ORIGINAL ARTICLES

IN VITRO PHOTODYNAMIC EFFICACY OF POLYCATIONIC PHOTOSENSITIZERS BASED ON LONGWAVE PHTHALOCYANINES

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Abstract

Polycationic photosensitizers have previously demonstrated high *in vitro* efficacy against lung cancer cells, including cancer stem cells, and low dark cytotoxicity. Polycationic phthalocyanines have high quantum yield of singlet oxygen and photostability. In addition, it is possible to relatively simply introduce different metal-complexing agents and substituents into phthalocyanine macrocycles, which enables varying their photophysical characteristics. In this work, we studied photophysical properties of photosensitizers based on polycationic phthalocyanine derivatives with different chemical structure with strong absorption in the long wavelength region (680–690 nm). The studied photosensitizers exhibit negligible aggregation in the 1–100 μM concentration range and show very high phototoxicity in an *in vitro* study on A549 lung carcinoma cells (IC_{50} of 60–100 nM for $ZnPcChol_8$ and 100–300 nM for $4\alpha ZnPc^{4+}$ and $4\alpha\beta ZnPc^{4+}$, depending on the light dose), and low dark cytotoxicity.

Keywords: polycationic photosensitizer, A549 lung carcinoma, photodynamic therapy

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For citations: Romanishkin I.D., Meerovich I.G., Bunin D.A., Skobeltsin A.S., Akhlyustina E.V., Levkin V.V., Kharnas S.S., Kogan E.A., Zhi-Long Chen, Meerovich G.A., Gorbunova Yu.G., Reshetov I.V. *In vitro* photodynamic efficacy of polycationic photosensitizers based on polycationic phthalocyanines, *Biomedical Photonics*, 2025, vol. 14, no. 3, pp. 24–29. doi: 10.24931/2413–9432–2025–14–3–24–29

ФОТОДИНАМИЧЕСКАЯ ЭФФЕКТИВНОСТЬ *IN VITRO* ПОЛИКАТИОННЫХ ФОТОСЕНСИБИЛИЗАТОРОВ НА ОСНОВЕ ДЛИННОВОЛНОВЫХ ФТАЛОЦИАНИНОВ

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Резюме

Поликатионные фотосенсибилизаторы ранее продемонстрировали высокую эффективность *in vitro* против клеток рака лёгкого, в том числе против раковых стволовых клеток, при низкой темновой цитотоксичности. Поликатионные фталоцианины имеют высокий квантовый выход фотогенерации синглетного кислорода и фотостабильность. Кроме этого, возможно относительно простое введение различных металлов-комплексообразователей и заместителей во фталоцианиновые макроциклы, что дает возможность варьирования фотофизических характеристик. В данной работе мы изучали фотофизические свойства фотосенсибилизаторов на основе поликатионных производных фталоцианинов с различной химической структурой, обладающих интенсивным поглощением в длинноволновой области (680–690 нм). Исследованные фотосенсибилизаторы проявляют незначительную агрегацию в диапазоне концентраций 1–100 мкМ и демонстрируют очень высокую фототоксичность в исследовании *in vitro* на клетках карциномы лёгкого A549 (IC_{50} 60–100 нМ для $ZnPcChol_8$ и 100–300 нМ для $4\alpha ZnPc^{4+}$ и $4\alpha\beta ZnPc4^{+}$ в зависимости от дозы света), а также низкую темновую цитотоксичность.

Ключевые слова: поликатионный фотосенсибилизатор, карцинома лёгкого A549, фотодинамическая терапия.

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Для цитирования: Романишкин И.Д., Меерович И.Г., Бунин Д.А., Скобельцин А.С., Ахлюстина Е.В., Левкин В.В, Харнас С.С., Коган Е.А., Chen Z.-L., Меерович Г.А., Горбунова Ю.Г., Решетов И.В. Фотодинамическая эффективность *in vitro* поликатионных фотосенсибилизаторов на основе длинноволновых фталоцианинов // Biomedical Photonics. – 2025. – Т. 14, № 3. – С. 24–29. doi: 10.24931/2413–9432–2025–14–3–24–29

Introduction

Photosensitizers (PSs) used in clinical practice for photodynamic therapy (PDT) of tumors usually are anionic or electroneutral compounds; monocationic phenothiazines, in particular, Methylene Blue, are used mainly for antibacterial PDT (for photodynamic treatment of local pathological foci with bacterial lesions) [1–4]. Some works, in particular, [5–7], discuss the influence of charge sign of PS molecules in biological fluids, which are aqueous compositions, on its antitumor efficacy. It was discovered that polycationic chlorin conjugates had a significantly higher photodynamic efficacy against ovarian cancer cells compared to polyanionic ones [7].

Recently, a number of polycationic photosensitizers based on heterocyclic compounds with high antitumor efficacy have been developed [8–16].

The aim of the present work is to comparatively investigate the photophysical properties and phototoxicity of long-wavelength photosensitizers based on polycationic phthalocyanines with different structure and charge.

Materials and methods

Long-wavelength polycationic phthalocyanines with excitation in the spectral range of 680–690 nm with different composition and/or structure, were used as PSs (Fig.1):

- zinc octakis(cholinyl)phthalocyanine ($ZnPcChol_8$), synthesized at the Institute of Organic Intermediates and Dyes (Russia) [11].
- having the same composition but different structure, symmetric $4\alpha ZnPc^{4+}$ – tetraiodide 1,8(11),15(18),22(25)-tetrakis(4-((diethylmethylammonium)methyl)phenoxy) zinc phthalocyaninate and asymmetric $4\alpha\beta ZnPc^{4+}$ – tetraiodide 1,2,3,4-tetrakis(4-((diethylmethylammonium)methyl)-phenoxy)phenoxy) zinc phthalocyaninate, synthesized at Frumkin Institute of Physical Chemistry and Electrochemistry [14, 15].

$ZnPcChol_8$ is well soluble in both water and phosphate buffer saline (PBS). Since $4\alpha ZnPc^{4+}$ and $4\alpha\beta ZnPc^{4+}$ have low water solubility, dimethyl sulfoxide (DMSO) was used as their solvent with consideration that DMSO content should not exceed 1% in solutions for intravenous administration. Therefore, PS solutions in PBS containing no more than 1% of DMSO were used in the experiments. PS solutions with concentrations of 1, 3, 10, 30, 100 μM were prepared, as well as samples where inactivated fetal bovine serum (FBS) was used as the solvent and added to 1 mM PBS solution to obtain similar PS concentrations.

Comparative PS studies were performed on solutions of both $4\alpha ZnPc^{4+}$ and $4\alpha\beta ZnPc^{4+}$ pre-dissolved in DMSO, and $ZnPcChol_8$ in PBS and in its composition with FBS.

Absorption characteristics were investigated in a Hitachi-3410 dual-beam spectrophotometer (Hitachi, Japan) [17].

The shape of spectra and fluorescence intensity of polycationic phthalocyanines were studied using LESA-01-BIOSPEC spectrometer (Biospec, Moscow) with an extended dynamic range of the recorded signal [18] under excitation by a 633 nm laser. The data were analyzed with UnoMomento software (Biospec, Russia). To estimate the fluorescence lifetime, the PSs were excited by picosecond laser pulses with a wavelength of 637 nm, and the fluorescence was recorded using a spectroscopic complex with a streak camera (Hamamatsu, Japan). The fluorescence decay curves of the PSs were analyzed using Maximum Entropy Method to obtain lifetime values of individual fluorescence components [19].

To investigate the effect of PS composition on the survival of A549 human lung cancer cells, the cells were cultured in DMEM medium (PanEco, Russia) containing 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin supplemented with 10% fetal calf serum (Capricorn, Germany) at 37°C and 5% CO_2. To study cytotoxicity, cells were seeded into 96-well plates at 8000 cells per 100 μl of growth medium. After 24 h, the growth medium was replaced with the one containing the studied phthalocyanines in chosen concentrations ranging from 10^{-4} to 10^{-8} M. Cells were incubated with PS for 3 hours, after which they were washed from PS twice with growth medium. The irradiation of cell lines was carried out immediately after washing the cells, using light source with multiple LEDs [20]. After that, the plate was placed for a day in an incubator with a temperature of 37°C and 5% CO_2 content. Cell plates without irradiation (dark mode) after washing were left for 24 h in incubator at 37°C and 5% CO_2. Cell survival after photodynamic exposure with the studied PS was determined using the MTT test. Optical density of the stained solution was evaluated at 540 nm in multimodal plate spectrophotometer Clariostar plus (BMG, Germany). Statistical data processing was performed using GraphPad software.

Results and discussion

The absorbance dependence of $ZnPcChol_8$, $4\alpha ZnPc^{4+}$ and $4\alpha\beta ZnPc^{4+}$ solutions in PBS is linear up to 100 μM (Fig. 2), the shape and full width at half-maximum at high and low concentrations show no notable differences. The values of molar absorption coefficients maxima for these PSs are presented in Table 1.

Studies of PS solutions showed high fluorescence intensity of $ZnPcChol_8$ in water and PBS, while the fluorescence intensity of $4\alpha ZnPc^{4+}$ and $4\alpha\beta ZnPc^{4+}$ was low, presumably due to the aggregation of molecules of these compounds in such compositions. However, the addition of 10% FBS led to a significant increase of fluorescence of $4\alpha ZnPc^{4+}$ and $4\alpha\beta ZnPc^{4+}$. The disaggregation and monomerization of these compounds are also confirmed by measurements of fluorescence lifetime, which does not change in the concentration range < 30 μM. $ZnPcChol_8$ in water and PBS is characterized at all concentration values by a single fluorescence lifetime value of 2.3 ns, close to that of the

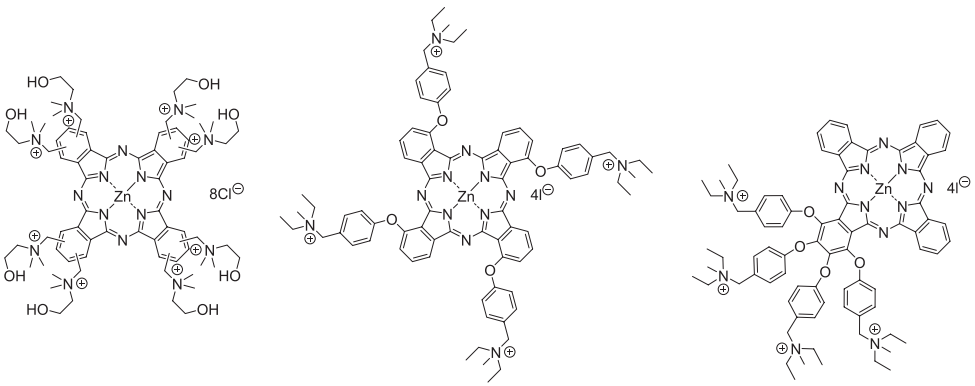

Рис. 1. Структурные формулы исследуемых фталоцианинов: $ZnPcChol_8$ (слева), $4\alpha ZnPc^{4+}$ (посередине), $4\alpha\beta ZnPc^{4+}$ (справа).
Fig. 1. Structural formulae of the studied photosensitizers: $ZnPcChol_8$ (left), $4\alpha ZnPc^{4+}$ (middle), $4\alpha\beta ZnPc^{4+}$ (right).

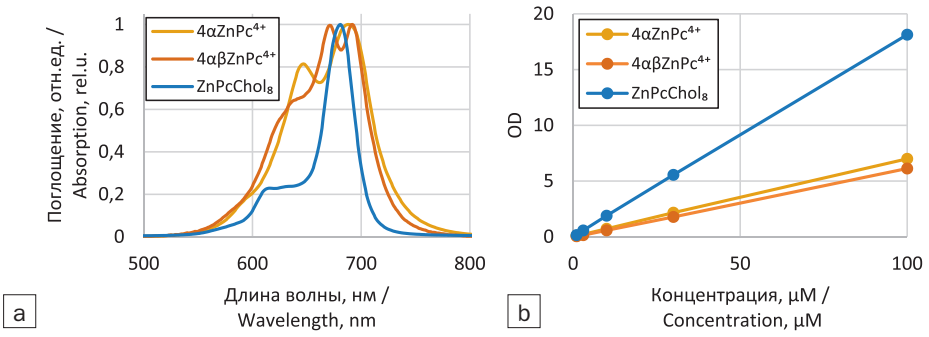

Рис. 2. Характеристики поглощения изучаемых ФС: a – нормализованные спектры поглощения; b – зависимость оптической плотности растворов ФС от их концентрации.
Fig. 2. Absorption characteristics of the studied PSs: a – normalized absorptions spectra; b – concentration dependency of PS solutions optical density.

Таблица 1
Значение максимума коэффициента молярного поглощения исследуемых фотосенсибилизаторов и его длина волны в фосфатно-солевом буфере

Table 1
Molar absorption coefficient maxima of the studied PSs and their wavelengths in PBS

Фотосенсибилизатор Photosensitizer	Длина волны максимума, нм Wavelength of maximum, nm	Коэффициент молярного поглощения, $см^{-1}·М^{-1}$ Molar absorption coefficient, $cm^{-1}·M^{-1}$
$ZnPcChol_8$	683	$12.2×10^4$
$4αZnPc^{4+}$	688	$7.0×10^4$
$4αβZnPc^{4+}$	682	$6.1×10^4$

monomeric solution at low concentration. Components with shorter radiative lifetimes (in the range of 0.5–1.3 ns) appear in aqueous compositions of $ZnPcChol_8$ with FBS (Fig. 3a), but their fraction is negligible, especially in the biologically important range < 30 μM. Whether this may be a sign of weak aggregation or a result of these PS binding with blood proteins requires additional study.

$4αZnPc^{4+}$ in PBS with DMSO and in its composition with FBS, besides the main monomeric component with radiative lifetimes in the range of 2.4–3.3 ns, contains components with shorter lifetimes (0.34 and 1.05 ns) corresponding, presumably, to aggregated states, but their fraction is also negligible (Fig. 3b). These results show that $4αZnPc^{4+}$ slightly aggregates in the studied compositions.

The asymmetric photosensitizer $4αβZnPc^{4+}$ in PBS with DMSO has, in addition to the main monomer component with an emission lifetime of 2.3 ns, a larger fraction of a component with a short lifetime corresponding to the aggregated state (Fig. 3c). However, in the aqueous composition with FBS, this fraction also decreases. The reduction of aggregation in aqueous composition with FBS is due to the disaggregating effect of FBS proteins [21]. Considering that in in vitro studies cell cultures contain up to 10% FBS, and in vivo the PS solution will be administered intravenously (i.e., directly into blood plasma), it can be expected that these PS will be in a predominantly is a disaggregated state, and their photodynamic efficiency will be high.

The photodynamic efficacy of the studied phthalocyanine PSs against A549 lung cancer cells was evaluated by the ratio of cell survival after sensitization with different concentrations of the photosensitizer after irradiation with dose densities of 10 J/cm² and 30 J/cm² (phototoxicity) and without irradiation (cytotoxicity). The phototoxicity was very high, with IC_{50} values at both light dose densities lying in the submicromole range (Table 2).

In all experiments, cytotoxicity was very low, IC_{50} values for the dark control were not reached even at concentrations of 100 μM, which allows these photosensitizers to be classified as non-toxic substances.

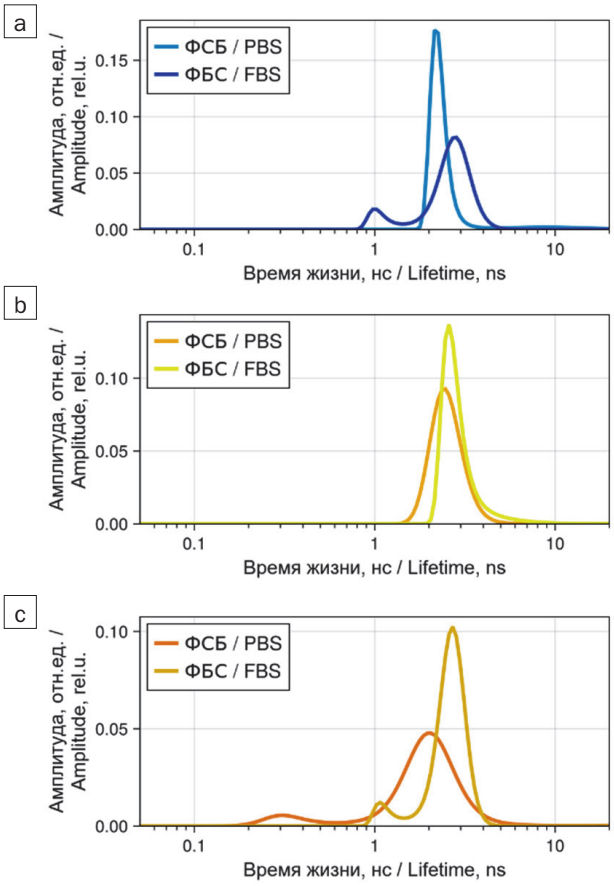

Рис. 3. Распределение времён жизни флуоресценции исследованных фотосенсибилизаторов с концентрацией 10 μM: a – $ZnPcChol_8$; b – $4αZnPc^{4+}$; c – $4αβZnPc^{4+}$ в фосфатно-солевом буфере и в фетальной бычьей сыворотке.
Fig. 3. Distribution of fluorescence lifetimes of the studied photosensitizers with 10 μM concentration: a – $ZnPcChol_8$; b – $4αZnPc^{4+}$; c – $4αβZnPc^{4+}$ in phosphate buffer saline and in fetal bovine serum.

Таблица 2
Значения IC_{50} для $ZnPcChol_8$, $4αZnPc4^+$ и $4αβZnPc^{4+}$ против клеток рака легкого A549

Table 2
IC_{50} values for $ZnPcChol_8$, $4αZnPc^{4+}$ and $4αβZnPc^{4+}$ against A549 lung cancer cells

Фотосенсибилизатор Photosensitizer	IC_{50} (мкМ) при разных плотностях дозы света IC_{50} (μM) at different dose densities	
	10 Дж/см² 10 J/cm²	30 Дж/см² 30 J/cm²
$ZnPcChol_8$	0.16±0.03	0.06±0.01
$4αZnPc^{4+}$	0.19±0.01	0.11±0.01
$4αβZnPc^{4+}$	0.33±0.04	0.25±0.04

High in vitro photodynamic efficiency of polycationic PSs against cancer cells is due, in our opinion, to several factors:

- high values of extinction and quantum yield of reactive oxygen species [11, 14, 15] in aqueous compositions, maintained in a wide range of concentrations values due to the absence of aggregation because of electrostatic repulsion of the PS ions from each other;
- effective binding of polycationic PS molecules to the surface of tumor cells, which has a significant negative charge due to the Warburg effect [22], internalization of their molecules into cells and photodynamic destruction of their organelles [5–7, 16].

Increasing the light dose density leads to an increase in phototoxicity, but this increase is not proportional to the light dose density. This may be due to the fact that the studies are performed on an adherent culture of A549 cells, which are localized near the bottom of the wells of the plate during irradiation. The study is conducted at high power density (about 50 mW/cm^2), and the rate of oxygen utilization in the effective photodynamic process under such exposure is higher than its diffusion rate through the liquid layer in the well. This leads to a significant decrease in the partial pressure of oxygen in the zone of photodynamic action on cells, and the effectiveness of photodynamic reaction will decrease, resulting in lower effect of photodynamic action on the light dose density [23]. The higher phototoxicity of $ZnPcChol_8$ compared to $4\alpha ZnPc^{4+}$ and $4\alpha\beta ZnPc^{4+}$ can be explained, similarly to [16], by the fact that the antitumor efficacy of octacationic photosensitizers is higher compared to tetracationic ones. In addition, in in vitro photodynamic efficiency studies in cell culture monolayers, the fraction of light energy absorbed by $ZnPcChol_8$-sensitized cells will also be higher [24] compared to $4\alpha ZnPc^{4+}$ and $4\alpha\beta ZnPc^{4+}$ sensitized cells due to the significantly higher extinction of $ZnPcChol_8$. Consequently, the dose-dependent photodynamic cell damage will also be higher.

The photodynamic efficiency of $4\alpha ZnPc^{4+}$ and $4\alpha\beta ZnPc^{4+}$ may in principle be lower compared to $ZnPcChol_8$ due to their partial aggregation in aqueous compositions, but in the range of their concentration values < 30 μM, the contribution of such aggregation to the IC_{50} value for these PSs is negligible.

Conclusion

Polycationic photosensitizers based on long-wavelength phthalocyanines with high extinction in the spectral range 682–689 nm demonstrate high in vitro phototoxicity in the submicromole range due to effective binding of polycationic PS molecules to the surface of tumor cells possessing significant negative charge, internalization of their molecules into cells and photodynamic destruction of their organelles, and high values of extinction and quantum yield of reactive oxygen species in a wide range of concentration values due to low or no aggregation of polycationic phthalocyanine molecules because of electrostatic repulsion of their ions in aqueous solutions.

Work of Romanishkin I.D., Bunin D.A., Skobeltsin A.S., Akhlyustina E.V., Kogan E.A., Meerovich G.A., Reshetov I.V. was supported by RSF grant N 24-45-00020.

Work of Zhi-Long Chen was supported by NSFC grant N 22361132541.

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