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Solubilization of the photosensitizers $TPCS_{2a}$ and $TPPS_{2a}$ in aqueous media evaluated by time-resolved fluorescence analysis

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Received November 4, 2011, accepted December 29, 2011

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Pharmazie 67: 598–600 (2012) *doi: 10.1691/ph.2012.1791*

The pH-dependent aggregation of the novel photosensitizer $TPCS_{2a}$ is investigated at low concentration ($c = 10^{-6}$ M) in aqueous media by means of time-correlated single-photon counting, and compared to that of the chemically related photosensitizer $TPPS_{2a}$. The efficacy of selected solubilizers, *i.e.*, various nonionic Pluronic block copolymers and the nonionic surfactant Tween 80, in inhibiting aggregation of the two photosensitizers is evaluated, which is important for the further formulation of $TPCS_{2a}$.

1. Introduction

The novel photosensitizer meso-tetraphenyl chlorin disulphonate ($TPCS_{2a}$) is used in the patented technology of photochemical internalization (PCI), as discussed elsewhere (Berg et al. 2006; Lilletvedt et al. 2010a,b, 2011a,b, Selbo et al. 2010). PCI is a new treatment modality against cancer at present in clinical trial phase I/II. $TPCS_{2a}$ is structurally related to the photosensitizer meso-tetraphenyl porphyrin disulphonate ($TPPS_{2a}$), which was used as a reference. Porphyrin-related compounds dissolved in aqueous solutions tend to form aggregates or dimers at pharmaceutical relevant concentrations and even at very low concentrations ($c < 10^{-6}$ M; Brown et al. 1976; Poderys et al. 2004). $TPCS_{2a}$ has to be solubilized into monomers by formulation technology prior to *i.v.* administration. Further, deaggregation is essential to obtain optimal bioavailability and a reproducible pharmacokinetic profile. Finally, as aggregation generally leads to reduced photoreactivity of photosensitizers (Krasnovsky Jr et al. 1990), monomerization is desirable in order to achieve an optimal biological response. Aggregation of porphyrin-related compounds is most extensive in aqueous acidic environment (White 1978). This particularly holds true 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). $TPCS_{2a}$ and $TPPS_{2a}$ display both these properties. Aqueous solution at low pH (pH 2.9) was therefore selected as the reaction medium. In our recent study, the UV-Vis absorption and steady-state fluorescence emission properties of $TPCS_{2a}$ and $TPPS_{2a}$ were analyzed in aqueous solution at varying pH, and some of the observed variations were interpreted to be due to aggregation (Lilletvedt et al. 2011a). In aggregates, the decay from the excited singlet state (S_1^*) by internal conversion is notably enhanced as compared to the corresponding monomer (Krasnovsky Jr et al. 1990). Thus, the extent of aggregation of a fluorescent species in solution may be quantitatively assessed by analyzing the time-resolved fluorescence decay distribution. Assuming that the stereoisomeric structures of the analyzed compound do not have distinct decay times, the number of various aggregates in solution is equal to the number of exponential decay components revealed in the fluorescence decay pattern. The relative amplitudes of the decay components will then coincide with the relative concentrations of the corresponding aggregates (Kelbauskas and Dietel 2002). A previous study on the fluorescence decay of $TPCS_{2a}$ and $TPPS_{2a}$ indicated that the above condition is merged for these photosensitizers (Lilletvedt et al. 2010a).

2. Investigations, results and discussion

In this work, aggregation of $TPCS_{2a}$ at low concentration ($c = 10^{-6}$ M) was investigated by time-resolved fluorescence as a function of pH, and compared to that of $TPPS_{2a}$. Deaggregation of the photosensitizers by the nonionic Pluronic block copolymers L44, F68, P123, F127 and the nonionic surfactant Tween 80 was evaluated at concentrations above or below the respective critical micelle concentration (CMC; Lilletvedt et al. 2011b), by reconstructing the fluorescence decay distributions through time-correlated single-photon counting (TCSPC; Becker 2006). The decay data obtained for $TPCS_{2a}$ and $TPPS_{2a}$ in different aqueous media are reported in the Table. At neutral pH, the decay pattern of both $TPCS_{2a}$ and $TPPS_{2a}$ could be fitted by a single exponential with long decay time τ_2. At acidic pH, an additional decay component with shorter decay time τ_1 was obtained. The latter decay time was dominant at pH 2.9 (Fig. for $TPCS_{2a}$). This is not caused by the equilibrium of ionic forms (pK_a sulphonate groups ≤ 1; inflection points of imino nitrogens = 3.9; Lilletvedt et al. 2011a). As aggregation is a phenomenon occurring preferentially in acidic aqueous media, the long-lived fluorescence decay component was interpreted to be due to the emission of monomers, and the short-lived one as due to emission of aggregates. The increase of fraction f_1 at lowering the pH value is in agreement with our interpretations of the steady-state absorption and fluorescence data (Lilletvedt et al. 2011a), as well as with previous observations on similar compounds (Brown 1976; Pasternack et al. 1972; Sutter et al. 1993). Addition of any of the solubilizers at concentrations above the critical micelle concentration (CMC) to acidic solutions at pH

Table: Decay times (τ_1 and τ_2) and relative amplitudes (f_1 and f_2) obtained by the fitting of the experimental fluorescence decay patterns of a) TPCS$_{2a}$ and b) TPPS$_{2a}$ in different aqueous media

(a) TPCS$_{2a}$

pH	Solubilizer	Solubilizer concentration	τ_1 (ps) ± s.d.	f_1 ± s.d.	τ_2 (ps) ± s.d.	f_2 ± s.d.
6.3				0	8081 ± 5	1
4.9			622 ± 32	0.162 ± 0.003	8122 ± 29	0.838 ± 0.003
2.9			342 ± 4	0.906 ± 0.001	6945 ± 141	0.094 ± 0.001
2.9	Pluronic L44	< CMC	453 ± 3	0.459 ± 0.003	8522 ± 5	0.541 ± 0.003
		> CMC		0	10023 ± 10	1
2.9	Pluronic F68	< CMC	583 ± 4	0.206 ± 0.003	9135 ± 16	0.794 ± 0.003
		> CMC		0	9940 ± 9	1
2.9	Pluronic P123	> CMC		0	9352 ± 5	1
2.9	Pluronic F127	< CMC	567 ± 13	0.166 ± 0.006	8880 ± 19	0.834 ± 0.006
		> CMC		0	10186 ± 8	1
2.9	Tween 80	> CMC		0	11132 ± 66	1

(b) TPPS$_{2a}$

pH	Solubilizer	Solubilizer concentration	τ_1 (ps) ± s.d.	f_1 ± s.d.	τ_2 (ps) ± s.d.	f_2 ± s.d.
6.3				0	7901 ± 22	1
4.9			2397 ± 21	0.251 ± 0.001	10350 ± 15	0.749 ± 0.001
2.9			2641 ± 42	0.740 ± 0.010	9606 ± 582	0.260 ± 0.010
2.9	Pluronic L44	< CMC	2612 ± 29	0.445 ± 0.003	8115 ± 158	0.555 ± 0.003
		> CMC		0	11234 ± 10	1
2.9	Pluronic F68	< CMC	2395 ± 77	0.312 ± 0.004	9205 ± 16	0.688 ± 0.004
		> CMC		0	11545 ± 12	1
2.9	Pluronic P123	> CMC		0	9595 ± 38	1
2.9	Pluronic F127	< CMC	2555 ± 17	0.231 ± 0.002	11311 ± 23	0.769 ± 0.002
		> CMC		0	11734 ± 11	1
2.9	Tween 80	> CMC		0	13150 ± 80	1

2.9 resulted in recovery of single exponential decay patterns for both TPCS$_{2a}$ (as shown in the Fig. for addition of Tween 80) and TPPS$_{2a}$. This indicates that the Pluronics L44, F68, P123, F127 and Tween 80 are efficient inhibitors of aggregation through solubilization of the two photosensitizers. These observations support the deaggregation of TPCS$_{2a}$ and TPPS$_{2a}$ and verify the experimentally determined Pluronics CMC's presented in previous work (Lilletvedt et al. 2011a, b). Interestingly, even addition of solubilizer at concentration below CMC caused a significant decrease in f_1. This supports the presence of small complexes between Pluronic unimers and the photosensitizers, as previously suggested (Lilletvedt et al. 2011b; Steinbeck et al. 2004). The observed changes in the τ_1 and τ_2 values by a variation in the type of solubilizer are due to changes in the aqueous microenvironment of the photosensitizers (mainly polarity effects; Lilletvedt et al. 2010a). The discrepancy in τ_2 measured below and above CMC represents the physiochemical influence of the microenvironment, formed by interactions with Pluronics unimers versus incorporation into polymeric micelles at the low photosensitizer concentration investigated (Lilletvedt et al. 2011b). The different τ_1 and τ_2 values obtained for TPCS$_{2a}$ and TPPS$_{2a}$ reflect the impact of steric conformation and molecular electron distribution on photophysical reactivity as influenced by the medium (Lilletvedt et al. 2010a, b, 2011a, b).

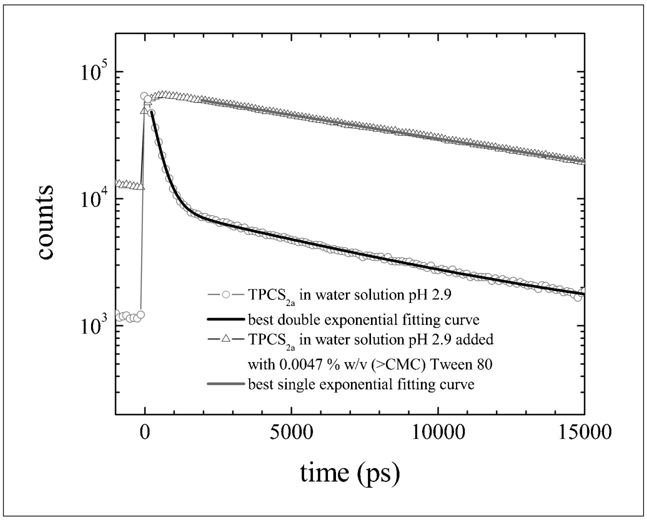

Fig.: Experimental fluorescence-decay distributions of TPCS$_{2a}$ in aqueous solution at pH 2.9 with (triangles) and without (circles) addition of Tween 80, together with the corresponding best-fitting curves

Aggregation of the novel photosensitizer TPCS$_{2a}$ and the reference compound TPPS$_{2a}$ was demonstrated in acidic aqueous media at micromolar concentrations by means of time-resolved fluorescence analysis. Solubilization by Pluronic F68, F127, P123, L44 or Tween 80 prevents aggregation of TPCS$_{2a}$ and TPPS$_{2a}$, and thus preserves their photoreactivity and biological effect.

3. Experimental

Di(monoethanolammonium) meso-tetraphenyl chlorin disulphonate (TPCS$_{2a}$) and di(triethylammonium) meso-tetraphenyl porphyrin disulphonate (TPPS$_{2a}$) were supplied by Synthetica AS, Oslo, Norway (purity $\geq$ 98.7%). Pluronics F68, P123 and F127 were purchased from Sigma Aldrich, Pluronic L44 (containing DL-alpha-Tocopherol at 100 ppm) was a gift from BASF Corp., USA. Tween 80 of Ph.Eur. quality was purchased from Merck. Aqueous samples of TPCS$_{2a}$ and TPPS$_{2a}$ ($c = 2 \times 10^{-6}$ M) at pH 6.3, 4.9, and 2.9 and constant ionic strength ($\mu = 0.01$) were prepared in water of p.a. grade (Emsure®, Merck, Darmstadt, DE) by addition of HCl and NaCl (n = 3). Assessments on the efficacy of the Pluronics and Tween 80 in preventing aggregate formation were performed at pH 2.9, in accordance with Lilletvedt et al. (2011a). The concentrations of the solubilizers were 0.0084–0.0087% w/v (< CMC) and 0.073–0.078% w/v (> CMC) for Pluronic L44; 0.0075–0.0092% w/v (<CMC) and 0.0919–0.0975% w/v (> CMC) for Pluronic F68; 0.000829–0.001% w/v (> CMC) for Pluronic P123; 0.00088% w/v (< CMC) and 0.00191–0.00196% w/v (>CMC) for Pluronic F127; and 0.0045–0.0047% w/v (> CMC) for Tween 80. The CMC values were taken from Lilletvedt et al. (2011b). A fluorescence decay distribution was acquired for each parallel (n = 3) with a TCSPC apparatus whose overall temporal resolution is 30 ps and which is exhaustively described elsewhere (Lilletvedt et al. 2010a). The fluorescence decay distributions $F(t)$ were fitted to multi-exponential decays above a constant background, $F(t) = y_0 + \sum_{i=1}^{n} A_i \exp\left(-\left(t - t_{exc}\right)/\tau_i\right)$, by minimizing the chi-square value through a Levenberg-Marquardt algorithm with a commercial data-analysis software (Origin 7). For each decay, the number of exponential components was established by adding, one by one, exponential components to the fitting function until the fitting routine converged to yield two components of equal time constant. Either single-exponential decays ($n = 1$) or double-exponential decays ($n = 2$) were obtained. Exemplary decay patterns are displayed in the Fig. together with their best-fitting curve. The decay times τ_1 and the corresponding relative amplitudes $f_i = A_i/(A_1 + A_2)$ reported in the Table are the average of the values obtained by the fit of the parallels, with errors given by the corresponding standard deviations.

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