

Photodynamic inactivation of *Pseudomonas aeruginosa* bacterial biofilms using photosensitizers based on octacationic derivatives of phthalocyanines and bacteriochlorins

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Abstract. Antibacterial photodynamic therapy is a promising method of treating local infected foci, especially surgical and burn wounds, trophic and diabetic ulcers. This work explores the photophysical and antibacterial properties of novel phthalocyanine- and synthetic-bacteriochlorin-based octacationic photosensitizers (PS). The results of the study confirm their low degree of aggregation at high concentrations, as well as high efficiency of photodynamic treatment of Gram-negative bacterial biofilms.

1. Introduction

Infected long-term non-healing complicated skin and mucosa wounds, trophic ulcers, pressure sores and ulcers of diabetic feet present serious treatment problems. Recently, evidence connecting the chronicity of wounds with biofilms and polymicrobial communities has been found. These are usually multi-species and include both Gram-positive and Gram-negative bacteria [1].

Antibacterial photodynamic therapy (APDT) is a promising way to treat infected surgical and burn wounds, trophic and diabetic ulcers [2,3]. Photodynamic effect can effectively inactivate bacterial cells without causing any drug resistance in response to the treatment and does not damage the patient's normal microflora as compared to antibiotics due to local irradiation [4,5]. Even after 20 consecutive cycles of partial destruction of bacterial flora and its regrowth, no resistance to PS and APDT has been observed [6,7]. Almost all pathogenic microorganisms, including antibiotic-resistant strains of bacteria, are susceptible to APDT [8].

An effective PS must kill both Gram-positive and Gram-negative bacteria both in planktonic and in biofilm states. The sensitivity of Gram-negative bacteria to APDT is much lower than that of Gram-positive bacteria. Gram-negative bacteria have an additional structural element - an outer 10-15 nm thick membrane, which is external to the peptidoglycan network and has a very heterogeneous composition (porin-function proteins, lipopolysaccharide trimers and lipoproteins which create an external pseudosurface of tightly packed negative charges) [9]. This highly organized system prevents large

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molecules from penetrating and facilitates drug resistance. The Gram-negative bacteria effectively interact only with polycationic PS with a small molecules size and weight. The most likely mechanism to ensure the association of cationic PS with microbial cells is the electrostatic interaction of positively charged molecules with negatively charged cell wall sites [10]. Inactivation of Gram-negative bacteria is, therefore, the most important and difficult goal for photodynamic treatment of infected foci [11].

The efficiency of bacterial photodynamic inactivation depends on the properties of PS molecules — their size, mass [12], and the number and configuration of the cationic groups. High concentrations of PS should be used for sensitization to ensure correct inactivation of bacteria. The aggregation of tetrapyrrole molecules [13–15] leads to a decrease in absorption intensity, the lifetime of the excited state, the fluorescence quantum yield, as well as a change in the shape of the absorption and fluorescence spectra. Coulomb repulsion of cationic PS molecules can partially reduce the aggregation. An increase in the number of cationic group n leads to a reduction in the degree of aggregation of aquatic solutions and, therefore, to an increased fluorescence and singlet oxygen quantum yields [16–18]. The rise of their activity with the increase of n may be both due to the dissociation of inactive dimer complexes and the increase of the bacteria binding efficiency.

It should be noted that the depth of the *P. aeruginosa* infectious lesions foci can reach 12-15 mm [19]. Therefore, PS, the excitation of which can be carried out in 720-850 nm spectral range, known as the "biological tissue transparency window," must be used for achieving proper photodynamic effect on such foci. In addition, a number of pigments (especially, pyocyanin, pyoverdine, pyorubrin, pyomelanin) are produced by *P. aeruginosa* bacteria during their lifetime. [20]. The presence of these pigments in wound discharge results in a large absorption in the 660-740 nm spectral range. Due to this, APDT with the red spectral range photosensitizers may be ineffective due to the large loss of exciting radiation caused by absorption of both the pigments and the hemoglobin in host tissues.

This work is the part of a research series devoted to the photophysical study of photosensitizers for antibacterial photodynamic inactivation. This article focuses on the analysis of octacationic compounds ZnPcChol_8 and $(3\text{-PyEPy})_4\text{BCBr}_8$. The photophysical properties of the compounds and their effectiveness were analyzed, and their efficacy on one of the most common biofilms in clinical practice (*P. aeruginosa*) was verified.

2. Materials and Methods

In our work, we studied original tetrapyrrole-derivative-based polycationic photosensitizers synthesized in Organic Intermediates and Dyes Institute (Russia) for photodynamic inactivation of bacteria and their biofilms: zinc octakis(cholanyl)phthalocyanine ZnPcChol_8 (1678 Da, local optical absorption maximum at 677 nm) and octacationic bacteriochlorin derivative $(3\text{-PyEPy})_4\text{BCBr}_8$ (1691 Da, local optical absorption maximum at 763 nm) [12,21–23].

The novel PS were studied in vitro by absorption and fluorescence spectroscopy for signs of aggregation at various concentrations. The absorption studies were carried out on a two-beam spectrophotometer "Hitachi U-3410" (Hitachi, Japan). The fluorescence intensity was measured with fiber-optic spectrometer "LESA-01-Biospec" (BIOSPEC, Russia). The PS absorption was measured in the concentration range between 0.001 mM and 0.1 mM. The fluorescence of $(3\text{-PyEPy})_4\text{BCBr}_8$ was excited using 532 nm CW laser, which matches its Q_2 absorption band. The ZnPcChol_8 fluorescence was excited using 632.8 nm He-Ne laser.

To evaluate photo-bleaching, we studied the intensity and shape of the PS fluorescence spectra before and after the irradiation process, as well as at certain intermediate moments of the irradiation process. The evaluation of fluorescence lifetime was carried out using Hamamatsu streak-camera system [12,24,25].

The microbiological studies were carried out on *P. aeruginosa* 32 clinical isolate. The methodology is thoroughly described in [25].

3. Results

No signs of aggregation were observed in the absorption spectra of the studied PS in the range of concentrations up to 0.2 mM (Figure 1). The bathochromic shifted dimer band [18] in ZnPcChol_8 spectrum is absent even at high concentrations. The absorption is in the linear dependence with concentration and agrees with optical extinction values determined at low concentrations. Such dependence suggests low degree of aggregation at higher concentrations.

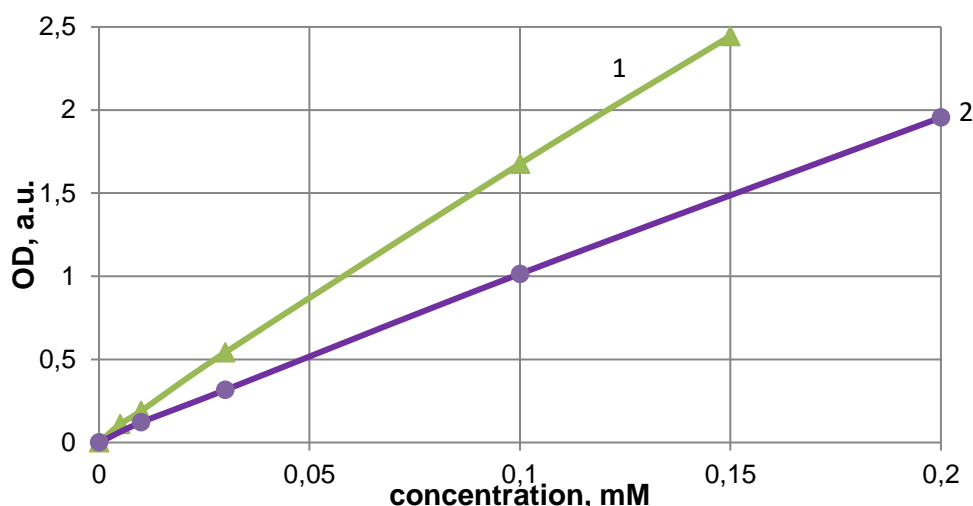


Figure 1. The dependence of optical density of ZnPcChol_8 (1) and $(3\text{-PyEPy})_4\text{BCBr}_8$ (2) water solution on their concentration (in cell 1 mm long)

The studied PS fluorescence band is narrow and intense in water. The dependence of the fluorescence intensity on the concentration increases to a concentration of 0.1 mM (up to 0.03 is linear, further is sublinear) (Figure 2). This suggests low degree of reabsorption in this range of concentrations.

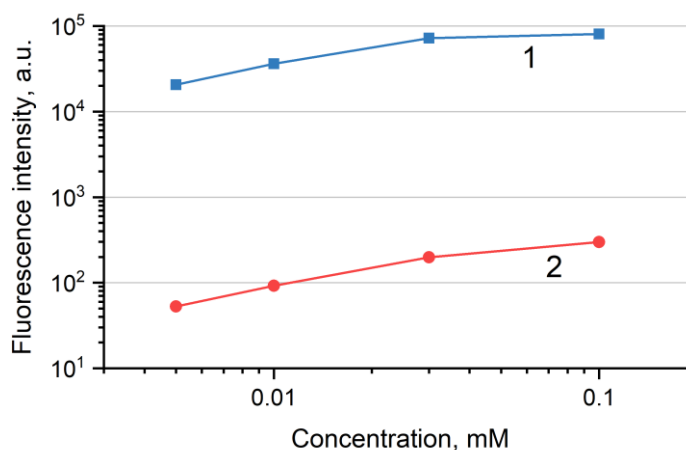


Figure 2. Fluorescence intensity of ZnPcChol_8 (1) and $(3\text{-PyEPy})_4\text{BCBr}_8$ (2) in water

P. aeruginosa bacteria produce pigments during biofilm growth. Analysis of the culture medium reveals an intense absorption band with maximum at 690–700 nm and half-width of 60 nm at 24 hours [25]. The absorption spectra of pigments in the biofilms of clinical isolate of *P. aeruginosa* 32 are presented in Figure 3. The optical density of pigments in culture medium of *P. aeruginosa* 32 biofilms grown in LB broth reaches higher values at 690 nm (up to 1.8). These wavelengths are close to absorption maxima of a variety of PS (for example, ZnPcChol_8 used in this study). It should be noted

that the concentration of pyocyanin in the wound discharge in case of *P. aeruginosa* growth is much higher compared to biofilm growth in LB broth [20].

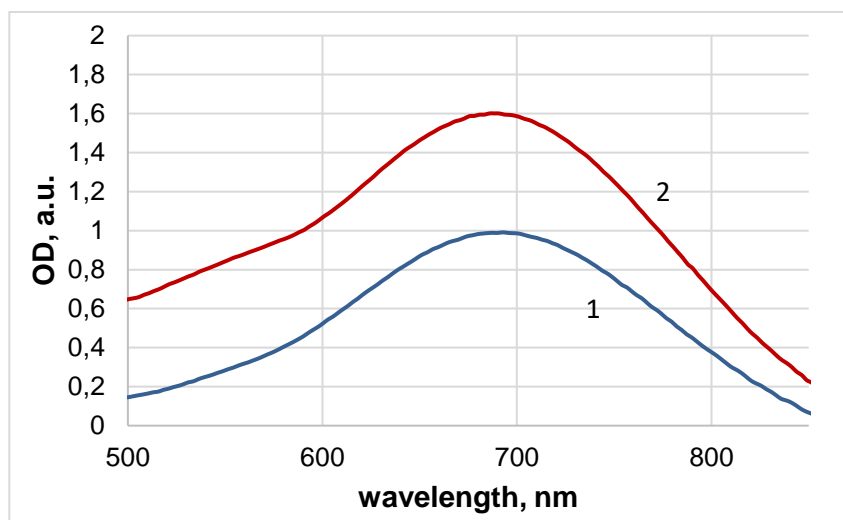


Figure 3. The pigment absorption spectrum in the culture medium during the growth of the biofilms of *P. aeruginosa* 32 clinical isolates (1) 24 hours after the beginning of growth; (2) 72 hours after the beginning of growth.

Studies of photobleaching (the PS fluorescence dependence on the radiation dose) showed that the speed of ZnPcChol8 photobleaching is low up to a dose of 100 J cm^{-2} , however, the fluorescence intensity of $(3\text{-PyEPy})_4\text{BCBr}_8$ decreased e times by 50 J cm^{-2} .

The results of *P. aeruginosa* 32 biofilms APDT are shown in Figure 4.

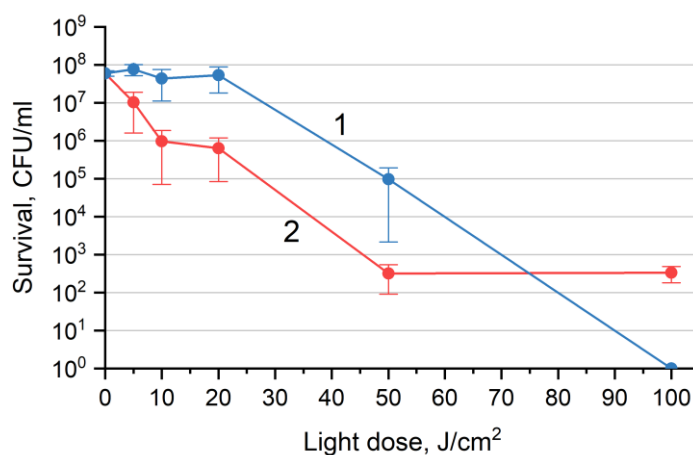


Figure 4. Photoinactivation of *P. aeruginosa* biofilms after 60 min of incubation with aqueous solutions of the studied PS with concentration of $250 \mu\text{M}$ depending of light dose:

1 – ZnPcChol₈; 2 – $(3\text{-PyEPy})_4\text{BCBr}_8$

Effectiveness of APDT in biofilms greatly depends on light dose and PS concentration during incubation. For $(3\text{-PyEPy})_4\text{BCBr}_8$ it reaches 10^5 times reduction in CFU at a low (50 J cm^{-2}) light dose. The effectiveness of inactivation of the *P. aeruginosa* bacteria in biofilms using ZnPcChol₈ is greatly reduced due to high light absorption by pigments in biofilm at around 680 nm , where ZnPcChol₈ is

excited. Light loss for the excitation of (3-PyEPy)₄BCBr₈ is less compared to ZnPcChol₈, since its absorption lies at a wavelength of 760 nm. However, at a higher radiation doses (up to 100 J cm⁻²), complete inactivation of *P. aeruginosa* biofilms is achieved due to higher photostability of ZnPcChol₈.

Fluorescent microscopy of biofilms after APDT and Live/Dead staining visualize the bacteria that are either dead or have higher membrane permeability, which is the first step of cell damage under action of reactive oxygen species. Live/Dead staining is not quantitative reaction, but this experiment confirms the data of plating experiments. The stained microscopic images for *P. aeruginosa* biofilms are presented in Figure 5. At high (>30 J cm⁻²) doses of light, these results demonstrate the high efficacy of all the studied PS for APDT of *P. aeruginosa* in biofilms. However, at low (10 J cm⁻²) light doses only the bacteria in biofilms after APDT using (3-PyEPy)₄BCBr₈ significantly lower than using ZnPcChol₈, which confirms the conclusion that APDT with (3-PyEPy)₄BCBr₈ is more effective at low doses of light compared to ZnPcChol₈.

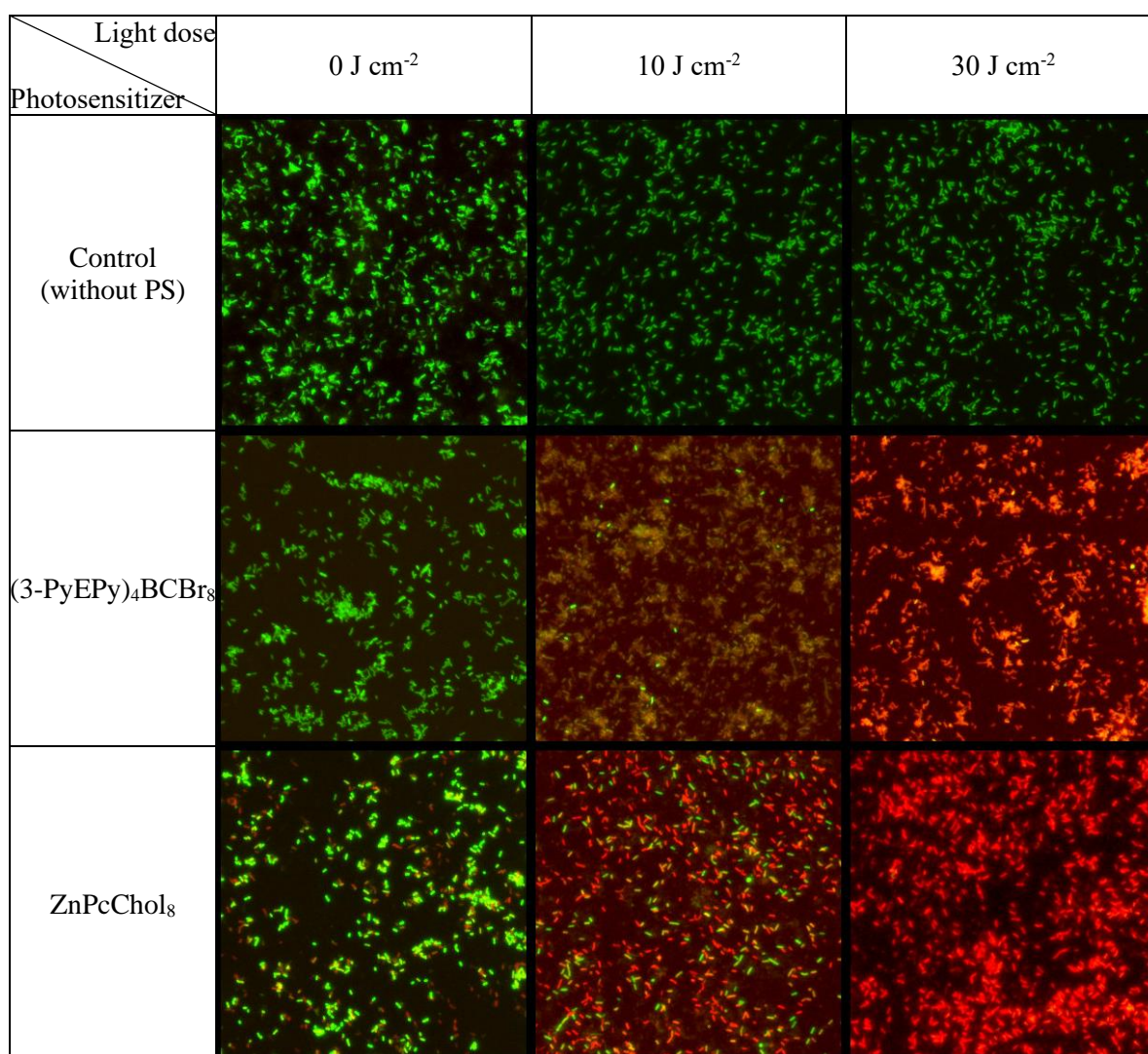


Figure 5. The microphotos of *P. aeruginosa* biofilm before and after the APDT using (3-PyEPy)₄BCBr₈ and ZnPcChol₈ cationic PS with light doses of 10 and 30 J cm⁻².

4. Discussion

The antimicrobial photodynamic therapy of Gram-negative bacterial *P. aeruginosa* biofilms can achieve highly effective results *in vitro* by using photosensitizers based on the derivatives of polyacationic phthalocyanines and synthetic bacteriochlorins.

Inactivation of bacteria in biofilms depends on light dose and reaches about 5 orders of magnitude with (3-PyEPy)₄BCBr₈ at low (<50 J cm⁻²) light dose. The effectiveness of inactivation of the *P. aeruginosa* bacteria in biofilms using ZnPcChol₈ is lower. However, at a higher radiation dose (up to 100 J cm⁻²), complete inactivation of *P. aeruginosa* biofilms is achieved due to the high photostability of ZnPcChol₈.

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