Molecular interactions and solubilization of structurally related meso-porphyrin photosensitizers by amphiphilic block copolymers
(Pluronics)

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Abstract

The influence of four Pluronics block copolymers (i.e. F68, P123, F127, and L44) on the aggregation and solubilization of five structurally related meso-tetraphenyl porphyrin photosensitizers (PS) as model compounds for use in Photodynamic Therapy of cancer (PDT) was evaluated. Interactions between the PSs and Pluronics were studied at micromolar concentration by means of UV-Vis absorption spectrometry and by kinematic viscosity (η) and osmolarity measurements at millimolar concentrations. Pluronic micelles were characterized by size and zeta potential (ζ) measurements. The morphology of selected PS-Pluronic assemblies was studied by atomic force microscopy (AFM). While hydrophobic 5,10,15,20-Tetrakis(4-hydroxyphenyl)porphine (THPP) seemed to be solubilized in the Pluronic micellar cores, amphiphilic di(monoethanolammonium) meso-tetraphenyl porphine disulphonate (TPPS2a) was likely bound to the micellar palisade layer. Hydrophilic PSs like 5,10,15,20-Tetrakis(4-trimethylammoniumphenyl)porphine (TAPP) seemed to form complexes with Pluronic unimers and to be distributed among the micellar coronas. TPPS2a aggregated into a network which could be broken at Pluronic concentration >cmc, but would reconstitute in the presence of tonicity adjusting agents, e.g. sodium chloride (NaCl) or glucose.

Introduction

Photodynamic therapy (PDT) utilizes a combination of non-toxic dyes (photosensitizers; PS), molecular oxygen and visible light of appropriate wavelength, in order to treat, e.g. cancer and localized bacterial infections. The photosensitization process involves energy transfer to molecular oxygen and free radical and electron exchange reactions, initiated by light absorption by the PS. The reactive oxygen species formed will cause damage to diverse cell structures in their direct surroundings. Porphyrins, commonly employed as PSs in PDT, tend to aggregate in aqueous solutions even at very low concentrations (<1 μM). Meso-tetraphenyl chlorin disulphonate (TPCS2a) even forms a gel at pharmacologically relevant concentrations (i.e. mM-range). Solubilization by amphiphilic copolymers, e.g. Pluronics that spontaneously form nanoparticulate carriers (10–100 nm) in aqueous media is one possible strategy to improve PS bioavailability. Several Pluronics are approved for oral or intravenous administration by the US Food and Drug Administration by virtue of their high solubilizing capability, low toxicity and prolonged in vivo circulation time prior to dissociation. Use of drug delivery systems of appropriate size and low clearance may lead to enhanced accumulation of antineoplastic agents in tumor tissues.

The five model PSs selected in the present study are structurally related porphyrin compounds, with different substituents (Figure 1). They are characterized by various lipophilicity (logP values ranging from −4.54 to 10.1), charge and charge distribution. TAPP is positively charged in aqueous media, THPP is uncharged at physiological pH, TSPP, TCPP and TPPS2a are present as mixtures of a negatively charged free base and a zwitter ion. The aqueous solubilities and aggregation states of these PSs have been demonstrated to depend on medium properties, such as, e.g. ionic strength or pH. Porphyrin aggregation can be depicted by a broad and flattened absorption band in the UV-Vis absorption spectrum (i.e. the Soret band). A split Soret band represents a mixture of differently charged free base and zwitter ion. Upon addition of a co-solvent or nonionic surfactant the PSs may dissolve and be present as monomers in solution, exhibiting a narrow and intense Soret band and the four bands of lower intensity (Q-bands).

Previous studies on Pluronic interactions with meso-tetraphenylporphyrin were performed by employing NMR spectroscopy or Atomic Force Microscopy (AFM). In another study, we presented the influence of formulation by various Pluronics on dark cytotoxicity, photocytotoxicity and localization of the four model photosensitizers in cancer cells. The employed Pluronics differ in regard to their hydrophobicity, size of polyethylene oxide block (EO) and polypropylene oxide block (PO) and molecular weight (Mw; Figure 1). Briefly, Pluronics influenced cellular uptake of the PSs, changed PS.

Keywords

Cancer, nanoparticles, photodynamic therapy, photosensitizer, pluronic

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localization in different cellular organelles and stimulated PS transport to the nucleus. While some Pluronics enhanced photokilling of cancer cells in vitro, others attenuated PS activity. For lipophilic Pluronics, an inverse correlation was found between the photodynamic activity of PS-Pluronic formulations and Pluronic concentration. Of the four selected Pluronics (i.e., L44, F68, F127 and P123), P123 is the most promising in regard to improving photocytotoxic PS potential in cancer cells.

The aim of current study was to elucidate specific PS-Pluronic interactions at different concentration levels, assess the PS-Pluronic affinity and determine the locus of solubilization in Pluronic micelles. Moreover, the influence of solubilize on the micellization process was studied and solubilization capacity of the selected Pluronics was assessed. Finally, PS-Pluronic formulations containing P123 and F127 were developed and their morphology was analyzed, including variables important for drug delivery, i.e. particle size, surface morphology and ζ-potential. In the present study, the influence of the selected Pluronics on the aggregation and solubilization of structurally related meso-tetraphenyl porphyrin photosensitizers was studied by use of kinetic viscosity (η). The spectral changes in PS UV-Vis spectra and variation in cmc values were used to evaluate Pluronic-PS affinity. Dynamic light scattering and electrophoretic mobility measurements were employed to characterize the Pluronic micelles and their interactions with PSs. AFM was utilized to study the morphology of PS-Pluronic assemblies in selected formulations.

Materials and methods

Materials

5,10,15,20-Tetakis(4-trimethylaminophenyl) porphine tetra-chloride (TAPP, Mw = 989.02 g/mol); 5,10,15,20-Tetakis(4-hydroxyphenyl) porphine (THPP, Mw = 678.74 g/mol); 5,10,15,20-Tetakis(4-sulphonaphthophenyl) porphine dihydrochloride (TSPP, Mw = 1007.92 g/mol); 5,10,15,20-Tetakis(4-carboxyphenyl) porphine (TCP, Mw = 790.8 g/mol; purity >97%) were purchased from Frontier Scientific (Logan, UT). Di(monoethanolammonium) meso-tetraphenyl porphine disulphonate (TPPS2a, Mw = 977.24) was synthesized by Sochinaz SA (Vionnaz, Switzerland) and kindly provided by PCI Biotech AS (Oslo, Norway). The compounds were stored desiccated at + 4 °C and used as received. Pluronics® F68, P123, F127 and L44 were purchased from Sigma Aldrich (St. Louis, MO). Pluronic L44® contained 100 ppm DL-alpha-Tocopherol as an antioxidant. The water used was EMSURE® water (declared metal ion content, e.g., Cu ≤0.0004 mg/L, Fe ≤0.001 mg/l) purchased from Merck (Darmstadt, Germany) or sterile water for irrigation (USP) from B. Braun Melsungen AG, Germany. All other reagents were of analytical grade. Milllex® GP 0.22 μm syringe filters were from Millipore (Corrigtwhill, Co. (Cork, Ireland)); Spartan® 0.45 μm syringe filters (13/0.45 RC) were from Whatman (Springfield Mill, UK); disposable 10 × 10 × 45 polystyrol/polystyrene sizing cuvettes were from Sarstedt AG & Co. (Nümbrecht, Germany); disposable 70 μl microwettes were from Plastibrand (Brand GMBH, Wertheim, Germany). The Zeta potential transfer standard (−68 ± 6.8 mV) and DipCell® electrode assembly were purchased from Malvern Instruments Ltd. (Malvern, UK). The computer software MarvinView5.3.1 (ChemAxon, Budapest, Hungary) was used to calculate logP values.

UV-Vis absorption measurements

TAPP and TSPP stock solutions (0.25 mM) were prepared in ESMURE® water. Stock solutions of THPP and TCP (0.25 mM) were prepared in methanol and stored in the dark at (+4 °C) for maximum 4 days. Pluronic stock solutions in Esmure® water (0–36 mM) were prepared by shaking overnight (150 rpm; Edmund Büchler), at ambient temperature (25 ± 1°C). The samples were prepared by mixing aliquots of Pluronic stock solution and PS stock solution and diluting the sample to 10 ml with ESMURE® water. The PS concentration was constant (5 μM) and the investigated Pluronics concentration ranges were: F68 (0–12 mM), L44 (0–36 mM), F127 (0–0.3 mM), P123 (0–9.1 mM) (10–14 concentration levels, n ≥3). The methane content provided by THPP and TCP stock solutions never exceeded 2% (v/v) of the aqueous samples. Absorption spectra of the four PSs (5 μM) were recorded from 290 to 700 nm on a Shimadzu UV-2401PC spectrophotometer. The accuracy in wavelength determination was ± 0.5 nm. The measurements were performed at ambient temperature (20 ± 1°C). The samples were protected from light and measured immediately after preparation.

Determination of kinematic viscosity

Samples containing TAPP, TSPP or TPPS2a (10 mM) (n = 3) were prepared in ESMURE® water, Pluronic aqueous samples (2–25 mM) or Pluronic aqueous isotonic samples containing NaCl or glucose by shaking overnight (125 rpm, Edmund Bühler, Hechingen, Germany) at 25 ± 1°C. Samples containing THPP (0.5–10 mM) or TCP (0.5 mM) (n = 3) were prepared by solvent evaporation18. Appropriate volumes of THPP stock solutions (0.2–2.5 mM) and Pluronic stock solutions (0.2–2.5 mM) in acetone were mixed in a round bottle (100 ml). The acetone was evaporated (EL 131 Rotavapor equipped with 461 water bath, Büchi, Flawil, Switzerland, 100 rpm) at 40 ± 1°C during light protection and Pluronics-PS formed a film. 3 ml of ESMURE® water, isotonic saline or glucose in ESMURE® water was immediately added to the round bottle and the film was dissolved by agitation (125 rpm, Edmund Bühler) for 30 min at 25 ± 1°C. All the samples were filtered (Spartan® 0.45 μm), protected from light and allowed to equilibrate for 30 min at 25 ± 1°C, prior to measurements.

The kinematic viscosity was measured by an Ostwald capillary viscometer (Schott Type 516 10/I; sample volume = 2 ml;
The particle size and \( \zeta \)-potential were determined by use of a Zetasizer (Malvern Nanosizer ZS, Malvern Instruments, UK), equipped with 633 nm laser, at 25 ± 0.1 °C. Lower detection limit is 0.3 nm for particle size measurements, lower particle diameter is 3.8 nm for zeta potential measurements. Samples \( (n = 1) \) were equilibrated for 120 s before each measurement \( (n \geq 5) \). Automatic attenuation was used. The refractive index of polystyrene particles, \( \zeta \)-potential = −68 mV) prior to measurements. Quality of data was determined by assessing count rate and attenuation level.

Results

UV-Vis absorption measurements and estimation of cmc values

Representative absorption spectra of THPP, TAPP and TSPP in water and in the presence of a low concentration F68 (i.e. 20 \( \mu \)M, where monomers are predominant) and high concentration of F68 (i.e. 240 \( \mu \)M, where micelles are expected) are presented in Figure 2. Data for other PS-Pluronic formulations are presented in the supplementary material (Figures S1–S4 and Table S1). Upon increasing the F68 concentration, the broad and flattened Soret band of THPP gradually became narrow and intense; no apparent isosbestic point was observed (Figure 2A). Absorbance at the Soret band maximum (422 nm) increased with increasing Pluronic concentration resulting in a pronounced break when plotted on a semi-logarithmic scale (Figure 2B). The break was taken as a result of micellization and recorded as the cmc-value of F68 in the presence of THPP (Table 1). A similar gradual increase in THPP absorbance upon increasing concentrations of F127, P123 and L44 was observed, but cmc values could not be estimated (Figure S1). Absorption spectra with split Soret bands were observed for the anionic TCPP and TSPP in water (Figures 2C, S2 and S3). The absorption spectra of TCPP at low concentrations of Pluronics were markedly different from the absorption spectra recorded at high Pluronics concentrations (Figure S3). Systematic changes in the TCPP absorbance or an isosbestic point were not observed, thus estimations of cmc-values were not performed. Addition of Pluronics to TSPP resulted in a gradual decrease in the absorbance measured at 434 nm and a corresponding increase at 414 nm. An isosbestic point was observed at 424 nm. The Pluronic cmc values in the presence of TSPP were taken as the Pluronic concentrations, where TSPP absorbance levels off, as depicted by the crossing of two trend lines (Figures 2D and S2; Table 1).

Addition of F68 to TAPP resulted in a small decrease in the Soret band intensity and a slight shift of the peak from 412 nm to 415 nm (Figure 2E) and shifts in Q-bands (Table S1). The TAPP Soret band absorption reached a plateau at F68 concentrations above ~65 \( \mu \)M (Figure 2F). Similar spectral changes were recorded at increasing concentrations of F127, L44 and P123 (Figure S4). The cmc values were taken as the Pluronic concentrations where no further decrease in TAPP absorbance could be detected as depicted by the crossing of two trend lines (Figures 2F and S4; Table 1). The cmc values of Pluronics in the presence of TPPS2a were not determined experimentally, assuming that the previously determined cmc values in the presence of the structural analogue chlorine TPCS2a is representative for TPPS2a.

Evaluation of aggregation by osmolarity measurements

Assuming fully dissolved and dissociated PS, the theoretically calculated osmolarity will show a linear increase when plotted...
against PS concentration, with slopes \( \sim 1 \) osmol/mol (THPP and TCPP); \( 3 \) osmol/mol (TSPP and TTPS2a) and \( 5 \) osmol/mol (TAPP) in Emsure\textsuperscript{®} water. The experimentally determined osmolarities of the PSs (0–10 mM) in Emsure\textsuperscript{®} water (0–10 mM; 6 concentration levels; \( n = 9 \); RSD \( \leq 5.4\% \)) increased linearly with PS concentration with a calculated slope of 2.8 osmol/mol (\( R^2 = 0.989 \)) for TAPP and 0.8 osmol/mol (\( R^2 = 0.949 \)) for TTPS2a. The increasing osmolarity of TSPP exhibited linearity (\( R^2 = 0.951 \)) with a calculated slope of 0.3 osmol/mol until a plateau was reached at concentrations \( \geq 7.5 \) mM. Osmolarities of TCPP and THPP could not be determined experimentally without solubilizer due to their low aqueous solubility.

Table 1. Estimated Cmc values (\( \mu M \)) determined for Pluronics F68, L44, P123, F127 in presence of the selected porphyrins (5 \( \mu M \)); n.d. = not determined.

| Porphyrin (5 \( \mu M \)) | F68 | L44 | F127 | P123 |
|--------------------------|-----|-----|------|------|
| THPP                     | \( \sim 60 \) | n.d. | n.d. | n.d. |
| TAPP                     | \( \sim 65 \) | \( \sim 440 \) | \( \sim 200 \) | \( \sim 400 \) |
| TSPP                     | \( \sim 65 \) | \( \sim 630 \) | \( \sim 16 \) | \( \sim 20 \) |

Evaluation of aggregation by kinematic viscosity measurements

While the kinematic viscosity (\( \nu \)) of TAPP and TSPP in plain aqueous solutions showed a small increase with increasing concentration, the viscosity of TTPS2a increased substantially \( >5 \) mM (Figure 3A). Aqueous solutions of TTPS2a \( >10 \) mM formed a stiff gel, at 25 °C. The \( \nu \) of TCPP and THPP in pure water could not be determined due to their low aqueous solubility. A linear increase (\( R^2 \geq 0.986 \)) in \( \nu \) of the Pluronics was detected in water (Figure S5A). Concentration selected for further investigations was 2 mM; L44 was excluded from further studies due to high cmc values (Table 1).

TCPP and THPP could not be formulated with Pluronics by direct dissolution. The use of a cosolvent (\( \leq 5\% \) ethanol) yielded solutions of low concentration and stability (determined by visual phase separation). Thus samples containing THPP and TCPP were prepared by solvent evaporation. The \( \nu \) of PS-Pluronic formulations containing TCPP, TAPP or TSPP was higher than the respective plain Pluronic samples (Figure 3B), although only slightly for TCPP-Pluronic combination. The experimentally determined \( \nu \) of the TSPP formulations followed the same order as the Pluronics samples in Emsure\textsuperscript{®} water (i.e. F127 > F68 > P123), and were relatively close to the sum of the contribution from TSPP and the representative Pluronic.
The increase in the \( \mu \) of PS-Pluronic formulations compared to the sum of the viscosities measured separately implied interactions between solubilizer and solubilizate (Figure 3B). P123 was selected to study the PS-Pluronic formulations at higher Pluronic concentration, i.e. from 2 up to 25 mM and in combination with isotonic glucose or saline (Figure 3C and D). In accordance with the previous experiment (Figure 3B), the presence of 10 mM TAPP, TSPP or TPPS\(_2\)a increased the \( \mu \) of P123 samples in the concentration range 5–25 mM (Figure 3D). The \( \mu \) of 10 mM TPPS\(_2\)a was markedly reduced in presence of increasing P123 concentration in the 0–5 mM range. The \( \mu \) of 10 mM THPP could not be measured at 2 mM P123, due to visible precipitation.

Glucose and sodium chloride at isotonic concentration caused only slight changes of the \( \mu \) of the PS-P123 formulations when compared to the \( \mu \) measured in the formulations without tonicity adjusters. The exception was the \( \mu \) of the TPPS\(_2\)a–P123 formulation adjusted with NaCl, which was too high to be measured by the employed method, due to formation of a stiff gel. The \( \mu \) of the TPPS\(_2\)a–P123 formulation, adjusted with glucose was dependent on the P123 concentration at low glucose content (Figure 3C). Addition of NaCl caused precipitation of TSPP, both in the presence and the absence of P123.

Particle size measurements
F127 and P123 were selected for the investigation of particle size. They are known to form spherical micelles\(^5\) with well-defined cores >cmc, as also shown by the monodisperse size distribution of 2 mM P123 (18 ± 0.1 nm; PDI = 0.026; Figure 4A). Two size populations were observed in 2 mM aqueous sample of F127 (mean intensity peaks at 35 nm and 6 nm; Figure 4B).

The mean particle size of P123 micelles was decreased only in the presence of 0.1 mM TPPS\(_2\)a (Figure 4A). The mean value of the upper intensity peak of F127 increased /C\^12 nm upon addition of 0.1 mM TAPP (Figures 4B and S5B) or TSPP, but decreased /C\^5 nm upon addition of TPPS\(_2\)a (Figure 4B). The hydrophilic TAPP, hydrophobic THPP and amphiphilic TPPS\(_2\)a were selected for further study of the influence of PS concentration on particle size (Figure 4C). The particle size of P123 decreased with a minimum observed at 0.2 mM TPPS\(_2\)a. Only the upper F127 intensity peak was present at THPP concentrations >0.2 mM, while the lower intensity peak was not detectable (data not shown). Addition of isotonic glucose had only a slight influence on P123 micellar size and size distribution, while isotonic saline had no detectable effect.

Zeta potential (\( \zeta \)-potential) measurements
Addition of 0.1 mM TPPS\(_2\)a to 2 mM F127 changed the \( \zeta \)-potential significantly, from \(-2.1 ± 0.6 \text{ mV}, \text{ to } -11.0 ± 1.6 \text{ mV} (n = 5)\). Addition of 0.1 mM TAPP to 2 mM F127 increased the \( \zeta \)-potential to a very small extent, but this was not consistent and not statistically significant (0.8 units; \( p = 0.07 \)). Addition of other PSs (0.1 mM) resulted in \( \zeta \)-potential values overlapping with the \( \zeta \)-potential of Pluronic particles within standard deviation.
AFM images

TPPS2a and TSPP aggregates showed structural changes as a function of PS concentration (Figure 5A–F). Dry 0.1 mM TPPS2a yielded small cylindrical particles (Figure 5A), while the 1 mM sample showed the presence of irregular particles, as well as long strain-like structures (Figure 5B). At 10 mM a highly organized network was observed, composed of elongated wire-like structures (Figure 5C). Dry 0.1 mM TSPP contained small, elongated structures (Figure 5D), while the 1 mM and 10 mM TSPP samples yielded long, needle-shaped structures (Figure 5E and F). TAPP showed a pattern, attributed to dried crystals or irregular particles. The sample of THPP (0.1 mM) resulted in well-defined particles (Figure 5H) forming irregular bubbles, composed of smaller spherical particles. Similar structures were observed for 0.1 mM TCPP (Figure 5I).

The 2 mM P123 yielded very small and well-defined spherical particles (Figure 6H). The presence of spherical particles of diameter comparable with blank P123 samples was also observed in formulations composed of 2 mM P123 and 0.1 mM TAPP, TPPS2a (Figure 6A) and TSPP (Figure 6B). Larger particles of similar morphology were present in 2 mM P123 containing 1 mM TPPS2a (Figure 6C). The combination of 2 mM P123 and 10 mM TPPS2a exhibited irregular, bulky structures as well as dendritical structures (Figure 6E and G). 2 mM P123 containing 1 mM or 10 mM TSPP (Figure 6D and F) were similar to plain TSPP samples (Figure 5E and F). Formulations of THPP or TCPP and 2 mM P123 formed round drops of several tens of micrometers during drying. The presence of small spherical particles in the background was observed (data not shown).

Discussion

Aggregation of PSs in aqueous media

The evaluated PSs behave as weak acids, when dissolved in pure water. pH of the un-buffered medium, ionic strength, high PS concentration as well as several other parameters, will influence PS aggregation, which has been thoroughly evaluated². Even though neutral-alkaline pH may be used to increase the solubility of THPP and reduce aggregation of TCPP and TSPP, in this study ESMURE® water was used in order to reduce number of variables (i.e. ionic strength, buffer salts, metal ions) that could affect PS-Pluronic interactions. Hydrophilic TAPP (logarithm of partition coefficient, logP_\text{v/w} \approx -4.54) molecules are expected to exhibit strong intermolecular repulsion, due to their positive charge. However, the v of TAPP in plain aqueous solution was low and the osmolarity was half of the expected (calculated) value (Section ‘‘Evaluation of aggregation by osmolarity measurements’’), and therefore some aggregate formation >0.1 mM cannot be ruled out (e.g. as dimers or a mixture of monomers and smaller aggregates). The AFM images of the dried samples showed small particles tightly packed on the mica (Figure 5G). The distribution pattern of the particles was most probably a consequence of the drying and not aggregation.

The hydrophilic TSPP (log P_\text{v/w} \approx 0.08 at the isoelectric point, pI) forms aggregates of higher order, as shown by the low osmolarity detected in plain aqueous solutions (Section ‘‘Evaluation of aggregation by osmolarity measurements’’) and indicated by AFM images of the dried samples (Figure 5D). Aqueous samples of TSPP contain a mixture of monomeric free base and aggregating zwitterions even at mM concentrations². The structure of TSPP aggregates apparently did not change much with increasing TSPP content, as depicted by a low increase in v and osmolarity in the concentration range 1–10 mM (Figure 3A; Section ‘‘Evaluation of aggregation by osmolarity measurements’’) and visualized by AFM (Figure 5D–F). The two-step v increase (Figure 3A) suggests that the amphiphilic TPPS2a aggregation process includes smaller intermediates which form a more complex network-like structure at increased concentrations, as supported by the AFM-pictures (Figure 5A–C).

In plain water, TCPP forms a mixture of monomeric free base and lipophilic, aggregating zwitterion (logP_\text{pI} \approx 7.2), limiting aqueous solubility². Thus, dried aqueous solutions of TCPP showed a clear aggregation-pattern as small, rounded particles
prolonged into chains (Figure 5I). The uncharged, highly lipophilic THPP (logP ≈ 10.1) has an even lower solubility showing the presence of spherical particles (Figure 5H), which is in accordance with previous reports. TCPP and THPP aggregation was not studied above 0.1 mM PS concentration due to their low solubility in water.

Calculation of cmc values and evaluation of porphyrin-Pluronic interactions

The cmc estimated for F68 (Table 1) was not dependent on the PS properties, which indicates that the hydrophilic F68 unimers did not form stable complexes with any of the porphyrins below cmc. The cmc was close to previous reports. The cmc estimated for the other Pluronics in the presence of TSPP (Table 1) were comparable to values determined by the pyrene solubilization method, as it is generally acknowledged that cmc-values can vary up to 10 times depending on the method employed. The spectroscopic measurements suggested minor changes in the polar environment surrounding TAPP in the presence of Pluronic unimers (Figures 2E and S4). Although interactions between TAPP and Pluronic unimers may be thermodynamically favorable, as indicated by the large cmc values (Table 1), it should be noted, that the change in TAPP absorbance as a function of Pluronic gradient was small (∼10% decrease), and determination of cmc is associated with inaccuracy.

THPP was partially solubilized by all the selected Pluronic unimers as indicated by a more narrow and intense Soret band < cmc (Figures 2A and S1), but it was possible to determine the cmc value for F68 only. F68 exhibits a marked polarity difference between its hydrophilic unimers (containing 84% EO units) and an apolar micellar core (composed almost exclusively of PO units). Thus, micellization will cause a substantial increase of THPP solubility, as indicated (Figure 2B). The more hydrophobic F127, P123 and L44, will enhance THPP solubility by a concentration-dependent change of micellar aggregation number, size and shape, characterized by a gradually less-polar milieu of the micellar core. As a result, the absorbance at the THPP Soret band increased gradually (Figure S1). Cmc estimation was difficult by use of TCPP, due to the presence of several PS species of various charge and aggregation states (Figure S3).

Solubilization of PSs by Pluronic nanocarriers

Intermicellar interactions are reported to be mediated mainly by the hydrophilic micellar coronas, filled with highly-ordered water molecules bound between EO chains, and to some extent within the micelle core. This water will be a hindrance to the supercooling process in the osmolarity measurements. As the water binding effect was negligible at 2 mM Pluronic in the absence of PSs, this concentration was used in most studies. A 2 mM P123 sample shows the presence of ∼20 nm micelles (Figure 4A), as also previously reported. F127 shows a bimodal distribution (Figure 4B) corresponding to the reported mixture of micelles at ∼30 nm and unimolecular micelles at ∼5 nm. As plain aqueous Pluronic samples showed an increase of υ upon increasing concentration (Figure S5A), measurements >2 mM were only performed for P123, that exhibits a low increase in υ (Figure 3D) up to gelling point (∼30 mM at 25°C). THPP was solubilized by Pluronic unimers and micelles, as depicted by the steep increase of the THPP Soret band absorptivity with increasing Pluronic concentration (Figures 2B and S1).
However, the high absorptivity of the THPP Soret band in the presence of P123 and F127 at μM concentration suggests, that THPP will likely exhibit strong interactions with Pluronics possessing high average number of propylene oxide groups (NPO). 2 mM F68 yielded an unstable formulation with THPP (observed as visible precipitation 1 h after sample preparation), probably due to the short lipophilic PO segment (Figure 1). F127 and P123 have similar N PO and cmc (Figure 1, Table 1) and should subsequently exhibit comparable solubilization of the hydrophobic THPP. Still, the N of 2 mM F127 was reduced upon THPP addition, while it had the opposite effect on 2 mM P123 (Figure 3B). A similar variation in behavior of these two Pluronics upon addition of a hydrophobic probe was associated with changes of the size of the hydrophobic micellar core. Spectroscopic measurements showed that Pluronics preferentially solubilize TSPP free base, which led to a decrease in zwitter ion concentration as detected at 434 nm and a clear isosbestic point at 424 nm (Figures 2C and S2). At low concentrations (≤0.1 mM) TSPP will be associated with Pluronic unimers and partly be located in the hydrophilic surface of the micelles. This may explain the increase in F127 micellar size upon TSPP addition (Figure 4B). While 0.1 mM TSPP became disaggregated following P123 addition (Figure 6B), 1 mM or 10 mM TSPP samples contained aggregates (Figure 5E and F) which were not affected by P123, as shown by AFM-imaging (Figure 6D and F) and ζ measurements (Figure 3B). TSPP was able to strengthen the network formed by P123 micelles and enhance its ζ (Figure 3D), probably due to the reduced water activity, caused by TSPP aggregates in the aqueous intermicellar space and a subsequent increase in effective concentration of polymer in the system.

A preferential interaction between TAPP and unimers of P123 and F127 was suggested by the detected cmc (Table 1). The fact that the size of the unimer complex is close to the detection limit of electrophoretic mobility may partly explain why the formulations of TAPP did not show a reproducible change in ζ-potential as compared to plain Pluronic samples.
The measured ζ-potential of F127 micelles in the presence of the amphiphilic TPPS2a (Figure 1) indicates that the unsubstituted phenyl rings were located towards the micellar core, while the charged sulphonic groups were located in the hydrophilic corona. The micelles obtained a better aqueous solubility by introduction of surface charge, while their core was stabilized by presence of the hydrophobic porphine ring. Such an arrangement will promote the formation of numerous small micelles by the large surface to volume ratio. However the amount of molecules, accommodated at the micellar interface (palisade layer) will be limited. At increased TPPS2a concentration the PS molecules are increasingly located in the micellar core. Thus the size of P123 micelles was increased (Figure 4C) and spherical micelles were replaced by larger structures with a tendency to bind to each other (Figure 6A, C, E and G). Transition of spherical micelles to rod- or worm-like structures probably induced an increased v at P123 concentration >10 mM (Figure 3D). Formation of the TPPS2a network was hindered in presence of Pluronics as depicted by a steep decrease in v upon increasing P123 concentration (Figure 3C).

Lack of a clear isosbestic point in TCPP spectra following Pluronic addition suggests the presence of PS aggregates during solubilization at low Pluronic concentration. A mM Pluronic content was needed to increase TCPP solubility and hinder aggregation (Section “AFM images”). Solubilization of 0.5 mM TCPP was achieved at Pluronic/PS molar ratio 4:1. It was however not possible to solubilize 10 mM TCPP, even with the highest P123 concentration used in this study (i.e. 25 mM). The results suggested that TCPP is not well suitable for formulation by Pluronics.

**Influence of isotonic media**

Isotonic NaCl caused precipitation of 10 mM TSPP, probably due to salting out of TSPP aggregates, which is in accordance with the salt-induced aggregation, reported in our previous paper. P123 (2 mM) was not able to prevent this process, which is in accordance with the observed low solubilization of TSSP (Figure 6D and F). Addition of saline caused a substantial increase in v of the TPPS2a – P123 formulation (above detectable range), while isotonic glucose had a weaker, but detectable effect (Figure 3B), dependent on the P123 concentration (Figure 3C). Salts can dehydrate the EO blocks and compact the micelles, leading to reduced interactions between the amphiphilic TPPS2a and the hydrophilic P123 corona. TPPS2a may then subsequently be expelled outside the P123 micelles, leading to the recreation of the TPPS2a network. An increasing number of micelles by increased P123 concentration were sufficient to eliminate the effect of glucose and solubilize TPPS2a (Figure 3C). Presence of toxicity adjusting agents is an important variable in the development of TPPS2a and TSPP formulations, while it seems not to affect the other PS-Pluronic assemblies.

**Conclusion**

The five evaluated PSs of various charge, charge distribution and polarity differ in regard to their aqueous solubility, aggregation in aqueous media, and interaction with the selected amphiphilic copolymers. While hydrophobic THPP and amphiphilic TPPS2a exhibit strong interactions with the Pluronics, the hydrophilic PSs are only loosely bound. THPP seems to be solubilized in the Pluronic micellar cores; TPPS2a is likely bound to the micellar palisade layer with the lipophilic part located in the core. Therefore, Pluronics with low cmc values are good solubilizers for the PS-Pluronic formulation. As TAPP, TCP and TSPP seem to be distributed among the micellar coronas and complexes with Pluronic unimers, Pluronics with high cmc values may be preferable for solubilization of these PSs. P123 seems to be the most efficient solubilizer among the evaluated Pluronics, which may partly explain the improvement of in vitro PS-photocytotoxicity in cancer cells by formulation with P123. 2 mM P123 is sufficient to solubilize 2 mM THPP, 0.5 mM TCP, 10 mM TPPS2a, but only 0.1 mM TSPP. At Pluronic concentration > cmc, the TPPS2a aggregate network is broken. Addition of isotonic NaCl or glucose influences the TPPS2a-Pluronic interactions, leading to reconstruction of the PS-aggregate network. An increased P123 concentration can be employed to solve this problem. The results from the current study help to explain Pluronic effects on PS photodynamic activity in vitro and may be used in the further development of PS-Pluronic formulations for preclinical studies.

**Declaration of interest**

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Supplementary material available online
Supplementary Figures S1–S5 and Table S1