NANOSTRUCTURED PHOTOSENSITIZER BASED ON A TETRACATIONIC DERIVATIVE OF BACTERIOCHLORIN FOR ANTIBACTERIAL PHOTODYNAMIC THERAPY

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Making antibacterial PDT more effective is a task that calls for the development of photosensitizers (PS) based on polycationic synthetic bacteriochlorins and subsequent analysis of properties of such photosensitizers. This study aimed to explore photophysical and antibacterial properties of the nanostructured PS based on 3-$Py_4BSHp_4Br_4$, tetracationic amphiphilic derivative of synthetic bacteriochlorin. The PS was solubilized in a 4% Kolliphor ELP to obtain its nanostructured dispersion. We researched the absorption and fluorescence spectra intensity and profiles, studying concentrations from 0.001 to 0.2 mM, and found that the aggregation level of the PS in question is low throughout the range investigated while the *S. aureus* (gram-positive) and *P. aeruginosa* and *K. pneumoniae* (gram-negative) PD inactivation effectiveness is high.

Keywords: photosensitizer, cationic bacteriochlorin, aggregation, nanostructured dispersion, fluorescence, antibacterial photodynamic therapy

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Received: 31.08.2018 **Accepted:** 27.09.2018

DOI: 10.24075/brsmu.2018.087

НАНОСТРУКТУРИРОВАННЫЙ ФОТОСЕНСИБИЛИЗАТОР НА ОСНОВЕ ТЕТРАКАТИОННОГО ПРОИЗВОДНОГО БАКТЕРИОХЛОРИНА ДЛЯ АНТИБАКТЕРИАЛЬНОЙ ФОТОДИНАМИЧЕСКОЙ ТЕРАПИИ

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Задача повышения эффективности антибактериальной ФДТ делает актуальными создание и исследование фотосенсибилизаторов (ФС) на основе поликатионных синтетических бактериохлоринов. Целью работы было изучить в широком диапазоне концентраций фотофизические и антибактериальные свойства наноструктурированного ФС на основе тетракатионного амфифильного производного синтетического бактериохлорина 3-$Py_4BCHp_4Br_4$. Наноструктурированную дисперсию ФС получили путем его солюбилизации в 4%-м Kolliphor ELP. Исследование интенсивности и формы спектров поглощения и флуоресценции в диапазоне концентраций от 0,001 до 0,2 мМ продемонстрировало низкую агрегацию этого ФС во всем диапазоне и высокую эффективность фотодинамической инактивации грамположительных бактерий *S. aureus* и грамотрицательных бактерий *P. aeruginosa* и *K. pneumoniae*.

Ключевые слова: фотосенсибилизатор, катионный бактериохлорин, агрегация, наноструктурированная дисперсия, флуоресценция, антибактериальная фотодинамическая терапия

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Статья получена: 31.08.2018 **Статья принята к печати:** 27.09.2018

DOI: 10.24075/vrgmu.2018.087

Antibacterial photodynamic therapy (APDT) is a promising mode of treatment of localized infection sites: surgical and burn wounds, trophic and diabetic ulcers, etc [1, 2]. Unlike antibiotic therapy, APDT destroys cells of bacteria without promoting their resistance to the treatment [3–6]. Most pathogens, including antibiotic-resistant strains of bacteria, are susceptible to APDT [7].

Localized infection sites, infected wounds in particular, most often contain *Staphylococcus aureus* (*S. aureus*) Gram-positive bacteria, *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Klebsiella pneumoniae* (*K. pneumoniae*) Gram-negative bacteria, the strains of which may be resistant to many types of antibiotics, cause chronic infections and various dangerous consequences for the patients [8].

Gram-positive and Gram-negative bacteria are fundamentally different in their structure and sensitivity to drug exposure. The cell wall of Gram-positive bacteria is just a minor obstacle for most photosensitizers (PS). In Gram-negative bacteria, it

has an additional structural element, a 10–15 nm thick outer membrane, which is heterogeneous and consists of porin proteins, lipopolysaccharide trimers and lipoproteins that build an external pseudo-surface of densely packed negative charges [9–11]. Such a system prevents the humoral protective factors from penetrating and enables resistance to many drugs: only relatively hydrophilic compounds with a molecular weight below 700 Da diffuse through the porin channels, and as the size and weight of the molecules grow, the probability of their diffusion into Gram-negative bacteria decreases. Only cationic PSs effectively interact with Gram-negative bacteria [10, 11]. Cationic PS have another advantage: their highly concentrated aqueous compositions (solutions or nanodispersions) can be used for sensitization, since the Coulomb repulsion of molecules of cationic bacteriochlorins negatively affects their aggregation [12] and thus improves the efficacy of the photodynamic processes.

While selecting PS for APDT, it is necessary to take in account that some bacteria, e.g., *P. aeruginosa*, can infect the tissue up to the depth of 12–15 mm [13], which renders conventional antibacterial agents (solutions, ointments, gels) and PSs phototoxic only when excited with visible range light ineffective. Therefore, proper photodynamic treatment of such infected sites requires PSs excited by the light in near infrared spectral range, at the wavelengths of 720–850 nm, which cover the "biological tissue transparency window". In this connection, derivatives of cationic bacteriochlorins are being actively investigated for their potential to be PS in APDT. A number of studies are dedicated to researching the properties of polycationic derivatives of synthetic bacteriochlorins with a molecular weight of 1500–1800 Da; according to their results, such PSs are capable of inactivating both Gram-positive *S. aureus* bacteria and Gram-negative *P. aeruginosa* bacteria, but the minimum bactericidal concentrations of such photosensitizers are quite high (> 6 μM for *S. aureus* and about 25 μM for *P. aeruginosa*) [14].

Increasing the efficacy of APDT is a problem that requires development of PSs based on polycationic synthetic bacteriochlorins having smaller molecular size and mass. This study is aimed at exploring photophysical and antibacterial properties of the nanostructured PSs based on 3-$Py_4BCHp_4Br_4$, tetracationic amphiphilic derivative of synthetic bacteriochlorin meso-tetra-(1 heptyl-3-pyridyl)-bacteriochlorin tetrabromide.

METHODS

Compared to a derivative described in an earlier study [14], tetracathionic amphiphilic derivative of synthetic bacteriochlorin meso-tetra-(1-heptyl-3-pyridyl)-bacteriochlorin tetrabromide 3-$Py_4BCHp_4Br_4$, is less lipophilic and has the molecule of a smaller radius. The derivative was synthesized by meso-tetra-(3-pyridyl)-bacteriochlorin alkylation with heptyl bromide in nitromethane in an inert atmosphere. The nanostructured dispersion of 3-$Py_4BCHp_4Br_4$ was obtained through its solubilization in 4% Kolliphor ELP (BASF; Germany). Measurements taken with Zetasizer Nano Series ZS 3600 (Malvern Panalitical; UK) put the hydrodynamic size of nanoparticles within 12–14 nm.

We used Hitachi U-3410 dual-beam spectrophotometer (Hitachi; Japan) to study PS absorption in the concentration range of 0.001–0.1 mM and LESA-01-Biospec spectrum analyzer (BIOSPEC; Russia) to study the spectral-fluorescent properties. 532 nm laser was used to excite the fluorescence; this wavelength belongs to the bacteriochlorin derivative's Q2 band. To study the features of the spectral band shape we investigated spectral-fluorescent properties of the PS in cuvettes of various lengths (1 mm and 10 mm) and normalized its fluorescence spectral intensity to the fluorescence band maximum (reduced spectral maximum to 1). Thus, when analyzing the spectra, we could separate changes associated with reabsorption from those resulting from aggregation.

To measure the luminescence lifetime of aqueous compositions of the studied PSs, we used a time-resolved spectrometer. The spectrometer consisted of Picosecond Light Pulser PLP-10 (Hamamatsu; Japan), a fiber output pulse laser emitting 65 ps pulses at 637 nm; Jarrell-Ash fiber input polychromator (Division of Fisher Co; USA); Semrock LD01-785/10-12.5 optical filter (Semrock Inc; USA) at the input, which filtered out light outside of the bacteriochlorin derivatives luminescence band and minimized background noise. The resulting signal was approximated by the sum of several exponentials.

We used *S. aureus* 15, *P. aeruginosa* 32, *K. pneumoniae* 1556 clinical isolates to study the process of photoinactivation of planktonic bacteria. The bacteria were grown in LB nutrient broth or on 1% LB agar (Difco; USA). To determine the minimal bactericidal concentration (MBC) of PS applicable to plankton cultures, we incubated them with PS for 30 minutes and irradiated at the light energy density of 20 J/cm^2 (standard conditions). The initial titer of bacteria was $1 \cdot 10^8$ CFU/ml (Colony Forming Units per ml). The PS dilution pattern was double, starting at 1 mM. After incubation, the bacterial suspension was centrifuged for 5 min at 7000 rpm in the Eppendorf laboratory centrifuge (Eppendorf; Germany), PS removed, bacteria resuspended in saline; then the suspensions of each concentration, as well as the PS-free control samples, were poured into wells of the two 96-well flat-bottomed plates, 100 μl in each well. One plate was used for the irradiation experiment, the other served as a dark control.

SFD-M-760 LED source (ANO MIKEL; Russia) emitting at 760 nm (wavelength at maximum) and 35 nm FWHM was used for irradiation purposes. The power density was 22–25 mW/cm^2; irradiation lasted for 20 minutes. To control the power density, we used Coherent labmax (Coherent; USA) diaphragm laser power meter.

After irradiation, 50 μl of suspension from each well were added to LB agar Petri dishes, then incubated in the dark at 37 °C for 20 h. Examining the dishes, we looked for the samples that gave no growth, registered the corresponding PS concentrations and took the smallest of them as MBC.

RESULTS

By studying the dependence of 3-$Py_4BCHp_4Br_4$ absorption on its concentration in the nanodispersion, we aimed to evaluate the severity of the aggregation process. The operating absorption band of 3-$Py_4BCHp_4Br_4$ has a narrow spectral contour; its FWHM is about 22 nm, maximum at approximately 760 nm. According to the research, in contrast to polycationic phthalocyanines, there are no expressed signs of aggregation in the absorption spectra of the 3-$Py_4BCHp_4Br_4$ dispersion [15]: the shape of the absorption spectrum does not change as the concentration grows; the dependence of optical density on molar concentration is linear (Bouguer law satisfied) and consistent with the extinction values determined at low concentrations (Fig. 1).

To confirm the assumption that the studied PS shows low aggregation capabilities, we studied the spectral-fluorescent properties of its nanodispersion, focusing on the shape and intensity of fluorescence spectra, as well as radiative lifetime of the excited 3-$Py_4BCHp_4Br_4$ at high and low concentrations.

The analysis of the PS fluorescence spectra shows that increasing the cuvette length from 1 to 10 mm at low (0.005 mM) concentrations does not affect the shape of the spectral contour (Fig. 2, spectra 1, 2) and leads only to an insignificant (0.3 nm) shift of the spectrum maximum due to reabsorption. The fluorescence band remains narrow (27 nm).

At high (0.05 mM) concentrations that approximately correspond to the PS concentrations in blood plasma 1 hour after intravenous administration, reabsorption causes a long wavelength shift of the fluorescence band spectrum maximum that depends on the length of the cuvette: 1.5 nm in the cuvette 1 mm long, 3.4 nm — in a cuvette 10 mm long. The half-width of the fluorescence band also grows (by 1.1 nm in a 1 mm cuvette, by 4.3 nm in a 10 mm cuvette), but the shape of the spectral contour does not change, no additional bathochromically and hypsochromically shifted peaks appear there.

Studying the radiative lifetime with the help of the approach described earlier [16], we discovered two components. In water-based experiments, the dominant component has the average lifetime of 2.8 ns; its share is approximately 86%. In

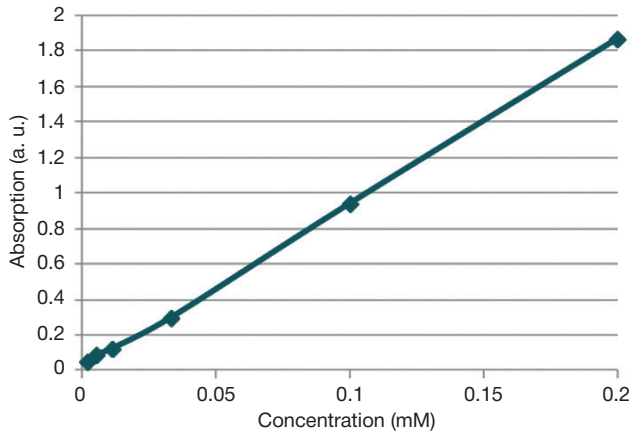

Fig. 1. Dependence of 4% Kolliphor ELP 3-$Py_4BCHp_4Br_4$ dispersion absorption on its concentration

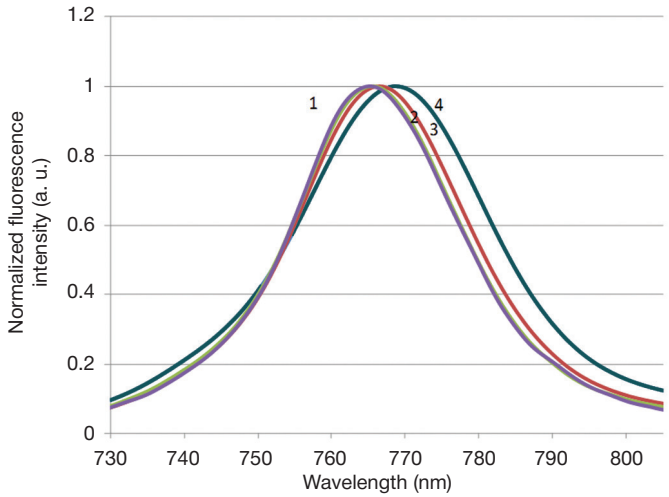

Fig. 2. Normalized fluorescence spectra of 3-$Py_4BCHp_4Br_4$ dispersions, various concentrations (spectra 1, 2 — 0.005 mM; spectra 3, 4 — 0.05 mM) and lengths of cuvettes (spectra 1, 3 — 1 mm; spectra 2, 4 — 10 mm)

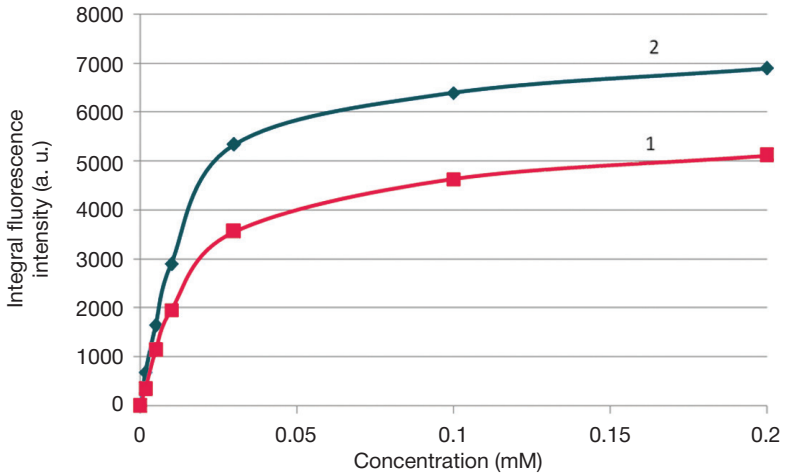

Fig. 3. Dependence of integral fluorescence intensity of 3-$Py_4BCHp_4Br_4$ aqueous compositions on their molar concentration: 1 — in water; 2 — in blood plasma

experiments with blood plasma, where aggregation is reduced, the dominant component has the average lifetime of about 2.9 ns, and its share is almost 100%.

The dependence of dispersion's integral fluorescence intensity on PS concentration is close to being linear up to 0.03 mM (Fig. 3); at higher concentrations, it becomes sublinear. The dependence pattern is the same for the 3-$Py_4BCHp_4Br_4$ composition in blood plasma. Furthermore, the shape of the curves remains almost unchanged, although the fluorescence intensity in blood plasma is 1.3–1.4 times higher than that in water.

Table below contains the discovered MBCs of 3-$Py_4BCHp_4Br_4$ at standard conditions.

DISCUSSION

Investigating absorption of the studied PS, we found that its aggregation values are low in the considered range of concentrations [15], shape and half-width of the absorption band spectrum therein do not change, and the absorption itself linearly depends on the concentration.

Analysis of the fluorescence band shape changes associated with increased concentrations and cuvette lengths allows an assumption that the main reason behind the phenomenon observed at higher concentrations of the researched PS is reabsorption, and contribution of aggregation, which also occurs, is insignificant. This is also backed by the investigation of radiative lifetime of the excited PS based on 3-$Py_4BCHp_4Br_4$ and dependence of the fluorescence intensity on concentration of 3-$Py_4BCHp_4Br_4$ in dispersion, especially in blood plasma [17–21].

These data lead to a conclusion that the efficacy of photodynamic processes at high concentrations of 3-$Py_4BCHp_4Br_4$ will not deteriorate, which allows using 3-$Py_4BCHp_4Br_4$ nanodispersions of such concentrations as sensitizers in APDT.

Compared to the previously described PSs based on cationic bacteriochlorins [14], 3-$Py_4BCHp_4Br_4$ offers significantly lower MBC values for Gram-positive *S. aureus* bacteria and Gram-negative *P. aeruginosa* bacteria in plankton state. The MBC for Gram-negative *K. pneumoniae* bacteria are also low.

CONCLUSIONS

The results of the research show that tetracationic PS based on the synthetic amphiphilic derivative of 3-$Py_4BCHp_4Br_4$ bacteriochlorin, the molecular size and weight of which are smaller, can photodynamically inactivate Gram-positive *S. aureus*, Gram-negative *P. aeruginosa* and *K. pneumoniae* bacteria. Investigation of photophysical properties of the PS in a wide range of concentrations revealed its low aggregation capability in water and blood plasma. The studies conducted allow a conclusion that the PS based on a nanostructured 3-$Py_4BCHp_4Br_4$ is promising as a component of protocols of photodynamic treatment of localized infections by Gram-positive and Gram-negative bacteria.

Table. 3-$Py_4BCHp_4Br_4$ MBC values, standard conditions (incubation time 0.5 h, exposure dose 20 J/cm^2)

Bacteria	*S. aureus*	*P. aeruginosa*	*K. pneumoniae*
MBC, μM	0.2	6.2	3.1

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