New cationic photosensitizers: photophysical properties and results of preliminary studies of antibacterial efficacy

E V Akhlyustina¹, G A Meerovich¹,², I G Tiganova³, E A Makarova⁴, N I Philipova¹, I D Romanishkin², N V Alekseeva³, E A Lukyanets⁴, Yu M Romanova³ and V B Loschenov¹,²

¹National Research Nuclear University MEPhI, Moscow, Russia
²Prokhorov General Physics Institute of the Russian Academy of Sciences, Moscow, Russia
³N.F.Gamaleya National Research Center of Epidemiology and Microbiology, Moscow, Russia
⁴Organic Intermediates and Dyes Institute, Moscow, Russia

E-mail: katya_ahlyustina@mail.ru

Abstract. Polycationic derivatives of synthetic bacteriochlorin with absorption in the near infrared range are promising for the creation of photosensitizers (PS) for antimicrobial photodynamic therapy. In the present work, the photophysical and antibacterial properties of PS based on tetracationic derivatives of synthetic bacteriochlorins: hydrophilic 3-Py₄BBr₄ in aqueous solution and amphiphilic 3-Py₄BC(OEt)₄Br₄ in the dispersion of Kolliphor ELP were studied. Analysis of absorption and fluorescence in a wide range of concentrations has demonstrated low aggregation of the PS over the entire range. A high efficiency of photodynamic inactivation of Gram-positive Staphylococcus aureus and Gram-negative Pseudomonas aeruginosa, Klebsiella pneumoniae and Acinetobacter baumannii bacteria was observed.

1. Introduction

Bacterial photodynamic inactivation (PDI) is a promising method of treatment of local infections, in particular, infected surgical and burn wounds, trophic and diabetic ulcers [1, 2]. Antimicrobial photodynamic therapy (APDT) is able to effectively destroy bacterial cells without developing resistance in response to treatment [3-6]. Almost all pathogenic microorganisms are responsive to APDT, including antibiotic resistant strains of bacteria [7].

In most local infected foci, in particular infected wounds, Gram-positive Staphylococcus aureus (S. aureus) and Gram-negative Pseudomonas aeruginosa (P. aeruginosa), Klebsiella pneumoniae (K. pneumoniae) and Acinetobacter baumannii (A. baumannii) bacteria were found [8].

Gram-positive and Gram-negative bacteria have fundamental differences in their structure and sensitivity to medical drugs. The cell wall of Gram-positive bacteria is not a permeability barrier for most photosensitizers (PS), the molecular mass of which usually does not exceed 1800 Da. The cell wall of Gram-negative bacteria has an additional outer membrane 10-15 nm in thickness [9]. It is external to the peptidoglycan network and has a very heterogeneous composition (proteins with a
porin function, lipopolysaccharide trimers and lipoproteins that create an external pseudosurface of tightly packed negative charges) [10, 11]. Such a highly organized system prevents the penetration of large molecules. Its regulatory properties trigger resistance mechanisms to many antibiotics. Only hydrophilic compounds with a molecular mass below 700 Da diffuse through the porin channels.

It is known that only cationic PSs interact efficiently with Gram-negative bacteria, while having high efficacy against Gram-positive bacteria. An additional benefit of cationic PS is the possibility of using highly concentrated aqueous compositions for sensitization. The aggregation of photosensitizers decreases the efficiency of photodynamic processes. Cationic PS can partially reduce their aggregation due to Coulomb repulsion of molecules [12, 13].

**Figure 1.** Absorption spectra: a) tissue; b) extract of *P. aeruginosa* bacteria culture liquid; c) BcH-4G (1) and BcH-4A (2) solution, normalized on maximum.
It should be borne in mind that the foci depth of infectious lesions can reach 12-15 mm for P. aeruginosa [14]. Therefore, for the proper photodynamic effect on such foci, it is necessary to use the APDT PS, excitation of which is carried out in the spectral range of the "biological tissue transparency window" (720-850 nm) (Figure 1a). Moreover, in the course of their life, P. aeruginosa bacteria produce in a detachable wound a number of pigments (in particular, pyocyanin, pyoverdin, pyorubrin, pyomelanin [15]). As a result, the detachable wound can have a strong absorption in the band with a spectral maximum of about 700 nm (Figure 1b). So, APDT with PS excited in red spectral range will be ineffective due to absorption. For APDT it is necessary to use PS excited in spectral range with wavelengths > 755 nm.

This work is dedicated to investigation of APDT effectiveness using the photosensitizers based on polycationic synthetic bacteriochlorins with wavelengths in a range of 759-763 nm, reduced size of the molecule and molecular weight.

2. Materials and methods

Tetracationic derivatives of bacteriochlorin: (meso-tetrakis[1-(2'-bromoethyl)-3-pyridil]-bacteriochlorin tetrabromide (3-Py3BcH(EtBr)2Br4) (hereinafter BcH-4G) [16] and meso-tetrakis(1-heptyl-3-pyridil)bacteriochlorin tetrabromide (3-Py7BcHBr4) (hereinafter BcH-4A) - were synthesized by alkylation of meso-tetra(3-pyridil)bacteriochlorin [17] with 1,2-dibromoethane or heptyl bromide, respectively, in nitromethane in an inert atmosphere for 2 hours. Studies of the hydrophilic substance BcH-4G were carried out in aqueous solutions, amphiphilic substance BcH-4A was solubilized in 4 % dispersion of Kolliphor ELP (BASF).

The PS absorption in a concentration range from 0.1 mM to 0.001 mM was studied using a two-beam spectrophotometer "Hitachi U-3410" (Japan), and spectral-fluorescence studies were performed using a spectrum analyzer LESA-01."Biospec" (BIOSPEC Russia). Fluorescence was excited by laser radiation at the wavelength of 532 nm matching the Q2-band of bacteriochlorin. To analyze changes in the shape of the fluorescence spectral band of the PS, the studies were performed in the cuvettes of various lengths (1 mm and 10 mm). For the convenience of the analysis, the fluorescence spectrum was normalized to the maximum intensity of fluorescence signal.

For the evaluation of PS fluorescence lifetime the Hamamatsu streak-camera with a time-resolution was used [18]. It comprised a pulsed laser source with fiber optic output of the Picosecond Light Pulser PLP-10 (Japan), generating pulsed laser radiation at the wavelength of 637 nm with a pulse duration of 65 ps, and the polychromator with fiber optic input and Semrock optical filter LD 01-785/10-12.5 inlet that transmits only the spectral region of fluorescence band derived bacteriochlorins and minimized the impact of third-party flashes. The image of the output slit of the polychromator was projected on the photocathode of the streak-camera, which included Streak scope C10627-13 (Hamamatsu, Japan) with an adjustable time scan from 1 ns to 10 ms in the direction perpendicular to the image of the slit. The image from the output screen of Streak scope was received by the input of Digital CCD Camera C9300-508. The synchronous delay generator C10647-01 with a repetition rate of up to 1.6 x 107 Hz, synchronized the launch of the laser source and a Streak scope, allowing registering a fluorescence signal with spectral and temporal decomposition. The time-correlated counting method of single photons was used in the measurement process. The signal was recorded on a 5 ns time scan. The received signal was approximated by the sum of several exponents. The number of exponents was chosen based on the quality of the approximation, which was determined by Pearson's criterion of agreement.

The photoinactivation of planktonic bacteria by each of the photosensitizers was carried out on clinical isolates S. aureus15, P. aeruginosa32, K. pneumonia1556, A. baumannii. Bacteria were cultured in LB-broth and 1% LB-agar (Difco, USA). The Minimal Bactericidal Concentration (MBC) of PS was estimated for standard conditions: incubation of bacteria with PS for 30 min, the dose of light 20 J/cm2. The initial titer of bacteria was 1x10^8 CFU/ml (Colony Forming Units per ml). Two-fold serial dilutions of PS were used, starting from 1 mM. After incubation the bacterial suspensions were centrifuged, and bacteria were resuspended in saline, aliquots of 100 µl from all samples and
control (without PS) were placed in two 96-well flat-bottom plates, one of them was irradiated. The other served as an unirradiated (“dark”) control. The SFD-M-760 LED with the emission maximum of 760 nm was used for samples irradiation. The power density was 22-25 mW/cm² and the duration of irradiation was 20 min. "Coherent Labmax" meter was used to control the power density. After irradiation, 50 µl from each well was plated on Petri dishes with LB agar and incubated in the dark at 37 °C for 20 h. The lowest concentration of PS, which did not give growth of colonies of bacteria, was taken for the Minimal Bactericidal Concentration (MBC) [19].

3. Results and discussion
3.1. Absorption spectra
Absorption spectra of BcH-4G and BcH-4A have a narrow absorption band (spectral half-width of about 22 nm) with a maximum of about 760 nm (Figure 1c). Studies in a wide range of concentrations (0.001-0.2 mM) showed that signs of aggregation in the PS’s absorption spectra are not expressed as opposed to polycationic phthalocyanines [20]. The shape of the absorption spectrum does not change with increasing concentration. The dependence of the optical density on the molar concentration is linear. It agrees with extinction values determined at low concentrations (Bouguer’s law holds). The results of the of the PS’s absorption suggest that the degree of their aggregation at high concentrations is low.

3.2. Results of spectral fluorescence studies
The shapes of the fluorescence spectra of BcH-4G and BcH-4A at various concentrations in cuvettes of various lengths are shown in Figure 2.

![Normalized fluorescence spectra of BcH-4G (a) and BcH-4A (b) at various concentrations](image)

**Figure 2.** The normalized fluorescence spectra of BcH-4G (a) and BcH-4A (b) at various concentrations (1, 2 – 0.005 mM, 3, 4 – 0.05 mM) in cuvettes of various lengths (1, 3 – 1 mm; 2, 4 – 10 mm).

Analysis of the spectra of BcH-4G and BcH-4A shows that an increase of cuvettes’ length from 1 to 10 mm at low (0.005 mM) values of the PS concentration does not affect the spectral shape (Figure 2, spectra 1, 2). This leads only to a slight (0.3-0.4 nm) shift of the spectral maximum due to reabsorption. The fluorescence band remains narrow (27 nm, respectively). At high (0.05 mM) concentrations of BcH-4G (approximately corresponding to the PS concentration in blood plasma
1 hour after intravenous administration), a long-wavelength shift of the spectral maximum occurs due to reabsorption. The shift depends on the length of the cuvette (by 2.2 nm - in a 1 mm cuvette and by 5.6 nm in a 10 mm cuvette). The half-width of the fluorescence band of BcH-4G increases at this concentration too: in the 1 mm cuvette - by 3.3 nm, and in the 10 mm cuvette - by 6.9 nm. However, the shape of the spectra remains approximately the same. This suggests that the observed phenomena at a high concentration of BcH-4G are mainly associated with reabsorption and weak aggregation.

The observed changes for BcH-4A are lesser than for BcH-4G. A long-wavelength shift of the spectral maximum of the fluorescence band occurs due to reabsorption at high concentrations (0.05 mM). The displacement depends on the length of cuvetttes (by 1.5 nm in a 1 mm cuvette and by 3.4 nm in a 10 mm cuvette). The half-width of the fluorescence band in the 1 mm cuvette increases by 1.1 nm, and in the 10 mm cuvette - by 4.3 nm. This increase is much lesser than for BcH-4G. This suggests that the observed changes occurring in BcH-4A at a high concentration are mainly associated with reabsorption, and aggregation also takes place but with a significantly smaller contribution [21].

Evaluation of the excited-state lifetime of BcH-4G in water and blood serum shows the presence of two components. In water, the component with a lifetime of 1.9 ns is dominant. Its share is estimated to be 73-75% of the total number of fluorescent photons. In serum, the lifetime increases to 2.4 ns; the share of the dominant component is higher (about 80-85% in the range below 0.1 mM). In studies of BcH-4A fluorescence in water, the component with an average lifetime of 2.8 ns is dominant; its share is approximately 86%. In serum, the dominant component with an average lifetime of about 2.9 ns is almost 100%.

The results of study of BcH-4G and BcH-4A aqueous solutions fluorescence intensity dependence on their concentration in water and serum are shown in Figure 3 (a, b).

![Figure 3](image_url)

**Figure 3.** Dependence of fluorescence intensity of BcH-4G(a) and BcH-4A(b) solutions on their concentration: 1 – in water; 2 – in serum.

The dependence of the fluorescence intensity of the BcH-4A solution on the concentration of PS is close to linear up to 0.03 mM in blood serum. At higher concentration it becomes sublinear. The same tendency is observed for the dispersion of BcH-4A in aqueous solution. The fluorescence intensity in water is approximately 1.3-1.4 times lower than the fluorescence intensity in serum.

The dependence of the fluorescence intensity of the BcH-4G solution in serum on PS concentration is also close to linear up to 0.03 mM. At a higher concentration it becomes sublinear. However, for the BcH-4G solution in water, sublinearity begins to appear even in the concentration
range 0.01-0.03 mM. The fluorescence intensity of BcH-4G in water is 1.5-2 times lower than the fluorescence intensity in serum.

Analysis of the BcH-4A and BcH-4G fluorescence in the concentration range confirms the assumption of a low degree of their aggregation at high concentrations, especially in serum [22]. This makes it possible to use these PS at high concentrations to sensitize pathological foci for APDT.

### 3.3. Antibacterial effectiveness of BcH-4A and BcH-4G

The results of the estimation of MBC value of BcH-4A and BcH-4G under standard conditions (time of incubation of bacteria with PS is 30 min, dose density is 25 J/cm²) are shown in Table 1.

#### Table 1. MBC of BcH-4A and BcH-4G for plankton bacteria, μM.

| Photosensitizer | Minimum bactericidal concentration for plankton bacteria, μM |
|-----------------|----------------------------------------------------------|
|                 | S. aureus | P. aeruginosa | K. pneumoniae | A. Baumannii |
| BcH-4A          | 0.2       | 6.2           | 3.1           |             |
| BcH-4G          | 1.6       | 3.1           | 6.2           | 6.2         |

BcH-4A and BcH-4G had significantly lower MBC for planktonic Gram-positive *S. aureus* bacteria and Gram-negative bacteria *P. aeruginosa* than PS based on cationic bacteriochlorins described previously [12,19]. Values of MBC for *K. pneumoniae* and *A. baumannii* are low too (close for value of MBC for *P. aeruginosa*).

### 4. Conclusion

The results of the research show that the investigated tetracationic photosensitizers based on the derivatives of synthetic bacteriochlorins with reduced molecular size and molecular weight have a high efficiency of photodynamic inactivation of Gram-positive bacteria *S. aureus* and Gram-negative bacteria *P. aeruginosa*, *A. baumannii* and *K. pneumoniae*. Studies of photophysical properties of PSs in a wide range of concentrations demonstrated a low aggregation of these derivatives in water and serum. The results of the studies allow us to conclude that the PSs are promising for photodynamic treatment of local infected foci caused by Gram-positive and Gram-negative bacteria.

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