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Original article

Photodynamic efficacy of water-soluble Si(IV) and Ge(IV) phthalocyanines towards *Candida albicans* planktonic and biofilm cultures

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ABSTRACT

Water-soluble phthalocyanine complexes of silicon (SiPc1) and germanium (GePc1) were synthesized. The absorbance of SiPc1 in water was with minor aggregation while GePc1 strongly aggregated in water. The fluorescence data in water showed low quantum yields of 0.073 (SiPc1) and 0.01 (GePc1) and similar lifetimes of 4.07 ns and 4.27 ns. The uptake of SiPc1 into *Candida albicans* cells was two orders of magnitude lower as compared to GePc1 and for both was dependent on the cell density. Fungal cells in suspension were completely inactivated after SiPc1 (1.8 μM) at soft light radiation (50 J cm^{-2}, 60 mW cm^{-2}). The fungal biofilm formed on denture acrylic resin was inactivated with 3 log after fractionated light irradiation.

1. Introduction

The antibiotic resistance of pathogenic microorganisms has forced the research efforts in the field of the photodynamic therapy (PDT) as an alternative, antimicrobial approach [1]. PDT for treatment of infections appears as a distinct technique in case of multidrug-resistant pathogens, although the fact that the method is still under research stage [2,3]. The photodynamic process utilises the photosensitizer (PS), the proper light from the visible and near IR spectra and surroundings of molecular oxygen. Upon irradiation, PS becomes excited and the absorbed energy is transferred to ground-state triplet oxygen to undergo electron alterations to highly reactive singlet oxygen (type II mechanism) which causes cell death. The alternative mechanism includes an electron or hydrogen transfer from the triplet state of PS to the cellular-associated biomolecules (type I mechanism). During the both processes the generated highly reactive oxygen species (ROSs) induce the membrane damages and consequence photoinactivation of pathogenic microorganisms. Most of PSs act by mechanism of singlet oxygen generation for the high cytotoxic effect [4].

The chemical aspects of PDT for treatment of microbial infections include the development of more efficient second generation photodynamic PS. Along with the well accepted phenothiazine and porphyrin derivatives, phthalocyanines (Pcs) have been of great interest during the last two decades [5–7]. The metal complexes of Pc (MPcs) characterize with intensive absorption maxima of the Q band around 675 nm and the red shifted fluorescence peak around 690 nm in organic solvents [8]. The Pc-molecule has strong lipophilic nature and on the other hand the flexible to tailoring chemical structure, which allows modifications. These include the substitution with suitable functional groups on the peripheral or non-peripheral positions of the macrocycle and axially to the coordinated metal ion. The substituents can strongly influence the hydrophobic nature and the overall charge distribution of MPcs. The combination of suitable bulky functional groups on the peripheral and axial positions influences on the water solubility and on the macromolecule being in monomeric state. The water-soluble MPcs are the better choice for PDT vs. their hydrophobic derivatives despite the fact of their aggregation in aqua media. The known MPcs with essential PDT activity have closed p- or d-electron configuration of the coordinated metal ions into ligand

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[9]. Apparently, have been reported well defined fluorescence quantum yields together with a long life-time of the triplet excited state of MPcs [8,9]. During the last decade, the PDT with MPcs, which are complexes of Zn(II), Al(III) and Si(IV) pass though all stages of investigations and were approved for clinical applications on a number of oncological and non-oncological conditions [10].

Recent studies on microorganisms reported different cationic substituents to the Pc-ligand that lead to an optimal hydrophobic/hydrophilic balance and charge distribution for improved uptake into microbial cells [11–14]. In addition the chemical structure is well accepted as a crucial factor for the good membrane diffusion, cellular localization and the final photodynamic response [15].

The oral infections are provoked by the increase amount of pathogenic microorganisms in the oral cavity and further formation of the biofilm on the tooth or the denture surfaces [16,17]. Our recent studies with numerous cationic, water-soluble MPcs coordinated with Zn(II), Ga(III), and In(III) metal ions and peripherally substituted with four or eight methylpyridyloxy functional group, suggested a potential value of MPcs for treatment of wide range of pathogenic microorganisms [18–20]. The inactivation of representative strains of pathogenic cells, all in planktonic phase was previously studied with MPcs (M = Ga, In, Al and Zn) [19]. However in case of biofilms of *Candida albicans* (*C. albicans*) the treatment with PDT has one limitation of the incomplete penetration depth into biomass [20]. The biofilms of *C. albicans* that were grown for 18 h on acrylic resin were not susceptible to PDT with phenothiazine dye methylene blue (MB) and also with the studied complexes of Ga(III) and In(III). The treatment of biofilms was effective only after complex of Zn(II) with four methylpyridyloxy-groups (ZnPcMe) and of Ga(III) with eight methylpyridyloxy-groups (GaPc2) [20]. Several authors reported techniques to improve the drug penetration into biofilms [21–23]. For example the combine action of PDT to destroy the polymeric matrix followed by antibiotic application [21]. The other is an ultrasound effect together with PDT to allow a low integrity of the biofilm by forming channels into the matrix [22] or the usage of selective to the pathogenic cells drug delivery nanoparticles for treatment of dental biofilms [23].

The present study aims the synthesis of tetra-methylpyridyloxy-substituted Si(IV)- and Ge(IV)-phthalocyanines (SiPc1 and GePc1) and their investigation as photodynamic sensitizers for inactivation of *C. albicans* as planktonic and biofilm cultures. Both quaternized MPcs were synthesized by modification of the previously described chemical procedure in order to obtain high yield and purity. The absorbance and the fluorescence properties were studied in water and in presence of additives that allow disaggregation, and in dependence on the temperature. The cellular uptake of SiPc1 and GePc1 into *C. albicans* cells in suspension was investigated in comparison. The penetration depth and the localization ability of water-soluble SiPc1 and GePc1 into 48 h fungal biofilm were assessed. The photodynamic responses of SiPc1 and GePc1 were compared to the photodynamically active ZnPcMe for *C. albicans* biofilms formed on denture acrylic resin.

2. Results and discussion

2.1. Synthesis

The starting compound 4-(3-pyridyloxy)phthalonitrile (**1**) was prepared by following a slightly modified procedure of Refs. [24,25]. The commercially available 4-nitrophthalonitrile and 3-hydroxypyridine were mixed in dry DMSO together with potassium carbonate as a base. The reaction mixture was stirred for five days at room temperature and the high purity product was finally isolated in a good yield (80%). The IR spectrum confirmed the presence of CN group with positioned at 2228 cm^{-1} sharp, narrow band and the aromatic ether group with characteristic vibrations at 1280 and 1253 cm^{-1}. The EI-MS spectrum showed a molecular ion peak at *m*/*z* 221 $[M]^+$ and two fragmentation ions peaks at *m*/*z* 127 $[M - C_5H_4NO^-]^+$ and at *m*/*z* 78 $[C_5H_4N]^+$.

4-(Pyridyloxy)-1,3-diiminoisoindoline (**2**) was obtained from the phthalonitrile (**1**) by bubbling with ammonia gas in the presence of sodium methoxide in methanol according to known modified procedure [26]. The attempts to purify product (**2**) by column chromatography were not successful. Hence, after solvent removal and drying under vacuum, the highly hygroscopic product was used as obtained in the next step. The reaction mixture soon after initiation turned slightly greenish. This observation is consistent with the fact that diiminoisoindolines display increased tendency towards cyclization and readily react to form phthalocyanines even at very mild conditions. The peak is very small in the ESI mass spectrum. The diiminoisoindoline is thermally not very stable and finally under heat forms the phthalocyanine. Therefore under conditions of the heating during ESI-MS also dimer as precursor of phthalocyanine is formed.

Diiminoisoindoline (**2**) was proved to be an excellent precursor for the synthesis of phthalocyanine complexes. In order to obtain SiPc1 at first the non-alkylated SiPc2 was prepared. The product (**2**) was reacted with $SiCl_4$ in anhydrous, freshly distilled quinoline (200 °C, 1 h). The formation of the SiPc2 proceeds quickly. The chlorine atoms exchange at the silicon was achieved by refluxing the reaction mixture with ammonium hydroxide at RT overnight. SiPc2 was synthesized in 54% yield. The electrospray mass spectrum of SiPc2 showed molecular ion peak at *m*/*z* 946 $[M^-]$. In the second step SiPc2 was converted into the positively charged, water-soluble derivative SiPc1. The reaction was carried out by stirring SiPc2 with an excess of methyl iodide in dry DMF. The water-soluble phthalocyanine SiPc1 was isolated in high yield (83%). The water-soluble complex GePc1 was obtained in a good yield of 88% via GePc2 (yield 77%). The electrospray mass spectrum of GePc2 showed *m*/*z* 992 $[M]^+$, 974 $[M - H_2O]^+$.[1] The ESI positive mass spectra of SiPc1 and GePc1 showed the molecular ions at *m*/*z* 251.3 $[M - 4I^-]^{4+}$ and *m*/*z* 263 $[M - 4I^-]^{4+}$, respectively.

IR spectra of SiPc1 and GePc1 showed intense bands at 1237–1278 cm^{-1} due to the presence of aromatic ether bonds (Ar–O–Ar) and the characteristic low intensity IR vibrations at 2941 and 2830 cm^{-1} and 1395 cm^{-1} of methyl groups.

The structures of all synthesized phthalocyanines were also assessed by ^1H NMR. The obtained proton spectra confirmed the structure assigned to the compounds. The presence of several regioisomers of the analysed substances resulted in a complicated multiplicity of the proton resonance signals especially in the aromatic region. SiPc1 signals in the interval 8.30–8.63 ppm could be attributed to the protons of the core aromatic structure of the phthalocyanine ring. The resonances of the aromatic protons of the R-constituents are subjected to significant deshielding effect of both oxygen from the bridge structure and the nitrogen in the heterocycle which are shifted downfield (9.00–9.77 ppm). The existence of at least two regioisomers of SiPc1 accounts for the two N-methyl group singlets at 4.47 and 4.49 ppm. The position and the shape of the hydroxyl proton signals could be quite different

[1] The mass spectrum of GePc2 has, due to various isotopes of Ge, a complicated calculated isotope pattern (including ^{13}C): *m*/*z* 988 (42.9%), 989 (27.0%), 990 (66.7%), 991 (55.1%), 992 (100.0%), 993 (54.7%), 994 (33.9%), 995 (14.4%), etc. Therefore also fragments were showed this complicated pattern. The OH groups at the Ge of GePc2 are reactive and can be protonated as we know. It is possible that in the solution of GePc2 for ESI some protonation at Ge–OH occurs followed by elimination of H_2O.

depending strongly on the chemical environment and the solvent medium. Even the absence of specific hydroxyl proton resonances is quite a common phenomenon especially in protic solvents as water or methanol. Even though as solvent for the NMR-studies, we used DMSO which in most cases tends to "fix" the proton signals, we were not able to observe the resonances of the two hydroxyl groups coordinated to Si(IV) as well as to Ge(IV). This most likely is due to the specific effect of the adjacent metal ion.

In addition the purity of all obtained phthalocyanines was proved by elemental analysis.[2] The data are consistent with the predicted structures as shown in the Experimental section.

2.2. Absorption spectra

The metal phthalocyanines SiPc1 and GePc1 exhibit the intensive Q-bands between 675 and 681 nm (Fig. 1a,b). The quaternized complexes are soluble in water, but only SiPc1 exists in great amount as monomer in aqua solutions with slight aggregation (Fig. 1a). The spectrum of SiPc1 in water shows the Q band at 675 nm, which is accompanied by a band around 643 nm attributed to the aggregated form of phthalocyanine. The disruption of aggregates may be achieved by changing the dielectric constant of the medium [27]. The well investigated sulfonated phthalocyanine derivatives were disaggregated by addition of cationic detergent [28]. A very effective approach to circumvent aggregation phenomenon is to employ surfactants, which form micelles in which Pc molecules may be disaggregated. The water-soluble cationic MPcs prepared during this work have charged groups at their periphery, such as quaternary pyridyl groups. The nature of the charge determines the choice of the detergent because the ionic Pc disaggregates better in micelles formed from surfactants with opposite charge [29]. For example, positively charged Pcs are bound to the anionic head group of the SDS at the micelle interface, while its long hydrophobic chain interacts with the phthalocyanine ring and screens it from forming aggregates. In contrast, with a positively charged detergent in water no disaggregation could be observed. Presumably, an addition of the oppositely charged to methylpyridyloxy-substituents attached to MPcs, an anionic sodium dodecyl sulfate (SDS) detergent reduced the aggregation (Fig. 1a). This was seen by a decrease of the band at 643 nm in spectra of SiPc1. The absorption spectra of SiPc1 in some organic solvents such as dimethylsulfoxide (DMSO) and tetrahydrofuran (THF) were also acquired (Fig. 1a).

In contrast, GePc1 showed a very strong aggregation in water (Fig. 1b). In the presence of SDS, the absorption spectrum is dominated by an intense absorption band at around 645 nm, which is characteristic of the strong aggregation. Owning to the knowledge that the aggregation behaviour of known MPcs depends on the concentration, on the metal ion into ligand, on the kind of substituents and on the polarity of solvent, and temperature, an increase of the temperature was applied. The progression of monomerization of GePc1 was observed in pyridine:water (1:1) at high

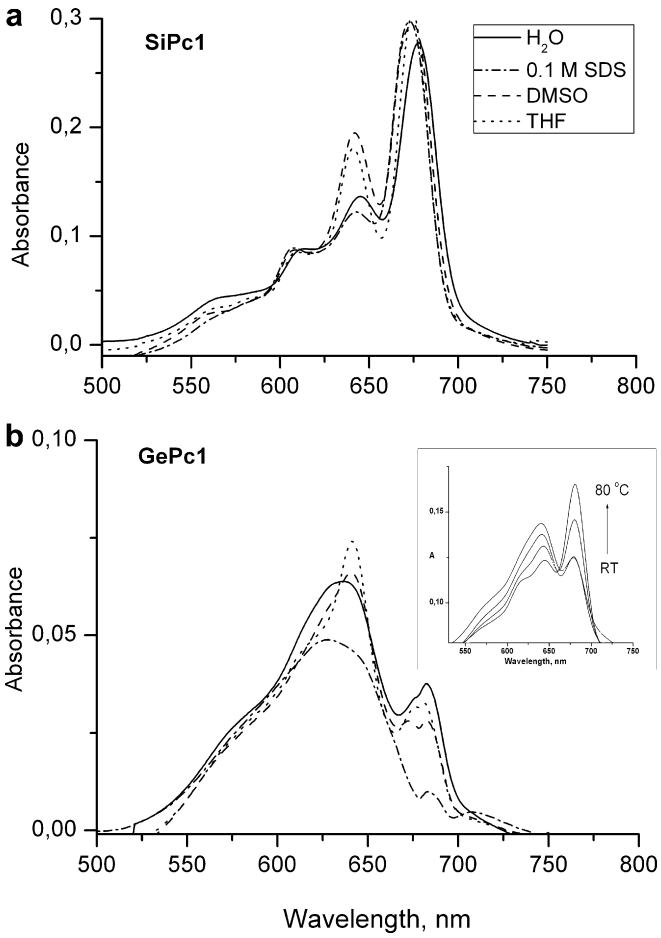


Fig. 1. Absorption spectra of SiPc1 (a) and GePc1 (b) recorded in water in concentrations of 1.2 μM and the spectra in 0.1 M SDS, and in organic solvents. Inset: the absorption spectra of GePc1 in water: pyridine (1:1, v/v) recorded at rising of temperature.

temperature (80 °C) with an enhancement of the red shifted peak (Fig. 1b, inset). The studied SiPc1 and GePc1 monomerized in binary solvent mixture of water containing pyridine (50%) but not a complete disaggregation was occurred in case of GePc1 (spectra are not shown). In the UV region a wide B band between 345 and 361 nm with half of the intensity of absorption of the Q band was observed. SiPc1 being of monomeric state was measured by absorbance in 0.1 M SDS in wide-ranging concentration ($0.5–9 \times 10^{-6}$ mol L^{-1}).

The recent studies of water-soluble MPcs coordinated with atoms of Ga(III) and In(III) reported that only quaternized Ga(III)-phthalocyanines are monomers in aqua surrounding [30–35]. This phenomenon was explained by the axial ligands such as chloride or hydroxyl groups to the large atom of gallium, which can prevent the aggregation [20,31,32]. However, in case of indium ion, it was shown that InPcs in water forms aggregates [31–34,36]. In the present work, the axial substitution with two hydroxyl groups to the coordinated metals Si(IV) and Ge(IV) was used. However, it was shown that only SiPc1 exists in high amount as monomeric in aqueous media.

2.3. Fluorescence study

The fluorescence emission spectra of SiPc1 and GePc1 in water and in the presence of an anionic detergent (SDS), and of Cremophor EL (CEL), and in organic solvents were acquired (Fig. 2a,b). The

[2] SiPc2 contains a very small amount of SiO_2 formed by hydrolysis of $SiCl_4$. As described in the Experimental section, the synthesis was carried out in freshly distilled quinoline, and $SiCl_4$ was added by a syringe under inert gas. However, even under these conditions a small rest of moisture may lead to hydrolysis, and then the very small amount of probably colloidal SiO_2 cannot be removed totally during the purification process. This is also the reason that the found C and *N* values are a little bit too small compared to the calculated values. The same is for GePc2. These very small amounts of SiO_2 and GeO_2 are inert and have no influence on the further reactions to the final SiPc1 and GePc1. Only these two compounds were used then for the further described investigations. Therefore, we decided to omit the Si and Ge values of the elemental analyses. In addition the combined analysis by UV–vis, NMR, IR, MS and elemental analysis is a good statement for the purity of the compounds.

fluorescence emission maxima are red shifted to 683 nm for SiPc1 and to 686 nm for GePc1 as compared to the absorption maxima 678 nm (SiPc1) and 637 nm and 684 nm (GePc1) in water.

The fluorescence maximum in water plus detergent (SDS) was more intensive due to formation of monomers of SiPc1 (Fig. 2a). The strong aggregation of GePc1 persisted even in the presence of detergent or CEL, which was determined also by the low intensity of fluorescence. The fluorescence maxima in organic solvents such as THF and DMSO were between 678 and 680 nm for SiPc1 and 684–686 nm for GePc1. The low intensity fluorescence maximum of GePc1 in all used solvents suggests a strong aggregation which was shown also by absorption spectra (Fig. 2b).

The fluorescence quantum yields (Φ_F) of SiPc1 and GePc1 in water and organic solvents were determined. The Φ_F values for SiPc1 were 0.073 in water to 0.101 in the presence of a detergent (SDS). In addition the Φ_F values for SiPc1 in DMSO (0.195), in THF and DMF (0.175 and 0.151) for SiPc1 were determined. The observation that Φ_F value of SiPc1 in water (0.101) is one order higher than that of GePc1 (0.01) suggests some quenching due to aggregation activities. It was estimated that the electronic configurations of the ground and excited states of SiPc1 and GePc1 were not affected by the applied excitation wavelengths (380 nm, 610 nm and 660 nm).

As is well-known a significant fraction of the excited MPcs in singlet state decays by fluorescence which is useful for diagnosis [37]. In present study both MPcs showed fluorescent quantum yields and life-times strongly affected by the solvent and by the formation of aggregates. In water solutions the Φ_F values were relatively low (0.01 for GePc1 and 0.101 for SiPc1) due to formation of dimers and higher oligomers. The aggregates of GePc1 were persisted even at low dye concentration and in some organic solvents (DMSO and THF).

The time-resolved fluorescence study of SiPc1 and GePc1 were carried out in DMSO (Fig. 3). The fluorescence decay of SiPc1 and GePc1 resulted in monoexponential curves with life-time (τ_F) of 4.273 ns (SiPc1) and 4.065 ns (GePc1), and 3.990 ns (ZnPc). The obtained values are independent on the excitation or the observation wavelengths. Fig. 3c presents data of ZnPc with τ_F value of 3.99 ns with monoexponential decay, which suggests the monomeric molecules in the volume.

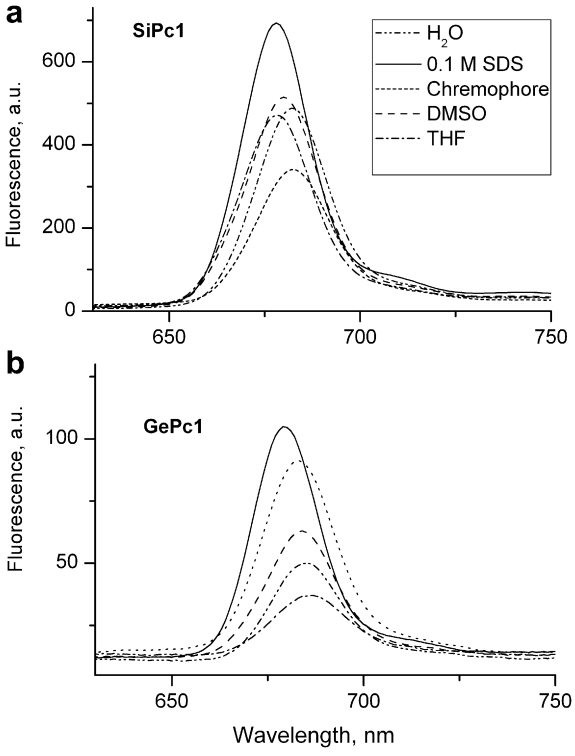


Fig. 2. Fluorescence spectra of SiPc1 (a) and GePc1 (b) in water in comparison to the spectra in presence of detergents and in organic solvents at excitation wavelength, λ_{exc}: 610 nm.

Most of quaternized ionic MPcs showed aggregation behaviour in water [30]. That is why Φ_F values of there complexes in water are for the mixture of the monomer-aggregated fractions. The obtained Φ_F values for SiPc1 and GePc1 are basically typical to those reported for related hydroxyl or mercaptopyridine substituted MPcs [31–35]. The phthalocyanine complexes such as the peripherally methylpyridyloxy-substituted GaPc, which exists as monomeric in water has relatively high values of Φ_F (0.24 in DMSO and 0.12 in water) suggesting not much of quenching of the excited singlet state by the substituents [31]. In case of quaternized tetra-2-mercaptopyridine substituted MPcs complexes of Ga(III) and In(III) (Qp-GaPc and Qp-InPc) which showed the mixture of monomer-aggregated MPc species in water, the Φ_F values were as expected lower (0.074 for Qp-GaPc and 0.0008 for Qp-InPc) than that in DMSO (0.12 and 0.021) [32]. Comparing the used solvents DMSO and water, the quaternized SiPc1 and GePc1 like the related PSs, were evaluated with larger Φ_F values in DMSO than in water. The phenomenon was explained by the higher viscosity of DMSO as a solvent than that of water [31,32]. This is according to the Foster–Hoffmann relationship ($\log \Phi_F = C + \chi \log \eta$) between Φ_F and the viscosity (η) of the solvents, and two constants, one temperature dependent (C) and one chromophore dependant (χ) [32].

The fluorescence life-time (τ_F) determines the average time of the molecule stays in the singlet exited state before quenching by fluorescence and its value correlates with the value of Φ_F [33]. The τ_F values for the previously studied hydroxyl and mercaptopyridine gallium-Pcs (1.57 ns or 1.93 ns in DMSO) were calculated higher when compare to the same substituted indium-Pc (0.06 ns or 0.36 ns) as a result of the very heavy indium metal [31,32]. The studied SiPc1 (4.27 ns) and GePc1 (4.07 ns) and also unsubstituted ZnPc (3.99 ns) in DMSO were evaluated by higher τ_F values than the same substituted GaPcs and InPcs in Refs. [31–33]. The last can be explained by the nature of the coordinated metals. Increases of the size of the central metal ion such as Ga, In or Sn have resulted in the reduced quantum yield and life-time of fluorescence [33]. The heavy atom effect encourages the intersystem crossing to the triplet state, hence diminished spin allowed fluorescence. The central metal effects on the photophysical and photochemical parameters were investigated in details by Moeno and Nyokong [34]. The authors' compared the trend of the heavy atom effect, which plays a major role in the results observed suggesting that the singlet excited state deactivates by intersystem crossing. However there are some exceptions of MPcs where the overall trend for τ_F does not match the trend in Φ_F [34]. The studied in this work quaternized MPcs showed the extremely low values of Φ_F obtained in water and by addition of a detergent (0.073 and 0.1 for SiPc1 vs. 0.01 and 0.02 for GePc1) which can be attributed to the high aggregation tendencies of SiPc1 and especially of GePc1. However the higher Φ_F value for SiPc1 obtained in DMSO (0.195) suggests reduction in aggregation. Considering the τ_F values of SiPc1 and GePc1 can be concluded that the quenching of the singlet excited state occurs thought the singlet state relaxation by fluorescence more probably than that observed for similarly substituted quaternized phthalocyanine complexes of Ga(III), In(III) and Sn(IV) metal ions [35].

2.4. Dark toxicity on cell cultures

Survival of the five cell cultures were evaluated after incubation with SiPc1 and GePc1 under dark conditions. The

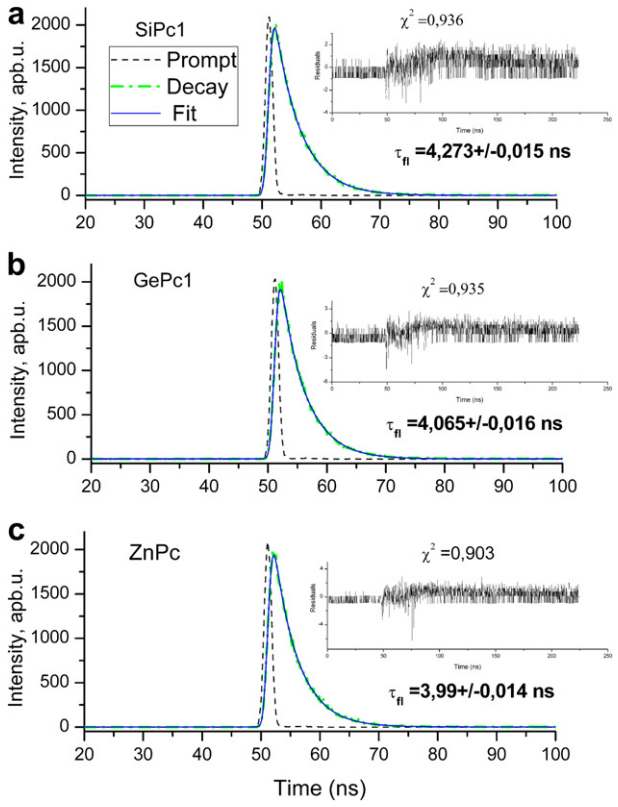


Fig. 3. Fluorescence decay of SiPc1 (a) and GePc1 (b), and ZnPc (c), all in DMSO (green dash dot), fit (blue solid line) and instrumental response (dash line) for λ_{exc} = 365 nm and fluorescence emission at 683 nm (SiPc1) and 685 nm (GePc1), and 681 nm (ZnPc).

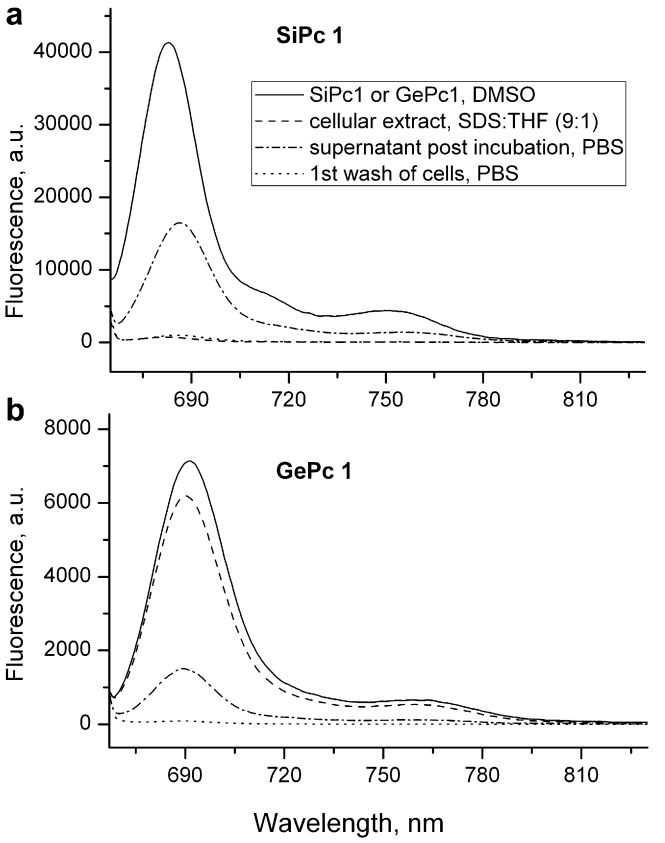


Fig. 4. Fluorescence spectra of samples of fungal cells (10^7 CFU mL^{-1}) incubated for 15 min with SiPc1 (a) and GePc1 (b) both at concentration 1.0 μM. The excitation light of 660 nm LED was applied.

concentration of 8 μM and incubation times of 2 h was found as optimal conditions to test dark toxicity. It was shown that up to 10 μM SiPc1 and GePc1 were not toxic for the used five cell lines. The viability was more than 75% for each tested cell line.

2.5. Uptake of Si(IV)- and Ge(IV)-phthalocyanines

The uptake of SiPc1 and GePc1 by the fungal cells was determined by using chemical extraction procedure. The quantification of the number of sensitizers molecules accumulated into *C. albicans* cells was carried out by the means of fluorescence measurements of the collected samples (Fig. 4). The estimation of the uptake of 1 μM SiPc1 and 1 μM GePc1 into fungal cells was made for the cellular suspensions with different densities (10^5–10^8 CFU mL^{-1}). Several supernatant samples were collected: (1) in PBS after incubation, (2) in PBS after cell wash, and (3) after cell extraction with a mixture of 2% SDS:THF (9:1). The collected samples were measured by using set-up for fluorescence measurements, which was described in Ref. [37].

The obtained results showed that SiPc1 and GePc1 as positively charged sensitizers are likely to accumulate into fungal cells (Fig. 5). An inverse behaviour of decreasing cellular accumulation of SiPc1 and GePc1 with increase of cell density was found. However the amount of SiPc1 into fungal cells was estimated to be lower than that of GaPc1 for all studied cell densities (10^5–10^8 CFU mL^{-1}). This phenomenon of an inverse dependence of the number of the bounded PS molecules per cell on the cell density was firstly reported by Demidova and Hamblin for *Escherichia coli* [38]. Recent studies on various pathogenic microorganisms treated by cationic MPcs confirmed that the decrease of accumulation occurred with an increase of the cell density in suspension [13,19,20,36]. The further study suggests that the insignificant uptake of SiPc1 seems not to influence on the photodynamic response towards *C. albicans* planktonic culture.

2.6. Confocal laser-scanning microscopy of biofilms

The water-soluble SiPc1 and GePc1 have a limited accumulation into the fungal cells of *C. albicans* and also the penetration in fungal biofilms as were studied by the confocal laser-scanning microscopy (CLSM). The fungal cells formed the biofilm on acrylic resins were detected by the green autofluorescence (exc: 488 nm; em:

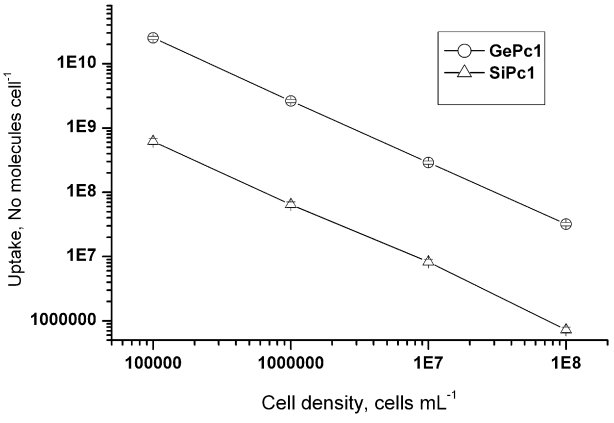


Fig. 5. Uptake of SiPc1(1.0 μM) and GePc1 (1.0 μM) in dependence on the cell density of *C. albicans* in suspension. The data are presented as means ± SD (n = 5).

500–580 nm). The red fluorescence of phthalocyanines (exc: 635 nm, em: 650–740 nm) into biofilm on slices of 0.150 μm was observed (Fig. 6a). The light transmission mode of 635 nm laser was used to visualize the cells (Fig. 6b). In addition the merge of the transmission mode and MPc fluorescence was applied to visualize the dye uptake (Fig. 6c). The fluorescence detection of SiPc1 into the cells suggests that SiPc1 accumulates close to the cellular wall and in some extent inside the cells. The fluorescence of GePc1 into biofilm was negligible as a result of the high aggregation of this PS in water even in the presence of the additive for disaggregation (CEL). The studied *C. albicans* biofilms formed for 48 h were measured for their thicknesses (23 ± 6 μm). The assessments of SiPc1 penetration depth into fungal biofilms were around 75% of the total biofilm thickness.

2.7. PDT on C. albicans planktonic cultures

Photodynamic antimicrobial activity of methylpyridyloxy-substituted SiPc1 and GePc1 against pathogenic fungus *C. albicans* in suspension (10^7 CFU mL^{-1}) of aqua buffered solution was investigated (Fig. 7). The phototoxic effects of SiPc1 and GePc1 towards *C. albicans* in planktonic phase for a range of concentrations (0.45–5.8 μM) showed a complete cell death for SiPc1 (1.8 μM). The inactivation with GePc1 was insignificant for the applied concentrations (0.45–5.8 μM). The effect of GePc1 was low (1 log) even at highest concentration (5.8 μM) applied. The dark toxicity of SiPc1 as well as of GePc1 was not observed at all treatment concentrations.

The newly investigated SiPc1 and GePc1 were compared to the powerful PS (ZnPcMe) which was used as a referent standard as well as ZnPc and MB towards fungal cells (Fig. 8). Nevertheless the lower uptake of SiPc1 as compare to ZnPcMe into *C. albicans* cells (unpublished data), the both PSs were determined as highly phototoxic to the fungal cells even at low drug concentrations. The applied experimental condition of removal of the non-bounded SiPc1 and respectively ZnPcMe before the light treatment showed that not only SiPc1 taken by the cells but also the free SiPc1 in suspension can contribute to the photodynamic action. For comparison reason, in all PDT experiments one and the same light dose (50 J cm^{-2}) and irradiance (60 mW cm^{-2}) from a 635 nm LED device were applied.

2.8. PDT on C. albicans biofilm cultures

The biofilms of *C. albicans* developed after 48 h were treated with SiPc1 and GePc1 (5.8 μM) for 1.5 h incubation period. The same light regime as for suspension (635 nm LED, 50 J cm^{-2} and 60 mW cm^{-2}) was used. In contrast to the planktonic phase of the same pathogenic cells where the concentration below 2 μM (SiPc1) and incubation time of 15 min were sufficient for full photoinactivation, for biofilms only 1 log cell death was achieved. Obviously in case of biofilm due to a limited penetration depth of SiPc1

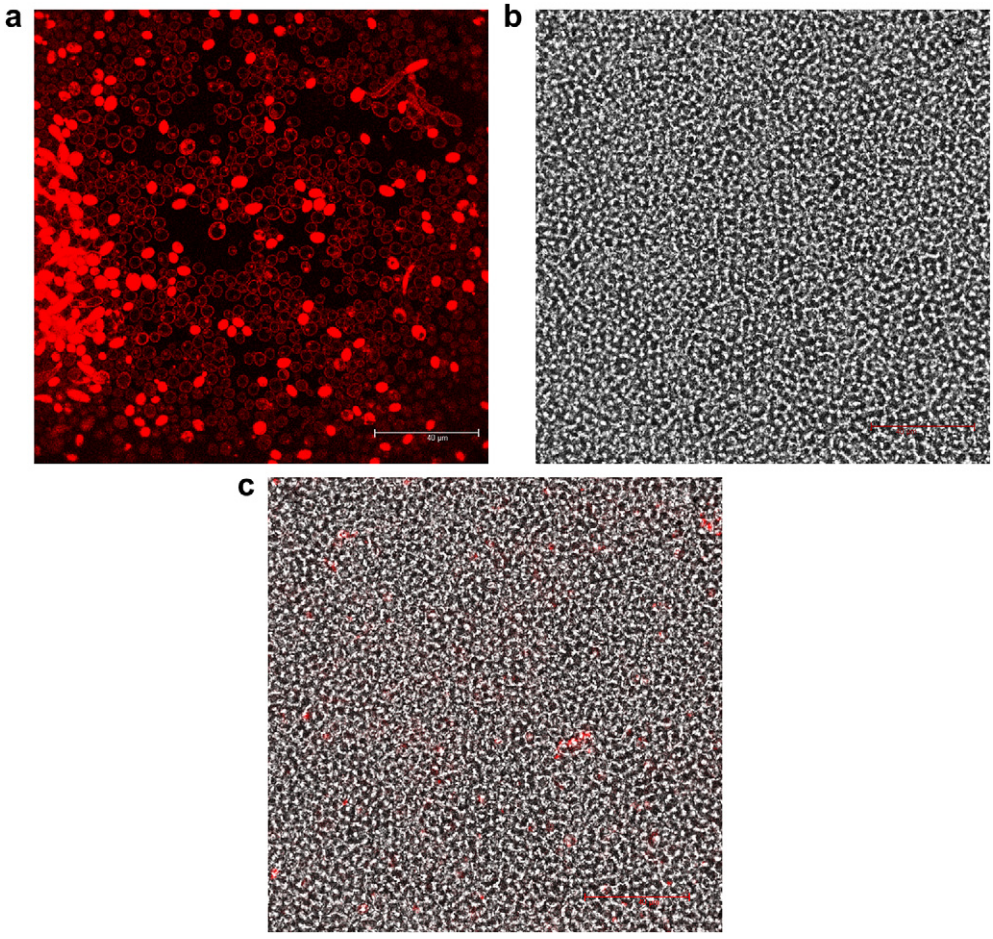


Fig. 6. Confocal images of *C. albicans* 48 h biofilm incubated for 1.5 h with 5.8 μM SiPc1. The slice of biofilm (0.150 μm) imaged by red fluorescence of SiPc1 (exc: 635 nm, em: 650–740 nm) into fungal cells (a); the cells imaged by the light transmission of 635 nm laser (b); the overlay of fluorescence of SiPc1 with the light transmission mode (c). Magnification: 63×. Scale bar: 40 μm.

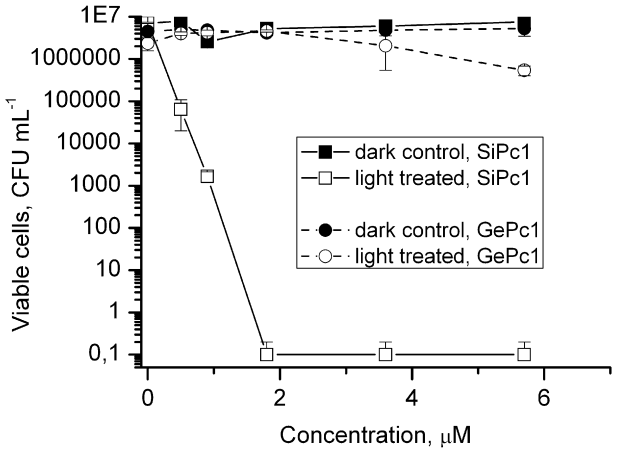


Fig. 7. Concentration dependence on the PDT effect on *C. albicans* planktonic cultures treated with SiPc1 and GePc1 and radiation of LED at 635 nm (60 mW cm^{-2}, 50 J cm^{-2}) and without light (dark control). The data are presented as means ± SD ($n = 5$).

and GePc1 and the strong aggregation behaviour of GePc1 the photodynamic response was negligible.

Among the previously studied by us water-soluble phthalocyanine complexes of Zn(II), Ga(III) and In(III) only tetra-substituted methylpyridyloxy ZnPcMe and octa-substituted methylpyridyloxy GaPc2 showed full effect towards 18 h fungal biofilms [20,36]. The different susceptibility of the fungal cells to the applied photosensitizers can be explained by several factors. The difference in electronic structure of the phthalocyanines due to metal ions coordinated into macrocycle. The maturation phase of fungal biofilms was a crucial factor for effectiveness of the treatment. The assessment of the PDT effect towards different stages of biofilm formation (18 h vs. 48 h) showed that the number of death cells decreased in case of 48 h biofilm and the efficacy dropped around 50%. This can be explained by the structure of the formed fungal biofilms, which during different stages of biofilm development characterizes with the matrix that varies in density by forming areas with water channels [16,17]. Singlet oxygen is known to affect directly polysaccharides, which exist in the extracellular matrix of a microbial biofilm [39]. The molecular oxygen supply seems to be significant in case of 18 h formed biofilm with an effective destruction of the extracellular matrix of a microbial biofilm. The penetration of the photosensitizer into the biofilms during different stages of biofilm growth is also a very important factor. Several approaches reported the application of conventional therapy together with PDT and the selective drug delivery with nanoparticles and the ultrasound wave for better drug penetration, all in order to improve a limited drug transfer through the biofilm channels [21–23].

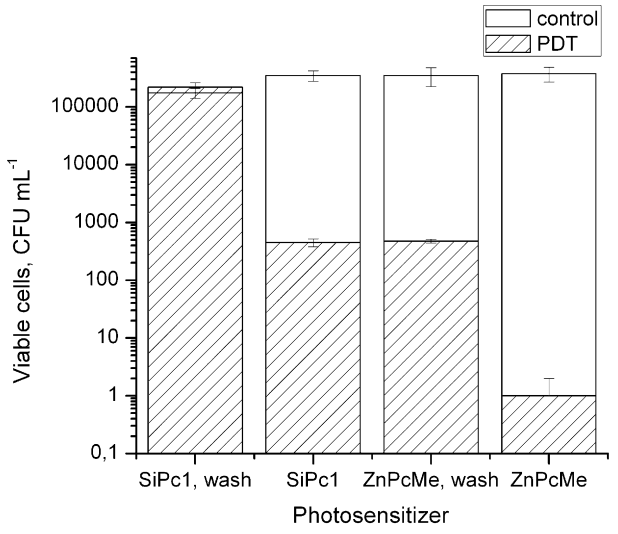


Fig. 8. Influence of uptake on the PDT effect of *C. albicans* planktonic cultures treated with SiPc1 and ZnPcMe, both in 1 μM concentration. The data present the survived cells without and after removal of the non-bounded PS and irradiated with 635 nm LED device (60 mW cm^{-2}, 50 J cm^{-2}). The data are presented as means ± SD ($n = 5$).

The increase of sensitizer penetration depth thought the biofilm by a new more practical for dental medicine approach was studied. The fractioned light application is well-known course of effective action in case of microbes [40]. The inactivation of fungal biofilms with 3 logs was achieved for SiPc1 and light radiation with light intensity of 60 mW cm^{-2} and total dose of 150 J cm^{-2}. The samples were irradiated three times within 2 h with constant light dose (50 J cm^{-2}) which was effective for fungal cells in suspension. The procedure was evaluated with three order improvement of the response (3 log) towards the single light radiation. The application of the fractioned radiation together with hydrogen peroxide appears an approach for full inactivation of fungal biofilms as was reported recently [41].

Comparisons of the PDT responses of SiPc1 and GePc1 on *C. albicans* planktonic and biofilm cultures with the results obtained with other MPcs with the same substitution [20,36] showed that the used as a standard ZnPcMe is more potent as antifungal photosensitizer for biofilms. This statement suggests that differing in molecular electronic structure of MPcs due to replacement of Zn(II) with Si(IV) or Ge(IV) tends to change sufficiently some photophysical and uptake properties efficiently but the influence on the photodynamic efficiency was in advantage to the complex with Si(IV) ion.

3. Conclusions

The phthalocyanine complexes of Si(IV) and Ge(IV) that after quaternization are soluble in water and in high degree monomeric (SiPc1) but strongly aggregated even at 80 °C (GePc1) were synthesized. The absorption and fluorescence spectra in the presence of an anionic detergent (SDS) and an emulsion (CEL) increase the monomers for SiPc1, but not for GePc1. The fluorescence quantum yields in water were between 0.101 (SiPc1) and 0.011 (GePc1) as a result of quenching process by aggregated forms. The cationic SiPc1 and GePc1 were taken up into fungal cells with two orders of magnitude higher for GePc1 as compare to SiPc1. The uptake behaviour followed the recent observation of enhancement of the number of dye molecules per one cell by lowering of cell density. *In vitro* studies on pathogen *C. albicans* as planktonic and biofilm cultures demonstrated that fungal suspension was completely inactivated at low concentration of SiPc1 (1.8 μM) and soft parameters of light irradiation (50 J cm^{-2}, 60 mW cm^{-2}). The effect for fungal cells after GePc1 was negligible (1 log) even at strong treatment conditions. The successful outcome of the photodynamic process can be achieved at low uptake of water-soluble SiPc1 when it circulated in the cellular suspension. GePc1 and SiPc1 showed incomplete penetration depth (~75%) into biomass of fungal biofilms (~23 μm thick). The reduced biofilm inactivation of 3 log (SiPc1) was achieved only after application of fractionated LEDs radiation (3 × 50 J cm^{-2}). In conclusion, water-soluble SiPc1 has a potential value for photodynamic inactivation of *C. albicans* in suspension and limited effect on mature fungal biofilms grown on denture acrylic resin.

4. Experimental

4.1. Chemicals

The chemicals for synthesis and spectroscopy were purchased from Sigma–Aldrich and Fluka. The spectrophotometric experiments were carried out in solvents DMSO and THF of spectroscopic grade. The synthesis of phthalocyanines was carried out in double distilled over CaH_2 quinoline. The other solvents such as dimethylformamide (DMF), dichloromethane (DCM), acetone (Ac), ethanol and pyridine of analytical grade were dried or distilled before using. The chemicals such as 3-hydroxypyridine, potassium carbonate, methyl iodide, $SiCl_4$ and $GeCl_4$ were used as purchased. The negatively charged SDS in concentration 0.1 M and CEL are products of Sigma. The stock solutions of the methylated complexes SiPc1 and GePc1, both in concentrations around 2 mM in DMSO, were prepared and stored in the dark and cool. Dilutions were prepared in sterile 0.01 M phosphate-buffered saline (PBS) to the final concentrations between 1 and 10 μM prior the experiments. The assessment of the drug concentration was carried out on the basis of the absorption spectra.

4.2. Equipment

Electron absorption spectra were recorded on a Jasco spectrophotometer (Japan) at room temperatures (RT). Fluorescence studies were carried out on a Jasco 6600 fluorimeter (Japan). Fluorescence decay curves were recorded in a time-correlated single photon counting using a Fluorolog-3 apparatus (Horiba Jobin Yvon) equipped for measurements of life-time. ^{1}H NMR (600 MHz) spectra were acquired on the spectrometer AVANCE AV600 II + NMR. Mass spectra were obtained on a Bruker Esquire LC (electronic ionization) spectrometer. Infrared spectra were recorded on KBr pellets using a BIORAD SPC-3200 FTS7 FT-IR spectrometer.

4.3. Synthesis

The starting phthalonitrile (4-nitrophthalonitrile) was synthesized and purified by known procedure [25]. The further reactions followed the modify pathway of synthesis described recently for GaPcs [20]. Similar reaction conditions were practical for preparation of Ge(III)- and Si(IV)-phthalocyanine derivatives (Scheme 1).

4.3.1. Synthesis of 4-(3-pyridyloxy) phthalonitrile (*1*)

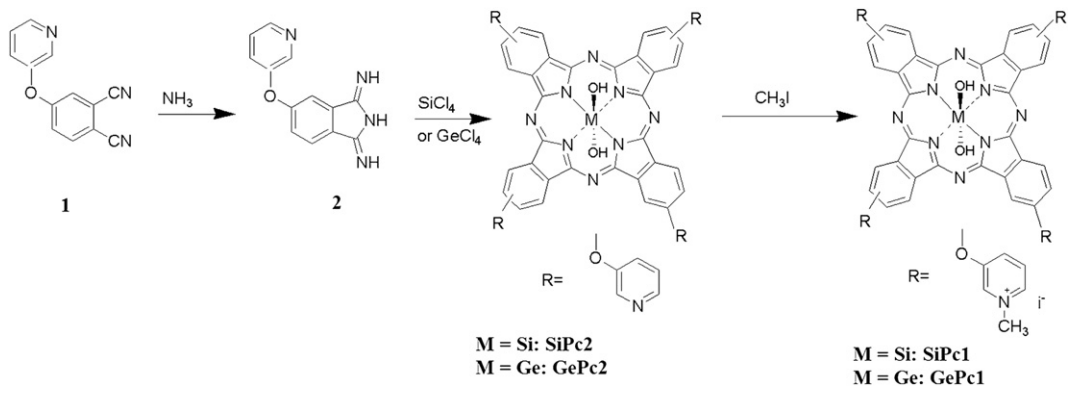
This synthesis is a modified version of the method described previously [24,25]. 4-Nitrophthalonitrile (8.7 g, 50 mmol) and an excess of 3-hydroxypyridine (**1**) (7.13 g, 75 mmol) were dissolved in 100 mL of dry DMSO under inert gas. A portion of dry potassium carbonate (13.8 g, 100 mmol) was added. After 48 h stirring at room temperature next portion of potassium carbonate was added (10 g, 72 mmol). After 72 h the mixture was poured to 500 mL of water. Precipitate was washed thoroughly with water. The isolated and dried product was dissolved in hot ethanol and active charcoal was added. After filtration ethanol solution was poured into water. Precipitate was filtered, washed with water and dried. Chromatography on silica gel with CH_2Cl_2 afforded solid product that was dissolved in Ac and slowly poured to water. The white crystals of high purity were obtained. Yield 8.73 g (80%). M = 221.22 g mol^{-1}, $C_{13}H_7N_3O$. IR (KBr): ν, cm^{-1} = 3107, 3070, 3039, 2228 (CN), 1596, 1574, 1566, 1487, 1424, 1314, 1280 (Ar–O–Ar), 1253 (Ar–O–Ar), 1216, 1084, 1025, 956, 892, 854, 820, 706, 615, 527. ^{1}H NMR (200 MHz, DMSO-d_6): δ = 8.52 (m, 1H), 8.40 (s, 1H), 8.12 (d, 1H), 7.9 (dd, 1H), 7.71 (m, 1H), 7.54 (m, 1H), 7.49 (dd, 1H). EI-MS (200 °C, 70 eV): m/z 221 $[M]^+$, 127 $[M^+ - C_5H_4NO]$, 78 $[C_5H_4N]^+$.

4.3.2. Synthesis of 4-(3-pyridyloxy)-1,3-diiminoisoindoline (*2*)

This method is a modified version of the synthesis described elsewhere [26]. 4-(3-Pyridyloxy) phthalonitrile (**1**) (1 g, 4.52 mmol) was added to a mixture of dry methanol (100 mL) and sodium methoxide (50 mg, 0.93 mmol). Anhydrous ammonia gas was bubbled through the stirred suspension for 45 min. The mixture was then refluxed and maintained at 110 °C for 3.5 h with continuous bubbling of ammonia gas. The reaction mixture became slightly greenish. After cooling the methanol was removed at reduced pressure. Any attempt to purify by column chromatography and isolate the product failed. Hence, the hygroscopic product was dried under vacuum at 80 °C and used directly without any purification. M = 238.25 g mol^{-1}, $C_{13}H_{10}N_4O$. ^{1}H NMR (200 MHz, DMSO-d_6): δ = 8.62 (m, 2H), 7.84 (d, 1H), 7.49 (m, 3H), 7.1 (dd, 1H), 3.79–3.59 (m, 3H, NH). EI-MS (205 °C, 70 eV): m/z 238 $[M]^+$, 222 $[M - NH_2^-]^+$, 78 $[C_5H_4N]^+$.

4.3.3. Synthesis of 2(3),9(10),16(17),23(24)-tetrakis-(3-pyridyloxy) phthalocyaninato dihydroxy-silicon (IV) (SiPc2) (mixture of regioisomers)

Dry 5-(3-pyridyloxy)-1,3-diiminoisoindoline (**2**) (0.270 g, 1.13 mmol) obtained from 4-(3-pyridyloxy)phthalonitrile (**1**) was placed in a 100 mL two-necked round-bottomed flask, equipped with magnetic stirrer and a reflux condenser. 10 mL of freshly distilled quinoline was added to the flask and $SiCl_4$ (0.5 mL, 4.35 mmol) was transferred with the aid of syringe under inert gas atmosphere at RT. The reaction was stirred at 180 °C for 15 min and than at 200 °C for 45 min. The formation of a green precipitate took place. After being cooled 20 mL of water solution of



Scheme 1. Synthetical pathway of preparation of water-soluble tetra-methylpyridyloxy-substituted Si(IV)-phthalocyanine, SiPc1 and Ge(IV)-phthalocyanine, GePc1.

ammonium hydroxide (25%) was added to the reaction mixture and stirring was continued overnight. After the water phase was evaporated the residue was diluted with DMF. The mixture was poured into 400 mL of water and the precipitate obtained was collected by filtration, washed thoroughly with DMF:water (1:2), with water only and then with Ac. The crude product was chromatographed on silica gel using CH_2Cl_2 to remove first yellow and greenish impurities. The desired compound was then eluted as a blue band using CH_2Cl_2:MeOH (2:1). After solvent evaporation a second chromatography was applied using $CHCl_3$ and then $CHCl_3$:MeOH (9:1) to get rid of impurities. Finally, the product was eluted with $CHCl_3$:MeOH (6:4). Removal of the solvent resulted in 0.578 g of blue solid. Yield: 54%. M = 946.97 g mol^{-1}. UV–Vis (DMF) λ, nm ($\varepsilon/10^5$): 676 (0.56), 608 (0.13), 345 (0.29). IR (KBr): ν, cm^{-1} = 3058, 1614, 1574, 1519, 1472, 1412, 1331, 1238 (C–O–C), 1125, 1079, 1060, 1021, 956, 820 (Si–OH), 758, 734, 706, 538. ^{1}H (600 MHz, DMSO-d_6) δ (ppm): 9.50–9.64 (m, 4H), 8.98–9.07 (m, 4H), 8.75–8.81 (m, 4H), 8.61–8.62 (m, 4H), 8.05–8.14 (m, 4H), 7.85–7.92 (m, 4H), 7.62–7.67 (m, 4H). Calc. for $C_{52}H_{30}N_{12}O_6Si$: C 65.95, H 3.19, N 17.74%; Found: C 65.74, H 3.23, N 17.21%. ESI-MS (negative ion mode) m/z: 946 [M$^-$].

4.3.4. Synthesis of 2(3),9(10),16(17),23(24)-tetrakis-[3-(N-methyl) pyridyloxy]-phthalocyaninato dihydroxy-silicon (IV) tetraiodide (SiPc1) (mixture of regioisomers)

2(3),9(10),16(17),23(24)-Tetrakis-(3-pyridyloxy)phthalocyaninato dihydroxy-silicon (IV) (SiPc2) in a quantity of 150 mg (0.158 mmol) was dissolved in DMF (10 mL). An excess of methyl iodide (2 mL, 32 mmol) was added and the solution was stirred for 19 h under nitrogen at 50 °C. The rest of methyl iodide was evaporated and the reaction product was precipitated with CH_2Cl_2, filtered off and washed with CH_2Cl_2, EtOH and Ac. The compound was dissolved in water and after filtration isolated by freeze-drying. Yield: 200 mg (83%). M = 1514.73 g mol^{-1}. UV–Vis (H_2O, pH 7, SDS 0.1 mol L^{-1}) λ, nm ($\varepsilon/10^5$): 675 (1.24), 642 (0.61), 609(0.18), 346(0.56). IR (KBr): ν, cm^{-1} = 3026, 2941 (CH_3), 2830 (CH_3), 1614, 1582, 1501, 1471, 1409, 1330, 1275(C–O–C), 1124, 1083, 1061, 966, 826(Si–OH), 756, 663, 541. ^{1}H (600 MHz, DMSO-d_6) δ: 9.73–9.77 (m, 2H), 9.66–9.70 (m, 2H), 9.38–9.47 (m, 8H), 8.98–9.00 (m, 4H), 8.63–8.70 (m, 4H), 8.30–8.38 (m, 8H), 4.49 (s, 6H), 4.47 (s, 6H). Calc. for $C_{56}H_{42}N_{12}O_6SiI_4$: C 44.39, H 2.80, N 11.10%; Found: C 43.69, H 2.99, N 11.26%. ESI-MS (positive ion mode) m/z: 251.3 [M − 4I$^-$]$^{4+}$.

4.3.5. Synthesis of 2(3),9(10),16(17),23(24)-tetrakis-(3-pyridyloxy) phthalocyaninato dihydroxygermanium (IV)(GePc2) (mixture of regioisomers)

Dry 4-(3-pyridyloxy)-1,3-diiminoisoindoline (**2**) (0.400 mg, 1.68 mmol) was placed in a 100 mL two-necked round-bottomed flask, equipped with magnetic stirrer and a reflux condenser. A freshly distilled quinoline (10 mL) was added to the flask and 0.5 mL of $GeCl_4$ (4.29 mmol) was transferred with the aid of syringe under inert gas atmosphere at RT. The reaction was stirred at 200 °C for 2 h. The formation of a green precipitate took place. After being cooled 20 mL of water solution of ammonium hydroxide (25%) and 20 mL of pyridine was added to the reaction mixture and stirring was continued overnight. The reaction mixture was poured into 500 mL of water and the green precipitate obtained was collected by filtration, washed thoroughly with DMF:water (1:2) with water only and then with Ac. The crude product was dissolved in a mixture of CH_2Cl_2:MeOH and chromatographed on silica gel using initially CH_2Cl_2:MeOH (9:1) to remove first impurities. The desired compound was then eluted as a blue-green band using CH_2Cl_2:MeOH (6:4). Removal of the solvent resulted in deep-blue solid. Yield (with respect to the phthalonitrile used): 1.29 g (77%). M = 991.50 g mol^{-1}. UV–Vis (DMF) λ, nm ($\varepsilon/10^5$): 680 (1.93), 612 (0.42), 365 (0.84). IR (KBr): ν, cm^{-1} = 3058, 1614, 1574, 1500, 1472, 1424, 1405, 1347, 1320, 1237 (C–O–C), 1123, 1086, 1055, 1022, 954, 810, 749, 734, 707, 644. ^{1}H (600 MHz, DMSO-d_6) δ (ppm): 9.48–9.59 (m, 4H), 8.98–9.06 (m, 4H), 8.76–8.82 (m, 4H), 8.61–8.63 (m, 4H), 8.05–8.15 (m, 4H), 7.86–7.93 (m, 4H), 7.61–7.68 (m, 4H). Calc. for $C_{52}H_{30}N_{12}O_6Ge$: C 62.99, H 3.05, N 16.95%; Found: C 62.54, H 3.08, N 16.26%. ESI-MS (positive ion mode) m/z: 992 [M]$^+$, 974 [M − H_2O]$^+$.

4.3.6. Synthesis of 2(3),9(10),16(17),23(24)-tetrakis-[3-(N-methyl) pyridyloxy]-phthalocyaninato dihydroxygermanium (IV) tetraiodide (GePc1) (mixture of regioisomers)

A quantity of 100 mg (0.1 mmol) of dihydroxygermanium 2,9,16,23-tetrakis-(3-pyridyloxy)phthalocyanine (GePc2) was dissolved in dry DMF (10 mL). An excess of methyl iodide (5 mL, 80 mmol) was added and the solution was stirred for 16 h under nitrogen at 50 °C. The methyl iodide was evaporated and the reaction product was precipitated with CH_2Cl_2 (400 mL), filtered off and washed with CH_2Cl_2, EtOH and Ac. The compound was dissolved in water and after filtration isolated by freeze-drying cycle. Yield: 138 mg (88%). M = 1559.25 g mol^{-1}, UV–vis (H_2O, pH 7, SDS 0.1 mol L^{-1}) λ, nm ($\varepsilon/10^5$): 678 (0.33), 643 (0.52), 613(0.48). IR (KBr): ν, cm^{-1} = 3024, 2941 (CH_3), 2831 (CH_3), 1613, 1582, 1500, 1469, 1405, 1385 (CH_3), 1346, 1275 (C–O–C), 1217, 1122, 1089, 1056, 966, 887, 742, 663. ^{1}H (600 MHz, DMSO-d_6) δ (ppm): 10.0–9.6 (m, 2H), 9.6–9.34 (m, 5H), 9.34–9.12 (m, 2H), 9.12–8.93 (m, 4H), 8.93–8.75 (m, 4H), 8.75–8.58 (m, 2H), 8.58–7.9 (m, 9H), 4.70–4.26 (m, 12H). Calc. for $C_{56}H_{42}N_{12}O_6GeI_4$: C 43.12, H 2.72, N 10.78%; Found: C 42.85, 2.92, 10.85%. ESI-MS (positive ion mode) m/z: 263 [M − 4I$^-$]$^{4+}$, 236.6 [M − 4I$^-$ − Ge(OH)$_2$ + 2H$^+$]$^{4+}$.

4.4. Steady state and time-resolved fluorometry

The fluorescence emission spectra were recorded at excitation wavelength 610 nm for the spectral region of 625–750 nm by using 1 cm quartz cuvette (Hellma) at RT. The fluorescence quantum yields (Φ_F) were calculated on the basis of absorption and fluorescence spectra of SiPc1 and GePc1 and by using a comparative method [28]. The equation (1) was employed for calculations:

$$\Phi_F = \Phi_{F(R)}\left(I A_{(R)} \eta^2\right) \Big/ \left(I_{(R)} A \eta_{(R)^2}\right) \quad (1)$$

where $\Phi_{F(R)}$ is the fluorescence yield of the reference standard (ZnPc, $\Phi_{F(R)}$ = 0.18 in DMSO [42]), I and $I_{(R)}$ are the integrated fluorescence of the sample (SiPc1 or GePc1) and the reference standard (ZnPc), A and $A_{(R)}$ are the absorbance of the sample and the reference, and η and $\eta_{(R)}$ are the refractive indices of the solvents employed in calculating of fluorescence quantum yields. The absorbances of the sample and the reference were kept under 0.05 at λ_{exc} in order to avoid the reabsorption effect.

The fluorescence lifetimes (τ_F) of SiPc1 and GePc1 and the used standard ZnPc were measured at 20 °C in 1 cm quartz cuvette with a stopper by the means of an SPEX Fluorolog-3 fluorometer (Horiba Jobin Yvon). The time-correlated single photon counting (TCSPC) mode of the apparatus equipped with the Fluorolog fitting software IBH program was used. Briefly, the set-up consists of the excitation source (light emitting diode NanoLED, 365 nm) with 1 MHz repetition rate with a linear polarizer. The fluorescence was detected by a peltier cooled photomultiplier tube (PMT) and the integrated electronics. A monochromator with a spectral width of 2 nm was used to select the required emission wavelength band. The response function of the system, which was measured with a scattering Ludox solution (DuPont), had a full width at half-maximum (FWHM) of about 500 ps for NanoLED. All decay curves were measured at the emission maximum of PSs and the lifetimes were obtained by

deconvolution of the decay curves with the Fluorolog fitting software IBH program. The measured decays were fitted to the convolution of single exponential functions and the low values of factor χ^2 and random distribution of residuals were used as criteria for well fit.

4.5. Cell cultures

The used cell lines were Calf trachea cell line, MDBK cell line, Vero cell line, Madin–Darby Canine Kidney Cells and primary tissue culture of chick embryo fibroblast. The cells were cultured at 37 °C in a humidified sterile atmosphere of 95% air and 5% CO_2. DMEM (Dulbecco Modified Eagles's Medium, Gibco BRL, USA) enriched with foetal calf serum (5% v/v) and 10 mM HEPES was used. The cells were incubated in 5% CO_2 at 37 °C.

4.6. Dark toxicity

The toxicity of SiPc1 and GePc1 in the absence of light was determined on monolayer cell cultures by the MTT colorimetric assay [43]. Cells were seeded in 96 well plates and cultured to 80–85% confluence. Further incubation with 0.25 μM–10 μM SiPc1 and GePc1 in the dark at 37 °C for three time intervals (15 min, 24 h and 48 h) was performed. PBS was used for washing and 200 μL DMEM with 5 mg mL^{-1} MTT was added to each well. Cell cultures were incubated for 4 h at 37 °C and 5% CO_2, then medium was discarded, the plates were dried and 0.1 mL of cold *iso*-propanol was added. Plates were mildly shaken for 10 min and the absorbance was read on spectrophotometer. The 50% cytotoxic concentration (CC_{50}) was calculated in comparison to the control group (Origin 6.1 software). The test was carried out in triplicate and the values were statistically validated.

4.7. Fungus

The fungus *C. albicans* 74 from the National bank for industrial microorganisms and cell cultures (NBIMCC) Bulgaria was used. Tryptic soy broth and tryptic soy agar media (Difco) were used. The fungal strain was grown aerobically at 37 °C. Cells were harvested by centrifugation and were resuspended in sterile PBS. Prior to the experiments they were diluted to the required cell densities from 10^8 to 10^5 cells per mL.

4.8. Sensitizers uptake

The fungal suspension with cellular densities between 10^5 and 10^8 cells per mL were incubated with SiPc1 or GePc1 (1.0 μM) for 15 min at 25 °C by gentle stirring in the dark. The supernatants were removed and stored for fluorescent measurements. The cells were washed with PBS in triplicate and the supernatants were collected for fluorescence study. The extraction mixture of THF: aqueous 2% SDS (1:9) was added to the sediment cells and the extraction was continued for 30 min by shaking in the dark. Then the samples were centrifuged, and the extracts were examined by fluorescence analysis. The uptake of SiPc1 and GePc1 into *C. albicans* cells was evaluated by the intensity of fluorescence maximum of the samples using the set-up described in Ref. [37]. The uptake data are presented as number of molecules per one fungal cell by processing the values of fluorescence intensities and referring to the recorded calibration curves for SiPc1 and GePc1.

4.9. Biofilm assay

Biofilm assay was performed on polymethylmethacrylate (PMMC) resin designed as discs with diameter of 10 mm and 1.5 mm thick. The acrylic discs were placed in commercial presterilized, polystyrene flat bottomed 12 well cell culture test plates (Switzerland). Standard suspension of *C. albicans* (1 mL, 10^7 CFU mL^{-1}) was prepared after serial dilutions and it was placed onto the surface of the discs in each well of the plate. The incubation was carried out for 1.5 h at 37 °C to promote cellular adherence to the acrylic surface. 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 of *C. albicans* an addition of 4 mL Tryptic soy broth (Difco Lab, MD, USA) was placed in each well. The plates were incubated for 48 h at 37 °C to form the biofilm.

4.10. Confocal microscopy study of biofilm

The biofilms of fungal cells were developed on the cover- slips covered with PMMC resin during 48 h incubation (37 °C). The biofilms were examined by a confocal laser-scanning microscope Leica Microsystems (Leica TCS SPE). The images were processed via the Leica LAS AF software provided with the CLSM. An oil immersion of 63× objective (NA = 1.23) was used. The biofilms of *C. albicans* were washed in PBS and covered with coverslips. The images of biofilm slices with thickness of 0.150 μm were scanned by the transmission mode using 635 nm laser radiations. The red fluorescence of SiPc1, GePc1 and ZnPcMe bounded to the cells of biofilm were pictured at excitation with 635 nm laser and fluorescence emission in the region 650–740 nm. The green autofluorescence of the fungal cells organized as biofilm were imaged at excitation 488 nm and emission between 500 and 580 nm. The penetration depth of MPcs into biofilm was examined by following the transmission of 635 nm laser and the native fluorescence emission from the fungal cells towards the red fluorescence emission.

4.11. Antimicrobial PDT

Samples of 1 mL fungal suspensions with cell density around 10^7 CFU mL^{-1} were incubated with SiPc1 and GePc1 in DMSO (5% CEL) for 15 min. The concentrations between 0.45 and 6.8 μM were used. The incubation was carried out at RT in the dark by gentle shaker. After incubation time was passed the aliquot (200 μL) from the suspension was placed in a standard palette where the light was applied. The light source was a newly developed LED device produced by ELO Ltd, Bulgaria. The device contains 25 super bright diodes at 635 nm with spectral half width of about 20 nm and safe light intensity more than 100 mW cm^{-2} for area of about 200 cm^2. The irradiance of 60 mW cm^{-2} was controlled with photometer (Spectra Physics, USA). The fungal biofilm grown for 48 h on PMMC discs were incubated for 1.5 h with SiPc1 (5.8 μM). After incubation the discs were irradiated with a single dose 50 J cm^{-2} and irradiance 60 mW cm^{-2}. After 1 h, in addition a second irradiation with the same dose was applied. The additional irradiation was applied within 2 h to total dose 150 J cm^{-2}. The results were evaluated towards three control groups: (1) without PS, but radiated, (2) with PS, but no light (dark toxicity), and (3) untreated bacterial suspension (without PS and light) or only biofilms grown for 48 h on acryl discs. The fungal suspension and the resuspended biofilm (0.1 mL) were serially diluted (10-fold) in PBS. The aliquots (0.1 mL) were spread over Trypticase® Soy agar and the number of colonies (CFU) was counted after 48 h incubation at 25 °C.

4.12. Statistics

Each experiment was carried out in triplicate and the data are presented as a mean ± 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.

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2011.07.015.

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