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Aggregation of water soluble octaanionic phthalocyanines and their photoinactivation antimicrobial effect *in vitro*

Dmitry B. Berezin,*[a] Vladimir V. Makarov,[a] Serafima A. Znoyko,[a] Vladimir E. Mayzlish[a] and Andrey V. Kustov[a,b]

[a] *Research Institute of Macroheterocycles, Ivanovo State University of Chemistry and Technology, 153000 Ivanovo, Russian Federation. E-mail: berezin@isuct.ru*

[b] *G. A. Krestov Institute of Solution Chemistry, Russian Academy of Sciences, 153045 Ivanovo, Russian Federation*

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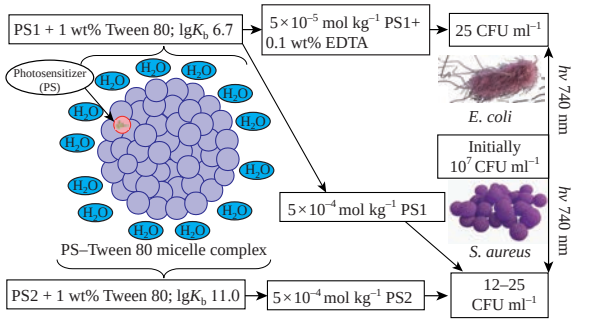
Aggregation of sulfophenylsulfanyl-substituted phthalocyanine and its aluminum complex has been investigated in aqueous solutions of DMF or nonionic surfactant Tween 80 by UV-VIS spectroscopy and DLS. Aluminum complex is less aggregated and 4.5 orders of magnitude stronger bound to the micelles of Tween 80 compared to a metal-free ligand. Under irradiation the phthalocyanine derivatives reveal a pronounced antimicrobial activity towards both Gram(+) and Gram(–) archive microflora *in vitro*.



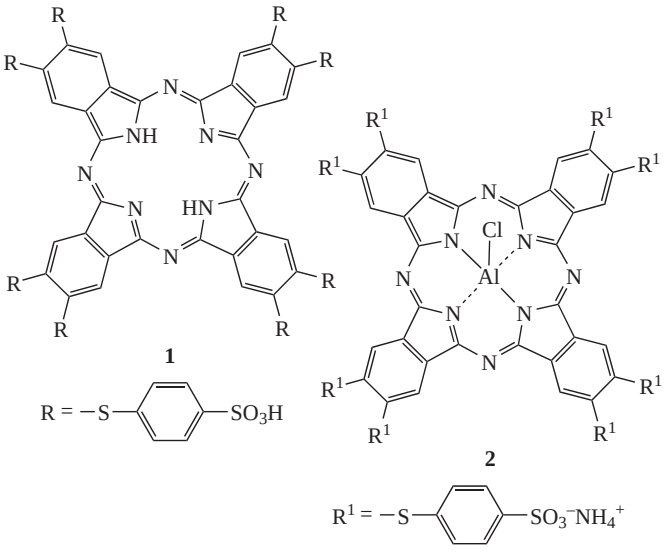
Keywords: antimicrobial photodynamic therapy, photosensitizer, water soluble phthalocyanine, aggregation, nonionic surfactant, microorganisms photoinactivation.

Worldwide spread of antibiotic resistance among microorganisms have led to the search of new therapeutic antimicrobial approaches. Photodynamic therapy (PDT) represents a well-known alternative way for treatment of infectious diseases,[1,2] consisting in combined effect of three factors, which are themselves non-toxic, namely organic photosensitizer, visible light and oxygen.[3] Pathogens are killed by the toxic reactive oxygen species produced by photosensitizer molecules located in an appropriate place and excited to the long-lived triplet state.[1,2] Tetrapyrrole compounds such as porphyrins, phthalocyanines, chlorins and bacteriochlorins as well as their analogues like corroles and the porphyrin isomers are currently considered as promising PDT pharmaceuticals, some of them being clinically approved drugs.[1–3] The generation of the reactive oxygen species including singlet oxygen $^1O_2$ represents a key feature of photosensitizers and is typically characterized by the value of quantum yield.[3] An efficient $^1O_2$ production in biological liquids or model aqueous solutions can be hampered by the low solubility of hydrophobic tetrapyrrole compounds as well as their aggregation. However, incorporation of photosensitizer molecules into appropriate nanocarriers such as micelles or liposomes results in their essential disaggregation followed by the increase of singlet oxygen production and leading to the increase of PDT efficacy.[3,4] Thus, such carriers can be successfully used in targeted drug delivery.[5]

We have explored the synthesis and properties of potential macroheterocyclic photosensitizers for antimicrobial PDT.[4,6–9] Here we present an investigation of the aggregation behavior of new phthalocyanine compounds **1** and **2**, their interaction with biocompatible nonionic surfactant Tween 80 as well as inactivation of opportunistic archive microflora *in vitro*.

Phthalocyanines **1** and **2** bearing eight 4-sulfophenylsulfanyl groups at positions 2, 3, 9, 10, 16, 17, 23 and 24 of fused benzene rings of the macroheterocycle (MHC) moiety were obtained according to the known procedure[10] and their structures were confirmed by ^{1}H NMR, MS, IR, UV-VIS spectra as well as elemental analysis (for details, see Online Supplementary Materials).

Absorption spectra of compounds **1** and **2** (Figure 1, Table S1) are typical of phthalocyanine macrocyclic chromophores and reveal bathochromic shifts of the intensive Q_x(0–0) band at ~700 nm (lgε = 4.4–4.9) up to 25 nm compared with the unsubstituted metal-free MHC.[11,12] The red spectral shift for compound **2** originates from both incorporation of eight thio bridges on the periphery of the molecule and the aluminum(III) atom insertion inside the macrocyclic core.[12,13] As was found,[10] fluorescence spectra



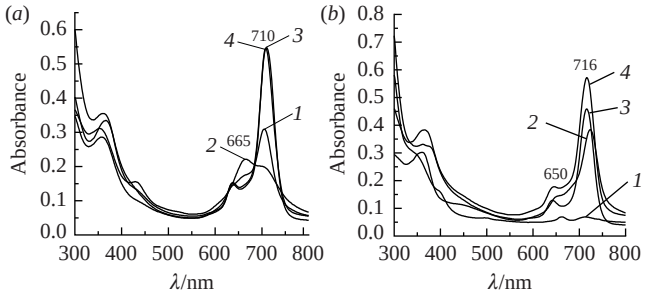


Figure 1 Absorption spectra of 7.3×10^{-6} mol kg^{-1} solutions of compounds (*a*) **1** and (*b*) **2** in (*1*) EtOH, (*2*) H_2O, (*3*) DMF and (*4*) 1 wt% aqueous Tween 80.

of compounds **1** and **2** in EtOH demonstrated single band at ~725 nm with a Stokes shift of 9–11 nm (*i.e.*, 175–210 cm^{-1}), which was typical of tetrapyrrolic macrocycles with a substantially planar and poorly polarized chromophore.[14]

As is known,[15] the distribution coefficient in 1-octanol–PBS buffer for Zn complexes of phthalocyanines bearing neutral or charged hydrophilic groups, which are typically used for antibacterial PDT, is in the range of 8–40 and corresponds to an acceptable hydrophilic–lipophilic balance. For compounds **1** and **2** at micromolar concentrations, despite their solubility in water, the UV-VIS spectra (Figure 1) demonstrate strong aggregation in both water and ethanol as well as complete breaking up of aggregates in pure DMF and in aqueous solution of biocompatible nonionic surfactant Tween 80. Titration of aqueous solutions of macrocycles **1** and **2** with DMF revealed lower aggregation trend for aluminum complex **2** compared with metal-free compound **1** [Figure 2(*a*)]. The UV-VIS spectral changes indicate an emergence of H-aggregates[16,17] at water content in the binary solvent mixture more than 40 wt% for free phthalocyanine **1** and at 90 wt% of water for aluminum complex **2**. Formation of these entities was confirmed[4,18] by blue shift of the longwave Q_x band in the UV-VIS spectra of aqueous solutions by 43 and 7 nm for compounds **1** and **2**, respectively, as well as by substantial decrease in the intensity of absorbance, namely the change in lgε value for macrocycle **1** from 4.87 in DMF or 1 wt% Tween 80 to 4.48 in water and for macrocycle **2** from 4.89 to 4.72, respectively. A resolved absorption band of H-aggregates in the UV-VIS spectrum is present only for metal-free phthalocyanine **1**, which demonstrates its higher tendency to assemble in aqueous solution.

The same considerations on the aggregation could be deduced from dynamic light scattering (DLS) data for diluted aqueous solutions of compounds **1** and **2**, where the monomodal particle distribution (100–3500 nm) for free phthalocyanine **1** as well as the formation of aggregates with two ranges of hydrodynamic diameters, namely 5–50 and 60–3500 nm, for metallocomplex **2** were revealed. Addition of Tween 80 to the aqueous solutions with the molar ratio surfactant–phthalocyanine of ~1:1 totally destroyed the large aggregates at the nano level [Figure 2(*b*)] due to complex formation between the phthalocyanine and Tween 80 molecules.

Next, an interaction of compounds **1** and **2** with the Tween 80 micelles was investigated *via* spectrophotometric titration and then quantitatively estimated using equation (1)[4]

$$\lg[(A–A_0)/(A_{max}–A_0)]/[1–(A–A_0)/(A_{max}–A_0)] = \\ = \lg K_b + n\lg\{m_T^{micellar} – n[m_{PS}(A–A_0)/(A_{max}–A_0)]\}, \quad (1)$$

where K_b was the compound–Tween 80 equilibrium binding constant; n was the number of macrocycle-bound Tween 80 molecules; $m_T^{micellar}$ was molality of aggregated Tween 80 equalled to the difference between its analytical concentration and the critical micelle concentration (CMC), which was taken

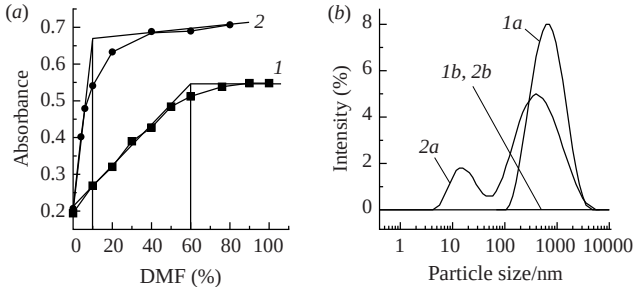


Figure 2 (*a*) Titration curves for 7.3×10^{-6} mol kg^{-1} solutions of compounds (*1*) **1** at λ = 710 nm and (*2*) **2** at λ = 716 nm in H_2O–DMF system at room temperature, the points represent experimental data; (*b*) particle size distribution measured by DLS for 7.0×10^{-5} mol kg^{-1} aqueous solutions of compounds (*1*) **1** and (*2*) **2** at 298 K in the absence (*1a*,*2a*) and presence (*1b*,*2b*) of 7.0×10^{-5} mol kg^{-1} Tween 80.

as 1.5×10^{-5} mol kg^{-3}; m_{PS} was initial concentration of the photosensitizer; A_0, A_{max} and A represented initial, final and current optical densities of the macrocycle in aqueous Tween 80 solutions, respectively.[4] The obtained experimental curves are shown in Figure 3(*a*).

The model parameters K_b and n were evaluated *via* standard iteration mode [Figure 3(*b*) and Table 1]. Depending on the phthalocyanine derivative structure, there are two types of interaction with Tween 80 micelles, where one macrocycle molecule is in a close contact with one (as for compound **1**) or two (as for compound **2**) surfactant molecules [see Figure 3(*b*)]. The corresponding binding constants (see Table 1) are up to five orders of magnitude higher compared with the K_b values obtained for interaction of ionic and nonionic chlorins with Tween 80.[4,19] Binding of less aggregated aluminum complex **2** with a Tween 80 micelle is about 4.5 orders stronger than the association of free phthalocyanine **1**. Presumably, an axial ligand in the structure of compound **2** prevents π-stacking of the macrocycle moieties and thus favours further interaction with the micelle. Steric constraints of substituents in MHC molecules followed by their disaggregation in aqueous solutions have been already observed and discussed.[20]

The disaggregated form of photosensitizers in the presence of Tween 80 was further explored in antimicrobial PDT experiments. The photodynamic inactivation of three opportunistic microorganisms, namely Gram-positive bacterium *Staphylococcus aureus* (ATCC 6538-P, FDA 209-P strain), Gram-negative bacterium

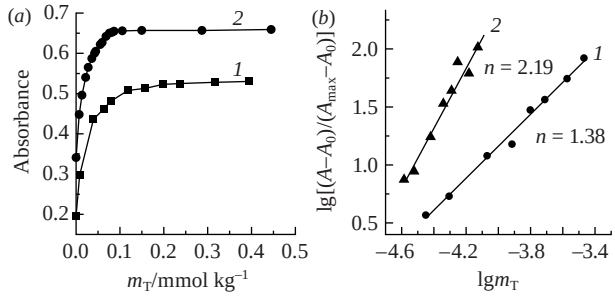


Figure 3 (*a*) Titration curves for 7.3×10^{-6} mol kg^{-1} solutions of compounds (*1*) **1** at λ = 708 nm and (*2*) **2** at λ = 716 nm with Tween 80 *vs.* molality of the surfactant m_T at room temperature, $m_T = m_T^{micellar} – n[m_{PS}(A–A_0)/(A_{max}–A_0)]$, the points represent experimental data; (*b*) linearization of experimental data according to the equation (1) model for compounds (*1*) **1** and (*2*) **2**.

Table 1 Parameters of equation (1) and regression statistics for compounds **1** and **2** at 7.3×10^{-6} mol kg^{-1}.

Compound	m_T/mol kg^{-1}	lgK_b	n	R^2	s_f
1	$(0.2–5.6) \times 10^{-4}$	6.69 ± 0.20	1.38 ± 0.05	0.993	0.013
2	$(0.4–8.2) \times 10^{-4}$	10.99 ± 0.99	2.19 ± 0.23	0.964	0.123

Table 2 PDT of opportunistic microflora in the presence of photosensitizers **1** and **2** after irradiation dose of 40 J cm^{-2} at λ = 740 nm.[a]

Compound	Pathogen	m_{PS}/mol kg^{-1}	Cell density (CFU ml^{-1})[b]
1	*S. aureus*	5×10^{-4}	25
		5×10^{-5}	10^7
		5×10^{-5}[c]	10^7
		5×10^{-4}	10^7
	C. albicans	5×10^{-5}	10^7
		5×10^{-5}[c]	10^7
		5×10^{-4}	10^7
	E. coli	5×10^{-5}	10^7
		5×10^{-5}[c]	25
2	*S. aureus*	5×10^{-4}	12
	C. albicans	5×10^{-4}	10^7
	E. coli	5×10^{-4}	10^7

[a] Incubation medium consisted of photosensitizer, 1 wt% Tween 80 and optionally disodium EDTA. [b] Cell density before the irradiation was 10^7 CFU ml^{-1}. [c] In the presence of 0.1 wt% disodium EDTA dihydrate.

Escherichia coli (M-17 strain) and fungus *Candida albicans* (ATCC 90028, CCM 8261 strain) was investigated. The first series of experiments at photosensitizer concentration $m_{PS} = 5\times10^{-5}$ mol kg^{-1} (Table 2) indicated that diluted phthalocyanine solutions did not allow photodynamic inactivation of the archive microflora. To attain better penetration of photosensitizer through the outer cell membrane of the microbes, an increase in the concentration by one order was undertaken or, alternatively, 0.1 wt% of disodium EDTA dihydrate was added to each macrocycle solution.[1,2,21] Maintaining the concentration of both photosensitizers at 5×10^{-4} mol kg^{-1} resulted in efficient photoinactivation of Gram-positive bacteria within five to six orders of the colony-forming units (CFU) count. Addition of disodium EDTA resulted in selective killing of Gram-negative pathogen even at smaller concentration of compound **1** (see Table 2 and the Online Supplementary Materials). It is quite a remarkable result especially taking into account the negatively charged surface of the photosensitizer molecule, which typically does not favour its interaction with a cell membrane. *C. albicans* was found to be resistant to the photoinactivation under all the conditions employed.

In summary, aggregation of potential phthalocyanine photosensitizers has been investigated using UV-VIS spectroscopy and DLS in water and aqueous solutions of nonionic surfactant Tween 80 as an appropriate carrier for drugs in biological media. The size of the phthalocyanine aggregates in water has been estimated and the photosensitizer–Tween 80 binding constants have been calculated. Our results clearly indicate that the metal-free phthalocyanine derivative reveals much higher tendency to form aggregates compared with the aluminium phthalocyanine complex in diluted aqueous solution. An addition to photosensitizer even small amount, for example equimolar, of Tween 80 induces disruption of the nanosized aggregates due to efficient binding of the macrocycle molecules to the carrier. Solutions of disaggregated photosensitizers have been tested for antibacterial PDT *in vitro*. Both phthalocyanine compounds investigated have been found to inactivate the archive strains of *S. aureus*. The addition of disodium EDTA, which is known to enhance the outer membrane permeability, is required to kill the Gram-negative pathogen.

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Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2020.09.023.

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