Phthalocyanines Structure Versus Photodynamic Effectiveness towards Pathogenic Microorganisms: Our Recent Experience

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Abstract. The present review paper aims to summarize our recent experience in research and development of new phthalocyanine complexes and investigations of the main photophysical, photochemical and photobiological properties which are related to antimicrobial photodynamic therapy (aPDT) as alternative method for inactivation of the resistant pathogens. The effect of functionalization of Zn(II) phthalocyanine (ZnPc) with biologically-active natural substances such as amino acids, sugars and steroids was studied in comparison to the basic ZnPc ring molecule. The structural features of the substitution groups were chosen to facilitate the main properties responsible for PDT outcome. For example, the linkage groups of amino acids tyrosine, phenylalanine, lysine and arginine have positive charge in physiological media to the better attachment to bacterial wall and some of them have a good fluorescence for a contribution to the visualization of the infected area. Also, ZnPcs linked to sugars and steroids was expecting to possess receptor specific selectivity. The physicochemical properties of the novel functionalized ZnPcs are presented in respect to their efficiency for a number of pathogenic bacterial and fungal species. Additionally, the complexes of two heavy metal ions such as lutetium(III) (Lu(III)) and tin(IV) (Sn(IV)) were synthesized and evaluated for antimicrobial PDT. These complexes were designed with the same structural skeleton as our previous water-soluble methylpiridyloxy-substituted phthalocyanine complexes with zinc (II), silicon (IV), germanium (IV), indium (III) and gallium (III), all with relatively promising antibacterial efficiency.

Keywords: Photosensitizers; Phthalocyanines; PDT; Photochemistry; Antibacterial photodynamic therapy; aPDT.

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1 Introduction

Photodynamic therapy (PDT) is well-accepted curative modality with fast outcome after treatment, which is applicable on different acute disorders including infections [1, 2]. The method is based on a local irradiation of the target spot of the problematic area associated with pathogens where the photosensitizer (PS) is preferably accumulated. The efficacy of PDT action depends on the saturation with molecular oxygen but usually the

atmosphere surrounding is adequate for the process. The procedure starts with a light absorption within visible or near infrared spectra, which initiates a physical conversion of generation of first singlet excited state PS^1. The absorbed energy of PS^1 may relax by radiative and non-radiative conversions with high input of the intersystem crossing transition which leads to production of the triplet excited state PS^3 [3]. The molecules in their triplet excited state can take part in the photochemical reactions with participation of the molecular oxygen placed in the vicinity of PS^3 as well as with the surrounded bioorganic molecules as part of the structure of pathogens. The progress of the photosensitization includes different reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide anion ($O^{2\bullet-}$), hydroxyl radical (•OH) and hydrogen peroxide, all causing the irreversible oxidative damage of membranes so that the development of drug resistance is not yet observed for photodynamic inactivation [4].

It is believed that the fast development of drug- and multi-drug resistance is associated with the over-use and often abuse to the presently available chemotherapeutic drugs [5]. The knowledge about the efficacy of compounds absorbing in the far-red spectral region on the local infections has more than hundred years' historical timeline. Though, the "Golden era" of discovery and common usage of antibiotics has limited the progress of development of PDT method towards pathogenic microorganisms. Since the time when the resistance problem was announced as a high social health problem, the PDT topic together with many other approaches are under consideration as alternative methods to the antibiotics treatment [6].

The structure of a PS has to be capable to accumulate in high amounts into pathogens to result in the pathogens inactivation after light irradiation with a specific wavelength [7, 8]. The research and development of new generation PS, which are especially designed for pathogenic microbes, have been under intensive research consideration [9, 10]. The interaction of a PS with the pathogenic microbial species depends on their morphological characteristics, which have the well-defined difference for both bacterial species. For example, the bacterial wall of Gram (−) bacteria is more complicated having an additional layer than that in Gram (+) bacteria, which can act as a permeability barrier to limit the drug penetration and the further inactivation. The most involved mechanisms of drug uptake are known to happen by the physical uptake of PS molecules to the membranes due to hydrophobic and charge interactions [11]. These mechanisms can act together with the membrane's receptors-interactions, which are likely delivered the sufficient amount of PS to the target cells. The studies also present the selectivity of the uptakes in randomly arranged pathogens over the surrounding organized mammalian cells [12–14]. The specific structural characteristics of the particular pathogenic specie of one group of pathogens with common features need to be considered in strength designed PS's. The focus in investigations is made on the biologically-active functional groups for substitution of PS with a goal for improvement of the chemical, physicochemical and photoinactivation PS properties [15].

For these reasons, the new generation photosensitive drugs used for antimicrobial PDT (aPDT) or photodynamic inactivation (PDI), as well as the photodisinfection of medical devices related to human activity and treatment, have a well-defined chemical structure, cationic charge and hydrophilic or amphiphilic nature [16]. Nowadays only few numbers of metal coordinated phthalocyanine complexes (MPcs) of zinc (ZnPc), aluminum ($AlPcS_n$) and silicon (Pc4) are clinically approved for PDT of different tumor localization [17]. However, the knowledge about these drugs suggested the low uptake capacity and non-selective efficiency on pathogenic bacteria and fungi [18]. On the other hand, taking into account the structural flexibility and the advanced optical properties of MPcs, such as the long wavelength of absorption (> 680 nm), the high molar absorptivity (> 10^5 $M^{-1}\cdot cm^{-1}$), lack of dark toxicity and relatively high photocytotoxicity, the promising MPcs with advanced properties towards pathogenic species are under development [19, 20].

The review presents new research studies, together with already published results, in order to give a general picture of the effects that functionalizing biocompatible groups have on the overall properties of MPcs photosensitizers when used against pathogens.

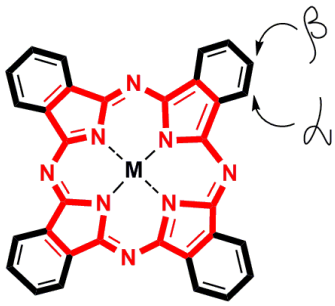


Fig. 1 Chemical structure of phthalocyanine complex (MPc) with the positions for conjugation.

2 Biologically-active groups to Zn(II)-phthalocyanines towards pathogens

The phthalocyanine molecule possess structural characteristics of symmetrical planar skeleton with sixteen positions for substitutions on the ring macrocycle and one or two axial positions for substitutions to the coordinated metal ions (M) (Fig. 1).

The peripherally substituted MPcs are assumed as more likely to interact with the target cells because of the extent area of these groups as well as the axial substitutions with bulky groups, which influence on the aggregation behaviour and arrangement of photo-inactive molecular associates [21].

2.1 Amino acids substituted Zn(II)-phthalocyanines

The chemical conjugates of phthalocyanines (Pc) with amino acids have been of research interest during more

than two decades [22–24]. The intention in the beginning was the improvement of solubility of highly hydrophobic Pc molecule. The further studies aim modifications of properties of absorption, fluorescence and singlet oxygen generation [25, 26]. In addition, some new essential biological properties appear related to the cell-specificity, the uptake and the photocytotoxicity. In our studies, four amino acids tyrosine, phenylalanine, lysine and arginine were selected to advance the properties of ZnPcs as photosensitizers [27]. The non-essential amino acids phenylalanine, lysine and arginine (for adolescent) are well documented with their positive physiological effects, curative action and the wide usage in medicine as prodrugs [28]. Next to their biological functions, they also have some specific properties as, for example, is the detectable fluorescence of tyrosine and phenylalanine [29]. Other selected amino acids are lysine and arginine, which are well documented with their ability of excellent penetration and interaction with cell membranes [30, 31]. These properties were expected to affect the physicochemical properties and the easy localization of ZnPc's functionalized with amino acids in sufficient amount for an effective pathogen's photo-inactivation. Ultimately the conjugation of two biologically-active molecules was expected to lead to a photosensitive compound with synergistic action on the target. Presently, treatments of acute infections related to pathogenic species have been settled with the chemotherapy with conventional and newly developed antibiotics [32]. Even so they are known to induce several side effects due to non-selectivity of uptakes, which can lead to harmful toxicity in healthy host tissues and the fast development of drug resistance to the treatment regime [33].

2.1.1 Synthesis of ZnPcs linked to amino acids

New bioconjugates of Zn(II) phthalocyanine with four and eight amino acids were synthesized. Two molecules of different origin were connected by amide bond between carboxyl groups of tyrosine, phenylalanine, lysine and arginine, and amino (aminophenoxy) group of phthalocyanine placed on four and eight peripheral positions to the Pc ring in the presence of coupling agents (Fig. 2).

The conjugation was performed using multi-step procedure to prepare two starting tetra- and octa-aminophenoxy- substituted Zn(II) phthalocyanines. Briefly, the used synthetic pathway presented in Fig. 3 starts with synthesis of dinitriles with nitrophenoxy group by following the reaction step of cyclotetramerization in the presence of zinc salt, the next step by reaction of reduction of nitro groups to amino groups were carried out according to the literature [34–36].

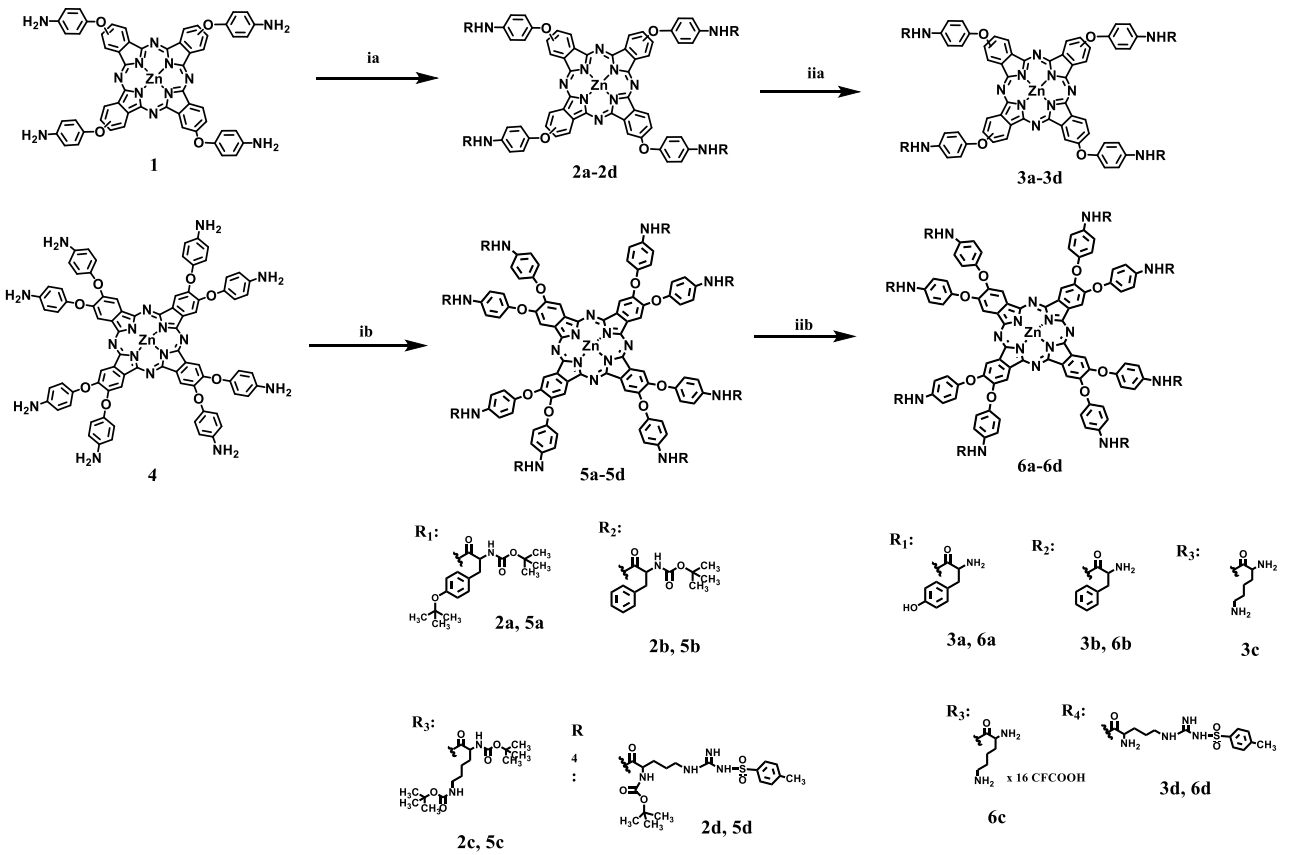


Fig. 2 Synthesis of Zn(II) phthalocyanines linked to amino acids (tyrosine, phenylalanine, arginine and lysine). Reactions and conditions: (ia) protected amino acid-OH, DMTMM, NMM, THF, ice bath, rt; (ib) protected amino acid-OH, DMTMM, NMM, DMF, ice bath, rt; (iia) TFA, THF, rt; (iib) TFA, DMF, rt.

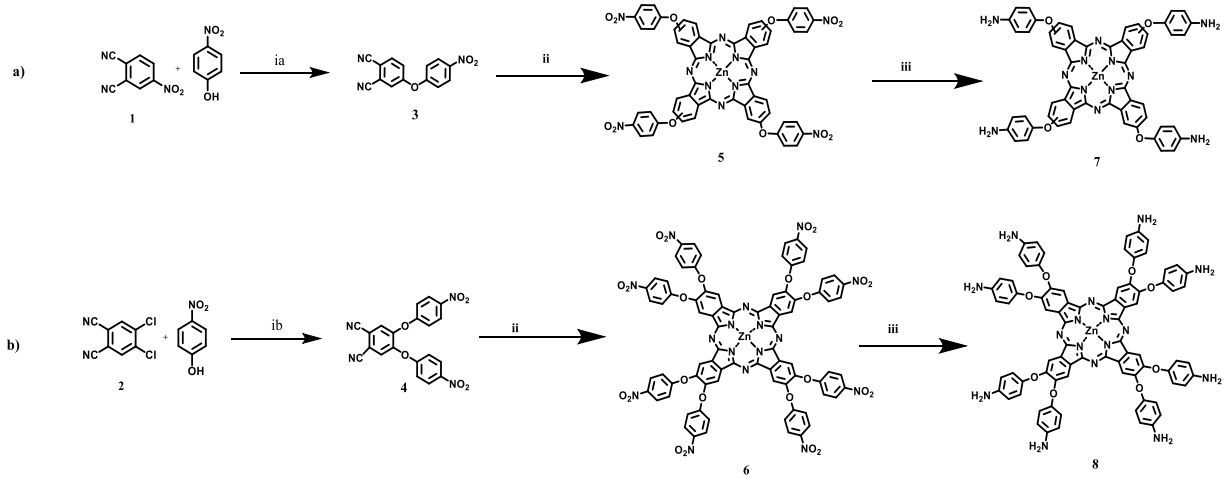


Fig. 3 Synthesis Zn(II) phthalocyanines for conjugation: (ia) K2CO3, dry DMF, RT, Ar; (ib) K2CO3, dry DMF, 90 °C, Ar; (ii) $Zn(OAc)_2$, DBU, 1-pentanol; (iii) dry DMF, Na_2Sx9H_2O.

Zn(II) conjugates with the selected amino acids were obtained with a relative yield: 44% for lysine conjugates to 87% for phenylalanine and tyrosine derivatives. The conjugates ZnPc with selected amino acids were analysed, with such known spectroscopic techniques as IR, ^{1}HNMR, MS and UV–Vis spectroscopy. They showed excellent solubility in most organic solvents. The aimed high solubility was observed for two ZnPc conjugates with lysine and arginine (Fig. 2-6a and 6c) showing water solubility.

2.1.2 Physicochemical properties

Electronic absorption spectra of the conjugates with amino acids and basic photophysicochemical properties were measured in Dimethyl Sulfoxide (DMSO) and data are collected in Table 1. The absorption spectra were all similar, which is characteristic for non-aggregated metalphthalocyanines. They showed a B band at 352 to 366 nm, a band at 617 nm and intensive sharp Q-band at 680 to 683 nm, which strictly followed the Lambert–Beer law. The amino acids lead to red shifts of the absorption maxima with 10 to 12 nm compared to unsubstituted ZnPc but the type of the amino acids less influence the position of the absorption maxima of the compounds. Upon excitation at 360 nm, 610 nm and 660 nm they showed relatively high fluorescence emissions at 690 to 694 nm but with low fluorescence quantum yield (Φ_F) from 0.018 – 0.069 except for tetra tyrosine substituted Zn(II) phthalocyanine ($\Phi_F = 0.12$). The lower values of the fluorescence quantum yield of the phthalocyanines may be due to the physical quenching by the bulky substituents on the amino acids in addition to the influence of free amino groups on peripheral positions of the ZnPc ring. The high values of the fluorescence quantum yield of the tetra tyrosine substituted ZnPc, which is comparable with the fluorescence quantum yield of unsubstituted ZnPc, is may be due to the contribution in the total fluorescence of the phthalocyanine and tyrosine molecules. Recently Zhang et al. [37] showed that the introduction of NH_2- groups to ZnPc may have different effects than the other studied substituents (−$OCH(CH_3)_2$, H, NO_2). This group can incite numerous roles: a good electron donor, a strong proton acceptor, a hydrogen bonding formation ability and a good ligand for coordination of metal ions. Moreover, the amino-substituted ZnPc may show very low fluorescence quantum yield as the new Zn(II) phthalocyanines with tyrosine, arginine, lysine and phenylalanine except tetra tyrosine substituted Zn(II) phthalocyanine on symmetrical peripheral positions presented but the fluorescence lifetime does not decrease significantly.

The lifetime of the bio-conjugates is comparable with lifetime of the unsubstituted ZnPc. The lower values of the fluorescence quantum yield and not so significant lowering of the lifetimes of new compounds compared to unsubstituted ZnPc can be explained by the effect of quenching of the unprotected NH_2-groups, which is in contribution to a high electron density of the molecules. Therefore, it is more likely to occur the photo-induced electron transfer (PET) and as a consequence is decrease of fluorescence yields. The protection with tetra-butoxycarbonyl group (Boc), which is electron withdrawing group can reduce the electron density of nitrogen atom and this leads to less effective quenching of the singlet excited state of the conjugates through PET [37]. This observation supports the reductive quenching of amino groups, which disfavour the intersystem crossing based on the intramolecular PET effect.

The efficiency of the Zn(II) conjugates with amino acids in generating singlet oxygen, as reflected by the rate of decay of the singlet oxygen quencher 1,3 - diphenylisobenzofuran (DPBF) in DMSO, was also examined. Tetra conjugates showed higher values according to their octa analogues as tetra tyrosine and phenyalanine conjugates ($\Phi_\Delta = 0.63$ and 0.71), with exception of tetra lysine conjugates, which has lower value of singlet oxygen ($\Phi_\Delta = 0.36$) than its octa analogue ($\Phi_\Delta = 0.57$) and almost same values with arginine compounds – ($\Phi_\Delta = 0.39$) for tetra compound and ($\Phi_\Delta = 0.40$) for octa- substituted ZnPc derivatives.

Table 1 Physicochemical properties of Zn(II) phthalocyanines linked to amino acids in DMSO.

PS	λ_{abs} (nm), (log ε)	Emission λ_{em} (nm)	Φ_F	τ_F (ns)	Φ_Δ	dC/dt (M/s)
3a	680 (3.02), 352 (–)	692	0.12	2.91	0.63	3.1×10^{-10}
6a	680 (4.36), 364 (3.94)	689	0.04	2.03	0.38	32×10^{-10}
3b	682 (4.23), 353 (3.93)	693	0.069	2.82	0.71	5.63×10^{-10}
6b	683 (4.17), 357 (3.88)	690	0.018	2.67	0.23	3.5×10^{-10}
3c	683 (3.63), 356 (3.38)	693	0.047	2.85	0.36	13.5×10^{-10}
6c	681 (5.28), 360 (4.86)	690	0.03	–	0.57	2.20×10^{-10}
3d	682 (4.27), 352 (3.97)	694	0.055	2.89	0.39	1.53×10^{-10}
6d	682 (4.02), 355 (3.73)	690	0.038	2.56	0.40	7.89×10^{-10}
ZnPc*	672 (5.21), 341 (2.61)	679	0.20	3.99	0.67	0.94×10^{-10}

The formation of molecular singlet oxygen does not affect the intensity of the Q-band of the conjugates, which suggests that there is no photooxidative effect on the phthalocyanine molecules during their irradiation. The option of singlet oxygen physical quenching is more probable by the eight peripheral substituents than four groups on ZnPc ring molecule. The physical quenching by bulky substituents was known to diminish the singlet oxygen quantum yields of some other substituted phthalocyanines [38]. The low values of the conjugates (0.23–0.40) correlate to the quenching of excited state due to PET effect of the amino groups closed to ZnPc (phenylamine and benzylamine groups) resulting in the lower singlet oxygen generation. This effect can be inhibited by the distance of NH_2 to the Pc-molecule, acidity of media and protonation of amino groups [39].

The photostability study of the tetra and octa Zn(II) phthalocyanines with the amino acids tyrosine, phenylalanine, lysine and arginine was carried out by following the absorption spectra during irradiation (60 mW·cm^{-2} at 665 nm wavelength). All studied compounds were as effective as unsubstituted ZnPc which showed relatively high stability at the applied light. The results are summarized in Table 1 showing the rates (dC/dt) in one of the same order of magnitude. The obtained rates suggested an optimal photostability without formation of the by-products due to irradiation. The collapse of the absorption spectra without any distortion of the shape which suggested the lack of structural alteration of phthalocyanine molecules in the spectra of irradiation was observed for all studied phthalocyanines.

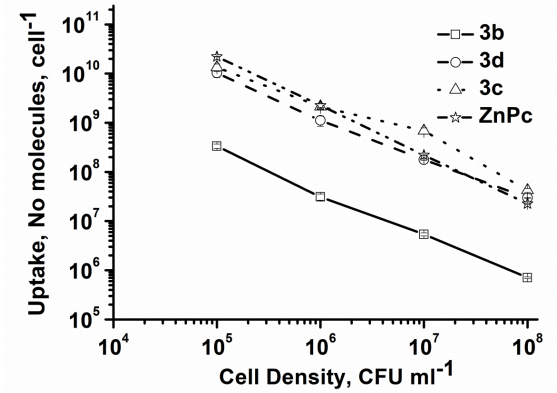
2.1.3 Uptake and photoinactivation efficacy

Uptake of the Zn(II) conjugates with phenylalanine, lysine and arginine (Fig. 2–3b–3d and 6b–6d) on fungal cells *C. albicans* were studied by using chemical extraction method which included incubation of the studied conjugates in the cellular suspensions with different cell density (10^5–10^8 Colony-forming units/mL (CFU/mL)). The collected supernatants with the extraction mixtures were measured the fluorescence between 650–800 nm with λ_{ex} = 637 nm. The results obtained are presented as number of molecules (No), uptake in a one bacterial cell (No / cell). Fig. 4 shows that Zn(II)Pc linked to amino acids have relatively high uptakes with values two order of magnitute higher for 3c and 3d (10^{11}) than for 3b (10^9). This low uptake of 3b is probably due to its low solubility at the extraction mixture (THF / SDS). The octa- compounds showed similar values (10^{11} No / cell). The compound 6b showed the highest level of uptake among the octa- compounds. The typical inverse behaviour of decreasing of the accumulation of the studied conjugates with increasing of the cell density (10^5–10^8 CFU / mL) of suspensions was observed as in for the other pathogens. The obtained results are in agreement with the observation that the cellular accumulation depends on the cell density of suspension [40].

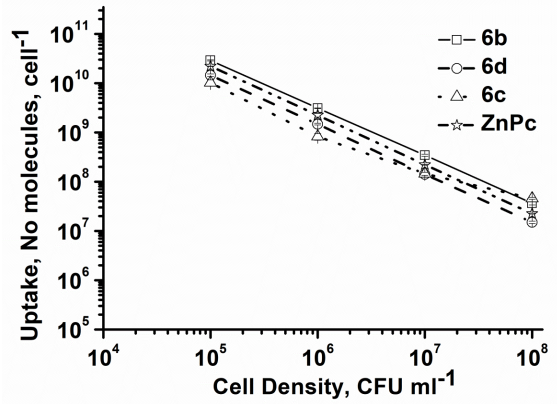
It is well studied that the cell membrane can bind more effectively the cationic compounds [41]. According to the obtained results with ZnPcs with phenylalanine, lysine and arginine the relatively high level of binding is a result of the amino groups which facilitate the binding to cell membrane of *C. albicans*. The interactions can occur by the hydrophobic nature of ZnPc or some physical force. On the other side lysine and arginine are able at physiological pH to act as cationic ZnPcs with higher accumulation and retention time of these compounds in membranes.

The photoinactivation study with Zn(II)Pcs with amino acids showed the lack of dark toxicity and low

photocytotoxicity (~2log) for *Candida albicans* (Fig. 5). The study was carried out at concentration of 10 μM and irradiation on 665 nm wavelength by Light emitted diode (LED) (light dose 50 J·cm^{-2} at 60 mW·cm^{-2} light intensity). Only both arginine substituted compounds (3b, 3d and 6d) showed complete photoinactivation, followed from lysine substituted 3c. These results are supported by the properties of singlet oxygen generation and the uptakes.



(a)



(b)

Fig. 4 Uptake of a) 3b, 3c, 3d; b) 6b, 6c, 6d (5.5 μM) in dependence on the cell density of *Candida albicans* in suspension with incubation time 20 min. The data are presented as means ±SD (n=3).

The carbohydrates such as galactosa and furanosa chemically linked to ZnPc were studied with low efficiency towards pathogenic microorganisms [42]. The low efficiency was also obtained with ZnPc conjugated with steroid [43].

2.1.4 In vitro photocytotoxicity

Photocytotoxicity assays were carried out with new conjugates (Fig.2–3a, 6a and 3c) in comparison with unsubstituted ZnPc and tetra aminophenoxy Zn(II) phthalocyanine (TZnPcNH$_2$) on two human breast cancer cells lines (MDA-MB-321 and MCF-7) and normal human cell line (MCF-10A). All of the studied conjugates (3a, 6a and 3c) showed no dark toxicity in a wide concentration range (0.15–20 μM) at irradiation with LED 665 nm and light parameters used in PDT study (50 J·cm^{-2} and 60 mW·cm^{-2}) [44]. Both tyrosine conjugates (tetra- and octa-) showed similar photocytotoxicity for concentrations up to 10 μM and with moderate photocytotoxicity at concentrations over 10 μM (37% viability for MCF-7 and 50% for MDA-MB-231). The healthy cell line (MCF - 10A) were not affected by irradiation for concentration between 1–10 μM from both tyrosine conjugates. Only unsubstituted ZnPc showed strong photocytotoxic effect towards tested cell lines. The amino acids – conjugates (3a and 6a) and ZnPc showed negligible photocytotoxic effect on normal cells (~80% viable cells) at concentrations bellow 10 μM and significant photocytotoxicity for concentrations over 10 μM for tumour cell lines (MDA-MB-231 and MCF-7). The interesting result is that the both aminophenoxy Zn(II) phthalocyanines used for conjugation showed very low phototoxic potential towards MDA-MB-231 tumour cells even at high dye concentrations and a lack of photocytotoxicity towards the cell lines MCF-7 and MCF- 10A.

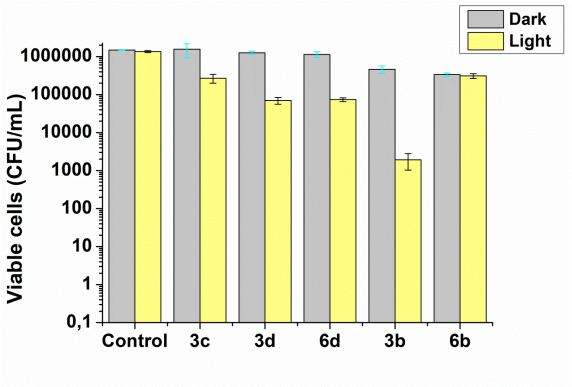


Fig. 5 Photocytotoxicity of 3b, 6b, 3c, 3d, 6d on *Candida albicans*.

3 Zn(II) phthalocyanines bearing both charges towards pathogens

The present knowledge about the influence of charge on the properties related to PDT efficacy is that the cationic photosensitizers including phthalocyanine complexes (MPcs) are better than anionic and neutral MPcs [45]. An interesting group of charged compounds which appears promising as photoantimicrobials are zwitterionic photosensitizers. They have the advantage of water solubility and low aggregation capability. Typical examples of zwitterions are amino acids with one amine and one carboxylic group where the basic NH$_2$- group is strong enough to deprotonate acidic-COOH group in solution [46]. These substituents have characteristic of a dipolar ion with positive and negative parts so that the net charge of the molecule is zero.

The zwitterionic Zn(II) phthalocyanines were synthesized by the treatment with 1,3-propanesultone at nitrogen of pyridiloxy group by following the well-known procedure [47]. Briefly, the reaction starts from dinitriles prepared from commercially available 4- and 3- nitrophthalonitrile in reaction with 3-hydroxypyridine in DMSO using potassium carbonate as a base. The both

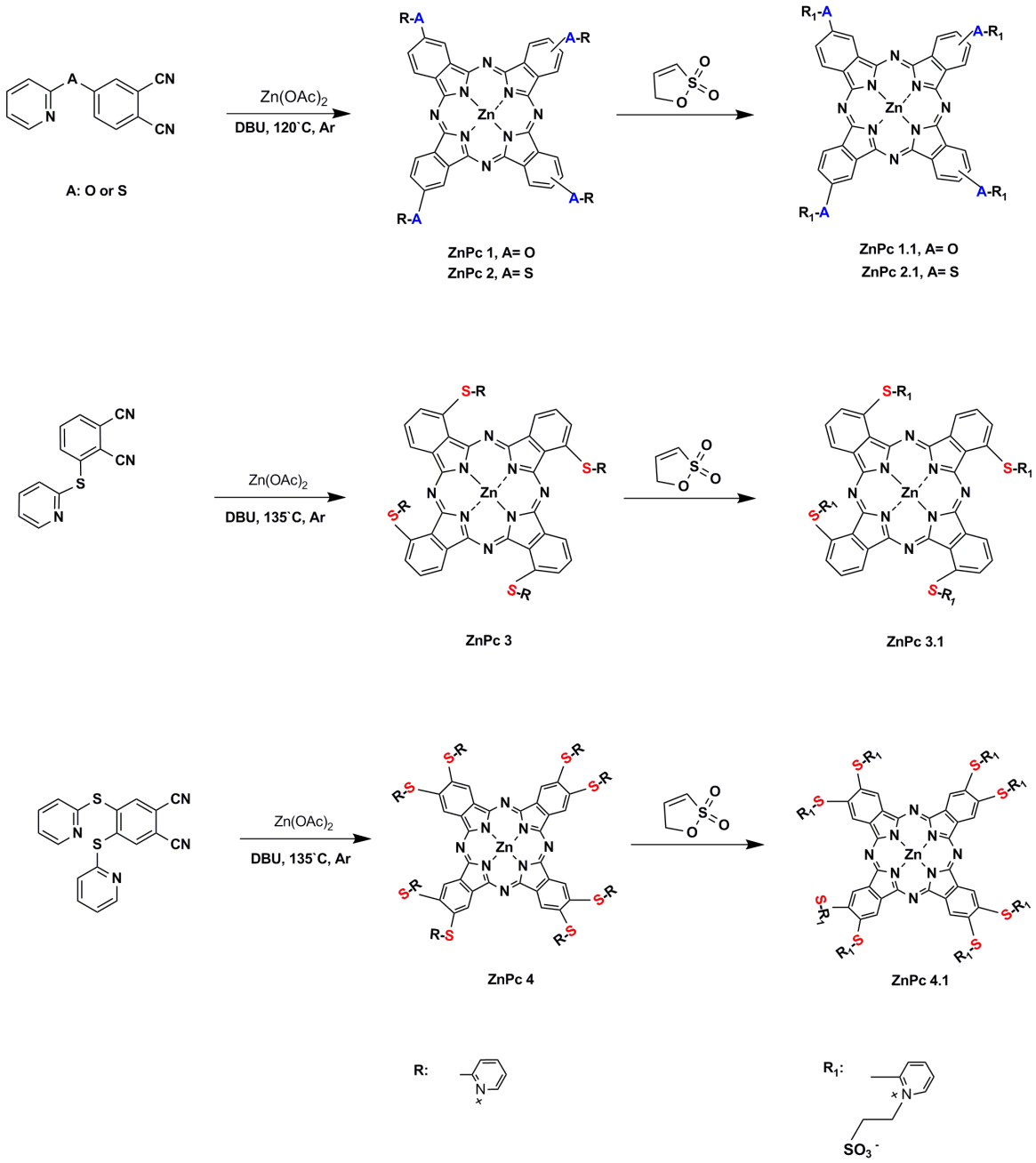


Fig. 6 Synthesis tetra-(3-/ 4-) pyridiloxy and pyridilsulfur Zn(II) phthalocyanines (ZnPc 1-4). Conditions: Zn(OAc)2, DBU, 1-pentanol and the derivatives ZnPc 1.1 – 4.1 after propanesulton in dry DMF.

reactions are productive for the chosen conditions (48 h, room temperature (RT)) and give the high purity products with approximately 80% yields. The physicochemical studies of differing in the positions zwitterionic ZnPcs suggested that they have promising photoproperties for biomedicine [48]. Nowadays together with different zwitterionic compounds such as carboxybetaine, phosphocholine, sulfobetaine also many phthalocyanines bearing selected as useful substituents have been synthesized and studied for PDT efficacy [37].

Our studies with the structurally different ZnPcs namely as the numbers of substituents (four and eight), the bridging atoms (sulfur or oxygen) and the positions of the substitution groups (Fig. 6). The peripherally substituted ZnPcs are as followed: tetra- 2-(N-propanesulfonic acid) oxypyridine (ZnPc1.1); tetra- 2-(N-propanesulfonic acid) mercaptopyridine (ZnPc2.1) and non-peripherally substituted compound is tetra- 2-(N-propanesulfonic acid) mercaptopyridine (ZnPc3.1), and octa- 2-(N-propanesulfonic acid) mercaptopyridine (ZnPc4.1). The bridging atom of sulfur add to absorption a red shift of approximately 10 nm (684 nm) as compared to the oxygen bridging atom in ZnPc1.1 (674 nm).

Two model pathogenic bacterial strains were tested, the both associated with the acute infections to humans namely a Gram (+) pathogenic bacterium *Enterococcus faecalis (E. faecalis)* and the Gram (–) bacterium *Pseudomonas aeruginosa* (*P. aeruginosa*), which is

known as a hardest to the inactivate, due to bacterial wall consisting of one additional layer of phosphatidyl-ethanolamines [49]. However, the Gram (–) species are also known with low susceptibility to the conventional therapy and with fast development of resistance to the treatment [50].

3.1 Uptake and localization study

The studied zwitterionic ZnPcs showed relatively high uptakes on resistant pathogenic bacterial strains of Gram (+) *E. faecalis* and Gram (–) *P. aeruginosa* as suspensions with different cell densities and as early stage biofilms formation [51]. It is assumed that the uptakes occur by a non-chemical attachment to the bacterial species in suspension or organized as biofilms. These compounds showed a high tendency of aggregation in water and buffered solution which can lead to ineffective photodynamic process. A Gram (–) *P. aeruginosa* was observed with less amount of the tested ZnPc1.1-4.1 with approximately one order of magnitude that the Gram (+) specie. The typical inversely proportional behaviour for the uptake values at the increasing cell densities was observed. This phenomenon is typical for any suspensions and repeated independently on the applied PS and type of treated bacteria since the first report for the Gram-negative *Escherichia coli* [52]. Many different pathogens and photosensitizing compounds were studied with similar accumulation behaviour [53, 54]. This may occur due to higher competition between the ZnPc molecules to bind to one bacterial cell with increasement of the bacterial density and as a consequence with the fewer available binding sites. The knowledge that the binding ability of the incubated ZnPcs can occur by hydrophobic or electrostatic interactions, or hydrogen bounding is valid also for zwitterionic ZnPcs, which possess the both charges in one substitution group. Obviously, this characteristic tends to improve the uptakes showing the almost equal values for octa-substituted ZnPc 4.1 for the both bacterial species.

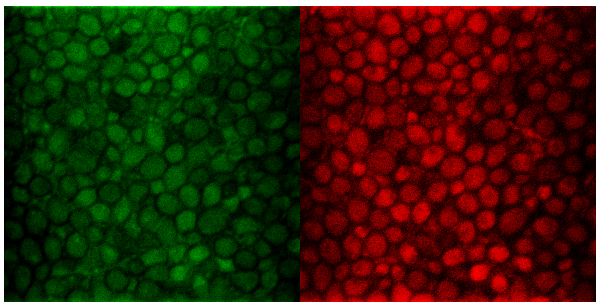


Fig. 7 The typical fluorescence confocal images of the slice of *C. albicans* biofilm (excitation 488 nm; emission 530–580 nm for the native fluorescence and excitation 635 nm; emission 660–720 nm for ZnPc1.1).

The biofilm's penetration and localization of zwitterionic ZnPcs in the fungal biofilms were evaluated by means of confocal laser scanning fluorescence microscopy (Fig. 7). The study is based on the fluorescence difference between the natural chromophores of cells which can be visualized by excitation with lasers 365 nm and 488 nm to image the typical green fluorescence in the region of emission 520–580 nm. The red fluorescence of ZnPc1.1-4.1 was observed at excitation with laser 635 nm for the emission spectra 660–740 nm as previously observed for bacterial biofilms [55, 56]. By the overlapping of the green channel and red channel of fluorescence signals was observed the significant ZnPc1.1 spreading throughout the biofilms. This was shown by two modes of observation, namely the fluorescence and transmittance. Among the studied ZnPcs, only non-peripheral ZnPc3.1 was observed with limited penetration in biofilms. The observed sufficient accumulation of ZnPc1.1, 2.1 and 4.1 into pathogenic bacterial biofilms suggested their potential for sufficient photodynamic inactivation. The different structures of the photoactive compounds influence the membrane permeability and on the uptakes [57].

3.2 Photoinactivation and dark cytotoxicity study

The cells were evaluated with saturation enough to expect the high generation of ROS products, which affect easily the membranes in the close vicinity of ZnPc4.1 with eight substitution groups was studied to have higher inactivation of planktonic and biofilm cultured. The photodynamic inactivation studies with zwitterionic ZnPc1.1-4.1 suggested different effectiveness of these compounds towards pathogenic bacteria, namely a complete photoinactivation with 6 μM ZnPc4.1 for the tested model strains (*E. faecalis* and *P. aeruginosa*). There was observed a difference in the activity of the tetra-substituted ZnPc1.1-3.1 with the lack of any effect of non-peripheral ZnPc3.1. The Gram (–) bacterium *P. aeruginosa* was not inactivated even at high drug concentration such as 30 μM ZnPcs. Two peripherally substituted ZnPc1.1 and ZnPc2.1 were evaluated with significant inactivations (> 3logs). The studies on normal cell line suggested the lack of dark toxicity but after irradiation the phototoxicity occurred. The irradiation was carried out with light source LED 665-nm on normal cells (Balb / c, 3T3) incubated with ZnPc1.1-4.1. The promising features of the zwitterionic ZnPcs are the lack of dark toxicity and a high phototoxicity at therapeutic light exposure on the normal cells.

In conclusion, the zwitterionic ZnPcs have charges influence on the uptakes and localization in an advanced way than the cationic ZnPcs derivatives [39, 58]. Moreover, the resistant Gram (–) *P. aeruginosa* showed promising results with the octa-substituted ZnPc4.1 as a result of the optimal physicochemical properties of singlet oxygen generation. It was still low-effective to inactivate an early-stage biofilm even at high doses of ZnPcs with the response below 3logs. The problem with pathogens resistance and not so efficient treatment of biofilms with PDT approach features the needs for improvement of the applied procedure in order to have better PS distribution on the grown biofilms and the effective inactivation of the resistant pathogens.

4 Lu^{3+} and Sn^{4+} phthalocyanines towards pathogenic species

The insertion of non-transition metal or semi-metal diamagnetic closed shell ions in the phthalocyanine core is well documented way for improvement of the main physicochemical properties associated with the excited states of the molecules [59]. The knowledge about the effect of the large atoms in regards to the photophysical properties of the phthalocyanine complexes (MPcs) is also well documented [60]. However, there are limited studies about the impact of the large metal ions (Lu^{3+} and Sn^{4+}) on the PDI efficiency. On one side, there is a steric hindrance effect due to the physical placement of large atoms out of the plane of macrocycle, which tends to lower the aggregation capacity of MPcs. On the other hand, the high coordination number of these metals permits attachment of one or more axial bulky groups of substituents, which also can decrease the aggregation behavior of the complexes.

Phthalocyanine complexes of lutetium ion (Lu^{3+}) have been of research interest due to their unique physicochemical properties and the ability for coordination of two or more Pc molecules [61, 62]. In regards to biomedical application, there is only one known Lu(III) complex, namely Lu(III)-texapyrine (Lu-tex^{TM}), which after many years of investigations, was considered for PDT and for diagnosis of cancer [63]. Nowadays the Lu(III)Pcs which are designed for PDT should be monomolecular and with cationic charge [64]. In our recent studies, two new water-soluble cationic lutetium (III) phthalocyanines (LuPcs) were synthesized and were investigated as effective towards fungus *Candida albicans* [65]. These are peripheral and non-peripheral Lu(III) phthalocyanines with four pyridyloxy groups which after methylation were converted to their water-soluble derivatives LuPc 1.1 and 1.2 (Fig. 6) [66].

The existing studies with tin (Sn(IV)) complexes for PDT are with purpurins, which were reported with an excellent PDT efficacy more than the related zinc complexes [70]. During 90s years this research resulted in a new PDT drug ($SnEt_2$) with very high potential to replace the originally clinically accepted porphyrin derivatives [67]. The known Sn(II/ IV) phthalocyanine complexes were explored with relatively high quantum yield of the triplet excited state and the favourable photo-physicochemical properties [68, 69]. In the similar way, two new Sn(IV) phthalocyanines (Sn4+Pcs) with hydroxyl groups on axial positions were synthesized. The obtained water-soluble Sn(IV)Pcs are the corresponding compounds to Lu(III)Pcs, which are differing in the positions of methylpyridyloxy groups in non-peripheral (SnPc 2.1) and peripheral (SnPc 2.2) positions (Fig. 8).

4.1 Synthesis of LuPcs and SnPcs

The dinitriles, which are differed in the position of pyridyloxy- group namely 4-(3-pyridiloxy) nitrophthalonitrile (1) and 3-(3-pyridiloxy) phthalonitrile (2) were used as monomers for cyclotetramerization [70]. The synthetic procedure was previously applied for synthesis of other complexes of MPcs with N- quaternized atom. The CN group was confirmed with the sharp narrow band at 2228 cm^{-1} and the aromatic ether group with vibrations at 1280 and 1253 cm^{-1} in the IR spectrum.

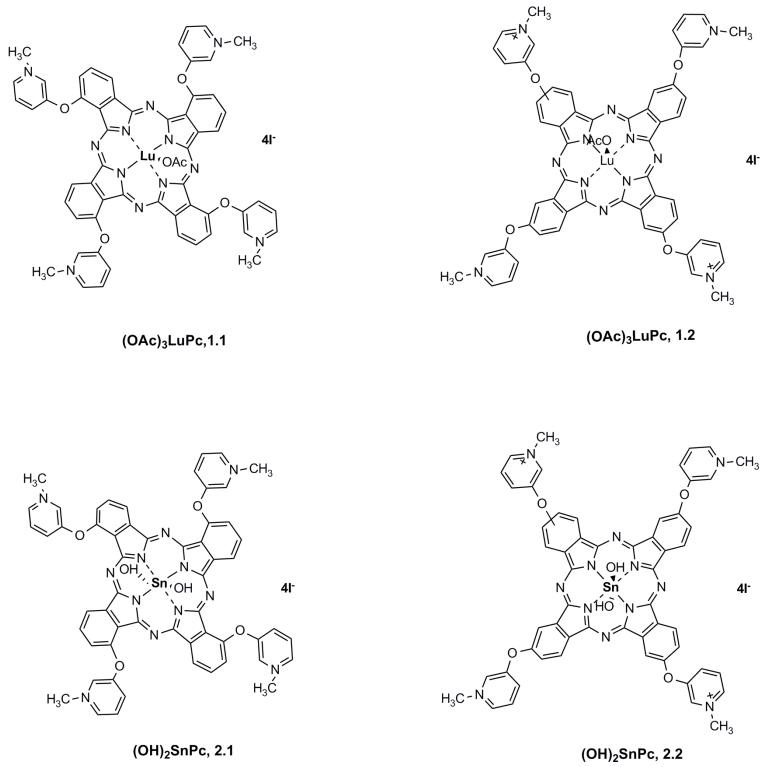


Fig. 8 Chemical structures of the studied MPcs coordinated with heavy metal ions of Lu(III) and Sn(IV).

The molecular ion peak at *m/z* 221 $[M]^+$ and two fragmentation ions peaks at *m/z* 127 $[M\text{-}C_5H_4NO^-]^+$ and at *m/z* 78 $[C_5H_4N]^+$ were obtained by means of MS spectroscopy. The cyclotetramerization reactions were carried out by addition of anhydrous salt of lutetium acetate and tin chloride by reflux in freshly distilled 1-pentanol, DBU in argon atmosphere. Another route involves Pc ring formation by the initial step of dissolved lithium pieces in n-pentanol. It was noticed the precipitation of the green product in 1-pentanol at the time of exchange of metal ions. The characteristic for the synthesis of Lu(III)Pcs is that the product was obtained as a mixture of three products, namely Li_2Pc, double-decker $LuPc_2$ and the mono-molecular LuPc (~60%) plus other impurities. The purification by column flash chromatography resulted in positional (region) isomers of desired product. The chemical characterization performed by the routine analyses showed ^1H NMR spectra with shifting of the signals depending to the position of the substituents and for CH_3 groups the recorded signals corresponded to the structures. IR spectra showed intense bands at 1237 cm^{-1} due to the presence of aromatic ether bonds (Ar-O-Ar). The mass spectra (MALDI-TOF) is characterized with a molecular peak *m/z* 1214.718 [M-OAc+DHB] for 1 and *m/z* 1214.398 [M-OAc+DHB] for 2. The final step of alkylation reaction was carried out with an excess of dimethyl sulphide or methyl iodide in DMF, which converted LuPcs into water-soluble derivatives 1.1 and 1.2. in relatively high yields (79–93%) and purity [71].

The similar synthetic procedure was followed to prepare the water-soluble SnPcs (Fig. 6). The specificity in the synthesis of tin complexes is that two different complexes Sn(II)Pcs or Sn(IV)Pcs can be obtained in dependence on the molar ratio between the starting monomer (dinitriles) and the used salt ($SnCl_4$). The complex Sn(IV)Pc was prepared by using an equal ratio between both mixed solids. By addition of an excess of tin salt, Sn(II)Pc was synthesized. Taking into account our further experiments, we have prepared Sn(IV)Pcs, which are assumed to be more capable to limit the aggregation due to axial substituents of chlorine converted to hydroxyl group by pyridine in ammonium hydroxide treatment. The reactions were carried out in 1-pentanol for 5 h with lithium granules to facilitate the formation of Pc ring and then the tin metalation was performed with the same equivalent of SnCl4 as for the starting dinitrile at reflux for one additional hour. The purification of SnPcs 1 and 2 was carried out with dichloromethane (DCM) and methanol (MeOH) in increasing of gradient of MeOH. The water-soluble SnPcs 3 and 4 were washed in a series of solvents with different polarity and then dried on glass oven overnight. Four new Sn(IV)Pcs were chemically characterized with the routine analytical techniques. 1H NMR spectra corresponded to the predicted structures with a typical shifting of the signals depending to the position of the substituents. The signals of CH_3 groups in the investigated compounds are well corresponding to the protons of four methyl groups. IR spectra showed the lack of the peak around 2230 cm^{-1} (C≡N) and the appearance of new peak at 3304 cm^{-1} for the complex. The aliphatic hydrocarbons showed stretching at 2910–2985 cm^{-1} and for aromatic C–H at 3015 to 3035 cm^{-1}. The mass spectra were obtained with the signals of M+ (1382) for SnPc2.1 and (1225) for SnPc2.2 which are *m/z* of these compounds.

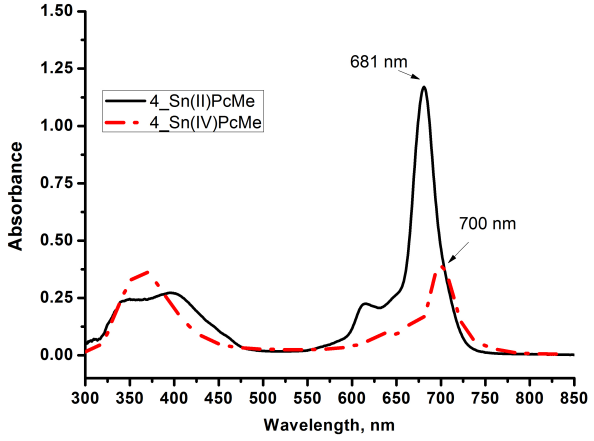
4.2 Physicochemical properties

Lutetium (Lu(III)) and Sn(IV) phthalocyanine complexes, which are differing only in the inserted metal ions were characterized with similar photo-physicochemical properties. Both complexes have red shifted absorption Q-band as compared to the same MPc with zinc metal ion (ZnPcMe). The absorption maxima were determined for the peripheral LuPc2 and SnPc2 at 675 nm and 678 nm in dimethylsulphoxide (DMSO) and even more shifted to 684 nm and 687 nm for non-peripheral LuPc4 and SnPc4 together with the low intensity peaks around 610 nm and the low-intensity B-bands between 360–367 nm. The quaternization of pyridyloxy-group leads to compounds with increased solubility but also to formation of the photo- inactive aggregates. For example, the absorption spectra of LuPcs (2 and 4) in water or buffer showed aggregation with a low intensity splitted Q-bands with appearance of the second band (681 nm for 2 and 692 nm for 4). Thus was previously observed with the other studies by us Mpcs (M: Zn, Al, In, Si, and Ge) with an exception of Ga(IV) Pcs, which exist as monomeric molecules in water [72]. The limitation of the formation of non-photoactive aggregates is possible by addition of an anionic detergent or some hydrophobic vehicle molecules [73]. The undesirable tendencies of formation of photo- inactive associates in water are also minimal for experimental studies in buffered media of the fungal cell suspensions.

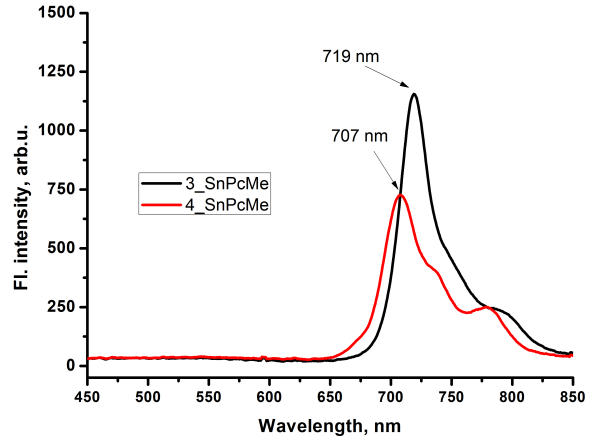
The fluorescence emission maxima were recorded at 704 nm and 721 nm for LuPcs and at 707 nm and 719 nm for SnPcs in DMSO (Fig. 9). The fluorescence spectra of Lu(III) Pcs and Sn(IV) Pcs showed a long red shift of the emission maximum (29–36 nm) as compared to the absorption maxima of Q-bands. The proximity of the wavelength in absorption and excitation spectra is indicative for the nuclear configurations of the ground and excited states, which are not affected by the excitation wavelength (365 nm, 635 nm, and 660 nm).

The fluorescence lifetime (τ_F) of a compound suggests the time interval, in which it can participates in photosensitization process. The fluorescence decay curves were mono-exponential and typical for non-aggregated molecular species. The fluorescence properties of quantum yield and lifetime of the complexes with the both metals (Lu^{3+} and Sn^{4+}) and the same substituted zinc complex (ZnPcMe) showed that the heavy atom ions complexes have relatively low quantum yields (0.01–0.08) and also lifetimes (2.24 ns for LuPc1.2 and 3.27 ns for LuPc1.1) as compared to the same properties of ZnPcMe, which have higher values (respectively 0.2; 3.99 ns). The very fast quenching of the fluorescence can be explained also by the presence of bulky substituents as well as the differences in the location of substitution groups so that the non-peripheral complexes underwent a faster quenching of the

fluorescence signal showing the shorter life-time than the peripheral derivatives.



(a)



(b)

Fig. 9 (a) Absorption spectra of Sn(II) vs Sn(IV) phthalocyanines, 2.2 and (b) fluorescence spectra of both new Sn(IV) phthalocyanines (2.1 and 2.2).

The capability of the both complexes of Lu(III) and Sn(IV) to produce singlet oxygen was measured in DMSO solutions in order to avoid some aggregation. The used indirect photochemical method is based on the oxidation of a chemical substrate 1,3-Diphenylisobenzofuran (DPBF) from the generated in the reaction vesicle molecular singlet oxygen during specific light irradiation. The calculations were performed referred to the quantum yield of the standard compound (ZnPc, 0.67 in DMSO) [74]). The values of singlet oxygen quantum yield were similar, namely 0.32 and 0.35 for LuPcs (1.1 and 1.2). In conclusion there is no significant difference in the singlet oxygen quantum yields by the replacement of Zn(II) and Al(III) with the large atom ions of Lu(III) and Sn(IV). The studies showed that there was significant decrease in the fluorescence quantum yields of Lu(III)- and Sn(IV) Pcs but not so much in their singlet oxygen quantum yields of importance for PDI efficacy.

4.3 Uptake and localization of Lu(III) phthalocyanines

The promising physicochemical properties of the water-soluble cationic Lu(III)- and Sn(IV)- phthalocyanines are the base for their photobiological investigations on pathogenic species. The expansion of the sensitiser uptake is known to depend on the structural modifications, one of which is the lack of symmetry in large atom's coordinated MPc molecules. *Candida albicans* is the most prevalent pathogen representing about 60% of all yeasts isolated in clinical samples. The uptake and localization studies were performed on the suspensions of following the fluorescence. The molecules attached to *C. albicans* cells were calculated on the basis of the fluorescence intensity of the spectra taken from extracted samples and referring to the calibration curves recorded for the same compound in the extraction mixture [74, 75]. The uptakes of LuPcs (3 μM) for different cellular suspensions (10^5 – 10^8 CFU mL^{-1}) followed the typical decrease of uptake with increase of the cell density. The samples were taken from the supernatant (1) in Phosphate-buffered saline (PBS) after incubation, (2) in PBS after 1st and 2nd cell wash, and (3) after cell extraction with a mixture of 2% Sodium dodecyl sulphate (SDS): Tetrahydrofuran (THF) or Dimethylformamide (DMF) (9 : 1) depends on the solubility of the studied compound. The obtained line dependence suggests that both Lu(III)Pcs and Sn(IV)Pcs are reliable to uptake into fungal cells and in amounts, which are not in a dependence on the coordinated metal ions. The typical inverse dependence of the decrease of the number of molecules accumulated into fungal cells with increase of the cell density was observed with a steep slope for the both LuPcs.

It is well accepted that cationic dyes are more likely to taken up by the bacterial and fungal species as a result of the nature of the cell wall and the binding process tend to occur via an electrostatic mechanism of interactions [76, 77]. The fluorescence confocal microscopy studies showed that the water-soluble cationic LuPcs have the surface localization in fungal cells with relatively high amount and in biofilms with extension inside the fungal cells. The full penetration into 48-h developed fungal biofilm occurred preferably on the cell wall and in the cytoplasm. The green fluorescence taken at excitation 488 nm and emission 500–580 nm shows the native fluorescence of the biofilm. The typical red fluorescence was observed for excitation with laser 635 nm and emission 660–740 nm. The formed *C. albicans* biofilms were determined with thicknesses between 17 and 23 μm and the scans on the slices showed the fluorescence within the whole biomass suggesting the full penetration of LuPcs.

The confocal fluorescence study of biofilms with Sn(IV) Pcs was not possible perhaps of the poor fluorescence intensity of the tin containing photosensitizers. The reason may be due to an improper excitation spectrum, which has limited light penetration and in case of fungal cells the cells inactivation may have happened layer by layer till the whole biofilm destruction

in a single step of irradiation. The phthalocyanine complexes with the same substituents but with different metal ions (ZnPcMe, SiPc1, GePc1, GaPc1 and GaPc2) were with approximately 2/3 limited penetration on the basis of the entire biofilm [66, 74]. The observation that Lu (III)Pcs completely penetrate into 48-h fungal biofilm can be explained with the cationic charge of the methylpyridyloxy groups as well as with some loss of planarity of the Pc ring due to large size of Lu atom. Even so the fungal biofilms incubated with LuPcs demonstrated the full penetration depth after the proper light excitation (635 nm) but very low photodynamic response (< 3 log) on the studied biofilms. [74]. The uptake and localization behaviour of Lu(III)Pcs seems to be in advance to their application in optical diagnosis. The complexes with large atom ions such as lutetium and tin together with the charge feature are an effective structure approach for improvement of the photosensitizer's penetration and selectivity for better photodynamic inactivation.

4.4 In vitro study on C. albicans

The phthalocyanine complexes with large atoms of Lu(III) and Sn(IV) were studied for their antimicrobial photodynamic efficacy on the pathogenic fungus *C. albicans* as suspension with density of approximately 10^7 CFU·mL^{-1} [78]. The studies were carried out with a wide concentration range of Lu(III)- and Sn(IV)-Pcs (1 μM – 30 μM). The photodynamic response towards *C. albicans* was lower than 3 log for 1–20 μM MPcs. The photoinactivation studies suggested that despite the significant uptake of both kinds of MPcs tested in these concentrations, the complete photo-inactivation was observed over 20 μM at the optimal tested light parameters (50 J·cm^{-2} and 60 mW·cm^{-2}). Lu(III)Pcs as well as Sn(IV)Pcs showed lack of dark toxicity for the studied concentrations. Considering the impact of the coordinated metal ion, the PDT effect of water-soluble LuPcs was studied in comparison to Zn(II) phthalocyanine with the same functional groups as substituents (ZnPcMe). At as low concentration as 3 μM MPcs the lower uptake was determined for the both LuPcs as compared to the uptake of ZnPcMe for *C. albicans* within a range of cell density (10^5–10^8 CFU / mL). Zn(II) and Si(IV) coordinated complexes were studied with higher phototoxic effects towards *C. albicans* for lower concentrations than that needed with LuPcs and SnPcs. All PDT studies were performed without cell washing after incubation followed by exposure with mild light dose (50 J·cm^{-2}) from a specific light source (LEDs emitted at 635 or 665 nm). The relatively high singlet oxygen quantum yields and high uptake in cells resulted in low PDI effect which can be explained with the lower cell susceptibility to singlet oxygen and another ROS.

The comparisons of the PDT responses of Lu(III)Pcs versus Sn(IV)Pcs on *C. albicans* planktonic and biofilm showed that ZnPcMe, which has the same substituents and differs only with Zn(II) coordination showed the photoinactivation with higher activity towards pathogens in suspension as well as biofilms. The molecular electronic structure due to replacement of Zn(II) with Lu(III) or Sn(IV) has changed the photophysical parameters but doesn't influence in a positive way on the photodynamic efficiency. The much harsh photodynamic conditions are needed together with repetitive application of the procedure in order to be effective the treatment of pathogens biofilms.

5 Conclusions

The knowledge of the structural characteristics, composition, location and nature of the substituents, the charge of the molecules and the effect these features have on the pharmacokinetic characteristics of phthalocyanine complexes used as photosensitive compounds for PDT of tumour cells and PDI of pathogenic microorganisms are essential.

The biologically active substituents used to increase selectivity of phthalocyanine complexes with respect to the treated objects give specific characteristics to these compounds both in terms of accumulation and in terms of localization in cellular structures and in the ways of treating the objects in order to increase the efficiency of light-activated photodynamic reactions.

The coordination of the phthalocyanine complex by means of large metal ions provides additional opportunities both for optimizing the physicochemical and pharmacokinetic characteristics of these complexes and for realizing compounds with different hydrophobicity. It is also a promising method for increasing their efficiency and reducing the toxicity of phthalocyanine complexes in their functionalization with bioactive conjugates such as sugars, sterols and amino acids.

In the use of phthalocyanine complexes for PDI of pathogenic microorganisms, the realization of compounds with affinity to the potential of bacteria membrane is of extremely importance for efficiency.

In conclusion, in terms of the realization of even more effective phthalocyanine photosensitizers, in-depth additional and appropriate studies are necessary to link the effectiveness of PDT with the structure of the compounds.

Disclosures

All authors declare that there is no conflict of interests in this paper.

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