PHOTODYNAMIC INACTIVATION OF BACTERIA AND BIOFILMS USING CATIONIC BACTERIOCHLORINS

1Meerovich G.A., 2Tiganova I.G., 3Makarova E.A., 4Meerovich I.G., 5Romanova Ju.M., 2Tolordova E.R., 2Alekseeva N.V., 2Stepanova T.V., 2Koloskova Yu., 2Luk’anets E.A., 3Krivospitskaya N.V., 5Sipailo I.P., 5Baikova T.V. 1Loschenov V.B., 2Gonchukov S.A.

1A.M. Prokhorov General Physics Institute, Moscow, Russia
2N.F. Gamaleya Federal Research Center for Epidemiology and Microbiology, Moscow, Russia
3Organic Intermediates and Dyes Institute, Moscow, Russia
4Federal Research Centre “Fundamentals of Biotechnology” of the Russian Academy of Science, Moscow, Russia
5National Research Nuclear University MEPhI, Moscow, Russia
gonchukov@mephi.ru

Abstract
This work is devoted to the study of two new synthetic bacteriochlorins with four and eight cationic substitutes as the photosensitizers in the photodynamic process. The spectral and antibacterial properties of these photosensitizers in saline solution were investigated. It is shown, that the aggregation ability decreases and the antibacterial efficiency grows as the cationic substitute number increases.

1. Introduction
As it is known, the photodynamic process (PDP) is widely used in the treatment of different illnesses. Photodynamic therapy (PDT) is based on the cytotoxic mechanism of PDP that leads to the loss of cells vital function. A key element of this process is a photosensitizer (PS), the properties of which determine the treatment effectiveness. A number of PS are used in medical practice. However, the quest for more effective PS continues. Strong absorption in the transparency spectral region of biological tissue, a high quantum yield of the triplet state for the singlet oxygen formation, a good solubility in water, as well as the available synthesis are the most important requirements to PS. Cancer treatment is traditionally given a lot of attention [1,2]. PDP also has a great potential in the fight against pathogenic bacteria [3,4], because PS can possess the property of selectively interaction with a bacterium. Cationic bacteriochlorins can meet these requirements [5-7].

Unlike antibiotics, all of which act on a specific target in a microbial cell, the photodynamic antibacterial process is a nonspecific damage of cellular components potentially subjected to oxidative reactions. In so doing the bacteria do not develop resistance...
to PD treatment, bactericidal effect has an addressable character and has no systemic effects on the body flora.

Antibacterial efficiency should increase with PS concentration increasing. But, at the same time, it may be accompanied by an aggregation of PS, that can significantly reduce the treatment effectiveness. The phenomenon of PS aggregation plays an essential negative role in the PDP [8-11]. However, it should be expected that the Coulomb repulsion will help reduce of this factor influence in the case of cationic bacteriochlorins using [12].

The study of PDP antibacterial effect on the bacterial biofilms, which are formed during severe infectious diseases development, is of considerable interest. Normally biofilms are surrounded by a matrix which protects the bacteria from exposure to antibiotics and immune protection factors [13,14]. Biofilms formed by gram-negative bacteria *Pseudomonas aeruginosa*, peculiar to the nosocomial infections, possess a very high resistance [15]. These biofilms can be inactivated only by cationic PS using [5-7]. The inactivate efficiency of gram-negative bacteria essentially depends on the number of positive charges in the molecular structure of cationic PS [16].

Antibacterial efficiency of both gram-positive and gram-negative bacteria using synthetic cationic bacteriochlorins was demonstrated in the works [17-19]. However the bacteriochlorin derivatives, used in these studies, have no more than four cationic substitutes.

Two original synthetic cationic bacteriochlorins: 5,10,15,20-Tetrakis [1-(4'-bromobutyl)-3-pyridyl] bacteriochlorin tetrabromide [(3-PyBuBr)4BCBr4] and 5,10,15,20-Tetrakis [1-(4'-pyridiniobutyl)-3-pyridyl] bacteriochlorin octabromide [(3-PyBuPy)4BCBr8]), which have four and eight cationic groups (BCl -4 and BCl-8) are under consideration in this work. These BCls have been previously synthesized for the cancer therapy [20,21]. In this work the possibilities of these BCls for inactivating of plankton (free floating) and biofilm cultures of gram-negative *Pseudomonas aeruginosa* are studied.

### 2. Materials and Methods

#### 2.1. Sample preparation

BCl-4 and BCl-8 photosensitizers were synthesized according [20,21].

#### 2.2. Spectroscopic instrumentation

The absorbance spectra were measured with the help of Hitachi-S3410 and Cary-300 UV-Vis spectrometers. Fiber-optic spectrometer LESA-5 was used for the spectral analysis of fluorescence, excited by Nd:YAG CW laser at the wavelength of 532 nm.

#### 2.3. Antibacterial efficiency measurements

Biological studies were done on bacterial cultures of *Pseudomonas aeruginosa* in plankton and biofilm forms. Bacterial biofilms were grown on the glass slides (8x12 mm) in a Petri dish filled with the LB nutrient broth for 20 h at 37°C. The slides were put in the wells of 24-well plate, washed and exposed to PS in saline for 1 h at 37°C. After incubation samples were washed with fresh saline and irradiated using LPhD-03-Biospec arc lamp source with narrow-band filter (670-840 nm) or LED matrix source with the wavelength of 761 nm. Power density in spectral range of PS absorption was 10-20 mW/cm². Then serial dilutions were plated to determine viable cells using colony-forming unit (CFU) microbiological method.
Effectiveness of photodynamic antibacterial chemotherapy (PACT) was demonstrated by fluorescence microscopy using Nikon H600L microscope. Biofilms of bacteria were grown on cover slips for 3 h, incubated with PS, washed and irradiated. Three cover slips were stained with Live/Dead Biofilm Viability Kit (“Invitrogen”), and pictures were taken with 800× magnification, with subsequent overlay of images acquired with green and red filters.

3. Results and discussion

As shown on figures 1 and 2, the absorption spectra for both bacteriochlorins are similar with maxima at 763-765 nm. The analysis of experimental data showed that shapes of spectra and positions of maxima did not significantly change with concentration. The concentration dependence of absorption intensity for BCl-8 is very close to predicted by Bouguer's law, while for BCl-4 it is possible to observe some sublinearity at concentrations higher than 0.12 mM.

**Figure 1.** Absorption spectra of BCl-4 in saline solution for concentrations: 0.02 mM (1), 0.06 mM (2), 0.12 mM (3) and 0.2 mM (4).

**Figure 2.** Absorption spectra of BCl-8 in saline solution for concentrations: 0.02 mM (1), 0.06 mM (2), 0.12 mM (3) and 0.2 mM (4).
The fluorescence spectra of bacteriochlorins at different concentrations are presented in figures 3 and 4. As can be seen, the main spectral lines take place in the region of 770-780 nm. The comparison of the absorption and fluorescence spectra shows that they significantly overlap. Stokes shifts do not exceed a few nanometers and slightly increase with the concentration: 8.4-13.2 nm for BCl-4 and 9.1-15.3 nm for BCI-8.

**Figure 3.** Fluorescence spectra of BCI-4 in saline solution for concentrations: 0.02 mM (1), 0.06 mM (2), 0.12 mM (3) and 0.2 mM (4). Excitation wavelength is equal to 532 nm.

**Figure 4.** Fluorescence spectra of BCI-8 in saline solution for concentrations: 0.02 mM (1), 0.06 mM (2), 0.12 mM (3) and 0.2 mM (4). Excitation wavelength is equal to 532 nm.

The intensity of BCI-8 fluorescence at high concentrations (>0.1 mM) shows the sublinear growth (Fig.5), with its spectral maximum shifting to longer wavelengths, while the halfwidth of fluorescence band does not noticeably change. Together with linear concentration growth of absorption, this behaviour is closer to the case of re-absorption of fluorescence in concentrated solutions than behavior connected to aggregation of PS molecules [11]. On the other hand, in BCI-4 solutions the sublinearity of concentration dependence on fluorescence starts much earlier, at the concentrations above 0.05 mM, coming by the level of 0.14 mM to saturation. Combined with behaviour of absorption, this allows us to assume that BCI-4 does express some (not significant) aggregation of molecules at the
upper limit of the concentration range used in this study. In general, it can be concluded that the aggregation process in solutions of both bacteriochlorins is minimized due to the Coulomb repulsion, allowing us to expect high antibacterial photodynamic efficiency of both photosensitizers.

![Graph](image)

**Figure 5.** The dependences of fluorescence intensity on the bacteriochlorin concentration for BCI-8 (1) and BCI-4 (2)

The results of PDP investigation against gram-negative bacteria in the plankton state and in the biofilms are demonstrated in fig. 6 and 7. As can be seen in fig.6, the photodynamic inactivation is efficient even at photosensitizers dose of 0.005 mM. Under these conditions, irradiation up to the light dose of 8 J/cm² is sufficient for decrease of viability bacteria with BCI-4 and BCI-8 by 4 and 5 decimal orders respectively. Fig.7 shows that antibacterial photodynamic treatment with PS under study is also efficient against biofilms of *Pseudomonas aeruginosa*. The antibacterial efficiency of BCI-8 is higher than for BCI-4.

![Graph](image)

**Figure 6.** The dependences of CFU on the light dose for *Pseudomonas aeruginosa* plankton bacteria at 0.005 mM solutions using BCI-4 (1) and BCI-8 (2) photosensitizers.
Figure 7. The dependences of CFU on the concentration for *Pseudomonas aeruginosa* in the biofilms after irradiation (light dose 50 J/cm²) for BCI-4 (1) and BCI-8 (2) photosensitizers.

The photodamage of bacteria in biofilms after PDP was demonstrated by fluorescent microscopy using Live/Dead Biofilm Tracer, as shown on fig.8. Live/Dead Tracer includes two components: SYTO 9 traces the DNA with the green fluorescence in all bacteria of biofilm, while propidium iodide provides intense green fluorescent marking of DNA only in cells with damaged membranes. Overlay of images of red and green fluorescence images of cells stained with this techniques allows the undamaged bacteria (with green fluorescence) from the damaged ones (with red sum fluorescence) to be distinguished.

Figure 8. The microphotos of *Pseudomonas aeruginosa* biofilm before (left) and after (right) PDP.

4. Conclusion

It was shown that the photodynamic treatment with new synthetic cationic photosensitizers 5,10,15,20-Tetrakis [1-(4’-bromobutyl)-3-pyridyl] bacteriochlorin tetrabromide and 5,10,15,20-Tetrakis [1-(4’-bromobutyl)-3-pyridyl] bacteriochlorin octabromide with high cationic substitute number provides efficient inactivation of gram-negative *Pseudomonas aeruginosa* bacteria both in plankton and biofilm forms. The high
positive charge of molecules of these photosensitizers can realize the efficiency of the photodynamic antibacterial therapy.

References
[1] Gelfond M L 2007 Practical oncology 8 204 in Russian
[2] Chissov VI Sokolov VV and Filonenko EV 1998 Russian Chemical Journal XLII 5
[3] Jori G and Brown S 2004 Photochem. Photobiol. Sci. 3 403
[4] Hamblin MR and Hasan T 2004 Photochem. Photobiol. Sci. 3 436
[5] Malik Z Ladan H and Nitzan Y 1992 J. Photochem. Photobiol. 14 262
[6] Minnock A et al 1996 J. Photochem. Photobiol. 32 159
[7] Brusov SS et al 2014 Russian Journal of Biotherapy 4 59
[8] Meerovich GA et al 2013 Russian Journal of Biotherapy 3 45
[9] Ma L Moan J and Berg K 1994 Intern. Journ. of Cancer. 57 883
[10] Edrei R et al 1998 Journ. of Porphyrins and Phthalocyanines 2 191
[11] Dhami S et al 1995 Photochemistry and Photobiology 61 341
[12] Makarov DA et al 2009 Russian Journal of Physical Chemistry A 83 1044
[13] Donlan RM and Costerton JW 2002 Clin. Mic. Rev. 15 167
[14] Pace JL et al 2005 CRC journal 7 109
[15] Hassett DJ Sutton MD and Schurr MJ 2009 Trends Microbiol. 17 130
[16] Strakhovskaya MG et al 2009 Biochemistry 74 1305 in Russian
[17] Huang L et al 2010 Antimicrobial Agents and Chemotherapy 54 3834
[18] Schastak S et al 2010 PLOS ONE 5 1
[19] Schastak S et al 2008 Methods Find Exp Clin. 30 129
[20] Dudkin SV et al. 2013 Russian patent № 2476218
[21] Dudkin SV et al. 2013 Russian patent № 2479585