

# Photodynamic inactivation of pathogenic species *Pseudomonas aeruginosa* and *Candida albicans* with lutetium (III) acetate phthalocyanines and specific light irradiation

Vanya Mantareva<sup>1</sup> · Vesselin Kussovski<sup>2</sup> · Mahmut Durmuş<sup>3</sup> · Ekaterina Borisova<sup>4</sup> · Ivan Angelov<sup>1</sup>

Received: 24 March 2016 / Accepted: 28 June 2016 / Published online: 16 July 2016  
© Springer-Verlag London 2016

**Abstract** Photodynamic inactivation (PDI) is a light-associated therapeutic approach suitable for treatment of local acute infections. The method is based on specific light-activated compound which by specific irradiation and in the presence of molecular oxygen produced molecular singlet oxygen and other reactive oxygen species, all toxic for pathogenic microbial cells. The study presents photodynamic impact of two recently synthesized water-soluble cationic lutetium (III) acetate phthalocyanines (LuPc-5 and LuPc-6) towards two pathogenic strains, namely, the Gram-negative bacterium *Pseudomonas aeruginosa* and a fungus *Candida albicans*. The photodynamic effect was evaluated for the cells in suspensions and organized in 48-h developed biofilms. The relatively high levels of uptakes of LuPc-5 and LuPc-6 were determined for fungal cells compared to bacterial cells. The penetration depths and distribution of both LuPcs into microbial biofilms were investigated by means of confocal fluorescence microscopy. The photoinactivation efficiency was studied for a wide concentration range (0.85–30  $\mu\text{M}$ ) of LuPc-5 and LuPc-6 at a light dose of 50  $\text{J cm}^{-2}$  from red light-emitting diode (LED; 665 nm). The PDI study on microbial biofilms

showed incomplete photoinactivation (<3 logs) for the used gentle drug-light protocol.

**Keywords** Photodynamic inactivation · Lutetium (III) phthalocyanines · Blue diode laser · Red light-emitting diode · *Candida albicans* · *Pseudomonas aeruginosa*

## Introduction

The increasing incidence of microbial infections, coupled with the growing resistance towards conventional antibiotic-associated therapy and the side effects of the chemotherapeutic drugs, has forced the research and development of alternative therapeutic strategies [1, 2]. Historically, photodynamic therapy (PDT) was well developed starting from the beginning of the twentieth century as a curative procedure for tumor treatment [3]. In the recent years, research interest to PDT has been reinforced because of the fast development of drug resistance of the pathogenic microorganisms [4]. The mechanism of PDT action on pathogenic cells is the same which acts also on tumor cells with an essential role of type II photocatalytic mechanism [5]. It involves an energy transfer from the triplet state of a photosensitizer (PS) to the molecular oxygen in the ground triplet state which converts to molecular singlet oxygen [6]. Additionally, reactive oxygen species (ROS) can be produced by type I mechanism which happens through an electron or proton transfer from the triplet state of PS to the biomolecules which are in the vicinity of the PS pathway. The produced radicals are reacting with atmospheric oxygen with generation of oxidized products such as superoxide radical anion, hydrogen peroxide, or hydroxyl radical. The consequences of both mechanisms are oxidative reactions which action is limited to a few microns around the area of local light application. The parts of microbial cells such as cell wall, lipid

✉ Vanya Mantareva  
mantareva@yahoo.com

<sup>1</sup> Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Acad. G. Bonchev str., Bl. 9, 1113 Sofia, Bulgaria

<sup>2</sup> The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Acad. G. Bonchev str., Bl. 26, 1113 Sofia, Bulgaria

<sup>3</sup> Department of Chemistry, Gebze Technical University, P. O. Box 141, Gebze, Kocaeli 41400, Turkey

<sup>4</sup> Institute of Electronics, Bulgarian Academy of Sciences, Tzarigradsko chaussee blvd, 1784 Sofia, Bulgaria

membranes, enzymes, or nucleic acids which are in the singlet oxygen and in the surrounding environment of ROS are the target of the generated cytotoxic species [7]. The development of resistance to photodynamic inactivation (PDI) seems very unlikely because of the harmful singlet oxygen which is causing irreversible cell death [8]. The photodynamic method for control and inactivation of multispecies microbial biomass has several advantages compared to known antimicrobial drugs: (1) local application of a PS and light, (2) lack of specificity of PS to the pathogenic genus, (3) minimal or non-resistance against PS, and (4) the local application of light only on the site of the lesion which leads to fast results a short time after treatment [9].

The metal phthalocyanines (MPcs) belong to second-generation photosensitizers for cancer PDT [10–12]. The optical properties of MPcs such as far-red high-intensity absorption ( $>670$  nm,  $>10^5$  mol<sup>-1</sup> cm<sup>-1</sup>) and red-shifted fluorescence ( $>680$  nm) with relatively high singlet oxygen quantum yield ( $>0.3$ ) are favorable for PDI application. Sulfonated aluminum (III) phthalocyanine (Photosens®) is the first clinically accepted metal phthalocyanine for treatment of a variety of cancer localizations [13, 14]. Another phthalocyanine on clinical stage is silicon phthalocyanine (Pc4) which has been studied as photosensitizer for decontamination of blood transfusion [15].

PDI studies suggested advantages of cationic PSs for inactivation of pathogenic microorganisms [16–18]. They can bond to the negatively charged outer membrane of the fungal and bacterial cellular wall by electrostatic interaction and by direct mechanism of cellular inactivation straight to affect the outer cell membranes [19, 20]. On the other hand, the positive charge of the PS affects the permeability barrier of the highly organized outer membrane, which allows the PS penetration and localization in the cytoplasm [21]. The lutetium texaphyrin derivatives are used for radiation and PDT on clinical stage [22, 23]. Two new lutetium (III) acetate phthalocyanines (LuPcs) were recently synthesized and characterized with photophysical properties which are suitable for PDI application [24].

At the beginning of the clinical PDT for the tumor treatment it involves the use of lasers which are not easy to operate, and they are expensive for maintenance. However, the coherence of laser light is not a crucial property for PDT. In recent years, the non-coherent light sources have become available and useful in the experimental as well as for clinical PDT [25]. They are relatively inexpensive, stable, and easy to operate and require simple maintenance but differ fundamentally from the lasers in their output characteristics.

Nowadays, the available treatments for *Candida albicans* (*C. albicans*)-associated infections are mainly based on the conventional antifungal drugs, mostly with high toxicity to the whole body [26]. The fungal strain studied in the present work belongs to *Candida* species, the pathogens associated with the opportunistic fungal diseases, particularly in immuno-compromised

patients and denture wearers [27]. The bacterial strain *Pseudomonas aeruginosa* (*P. aeruginosa*) is a Gram-negative bacterium which is known for its very low susceptibility to the conventional antibiotic therapy [28].

The study presents the uptake of two water-soluble cationic lutetium (III) acetate phthalocyanines LuPc-5 and LuPc-6 for cellular suspensions of bacterial *P. aeruginosa* and of fungal *C. albicans* cells by observation of the fluorescence signal obtained at excitation from one LD at 365 nm and emission between 660 and 750 nm. The localization and penetration depth of both LuPcs were studied in 48-h biofilms by using laser excitation from a confocal laser fluorescence microscope. In vitro photodynamic inactivations were evaluated for a wide concentration range of LuPcs at irradiation with therapeutic light (LED 665 nm) applied on cells as planktonic and biofilm cultures.

## Materials and methods

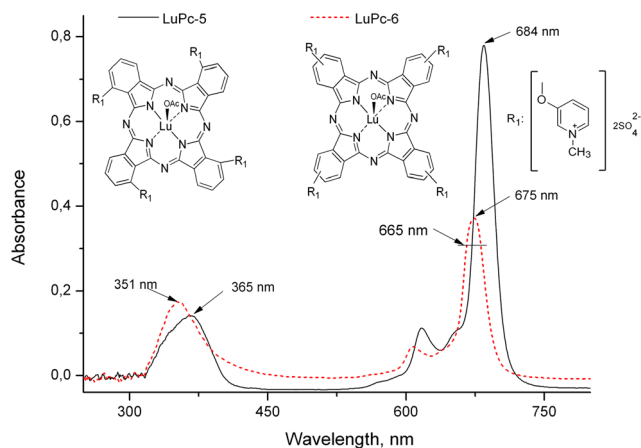
### Photosensitizers and chemicals

The photosensitizers used in this study are two Lu(III) acetate phthalocyanines with four methylpyridyloxy groups which differ in the position on the macrocycle, namely, non-peripheral ( $\alpha$  position) for LuPc-5 and peripheral ( $\beta$  position) for LuPc-6 (Fig. 1, inset). The synthesis and chemical characterization of LuPcs are recently described [24]. The stock solutions of LuPc-5 and LuPc-6 (2 mM) were freshly prepared in dimethyl sulfoxide (DMSO) and diluted prior the experiments. The dilution was made in sterile 0.01 mM phosphate-buffered saline (PBS). The control of the concentrations was carried out spectrophotometrically in DMSO of spectroscopic grade (Sigma-Aldrich). All used chemicals for PBS preparation and for the extraction procedure during the uptake study such as sodium dodecyl sulfate (SDS) and tetrahydrofuran (THF) are products of Sigma-Aldrich.

### Microbial strains and culture conditions

The fungus *C. albicans* strain no. 74 and the Gram-negative *P. aeruginosa* 1390 were taken from the National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC), Sofia, Bulgaria. Brain Heart Infusion Broth (Difco, BD Diagnostic Systems, and Sparks, MD) for *P. aeruginosa* and Tryptic Soy Broth and Tryptic Soy Agar media (Difco) for *C. albicans* were used. Both microbial strains were grown aerobically at 37 °C. The cells were harvested by centrifugation, and then they were resuspended in sterile PBS buffer. The cell density was checked by absorbance at 600 nm,  $A = 0.490$  for  $10^9$  cells per milliliter.

The Gram-negative strain *P. aeruginosa* 1390 used in our study was tested for drug resistance against a variety of drug classes which included penicillins (amoxicillin and penicillin);



**Fig. 1** UV-visible spectra of Lu(III) acetate phthalocyanines (LuPc-5 and LuPc-6) in dimethylsulfoxide with their chemical structures (inset)

polypeptide antibiotic (bacitracin); glycopeptide antibiotic (vancomycin); tetracycline (doxycycline); sulfonamide (trimethoprim); aminoglycoside (kanamycin); and others (lincomycin, nalidixic acid and rifampin, novobiocin). The results showed sensitivity of *P. aeruginosa* to the groups of antibiotics: cephalosporins (ceftriaxone); aminoglycosides (amikacin, tobramycin, gentamicin); and other (chloramphenicol). The used strain *P. aeruginosa* 1390 has comparable values of sensitivity to the above drugs to those published from the commercially available strains specified under the name ATCC® Drug-Resistant *Pseudomonas aeruginosa* Panel (ATCC® MP-23™) [29].

### Light sources and equipment

An experimental setup (Scheme 1) is arranged on the basis of several irradiation devices such as diode lasers (DLs) and light-emitting diodes (LEDs). The setup is configured to be usable for experimental PDT investigations with different photosensitizers. This setup allows controlling the bleaching and measurements of spectral properties of different photoactive compounds by several irradiation modalities. In case of PDT experiment, the irradiation mode of two different light sources with maximum at 635 and 665 nm can be used in dependence of the absorption band of the applied PS (Fig. 1). The light sources have a full width of half maximum (FWHM) of 25 nm with maximum at 635 and 665 nm, respectively. The working regimes of the LED sources (power and distance to the target) and illumination duration ( $t_{exc}$ ) were evaluated for the light dose ( $D$ ) needed at the applied wavelength in accordance with the equation  $D = I \times t_{exc}$ , where  $I$  is the measured light intensity on the sample position. The maximal power density at a distance of 10 cm from the irradiated sample is up to 200 mW cm<sup>-2</sup> for a 635-nm LED and up to 100 mW cm<sup>-2</sup> for the second 665-nm LED at an area of 25 cm<sup>2</sup> (ELO Ltd., Sofia, Bulgaria).

The spectral measurements are based on the use of a QE 65000 spectrophotometer as it is shown in Scheme 1. As excitation sources for fluorescence measurements, we used continuous-wave (CW) diode lasers with different wavelengths. The excitation power applied at the excitation wavelengths for fluorescence measurements was constant at about 50 mW. Different delivery components (light guide, lenses (L), several optical filters (F), and polarizer) to obtain light intensity in the linear range of the QE 65000 spectrophotometer were used. Several optical fibers (LG) to deliver the excitation and registered signals in the spectral range 275–800 nm were used.

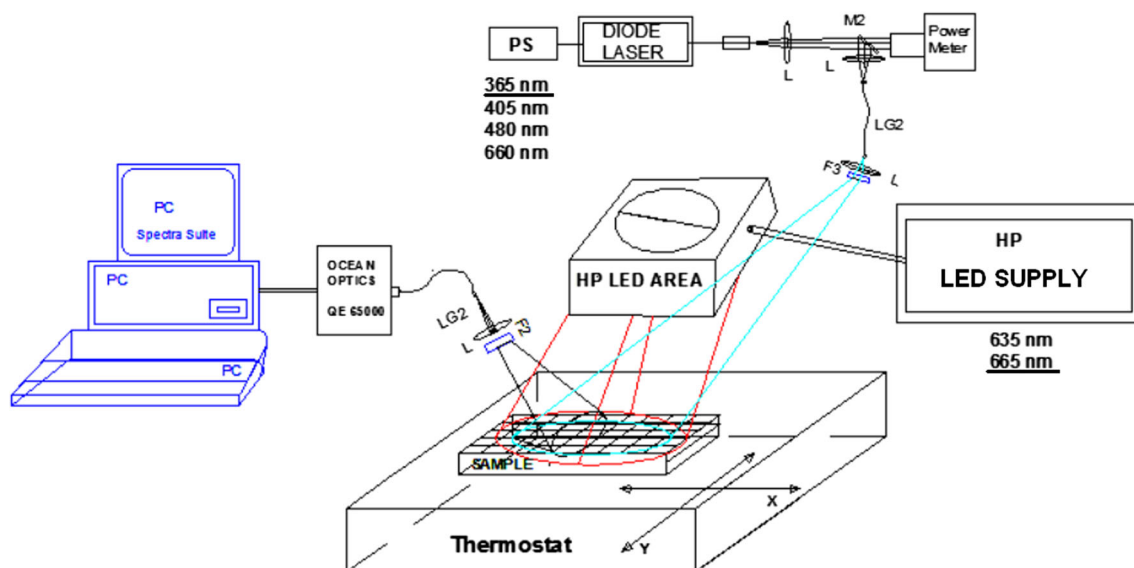
The absorbance and fluorescence spectra were recorded using a fiber-optic QE 65000 microspectrometer (Ocean Optics Inc., Dunedin, FL, USA). The spectral resolution of the microspectrometer was approximately 1 nm. The spectra were recorded using the microspectrometer specialized software Spectra Suite (Ocean Optics Inc., Dunedin, USA). The data were analyzed and graphically represented by means of computer programme Origin 8.0 (Microcal Software, Inc., Northampton, MA, USA). The assessment of the drug concentration was performed on Shimadzu UV-Vis 3000 apparatus (Japan). The confocal laser scanning microscope (CLSM) of Leica Microsystems (Leica TCS SPE) equipped with Leica LAS AF software was used.

### Uptake study

The suspensions were serially diluted to the required densities from 10<sup>8</sup> to 10<sup>5</sup> cells per milliliter prior to the experiments. The uptake study was carried out following the chemical extraction procedure. The quantification of the number of molecules accumulated into bacterial and fungal cells was evaluated by means of fluorescence measurements of the collected samples. The pathogenic cells were incubated for 15 min with 3 μM LuPc-5 and LuPc-6 in the dark. The collected samples for fluorescence measurements were (1) the supernatant of PBS after incubation, (2) the PBS after the first and (3) after the second cell wash, and (4) the supernatant after extraction with THF: 2 % SDS (1:9). The fluorescence spectra of the collected samples were measured by using an elaborated experimental setup with an excitation source DL of 365 nm (Scheme 1). The samples (1–4) were diluted with DMSO, and the fluorescence maxima were recorded in the 660–750-nm region. The results were presented as the number of molecules of LuPc-5 or LuPc-6 per one cell by processing the obtained values of fluorescence intensities and referring to the calibration curves taken for both LuPcs in the solvent mixtures.

### Biofilm development

The microbial pathogenic biofilms of each of the studied pathogenic strains (*P. aeruginosa* and *C. albicans*) were cultured



**Scheme 1** Experimental setup of fiber optics and several light sources with elaborated Ocean Optics QE 65000 spectrometer, used in the uptake and in vitro photodynamic studies and applicable for different photosensitizers

on coverslips, which were placed in commercial pre-sterilized polystyrene flat-bottomed 12-well cell culture test plates (Switzerland). A standard cellular suspension ( $1 \text{ mL}$ ,  $10^7 \text{ CFU mL}^{-1}$ ) prepared after serial dilutions was applied onto the surface of the discs placed in each well of the plate followed by incubation for 1.5 h at  $37^\circ \text{C}$  to promote cellular adherence to surface of the discs. The blank control wells with the discs at the same conditions but without bacterial cells were inoculated. After the initial adhesion phase, the cell suspensions were aspirated and the discs were gently washed with PBS to remove loosely adherent cells. In the biofilm phase formation, an addition of 4 mL Tryptic Soy Broth (Difco Laboratories, MD, USA) was placed in each well. The plates were covered, and the incubation continued for 48 h at  $37^\circ \text{C}$  to form the biofilms for analyses.

### Confocal laser scanning microscopy

The biofilm images were processed via the Leica LAS AF software provided with the equipment. The characteristics of biofilm were studied following the protocol of our recent studies [30, 31]. The oil immersion of  $\times 63$  objective ( $\text{NA} = 1.23$ ) was used. The thickness of the biofilm was evaluated by the native fluorescence of the cells (excitation 488 nm, emission 520–580 nm). The microbial biofilms were incubated in the dark for 1.5 h with  $20 \mu\text{M}$  LuPcs. The study includes measurements of the biofilm thicknesses and LuPc localization and penetration depths into the biomass. The samples were washed in PBS, and then they were covered with coverslips. The fluorescence images of the biofilms were obtained by excitation with a 633-nm laser, and the emission was taken between 660 and 740 nm to image the localization and penetration depth of LuPcs throughout the biofilm thicknesses. The whole biofilm

was scanned on slices of  $0.100 \mu\text{m}$  each by following the fluorescence and transmittance modes. The co-localization of LuPcs in the cells organized in the biofilm was evaluated by following both channels.

### In vitro PDI study

The cells in suspensions ( $1 \text{ mL}$ ) were incubated with LuPcs for 15 min, by using the freshly prepared stock solutions. The incubation was carried out on a magnetic stirrer with wide concentration range (0.85, 1.70, 3.40, 6.80, 12, 20, and  $30 \mu\text{M}$  LuPc-5 and LuPc-6) in the dark at room temperature. The cell suspensions for experiments were with cell densities between  $10^6$  and  $10^7 \text{ CFU}$  per milliliter in PBS. After incubation time was passed, an aliquot ( $200 \mu\text{L}$ ) of the suspension was placed in a standard palette. The power densities applied on the samples were calculated according to the power measured on the surface of the irradiated cells with a negligible decrease (5 %) inside the thin layer of the cells as suspension as well as for the biofilm cultures. The irradiation from a 665-nm LED was adjusted at a distance of 20 cm to achieve the power density of  $60 \text{ mW cm}^{-2}$ . The requested dose of  $50 \text{ J cm}^{-2}$  was reached for an irradiation time of 12 min. The samples of four control groups were collected: (1) with photosensitizer, but no light (dark toxicity), (2) without photosensitizer, but illuminated, (3) only bacterial suspension (no photosensitizer, no light), and (4) bacteria incubated only with DMSO (5 % from the total volume). After irradiation, the aliquot of cells ( $0.1 \text{ mL}$ ) was taken off and serially diluted (10-fold) with PBS. The same action was repeated for the control samples (1–4). Aliquots ( $0.1 \text{ mL}$ ) were spread over Trypticase<sup>®</sup> Soy Agar, and the number of colonies (CFU) was counted after a 48-h incubation.

## Statistics

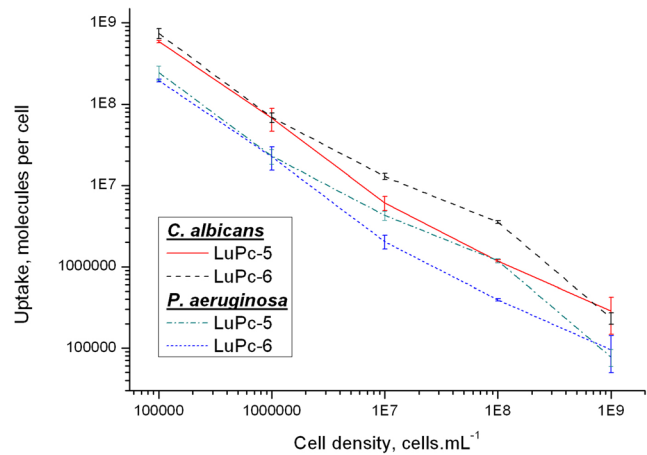
The uptake and in vitro experiments were carried out in triplicate, and the data were presented as a mean  $\pm$  standard deviation (SD). The difference between two means was compared by a two-tailed unpaired Student's test. The values of  $p < 0.05$  were considered as significant.

## Results and discussion

### Uptake

Two water-soluble cationic Lu (III) acetate phthalocyanines, which are presented with the absorption spectra in Fig. 1, were recently synthesized and characterized as photosensitizers suitable for PDI. The absorption, fluorescence, and photochemical properties of LuPc-5 and LuPc-6 were determined with promising values [24]. Both complexes, apart from the formation of photoinactive aggregates in pure water, show a tendency to monomerize by addition of some detergent [24]. The larger atom of lutetium coordinated in the macrocycle of phthalocyanine contributed to properties of the triplet excited state which relates to the singlet oxygen quantum yields of LuPcs (0.3–0.4).

The uptake of LuPc-5 and LuPc-6 was evaluated for *P. aeruginosa* and *C. albicans* cellular suspensions (Fig. 2). The results are presented as a number of LuPc molecules attached to one bacterial or fungal cell on the basis of the fluorescence intensity of the collected samples referring to the calibration curves. The results suggested that LuPc-5 and LuPc-6 are likely to accumulate with lower amount in the Gram-negative bacterial cells than in fungal cells. Both LuPc values of uptake are inversely proportional with densities. Namely, the number of LuPc molecules per one cell decreases with an increment of the cell density. This phenomenon of the opposite dependence of the number of PS molecules on the cell density of suspension was firstly reported for the Gram-negative *Escherichia coli* [16]. Further studies on the various bacterial and fungal strains confirmed that the uptake of any applied PS decreases with an increment of cellular density [17–19]. The cationic MPc was proved that can be more easily taken up by the cells as a result of the nature of the membrane bilayers [20]. Having in consideration that the binding process usually occurs via an electrostatic mechanism of interaction, the positively charged PSs are taken up usually in higher amount into the cells than the neutral and negatively charged PSs [19, 32]. The permeability of the membranes is also influenced by dipole moment of PS as was shown in a recent study with the mono-substituted molecules which were evaluated with better uptake for cells [33]. However, another study suggests that the photodynamic effect has no correlation to the



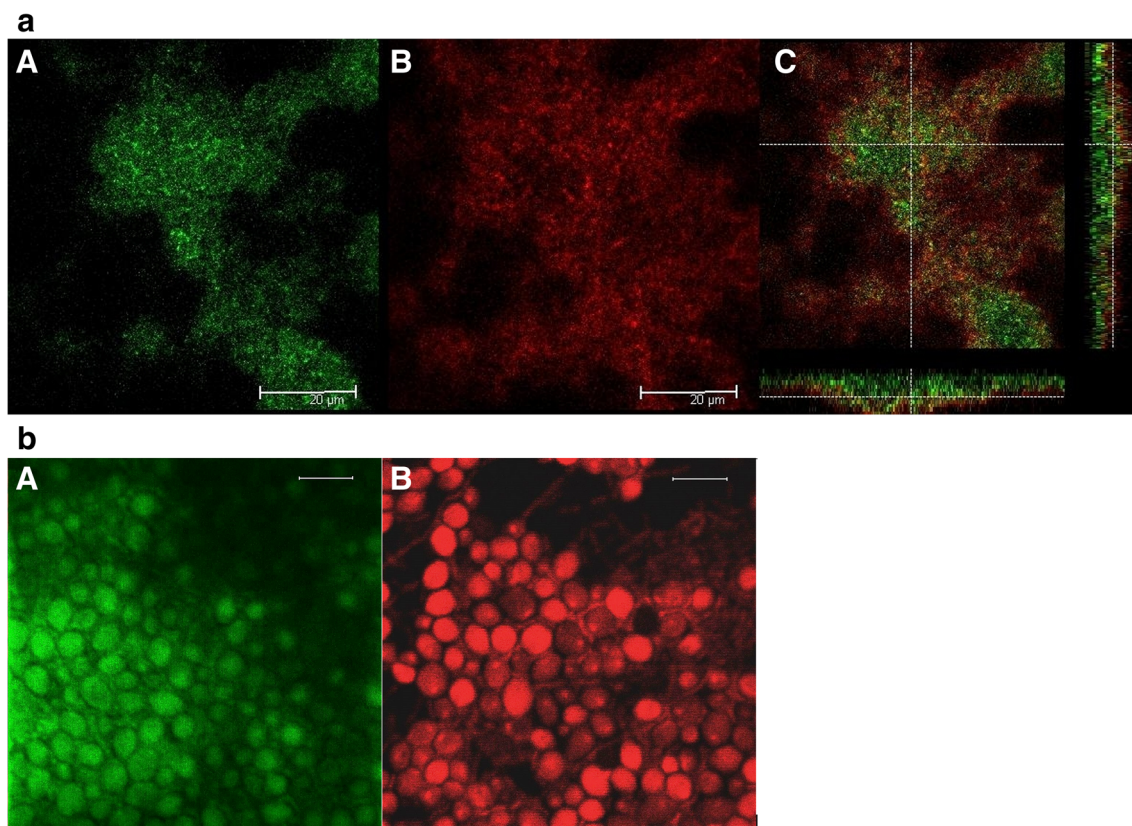
**Fig. 2** Uptake of Lu(III) acetate phthalocyanines (LuPc-5 and LuPc-6) after a 15-min incubation of cellular suspensions of *P. aeruginosa* and *C. albicans* cells with different density. Each point is the mean  $\pm$  standard deviation (SD) of three experiments with  $p < 0.05$  for the presented data

uptake efficiency as was reported for mono- and tetra-substituted indium (IV) phthalocyanines [34].

### Biofilm study

The biofilms were characterized by the excitation wavelength typical for the native cell chromophores and the fluorescence of the applied LuPcs (Fig. 3a, b). The native cellular fluorescence (A) was used for visualization of the formed biofilms (excitation 488 nm; emission 520–580 nm). The typical red fluorescence of LuPc-5 was observed for the spectrum 660–740-nm at excitation with laser at 633 nm (Fig. 3a, b (B)). The fluorescence detection of LuPc-5 in the biofilm slices suggested that it is localized into the cells and also the matrix of the formed biofilm. There are parts of the biofilm where LuPc-5 and LuPc-6 showed a full penetration (100 %) and others with limited penetration (75 %). This was proven by scanning the whole biofilm by fluorescence mode. The signal of red fluorescence typical for LuPcs was determined throughout the whole biofilm with thickness between 7 and 13  $\mu\text{m}$  (Fig. 3a, C). The obtained images showed a high colocalization of the incubated LuPcs which means the overlap of the native (green) and LuPc (red) fluorescence signals. The non-peripherally substituted LuPc-5 was evaluated with limited penetration (up to 3–7  $\mu\text{m}$ ) in some regions of the biofilm to full depth of penetration (7–13  $\mu\text{m}$ ) in other parts of the biomass as was seen by z-images.

The studied 48-h developed *C. albicans* biofilms were measured with full penetration of LuPc-5 into the biomass which was observed by fluorescence mode (Fig. 3b). The developed 48-h fungal biofilms were measured with thicknesses between 14 and 21  $\mu\text{m}$ . The accumulation of LuPcs into bacterial and fungal biofilms suggested an efficient photodynamic inactivation due to the close distance between the



**Fig. 3** Confocal laser scanning images of *P. aeruginosa* biofilm (**a**), *C. albicans* biofilm (**b**), and at excitation at 488 nm (**A**) and emission at 520–580 nm (green native fluorescence) and at excitation at 633 nm (**B**)

and emission at 660–740 nm (red photosensitizer fluorescence) as well as the overlapping of the images (**a**, **C**). Scale bar 20  $\mu$ m

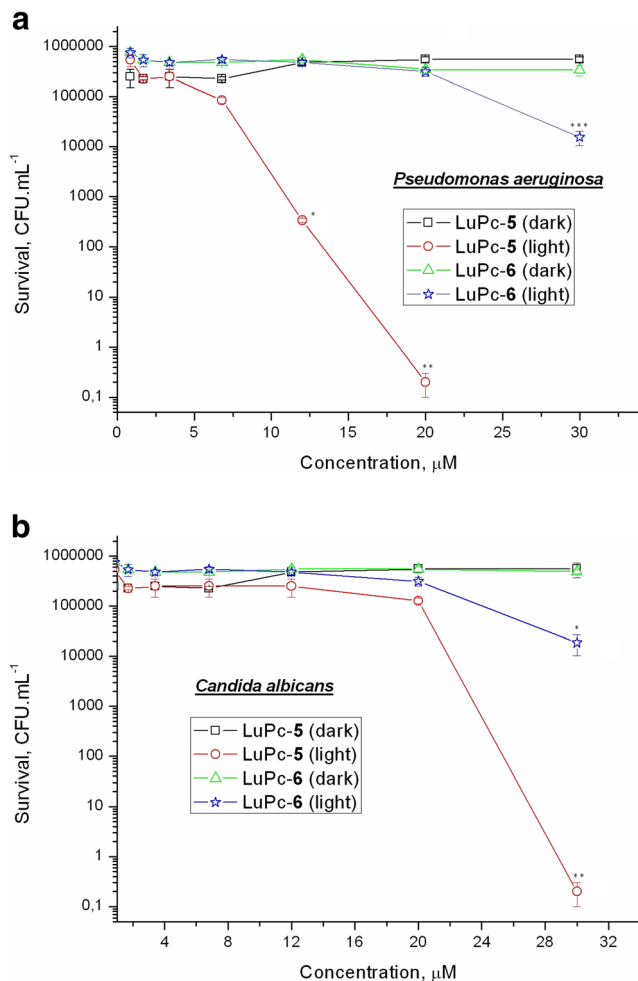
cell membranes where the photosensitizer is localized as well as the generated singlet oxygen and other ROS during the light exposure. The observed complete penetration of LuPc-5 and LuPc-6 in biofilms can be due to the cationic charge of the substituents. The large lutetium atom is positioned out of the plane of the phthalocyanine molecule which prevents the formation of photoinactivate aggregates and favors the photodynamic process.

### In vitro PDT

The setup for PDT treatment shown in Scheme 1 is general for PDT studies and has more opportunities; some of them are not applied in the present investigations, but the used light sources are specified in the “Material and methods” section. The photodynamic efficacy of two water-soluble cationic LuPc-5 and LuPc-6 was studied at constant red LED irradiations for a wide concentration range of LuPcs towards bacterium *P. aeruginosa* (Fig. 4a) and fungus *C. albicans* (Fig. 4b). The full photoinactivation was achieved with 20  $\mu$ M LuPc-5 for *P. aeruginosa* and 30  $\mu$ M LuPc-5 for *C. albicans*. This study suggests that the high uptake of LuPcs in the bigger-by-size *C. albicans* cells needs higher concentration for full inactivation than for the Gram-negative bacterium *P. aeruginosa*. The photoinactivation with the

peripherally substituted LuPc-6 was not so significant (2 logs) as with LuPc-5 for *C. albicans* (Fig. 4b). Both LuPcs showed no dark toxicity within the wide treatment concentration range for the bacterium as well as for the fungus.

The recent study with tetra- and mono-substituted indium phthalocyanines (InPcs) showed a higher effect towards *E. coli* after PDT with a tetra-substituted InPc, suggesting that the number of positive charges can play a more important role than the symmetry of the Pc molecule [34]. The comparison of the PDT efficacy of LuPc-5 and LuPc-6 on microbial pathogens *P. aeruginosa* and *C. albicans* with the previously studied unsubstituted Zn(II) phthalocyanine (ZnPc) and Zn(II) phthalocyanine with methylpyridyloxy substituents (ZnPcMe) showed a higher activity for the complexes with zinc [17, 19]. Thus, it suggests that the difference in molecular electronic structure due to replacement of Zn(II) with Lu(III) leads to improved physicochemical properties of MPc, but it does not influence the PDT efficiency. The comparative study with different metal complexes of phthalocyanine (Ga, In, Si, Ge) showed that coordination with gallium (GaPc1) and silicon (SiPc2) results in high phototoxic effect and full inactivation of *C. albicans* as planktonic and biofilm cultures [35]. In case of LuPc-5, the presence of nuclear membrane in the much-bigger-in-size fungal cells seems to be an obstacle for



**Fig. 4** Survival of pathogenic cells ( $\sim 10^6$  CFU mL $^{-1}$ ) dark incubated for 15 min with different concentrations of LuPc-5 and LuPc-6 followed by irradiation with LED at 665 nm for bacterium *P. aeruginosa* (a) and for fungus *C. albicans* (b). The points are presented as the mean  $\pm$  SD of three experiments with  $p < 0.05$  (\* $p = 0.006$ , \*\* $p = 0.008$ , \*\*\* $p = 0.03$ ) for bacterial cells and with  $p < 0.05$  (\* $p = 0.008$ , \*\* $p = 0.002$ ) for fungal cells

intracellular accumulation and the full inactivation was achieved for high LuPc-5 concentration. The applied PDI protocol on the 48-h biofilms showed low PDI efficacy (<3 logs) compared to effect in suspension.

## Conclusions

The uptake and localization study of two water-soluble cationic Lu(II) acetate phthalocyanines were evaluated for bacterial (*P. aeruginosa*) and fungal (*C. albicans*) cells with greater accumulation in fungal cells compared to bacterial cells and with full penetration (100 %) into fungal biofilms. The complete photoinactivation was achieved with 20  $\mu$ M LuPc-5 for bacterium and with 30  $\mu$ M LuPc-5 for fungus. The applied gentle irradiation from LED at 665 nm (50 J cm $^{-2}$ ) was

sufficient for the obtained high PDI efficacy for planktonic cultures. The used protocol was identical for biofilms and for the suspension, but the photoinactivation of the 48-h bacterial and fungal biofilms was not efficient (<3 logs).

**Acknowledgments** The authors thank Dr. Rumen Dimitrov for CLSM measurements. The work was supported by the project of the Bulgarian Academy of Sciences and partly by the Bulgarian National Science Fund (B02/9/2014).

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

## References

1. Maisch T (2007) Anti-microbial photodynamic therapy: useful in the future? *Lasers Med Sci* 22:83–91
2. Kessel D (2004) Photodynamic therapy: from the beginning. *Photodiagn Photodyn Ther* 1:3–7
3. Yano S, Hirohara S, Obata M et al (2011) Current states and future views in photodynamic therapy. *J Photochem Photobiol C* 12:46–67
4. Luksiene Z, Zukauskas A (2009) Prospects of photosensitization in control of pathogenic and harmful microorganisms. *J Appl Microbiol* 107:1415–1424
5. Plaetzer K, Krammer B, Berlanda J, Berr F, Klieslich T (2009) Photophysics and photochemistry of photodynamic therapy: fundamental aspects. *Laser Med Sci* 24:259–268
6. Delattin N, Cammue BPA, Thevissen K (2014) Reactive oxygen species inducing antifungal agents and their activity against fungal biofilms. *Future Med Chem* 6(1):77–90
7. Maisch T, Bosl C, Szeimies R-M, Lehn N, Abels C (2005) Photodynamic effects of novel XF porphyrin derivatives on prokaryotic and eukaryotic cells. *Antimicrob Agents Chemother* 49(4): 1542–1552
8. Spesia MB, Caminos DA, Pons P, Durantini EN (2009) Mechanistic insight of the photodynamic inactivation of *Escherichia coli* by a tetracationic zinc(II) phthalocyanine derivative. *Photodiagn Photodyn Ther* 6:52–61
9. Quishida CCC, Oliveira Mima EG, Dovigo LN, Jotge JH, Bagnato VS, Pavarina AC (2015) Photodynamic inactivation of a multispecies biofilms using photodithazine and LED light after one and three successive applications. *Lasers Med Sci* 30:2303–2312
10. Sekkat N, van der Bergh H, Nyokong T, Lange N (2012) Like a bolt from the blue: phthalocyanines in biomedical optics. *Molecules* 17: 98–144
11. Junqueira JC, Jorge AOC, Barbosa JO et al (2012) Photodynamic inactivation of biofilms formed by *Candida* spp., *Triposporon mucoides*, and *Kodamaea ohmeri* by cationic nanoemulsion of zinc 2,9,16,23-tetrakis (phenylthio)-29H, 31H-phthalocyanine (ZnPc). *Lasers Med Sci* 27:1205–1212
12. Ribeiro APD, Andrade MC, Bagnato VS et al (2015) Antimicrobial photodynamic therapy against pathogenic bacterial suspensions and biofilms using chloro-aluminium phthalocyanine encapsulated in nanoemulsions. *Lasers Med Sci* 30:549–559
13. Filonenko VE (2015) The history of development of fluorescence diagnosis and photodynamic therapy and their capabilities in oncology. *Russian J General Chemistry* 85(1):211–215

14. Maduray K, Odhav B, Nyokong T (2012) In vitro photodynamic effect of aluminium tetrasulfophthalocyanines on melanoma skin cancer and healthy normal skin cells. *Photodiagn Photodyn Ther* 9:32–39
15. Ben-Hur E, Zuk MM, Kenney ME et al (1996) Action spectra (660–700 nm) for virus inactivation and red cell damage photosensitized by the silicon phthalocyanine Pc4. *Lasers Med Sci* 11:221–225
16. Demidova TN, Hamblin MR (2005) Effect of cell-photosensitizer binding and cell density on microbial photoinactivation. *Antimicrob Agents Chem* 49:2329–2335
17. Kussovski V, Mantareva V, Angelov I et al (2009) Photodynamic inactivation of *Aeromonas hydrophila* by cationic phthalocyanines with different hydrophobicity. *FEMS Microbiol Lett* 294:133–140
18. Mantareva V, Kussovski V, Angelov I et al (2011) Non-aggregated Ga(III)-phthalocyanines: synthesis and photodynamic effect on pathogenic microorganisms planktonic and biofilms cultures. *Photochem Photobiol Sci* 10:92–102
19. Mantareva V, Kussovski V, Angelov I et al (2007) Photodynamic activity of water-soluble phthalocyanine zinc(II) complexes against pathogenic microorganisms. *Bioorg Med Chem* 15:4829–4835
20. Segalla A, Borsarelli CD, Braslavsky SE et al (2002) Photophysical, photochemical and antibacterial photosensitizing properties of a novel octacationic Zn(II)-phthalocyanine. *Photochem Photobiol Sci* 1:641–648
21. Kassab K, Fadeel DAE, Fadel MV (2013) Topical photodynamic therapy using transferrin Al phthalocyanine tetrasulfonate: in vitro and in vivo study. *Lasers Med Sci* 28:1352–1361
22. Sessler JL, Miller RA (2000) Texaphyrins: drugs with diverse clinical applications in radiation and photodynamic therapy. *Biochem Pharmacology* 59:733–739
23. Young SW, Woodburn KW, Wright M et al (1996) Lutetium texaphyrin (PCI-0123): a near-infrared, water-soluble photosensitizer. *Photochem Photobiol* 63(6):892–897
24. Mantareva V, Durmuş M, Aliosman M, Stoineva I, Angelov I (2016) Lutetium (III) acetate phthalocyanines for photodynamic therapy applications: synthesis and photo physicochemical properties. *Photodiagn Photodyn Ther* 14:98–103
25. Rosa LP, da Silva FC, Viana MS, Meira GA (2016) In vitro effectiveness of 455-nm blue LED to reduce the load of *Staphylococcus aureus* and *Candida albicans* biofilms in compact bone tissue. *Lasers Med Sci* 31:27–32
26. Hunter KD, Gibson J, Lockhart AP, Bagg J (1998) Fluconazole-resistant *Candida* species in the oral flora of fluconazole-exposed HIV-positive patients. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 85:558–564
27. Sullivan D, Haynes K, Bille J et al (1997) Widespread geographic distribution of oral *Candida dubliniensis* strains in human immunodeficiency virus-infected individuals. *J Clin Microbiol* 35:960–964
28. Prochnow EP, Martins MR, Campagnolo CB, Vianna RC, Villetti MA, Kantorski KZ (2015) Antimicrobial photodynamic effect of phenothiazinic photosensitizers in formulations with ethanol on *Pseudomonas aeruginosa* biofilms. *Photodiagn Photodyn Ther* 13: 291–296
29. ATCC Products (2016) Drug-Resistant *Pseudomonas aeruginosa* Panel (ATCC® MR-23™). <http://www.lgcstandards-atcc.org/Products/All/MP-23.aspx>
30. Prasanth CS, Karunakaran S, Paul A, Kussovski V et al (2014) Antimicrobial photodynamic efficiency of novel cationic porphyrins towards periodontal Gram-positive and Gram-negative pathogenic bacteria. *Photochem Photobiol* 90(3):628–640
31. Mantareva V, Angelov I, Kussovski V et al (2011) Photodynamic efficiency of water-soluble Si(IV) and Ge(IV) phthalocyanines towards *Candida albicans* planktonic and biofilm cultures. *Eur J Med Chem* 46: 4430–4440
32. Scalise I, Durantini E (2005) Synthesis, properties, and photodynamic inactivation of *Escherichia coli* using a cationic and a noncharged Zn(II) pyridyloxy phthalocyanine derivatives. *Bioorg Med Chem* 13(8):3037–3045
33. Ke MR, Eastel JM, Ngai KLK et al (2014) Photodynamic inactivation of bacteria and viruses using two monosubstituted zinc(II) phthalocyanines. *Eur J Med Chem* 84:278–283
34. Osifeco OL, Durmuş M, Nyokong T (2015) Physicochemical and photodynamic antimicrobial chemotherapy studies of mono- and tetra- pyridyloxy substituted indium (III) phthalocyanines. *J Photochem Photobiol A Chem* 301:47–54
35. Mantareva VN, Angelov I, Wohrle D, Borisova E, Kussovski V (2013) Metallophthalocyanines for antimicrobial photodynamic therapy: an overview of our experience. *J Porphyrins Phthalocyanines* 17:399–416