts of nitrogen atoms, i.e., imino (-N=) and pyrrole (-NH-). The two imino nitrogens are capable of accepting protons, while the two pyrrole nitrogens can in theory act as proton acceptors and donors (Phillips 1963). Therefore, the core of these porphyrin-type molecules may undergo several protonation steps involving both nitrogen types (Cunderlikova et al. 2001). However, deprotonation of the pyrrole nitrogens is presumed to occur only at extreme alkaline conditions, i.e., pH > 14 (Clarke et al. 1973; Cunderlikova et al. 2001). In addition, considering steric, electrostatic and resonance energy the effective basic centres are limited to the two imino nitrogens (Phillips 1960). Thus, in this study protonation of the imino nitrogens was investigated. $TPPS_{2a}$ and $TPCS_{2a}$ are both dianionic in the extended pH area relevant to this study (pH 2–12), due to their two sulphonate groups which have pK_a values ≤ 1 (Pasternack 1972; Maiti et al. 1998). The free base of $TPPS_{2a}$ and $TPCS_{2a}$ will therefore be dianionic (Lilletvedt et al. 2010a, Pasternack 1972). The absorption spectra of free base chlorins (e.g., $TPCS_{2a}$) and porphyrins (e.g., $TPPS_{2a}$) are characterized by a very intense band around 400 nm (the Soret band) and four additional minor absorption bands in the visible region (Q bands; Buchler 1978). The chlorins possess a characteristic Q band in the red part of the spectrum, which is more intense than the corresponding band of the porphyrins, and which is utilized in PCI technology (Sternberg et al. 1998; Gouterman 1979; Berg et al. 1999). A more detailed description of the spectroscopic properties of $TPPS_{2a}$ and $TPCS_{2a}$ has been published (Lilletvedt et al. 2010a, b).

$TPCS_{2a}$ and $TPPS_{2a}$ are rigid molecules, which in previous studies showed limited interactions with the surroundings in both the ground state (S_0) and the first excited state (S_1*; Lilletvedt et al. 2010a, b). In this study, the ionization constants (pK_a) and



(A) (B)

Fig. 1: Structural formulas of the chemically related compounds $TPCS_{2a}$ (A) and $TPPS_{2a}$ (B). Both compounds are presented in the protonated form relevant for this article. $TPCS_{2a}$ consists of three different stereoisomers, depending on the location of the double bonds in the molecular core

distribution coefficients (log D) of $TPCS_{2a}$ and $TPPS_{2a}$ were evaluated as these are crucial properties for cellular uptake and localization *in vivo*, i.e., physiological effect, and in further drug development and formulation. For determination of pK_a, several methods are reported, like UV spectroscopy (Asuero et al. 1986), potentiometric titration (Rosenberg and Wagenknecht 1986) and HPLC techniques (Gonzalez 1993). UV-Vis and fluorescence spectroscopy were chosen in the present work as the compounds contain chromophores that change with the extent of ionization. Aqueous media were made without presence of organic cosolvents, which can have profound effects on the pK_a values (Prankerd 2007). The ionic strength which may influence the measured pK_a, was kept constant during the measurements. Hydrophobicity is known to be a main determinant of the affinity of porphyrins or chlorins for lipid membranes (Ricchelli 1995), and the membrane localization of the photosensitizer is crucial in the PCI technology. In pharmaceutical studies, 1-octanol/water has been implemented as a model for determination of partition (log P) and distribution (log D) coefficients (Leo et al. 1971; Steele 2004). The most commonly used method is the shake flask method, which provide a direct experimental determination of log P in the range between –2 and 4 (OECD 1995), and was the method of choice in the present study. The concentration of the compound should be analyzed in both phases (OECD 1995, OECD 2004), and was performed by UV-Vis photometry in this study.

Porphyrin-related compounds dissolved in aqueous solutions tend to form aggregates in general, e.g., at $c = 10^{-4}$ M (Poderys et al. 2004), and dimers at low concentrations, e.g., $c < 10^{-6}$ M (Brown et al. 1976). This is especially recognized for synthetic porphyrins with anionic side chains and for para-substituted compounds e.g., $TPPS_4$ and $TPPS_3$ (Brown et al. 1976; Pasternack et al. 1972; Sutter et al. 1993). It is the planarity and the large surface area that favour stacking of these compounds (Rubires et al. 1999). Porphyrins have been shown to dimerize in neutral solutions, e.g., $TPPS_4$, but in acidic solution the compounds aggregate more extensively (White 1978). The aggregates may be of different types like edge-to-edge (J-aggregates) or face-to-face (H-aggregates; Ribo et al. 1994), as shown in Fig. 2. Addition of surfactants (Pottier and Kennedy 1990), like the non-ionic Tween 80, is used to disaggregate the compounds, since photosensitizer aggregation in general leads to reduced photoreactivity (Krasnovsky et al. 1990). As part of the present work, possible aggregation processes that might be revealed were evaluated. This provides valuable knowledge for further drug formulation work in relation to the PCI technology.

2. Investigations, results and discussion

2.1. Determination of spectral inflection points as an estimate of pK_a values

UV-visible absorption spectra of aqueous solutions of the photosensitizers $TPCS_{2a}$ and $TPPS_{2a}$ at different pH values (pH 2-6) are shown in Figs. 3 and 4. The spectral changes obtained upon acidification are indicated by arrows. Even though the concentration of $TPCS_{2a}$ and $TPPS_{2a}$ used is low ($c = 2 \times 10^{-6}$ M), aggregation cannot be excluded in these experiments. Thus, the

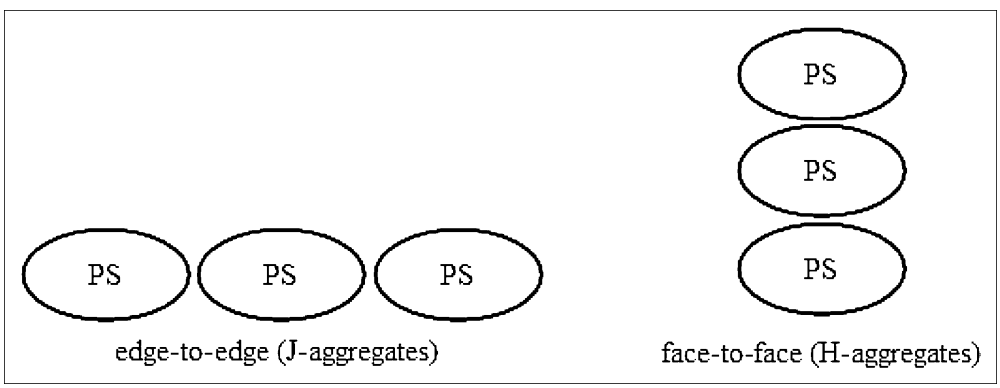


Fig. 2: Possible aggregation types of porphyrin-like photosensitizer molecules (PS). Edge-to-edge (J-aggregates) and face-to-face (H-aggregates) aggregates are presented. The figure is adapted from Ribo et al. (2004)

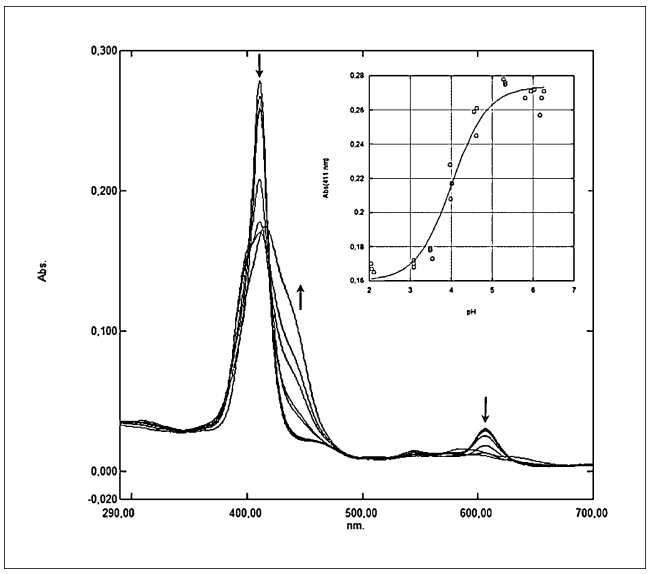


Fig. 3: Absorption spectra of aqueous samples of $TPCS_{2a}$ in the pH range 2.1-6.2. The arrows indicate spectral changes observed from neutral to acidic samples. Inset: example of plot for $TPCS_{2a}$ showing curve fitting for absorbance (Abs) at 411 nm as a function of pH (chi-square (χ^2) = 0.0015). The pH of the samples was 2.1; 3.1; 3.5; 4.0; 4.6; 5.3; 5.9; and 6.2, respectively. The data is given by circles (◯), while the solid line is the fitted curve

term inflection point is used to denote the acidity constants. The selected wavelengths used for the determination of the inflection points were 411, 444, and 606 nm in the case of $TPCS_{2a}$ and 399, 435 and 649 nm for $TPPS_{2a}$. For the latter compound, there is a decrease in absorbance at 399 nm upon acidification with a concomitant absorbance increase at 435 nm. This is assumed to correspond to the conversion of the porphyrin $TPPS_{2a}$ from the dianionic free base (i.e., ionized sulphonate groups) to the zwitterionic diprotonated form where both the porphyrin core and sulphonate groups are ionized. This in accordance with the spectral changes published for the porphyrins, $TPPS_3$ (Pasternack 1972) and THPP (Guo 2008) upon protonation. The same trend is also observed for the chlorin $TPCS_{2a}$, where a decrease in pH leads to decrease in absorbance at the Soret band at 411 nm and a concomitant increase at 444 nm. Further, $TPPS_{2a}$ shows an

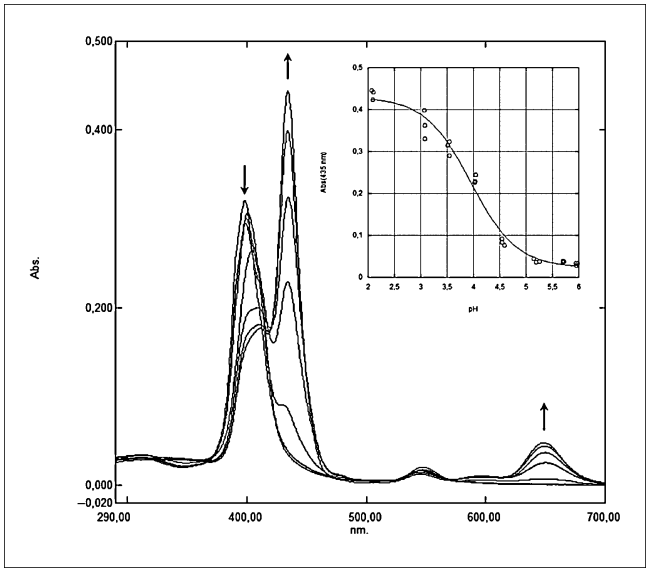


Fig. 4: Absorption spectra of aqueous samples of $TPPS_{2a}$ in the pH range 2.1-6.0. The arrows indicate spectral changes observed from neutral to acidic samples. Inset: example of plot for $TPPS_{2a}$ showing curve fitting for absorbance (Abs) at 435 nm as a function of pH (chi-square (χ^2) = 0.0086). The pH of the samples was 2.1; 3.1; 3.5; 4.0; 4.6; 5.2; 5.7; and 6.0, respectively. The data is given by circles (◯), while the solid line is the fitted curve

Table 1: Inflection points and estimated pK_a values of the imino nitrogens located in the core of $TPCS_{2a}$ and $TPPS_{2a}$ in aqueous solution

Compound	λ (nm)[1]	Inflection point (n = 3)	Estimated pK_a values[2]
$TPCS_{2a}$	411, 444, 606	3.9 ± 0.3	3.8 ± 0.3
$TPPS_{2a}$	399, 435, 649	3.9 ± 0.1	3.8 ± 0.1

[1] The wavelengths used for the determination of the inflection points.
[2] The calculated acidity constants were corrected for the effect of ionic strength ($\mu = 0.01$) according to Sinko (2006)
The variation is given as ± max/min values. The value of chi-square ($\chi 2$) is ≤ 0.0086 for the curve fitting.

increase in absorbance at 649 nm at decreasing pH. The opposite effect is present for $TPCS_{2a}$ at 606 nm, as shown in Fig. 3, likely due to aggregation which is further discussed Section 2.3. The absorption spectra of corresponding alkaline samples were obtained (data not shown), but the spectral differences observed were most likely not due to deprotonation. In the literature, only the pK_a of the imino nitrogens of the core is reported for similar compounds, e.g., $TPPS_4$, $TPPS_3$ (Escudero et al. 2005), *m*-THPC (Cunderlikova et al. 2001), HpIX (Cunderlikova et al. 2001) and hematoporphyrin (Brault et al. 1986). Therefore, the red shift in Soret band maximum of $TPPS_{2a}$ measured in the alkaline samples (i.e., 399 and 412 nm in neutral and alkaline samples (pH 6.2; 12.7), respectively; data not shown) and the minor spectral changes for $TPCS_{2a}$ (data not shown) might be due to processes that at present are not fully understood.
The absorbances at the selected wavelengths (n = 3) for spectral characterization as indicated in Figs. 3 and 4, were further used for the determination of the spectral inflection points. By combining the equation of the acid dissociation constant and Beer's law, assuming b = 1 and keeping the total concentration (C_{tot}) constant, a final equation was deduced for an acid (HB) - base (B) pair:

$$A = \left[\frac{10^{pH-pKa}}{10^{pH-pKa} + 1} \times (\varepsilon_B - \varepsilon_{HB}) + \varepsilon_{HB} \right] \times C_{tot} \qquad (1)$$

where A = measured absorbance, ε_B = molar absorbance for the base and ε_{HB} = molar absorbance for the acid. Following, the course of the curve will be described by the three parameters, ε_B, ε_{HB} and pK_a, which were determined by nonlinear regression. The insets in Figs. 3 and 4 give the plot of the absorbance at selected wavelengths as a function of pH with corresponding curvefitting for $TPCS_{2a}$ and $TPPS_{2a}$, respectively.
One inflection point was localized at pH 3.9 for both compounds, as given in Table 1. This likely correlates with two indistinguishable pK_a values of the imino nitrogens located in the molecular core, which are common in *meso*-substituted porphyrins (Escudero et al. 2005; Cunderlikova et al. 2004; Hibbert and Hunte 1977). The effect of ionic strength on the acidity constants was minor (Table 1). The colour of the aqueous solutions of $TPCS_{2a}$ and $TPPS_{2a}$ were observed to vary from pale yellow-greenish in neutral solution to bright yellow-green upon acidification. This corresponds to previous reports in the literature, where the colour of a solution of the positively charged porphyrin core is reported to be different from the free base (Fleischer 1970; Pasternack et al. 1972). The increase in colour intensity was not present in the case of the alkaline samples. Further, the pK_a of similar porphyrins is reported to be e.g., pK_a 5.0 for $TPPS_4$ or $TPPS_3$ in water (Escudero et al. 2005), around 2.3 for *m*-THPC in PBS (Cunderlikova et al. 2001), around 3.0 and 5.9 for HpIX in PBS (Cunderlikova et al. 2001) and 2.9 and 4.7 for hematoporphyrin in aqueous solutions (Brault et al.

Table 2: *In silico* determination of pK_a, log P and log D for $TPCS_{2a}$ and $TPPS_{2a}$

Compound	Parameter	Theoretical Value	Reference
$TPCS_{2a}$	pK_a		
	–N =	4.46; 5.90	http://www.chemaxon.com/marvin/sketch/index.php
	–NH-	14.47; 14.95	
	Log P	3.77	VG method http://www.chemaxon.com/marvin/sketch/index.php
	Log P	3.65	KLOP method http://www.chemaxon.com/marvin/sketch/index.php
	Log P	4.69	PHYS method http://www.chemaxon.com/marvin/sketch/index.php
	Log P	6.83	ChemBioDraw Ultra 11.0; www.cambridgesoft.com
	Log D (pH 6.5)	3.67	VG method http://www.chemaxon.com/marvin/sketch/index.php
	Log D (pH 6.5)	3.55	KLOP method http://www.chemaxon.com/marvin/sketch/index.php
	Log D (pH 6.5)	4.59	PHYS method http://www.chemaxon.com/marvin/sketch/index.php
$TPPS_{2a}$	pK_a	4.46; 4.48	http://www.chemaxon.com/marvin/sketch/index.php
	–N =	14.36;	
	–NH-	14.61	
	Log P	4.59	VG method http://www.chemaxon.com/marvin/sketch/index.php
	Log P	4.66	KLOP method http://www.chemaxon.com/marvin/sketch/index.php
	Log P	5.25	PHYS method http://www.chemaxon.com/marvin/sketch/index.php
	Log P	6.89	ChemBioDraw Ultra 11.0; www.cambridgesoft.com
	Log D (pH 6.5)	4.58	VG method http://www.chemaxon.com/marvin/sketch/index.php
	Log D (pH 6.5)	4.65	KLOP method http://www.chemaxon.com/marvin/sketch/index.php
	Log D (pH 6.5)	5.24	PHYS method http://www.chemaxon.com/marvin/sketch/index.php

The VG calculation method was derived from Viswanadhan et al. (1989), while log P data from Klopman et al. was applied in the KLOP method (Klopman et al. 1994). log P data from PHYSPROP© database was used in the PHYS method

1986). The protonation of the imino nitrogens studied in our work is accordingly the most important in a physiological context. In Table 2, the *in silico* pK_a values of $TPCS_{2a}$ and $TPPS_{2a}$ are calculated by use of MarvinSketch software (ChemAxon). The models indicate that the protonation of the imino nitrogens is in the same pH range as the experimentally detected inflections points, while the protonation of the pyrrole nitrogens is outside the experimentally measured pH range of this study. In alkaline samples, inflection points could also be detected for $TPCS_{2a}$ at pH 7.7 ± 0.1 (λ: 454 and 638 nm; data not shown) and for $TPPS_{2a}$ at pH 10.2 ± 0.2 (λ: 412, 517 and 586 nm; data not shown). These spectral differences do most likely not correlate with pK_a values; as to the best of our knowledge no similar results are presented in previous literature. The fluorescence data given in Sec. 2.2. support this.

It is well-known that *meso*-sulfonatophenyl substituted porphyrins self-assemble into aggregates in acidic aqueous media (Akins et al. 1996). Further, porphyrins are known to form J-aggregates (Fig. 2) in acidic aqueous solution (Poderys et al. 2004; Rubires et al. 1999), which have been explained by attraction between the positively charged molecular core (protonated imino nitrogens) and the negatively charged sulphonate groups (Rubires et al. 1999), as illustrated for $H_4^{2+}TPPS^{4-}$ by Ribo et al. (1994). Therefore, the spectral shifts observed in acidic environments might be due to reduced total molecular charge upon protonation of the imino nitrogens in the core followed by self-association and aggregation (Giovannetti et al. 2010). Aggregate formation is indicated for similar compounds by a red shift of the Soret band in the absorption spectrum compared to the monomer (Lambert et al. 1986; Ou et al. 2007; Poderys et al. 2004; Sutter et al. 1993), increased absorbance of the Q band in the red region of the absorption spectrum, e.g., $TPPS_{2a}$ (Poderys et al. 2004) and a colour change upon acidification of a neutral solution of the porphyrin, e.g., TPPS (Fleischer et al. 1971), $TPPS_3$, TCPP and TMPyP (Pasternack et al. 1972). All these spectroscopic characteristics are detected for $TPCS_{2a}$ and $TPPS_{2a}$. J-aggregates are known to exhibit a red-shifted absorption band compared to the monomer (Ou et al. 2007). A red shift in the Soret band is detected for both compounds as a function of a decrease in pH (Figs. 3 and 4). The shift is more prominent for $TPPS_{2a}$ than $TPCS_{2a}$. A new absorption peak is clearly appearing in acidic environment at 435 nm in the case of $TPPS_{2a}$ (Fig. 4). In Fig. 3, the arrow shows reduced intensity of the red Q band of $TPCS_{2a}$ in acidic samples. The opposite effect is demonstrated for $TPPS_{2a}$ (Fig. 4), where a red absorption peak is appearing upon aggregation in the presence of acid. This indicates different aggregation types of the two compounds, where the sterical structure of the planar $TPPS_{2a}$ versus the angeled $TPCS_{2a}$ might affect the aggregates formed.

In plain aqueous solution, it is suggested that $TPPS_{2a}$ does not stack, due to electrostatic repulsion caused by the negatively charged sulphonate groups (Poderys et al. 2004). This might also be the case for $TPCS_{2a}$. However, the compounds may exist as dimers, as previously shown for TPP (Udal'tsov et al. 2002), protoporphyrin (Brown et al. 1976), deuteroporphyrin, mesoporphyrin, protoporphyrin (Margalit and Rotenberg 1984)

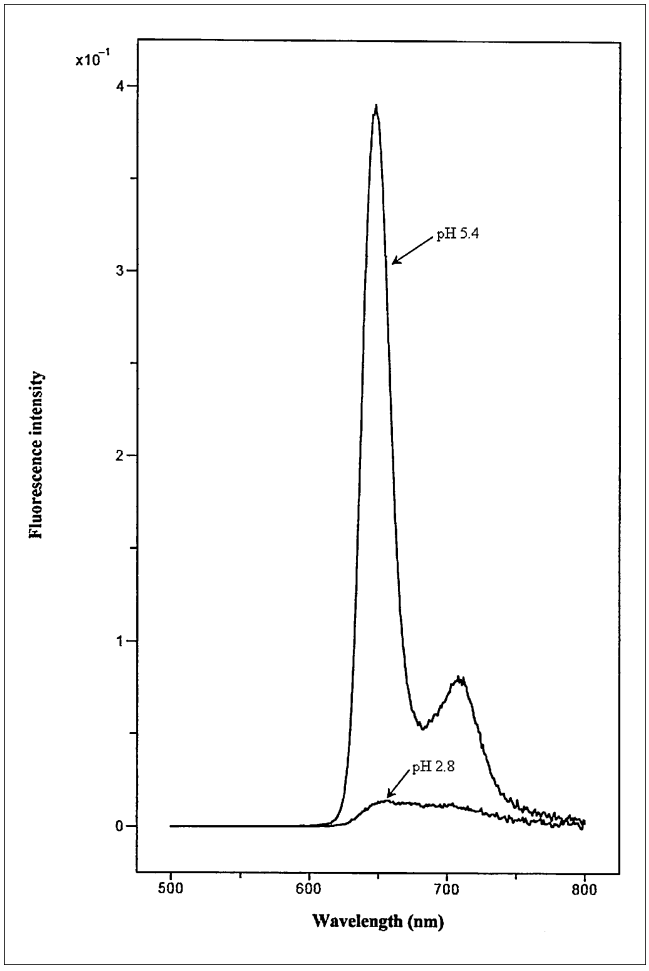


Fig. 5: Fluorescence emission spectra of aqueous samples of $TPCS_{2a}$ above (pH 5.4) and below (pH 2.8) the spectral inflection point (i.e., estimated pK_a value). Excitation wavelength: 413 nm

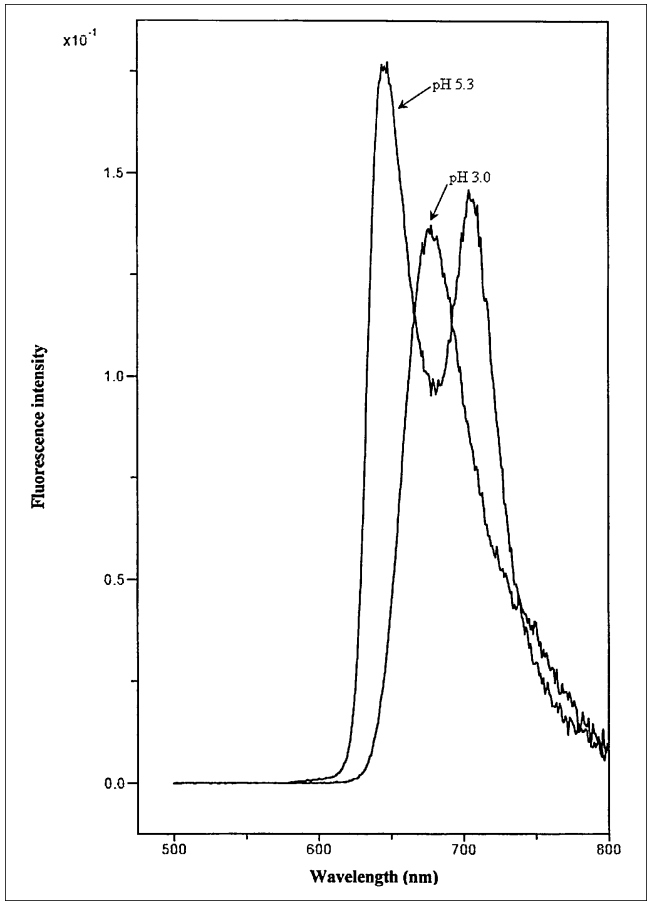


Fig. 6: Fluorescence emission spectra of aqueous samples of $TPPS_{2a}$ above (pH 5.3) and below (pH 3.0) the spectral inflection point (i.e., estimated pK_a value). Excitation wavelength: 413 nm

and haematoporphyrin (Margalit et al. 1983). In addition, both the comparable compounds $TPPS_4$ and $TPPS_3$ show monomer-dimer equilibrium near neutral pH and higher aggregates in acidic environment (Sutter et al. 1993). In total, the inflection points of $TPCS_{2a}$ and $TPPS_{2a}$ seem to reflect protonation followed by aggregation in acidic environment (Table 1).

2.2. Fluorescence emission above and below the inflection points

Experiments were performed to find possible changes in the fluorescence emission spectra upon protonation of $TPCS_{2a}$ and $TPPS_{2a}$. In acidic media below the inflection point at pH 3.9, the two imino nitrogens in the core are protonated (Fig. 1), and the total molecular charge is zero. The compounds might aggregate as discussed in Sec. 2.1, and further evaluated in Sec. 2.3. Significant changes in the fluorescence emission spectrum were observed for both compounds in acidic solution below the inflection point compared to the samples above the inflection point. A change in the fluorescence emission spectrum from two distinct maxima at 648 nm and 709 nm in a solution of pH 5.4 to a very weak fluorescence signal at 652 nm at pH 2.8 was measured for $TPCS_{2a}$ (Fig. 5). A change from two distinct maxima at 647 nm and 705 nm in a solution at pH 5.3 to only one maximum at 678 nm at pH 3.0 was measured for $TPPS_{2a}$ (Fig. 6). This is in accordance with the pH induced spectral changes of the absorption spectra discussed in Sec. 2.1. Protonation of imino nitrogens is reported to lead to a shift of the emission peaks (Srivastava et al. 1973), and the asymmetrical shape of the emission bands suggests that there is more than one emitting species (Akins et al. 1996). In general, formation of H-aggregates leads to fluorescence quenching. This is consistent with the observations on $TPCS_{2a}$ in acidic media (Fig. 5). Formation of J-aggregates leads in comparison to higher fluorescence intensity (Ou et al. 2007). Both fluorescence and singlet oxygen quantum yields of aggregated species are usually lower than those of monomeric molecules, which in general lower the photoactivating effect (Krasnovsky et al. 1990). On the contrary, the fluorescence does not seem to be quenched to the same extent for $TPPS_{2a}$ at low pH where the area under the emission curve (AUC) at pH 3.0 makes up $\sim$ 70% of the AUC at pH 5.3 (Fig. 6) compared to $TPCS_{2a}$ where the AUC at pH 2.8 makes up $\sim$ 10% of the AUC at pH 5.4 (Fig. 5). This might indicate formation of different aggregate structures of the two photosensitizers in acidic medium. The spatial difference between the structures of $TPCS_{2a}$ and $TPPS_{2a}$ should also be kept in mind in relation to possible aggregation, since the porphyrin $TPPS_{2a}$ is planar while all three stereoisomers of $TPCS_{2a}$ are twisted around the core (Lilletvedt et al. 2010a). Different aggregation types might therefore be formed for the two compounds, resulting in a difference in fluorescence properties. In alkaline solution, no change in the fluorescence emission properties was measured for neither $TPCS_{2a}$ nor $TPPS_{2a}$ when excited at the Soret band maximum (data not shown), which supports the assumption that deprotonation is not taking place in the actual pH range (Sec. 2.1). Alkaline samples were therefore not further evaluated.

2.3. Evaluation of aggregation by addition of surfactant

Experiments were undertaken to evaluate the effect of the non-ionic surfactant Tween 80 (0.006% v/v = 4 $\times$ cmc) on

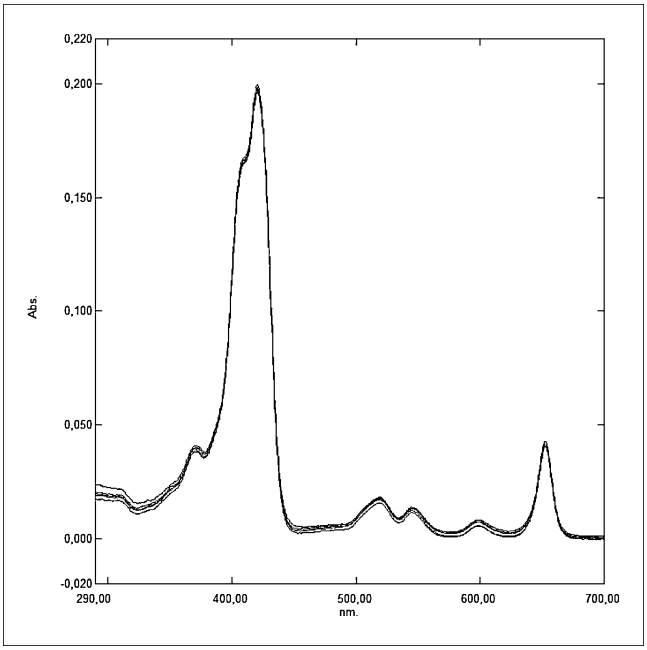


Fig. 7: Absorption spectra of aqueous samples of $TPCS_{2a}$ ($c = 1.2 \times 10^{-6}$ M) containing Tween 80 (0.006% v/v = 4 × cmc) at pH 3.1; 3.6; 4.1; 4.9; and 5.5

monomerisation of aggregates of $TPCS_{2a}$ and $TPPS_{2a}$ in aqueous solution. Tween 80 was added to the samples after acidification, and the compounds should therefore be in the protonated form. Initial observations indicated a major change in the colour of the solutions upon addition of a small amount of Tween 80. The bright yellow-green colour change observed upon acidification of the aqueous samples, was reversed by addition of Tween 80 leading to nearly colourless samples at the actual concentration of the photosensitizer.

As the addition of Tween 80 virtually eliminated the pH dependent spectral changes in the absorption spectra for both compounds in the range pH 3-5 (Figs. 7 and 8), i.e., the absorption shifts which were the basis for the determination of the inflection points in Sec. 2.1 (Figs. 3 and 4), were also removed by Tween 80 addition. However, a slight red shift (9–21 nm) in the Soret band maxima of both compounds was observed by addition of Tween 80 (Figs. 7 and 8), when compared to the corresponding

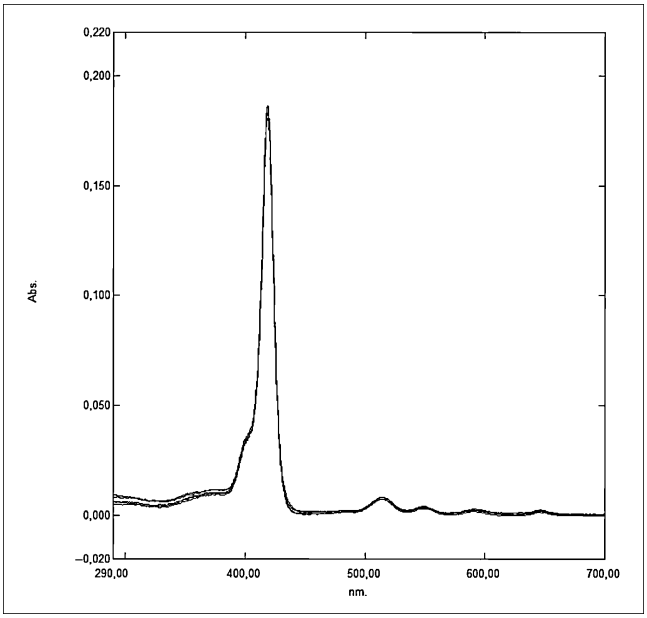


Fig. 8: Absorption spectra of aqueous samples of $TPPS_{2a}$ ($c = 4.2 \times 10^{-7}$ M) containing Tween 80 (0.006% v/v = 4 × cmc) at pH 3.0; 3.6; 4.1; 4.7; and 5.3

samples in Figs. 3 and 4. In addition, the Soret band of $TPCS_{2a}$ in the presence of Tween 80 (Fig. 7) possesses more structure than the corresponding band in Fig. 3. The Q bands of $TPCS_{2a}$ are more prominent in the presence of Tween 80, especially the red band at ~ 650 nm which is important for use in PCI technology. This is likely due to deaggregation of $TPCS_{2a}$, and a subsequent increase in absorption of the red Q band which is characteristic for the monomeric form of chlorins. The red aggregation band of $TPPS_{2a}$ discussed in Sec. 2.1, was absent when Tween 80 was added to the sample. Accordingly, no pH dependent spectral changes of the fluorescence emission spectra were observed after addition of Tween 80, when the compounds were excited at the Soret band maximum (420 nm); at other wavelengths of the Soret band (370 nm or 435 nm); or at the red Q band (650 nm; data not shown). The same excitation wavelengths were utilized for both compounds. The pH range chosen for this study was around the inflection points (i.e., estimated pK_a values) obtained for the aqueous solutions without Tween 80 (Sec. 2.1).

Protonation of the compounds might in theory be inhibited by the presence of Tween 80, through sterical and intermolecular interactions and/or change of pK_a values. The elimination of pH dependent spectral shifts induced by addition of the surfactant at concentrations above cmc is however, likely to be caused by a reduction in aggregate formation normally observed upon acidification of $TPCS_{2a}$ and $TPPS_{2a}$. Addition of surfactant can lead to monomerization by solubilization, which is shown for Hematoporphyrin IX in aqueous solution in the presence of sodium dodecyl sulfate (SDS; Pottier et al. 1988), and for TCPP, TPPS or TMAP in aqueous solution in the presence of Triton X-100 (Lambert et al. 1986). For these porphyrins, the surfactant addition also leads to a slight red shift in the Soret band and more prominent Q bands. The presence of dimers or aggregates in plain aqueous solution was confirmed by UPLC (Lilletvedt et al., unpublished results), where several peaks occurred in the chromatogram of $TPCS_{2a}$, while addition of Tween 80 removed several of the peaks.

However, there is a non-linear correlation between the absorbance at the Soret band maximum versus the AUC of the fluorescence emission spectra of the samples containing Tween 80, which is not fully understood at present. In plain water, the Q bands of $TPCS_{2a}$ are not as prominent as in organic solvents (Lilletvedt et al. 2010a, b) or in the presence of Tween 80 (Figs. 7 and 8), which indicates the presence of dimers in plain aqueous media. On the contrary, plots of the absorbance at Soret band maximum (A (413 nm): 0.029–1.042) versus concentration ($c = 1.1 \times 10^{-7}$–5.5×10^{-6} M) of $TPCS_{2a}$ dissolved in plain water are linear (Sec. 2.4 and 3.6), which indicates a majority of monomers at these concentrations (Pasternack et al. 1972). However, spectral variations that were observed for the Q bands when dissolving $TPCS_{2a}$ (Fig. 9 inset) or $TPPS_{2a}$ (data not shown) in plain water leads to the assumption of some extent of dimerization. Whether partial or full disaggregation takes place by addition of Tween 80 to the aqueous solutions is at present not clear. In conclusion, these results show that in the presence of a non-ionic surfactant like Tween 80, the spectroscopic properties of $TPCS_{2a}$ and $TPPS_{2a}$ in aqueous media are unaffected by pH. The addition of surfactant seems to stabilize aqueous samples of the two photosensitizers through hindrance of aggregation, which is important with regard to future formulation work and the use of $TPCS_{2a}$ in PCI technology.

2.4. Distribution of $TPCS_{2a}$ and the reference $TPPS_{2a}$ in 1-octanol/water

Log P describes the partition of unionized compounds between 1-octanol and water (Steele 2004). In the case of $TPCS_{2a}$ and

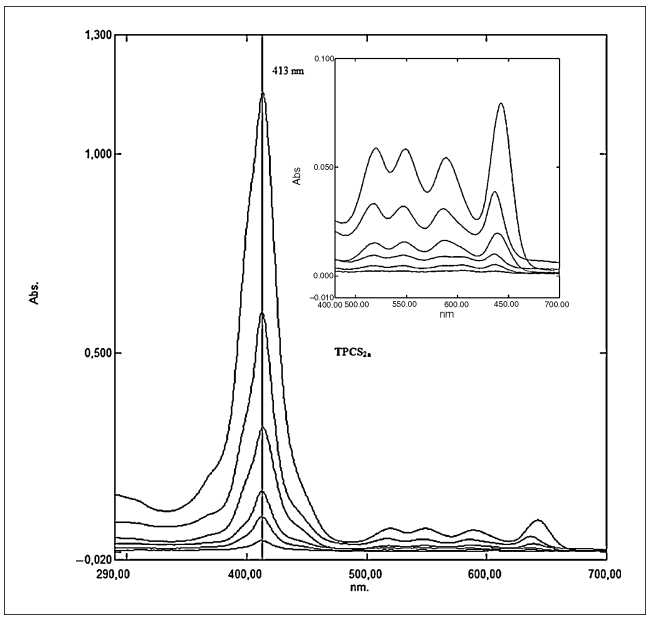


Fig. 9: Absorption spectra of aqueous samples of $TPCS_{2a}$ ($c = 1 \times 10^{-7}$–5×10^{-6} M) used in the determination of log D. The Soret band maximum at 413 nm gives a linear plot of absorbance versus photosensitizer concentration ($R^2 \geq 0.998$; data not shown). The inset shows the truncated Q bands

the reference compound $TPPS_{2a}$, the two sulphonate groups ($pK_a \leq 1$; Pasternack et al. 1972) will remain permanently ionized throughout the relevant pH range while the molecular core is uncharged, and the compounds are therefore amphiphilic. Hence, log D, which takes into account ionization (Steele 2004), is a more suitable descriptor of the distribution for these photosensitizers. Cunderlikova and coworkers determined the partition coefficient of $TPPS_{2a}$ to log P = 2 (pH ~ 6), which is reported to remain unchanged in the actual pH area, i.e., pH 4-8 (Cunderlikova et al. 2004). As a reference for the novel photosensitizer $TPCS_{2a}$, log D of $TPPS_{2a}$ was also evaluated in the present study. Shake flask measurements were performed in 1-octanol/water, resulting in log D = 1.5 (± 0.5 SD) for $TPPS_{2a}$ (n = 5), which is in the same range as the partition coefficient given by Cunderlikova et al. 2004). The high statistical variation was mainly due to extensive accumulation (~ 40%) of $TPPS_{2a}$ in the interface formed between 1-octanol and water. Corresponding measurements performed for $TPCS_{2a}$ resulted in log D = 0.4 (± 0.4 SD; n = 24). The accumulation in the interface was lower than for $TPPS_{2a}$ (~ 15%), but probably responsible for the high standard deviation. The sterical conformation of the photosensitizers thus seems to affect their lipophilicity, since the angeled $TPCS_{2a}$ possess lower log D than the planar $TPPS_{2a}$.

The *in silico* calculated log P and log D values of both $TPCS_{2a}$ and the reference $TPPS_{2a}$ (Table 2) indicate a molecular distribution shifted towards the 1-octanol phase, but the hydrophobicity is suggested to be higher than the experimental values obtained. However, the variation is extensive both for the *in silico* calculated values and for the experimentally measured partition coefficients of similar compounds determined by Cunderlikova et al. (2004), i.e., m-THPC (log P = 5.5); HpIX (log P = 1.5 at pH ~ 6); and mTHPP (log P = 5 at pH ~ 6). The results emphasize that both $TPCS_{2a}$ and $TPPS_{2a}$ are amphiphilic molecules, explaining the distribution and further restrain of diffusion across lipid membranes as observed in biological studies (Berg et al. 1990; Friberg et al. 2003; Kessel et al. 1987; Mojzisova et al. 2007; Mojzisova et al. 2009; Norum et al. 2009a; Selbo et al. 2006). Amphiphilicity of the photosensitizer is considered advantageous for the PCI technology, resulting in accumulation at the molecular target (i.e., cell membrane). $TPCS_{2a}$, which has been designed for PCI, appears to be an ideal amphiphilic compound with log D value close to zero.

3. Experimental

3.1. Materials

Di(monoethanolammonium) meso-tetraphenyl chlorin disulphonate ($TPCS_{2a}$) and di(triethylammonium) meso-tetraphenyl porphyrin disulphonate ($TPPS_{2a}$) were synthesized by Synthetica AS, Norway, (purity ≥ 98.7%) and used as received. The compounds were stored desiccated at +4 °C. The photosensitizer $TPCS_{2a}$ is specifically prepared for use in PCI. Aqueous stock solutions of HCl (0.001–0.1 M) and NaOH (0.0001–1 M) were used for pH regulation of the samples. 0.2 M NaCl solutions were utilized for adjustment of ionic strength. The metal ion content of the water used was specified (e.g., Cu ≤ 0.0004 mg/l, Fe ≤ 0.001 mg/l; p.a. grade, Emsure®, Merck). Tween 80 of Ph.Eur. quality was purchased from Merck. Methanol and 1-octanol were of spectroscopic grade from Merck and Fluka, respectively. Quinine sulphate (purity > 99%) was purchased from Fluka.

3.2. Equipment

UV-visible absorption spectra (290-700 nm) were recorded by a Shimadzu UV-2401PC spectrophotometer. The accuracy in wavelength determination was ± 1 nm. The computer program Kaleidagraph 4.0 (Synergy software, US) was used for the nonlinear curvefitting. A universal shaker (Edmund Büchler) and a centrifuge (MSE) were applied in the determination of the distribution coefficients. Fluorescence emission spectra were acquired on a PTI modular fluorescence system (PTI, London, Ontario, Canada). The excitation source was a 75 W xenon lamp. The monochromators were Model 101 with *f*/4 0.2-m Czerny-Turner configuration, whose entrance and exit slits were adjusted to 2 nm. Spectral correction of the emission light for the detector quantum efficiency, as well as correction for the excitation lamp spectral response, were automatically performed by the acquisition software (FeliX32 for Windows).

3.3. Determination of spectral inflection points

UV-visible absorption spectra (290–700 nm) of aqueous samples containing $TPCS_{2a}$ and $TPPS_{2a}$ were recorded as a function of pH 2-12 at ambient temperature in order to determine the inflection points. Three parallels were measured. The concentration of the photosensitizer was kept constant at 2×10^{-6} M, and the ionic strength (μ) was 0.01. Spectroscopic changes in the absorption spectra were observed as a function of pH. Data were recorded at several wavelengths ($n \geq 3$) and used in the calculation of the inflection points. Eq. 2 (Sec. 2.1) was utilized through nonlinear regression. The computer program Kaleidagraph 4.0 (Synergy software, US) was used for the curve fitting. The samples were wrapped in aluminium foil prior to the measurements.

3.4. Fluorescence emission above and below the inflection points

Stock solutions of $TPCS_{2a}$ and $TPPS_{2a}$ ($c = 1 \times 10^{-4}$ M) were made in methanol. The final aqueous samples held fixed concentration (4×10^{-7} M, A (Soret band maximum) < 0.21) and ionic strength (μ = 0.01). The pH of the samples (n = 3) was adjusted by HCl (Sec. 2.1) to 2.8 and 5.4 for $TPCS_{2a}$ and 3.0 and 5.3 for $TPPS_{2a}$, respectively. The excitation wavelength was 413 nm for both compounds, and the fluorescence emission wavelength range was 500-800 nm. The fluorescence experiments were performed at 25 ± 0.1 °C. The samples were wrapped in aluminium foil prior to the measurements. Quinine sulphate dissolved in 0.05 M H_2SO_4 was used as a reference (Velapoldi and Tønnesen 2004).

3.5. Measurements of aggregation in aqueous samples

Samples of $TPCS_{2a}$ and $TPPS_{2a}$ were made according to Sec. 3.3 (n = 3), i.e., ionic strength (μ = 0.01), but the absorbance at the Soret band maximum was adjusted to A < 0.2 (i.e., $[TPCS_{2a}] = 1 \times 10^{-6}$ M and $[TPPS_{2a}] = 4 \times 10^{-7}$ M) to perform fluorescence emission measurements. In addition, 0.006% v/v Tween 80 was added, which correlated to 4 x critical micelle concentration (cmc) in the final samples (Wan and Lee 1974). The pH interval covered was pH 3-5. The temperature of the samples was 25 ± 1 °C prior to the measurement. Absorption measurements and fluorescence emission measurements were performed according to Sec. 3.3 and 3.4, respectively. Additional absorption measurements were performed at the same photosensitizer concentration as used in Sec 3.3 ($c = 2 \times 10^{-6}$ M).

3.6. Determination of log D

The distribution coefficient (log D) of $TPCS_{2a}$ and the reference $TPPS_{2a}$ in 1-octanol/water was determined by the shake flask method (Steele 2004). 1-Octanol and water were initially mutually saturated with each other at ambient temperature, in accordance with the OECD shake flask method (OECD 1995; Franks et al. 1993). The photosensitizer (2×10^{-6}–8×10^{-6} M) was introduced in either the water or 1-octanol phase, and the initial concentration was kept low to avoid additional problems with aggregation. The aqueous phase was made without justification of pH or ionic strength. The pH of the plain water phase (pH = 6.3, Emsure®, Merck) was not affected by the low concentration of the dissolved compound, i.e., the physiologically important ionic form (dianionic free base) of the photosensitizer is present in the aqueous phase like in normal tissues and more acidic tumour tissues (Vaupel et al. 1989). The same bottle of water was used for the log D experiments, and the pH was sufficiently above the estimated pK_a values of $TPCS_{2a}$ and $TPPS_{2a}$ (> two pH units; determined in Sec. 2.1) to ensure uncharged molecular core (deprotonated imino nitrogens). Thus, log D of $TPCS_{2a}$ and the related $TPPS_{2a}$ was determined in a region where the compounds are not pH-sensitive, since both compounds possess the same pK_a value (Table 1). The aqueous phase did not contain salts, e.g., buffers, since that might complicate the measurements further through ion-pair formation and a subsequent increase in the estimated log D value (Dearden and Bresnen 1988).

Equal amounts of both phases were mixed and agitated for 1 h and 30 min (100 rpm), which was found sufficient for achievement of equilibrium conditions. The samples were sentrifugated for 10 min at 500 g, and left at ambient temperature for 1 h for temperature equilibrium prior to the spectrophotometric quantification. The concentration of $TPCS_{2a}$ and the reference $TPPS_{2a}$ was determined spectrophotometrically at the Soret band maximum (n = 6; A (Soret band maximum): < 0.8), when possible in both phases, by use of suitable calibration curves ($[TPCS_{2a}] = 1 \times 10^{-7}$–$5 \times 10^{-6}$ M; $[TPPS_{2a}] = 5 \times 10^{-8}$–$3 \times 10^{-6}$ M; 5-6 concentrations; 3 parallels at each concentration; $R^2 \geq 0.998$). Linear calibration curves in the selected concentration range justify quantification by UV-vis spectrophotometry. Accordingly, the distribution coefficients were calculated:

$$\log D = \log(c_{1\text{-octanol}}/c_{water}) \quad (2)$$

where $c_{1\text{-octanol}}$ and c_{water} are the total concentration of the photosensitizer in the respective solvent phase at the actual pH. The samples were wrapped in aluminium foil prior to the measurements.

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