Journal of Photochemistry and Photobiology A: Chemistry 214 (2010) 40–47

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology A: Chemistry

journal homepage: www.elsevier.com/locate/jphotochem

Time-domain evaluation of drug–solvent interactions of the photosensitizers $TPCS_{2a}$ and $TPPS_{2a}$ as part of physicochemical characterization

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ARTICLE INFO

Article history:
Received 29 March 2010
Received in revised form 1 June 2010
Accepted 6 June 2010
Available online 11 June 2010

Abbreviations:
α, solvent proton donor parameter
API, active pharmaceutical ingredient
β, solvent proton acceptor parameter
DMF, dimethyl formamide
DMSO, dimethyl sulfoxide
ε, solvent dielectric constant
ESICT, excited state intramolecular charge transfer
ESIET, excited state intramolecular electron transfer
ESIPT, excited state intramolecular proton transfer
EtOH, ethanol
Δf, solvent orientation polarizability
λ_{abs}, absorption peak wavelength
λ_{em}, fluorescence emission peak wavelength
λ_{ex}, excitation wavelength
MeOH, methanol
$^1O_2$, singlet oxygen
PCI, photochemical internalization
PDT, photodynamic therapy
π^*, solvent polarity value
ROS, reactive oxygen species
S_0, ground state
$S_1{}^*$, first excited singlet state
SOSG, singlet oxygen sensor green reagent
τ_I, intermediate fluorescence decay component
τ_L, longest fluorescence decay component
τ_S, shortest fluorescence decay component
TCSPC, time-correlated single-photon counting
$TPCS_{2a}$, meso-tetraphenyl chlorin disulphonate
$TPPS_{2a}$, meso-tetraphenyl porphyrin disulphonate

ABSTRACT

The patented photosensitizer meso-tetraphenyl chlorin disulphonate ($TPCS_{2a}$) is intended for use in the technology of photochemical internalization (PCI). The compound is advantageous with respect to the related meso-tetraphenyl porphyrin disulphonate ($TPPS_{2a}$), due to its high absorption in the red part of the absorption spectrum ($\lambda_{abs} \approx 650$ nm). We report a time-resolved fluorescence study of $TPCS_{2a}$ aimed to elucidate the susceptibility of the photosensitizer's excited state dynamics to properties of its environment, such as polarity and hydrogen bond formation. $TPPS_{2a}$ is used as a reference compound. Fluorescence decays with <30 ps temporal resolution of $TPCS_{2a}$ and $TPPS_{2a}$ in 14 organic solvents of varying polarity and amphiprotic properties were measured by time-correlated single-photon counting (TCSPC). Both compounds show triple exponential fluorescence decays in non-polar environment, i.e. $\tau_L \sim 7$ ns, $\tau_I \sim 2$ ns and $\tau_S \sim 0.5$ ns. The two shorter decay components, τ_I and τ_S, which we associated with two different intramolecular charge transfer mechanisms, readily disappear when the solvent polarity is slightly increased. The fluorescence decays of both compounds in any solvent of dielectric constant $\varepsilon > 7.58$ are well fitted by a single exponential model, with decay time roughly constant, $\tau_L \sim 10$ ns, and independent of the amphiprotic properties of the solvents. The present results allow concluding that the fluorescence decay pathways of $TPCS_{2a}$ and $TPPS_{2a}$ are only slightly affected by the environmental properties under consideration, as previously probed by steady-state measurements (Lilletvedt et al. [1]). Singlet oxygen ($^1O_2$) generation of the two photosensitizers were measured indirectly in water by applying the singlet oxygen sensor green (SOSG) reagent. Both photosensitizers generate $^1O_2$ to some extent upon excitation *in vitro*.

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1010-6030/$ – see front matter
doi:10.1016/j.jphotochem.2010.06.004

Keywords:
Photosensitizer
Porphyrin
Chlorin
Photochemical internalization
Fluorescence decay
Singlet oxygen

1. Introduction

Photochemical internalization (PCI) is a technology, which is intended for use in e.g. the medical treatment of cancer [2–4]. The technology is at present applied in a phase I/II clinical trial. The trial has so far been successful and promising in the treatment of a range of cancer types. Strong anti-tumor response has already been shown for patients with sarcoma, breast, head and neck cancer [2]. In PCI, tumor targeting is accomplished by administration of the patented photosensitizer meso-tetraphenyl chlorin disulphonate, $TPCS_{2a}$, and a conventional antineoplastic agent, at present bleomycin, followed by tumor-specific illumination with red light. A detailed explanation of the PCI technology can be found elsewhere [3–7]. The main advantages of this new technique are site-specific drug delivery and a more efficient utilization of drugs and, consequently, reduced side effects.

Porphyrins and their derivatives are commonly used as photosensitizers in photodynamic therapy (PDT) [5]. They produce reactive oxygen species (ROS), like singlet oxygen ($^1O_2$), upon excitation e.g. in the Soret band. This band is situated in the blue–violet part of the spectrum around 400 nm, where these compounds exhibit extraordinarily high molar absorption coefficients: when dissolved in methanol, the molar absorption coefficients of $TPCS_{2a}$ at the Soret band (ϵ_{416}) = 201 000 M^{-1} cm^{-1} and of $TPPS_{2a}$ (ϵ_{413}) = 503 000 M^{-1} cm^{-1} [1]. Porphyrins and the related compounds, chlorins, also possess four minor absorption bands in the visible region in the range 500–700 nm (Q bands). The chlorins exhibit a characteristic absorption band situated in the red part of the spectrum (~650 nm), which is more intense than the corresponding band of the porphyrins [8,9]. Red light has enhanced penetration depth in tissues as compared to blue–violet light [10], and is therefore preferred in PDT [8] and PCI technology [3]. In methanol, $TPCS_{2a}$ exhibits a distinctive Q band at 651 nm (ϵ_{651} = 41 000 M^{-1} cm^{-1}[1]). Structurally, a given porphyrin and its corresponding chlorin are differentiated only by a double bond in the core of the molecules, i.e. present in porphyrin and reduced in chlorin, as illustrated in Fig. 1. In this work, $TPCS_{2a}$ (Fig. 1a) is compared to its corresponding porphyrin, the photosensitizer meso-tetraphenyl porphyrin disulphonate ($TPPS_{2a}$, Fig. 1b), which is used as a reference substance. The double bond reduction of $TPPS_{2a}$ by formation of $TPCS_{2a}$ induces an alteration of the sterical molecular structure [1]. While the porphyrin $TPPS_{2a}$ is a planar compound, the chlorin $TPCS_{2a}$ exhibits notable structural twisting. As a consequence, $TPPS_{2a}$ consists of an extensively conjugated system [11], where the electrons are delocalized, while in $TPCS_{2a}$ the free flow of electrons might to some extent be reduced. In addition, the sulphonate groups attached to the core of the molecules are known for their electron-withdrawing effect, and will therefore also influence the intramolecular charge transfer [1,12].

Physicochemical characterization of an active pharmaceutical ingredient (API) is mandatory in the preformulation phase [13]. Assessing the spectroscopic properties of an API is particularly relevant when the API is intended to be used as a photosensitizer. We previously performed a thorough study of the steady-state spectroscopic properties of $TPCS_{2a}$ compared to $TPPS_{2a}$, which focused particularly on polarity and H-bonding effects [1]. We showed that the absorption and fluorescence emission spectra of both $TPCS_{2a}$ and $TPPS_{2a}$ in solution are virtually independent of the properties of the vehicle, which can be desirable in view of the application of $TPCS_{2a}$ *in vivo*. Elucidation of the excited-state dynamics of a tentative photosensitizer and characterization of its dependence upon the environmental properties is another important step in the preformulation phase. In this paper, the deactivation mechanisms of the first excited singlet state (S_1*) of both $TPCS_{2a}$ and $TPPS_{2a}$ are investigated in 14 organic solvents of varying polarity and hydrogen bonding capacity, by means of fluorescence decay measurements performed with a time-correlated single-photon counting (TCSPC) system endowed with <30 ps temporal resolution. We further determined the singlet oxygen ($^1O_2$) production upon excitation of $TPCS_{2a}$ and $TPPS_{2a}$ in aqueous solutions. $^1O_2$ is capable of oxidizing biological cell components and destroying cellular structures [14]. Production of $^1O_2$ can be observed directly by luminescence at 1270 nm [15–17]. In addition, it can be revealed indirectly by use of the singlet oxygen sensor green (SOSG) reagent. The SOSG reagent, made commercially available by Molecular Probes in 2004, is highly selective for $^1O_2$ and does not show any appreciable response to neither hydroxyl radical nor superoxide. In the presence of $^1O_2$, SOSG emits a characteristic green fluorescence with emission maximum at 525 nm [18]. The SOSG reagent is intended for use in aqueous environments, and is therefore used as an *in vivo* probe for the detection of $^1O_2$ [19].

2. Materials and methods

2.1. 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. Quinine sulphate (purity > 99%) was purchased from Fluka, Switzerland. All solvents were of p.a. grade. Ethyl acetate was dried over sodium sulphate before use. The SOSG reagent was purchased from Invitrogen, Norway. The metal ion content of the water used in these experiments was declared (e.g. Cu ≤ 0.0004 mg l^{-1}, Fe ≤ 0.001 mg l^{-1}; p.a. grade, Merck). Riboflavin was kindly provided by Weifa, Norway.

2.2. Methods

2.2.1. Fluorescence decay measurements

The samples (absorbance at the excitation wavelength < 0.08) were measured in a fluorimeter quartz cuvette with 10 mm path length. The samples were excited at 420 nm by the second harmonic output of a SESAM mode locked Ti:sapphire laser (Tiger-ps SHG, Time Bandwidth Products, Zurich, CH) operating at a 48 MHz repetition rate and delivering >20 mW average power. The duration of the pulses generated at the fundamental wavelength (840 nm) was 3.9 ps. Fluorescence emitted at 90° to the excitation beam at wavelengths above 600 nm was selected by a cut-off filter (LL-600, Corion, Holliston, MA), collected by a 20× microscope objective and focused onto the sensitive area of the detector. This was

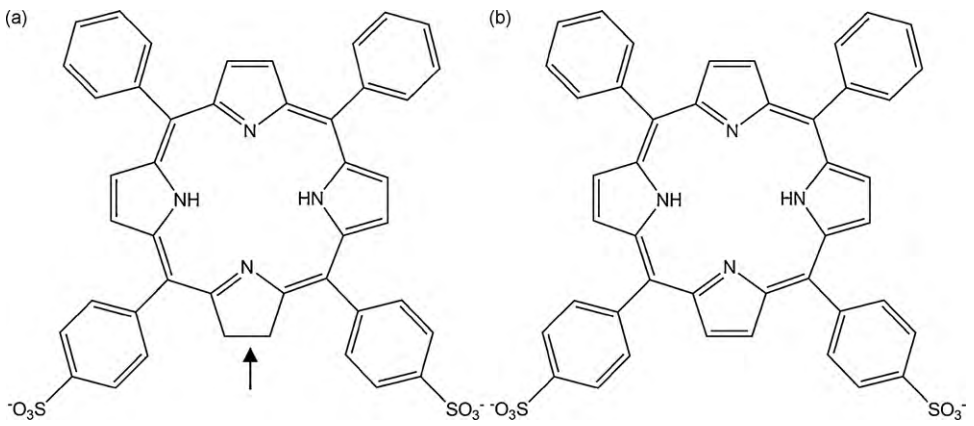

Fig. 1. Structural formulas of $TPCS_{2a}$ (a) and $TPPS_{2a}$ (b). One of three isomers is shown in (a). The structural difference between the two compounds is only a double bond in one pyrrole ring (indicated by an arrow).

Table 1
Solvent properties.

Category	Solvent	π^*	α	β	ε	n	$(\varepsilon-1)/(2\varepsilon+1)$	$(n^2-1)/(2n^2+1)$	Δf
Polar, protic (alcohols)	Ethylene glycol	0.92	0.90	0.52	37.70	1.431	0.480	0.206	0.275
	Methanol	0.60	0.93	0.62	32.66	1.328	0.477	0.169	0.309
	Ethanol	0.54	0.83	0.77	24.55	1.361	0.470	0.181	0.289
	2-Propanol	0.48	0.76	0.95	19.92	1.377	0.463	0.187	0.276
	1-Butanol	0.47	0.79	0.88	17.51	1.399	0.458	0.195	0.264
Highly polar, non-protic	Dimethyl sulfoxide	1.00	0.00	0.76	48.90	1.478	0.485	0.221	0.264
	Dimethyl formamide	0.88	0.00	0.69	37.60	1.430	0.480	0.205	0.275
	Acetonitrile	0.75	0.19	0.31	35.94	1.344	0.479	0.175	0.305
	Acetone	0.71	0.08	0.48	20.60	1.359	0.464	0.180	0.284
Slightly polar or non-polar, non-protic	Tetrahydrofuran	0.58	0.00	0.55	7.58	1.407	0.407	0.198	0.210
	Chloroform	0.58	0.44	0.00	4.81	1.446	0.359	0.211	0.148
	Ethyl acetate	0.45	0.00	0.55	6.02	1.372	0.385	0.185	0.200
	Diethyl ether	0.27	0.00	0.47	4.34	1.353	0.345	0.178	0.167
	Cyclohexane	0.00	0.00	0.00	2.02	1.426	0.202	0.204	-0.002

Solvent polarity (π^* parameter), proton donor property (α parameter) and proton acceptor property (β parameter) are adopted from Appendix 4 in Suppan and Ghoneim [34]. ε, dielectric constant; n, refractive index.
Solvent orientation polarizability, $\Delta f = (\varepsilon-1)/(2\varepsilon+1) - (n^2-1)/(2n^2+1)$ [20].

a single-photon avalanche diode with built-in active quenching circuitry (PDM50, Micro-photon-devices, Bolzano, IT), used in a single-photon timing apparatus utilizing a single card of an SPC 152 package (Becker & Hickl, Berlin, Germany). The time-to-amplitude conversion in our apparatus is started by the PDM50 timing output and stopped by the re-shaped output of a fast photodiode monitoring the excitation pulses. The output pulse-height spectra representing the experimental fluorescence decays, $F(t)$ (described in Eqs. (1)–(3)), cover a time range of about 16.5 ns with the resolution of 6.11 ps/channel. All fluorescence decays were collected up to 65,535 peak counts in strict single photon regime (detected photon rate < 100 kHz, i.e. one photon detected every 480 excitation pulses at most) by suitably attenuating the excitation beam. The background count rate was <200 Hz. The measurements were performed at ambient temperature.

Three identical samples were prepared for both $TPPS_{2a}$ and $TPCS_{2a}$ in each solvent, and one decay curve was acquired for each parallel. Each fluorescence decay was fitted, without deconvolving the system pulse response (full-width at half-maximum duration <30 ps), by minimizing the chi-square (χ^2) value through a Levenberg–Marquardt algorithm, to either a single exponential decay (Eq. (1)), double exponential decay (Eq. (2)) or triple exponential decay (Eq. (3)):

$$F_1(t) = y_0 + A_1 \exp\left(-\frac{t-t_0}{\tau_1}\right) \quad (1)$$

$$F_2(t) = y_0 + A_1 \exp\left(-\frac{t-t_0}{\tau_1}\right) + A_2 \exp\left(-\frac{t-t_0}{\tau_2}\right) \quad (2)$$

$$F_3(t) = y_0 + A_1 \exp\left(-\frac{t-t_0}{\tau_1}\right) + A_2 \exp\left(-\frac{t-t_0}{\tau_2}\right) + A_3 \exp\left(-\frac{t-t_0}{\tau_3}\right) \quad (3)$$

Apart from the decay constants τ_i and the amplitudes A_i, which have been left free to vary during the χ^2 minimization, two additional fitting parameters appear in these fitting functions: the constant background y_0, accounting for dark counts and environmental non-time-correlated light, which has also been left free to vary during the χ^2 minimization, and t_0, which represents the arrival time of the excitation pulses, and has been kept fixed at the time value corresponding to the channel at which the decay histogram had its maximum (65,535 counts). For each compound dissolved in any of the solvents, a value was attributed to each time constant, τ_i, and initial amplitude, A_i, equal to the average of the values obtained by fitting the experimental decays of the three parallels. The error on the τ_i is given by the standard deviation from the average value of the values obtained for the three parallels.

The relative amplitudes, f_i, reported in Tables 1 and 2, are calculated by means of Eq. (4):

$$f_i = A_i / \sum_j A_j \quad (4)$$

Table 2a
Spectroscopic properties of $TPPS_{2a}$ in pure solvents.

Solvent	λ_{abs} (nm)[a]	λ_{em} (nm)[a]	Φ_{Fl} [a]	τ_L (ps) (f_1)	τ_I (ps) (f_2)	τ_S (ps) (f_3)	τ_{av} (ps)	k_{Fl} (10^9 s^{-1})	k_{NR} (10^9 s^{-1})
Ethylene glycol	417	649, 715	0.129 ± 0.026	11,096 ± 93 (1)			11,096	0.012	0.078
Methanol	413	650, 715	0.038 ± 0.011	10,579 ± 44 (0.73)	2292 ± 2 (0.27)		8342	0.005	0.115
Ethanol	413	650, 715	0.041 ± 0.033	10,120 ± 82 (1)			10,120	0.004	0.095
2-Propanol	415	650, 717	0.062 ± 0.015	11,102 ± 68 (1)			11,102	0.006	0.084
1-Butanol	416	651, 718	0.073 ± 0.005	10,039 ± 89 (1)			10,039	0.007	0.092
DMSO	419	651, 718	0.110 ± 0.005	11,031 ± 91 (1)			11,031	0.010	0.081
DMF	418	651, 718	0.109 ± 0.009	10,464 ± 15 (1)			10,464	0.010	0.085
Acetonitrile	415	650, 717	0.076 ± 0.009	9355 ± 18 (1)			9355	0.008	0.099
Acetone	415	650, 717	0.034 ± 0.006	9589 ± 47 (1)			9589	0.004	0.101
Tetrahydrofuran	417	651, 718	0.062 ± 0.006	9769 ± 117 (0.73)	2559 ± 127 (0.26)	456(0.01)	7801	0.008	0.120
Chloroform	419	655, 720	0.009 ± 0.006	8124 ± 786 (0.54)	2267 ± 295 (0.24)	543 ± 15 (0.23)	5056	0.002	0.196
Ethyl acetate	415	650, 717	0.080 ± 0.026	9823 ± 1159 (0.61)	2491 ± 110 (0.34)	535 ± 4 (0.05)	6866	0.012	0.134
Diethyl ether	N.S.D.			8835 ± 927 (0.13)	2002 ± 130 (0.41)	562 ± 45 (0.45)	2222		
Cyclohexane	N.S.D.			7164 ± 70 (0.11)	2040 ± 21 (0.45)	538 ± 10 (0.43)	1937		

[a] The absorption and fluorescence emission maxima of $TPPS_{2a}$, λ_{abs} and λ_{em}, and the fluorescence quantum yields, Φ_{Fl}, are adopted from Lilletvedt et al. [1]. DMSO, dimethyl sulfoxide; DMF, dimethyl formamide; N.S.D., not spectrophotometrically detectable. The fluorescence decays, τ_L, τ_I and τ_S, are given ± standard deviation (ps), and relative initial amplitudes (f_1, f_2 and f_3, respectively). λ_{ex} = 420 nm. Average fluorescence decay time, τ_{av}, radiative and non-radiative decay rates, k_{Fl} and k_{NR}, are calculated from the Eqs. (5)–(7), respectively.

Table 2b
Spectroscopic properties of $TPCS_{2a}$ in pure solvents.

Solvent	λ_{abs} (nm)[a]	λ_{em} (nm)[a]	Φ_{Fl}[a]	τ_L (ps) (f_1)	τ_I (ps) (f_2)	τ_S (ps) (f_3)	τ_{av} (ps)	k_{Fl} (10^9 s^{-1})	k_{NR} (10^9 s^{-1})
Ethylene glycol	419	655	0.300 ± 0.011	9942 ± 14 (1)			9942	0.030	0.070
Methanol	416	654	0.200 ± 0.036	8462 ± 41 (1)			8462	0.024	0.095
Ethanol	417	654	0.245 ± 0.036	9046 ± 14 (1)			9046	0.027	0.083
2-Propanol	417	654	0.249 ± 0.041	8396 ± 956 (1)			8396	0.030	0.089
1-Butanol	418	655	0.262 ± 0.021	8890 ± 213 (1)			8890	0.029	0.083
DMSO	421	656	0.328 ± 0.022	10,156 ± 58 (1)			10,156	0.032	0.066
DMF	420	655	0.243 ± 0.047	9548 ± 30 (1)			9548	0.025	0.079
Acetonitrile	417	655	0.216 ± 0.011	8826 ± 16 (1)			8826	0.024	0.089
Acetone	417	654	0.196 ± 0.003	8943 ± 11 (1)			8943	0.022	0.090
Tetrahydrofuran	419	655	0.254 ± 0.014	9482 ± 130 (0.76)	2545 ± 38 (0.23)	474 ± 120 (0.01)	7796	0.033	0.096
Chloroform	N.S.D.			8444 ± 137 (0.83)	2301 ± 152 (0.15)	336 ± 36 (0.02)	7360		
Ethyl acetate	N.S.D.			8027 ± 521 (0.12)	1669 ± 78 (0.16)	383 ± 18 (0.72)	1506		
Diethyl ether	N.S.D.			7399 ± 165 (0.13)	2159 ± 20 (0.45)	537 ± 8 (0.41)	2159		
Cyclohexane	N.S.D.			7341 ± 112 (0.11)	2172 ± 16 (0.48)	583 ± 6 (0.42)	2089		

[a] The absorption and fluorescence emission maxima of $TPCS_{2a}$, λ_{abs} and λ_{em}, and the fluorescence quantum yields, Φ_{Fl}, are adopted from Lilletvedt et al. [1]. DMSO, dimethyl sulfoxide; DMF, dimethyl formamide; N.S.D., not spectrophotometrically detectable. The fluorescence decays, τ_L, τ_I and τ_S, are given ± standard deviation (ps), and relative initial amplitudes (f_1, f_2 and f_3, respectively). λ_{ex} = 420 nm. Average fluorescence decay time, τ_{av}, radiative and non-radiative decay rates, k_{Fl} and k_{NR}, are calculated from the Eqs. (5)–(7), respectively.

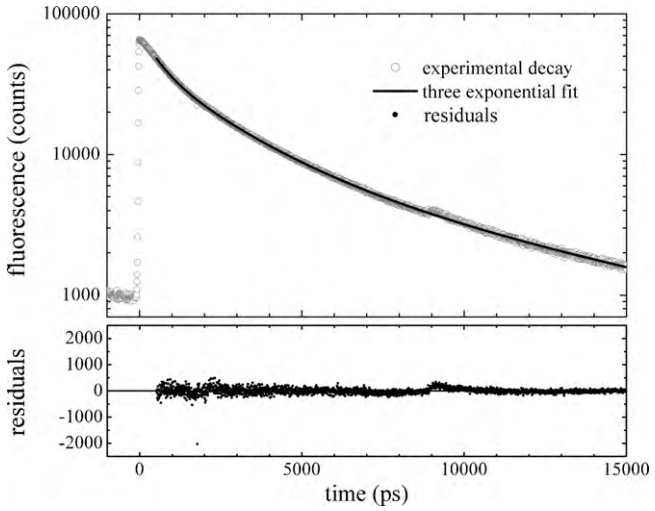

Fig. 2. Upper panel: exemplary decay of $TPCS_{2a}$ in diethyl ether (circles) and the best three-exponential fitting curve (line). Lower panel: residuals.

A typical decay is reported in Fig. 2, together with the relative fitting curve and a plot of the residuals.

2.2.2. Detection of $^1O_2$ by use of SOSG reagent

Aqueous solutions of $TPPS_{2a}$ and $TPCS_{2a}$ ($10^{-7}-10^{-6}$ M) containing SOSG reagent (1 μM) were prepared for determination of $^1O_2$ generation. The difference in fluorescence value between the samples and water containing SOSG reagent (1 μM) were calculated. A stock solution of SOSG was made in MeOH (5×10^{-5} M [18]). Riboflavin in water was used as a reference $^1O_2$ photosensitizer (87 μM [20]). The samples were filled in Falcon® polypropylene conical tubes (Becton Dickinson Labware, US), and illuminated for 6 min by a True-Lite® XDC radiation source (em: 300–800 nm). Immediately after illumination, the samples were excited at 480 nm, and fluorescence emission was detected at 525 nm (slit 10/10 [18,19]) by a Perkin Elmer LS50B fluorimeter.

3. Results and discussion

3.1. Deactivation from the first excited singlet state

The fluorescence decay distribution of the two photosensitizers $TPPS_{2a}$ and $TPCS_{2a}$ was measured in several organic solvents ($n=14$), possessing different polarity, proton donor and proton acceptor properties. The intention was to assess the effects of the surroundings (i.e. solvent) on the decay photophysics of $TPPS_{2a}$ and $TPCS_{2a}$. In this work, the polarity of the solvents is expressed in terms of three different parameters:

- the solvent dielectric constant (ε), which is a directly measurable, macroscopic, physical quantity proportional to the permanent solvent molecular dipole moment [21];
- the solvent orientation polarizability (Δf), which is a theoretical parameter calculated from ε and the refractive index (n). It also takes into account the solvent molecular polarizability [21];
- the solvent polarity value (π^*), which is an empirical parameter determined by the solvatochromic comparison method [22] and accounting for both permanent polarity and polarizability characteristics of the solvent.

The H-bond donating and accepting characters of the solvents are expressed in terms of the proton donor parameter (α) and the proton acceptor parameter (β), respectively, which are also empirically determined by the solvatochromic method [22,23]. The ε, Δf, π^*, α and β values of the solvents used in the present study are reported in Table 1. The solvents are divided into three main categories in order to simplify the discussion:

- alcohols (polar, protic): $\varepsilon \geq 17.51$, $\Delta f \geq 0.264$, $\pi^* \geq 0.47$, $\alpha \geq 0.76$ (i.e. ethylene glycol, methanol, ethanol, 2-propanol and 1-butanol);
- highly polar, non-protic: $\varepsilon \geq 20.6$, $\Delta f \geq 0.264$, $\pi^* \geq 0.71$, $\alpha \leq 0.19$ (i.e. dimethyl sulfoxide, dimethyl formamide, acetonitrile and acetone);
- non-polar or slightly polar, non-protic: $\varepsilon \leq 7.58$, $\Delta f \leq 0.210$, $\pi^* \leq 0.58$, $\alpha \leq 0.44$ (i.e. tetrahydrofuran, chloroform, ethyl acetate, diethyl ether and cyclohexane).

The fluorescence decay times of $TPPS_{2a}$ and $TPCS_{2a}$ in the above-mentioned organic solvents are shown in Tables 2a and 2b, respectively, together with their relative amplitudes. The fluorescence decays of both $TPPS_{2a}$ and $TPCS_{2a}$ are triple exponential in non-polar and slightly polar environment. The decay time values are roughly independent on the solvent. The values for the shortest decay component (τ_S) are in the range $(456)\ \text{ps} \leq \tau_S \leq (562\pm45)\ \text{ps}$ for $TPPS_{2a}$ and $(336\pm36)\ \text{ps} \leq \tau_S \leq (583\pm6)\ \text{ps}$ for $TPCS_{2a}$. The intermediate decay component (τ_I) has decay time values $(2002\pm130)\ \text{ps} \leq \tau_I \leq (2559\pm127)\ \text{ps}$ for $TPPS_{2a}$ and $(1669\pm78)\ \text{ps} \leq \tau_I \leq (2545\pm38)\ \text{ps}$ for $TPCS_{2a}$, respectively. The longest decay component (τ_L) has decay time values $(7164\pm70)\ \text{ps} \leq \tau_L \leq (9823\pm1159)\ \text{ps}$ for $TPPS_{2a}$ and $(7341\pm112)\ \text{ps} \leq \tau_L \leq (9482\pm130)\ \text{ps}$ for $TPCS_{2a}$, respectively. In the literature, only one long fluorescence decay (9.25 ns) was detected for the structurally similar H_2TPP dissolved in chloroform [24]. The decay becomes monoexponential for the other solvent categories, i.e. highly polar or polar, protic solvents, where only τ_L is detected, being $(9355\pm18)\ \text{ps} \leq \tau_L \leq (11102\pm68)\ \text{ps}$ for $TPPS_{2a}$ and $(8396\pm956)\ \text{ps} \leq \tau_L \leq (10156\pm58)\ \text{ps}$ for $TPCS_{2a}$, respectively. The monoexponential fluorescence decay observed in polar, protic solvents is in accordance with reported measurements of the monomeric form of chlorin e_6, hematoporphyrin and mesoporphyrin in ethanol [25], and several porphyrins like tetraphenylporphyrin and 5, 10, 15, 20-*tetrakis*(4-carbohydroxyphenyl)porphyrin in methanol or aceton [26]. The only exception to this pattern is the double exponential decay of $TPPS_{2a}$ in methanol, in which 27% of the molecules decay with $\tau_I = (2292\pm2)$ ps, which is rather similar to the τ_I of the remaining solvents. For both $TPPS_{2a}$ and $TPCS_{2a}$, the average fluorescence lifetime (τ_{av}) in each solvent was calculated from the decay data as Eq. (5):

$$\tau_{av} = \sum\nolimits_i \tau_i A_i / \sum\nolimits_i A_i \quad (5)$$

The obtained values are reported in Tables 2a and 2b for $TPPS_{2a}$ and $TPCS_{2a}$, respectively. The values of the absorption peak wavelength in the Soret band, λ_{abs}, the fluorescence emission peak wavelengths, λ_{em}, and the fluorescence quantum yield, Φ_{fl}, of $TPPS_{2a}$ and $TPCS_{2a}$, are taken from previously published steady-state measurements [1]. Due to inadequate solubility of $TPPS_{2a}$ in diethyl ether and cyclohexane, and correspondingly of $TPCS_{2a}$ in chloroform, ethyl acetate, diethyl ether and cyclohexane, our steady-state equipment did not possess sufficient sensitivity to determinate λ_{abs}, λ_{em} and Φ_{fl} in these solvents.

The values of the radiative decay rate constant, k_{FI}, and of the non-radiative decay rate constant, k_{NR}, were calculated, whenever possible, from Φ_{fl} and τ_{av}, by applying Eqs. (6) and (7):

$$k_{FI} = \frac{\Phi_{fl}}{\tau_{av}} \quad (6)$$

$$k_{NR} = \left(\frac{1}{\tau_{av}}\right) - k_{FI} \quad (7)$$

Table 3
Comparison of $TPPS_{2a}$ fluorescence lifetimes in various chloroform solutions.

Solvent	τ_L (ps) (f_1)	τ_I (ps) (f_2)	τ_S (ps) (f_3)	τ_{av} (ps)
Chloroform	8124 ± 786 (0.54)	2267 ± 295 (0.24)	543 ± 15 (0.23)	5056
Chloroform + 2% (v/v) EtOH	8089 ± 131 (0.81)	1166 ± 100 (0.19)		6774
Chloroform + 3% (v/v) BuOH	7972 ± 103 (0.89)	1059 ± 71(0.11)		7180

The fluorescence decays, τ_L, τ_I and τ_S, are given ± standard deviation (ps), and relative initial amplitudes (f_1, f_2 and f_3, respectively). Average fluorescence decay time, τ_{av}, is calculated from the Eq. (5). EtOH, ethanol; BuOH, 1-butanol.

The resulting values are reported in Tables 2a and 2b for $TPPS_{2a}$ and $TPCS_{2a}$, respectively. An interesting observation is that k_{NR} is similar for $TPPS_{2a}$ and $TPCS_{2a}$ when dissolved in equal solvents, while k_{FI} is approximately four times larger for $TPCS_{2a}$ compared to $TPPS_{2a}$ under the same conditions. Thus, the measured differences in the compounds' quantum yields primarily correspond to differences in the radiative decay rate. For $TPCS_{2a}$, k_{NR} dominates over k_{FI} in all solvents ($2 \leq k_{NR}/k_{FI} \leq 4.1$), and for $TPPS_{2a}$ the k_{NR} to k_{FI} ratio is even higher ($6.8 \leq k_{NR}/k_{FI} \leq 110$), indicating that $S_1{}^*$ deactivation of both compounds occur predominately via non-radiative pathways. The calculated values of k_{NR} obtained for $TPPS_{2a}$ in all solvents in which three exponential decays were measured and Φ_{fl} could be determined, namely tetrahydrofuran, chloroform and ethyl acetate, are significantly higher ($k_{NR} \geq (120 \pm 3) \times 10^6\ s^{-1}$) than k_{NR} in any solvent in which monoexponential fluorescence decay is observed ($k_{NR} \leq (100.7 \pm 0.6) \times 10^6\ s^{-1}$). Also for $TPCS_{2a}$, the maximum value of k_{NR} is detected in tetrahydrofuran, which is the only solvent where multiexponential decay was measured and Φ_{fl} could be assessed. This observation suggests that τ_S and τ_I in non-polar and slightly polar, non-protic environment are related to efficient non-radiative decay mechanisms, which are inhibited in highly polar and protic environments. The above conclusion is confirmed by the k_{NR} value obtained for $TPPS_{2a}$ in methanol ($k_{NR} = (115 \pm 1) \times 10^6\ s^{-1}$), where a significant amount of molecules decaying with decay time in the typical range of τ_I was detected. In methanol, the value of k_{NR} is lower than in any solvent where $TPPS_{2a}$ shows triple exponential fluorescence decay, but higher than in the solvents where single exponential decay is observed.

The results discussed above show that the $S_1{}^*$ decay photophysics of both $TPPS_{2a}$ and $TPCS_{2a}$ is slightly affected by the polarity of the surroundings. This might be explained by occurrence of intramolecular charge transfer reactions in the $S_1{}^*$ state, which were previously indicated for the two compounds by steady-state analysis [1]. Such reactions, implying either interchanges between different conformers or simple structural displacements, would be made possible in the $S_1{}^*$ state by conversion of the fluorescence excitation energy into free chemical potential energy, which is used to overcome the activation potential barrier. Thus, efficient non-radiative $S_1{}^*$ deactivation pathways would be provided. The formation of aggregates in organic solvents has been excluded, based on several facts: first, the concentrations of $TPPS_{2a}$ and $TPCS_{2a}$ used in this study are very low (maximum absorbance of samples at the Soret band peak < 0.08, corresponding to concentration $<10^{-7}$ M). This concentration range is described by others as non-aggregating (<1–50 μM [27,28]). Further, chlorin e_6, hematoporphyrin and mesoporphyrin were demonstrated to remain as monomers when dissolved in ethanol [25], and Andreoni et al. reported the presence of monomeric hematoporphyrin in alcoholic solutions [29]. The molar absorption coefficients of $TPPS_{2a}$ and $TPCS_{2a}$ in MeOH, given in the introduction, are also quite independent of the drug concentration at μM range (10^{-6}–10^{-7} M; RSD ≤ 5.5%, n = 18–24 [1]). And last, the spectral shapes of the compounds dissolved in MeOH do not change as a function of concentration (10^{-6}–10^{-7} M), as opposed to observations made in aqueous solutions, where aggregation induces changes in the Soret band and Q bands (data not shown). This phenomenon is at present under further investigation in our laboratories. Two possible excited state intramolecular charge transfer (ESICT) mechanisms are considered, which may be compatible with the molecular structures of both $TPPS_{2a}$ and $TPCS_{2a}$ and thereby provide a pathway for $S_1{}^*$ deactivation:

- an excited state intramolecular electron transfer (ESIET) from the central core towards the strong electron-withdrawing sulphonate groups;
- an excited state intramolecular proton transfer (ESIPT) between the tetrapyrrole nitrogens of the central core.

As the motion of electrons typically occurs on much faster time scales than protons, the measured τ_S value should be associated with the ESIET mechanism described above. Further, the measured τ_I value could be associated with the occurrence of the ESIPT mechanism. The measured τ_L value, due to its very high value (~10 ns), might characterize the intrinsic rate of decay of $TPPS_{2a}$ and $TPCS_{2a}$ from $S_1{}^*$ under the combined action of physical excitation energy dissipation modes (i.e. fluorescence emission, internal conversion and intersystem crossing). This is consistent with previously reported results on meso-tetraaryl porphyrins which exhibited a long decay component (10–15 ns) in a mixture of 3-methylpentane:isopentane accompanied by a reduction in lifetime as the molecular conformation was changed [30]. It is assumed that solvent relaxation, which usually occurs in the 10–100 ps time scale [21], takes place before fluorescence emission from $TPPS_{2a}$ and $TPCS_{2a}$. Consequently, the processes observed would be related to the vibrationally relaxed excited states of the molecules.

The non-polar solvent cyclohexane does not possess proton donor or proton acceptor properties. Thus, this solvent should not interfere with the decay mechanisms inherently displayed by either $TPPS_{2a}$ or $TPCS_{2a}$ in solution, and can therefore be used as a reference for comparison with the other solvents. A three exponential behavior is displayed for both compounds in cyclohexane. Moreover, the values of τ_S, τ_I, and τ_L and their relative amplitudes are almost identical for the two compounds. Therefore, the three different stereoisomers possible in the case of $TPCS_{2a}$ [1] do not seem to influence the fluorescence decay. The decays by means of ESIET (τ_S) and ESIPT (τ_I) in cyclohexane are equally probable (~0.4) and seem to be the dominating pathways for excitation energy dissipation in this solvent. Both ESIET and ESIPT appear to become of minor importance for both $TPPS_{2a}$ and $TPCS_{2a}$ upon an increase in solvent polarity. Indeed, τ_S and τ_I are detected in all the non-polar or slightly polar solvents, but disappear in highly polar solvents. If we assume ε to be the descriptor of the solvent polarity, the transition from triple to single exponential decay behavior occurs in correspondence with the largest increase in polarity (Tables 1, 2a and 2b). This observation is consistent also if the solvent polarity is described by means of Δf, although the increment in Δf between the most polar solvent exhibiting triple exponential decay (i.e. tetrahydrofuran, Δf = 0.210) and the least polar solvents exhibiting single exponential decay (i.e. DMSO and 1-butanol, Δf = 0.264) is less pronounced. However, the π^* solvent polarity values do not emphasize this trend. According to the π^* values, there

are two relatively polar solvents, namely chloroform ($\pi^* = 0.58$) and tetrahydrofuran ($\pi^* = 0.58$), where both $TPPS_{2a}$ and $TPCS_{2a}$ are decaying by a triple exponential behavior. Three less polar, but protic solvents, i.e. 1-butanol ($\pi^* = 0.47$), 2-propanol ($\pi^* = 0.48$), and ethanol ($\pi^* = 0.54$), induce a single exponential decay. This discrepancy could be ascribed to the difficulties in obtaining π^* in protic solvents [23]. It seems apparent that H-bonding properties have less influence than solvent polarity on the decay mechanisms of $TPPS_{2a}$ and $TPCS_{2a}$. However, it cannot be ruled out that intermolecular H-bonding between the drug molecule and the solvent partly restricts the ESIET and ESIPT processes. This is shown by comparing the decay data obtained in diethyl ether, ethyl acetate, chloroform and tetrahydrofuran, which have similar polarities but definitely different H-bonding properties (Table 1). The H-bond donor (chloroform) quenches the decay of $TPCS_{2a}$ via ESICT (τ_S and τ_1) more efficiently than the H-bond acceptors (diethyl ether, ethyl acetate and tetrahydrofuran; Table 2b). This is illustrated by the relative initial amplitudes, i.e. the sum of f_2 and f_3 is 0.17 for $TPCS_{2a}$ in chloroform compared to 0.88 in ethyl acetate and 0.89 in diethyl ether, respectively. It should be noted that the non-negligible H-bond accepting character of diethyl ether, ethyl acetate, and tetrahydrofuran seems to have minor importance in quenching ESICT decay mechanisms. The situation is more complicated for $TPPS_{2a}$ (Table 2a). In order to evaluate the influence of H-bond donating character in slightly polar solvents, chloroform was selected for investigation of $TPPS_{2a}$ in the presence of small amounts (2–3%, v/v) of ethanol or 1-butanol (Table 3). This amount of alcohol is too small to significantly change the polarity and the bulk properties of the solvent [21]. Still it was sufficient to increase the fraction of the long-decay time (f_1) from 0.54 to 0.81 or 0.89, respectively and to remove τ_S (Table 3). These experiments show that slightly polar H-bond donors also quench the decay of $TPPS_{2a}$ by ESICT (τ_S and τ_1), in accordance with the observations made for $TPCS_{2a}$.

For both $TPPS_{2a}$ and $TPCS_{2a}$, non-linear correlation (assessed by R^2) was observed for the average fluorescence decay time (τ_{av}) as a function of solvent polarity, when expressed as ε, Δf or π^*. By taking into account all the solvents ($n = 14$) for $TPPS_{2a}$, correlation indexes for the parameters ε, Δf and π^* were $0.59 \leq R^2 \leq 0.74$. As the correlation indexes for the non-protic solvents only ($n = 9$; $0.70 \leq R^2 \leq 0.88$), are not highly improved, the specific solvent effects of the environment seem to be of minor importance for the $S_1{}^*$ deactivation pathways of $TPPS_{2a}$. In the case of $TPCS_{2a}$, the plot of τ_{av} as a function of ε, Δf and π^* also emphasized the lack of correlation in all solvents ($n = 14$; $0.56 \leq R^2 \leq 0.63$) and in the non-protic solvents separately ($n = 9$; $0.51 \leq R^2 \leq 0.78$). In total, these observations indicate that the molecular dipole moments of $TPPS_{2a}$ and $TPCS_{2a}$ do not change appreciably upon excitation to the $S_1{}^*$ state, as previously suggested [1].

$TPPS_{2a}$ and $TPCS_{2a}$ behave differently in methanol, as reported above. The double exponential decay of $TPPS_{2a}$ might be explained by a specific solute–solvent interaction: the cavity size of porphyrins and chlorins would be sufficient for interactions with small molecules like methanol [1], but not for close interactions with alcohols of larger molecular size. Methanol might interact by hydrogen-bonding with the nitrogen and hydrogen moieties of the tetrapyrrole cavity of $TPPS_{2a}$, and reduce quenching by the ESIPT decay mechanism. The twisting of $TPCS_{2a}$ however, may prevent methanol from binding within the cavity, resulting in the observed single exponential decay.

3.2. Production of singlet oxygen

Porphyrins and structurally related compounds are known for their production of $^1O_2$ [31,32]. Formation of $^1O_2$ is reported for similar porphyrins, i.e. tetraphenylporphyrin and 5, 10, 15, 20-*tetrakis*(4-carbohydroxyphenyl)porphyrin [26], and for haematoporphyrin and related compounds [33]. $^1O_2$ production by $TPPS_{2a}$ and $TPCS_{2a}$ *in vivo* will be influenced by the surroundings. By comparing aqueous samples of $TPPS_{2a}$ and $TPCS_{2a}$ displaying equal absorbance (Abs) at their respective Soret band absorption maxima in the range Abs = 0.04–0.35, it was shown that $TPPS_{2a}$ gives a higher $^1O_2$ yield, determined by the SOSG fluorescence signal (I) at 525 nm as arbitrary units ($I_{525\,nm}$/Abs = 979 ± 510 STD, $n = 18$), than $TPCS_{2a}$ ($I_{525\,nm}$/Abs = 114 ± 86 STD, $n = 15$) under identical conditions. For neither of the two compounds, the sample absorbance (i.e. photosensitizer concentration) showed a linear correlation with the apparent amount of $^1O_2$ generated (i.e. SOSG fluorescence signal; $R^2 \leq 0.882$; data not shown). The reason for the non-linear relationship is not fully understood, but aggregation, e.g. formation of dimers, is more likely to occur even at low concentrations in aqueous environments. Aggregation as a function of microenvironment will be further evaluated in an upcoming study. In total, both photosensitizers generate $^1O_2$ in considerable amounts in aqueous solution, which should be relevant for the $^1O_2$ generation *in vivo*.

4. Conclusion

The deactivation pathways from the first excited singlet state ($S_1{}^*$) of the novel, patented photosensitizer $TPCS_{2a}$ have been studied in several organic solvents of different polarity and H-bonding properties, by means of time-resolved fluorescence measurements. The chemically related photosensitizer $TPPS_{2a}$ was studied as a reference compound. Both compounds have proven to interact with their environment primarily by polarity effects, while intermolecular H-bonding has been demonstrated to influence the excited-state dynamics only slightly. Two non-radiative decay mechanisms involving excited state intramolecular charge transfer were postulated to compete with internal conversion and intersystem crossing, in order to account for the triple exponential decays obtained in solvents of low polarity. These mechanisms were postulated to be: (1) electron transfer from the central core of the molecules towards the sulphonate groups and (2) proton transfer between the tetrapyrrole nitrogens of the core. However, the fluorescence decays became almost inert to the solvent properties as soon as polarity was moderately enhanced. Substantial insensitivity of the photophysical properties to the surroundings can be desirable for the photosensitizing properties *in vivo*. Singlet oxygen generation was measured in aqueous environment by applying the SOSG reagent method. Both compounds showed $^1O_2$ generation *in vitro*, which is essential for the utilization as a photosensitizer *in vivo*.

Acknowledgements

This work was supported by the Norwegian Research Council. Funding was also provided by the ERASMUS exchange program and the EMBIO program at the University of Oslo. The authors thank Prof. A. Andreoni for access to her lab and for providing assistance with the TCSPC measurements.

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