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Electrostatic Binding of Substituted Metal Phthalocyanines to Enterobacterial Cells: Its Role in Photodynamic Inactivation

M. G. Strakhovskaya[1]*, Y. N. Antonenko[2], A. A. Pashkovskaya[2,3], E. A. Kotova[2], V. Kireev[1,4], V. G. Zhukhovitsky[5], N. A. Kuznetsova[6], O. A. Yuzhakova[6], V. M. Negrimovsky[6], and A. B. Rubin[1]

[1]Department of Biophysics, Faculty of Biology, Lomonosov Moscow State University, 119991 Moscow, Russia; fax: (495) 939-1115; E-mail: marstr@biophys.msu.ru
[2]Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, 119991 Moscow, Russia; fax: (495) 939-3181; E-mail: antonen@genebee.msu.ru
[3]Faculty of Bioengineering and Bioinformatics, Lomonosov Moscow State University, 119991 Moscow, Russia; E-mail: PashkovskaiaAA@yandex.ru
[4]Faculty of Natural Sciences, Vilnius University, ul. Churlionis 21/27, Vilnius, LT-03101, Lithuania
[5]Botkin Moscow City Clinical Hospital, 2-oi Botkinskii Pereulok 5, 125101 Moscow, Russia
[6]Organic Intermediates and Dyes Institute (NIOPIK), ul. B. Sadovaya 1/4, 123995 Moscow, Russia; fax: (495) 254-7015; E-mail: lab32@niopik.ru

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Abstract—The effect of ionic substituents in zinc and aluminum phthalocyanine molecules and of membrane surface charge on the interaction of dyes with artificial membranes and enterobacterial cells, as well as on photosensitization efficiency was studied. It has been shown that increasing the number of positively charged substituents enhances the extent of phthalocyanine binding to *Escherichia coli* cells. This, along with the high quantum yield of singlet oxygen generation, determines efficient photodynamic inactivation of Gram-negative bacteria by zinc and aluminum octacationic phthalocyanines. The effect of Ca^{2+} and Mg^{2+} cations and pH on photodynamic inactivation of enterobacteria in the presence of octacationic zinc phthalocyanine has been studied. It has been shown that effects resulting in lowering negative charge on outer membrane protect bacteria against photoinactivation, which confirms the crucial role in this process of the electrostatic interaction of the photosensitizer with the cell wall. Electrostatic nature of binding is consistent with mainly electrostatic character of dye interactions with artificial membranes of different composition. Lower sensitivity of *Proteus mirabilis* to photodynamic inactivation, compared to that of *E. coli* and *Salmonella enteritidis*, due to low affinity of the cationic dye to the cells of this species, was found.

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Key words: photodynamic inactivation, photosensitizer, phthalocyanines, enterobacteria, lipopolysaccharides, fluorescence quenching, fluorescence correlation spectroscopy

Photodynamic inactivation of microorganisms is based on damage to cells by reactive oxygen species generated by exo- or endogenous sensitizers in photoexcited states [1]. In particular, zinc and aluminum phthalocyanines appeared to be efficient agents for photodynamic inactivation of bacteria [2-4]; their mechanism of action includes generation of cytotoxic singlet oxygen ($^1O_2$) [5].

The plasma membrane is one of main critical targets of cytotoxic destruction in cells of different origin [6, 7]. Irreversible damage to the membrane results in leakage of

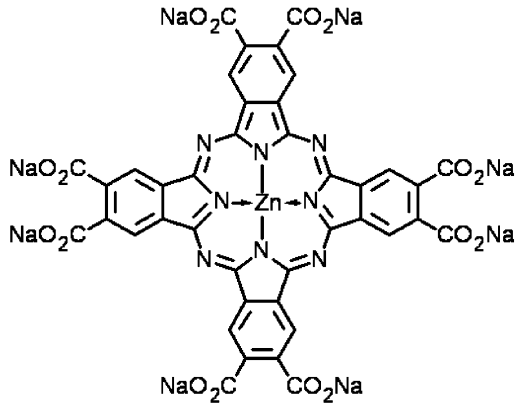
Abbreviations: $AlPc^{3-}$, trisulfonated aluminum phthalocyanine (Photosense); $AlPc^{4-}$, tetrasulfonated aluminum phthalocyanine; BLM, bilayer lipid membrane; CFU, colony-forming unit; DPhPC, diphytanoyl phosphatidylcholine; DPhPG, diphytanoyl phosphatidylglycerol; FCS, fluorescence correlation spectroscopy; Φ_Δ, quantum yield of singlet oxygen generation; LPS, lipopolysaccharides; Pc, phthalocyanines; PDI, photodynamic inactivation; $ZnPcPym^{4+}$, $AlPcPym^{4+}$, $ZnPcPym^{8+}$, and $AlPcPym^{8+}$, zinc and chloroaluminum tetrakis- and octakis(pyridiniomethyl)phthalocyanines; $ZnPcChol^{8+}$, zinc octakis(cholinyl)phthalocyanine; $ZnPc^{4-}$, tetrasulfonated zinc phthalocyanine.
* To whom correspondence should be addressed.

intracellular content, including ATP and K^+ ions, and inactivation of enzymes and transport systems [8]. Different lipophilic (protoporphyrin IX, unsubstituted phthalocyanines, etc.) and amphiphilic (hematoporphyrin, deuteroporphyrin, chlorines, etc.) dyes are efficient in photosensitization of cancer cells [9], yeasts [10], and Gram-positive bacteria [11]. However, uncharged lipophilic dyes as well as antibiotics and detergents [12] are not able to overcome the permeability barrier of Gram-negative cells – negatively charged outer cell membrane – and therefore they do not exhibit activity towards this bacterial group. Anionic hydrophilic dyes are inefficient and anionic amphiphilic dyes show low efficiency in photosensitization of Gram-negative bacteria [13], which may be due to their electrostatic repulsion from the negatively charged surface of the outer membrane. On the contrary, it was shown in a number of works that cationic dyes sensitize both Gram-positive and Gram-negative cells to visible light [4, 8, 13, 14].

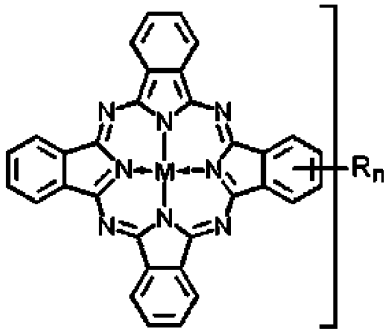
The charge on the outer membrane in Gram-negative bacteria originates mainly from lipopolysaccharides (LPS) composing its outer leaflet [15], and it depends strongly on acidity of the medium: at pH values below the cell isoelectric point in the pH range 1.5-4.5 [16] total cellular charge becomes positive, and it is negative at pH values above the isoelectric point (including the physiological pH values). Total negative charge of LPS is caused by high content of negatively charged groups in the central part of these macromolecules, namely, by phosphoric acid residues in D-glucosamine of lipid A and/or in core heptoses, by carboxyl groups of 3-deoxy-D-mannooctulosonic acid residues and acidic core saccharides (galactose, glucuronic acid) [15].

To study the relationship between efficiency of dye binding to Gram-negative bacterial cells and their photobactericidal activity, substituted zinc and aluminum phthalocyanines have been used in this work. This group of dyes was chosen because, along with high quantum yield of singlet oxygen generation, it includes a broad spectrum of polyanionic and polycationic compounds carrying up to eight ionic substituents. Structures and designations of phthalocyanines are given in Fig. 1. Physicochemical characteristics of these dyes were studied earlier [5].

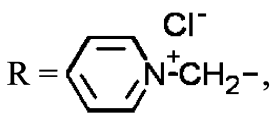
The goal of this work was to investigate electrostatic interaction of substituted metal phthalocyanines with enterobacterial cells as representatives of the Gram-negative species group and its relationship with photosensitization efficiency. Two groups of factors influencing dye interactions with the surface of bacterial cells and artificial membranes were studied. On one side, these are the sign of the charge and the number of ionic substituents in



$ZnPc^{8-}$



R = SO_3Na, n ~3, M = AlOH, – $AlPc^{3-}$;

R =  , n ~4, M = AlCl, – $AlPcPym^{4+}$; M = Zn, – $ZnPcPym^{4+}$;

n ~8, M = AlCl, – $AlPcPym^{8+}$; M = Zn, – $ZnPcPym^{8+}$;

R = $HOCH_2CH_2$-$\overset{\overset{CH_3}{|}}{\underset{\underset{CH_3}{|}}{N^+}}$-$CH_2$- Cl^-, n ~8, M = Zn, – $ZnPcChol^{8+}$

Fig. 1. Phthalocyanine structures and designations (*n* is average number of substituents R per molecule).

phthalocyanine molecules, and on the other side, these are factors that define the charge of the binding surface (lipid composition of membranes, content of bivalent ions Mg^{2+} and Ca^{2+} in the medium, pH, species peculiarities of LPS structure and charge).

MATERIALS AND METHODS

Zinc octacarboxyphthalocyanine ($ZnPc^{8-}$) was a single compound, while the other preparations were mixtures of differently substituted components with "*n*" mean extent of substitution. Sulfonated aluminum phthalocyanine (Photosense, $AlPc^{3-}$) produced by Federal Enterprise NIOPIK (Russia) was used in this work. Cationic zinc and chloroaluminum tetrakis- and octakis(pyridiniomethyl)phthalocyanines ($ZnPcPym^{4+}$, $AlPcPym^{4+}$, $ZnPcPym^{8+}$, $AlPcPym^{8+}$) and zinc octakis(cholinyl)phthalocyanine ($ZnPcChol^{8+}$) were synthesized as described in [5]. Values of quantum yields of phthalocyanine singlet oxygen generation in aqueous solutions (Φ_Δ) measured earlier in [17-19] are given in the table. Aluminum and zinc tetrasulfophthalocyanines $AlPc^{4-}$ and $ZnPc^{4-}$ were obtained from Porphyrin Products (USA). Gramicidin A (Sigma, USA) and lipids (Avanti Polar Lipids, USA) were used as well.

Experiments with Gram-negative bacterial strains (from the enterobacterial group) of clinical origin were carried out on *Escherichia coli*, *Salmonella enteritidis*, and *Proteus mirabilis*. Bacterial cultures grown at 37°C for 24 h on Trypcase Soy Agar (bioMerieux, France) were suspended in isotonic (0.9%) sodium chloride solution, and turbidity index was brought to 1 McF using a Densimat densitometer (bioMerieux). The photodynamic inactivation efficiency was estimated using the CFU decrease test. The number of colony-forming units (CFU) was determined by a standard procedure after plating on Trypcase Soy Agar of 0.1 ml from multiple dilutions of control and experimental (treated by photosensitizer in the dark and/or irradiated) samples and growing for 24 h at 37°C in a thermostat.

For determination of photobactericidal activity of different phthalocyanines, a bacterial bioluminescent test system based on the genetically engineered *E. coli* strain pXen7 was used, bioluminescence of which is due to the cloned complete *lux*-operon from luminescent soil entomopathogenic bacterium *Photorhabdus luminescens* [20]. Earlier we revealed a correlation between photosensitized quenching of test cultures of *E. coli* pXen7 bioluminescence and their inactivation as well as with inactivation of standard bacterial strain *E. coli* ATCC 25922 in CFU, which makes it possible to replace the routine method of CFU determination by the rapid method of measuring bioluminescence intensity [13, 20]. The mechanism of photosensitized quenching of bacterial bioluminescence is still not quite clear, but the fact that photodynamic inactivation (PDI) of bacteria corresponds to inhibition of bioluminescence is established for a number of dyes [13, 20, 21]. PDI was expressed via bioluminescence quenching ($(B_0 - B)/B_0$), where B_0 is bioluminescence intensity of cells irradiated with white light in the absence of dye, and B is the bioluminescence intensity of cells irradiated in the presence of dyes. Conditions of *E. coli* pXen7 growth, lyophilization, and rehydration were described previously [20]. Five microliters of phthalocyanine solutions in distilled water were added to 1 ml of a rehydrated bacterial sample. Bacterial suspensions ($3 \cdot 10^7$ CFU/ml) were incubated with dyes in the absence of light for 10 min at 20-22°C, and then they were irradiated using an EKOMP (Russia) device as a source of cold white light. Light intensity measured using an Ophir Optronics Ltd. device (Israel) was 50 mW/cm^2 at a distance of 5 cm from the light guide. The intensity of bacterial bioluminescence before and after irradiation was registered by a Biotox-6 luminometer (Russia) in 1.5-ml volume cell sample containing 1 ml of the sample under investigation at room temperature.

When effects of salts on PDI of the *E. coli* strain pXen7 bioluminescence were studied, the salts were introduced into bacterial suspensions, incubated for 5 min, and 1 μM $ZnPcChol^{8+}$ was added. After incubation for 10 min in the presence of the dye, the suspension was irradiated with white light.

When dependence of the photodynamic bioluminescence inactivation efficiency (B_0/B) on the acidity of the medium was studied, for biosensor *E. coli* pXen7 rehydration and preparation of $ZnPcChol^{8+}$ solutions, distilled water (pH 6.2) was used. Rehydrated bacteria (0.1 ml) were added to 0.9 ml of 0.067 M K-Na-phosphate buffer at different pH values, and then 5 μl of $ZnPcChol^{8+}$ solution was added to the final concentration of 1 μM, or 5 μl of distilled water in control. The pH values were measured using a pH meter in samples after addition of biosensor and photosensitizer and were 5.4-7.7. Control *E. coli* pXen7 cells exhibited stable bioluminescence level in this pH range. Spectral characteristics of $ZnPcChol^{8+}$ did not change in this pH range.

To determine spectrophotometrically the amount of dye bound during incubation with bacterial cells, the dye solution in 0.9% NaCl or bacterial suspension (10^8 CFU/ml) in 0.9% NaCl with the dye at the same concentration were incubated at room temperature and passed through filters with pore diameter 0.2 μm (Sarstedt, Austria). The amount of bound dye was calculated by the difference in filtrate absorption at 683 nm Q band maximum for $ZnPcChol^{8+}$ with molar absorption coefficient ε_{683} = 190,000 $M^{-1} \cdot cm^{-1}$ [5]. Absorption spectra were registered on a Hitachi-557 spectrophotometer (Japan).

The dye binding to bacterial cells was also studied using a set-up for fluorescence correlation spectroscopy (FCS) described earlier [22, 23]. It is a fluorescence microscope working in confocal regime. In this device, a

helium-neon (He-Ne) laser (633 nm) coupled with an Olympus IMT-2 microscope (USA), supplied with a water immersion objective 40×, 1.2 NA (Carl Zeiss, Germany), was used for dye excitation. Fluorescence signal passed through a corresponding dichroic separator and was projected on a 50-μm light guide core combined with an SPCM-AQR-13-FC avalanche photodiode (Perkin Elmer Optoelectronics, Canada). The signal was transformed using a Flex02-01D/C interface card (Correlator.com, USA). To tune the device, the light guide position with respect to the optical axis was adjusted before the experiment. For this purpose, the autocorrelation function for 10 nM $AlPc^{4-}$ solution was measured, which was characterized in optimum by three-dimensional diffusion time of about 500 μsec. Data were measured during 30 sec. Fluorescence was registered from the confocal volume located at a distance of 50 μm over a thin glass on which 60 μl of cell suspension was applied. To determine the dye fluorescence quenching, the fluorescence intensity was measured in the presence and absence of *E. coli* pXen7 without stirring. To determine the number of stained cells, measurements were made under stirring at 600 rpm. Each experimental point is the mean of three measurements.

A generally accepted method for quantitative processing of the fluctuating fluorescence signal $F(t)$ using the FCS method is calculation of the autocorrelation function, namely:

$$G(\tau) = \frac{\langle \delta F(t) \cdot \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2}, \qquad (1)$$

where $\langle F(t) \rangle$ is the mean fluorescence intensity and $\delta F(t) = F(t) - \langle F(t) \rangle$ is deviation from the mean. Measurements of autocorrelation function are usually used to determine diffusion coefficients of a substance, which are estimated by $G(\tau)$ curve fitting to the theoretical equation of three-dimensional diffusion [24]. However, in the case of objects as large as bacteria, statistically reliable measurement of $G(\tau)$ encounters some difficulties due to the necessity of signal accumulation over a very long time interval. In this work $G(\tau)$ was measured under stirring, when the dependence of G on τ is defined by the liquid flow rate caused by the stirrer. It should be pointed out that under conditions of dye binding to bacteria (like in the case with $AlPcPym^{8+}$), the observed characteristic bending of the $G(\tau)$ function in the absence of stirring is located in the time region of 10^{-1} sec, which was indicative of the presence of dye on objects with characteristic size of 1 μm.

When the cell suspension is stirred, the autocorrelation function indicates both the average time of residence of a fluorescent particle in the confocal volume and the average number of particles present in this volume. In our system, fluorescent particles are bacterial cells carrying bound dye molecules. The $G(\tau)$ value in the limit of low τ is used for quantitative estimation of an average number of fluorescent particles, namely, for suspension of identical particles, according to [24]:

$$G(\tau \to 0) = \frac{1}{N}, \qquad (2)$$

where N is the average number of fluorescent particles in the confocal volume.

Thus, under the suspension stirring conditions the FCS technique, namely measurement of autocorrelation function, also makes it possible to estimate dye binding not by a change in diffusion coefficient (as is done without stirring [25]), but rather by the change in the number of fluorescent particles. This approach was used in this work in studies of phthalocyanine binding to bacteria.

In experiments on gramicidin channel inactivation a planar bilayer lipid membrane (BLM) was formed of 2% lipid solution in decane according to Mueller et al. [26] on a hole 0.55 mm in diameter in a Teflon diaphragm separating two aqueous phases containing 100 mM KCl, 10 mM MES, 10 mM Tris, and 10 mM β-alanine, pH 7.0. In most cases, peptide solutions in different concentrations were added from the membrane *cis*-side. The *cis*-side is conventionally called the cell side to which positive potential is applied and from which the membrane is illuminated. Electric current through the membrane was measured using an amplifier Keithley 428 microammeter (USA). The analog signal was transmitted to a computer using a LabPC 1200 card (National Instruments, Inc., USA) and analyzed using the WinWCP Strathclyde Electrophysiology Software program elaborated by J. Dempster (University of Strathclyde, Great Britain). Electrodes made of chlorinated silver foil and submerged directly into the two aqueous phases were used to register membrane conductivity. An incandescent lamp with irradiation intensity near the membrane equal to 400 mW/cm^2 was used for membrane illumination by steady light. A light flash was produced by xenon flash lamp with energy of 0.3 J and the flash duration less than 3 msec. The photosensitized inactivation amplitude, which is the extent of lowering of the gramicidin-induced transmembrane current in response to the membrane illumination by visible light in the presence of photosensitizer, was determined using formula: $A = (I_0 - I)/I_0$, where I_0 and I are the stationary values of the current before and after illumination, respectively.

Electrophoretic mobility of liposomes was measured on a Zetasizer Nano device (Malvern, Great Britain) as described in [27]. Monolamellar liposomes from egg phosphatidylcholine or total *E. coli* lipid were prepared in 10 mM KCl, 5 mM MES, and 5 mM Tris, pH 7.0. The multilamellar mixture was passed through a Nucleopore polycarbonate filter with 0.1-μm pores using an Avanti Polar Lipids (USA) extruder.

RESULTS

Incubation of *E. coli* (10^8 CFU/ml) with $ZnPcChol^{8+}$ octacationic phthalocyanine solutions (0.2-2 μM) resulted in partial dye binding by cells. The highest extent of binding (16%) was observed at the total dye concentration of 1 μM and incubation time 10 min and did not increase after further increase in these parameters. Calculations have shown that under conditions of saturation, about 10^6 $ZnPcChol^{8+}$ molecules are bound per bacterial cell and do not pass into the filtrate, i.e. they are rather firmly retained by the cells. Taking into account that one *E. coli* cell contains $(2\text{-}3)\cdot10^6$ LPS molecules [28, 29], one molecule of bound $ZnPcChol^{8+}$ falls within 2-3 LPS molecules of outer membrane. The extent of binding of anionic dyes $ZnPc^{8-}$ and $AlPc^{3-}$ was much lower and varied in the range of 0-4% of initial concentration. In this case, the dye/LPS ratio did not exceed 1 : 10.

Binding to bacteria can result both in (i) a change in the spectrum and increase in intensity of fluorescence [30] as with cationic porphyrins, which is associated with the transfer of photosensitizer from aqueous medium to hydrophobic environment (outer membrane), and (ii) fluorescence quenching registered in cationic phthalocyanine [31]. In the case of interaction of cationic dyes acridine orange and safranin O with isolated polyanionic LPS, quenching of the dye fluorescence was observed due to, as the authors proposed, the aggregation of the dye upon its association with LPS [32]. The study of phthalocyanine binding to bacterial cells by the FCS technique has also shown a decrease in fluorescence signal of cationic compounds. The table shows data on the phthalocyanine fluorescence quenching by *E. coli* pXen7 cells as well as bactericidal action of a number of derivatives differing in charge on the macrocycle periphery, quantum yield of singlet oxygen generation (Φ_Δ), nature of central metal atom (Zn and Al), and cationic substituent (Pym and Chol). The bacteria caused very little quenching of fluorescence of negatively charged ions $ZnPc^{8-}$ and $AlPc^{3-}$. However, the fluorescence quenching efficiency sharply increased in the case of phthalocyanines having four and especially eight positively charged substituents. In the presence of *E. coli* cells of clinical isolate strain 2 in physiological medium, similar data were obtained concerning low efficiency of the anionic dye fluorescence quenching and high efficiency in the case of octacationic $ZnPcChol^{8+}$ (table). Supposedly, interaction with the cell wall LPS upon binding to bacterial cells is responsible for quenching of fluorescence of cationic phthalocyanines.

Comparison of the spectrophotometric and FCS data showed that for anionic phthalocyanines the relative amount of dye retained by *E. coli* (0-4%) and dye fluorescence quenching (2-5%) practically coincided, indicating interaction with cells. In the case of cationic $ZnPcChol^{8+}$ at the total concentration of 1 μM about 16% was retained by cells during filtration, while fluorescence quenching in suspensions of bacterial cells reached 60%, which may be indicative of the presence of relatively tightly and loosely bound dye fractions.

Quenching of fluorescence of aluminum and zinc octacationic phthalocyanine complexes was approximately the same, but in the case of tetracationic derivatives it was more efficient for $AlPcPym^{4+}$ (table). Although the nature of cationic substituents exerted a certain effect on the phthalocyanine fluorescence

Quenching of phthalocyanine fluorescence ($(F_0 - F)/F_0$) in the presence of *E. coli* pXen7 ($3\cdot10^7$ CFU/ml in distilled water), *E. coli* pXen7 photoinactivation according to the test of bioluminescence quenching ($(B_0 - B)/B_0$) by white light at a dose of 6 J/cm^2, and quantum yields of singlet oxygen generation (Φ_Δ) for phthalocyanines with different ionic substituents

Phthalocyanines (Pc)	$(F_0 - F)/F_0$, %	$(B_0 - B)/B_0$, %	Φ_Δ
$AlPc^{3-}$	2.8 ± 0.4 (2.0 ± 0.5)*	0	0.38 [17]
$ZnPc^{8-}$	4.3 ± 0.5 (5.0 ± 1.0)*	2 ± 1.0	0.57 [18]
$AlPcPym^{4+}$	47.0 ± 3.0	17 ± 2.0	0.20 [5]
$ZnPcPym^{4+}$	20.5 ± 1.5	29 ± 3.0	0.10 [5]
$AlPcPym^{8+}$	80.0 ± 6.0	52 ± 5.0	0.37 [19]
$ZnPcPym^{8+}$	77.0 ± 5.0	63 ± 5.0	0.45 [19]
$ZnPcChol^{8+}$	98.0 ± 2.0 (60.0 ± 7.0)*	87 ± 6.0	0.65 [19]

Note: Fluorescence and bioluminescence intensities were measured 10 min after addition of 1 μM dyes and incubation at 20-22°C.

* Data on the quenching of phthalocyanine fluorescence by cells of clinical *E. coli* isolate strain 2 (10^8 CFU/ml in 0.9% NaCl) are given in parentheses.

quenching by *E. coli* pXen7 cells, it was lower than that of the charge on the dye molecule.

Comparison of photobactericidal activity of $ZnPc^{8-}$, $ZnPcPym^{8+}$, and $ZnPcChol^{8+}$, present in aqueous solutions in monomeric form and having identical central atom and similar Φ_Δ, shows that positive charge at the macrocycle periphery has a crucial effect on the photoinactivation of *E. coli* pXen7 (table). A similar conclusion follows from comparison of data for $AlPc^{3-}$ and $AlPcPym^{8+}$. In this case, a correlation is observed between increase in fluorescence quenching and photoinactivation according to the test of bioluminescence quenching.

As shown in "Materials and Methods", measurement of autocorrelation function under stirring makes it possible to determine the number of fluorescent particles (see also [33]), i.e. in this case of stained cells. Figure 2 shows the results of measurement of $G(\tau)$ by the FCS technique for anionic $AlPc^{3-}$ and two cationic phthalocyanines, $AlPcPym^{4+}$ and $AlPcPym^{8+}$, in solutions and in *E. coli* pXen7 cell suspensions under stirring. It is seen that in the case of anionic dye the addition of bacteria does not change $G(\tau)$. These data show that $AlPc^{3-}$ practically does not interact with the cells. In contrast, in the case of $AlPcPym^{4+}$ and $AlPcPym^{8+}$ the amplitude of $G(\tau)$ increased after addition of bacteria. In this case, the number of fluorescent particles (stained cells, N) in the confocal volume (see expression (2)) was approximately 40% higher in the case of octacationic $AlPcPym^{8+}$ compared to tetracationic $AlPcPym^{4+}$, which corresponds to more efficient quenching of $AlPcPym^{8+}$ fluorescence in the presence of *E. coli* pXen7 cells (table).

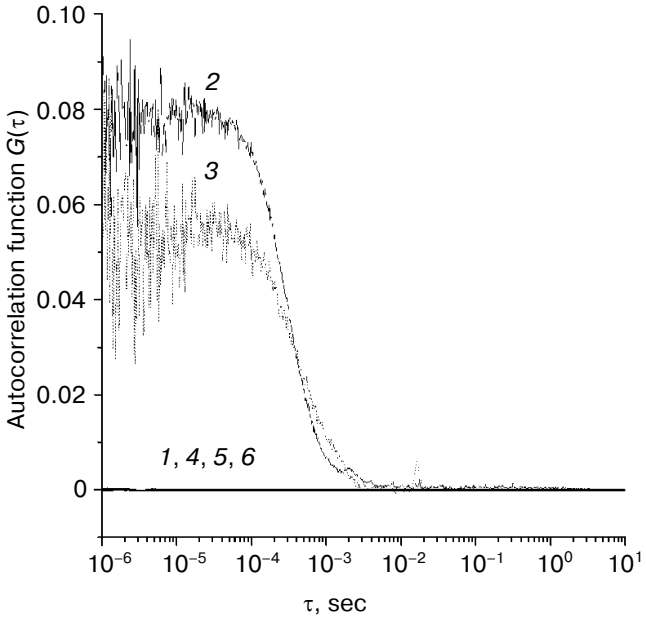


Fig. 2. Characteristic autocorrelation functions $G(\tau)$ for dyes (1 μM) in *E. coli* pXen7 cell suspension at a concentration of $3.3 \cdot 10^7$ CFU/ml (*1-3*) and control curves for dye solutions (*4-6*): *1*, *4*) $AlPc^{3-}$; *2*, *5*) $AlPcPym^{4+}$; *3*, *6*) $AlPcPym^{8+}$ under stirring.

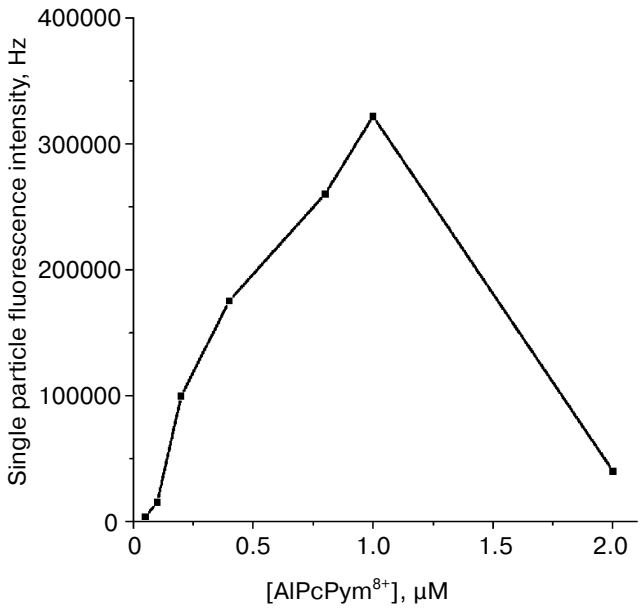


Fig. 3. Dependence of a single particle (stained cell) fluorescence intensity in confocal volume on $AlPcPym^{8+}$ concentration. Suspension density is $3.3 \cdot 10^7$ CFU/ml.

Using data on the suspension fluorescence intensity and N value, it is possible to determine the fluorescence intensity of a single stained cell. It is seen in Fig. 3 that in the cell suspension of constant density the increase in the total $AlPcPym^{8+}$ concentration to 1 μM resulted in practically linear growth of fluorescence intensity of individual particles – stained cells. This is probably indicative of filling of binding sites on the bacterial surface with the dye. The decrease in luminescence intensity per single particle along with increase in $AlPcPym^{8+}$ concentration from 1 to 2 μM might be due to the contribution of free dye molecules and their aggregates, i.e. particles of noncellular nature, to total number of fluorescent particles in the confocal volume. It should be noted that these data were obtained at a density of bacterial suspensions that provides practically complete binding of dye molecules up to the concentration of 1 μM. This follows, in particular, from direct proportionality of the dependence of N on suspension densities in the range of $3 \cdot 10^7$-10^8 CFU/ml (data not shown). In the case of lower concentrations of bacteria, a deviation from this dependence was observed.

As shown in a series of works by Antonenko et al. [22, 27, 34-36], it is convenient to use the method of photodynamic inactivation of gramicidin channels for estimation of photosensitizer efficiencies in a model membrane system [37]. The parameter measured in this case, relative amplitude of photosensitized inactivation, is the extent of lowering of the gramicidin-induced current

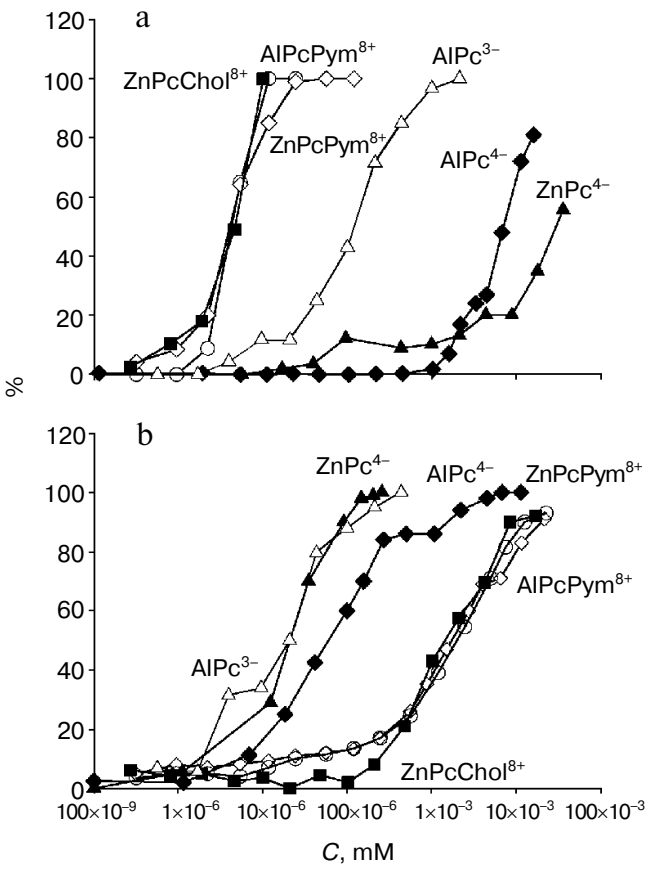


Fig. 4. Dependence of gramicidin A photoinactivation amplitude $(I_0 - I)I_0$ on the concentration of different dyes on a negatively charged membrane of DPhPC/DPhPG 70/30% mixture (a) and on neutral DPhPC membrane (b). Buffer solution (pH 7.0) contained 100 mM KCl, 10 mM MES, and 10 mM Tris.

through the model planar BLM in response to the illumination of the membrane by visible light in the presence of a photosensitizer. Figure 3 shows the dependences of the gramicidin photoinactivation amplitude, indicating the fraction of damaged gramicidin channels, on the concentration of different phthalocyanines. It is seen that on negatively charged membranes (Fig. 4a) containing 30% anionic lipids (diphytanoyl phosphatidylglycerol, DPhPG), zinc and aluminum cationic phthalocyanines, carrying eight positively charged substituents, exhibit photodynamic activity exceeding by three orders of magnitude that of zinc and aluminum anionic phthalocyanines having four or three negatively charged side groups. On neutral membranes consisting of 100% diphytanoyl phosphatidylcholine (DPhPC) (Fig. 4b), the activity of cationic zinc and aluminum phthalocyanines, on the contrary, is approximately two orders of magnitude lower than that of anionic phthalocyanines.

Data on photodynamic activity of charged phthalocyanines on planar BLM correlate with the liposome ζ-potential values obtained by measuring electrophoretic mobility, which suggest that zinc and aluminum cationic phthalocyanines bind much more efficiently than anionic phthalocyanines to negatively charged liposomes formed of *E. coli* lipid mixture (Fig. 5a) and poorly interact with liposomes of egg yolk phosphatidylcholine (Fig. 5b).

Taking into account efficient electrostatic attraction of Mg^{2+} and Ca^{2+} by negatively charged LPS of outer membrane of Gram-negative bacteria, the competitive binding of bivalent cations and polycationic phthalocyanines with bacterial cells could be expected. During preliminary incubation of cells with $MgCl_2$ and $CaCl_2$, but not with NaCl (in the range of 0.1-10 mM concentrations), photodynamic bioluminescence quenching with $ZnPcChol^{8+}$ decreased 3-4-fold (Fig. 6) compared to that for the dye in distilled water. Similar protective effects of NaCl were revealed at significantly higher concentrations (over 100 mM, data not shown). Resuspending *E. coli* pXen7 in 0.9% NaCl with following treatment by $ZnPcChol^{8+}$ resulted in 6-fold weakening of the photodynamic effect.

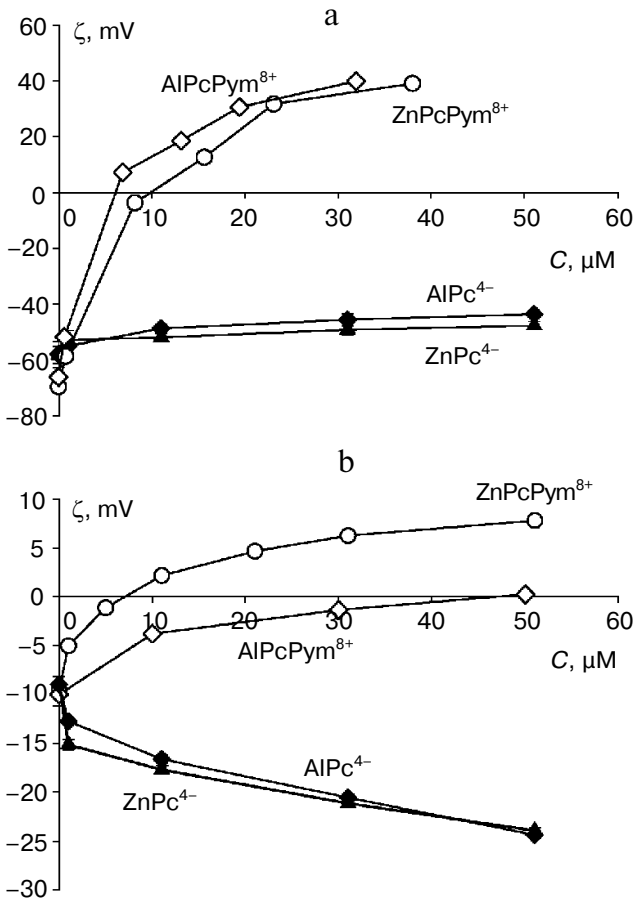


Fig. 5. Dependence of ζ-potentials of liposomes of *E. coli* lipids (a) and of egg yolk phosphatidylcholine (b) on dye concentration. Buffer solution contained 10 mM KCl, 5 mM MES, and 5 mM Tris, pH 7.0.

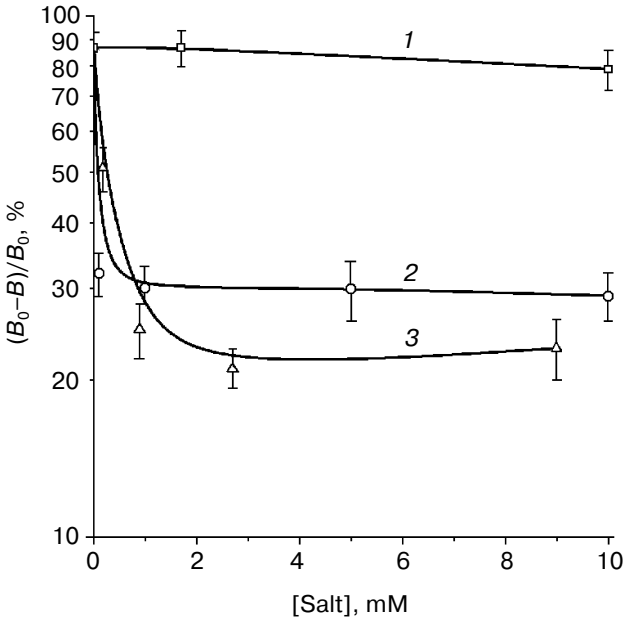


Fig. 6. Salt effects on photodynamic quenching of bioluminescence of genetically engineered *E. coli* strain pXen7: *1*) NaCl; *2*) $MgCl_2$; *3*) $CaCl_2$. Salts were added to the bacterial suspension 5 min prior to addition of 0.5 μM $ZnPcChol^{8+}$. After incubation with dye for 10 min, the suspension was irradiated by white light at a dose of 6 J/cm^2.

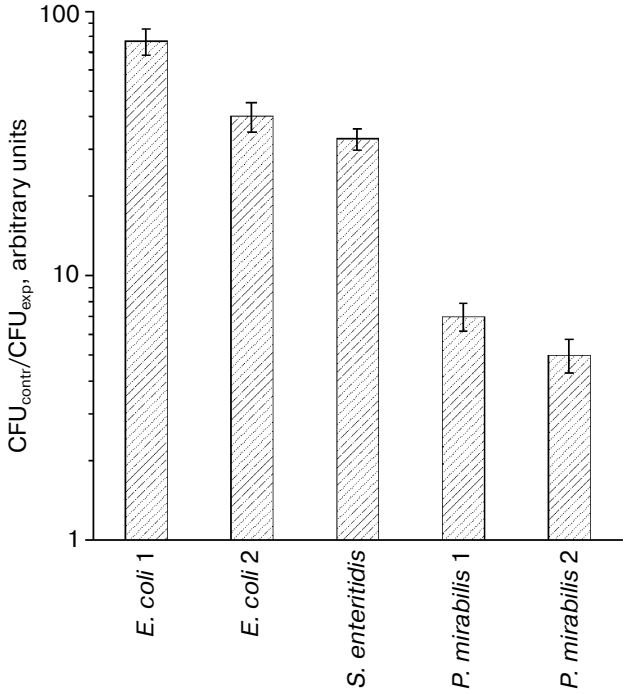


Fig. 8. PDI efficiency by the test of decrease of colony-forming ability (CFU_{contr}/CFU_{exp}) of different enterobacterial strains after 10 min incubation of suspension with initial density of 10^8 CFU/ml in 0.9% NaCl with 1 μM $ZnPcChol^{8+}$ and illumination by white light at a dose of 9 J/cm^2.

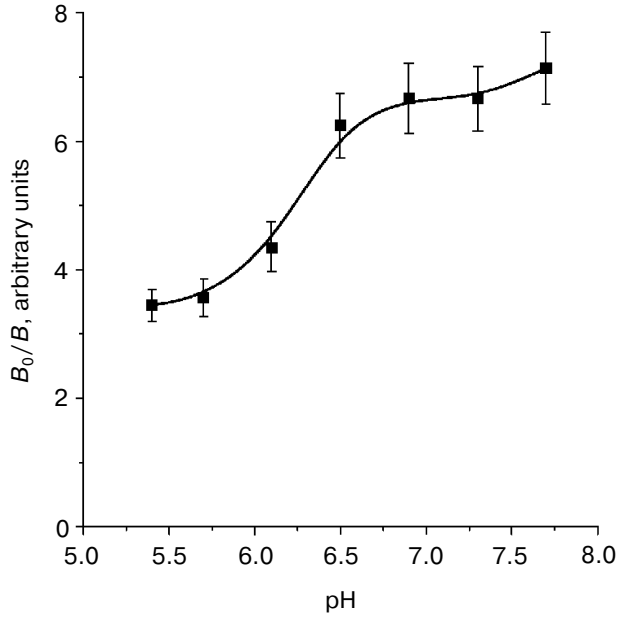


Fig. 7. Effect of pH on efficiency of photodynamic quenching of bioluminescence (B_0/B) of genetically engineered *E. coli* strain pXen7 in the presence of 1 μM $ZnPcChol^{8+}$ in the case of irradiation by white light at a dose of 6 J/cm^2.

Increasing the acidity of the medium decreased the efficiency of photodynamic quenching of *E. coli* pXen7 bioluminescence (Fig. 7). Maximal efficiency was observed at mildly alkaline pH values, the pH shift of 2 units from 7.7 to 5.7 lowering it by half.

Figure 8 shows that another factor that defines the efficiency of photobactericidal activity is the species to which the bacteria belong. Among five clinical isolates of the enterobacterial group the most resistant to photodynamic inactivation was *P. mirabilis*, *E. coli*, and *S. enteritidis*, which exhibited 5-15-fold higher sensitivity depending on the strain. Spectrophotometric determination of $ZnPcChol^{8+}$ concentration in filtrates showed that within the range of 0.2-1 μM *P. mirabilis* cells bound $ZnPcChol^{8+}$ to much lower extent than *E. coli*, the dye loss after 10 min incubation being 2-5%.

DISCUSSION

The LPS accumulation in the outer leaflet of the outer membrane and phospholipids in the inner leaflet creates a significant asymmetry in the negative charge distribution. It was shown on artificial bilayer membranes [38] that density of surface negative charges of the LPS

layer constitutes 3.33 elementary negative charges/nm^2, while that in phospholipid layer is one order of magnitude lower, only 0.31.

The high density of negative charges on the cell wall LPS in Gram-negative bacteria causes efficient electrostatic attraction of cations and polycations [29, 39, 40], including polycationic phthalocyanine molecules as is shown in this work. Electrostatic repulsion of phthalocyanine molecules having negatively charged substituents (sulfo and carboxy groups) results in polyanionic phthalocyanines having practically no affinity to the bacterial cells (table and Fig. 2). Results of experiments on model membranes having different charge (Figs. 4 and 5) also substantiate a significant role of electrostatic factors in interaction of polycationic zinc and aluminum phthalocyanines with bacterial cell wall. Thus, photodynamic activity of polycationic phthalocyanines measured by damage to gramicidin A peptide channel exceeded that for polyanionic phthalocyanines only if negative charge was present on the surface of the artificial membrane (Fig. 4).

The primary stage of antimicrobial action of many polycationic compounds (antimicrobial peptides, chitosan, etc.) is electrostatic interaction with bacterial cell walls [41]. The photosensitizer tight association with the biological target is also necessary for efficient photodynamic inactivation as suggested by a small diffusion radius of $^1O_2$ (less that 50 nm in biological medium) [42]. Our data concerning direct connection between the presence of positively charged substituents in phthalocyanine molecules and their photobactericidal action are in agreement with this assumption (table).

On the whole, phthalocyanine affinity to bacteria makes it possible to predict qualitatively their photobactericidal activity. In other words, only the dye bound to the bacterial cell is able to inactivate it.

To prevent mutual repulsion of negatively charged LPS and stabilize the outer leaflet of the outer membrane, LPS are bound via ionic bridges of Mg^{2+} and Ca^{2+}. It is known that chelating compounds cause disintegration [43] of this structure, disturb its barrier functions, and sensitize bacteria to various compounds. In accordance with this, we observed the most pronounced effect of photodynamic quenching of *E. coli* pXen7 bioluminescence upon cell suspension and photodynamic treatment in distilled water. The presence of Mg^{2+} and Ca^{2+} in the medium lowered photodynamic quenching of bioluminescence, i.e. protection of bacteria against inactivation (Fig. 6). This may be due, on one side, to direct competitive interaction of phthalocyanine polycations and bivalent cations with binding centers on the surface of the bacterial cell (with negatively charged LPS groups) and, on the other side, to enhanced cell wall permeability for dyes in the absence of stabilizing Mg^{2+} and Ca^{2+} cations.

The effect of the acidity of the medium on the photodynamic activity of the cationic dye ZnPcChol^{8+} (Fig. 7) is probably mediated by changing the number of charged groups on the surface of bacterial cells. Lowering the pH results in protonation of negatively charged LPS groups, decreasing the number of cell surface centers for electrostatic binding of positively charged dye molecules, and as a result decreasing the efficiency of photobactericidal action.

There is significant heterogeneity among enterobacteria concerning structure of the lipid A and LPS core polysaccharides. The modification of LPS is an important mechanism for development of resistance to cationic antimicrobial peptides in Gram-negative bacteria [44, 45]. The *P. mirabilis* bacterium exhibits high resistance to polymyxin B and protegrins, which is associated with incorporation into LPS of positively charged aminoarabinose molecules [46]. A similar feature is the observed relative resistance of these enterobacteria to photodynamic inactivation (Fig. 8). As is it shown in this work, *P. mirabilis* exhibits low sensitivity to photosensitization by octacationic phthalocyanine due to the weak binding of the dye by bacterial cells of this species. *Escherichia coli* cells able to bind 3-4-fold more of the dye are almost one order of magnitude more sensitive to photodynamic inactivation. The data show that both the number of positively charged substituents in phthalocyanine molecules and density of negative charges on the surface of bacterial cells influence the efficiency of dye binding and photobactericidal activity, which decrease under conditions of shielding, neutralization, and decrease of number of negatively charged LPS groups.

In conclusion, it is noteworthy that the data make it possible to predict the dye affinity to cells and photobactericidal activity based on the dye structure, physicochemical properties, and biological features of the chosen object and factors of the environment. The revealed mechanisms of the binding of differently charged phthalocyanines to Gram-negative bacterial cells can be useful in analysis of interaction with cells and investigation of antibacterial effects of different polar compounds.

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