

Phthalocyanines and Metal Phthalocyanines with Phosphoryl Groups: Supramolecular Ensembles, Photochemical and Photobiological Properties

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Based on the supramolecular approach, the conditions for the solubilization and disaggregation of phosphoryl-containing phthalocyanines and their metal complexes (I-IV) in the aqueous environment due to the formation of supramolecular ensembles are found. For the first time, zinc phthalocyaninate with phosphoryl-containing groups in the macrocycle (IV) was studied as a potential photosensitizer in various media. The supramolecular organization of Ps in solution, the singlet oxygen quantum yield Φ_{Δ}^{IV} in DMSO (0.48 ± 0.05) and an organized microheterogeneous medium based on cationic cetyltriphenylphosphonium bromide (0.54 ± 0.05) in combination with low cytotoxicity, sufficiently high phototoxicity (IC_{50} dose) and the formation of intracellular reactive oxygen species in vitro upon irradiation allow one to consider phosphoryl-containing phthalocyanines as deserving of attention and further research as compounds useful for PDT.

Keywords: Phthalocyanines, supramolecular ensembles, cetyltriphenylphosphonium bromide, cetyltrimethylammonium bromide, sodium deoxycholate, singlet oxygen, quantum yield, phototoxicity, reactive oxygen species, fluorescent microscopy.

Фталоцианины и металлофталоцианины с фосфорильными группами: супрамолекулярные ансамбли, фотохимические и фотобиологические свойства

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На основе супрамолекулярного подхода найдены условия сольubilизации и дезагрегации фосфорилсодержащих фталоцианинов и их металлокомплексов в водной среде благодаря образованию супрамолекулярных ансамблей. Впервые фталоцианинат цинка с фосфорил-содержащими группами в заместителях макроцикла был изучен как потенциальный ФС в различных средах. Супрамолекулярная организация ФЦ в растворе, квантовый выход

синглетного кислорода Φ_{Δ}^{IV} в ДМСО и организованной микрогетерогенной среде на основе катионного цетилтрифенилфосфоний бромида в сочетании с низкой цитотоксичностью, достаточно высокой фототоксичностью (доза IC_{50}) и образованием внутриклеточных активных форм кислорода *in vitro* при облучении позволяют рассматривать фосфорилсодержащие фтало- и металлофталоцианины как заслуживающие внимания и дальнейшего исследования в качестве соединений, полезных для ФДТ.

Ключевые слова: Фталоцианины, супрамолекулярные ансамбли, цетилтрифенилфосфоний бромид, цетилтриметиламмоний бромид, дезоксихолат натрия, синглетный кислород, квантовый выход, фототоксичность, активные формы кислорода, флуоресцентная микроскопия.

Introduction

Phthalocyanines (Pcs) and their supramolecular aggregates find application in molecular electronic devices and chemical sensors, catalysis, biology and medicine including photodynamic therapy (PDT).^[1,2] The PDT method involves three non-toxic components, namely, photosensitizer (PS), light and oxygen. PS accumulates in the tumor. When irradiated with light with a wavelength close to the maximum of its absorption band, with the participation of the triplet state of the PS reactive oxygen species (ROS) are generated, including singlet oxygen (1O_2). They have a cytotoxic effect on tumor cells (Scheme 1) leading to tumor destruction.^[3]

The main requirements to an ideal PS are the following:

1. Thoroughly characterized and available individual compounds; stability upon storage and use; low cytotoxicity; selectivity in respect of tumor cells; fast excretion.
2. Intense maximum in the visible and NIR ranges of electromagnetic spectrum (650–850 nm); intense fluorescence allowing diagnostics.
3. The lifetime of the triplet state, sufficient for the realization of the photodynamic effect; excitation energy not less than 94 kJ/mol necessary for the formation of 1O_2 .

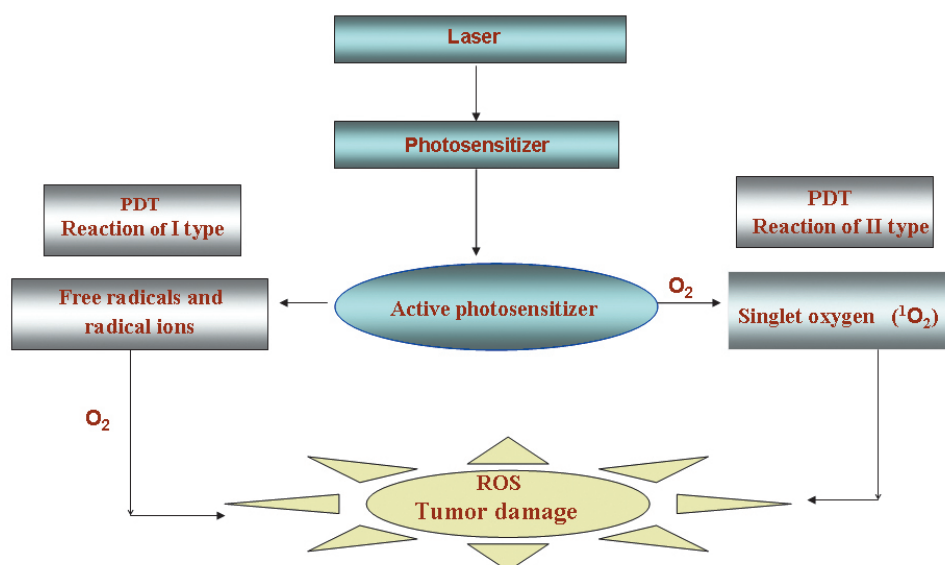
Such properties of Pcs as chemical stability, absorption spectrum with the maximum ($\epsilon \geq 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$) in the above mentioned range, low toxicity and others allow them

to be considered as potential PS in fluorescent diagnostics and PDT of tumors.^[4,5] Photophysical properties of diamagnetic non-transition metal (Al, Ga, Zn and others) phthalocyanines quite well meet the requirements of PDT and fluorescent diagnostics.^[6,7]

Generation of ROS (mainly 1O_2) requires the presence of phthalocyanine as a monomer and the absence of aggregation in physiological conditions. The presence of aggregates (mainly of *H*-type) decreases quantum yield of luminescence and lifetime of excited states.^[8] As a result, the formation of 1O_2 and other reactive species responsible for cell death is inhibited.^[9] Quantum yield of singlet oxygen and photostability of PS in oxidation conditions are important parameters for PDT. At the same time, high photostability of Pcs can be both advantage and shortcoming.

One of possibilities of preventing aggregation of Pcs in aqueous medium is the use of surfactants of various types to provide an environment for Pcs compatible with biological medium. For example, review^[10] was devoted to supramolecular interactions in redox transformations of an electroactive component (fullerene and its derivatives, transition metal complexes and metallophthalocyanines) embedded in an artificial lipid matrix, and their role in an aqueous medium.

This paper reports on the study of supramolecular organization of Pcs and Zn phthalocyaninate with substituents in the macrocycle bearing dioxiphosphoryl and oxy(alkoxy)



Scheme 1. Photodynamic action scheme.

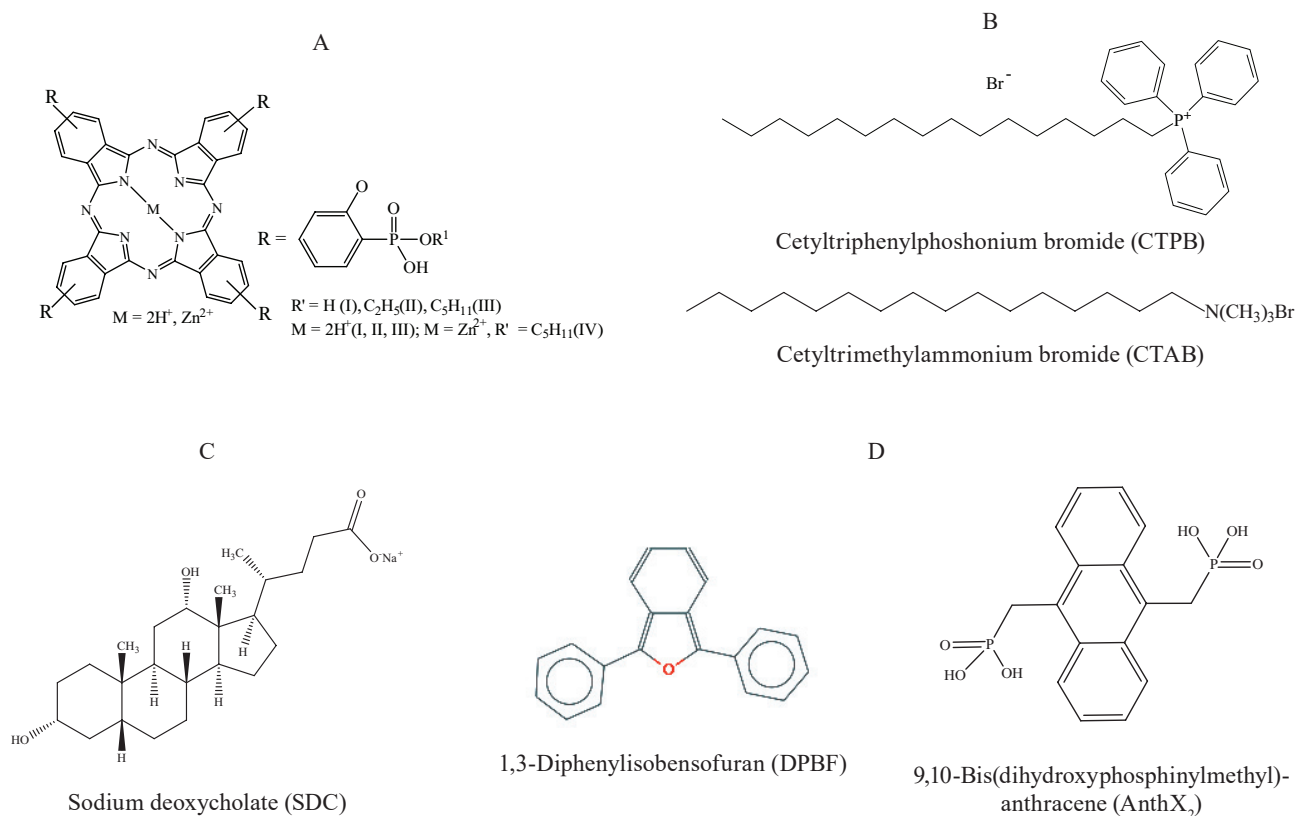


Figure 1. Structures of phthalocyanines and zinc phthalocyaninate with phosphonate and mono(alkyl)phosphonate substituents in the macrocycle (A), cationic CTPB and CTAB (B), anionic sodium deoxycholate (C), 1,3-diphenylisobenzofuran and 9,10-bis(dihydroxyphosphinylmethyl)anthracene (D).

phosphoryl groups in phosphate buffer and surfactant solutions, and their some properties. Photochemical properties of zinc phthalocyaninate were also analyzed in the presence of singlet oxygen quenchers, namely, 1,3-diphenylisobenzofuran (DPBF) and 9,10-bis(dihydroxyphosphinylmethyl)anthracene (AnthX₂) for DMSO and microheterogeneous aqueous medium based on cationic surfactants – cetyltriphenylphosphonium bromide (CTPB) and cetyltrimethylammonium bromide (CTAB), respectively. The structures of Pcs, surfactants and chemical traps for singlet oxygen are presented in Figure 1.

Experimental

General

Tetraphosphonic and tetra(monoalkyl)phosphonic acids of phthalocyanines (I, II, and III), and tetra(monoalkyl)phosphonic acid of zinc phthalocyaninate (IV) were obtained as described in.^[11] 9,10-Bis(dihydroxyphosphinylmethyl)anthracene was prepared according to procedure.^[12] Sodium deoxycholate (Aldrich, 98 %), 1,3-diphenylisobenzofuran (Aldrich, 97 %), cetyltriphenylphosphonium bromide and cetyltrimethylammonium bromide (Merck, 98 %) were used as received. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (Sigma-Aldrich) and DCFH₂-DA were purchased from Sigma-Aldrich. Solutions were prepared in bidistilled water. All reagents necessary for preparation of phosphate buffer (pH = 7.4) were extra-pure grade.

Samples of I-IV were added to known volume of phosphate buffer (PBS) and centrifuged after full dissolution. In spectral measurements, samples of surfactants of known concentration were placed in a cell filled with Pc solution. Measurements were carried out to constant value of optical density. The samples were protected from light.

Procedure for Analysis of Phosphoryl Containing Zn Phthalocyaninate as PS

1,3-Diphenylisobenzofuran and 9,10-bis(dihydroxyphosphinylmethyl)anthracene were used as singlet oxygen quenchers for DMSO and microheterogeneous medium of cationic surfactant. Freshly prepared solution comprising PS, quencher and solvent was used in each experiment. Cationic cetyltrimethylammonium bromide or cetyltriphenylphosphonium bromide in phosphate buffer were used for the water-PS system. Concentration of IV in DMSO and phosphate buffer was $X \cdot 10^{-6}$ M, where X was from 1 to 10, and concentration of AnthX₂ in phosphate buffer was $X \cdot 10^{-5}$ M, where X was from 1 to 10. Concentration of the quencher was equal or lower than $10 \cdot 10^{-5}$ M to avoid chain reaction of oxidation of DPBF in the presence of oxygen. The measurements were carried out in a 1.0 cm cuvette, and for aqueous system, a 96-well plate (200 μ L well capacity) was used, that allowed us to simultaneously study a series of solutions. Absorption spectra for quencher and PS in time were recorded with a Cary 60 UV-Vis spectrophotometer (Agilent Technologies Bayan Lepas Free, Malaysia) equipped with an interface for computer recording of spectra and thermostated cell holder, and with a multi-functional TECAN SPARK 10M spectrophotometer (Tecan, Switzerland)

in the dark at 24.3 °C. Solutions under study were irradiated with an ALKhT-ELOMED laser apparatus (Russia) ($\lambda = 670$ nm, optical radiation power equal to 0.3 W) in continuous mode by pulses of 1 to 50 seconds duration.

Quantum yield of singlet oxygen in DMSO or aqueous microheterogeneous medium of cationic surfactant (in the air, no additional oxygen treatment) was determined by a comparison method^[13] and calculated according to:

$$\Phi_{\Delta}^i = \Phi_{\Delta}^{\text{ref}} \frac{K^i \cdot I_{\text{abs}}^{\text{ref}}}{K^{\text{ref}} \cdot I_{\text{abs}}^i},$$

where K^i and K^{ref} are reaction rate constants for photobleaching of 1,3-diphenylisobenzofuran or 9,10-bis(dihydroxyphosphinyl methyl)anthracene under laser irradiation in the presence of PS and reference compound; $I_{\text{abs}} = I_0(\lambda) \cdot (1 - 10^{-A(\lambda)})$, where I_0 is laser radiation intensity at corresponding wavelength, and $A(\lambda)$ is optical density of studied macrocycle at corresponding wavelength. The value of $\Phi_{\Delta}^{\text{ref}}$ for PcZn used as a reference compound was 0.67 in DMSO,^[14] and $\Phi_{\Delta}^{\text{MB}}$ for MB in water (pH 7.4) was 0.39.^[15] K^i and K^{ref} were determined from the first-order kinetics equation linearized as $\ln C = \ln C_0 - k \cdot \tau$, where reaction rate constant was calculated as a slope of linear function to time axis: $-\text{tg} \alpha = k$. Concentration of 1,3-diphenylisobenzofuran and 9,10-bis(dihydroxyphosphinylmethyl)anthracene (C_i) was found from the Bouguer-Lambert-Beer law: $A(\lambda) = \varepsilon(\lambda) \cdot l \cdot C$, where $A(\lambda)$ is optical density; $\varepsilon(\lambda)$ is molar absorption coefficient; l is solvent layer thickness, and C is concentration of dissolved compound.

Fluorescence spectra for solutions of III and III/CTAB in phosphate buffer, and **IV** in micellar SDC solution were measured with a PerkinElmer LS55 spectrofluorimeter in 10 mm quartz cells in the air at room temperature. The slit width was 10 nm and detection angle was 90°. For **III**, excitation spectra were recorded at $\lambda_{\text{em}} = 710$ nm, and emission spectra were recorded at $\lambda_{\text{ex}} = 650$ nm. For **IV**, excitation spectra were recorded at $\lambda_{\text{em}} = 725$ and emission spectra were recorded at $\lambda_{\text{ex}} = 620$ nm. Position and shape of the emission band are not dependent on exciting light wavelength (578, 614, 650 and 675 nm). The bands which can be attributed to light scattering on microheterogeneous medium particles were not found upon excitation of **III** by light with wavelengths lying in the above mentioned range.^[16,17] No fluorescence was observed for all background solutions. The absorption spectra before and after the fluorescence measurements remained unchanged, that is, Pc did not decompose during photoexcitation in the spectrofluorimeter.

Spectrophotometric measurements were carried out with a Specord plus (Analytikjena, Germany) and a Specord M-40 spectrophotometer in 1, 2 and 10 mm quartz cells at room temperature. In some cases absorption spectra were deconvoluted into Gaussian components using Origin package.

Biological Experiment

Cell culture. Experiments were performed on HeLa cells (human cervix adenocarcinoma) received from the collection of vertebrata cell cultures (Saint Petersburg, Russia). The tumor cells were grown in EMEM medium (PanEco, Russia) containing 10 % embryonic whey (Biowest, France), penicillin (50 units/mL), and streptomycin (50 mg/mL) at 37 °C in 5 % CO₂ atmosphere.

Procedure for determination of cytotoxicity and photoinduced cytotoxicity is reported in.^[18] Cell viability was determined as a percentage of MTT staining of control (untreated) cells. IC₅₀ dose was calculated using MS Excel and Origin program packages. IC₅₀ dose was calculated using the analysis of median effect.^[19–21]

Determination of intracell content of ROS with DCFH₂-DA as detector in photoinduction conditions. Cells were sown in 96 well cultural plates with 25·10³/100 mL cells per well. Compound **IV** in PBS was added to incubation medium at final concentration

ranging from 0.195 to 100 mM. Cultural medium was removed 24 hours later and the cells were washed with PBS solution. DCFH₂-DA dye dissolved in PBS was applied to the cells at concentration of 25 mM and incubated in 5 % CO₂ for 30 min at 37 °C. Then the cells were washed once with PBS solution and irradiated for 30 min (ALKhT-ELOMED laser apparatus, $\lambda = 670$ nm) at optical power of 8 mW/cm². Fluorescence intensity was measured with a TECAN SPARK 10M multi-functional spectrophotometer at $\lambda_{\text{ex}} = 485$ nm and $\lambda_{\text{em}} = 535$ nm. Intracellular content of ROS with DCFH₂-DA was determined in different time intervals (from 30 to 120 min). A similar experiment was carried out as a control one in which no irradiation of cells was used. Relative intracellular content of ROS was calculated using MS Excel and Origin program packages.

Microscopy

Procedure for obtaining fluorescent micrographs is reported in.^[18] Accumulation of **IV** in cells was analyzed with an Axio Scope.A1 fluorescent microscope (Carl Zeiss, Germany) using A-Plan 40×/0.65 Ph2 and A-Plan 40×/0.65 M27 objectives. Samples were photographed using different sets of fluorescent filters, namely, Fs 49 DAPI (EX G 365, EM BP 445/50), Fs 45 HQ TexasRed (EX BP 560/40, EM BP).

Results and Discussion

Due to high solvability of DMSO which prevents π - π interaction between Pcs molecules, the absorption spectrum of solution **IV** corresponds to the monomeric state of compound (Figure 2, spectrum 1). Almost no deviation from the Bouguer-Lambert-Beer law is observed for optical density values at low (μM) concentration (see *Electronic Supporting Information*, ESI, Figure S1). The content of water of ~14 v/v % (DMSO/H₂O = 6:1) does not affect absorption spectra of monomer (Figure 2, spectrum 1), while further increase in medium polarity led to stacking aggregation of **IV** (spectrum 2).

1. Compounds I-IV in Phosphate Buffer and Micellar Solutions of Cationic CTAB and CTPB

Compounds **I-IV** are insoluble in water and transit into

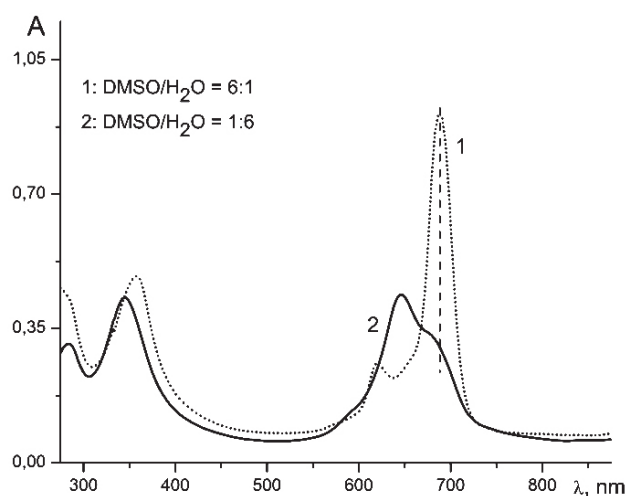


Figure 2. Absorption spectra for **IV** depending on a DMSO/H₂O ratio (v/v %).

solution in phosphate buffer (PBS) with $pH = 7.4$ similarly to 1,5-bis[2-(oxyethoxyphosphoryl)-4-ethylphenoxy]-oxapentane. The latter is in solution with $pH > 7$ as dianion.^[22] As an example, the spectrum for compound **III** ($M = 2H^+$, $R' = C_2H_5$) in PBS is shown in Figure 3. The maximum of its Q -band is in the range of absorption of H -dimers (spectrum 1, $\lambda_{max} = 628$ nm) as well as compounds **I** and **II**. The absorption spectra for **I-III** in PBS remained almost unchanged upon long-term storage, and the alkyl group in the phosphoryl fragment did not affect shape and position of the Q -band of dimer.

Absorption spectra show that **I-III** are in monomer state in micellar solutions of cationic CTAB or CTPB {[surfactant] \geq CMC (critical micelle concentration)} (see Figure 3, spectrum 3 for **III**, ESI Figure S2). For traditional cationic surfactants, spherical micelles are formed when their concentration in an aqueous solution is equal to approximately CMC. The values of CMC_{CTAB} and CMC_{CTPB} in water are $1.0 \cdot 10^{-3}$ and $0.16 \cdot 10^{-3}$ M, respectively,^[23,24] CMC_{CTAB} in PBS is $0.2 \cdot 10^{-3}$.^[25] The lower is CMC, the higher is stability of micelles.

Behaviour of compound **IV** is similar: it is a dimer in PBS (Figure 3, spectrum 2) and monomer with position and shape of the Q -band characteristic of metal PS in microheterogeneous medium (spectrum 4), respectively. A distinctive feature of the monomerization of compounds **I-IV** in a slightly alkaline medium is the high molar absorption coefficient {for example, $\log \epsilon_{686} = 5.15$ (**IV**) and $\log \epsilon_{710} = 5.37$ (**III**)} for Q -band. Polyvinylpyrrolidone does not affect the absorption spectra of monomer **IV** in system **IV**/CTAB/PBS.

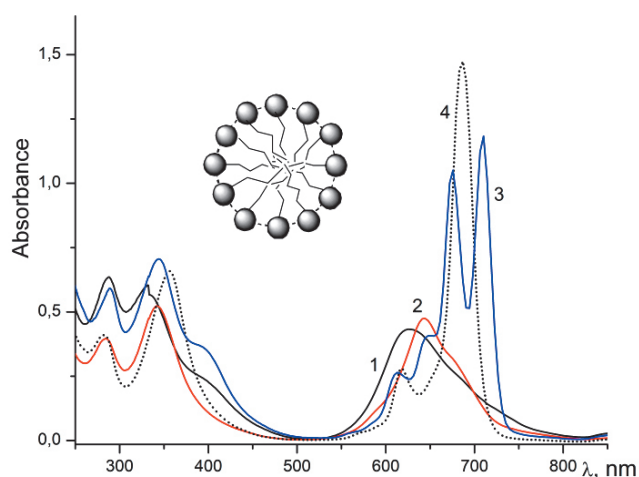


Figure 3. Absorption spectra for **III** and **IV** in PBS before (1 and 2) and after (3 and 4) addition of CTAB ([CTAB] \sim 5 CMC). Insert shows the view of spherical CTAB micelle.

Note that optical density of the Q -band of monomer **IV** (A_{686}) increases with a [CTPB]/[**IV**] ratio and attains maximum at ~ 25 . This corresponds to [CTPB] $\sim 1.55 \cdot 10^{-4}$ M (ESI, Figure S3) close to CMC.^[24] According to^[26] critical micelle concentration, hydrodynamic micelle radius and aggregation number were 0.1 mM, 2.1 nm and 29, respec-

tively, for CTPB in 0.25 mM aqueous solution. At [CTPB] $<$ CMC and [CTPB]/[**IV**] $<$ 25, the spectrum of monomeric Pc in CTPB solution changed in time indicating instability of a supramolecular ensemble.

2. Compounds **I-IV** in the Presence of SDC

Another interesting surfactant is biocompatible SDC. It turned out that the state of free metal **I** with four phosphoric acid groups in microheterogeneous polar medium depends on surfactant charge (Figure 4). Thus, compound **I** is mainly in aggregated form in solution of anionic SDC (spectrum 3), while in solution of cationic CTAB it is in monomeric form (Figure 4, spectrum 2). However, it should be noted that the presence of SDC in solution ([SDC] $>$ CMC) results in shift of the Q -band, which corresponds to H -aggregates and slight growth of optical density in the band range characteristic of metal free Pc monomer (spectra 1 and 3, respectively). Note that H_2Cr_8Pc was also in aggregated state in microheterogeneous SDC medium.^[27]

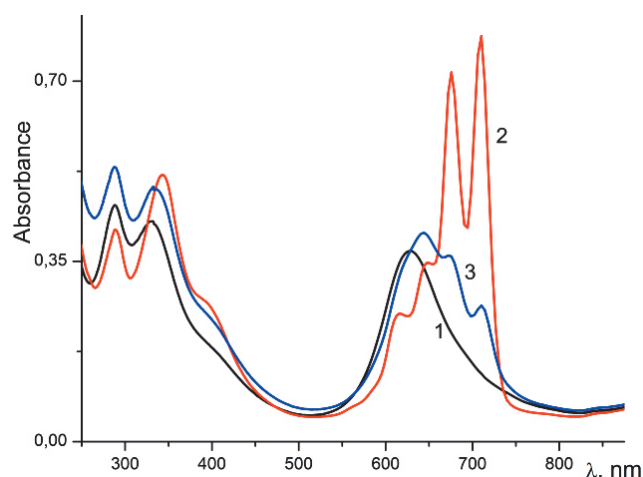


Figure 4. Absorption spectra for **I**/PBS (1), **I**/PBS/CTAB (2) and **I**/PBS/SDC (3).

Deconvolution of the absorption spectrum for the phthalocyanine **III** dimer in PBS using two Gauss functions ($R^2 = 0.9993$) suggests that at least two types of aggregates can be in solution. The addition of SDC to **III**/PBS solution ([SDC] $>$ CMC) leads to spectral changes (Figure 5, spectrum 3), and the spectrum is close to that for the **III**/CTAB/PBS system (Figure 3, spectrum 3), which can be considered as a four-component one ($R^2 = 0.9972$) with a $A_{647}/A_{618} \sim 1.3$ ratio for optical density of vibronic peaks. Absorption spectrum for **III**/PBS/SDC and its deconvolution ($R^2 = 0.9995$) by six Gaussian functions gives $A_{647}/A_{618} \sim 1.66$, that is evidence of the presence of $\sim 17\%$ of metal-free Pc in aggregated state additionally to the monomeric form in the **III**/PBS/SDC system (ESI, Figures S4 and S5). Note that degree of monomerization of metal free Pcs **I-III** in micellar solutions of SDC decreases in the row $R' = \text{pentyl} > \text{ethyl} \gg \text{H}$ (Figure 5). The behavior of compound **IV** in micellar solutions of SDC in PBS phosphate buffer was previously discussed.^[28]

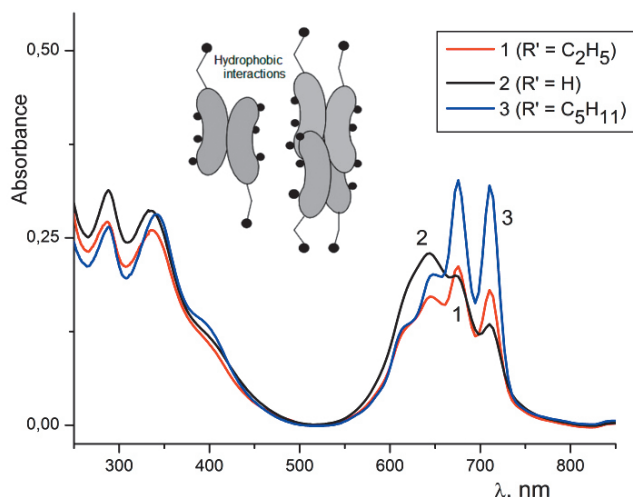


Figure 5. Absorption spectra for microheterogeneous **I**/SDC, **II**/SDC/ and **III**/SDC systems in phosphate buffer. The insert shows a schematic representation of small aggregates (micelles) of SDC with a hydrophobic “pocket” formed with the participation of hydrophobic interactions.

3. Photophysical Properties of **III**/CTAB/PBS and **IV**/SDC Systems

Figure 6 shows the normalized absorption, excitation, and fluorescence spectra for the **IV**/SDC/PBS system. It can be seen that the excitation spectrum **IV** repeats the absorption spectrum, and the emission spectrum is bathochromically shifted relative to the absorption spectrum. The Stokes shift is of the order of 10 nm and lies in the region characteristic of other phthalocyanines in solution in monomeric form.^[29]

The **III**/PBS system (Figure 3, spectrum 1) is inactive under excitation conditions ($\lambda_{\text{ex}} = 625 \text{ nm}$): *H*-dimers and larger aggregates show no fluorescence.^[30] Position of maximum in a fluorescence spectrum is independent of λ_{ex} , and I_{fl} grows with λ_{ex} and degree of its overlap with

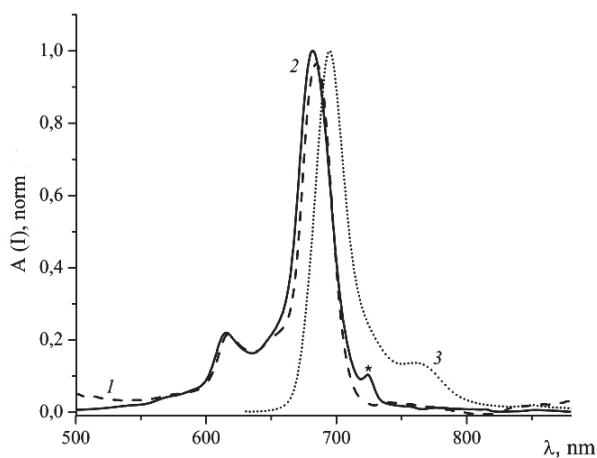


Figure 6. Normalized absorption, excitation ($\lambda_{\text{em}} = 725 \text{ nm}$) and fluorescence ($\lambda_{\text{ex}} = 620 \text{ nm}$) for system **IV**/SDC/PBS (1, 2, 3, respectively). $[\text{IV}] = 1.3 \cdot 10^{-6} \text{ M}$ and $[\text{SDC}] = 13.4 \cdot 10^{-3} \text{ M}$. An asterisk denotes an artifact that occurs when phthalocyanine molecules are excited by light with λ_{em} .^[28]

absorption spectrum of **III**, respectively. The excitation spectrum corresponds to the absorption spectrum for **III**, and the Stokes shift is close to 8 nm (Figure 7). The registration of the fluorescence spectrum of **III** in micellar solutions of cationic CTAB and **IV** in micellar solution of anionic SDC confirms their monomolecular state and screening surfactant molecules in an aqueous environment, respectively.

4. Photochemical Activity of **III**/CTAB/PBS and **IV**/CTAB/PBS Systems

4.1. Photostability

In the dark, absorption spectra for the **III**/CTAB(CTPB)/PBS and **IV**/CTAB(CTPB)/PBS remained unchanged within 2 weeks. When **III**/CTAB/PBS solution was irradiated by light ($\lambda = 578 \text{ nm}$) in the air, optical density of the sample decreased by $\sim 6 \%$ during 20 minutes. The rate of photodegradation of **IV** in microheterogeneous CTAB/PBS medium was higher. A similar result was obtained earlier for $\text{H}_2\text{cr}_8\text{Pc}$ /sodium dodecyl sulfate and Mgcr_8Pc /sodium dodecyl sulfate: metal free Pc was found to be more stable in micellar solutions under irradiation.^[27] Photobleaching of **III** and **IV**, and other Pc based systems^[31–33] was not accompanied by the appearance of new bands.

4.2. Photochemical Reactions

Among ROS generated under irradiation in the presence of Pcs in solution, singlet oxygen is now considered to be a major active (toxic) species.^[34] Singlet oxygen $^1\text{O}_2$ ($^1\Delta_g$) is a strong oxidizing agent for saturated C-H and unsaturated double bonds, sulfide, amino- and other electron donating groups in both organic and biomolecules. However, lifetime for $^1\text{O}_2$ in aqueous medium is shorter than 2 microseconds,^[35] *i.e.* by 1–2 orders shorter than in other solvents. Since this hinders direct detection of the formation of $^1\text{O}_2$ in aqueous solution under irradiation, quantum

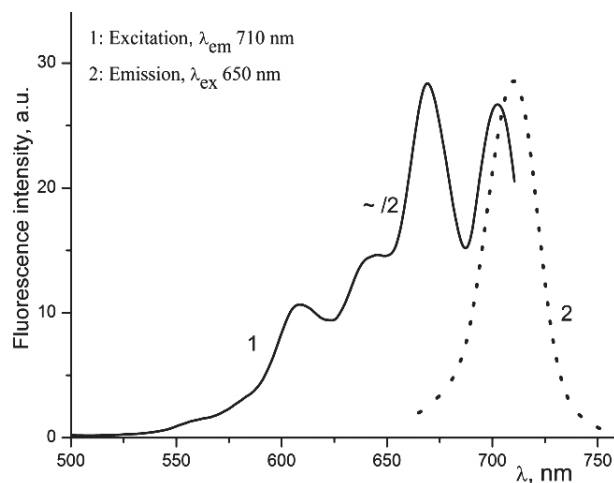


Figure 7. Excitation ($\lambda_{\text{em}} = 710 \text{ nm}$) and emission ($\lambda_{\text{ex}} = 650 \text{ nm}$) spectra for **III**/CTAB/PBS system.

efficiency ϕ_{Δ} of $^1\text{O}_2$ generation can be determined due to its photochemical reactions with various substrates.

In organic solvents, 1,3-diphenylisobenzofuran acts as a quencher of $^1\text{O}_2$, and in water, this function, along with other compounds of the anthracene series, is performed by 9,10-bis(dihydroxyphosphinylmethyl) anthracene (Figure 1).^[12,36] AnthX₂ slowly dissolves in water to concentration $\sim 10^{-4}$ M and well in PBS (pH 7.4). The solvent (water or buffer solution), as well as the presence of a cationic surfactant does not affect the absorption spectrum of AnthX₂. In the absence of light and at a temperature ~ 5 °C, AnthX₂/surfactant solutions in water and PBS were stable for a sufficiently long time.

4.2.1. Irradiation of 1,3-Diphenylisobenzofuran in DMSO in the Presence of IV

Oxidation of 1,3-diphenylisobenzofuran in organic solvents in the presence of Pcs sensitizing photochemical reactions involving $^1\text{O}_2$ (PDT of the 2nd type) is shown, for example, in.^[37–39] Photostability of PCs in solution is affected by both macrocycle charge and axial coordination of ligand with central ion of the macrocycle.^[31]

As it was already mentioned above, IV in the DMSO solution is in monomeric form (Figure 2), which determines the ability of the Pc to generate ROS and sensitize the oxidation of DPBF, respectively. Solvent quality (for example, the presence of water) essentially affects the state of IV. In a DMSO solution, photobleaching of the PS becomes noticeable only with a high degree of oxidation of the quencher used. Photostability of monomer IV in DMSO allowed one to determine quantum yield of formation of $^1\text{O}_2$ in the presence of Pc as photosensitizer. Figure 8 shows

changes in optical density of DPBF under irradiation in the presence of IV in DMSO. Kinetics of decrease in optical density of the peak with $\lambda_{\text{max}} = 417$ nm characteristic of DPBF measured in the presence of PcZn (reference compound, $\Phi_{\Delta}^{\text{PcZn}} = 0.67$ in DMSO^[14]) and IV, respectively, indicates higher photoactivity of PcZn as compared with the latter. The determined value of quantum yield of singlet oxygen for IV, $\Phi_{\Delta}^{\text{IV}}$ is 0.48 ± 0.05 .

4.2.2. Oxidation of AnthX₂ in Microheterogeneous Aqueous Medium of CTAB (CTPB)/PBS in the Presence of III or IV under Irradiation

Surfactants affect photophysical and photochemical properties of organic molecules, in particular, spectral-luminescent parameters and aggregation state of dyes change (see, for example,^[40]). Figure ESI S6 shows spectral changes in solution of IV/AnthX₂/CTAB under irradiation of light with 670 nm wavelength positioned close to absorption maximum for monomer IV. It is seen that optical density of AnthX₂ trap at 300–450 nm and the Q-band of monomer IV at 686 nm decreases in microheterogeneous medium (CTAB micelles), *i.e.* the effect of photoinduced ROS is accompanied by the formation of AnthX₂ peroxide (addition of $^1\text{O}_2$ in positions 9 and 10 of the anthracene molecule) and photodecomposition of IV.^[41] Time interval in which $A_{\text{mon}}^{686} = \text{const}$ is almost absent.

Behaviour of AnthX₂ in more stable microheterogeneous IV/CTPB system^[42] is shown in Figure 9. To estimate quantum yield of $^1\text{O}_2$ in microheterogeneous medium of cationic CTPB we used aqueous solution of methylene blue (MB) at pH = 7.4. The $\Phi_{\Delta}^{\text{MB}}$ value is (0.39 ± 0.04) .^[15]

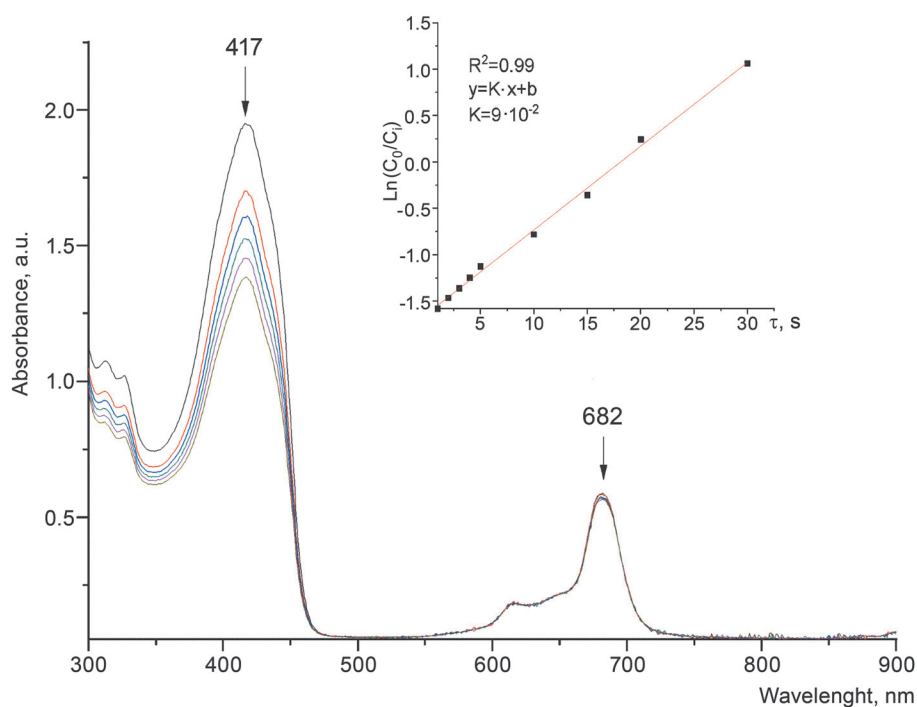


Figure 8. Changes in optical density (formation of $^1\text{O}_2$) in absorption range for quencher (DPBF) under laser irradiation in the presence of IV (DMSO, 670 nm, irradiation time was 60 seconds).

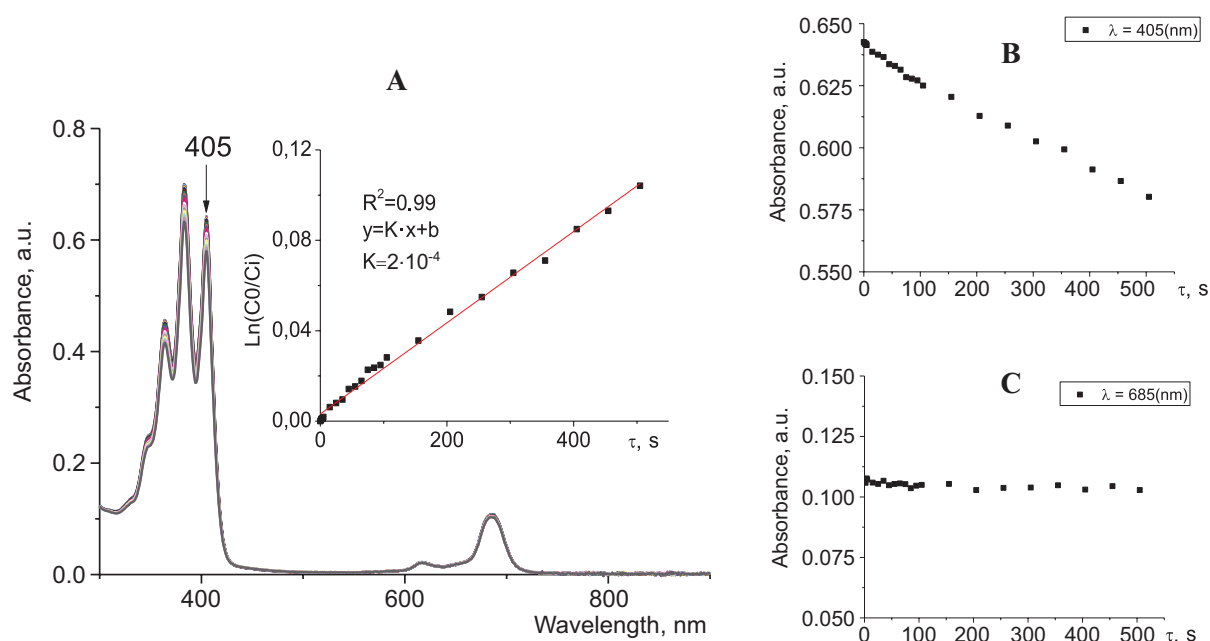


Figure 9. Changes in absorption spectra in **IV**/AnthX₂/PBS under irradiation in microheterogeneous medium of cationic CTPB. Insert shows kinetics of photoinduced oxidation of AnthX₂ (A). Changes in optical density of AnthX₂ and **IV** in microheterogeneous medium of cationic CTPB in time (B and C, respectively).

Besides, according to absorption spectra, **IV** is in monomeric form in both media (DMSO and CTPB micelles). Long wavelength maximum of the *Q*-band of this form is almost at the same wavelength. Thus, the estimated value of quantum yield of ¹O₂ for **IV** in microheterogeneous aqueous medium was 0.54±0.05, which is comparable with $\Phi_{\Delta}^{\text{IV}}$ in DMSO solution. This confirms the stability of monomeric state **IV** in micelles and retention of its ability to photodynamic action in media close to biological ones. It should also be noted that optical density in the *Q*-band range (685 nm) decreased by ~ 15 % after 30 min laser irradiation of AnthX₂/**IV**/CTPB/PBS, *i.e.* ~ 85 % of monomer **IV** remained in solution (Figure 9D). Photobleaching of Zn phthalocyaninate with menthol substituents in the macro-

cycle (ZnMintPc) and simultaneous oxidation of ¹O₂ trap in water-micellar solutions were reported, for example, in.^[43] Quantum yield of singlet oxygen for ZnMintPc depended on Pc state in microheterogeneous medium.^[44]

The experimental data on supramolecular organization, photostability and formation of ¹O₂ in the presence of **IV** in various media allowed us to investigate its photodynamic behaviour as a photosensitizer on HeLa cells. The first stage of our biological research involves the study of the uptake of the initial and previously unexplored **IV** by the cells HeLa (system **IV**/PBS), its dark and light toxicity, as well as the formation of reactive oxygen species in the presence of **IV** upon irradiation.

5. Accumulation of **IV** in HeLa Cells

One of components of culture medium is bovine serum albumin (BSA). Absorption spectra for **IV** in aqueous solution of BSA/PBS are close to those in EMEM (BSA ~ 10 %) (Figure 10A, spectra 1 and 2) but are different from the spectrum for **IV** in pure PBS (spectrum 3). [BSA]/[**IV**] molar ratio showed no noticeable effect on absorption spectra for **IV**. Absorption spectrum for **IV**/BSA/PBS solution can be considered as a sum of components including, along with aggregates of various compositions, apparently also some monomeric form of Pc (ESI, Figure 7). The difference in optical density values for solutions of **IV** before applying on cells and solutions measured after incubation was insignificant (Figure 10b). According to the absorption spectra (Figure 10), compound **IV**, like other Pcs (see, for example,^[45]), binds to bovine serum albumin, but the presence of the latter shows no noticeable effect on the degree of **IV** accumulation by HeLa cells when applying.

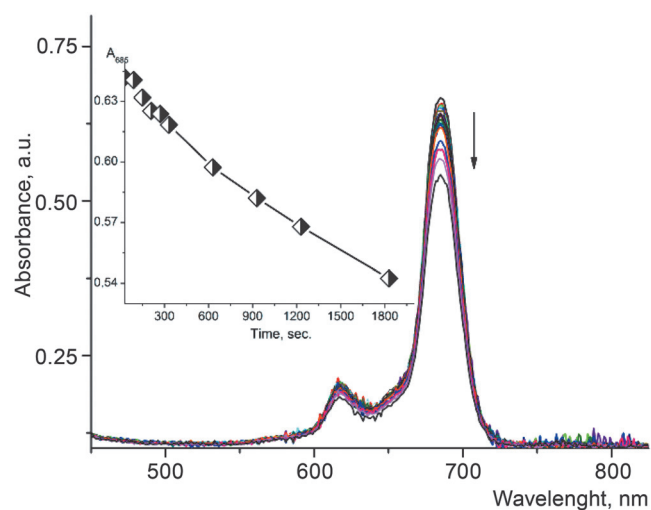


Figure 9D. Changes of absorption spectra in the **IV**/PBS/CTPB system in time under irradiation (laser, $\lambda = 670$ nm).

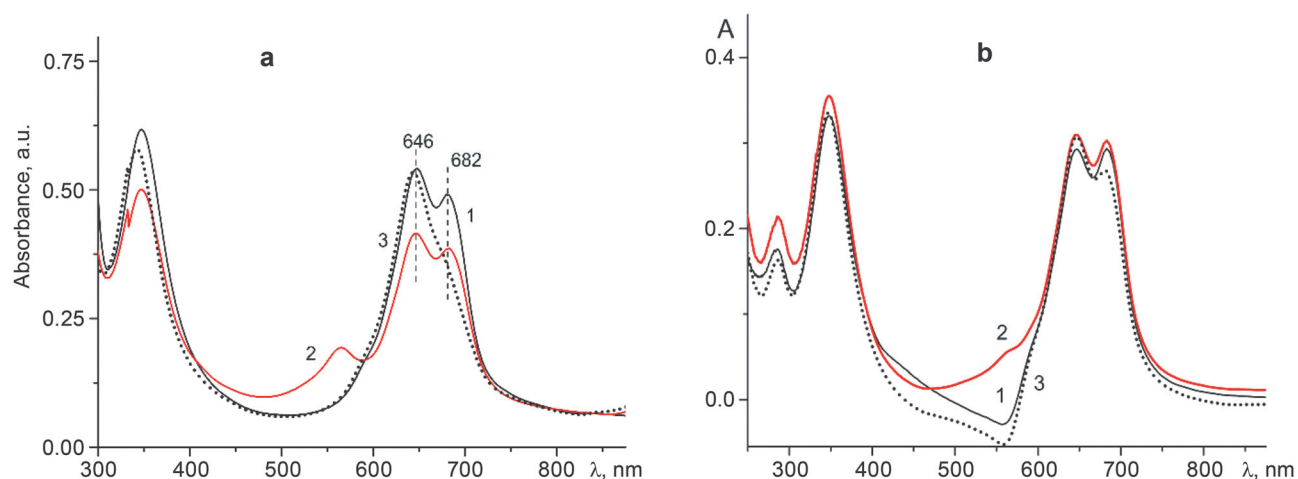


Figure 10. Absorption spectrum for **IV** in aqueous solution of BSA (1), aqueous solution in culture medium EMEM (2) and phosphate buffer (3) (a). Absorption spectrum for solutions taken from Vero (1) and Hela (2) cells after their incubation with **IV** (50 μM) and absorption spectrum for **IV** (50 μM /EMEM before incubation (3) (b).

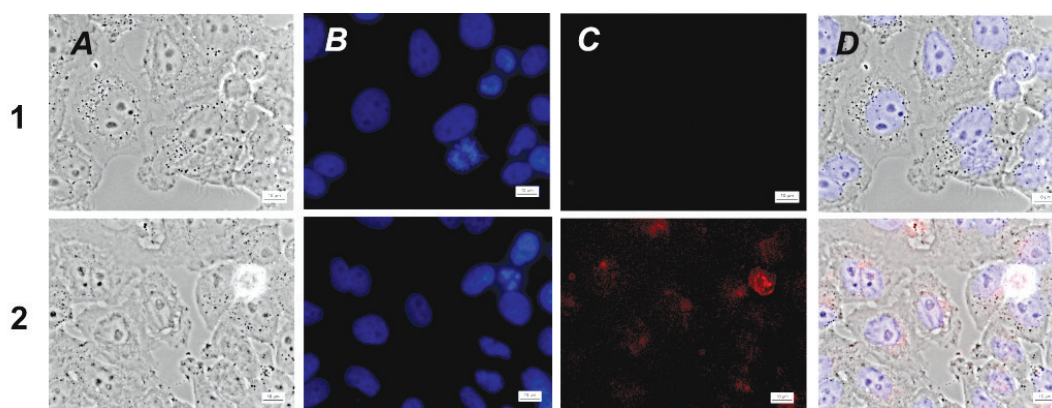


Figure 11. Micrographs of HeLa cells without **IV** (control) and in the presence of **IV**/SDC (panels 1 and 2, respectively). The cells were incubated with **IV** (50 μM)/SDC (5 μM) in culture medium during 24 hours. *A* – phase contrast, *B* – nuclei stained with DAPI, *C* – accumulation and localization of **IV**, and *D* – merger. Scale bar 10 μm .

Intracellular uptake and localization of **IV** was estimated by fluorescent microscopy. Note that accumulation of **IV** in cells was detected only upon introducing a small and nontoxic (in respect of cells) amount of SDC (5 μM) in incubation medium. Phase contrast showed, firstly, that HeLa cell morphology is not violated upon incubation with solution of **IV** at an initial concentration of 50 μM and 5 μM of SDC during 24 hours (Figure 11, column A, panels 1 and 2). Secondly, well-observable red fluorescence, weak for 50 μM of **IV**, (column C, panel 2) is evidence of accumulation of **Pc** by cells and its active fluorescent state. It should also be noted that cells incubated with **IV** were then repeatedly washed with warm PBS to fully remove unabsorbed **Pc**.

6. Toxicity of **IV** and Determination of Intracellular Content of ROS under Irradiation

Like other Zn-containing **Pcs**,^[46] **IV** shows no cytotoxicity in respect of HeLa cells at concentration of $0.195 \div 100$

μM without laser activation. Upon irradiation of cells in the presence of **IV** (diode laser, 670 nm), light toxicity of **IV** (IC_{50} dose) was 6.44 (Figure 12).

To estimate the formation and accumulation of ROS we used 2',7'-dichlorodihydrofluorescein diacetate (DCFH₂-DA) dye, which hydrolyzes in the presence of intracellular esterases to form DCFH₂. The later is oxidized by ROS to fluorescent 2',7'-dichlorofluorescein (DCF).^[47]

Incubation of cells with solution of **IV** and further treatment by DCFH₂-DA provides the formation and growth of ROS content under irradiation (photodynamic effect) as compared with control cells (Figure 13). Fluorescence intensity remained almost unchanged at [**IV**] = 50 μM and 100 μM . The values of $I_{\text{fl}}^{\text{DCF}}$, which can be evidence of the formation of ROS under irradiation, were relatively low even at such high concentrations of **IV**.

This can be due to low phototoxicity of **IV** and determination of intracellular content of ROS under irradiation level of accumulation of **IV** (see above) or its aggregated (non-fluorescent) state in cells. The shape of curves of $I_{\text{fl}}^{\text{DCF}}$ vs

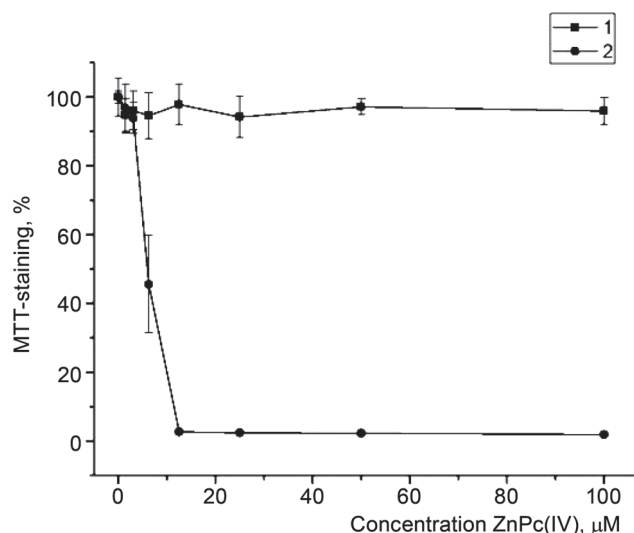


Figure 12. Dark and light toxicity of compound **IV** in HeLa cells depending on the concentration of PS in the incubation medium (black squares and circles, respectively).

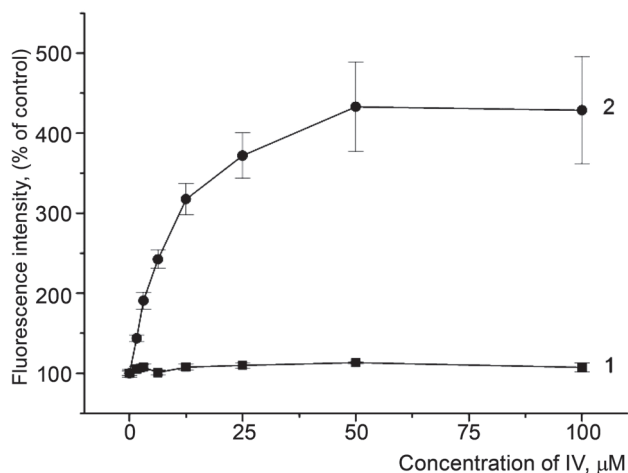


Figure 13. Changes in the DCF fluorescence intensity in HeLa cells in the presence of **IV** without irradiation (1) and under irradiation (2) at a concentration of 1.6 to 100 μM in the incubation medium. Irradiation conditions: $\lambda = 670 \text{ nm}$, 8 mW/cm^2 , 30 min.

time dependence is almost similar for irradiated and control cells (Figure 14) that is evidence of intracellular formation of ROS at selected concentrations of **IV** during irradiation and after exposure. This feature is characteristic of **IV** and is essentially different from the $I_{\text{fl}}^{\text{DCF}}$ vs time dependence obtained for HeLa cells in the presence of octa-crown containing Mg phthalocyaninate.^[48]

From the one hand, the decrease in optical density of AnthX₂ (¹O₂ quencher) indicates the formation of singlet oxygen *in vitro* in the presence of **IV** under irradiation (Figure 8 and 9). On the other hand, DCF intensity growth after switching off light indicates the formation of ROS after photodynamic effect (Figure 14).

One can suppose that in the presence of DCFH₂ (ROS indicator) which is able to reduce the compounds with

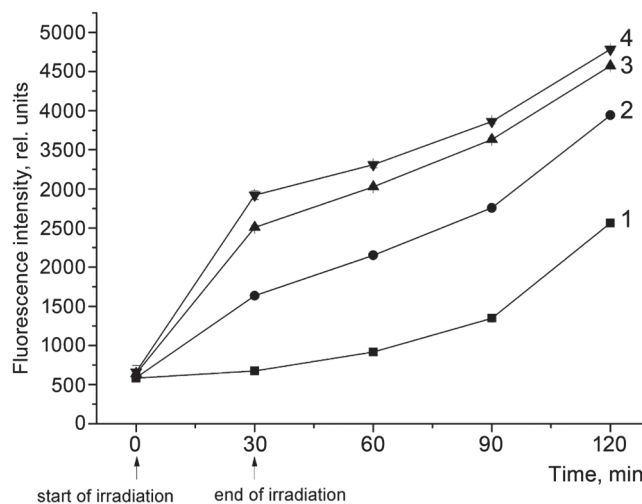


Figure 14. Photoinduced changes in fluorescence of DCFH₂-DA detecting ROS in HeLa cells in control (1) and in the presence of **IV** at concentration of 6.2 μM (2), 25 μM (3) and 50 μM .

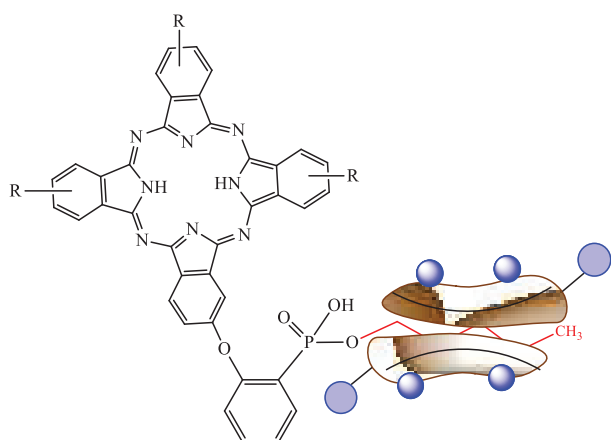
oxidation potentials lying in a wide potential range,^[49] the cells are still alive after photodynamic effect in the presence of **IV** accumulated by the cells *in vitro*, and retain ability to hydrolysis of DCFH₂-DA and the formation of ROS similarly to control cells. This proves that ROS can apparently be produced in photodynamic process involving **IV** as photosensitizer with the participation of PDT reactions of both types (Scheme 1). The level of formation of intracellular ROS under irradiation in the presence of **IV** (see above) is in agreement with low accumulation of Pc and weak red fluorescence, respectively. This means that only a small amount of **IV** from the taken is absorbed by the cells during incubation.

Discussion

Supramolecular chemistry in water – non-covalent assembly of simple ‘building’ blocks to higher-level ordered structures – now attracts great attention. Conditions of solubilization and disaggregation of phosphoryl containing phthalocyanines and their metal complexes in aqueous medium accompanied by the formation of supramolecular ensembles were found based a supramolecular approach – use of surfactants of different nature.

Sodium deoxycholate is a biocompatible micelle forming compound. Its properties are strongly different from those of conventional surfactants. SDC is specified by the ability to form primary aggregates (micelles) through hydrophobic interactions (Figure 5, insert), while hydrogen bonds are involved in the formation of large SDC aggregates (secondary micelles).^[50] Apparently, due to the solubilization of R' in small hydrophobic cavities of SDC (Scheme 2), alkyl group of the –(PO(OH)(OR')) fragment of macrocycle substituent provides monomerization of **II** and **III** by micelles (aggregates) of anionic SDC. Stability of such supramolecular ensemble is dependent on alkyl chain length (4.6 and 9.04 Å for ethane and pentane, respectively) and its

correspondence of linear size of SDC micelles: deoxycholic acid molecule length is 20–21 Å from head to tail. Indeed, degree of monomerization of metal free **I–III** changes in the row: pentyl > ethyl >> H. The state of **IV** in form of monomer in solutions of bile salts (sodium deoxycholate and others) was also proved by fluorescence spectroscopy.^[28]



Scheme 2. Assumed encapsulation model for alkyl groups of phosphoryl fragment in substituent in macrocycles of **II** and **III** by hydrophobic cavities of SDC. Solubilization of only one alkyl group is shown.

Compound **IV** is soluble in aqueous PBS and can carry up to four negative charges. The high hydrophilicity of the compound **IV** and the charge density, as well as the predominantly aggregated state of the Ps in the culture medium (Figure 10), may inhibit its penetration through the plasma membrane of HeLa cells and lead to the observed relatively low photodynamic activity. Thus, Photosens (hydroxyaluminium sulfophthalocyaninate with the number of sulfogroups ~ 3.4), a compound with a high degree of hydrophilicity, is distinguished by the slowest cell accumulation *in vitro* of transitional cell bladder cancer (T24) and human liver adenocarcinoma (SK-HEP-1). Its photodynamic activity was the lowest of the sequence of Ps – Photosens, Holosens and Phthalosens.^[29]

Diphilicity of Ps has a decisive influence on their interaction with the bilayer. For example, the presence of a cholesterol fragment promotes the absorption of ZnPc molecules by the membrane and determines its position inside the membrane.^[51] Participation of BS in binding and delivery of drugs to biological targets is discussed, in particular, in.^[52,53] In our case, sodium deoxycholate was found also to be useful at absorption of **IV** by HeLa cells (see above).

Since Pcs and metal Pcs are used generally in aerobic conditions, their photostability is an important factor in PDT. It was shown in^[31] that photobleaching rate for both anionic and cationic Zn phthalocyaninates in aqueous solution is dependent on concentration of oxygen. Micellar membranes are able to create diffusion limitation for photochemical reactions at solubilization of Pcs in microheterogeneous medium. Diffusion of $^1\text{O}_2$ in micelles is lower than in solution^[54] that can provide its interaction with Pc and photodegradation of the latter.

Since each participant involved into the process can be in micelle, O_2 transfer in sequence of micelle \rightarrow volume \rightarrow micelle (target), affects the rate of oxidation.^[33] Possible instability of Pc monomer in multi-component aqueous microheterogeneous medium under irradiation by light and participation of CTPB (CTAB) ~ AnthX₂ ~ Pc fragments (particularly, C₁₆H₃₃-group of surfactant) in redox processes, which can be initiated by photogenerated ROS, are assumed to be important in view of photodegradation of Pc (PS) in microheterogeneous medium.

For the first time, zinc phthalocyaninate with phosphoryl groups in substituents of the macrocycle has been analyzed as potential photosensitizer in different media such as DMSO and microheterogeneous medium of cationic surfactant. The conditions for the formation of supramolecular ensembles in microheterogeneous media (cationic and anionic surfactants) with the predominant formation of Pc monomer as major product have been found. The state of **IV** as monomer in polar medium is preferable for fluorescent diagnostics and photodynamic therapy. Quantum yield of singlet oxygen for **IV** in DMSO (0.48 ± 0.05) and microheterogeneous medium based on cationic CTPB (0.54 ± 0.05) is close to known values.^[55–58]

Initial compound **IV** shows low cytotoxicity, high enough phototoxicity (IC₅₀ dose) and photostability, and participates as PS in the formation of intracellular ROS under irradiation *in vitro*. Thus, phosphoryl containing Pcs deserve further research including determination of quantum yield of fluorescence and lifetime of triplet state, and increase of degree of accumulation by cells as well as the SDC and CTPB effect on photodynamic activity.

Conclusion

Based on supramolecular approach, conditions for solubilization and disaggregation of phosphoryl containing phthalocyanines and their metal complexes in aqueous medium due to the formation of supramolecular ensembles have been found. Zinc phthalocyaninate **IV** with phosphoryl groups in substituents of the macrocycle has been analyzed for the first time as potential photosensitizer in different media. Supramolecular assembly of Pcs in solution, quantum yield of singlet oxygen $\Phi_{\Delta}^{\text{IV}}$ in DMSO and organized microheterogeneous medium based on cationic cetyltri-phenylphosphonium bromide in combination with low cytotoxicity of **IV**, high enough phototoxicity (IC₅₀ dose) and formation of intracellular active oxygen species *in vitro* at irradiation allow phosphoryl containing phthalocyanines and metal phthalocyanines to be considered as deserving further research as compounds useful in PDT.

Conflict of Interest. The authors declare no competing financial interest.

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